ADVANCES AND CHALLENGES OF RNAi BASED TECHNOLOGIES FOR PLANTS - VOLUME 2

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ADVANCES AND CHALLENGES OF RNAi BASED TECHNOLOGIES FOR PLANTS - VOLUME 2

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Table of Contents

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EDITORIAL
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Editorial: Advances and Challenges of RNAi Based Technologies for Plants—Volume 2

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Editorial on the Research Topic

Advances and Challenges of RNAi Based Technologies for Plants—Volume 2

The greatest challenge for farmers is to ensure sufficient and safe food in an economically, socially, and environmentally sustainable way. Plant pests and diseases are among the main problems affecting crop species, which are mainly controlled by the application of chemical pesticides (Schumann and D'Arcy, 2010). There is strong social and political pressure in Europe to decrease the use of agrochemicals, as also highlighted in the recently published European Green Deal, where a 50% decrease in the use of chemical pesticides is expected before 2030. However, recent estimations outline that the implementation of the EU policy can lead to a significant decline in EU agricultural production, together with a non-negligible increase in prices of agrifood products (Barreiro-Hurle et al., 2021): innovation is crucial to combine farm productivity with the goal of agrochemical reduction. One possible strategy is the employment of crop varieties resistant and/or tolerant to diseases through classical breeding techniques, but, besides the limited durability of this type of resistance, this solution is not always feasible due to the lack of resistance genes (McDonald, 2014; Willocquet et al., 2017).

The implementation of specific genetic improvement programs, including the application of new breeding techniques (NBTs), represents a faster and more precise strategy than conventional breeding for ensuring durable crop protection and yield improvement (Podevin et al., 2013; Limera et al., 2017; Sabbadini et al., 2021). As an additional tool, RNA interference (RNAi), an evolutionarily conserved regulatory process used by eukaryotes to regulate the expression of endogenous genes and also functioning in plants as a defense mechanism against pathogens, has been successfully exploited to confer disease protection and improve traits of agronomic interest (Limera et al., 2017; Rosa et al., 2022). Short-range movement of RNAi-mediating small non-coding regulatory RNAs (sRNAs) between cells occurs through plasmodesmata, whereas the long-distance movement occurs via the vascular systems (Tang et al., 2022). sRNAs can move between cells as naked molecules, bound to RNA binding proteins or enclosed in extracellular vesicles (EVs) (Tang et al., 2022).

The expression in plants of double strand RNA (dsRNA)-generating constructs permits the silencing of genes of invading pests and pathogens (host-induced

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gene silencing, HIGS) (Nowara et al., 2010). Two papers in this Research Topic are dealing with HIGS. Huang et al. (2022) used omics techniques to compare RNAi-based genetically modified maize lines resistant to a hemipteran insect with the parental line and found no specific unintended effects. The long-term efficacy and the stability of small RNA (sRNA) profiles of the RNAi-mediated virus resistant "Honeysweet" plum, as well as the absence of adverse health effects, is reported by Singh et al. (2021).

Most papers in this Research Topic are on exogenous application, usually by spraying, of sRNAs or dsRNAs on plant surfaces (spray-induced gene silencing, SIGS). The review *Lab-to-Field Transition of RNA Spray Applications – How far are we?* by Rank and Koch (2021) gives an overview on recent advances of exogenous RNA applications for the control of viruses, fungi and insects and for silencing of plant genes, and also points to challenges for agricultural uses of this approach.

A communication mechanism based on sRNA trafficking via EVs exchange between some fungal pathogens and their respective host plants, called bi-directional cross-kingdom RNAi which seems to modulate host immunity and pathogen virulence during infection processes, has recently been demonstrated (Wang et al., 2016; He et al., 2021). This discovery also enabled further developments of strategies for crop disease management based on SIGS.

Fungal/oomycete-associated diseases, including "gray mold" and "downy mildew" (caused by *B. cinerea* and *P. viticola*, respectively) are the most serious grapevine diseases worldwide, causing severe losses in crop yields and grape quality (Pearson and Goheen, 1988; Pons et al., 2018). They are generally controlled with a high use of pesticides, that in Europe is reaching the amount of 68,000 tons/year of fungicides to control grape diseases, equaling 65% of all fungicides used in agriculture (Eurostat Report, 2007).

Grape breeding programs aiming at the introgression of resistance traits from wild species to cultivated varieties have the limit to lose trait specificity of clones identified in important vine cultivars. Target sequences coding for Dicer and Dicer-like (*DCL)* proteins have been identified as crucial genes for the pathogenicity of *B. cinerea* and *P. viticola* and used in SIGS approaches to control these two severe pathogens (Wang et al., 2016; Capriotti et al., 2020; Haile et al., 2021). A SIGS approach against the oomycete *P. viticola* was shown to work effectively both as preventive and therapeutic treatment (Haile et al., 2021). A different approach for downy mildew control is presented by Marcianò et al. (2021) who silenced a grapevine susceptibility gene by exogenous dsRNA application, resulting in reduced disease severity after artificial *P. viticola* inoculation. In this study, control of downy mildew was achieved through the exogenously induced silencing of a plant-host gene, the *VviLBDIf7* gene encoding the putative ortholog of a transcription factor (TF) belonging to the LOB (lateral organ boundaries) family of TF, acting as repressor of jasmonate-mediated defense responses and known to be involved in plant organ development and stress response in many plant species. This evidence represents an alternative SIGS application, implying a deep knowledge of the genetic mechanism underneath the plant susceptibility and the need of new investigations to clarify any possible undesirable drawback on plant development and productivity. On the other hand, since susceptibility genes can be highly conserved among plant species and can work in response to different pathogens, once developed in a particular plant species, this approach can inspire alternative control strategies in various crop systems to provide broad protection.

Regarding the application of RNAi to control important pest insects, Willow and Veromann (2021) reviewed dietary RNAi studies in coleopterans. They discuss the impediments to our current understanding of RNAi sensitivity in this important insect order, and identify critical future directions for research in this area, with an emphasis on using plant biotechnology approaches. Joga et al. (2021) report that RNAi is an emerging approach that can be used for forest protection. Forests are of immense importance due to their socio-economic and ecosystem services, and Coleopteran Forest pests such as Emerald ash borer (*Agrilus planipennis*), Asian longhorn beetles (*Anoplophora glabripennis*) and bark beetles have taken advantage of ongoing climate change, causing severe damage to the forests worldwide. Current management strategies have been unable to keep pace with these forest insect population infestations. Joga et al. (2021) focus in their review on recent innovations in RNAi delivery that can be deployed against forest pests, for instance cationic liposome-assisted (lipids), nanoparticle-enabled (polymers or peptides), symbiont-mediated (fungi, bacteria, and viruses), and plant-mediated deliveries (trunk injection, root absorption). They consider barriers to further developing RNAi for forest pest management and suggest future directions of research in order to develop the use of RNAi against wood-boring coleopterans.

One of the most alluring aspects of RNAi technology is its predicted minimal impact on the environment, due to high target selectivity and the short persistence of the active molecules in the environment. However, in order to exclude possible off-target effects and effects on non-target organisms and due to the limited knowledge of most eukaryotic genomes, the combination of bioinformatics and ecologically sound bioassays using selected focal species are deemed necessary to support biosafety claims of RNAi applications (Christiaens et al., 2022). In addition, it should be noted here that the exploitation of RNAi to improve crop health is a fast-growing market and while GM RNAi plants are being assessed using the existing regulatory framework, appropriate safety evaluations, and authorization procedures for SIGS-based products are less clear so far (De Schutter et al., 2022). Externally applied RNA products must be regulated according to pesticide regulations. Regulatory frameworks for plant protection products and considerations for the authorization of dsRNA-based pesticides for plant protection in the United States and in the European Union are outlined by Dietz-Pfeilstetter et al. (2021).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

Barreiro-Hurle, J., Bogonos, M., Himics, M., Hristov, J., Pérez-Domiguez, I., Sahoo, A., et al. (2021). *Modelling Environmental and Climate Ambition in the Agricultural Sector With the CAPRI Model. Exploring the Potential Effects of Selected Farm to Fork and Biodiversity Strategies Targets in the Framework of the 2030 Climate Targets and the Post 2020 Common Agricultural Policy*. Luxembourg: Publications Office of the European Union.

Capriotti, L., Baraldi, E., Mezzetti, B., Limera, C., and Sabbadini, S. (2020). Biotechnological approaches: Gene overexpression, gene silencing, and genome editing to control fungal and oomycete diseases in grapevine. *Int. J. Mol. Sci.* 21, 5701. doi: 10.3390/ijms21165701

Christiaens, O., Sweet, J., Dzhambazova, T., Urru, I., Smagghe, G., Kostov, K., et al. (2022). Implementation of RNAi-based arthropod pest control: environmental risks, potential for resistance and regulatory considerations. *J. Pest Sci.* 95, 1-15. doi: 10.1007/s10340-021-01439-3

De Schutter, K., Taning, C. N. T., Van Daele, L., Van Damme, E. J. M., Dubruel, P., and Smagghe, G. (2022). RNAi-based biocontrol products: Market status, regulatory aspects, and risk assessment. *Front. Insect. Sci.* 1, 818037. doi: 10.3389/finsc.2021.818037

Dietz-Pfeilstetter, A., Mendelsohn, M., Gathmann, A., and Klinkenbuß, D. (2021). Considerations and Regulatory Approaches in the USA and in the EU for dsRNA-Based Externally Applied Pesticides for Plant Protection. *Front. Plant Sci.* 12, 682387. doi: 10.3389/fpls.2021.682387

Eurostat Report (2007). *The use of plant protection products in the European Union – Data 1992-2003*. Available online at: https://ec.europa.eu/eurostat/documents/3217494/5611788/KS-76-06-669-EN.PDF

Haile, Z. M., Gebremichael, D. E., Capriotti, L., Molesini, B., Negrini, F., Collina, M., et al. (2021). Double-stranded RNA targeting dicer-like genes compromises the pathogenicity of plasmopara viticola on grapevine. *Front. Plant Sci.* 1218, 667539. doi: 10.3389/fpls.2021.667539

He, B., Cai, Q., Qiao, L., Huang, C. Y., Wang, S., Wang, S., et al. (2021). RNA-binding proteins contribute to small RNA loading in plant extracellular vesicles. *Nat. Plants* 7, 342-352. doi: 10.1038/s41477-021-00863-8

Huang, C., Zhi, W., Pengyu, Z., Chenguang, W., Chaonan, W., Wenjie, X., et al. (2022). RNA interference-based genetic engineering maize resistant to apolygus lucorum does not manifest unpredictable unintended effects relative to conventional breeding: short interfering RNA, transcriptome, and metabolome analysis. *Front. Plant Sci.* 13, 745708. doi: 10.3389/fpls.2022.745708

Joga, M. R., Mogilicherla, K., Smagghe, G., and Roy, A. (2021). RNAi against wood-boring forest pests RNA interference-based forest protection products (FPPs) against wood-boring coleopterans: hope or hype? *Front. Plant Sci.* doi: 10.3389/fpls.2021.733608

Limera, C., Sabbadini, S., Sweet, J. B., and Mezzetti, B. (2017). New biotechnological tools for the genetic improvement of major woody fruit species. *Front. Plant Sci.* 8, 01418. doi: 10.3389/fpls.2017.01418

Marcianò, D., Ricciardi, V., Fassolo, E. M., Passera, A., Bianco, P. A., Failla, O., et al. (2021). RNAi of a putative grapevine susceptibility gene as a possible downy mildew control strategy. *Front. Plant Sci.* 12, 667319. doi: 10.3389/fpls.2021.667319

McDonald, B. A. (2014). Using dynamic diversity to achieve durable disease resistance in agricultural ecosystems. *Trop. Plant Pathol.* 39. doi: 10.1590/S1982-56762014000300001

Nowara, D., Schweizer, P., Gay, A., Lacomme, C., Shaw, J., Ridout, C., et al. (2010). HIGS: Host-induced gene silencing in the obligate biotrophic fungal pathogen Blumeria graminis. *Plant Cell* 22, 3130–3141. doi: 10.1105/tpc.110.077040

Pearson, R. C., and Goheen, A. C. (1988). Compendium of Grape Diseases. St. Paul: APS Press. p. 96.

Podevin, N., Davies, H. V., Hartung, F., Nogué, F., and Casacuberta, J. M. (2013). Site-directed nucleases: a paradigm shift in predictable, knowledge-based plant breeding. *Trends Biotechnol.* 31, 375-383. doi: 10.1016/j.tibtech.2013.03.004

Pons, A., Mouakka, N., Deliere, L., Crachereau, J. C., Davidou, L., Sauris, P., et al. (2018). Impact of Plasmopara viticola infection of Merlot and Cabernet Sauvignon grapes on wine composition and flavor. *Food Chem.* 239, 102-110. doi: 10.1016/j.foodchem.2017.06.087

Rank, A. P., and Koch, A. (2021). Lab-to-field transition of RNA spray applications - How far are we? Front. Plant Sci. 12, 755203. doi: 10.3389/fpls.2021.755203

Rosa, S., Pesaresi, P., Mizzotti, C., Bulone, V., Mezzetti, B., Baraldi, E., et al. (2022). Game-changing alternatives to conventional fungicides: small RNAs and short peptides. *Trends Biotechnol.* 40, 320-337. doi: 10.1016/j.tibtech.2021.07.003

Sabbadini, S., Capocasa, F., Battino, M., Mazzoni, L., and Mezzetti, B. (2021). Improved nutritional quality in fruit tree species through traditional and biotechnological approaches. *Trends Food Sci. Technol.* 117, 125-138. doi: 10.1016/j.tifs.2021.01.083

Schumann, G. L., and D'Arcy C. J. (2010). *Essential Plant Pathology, 2nd Edn.* doi: 10.1094/9780890546710

Singh, K., Callahan, A. M., Smith, B. J., Malinowski, T., Scorza, R., Jarošová, J., et al. (2021). Long-term efficacy and safety of rnai-mediated virus resistance in 'honeysweet' plum. *Front. Plant Sci.* 12, 726881. doi: 10.3389/fpls.2021.726881

Tang, M., Bai, X., Wang, J., Chen, T., Meng, X., Deng, H., et al. (2022). Efficiency of graft-transmitted JcFT for floral induction in woody perennial species of the Jatropha genus depends on transport distance. *Tree Physiol.* 42, 189-201. doi: 10.1093/treephys/tpab116

Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P. H. J., Da Huang, H., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants.* 2, 16151. doi: 10.1038/nplants.2016.151

Willocquet, L., Savary, S., and Yuen, J. (2017). Multiscale phenotyping and decision strategies in breeding for resistance. *Trends Plant Sci.* 22, 420–432. doi: 10.1016/j.tplants.2017.01.009

Willow, J., and Veromann, E. (2021). Highly variable dietary rnai sensitivity among coleoptera. *Front. Plant Sci.* 12, 790816. doi: 10.3389/fpls.2021.790816

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ORIGINAL RESEARCH
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Double-Stranded RNA Targeting Dicer-Like Genes Compromises the Pathogenicity of *Plasmopara viticola* on Grapevine

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Downy mildew caused by *Plasmopara viticola* is one of the most devastating diseases of grapevine, attacking all green parts of the plant. The damage is severe when the infection at flowering stage is left uncontrolled. *P. viticola* management consumes a significant amount of classical pesticides applied in vineyards, requiring efficient and environmentally safe disease management options. Spray-induced gene silencing (SIGS), through the application of exogenous double-stranded RNA (dsRNA), has shown promising results for the management of diseases in crops. Here, we developed and tested the potential of dsRNA targeting *P. viticola Dicer-like* (*DCL*) genes for SIGS-based crop protection strategy. The exogenous application of *PvDCL1/2* dsRNA, a chimera of *PvDCL1* and *PvDCL2*, highly affected the virulence of *P. viticola*. The reduced expression level of *PvDCL1* and *PvDCL2* transcripts in infected leaves, treated with *PvDCL1/2* dsRNA, was an indication of an active RNA interference mechanism inside the pathogen to compromise its virulence. Besides the protective property, the *PvDCL1/2* dsRNA also exhibited a curative role by reducing the disease progress rate of already established infection. Our data provide a promising future for *PvDCL1/2* dsRNA as a new generation of RNA-based resistant plants or RNA-based agrochemical for the management of downy mildew disease in grapevine.

Keywords: *Dicer-like* genes, double-stranded RNA (dsRNA), *Plasmopara viticola*, spray-induced gene silencing, *Vitis vinifera*

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is an important fruit crop cultivated worldwide for fresh and dry fruit consumption and for wine production. Wine production trend is increasing yearly with the world wine trade worth getting about US $40 billion in the year 2018; Italy, France, and Spain being the largest wine-producing countries, contributing half of the world production.[1] Grapevine production is affected by several pre- and post-harvest pathogens that affect quality during production and processing. Some of the economically important diseases of the crop are

[1]http://www.oiv.int/

gray mold, powdery mildew, and downy mildew caused by *Botrytis cinerea*, *Erysiphe necator*, and *Plasmopara viticola*, respectively. The obligate biotrophic oomycete *P. viticola* attacks all green parts of grapevine, and the damage is severe if the infection occurring during flowering is not managed. Surprisingly, all cultivated European *V. vinifera* cultivars are susceptible to the pathogen (Armijo et al., 2016), which makes the management of downy mildews in vineyard and other crops rely on synthetic fungicides. As a result, its management, together with powdery mildew, consumes about two-thirds of all synthetic fungicides sprayed for disease management of crops in the European Union (Eurostat., 2007). With such heavy reliance on agrochemicals to control *P. viticola*, not only pathogen strains have developed resistance to several fungicides (Gisi and Sierotzki, 2008), but there also exist social concerns about environment and human health, which makes it urgent to find alternative control strategies.

The findings that exogenous small RNAs (sRNA) and double-stranded RNA (dsRNA) trigger posttranscriptional gene silencing (Fire et al., 1998; Hamilton and Baulcombe, 1999) have opened new avenues to exploit the gene silencing mechanism as a new class of regulatory molecules during plant–pathogen interaction. The gene silencing occurs *via* RNA interference (RNAi) machinery, a natural biological process conserved in most eukaryotes where sRNA molecules regulate gene expression by targeting specific endogenous messenger RNA molecules in a sequence-specific manner (Vaucheret and Fagard, 2001; Castel and Martienssen, 2013). The silencing signals of sRNA are bidirectional cross-kingdom, moving from the host to its interacting organism, and *vice versa* (Tomilov et al., 2008; Weiberg et al., 2015; Wang et al., 2016; Cai et al., 2018).

The involvement of sRNAs in the crosstalk between plant hosts and their fungal and oomycete pathogens has also been suggested (Weiberg et al., 2013; Brilli et al., 2018), implying that exploiting the RNAi mechanisms of both the hosts and the pathogens can represent a new strategy in fungal and oomycete disease management. Transgene-derived artificial sRNAs inducing gene silencing, called host-induced gene silencing (HIGS), have been observed providing resistance to plants against fungi (Nowara et al., 2010; Koch et al., 2013; Zhu et al., 2017) and oomycetes (Vega-Arreguin et al., 2014; Jahan et al., 2015). Interestingly, recent findings revealed that the external application of dsRNA also conferred host plant resistance to fungal pathogens by silencing targeted genes (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018; Nerva et al., 2020), an approach referred to as spray-induced gene silencing (SIGS).

The exogenous application of dsRNAs targeting *Dicer-like* (*DCL*), *lanosterol 14α-demethylase*, *chitin synthase*, and *elongation factor* genes of *B. cinerea* negatively affected its pathogenicity in multiple hosts (Wang et al., 2016; Nerva et al., 2020). Similarly, spraying of dsRNA targeting three *cytochrome P450* genes of *Fusarium graminearum* inhibited fungal growth at sprayed and distal parts of detached barley leaves (Koch et al., 2016). While these research findings provided proof that SIGS-based plant protection is effective against targeted pathogens, there is also indication that the effects of dsRNA can be reproduced on closely related pathogens based on sequence homology (McLoughlin et al., 2018). According to McLoughlin et al. (2018), dsRNA targeting *SS1G_05899* and *SS1G_02495* genes of *Scelerotinia sclerotiorum*, both involved in redox reaction, restricted the progress of the pathogen on a susceptible *Brassica napus* cultivar. Remarkably, the cultivar was also resistant to *B. cinerea* when treated with dsRNA targeting *BC1G_01592* and *BC1G_04955*, the *B. cinerea* homologs to *SS1G_05899* and *SS1G_02495*, respectively. Such results provide compelling evidence about the adaptability and flexibility of SIGS technology in crop disease management. In this study, we investigate the potential of dsRNA targeting *P. viticola DCL* genes for SIGS-based crop protection strategy. We show that the application of dsRNA targeting *PvDCL1/2* extremely reduces the pathogenicity of *P. viticola* and the expression level of the targeted genes, indicating that RNAi-based control strategy can indeed represent a promising alternative to hazardous agrochemical application to manage downy mildew disease of grapevine.

MATERIALS AND METHODS

Design and Production of dsRNA and Rate of Application

Plasmopara viticola genes encoding two Dicer-like proteins, as defined by the presence of a Dicer dimerization domain, corresponding to *PVITv1_T038441* and *PVITv1_T003331*, hereafter referred to as *PvDCL1* and *PvDCL2*, respectively (Brilli et al., 2018), were selected. For RNAi, 258- and 257-bp fragments of *PvDCL1* and *PvDCL2* sequences, respectively (**Supplementary Table 1**), were chosen as target, and the corresponding chimeric dsRNA molecule (*PvDCL1/2*, 515 bp) was chemically synthesized by AgroRNA (Genolution Inc., Seoul, Republic of Korea; **Supplementary Figure 1**). DsRNA targeting *B. cinerea DCL 1* and *2* genes, *BcDCL1/2* (490 bp; Wang et al., 2016), produced in the same way, was used as the negative control. After assaying different dsRNA concentrations, 75, 100, or 125 ng μl^{-1} concentrations of dsRNA were used for spot inoculation in a total volume of 50 μl.

Plant Material and *Plasmopara viticola* Inoculation

Seedlings of *V. vinifera* cv. Trebbiano were raised in growth chamber at 22°C ± 1°C and 12/12 h light cycle. *P. viticola* (strain 465, belonging to University of Bologna collection) was maintained on grapevine leaves at 22°C ± 1°C and 12/12 h of photoperiod. Sporangia were harvested in distilled water and filtered through cheesecloth. Sporangia concentration was determined using hemocytometer.

Fully expanded third and fourth leaves from 6–8-week-old grapevine seedlings were detached and immediately placed on wet absorbing paper in a plastic box. Detached leaves were surface sterilized for 1 min with 70% ethanol and then rinsed three times with sterile water. For assaying dsRNA as preventive treatment, the abaxial side of each leaf was treated with three droplets of 50 μl of either dsRNA or water. After 2 h, 7.5 μl

of a 1 × 10^5 ml^{-1} sporangia solution was placed on top of the droplets. Disease progress was evaluated until 14 days post inoculation (dpi) in five biological replicates. A single leaf was considered a biological replicate.

For assaying dsRNA as curative treatment, each leaf was first challenged by the pathogen by applying four droplets of 7.5 µl of a 1 × 10^5 ml^{-1} sporangia solution, and after 7 dpi, when a visible sign of *P. viticola* was observed, 50 µl of either dsRNA or water was placed on top of each spot of the progressing pathogen. Disease progress was evaluated until 14 dpi, i.e., 7 days post treatment (dpt) of either dsRNA or water, in three biological replicates.

To assess the progress of the pathogen, leaf area covered by *P. viticola* (in square millimeters) was measured from the digital images using the free software ImageJ program.[2] Leaf area covered by the pathogen, area under disease progress curve (AUDPC), and disease progress rate data were analyzed using analysis of variance. Means were separated by Tukey's honestly significant difference test.

[2]http://imagej.nih.gov/ij/

RNA Extraction and Quantitative PCR Analysis

Leaves that were treated in the preventive assay were collected at 7 dpi, in three replicates, immediately frozen in liquid nitrogen, and kept at −80°C until use. RNA was extracted using a rapid cetyltrimethylammonium bromide (CTAB) method (Gambino et al., 2008). First-strand cDNA was synthesized from 1 µg of total RNA, pretreated with TURBO DNA-free Kit™ (Invitrogen, CA, United States), using ImProm-II Reverse Transcriptase (Promega), following the manufacturer's guide. Quantitative PCR (qPCR) was performed in an MX3000 thermocycler (Stratagene,

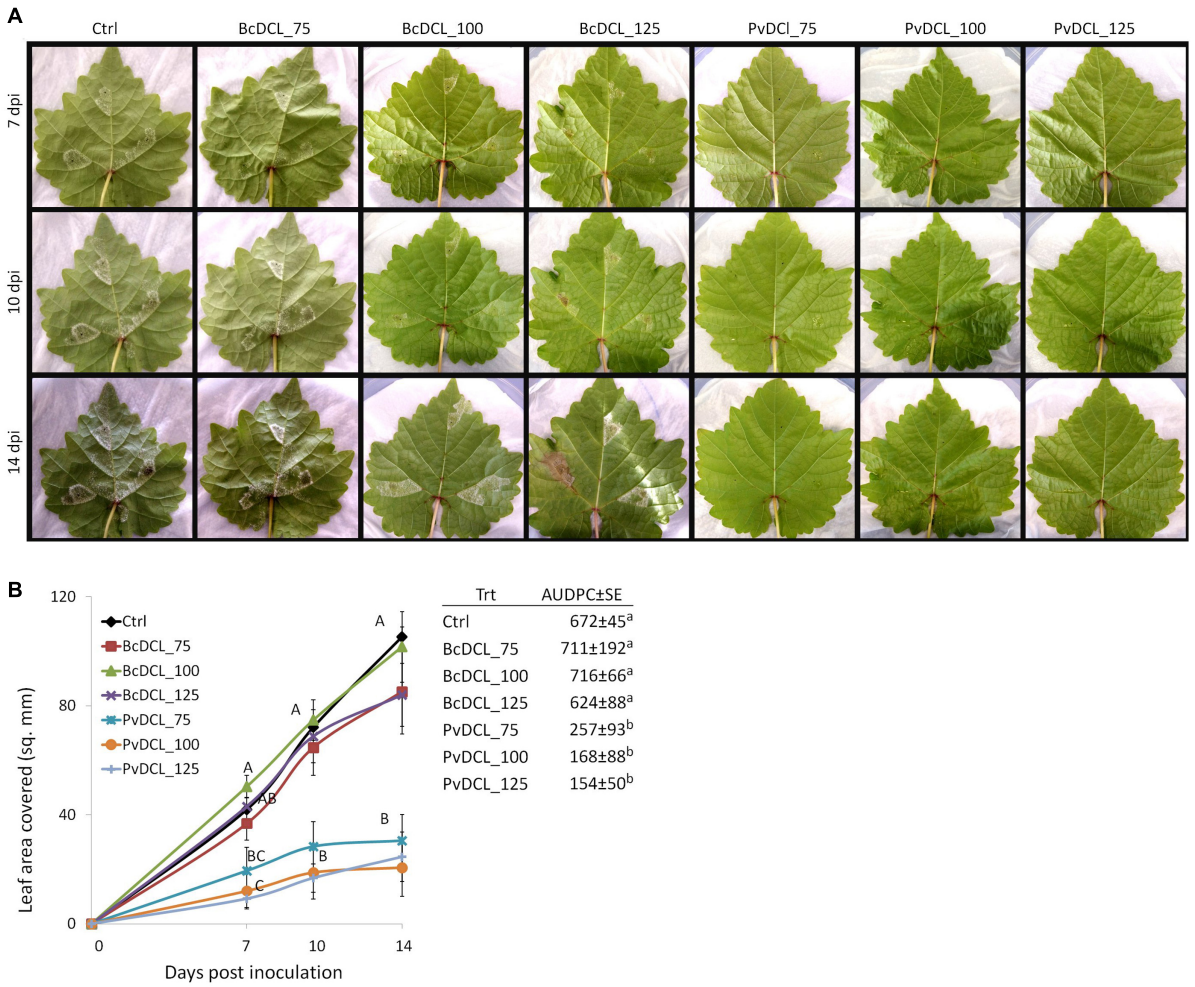

FIGURE 1 | Externally applied *PvDCL1/2* double-stranded RNA (dsRNA) on detached grapevine leaves and *Plasmopara viticola* infection. **(A)** Progress of *P. viticola* on grapevine leaves at 7, 10, and 14 days post inoculation (dpi). Leaves were treated with 50 µl of water (ctrl) or dsRNA [75, 100, or 125 ng μl^{-1} of dsRNA of *BcDCL1/2* (BcDCL_75/100/125) and *PvDCL1/2* (PvDCL_75/100/125)] before being inoculated with 7.5 µl of a 1 × 10^5 ml^{-1} sporangia. **(B)** Disease progression of *P. viticola* expressed as leaf area covered and as area under the disease progress curve (AUDPC ± SE, mm^2 × day) through 14 dpi. Error bars indicate standard error. Means at each dpi and AUDPC followed by a common letter are significantly not different according to Tukey's honestly significant difference test ($P \leq 0.05$).

CA, United States) using 0.25 μl of cDNA and 200 nM of specific forward and reverse primers (**Supplementary Table 2**) in a total volume of 12.5 μl using Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas). Each amplification reaction was run in duplicate. The cycling parameters were as follows: 5 min at 95°C, 40 cycles of 15 s at 95°C, 25 s at 61°C, and 30 s at 72°C. A melting curve was established from 55°C to 90°C by changing 0.5°C every 10 s. For normalization, *P. viticola* elongation factor eIF1b was used. Each primer pair's amplification efficiency was calculated using LinReg (Ruijter et al., 2009). The amplification efficiency value obtained was used to calculate the relative quantity (RQ) and normalized RQ (NRQ) according to Hellemans et al. (2007). Statistical analyses of the qPCR results were made after log2(NRQ) transformation (Rieu and Powers, 2009). Statistical significance was calculated by Tukey's honestly significant difference test.

RESULTS

Spray-Induced Gene Silencing of *Plasmopara viticola* DCL Genes Hampers Disease Development

Preliminary inoculation assay was conducted to determine a baseline concentration of *PvDCL1/2* dsRNA that could affect *P. viticola DCL1* and *DCL2* genes and consequently inhibit its germination and/or colonization of grapevine leaves. After the treatment with 10 and 50 ng μl^{-1} *PvDCL1/2* dsRNA and water, as control, detached grapevine leaves were challenged with *P. viticola* sporangia. Inoculated leaves were monitored for 2 weeks. Sign of *P. viticola* infection was conspicuous around the inoculation spot starting from the fifth dpi, mostly on control and on leaves treated with 10 ng μl^{-1} *PvDCL1/2* dsRNA, where white fluffy growth of sporangiophores and sporangia appeared. At 14 dpi, the rate of disease progress was relatively slower in leaves that received 50 ng μl^{-1} of *PvDCL1/2* dsRNA (**Supplementary Figure 2**), indicating that pathogen control efficiency can increase with higher concentrations.

Therefore, the ability of *PvDCL1/2* dsRNA to control *P. viticola* growth in preventive treatment was assessed using higher concentrations (i.e., 75, 100, and 125 ng μl^{-1}). Treatments with *BcDCL1/2* targeting *B. cinerea DCL1* and *DCL2* genes and water were used as controls. As shown in **Figure 1A**, the fluffy growth of sporangiophores was quite visible on control leaves treated with either water or *BcDCL1/2* dsRNA at the three different concentrations. On the contrary, the pathogen's progress was substantially low or null on leaves that received *PvDCL1/2* dsRNA. As a consequence, the area covered by *P. viticola* and the AUDPC values at 7, 10, and 14 dpi were significantly and consistently lower in leaves treated with *PvDCL1/2* dsRNA than in those treated with *BcDCL1/2* dsRNA or water (**Figure 1B**), confirming that *PvDCL1/2* dsRNA hampered *P. viticola* growth. To confirm that the inhibition of *P. viticola* growth by *PvDCL1/2* dsRNA was due to the downregulation of *PvDCL1* and *PvDCL2* genes, their expression, normalized to *P. viticola* elongation factor eIF1b, was quantified at 7 dpi using qPCR. We found that the relative expression of both *PvDCL1* and *PvDCL2* was reduced as compared to the controls (**Figure 2**). Compared with water and *BcDCL1/2*-treated leaves, the NRQs of *PvDCL1* and *PvDCL2* transcripts in leaves treated with 100 ng μl^{-1} concentration of *PvDCL1/2* dsRNA were reduced on average by 48 and 44%, respectively, which is in line with the concept of RNAi-based sequence-specific silencing *via* SIGS.

Spray-Induced Gene Silencing of PvDCLs Shows a Curative Effect Against *Plasmopara viticola*

The observed protective effect of *PvDCL1/2* dsRNA prompted us to check whether the dsRNA also has a curative effect against *P. viticola*. Detached leaves were initially inoculated with *P. viticola* sporangia, and then, once the infection has been established (i.e., 7 dpi), dsRNA was applied [i.e., the time of either dsRNA or water application is marked as 0 day post treatment (dpt)]. At each inoculation spot, 50 μl of dsRNA or water was added on top of the growing mycelia. At 4 dpt, the progress of the pathogen stagnated in most of the treatments, with more

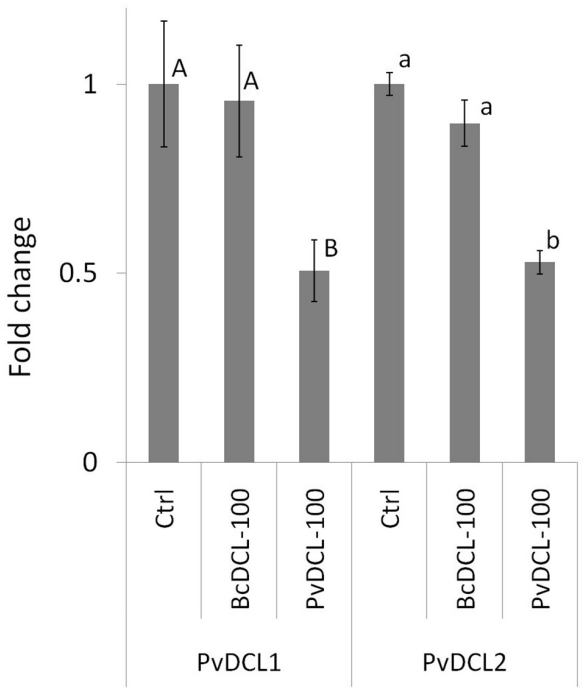

FIGURE 2 | Expression profiles of *PvDCL1* and *PvDCL2* following *Plasmopara viticola* inoculation on leaf samples treated with 50 μl of water (ctrl) or 100 ng μl^{-1} of double-stranded RNA (dsRNA) of either *BcDCL1/2* (BcDCL-100) or *PvDCL1/2* (PvDCL-100). Gene expression level was determined by quantitative PCR (qPCR). Bars represent fold change of dsRNA-treated sample relative to ctrl sample at 7 days post inoculation. Normalization based on the expression levels of elongation factor, *PvelF1b*, was carried out before calculating fold changes. Error bar represents standard error of the mean of three biological replicates. Expression values followed by a common letter are significantly not different among samples, according to Tukey's honestly significant difference test ($P \leq 0.05$), using one-way ANOVA of log2 [normalized relative quantity (NRQ)].

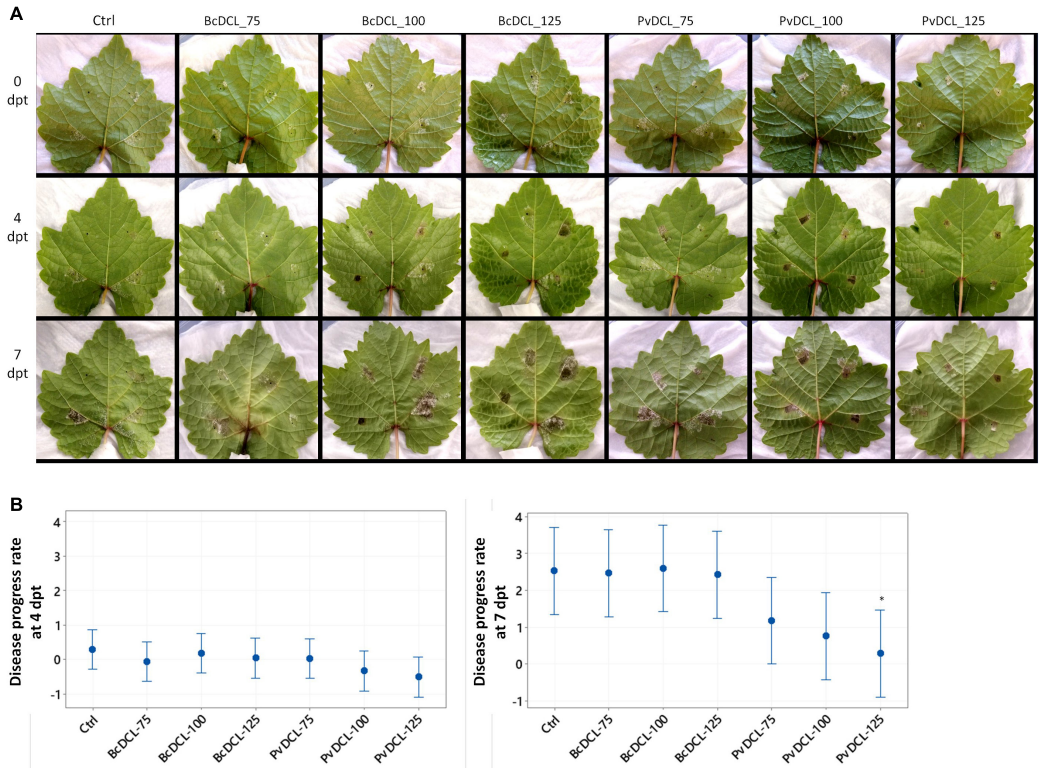

FIGURE 3 | Progress of *Plasmopara viticola on g*rapevine leaves after being treated with *PvDCL1/2* double-stranded RNA (dsRNA). **(A)** Progress of already established *P. viticola* infection after receiving dsRNA treatments. Leaves were treated with 50 μl of water (ctrl) or dsRNA [75, 100, or 125 ng μl^{-1} of dsRNA of *BcDCL1/2* (BcDCL_75/100/125) or *PvDCL1/2* (PvDCL_75/100/125)] 7 days after being inoculated with 7.5 μl of a 1 × 10^5 ml^{-1} sporangia [i.e., 0 days post treatment (dpt) of dsRNA]. **(B)** Disease progress rate at 4 and 7 dpt, computed by taking leaf area covered by *P. viticola* at 7 dpi (0 dpt) as a reference. Bars are 95% confidence interval, and asterisks (*) indicate statistically significant differences according to Tukey's honestly significant difference test ($P \leq 0.05$).

pronounced effect on leaves that received 100 and 125 ng μl^{-1} of *PvDCL1/2* dsRNA (**Figure 3A**). After 4 dpt, recovering of pathogen growth was more apparent on all leaves. At 7 dpt, the disease advanced more on leaves treated with *BcDCL1/2* and water than on those treated with *PvDCL1/2*, especially at the highest concentration (**Figure 3A**). Computing the rate of disease progress, taking diseased area at 7 dpi (0 dpt) as a reference, the disease progress rate was relatively slower on leaves treated with *PvDCL1/2*, with more pronounced effect at 7 dpt (**Figure 3B**). The result shows that the *PvDCL1/2* dsRNA can also hamper the expansion of already established downy mildew disease.

Compared to the preventive application, where all the three concentrations of *PvDCL1*/2 inhibited the growth of the pathogen significantly, when used as curative treatment, the rate of the pathogen growth was reduced significantly only at the highest concentration of *PvDCL1/2* dsRNA. These data show that the exogenously applied dsRNA targeting *PvDCL1*/2 has both promising protective and curative effects.

DISCUSSION

In grapevine cultivation, downy mildew, caused by *P. viticola*, is among the major diseases requiring repeated applications of pesticides within a growing season. In this study, we show that external application of long non-coding dsRNA, 515 bp long, targeting the two *DCL* genes of *P. viticola* reduced the progress of the pathogen on grapevine leaves. Transcript level reduction of the target genes, *PvDCL1* and *PvDCL2*, suggests specific RNA silencing effect triggered by *PvDCL1/2* dsRNA. To our knowledge, this is the first report showing the potential of exogenously applied RNAi molecules as an effective strategy for oomycete management in crops. The results presented further support the use of SIGS-based strategy for fungal pathogen management (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018; Nerva et al., 2020).

Non-coding sRNA molecules derived from plant pathogens could play a role in suppressing host immunity (Weiberg et al., 2013; Brilli et al., 2018) and hence could be regarded as additional classes of effectors, besides protein coding effector genes studied so far. It has been demonstrated that *B. cinerea* sRNAs (Bc-sRNAs) triggered the silencing of *Arabidopsis* and tomato targets involved in host immunity, such as *mitogen-activated protein kinase 1* (*MPK1*), *MPK2*, *peroxiredoxin*, and *cell wall-associated kinase* genes. Once they have entered the plant cell, Bc-sRNAs hijack the host's RNAi machinery, binding to Argonaute 1 (AGO1) protein and directing the silencing of host immunity genes (Weiberg et al., 2013). Accordingly, the *ago1* mutant

Arabidopsis exhibited reduced susceptibility to *B. cinerea*, and the expression of sRNAs that target *B. cinerea DCL1* and *DCL2* in *Arabidopsis* and tomato led to the silencing of the *BcDCL* genes and affected the fungal pathogenicity and growth, also when exogenously applied on different organs and tissues (Weiberg et al., 2013; Wang et al., 2016). In addition, *dcl1 dcl2 B. cinerea* double mutant, which is unable to produce sRNAs, displayed a stunted pathogenicity on several hosts (Weiberg et al., 2013; Wang et al., 2016). In a recent study, it was observed that during *V. vinifera–P. viticola* interaction, the sRNA profile of *P. viticola* showed enrichment in 21- and 25-nt sRNAs, which were also abundantly expressed in sporangia (Brilli et al., 2018). According to the study, the presence of DCLs, AGOs, and RNA-dependent RNA polymerase confirms the existence of RNA silencing machinery in *P. viticola*, which is active during its interaction with grapevine (Brilli et al., 2018).

The fact that the external application of *PvDCL1/2* dsRNA extremely reduced the pathogenicity of *P. viticola*, coupled with the observed reduction in *PvDCL1* and *PvDCL2* transcript levels, might suggest that the pathogen can take up external dsRNA and that the RNAi machinery is active during the infection process. Similarly, reduced disease symptoms and sequence-specific silencing of target genes were also observed in *B. cinerea*, *F. graminearum*, and *S. sclerotiorum* (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018), following the external application of dsRNA.

Reduced pathogenicity of plant pathogens due to sRNA and dsRNA has put forward the considerations of RNAi-based technology as a new plant protection method, at least for those pathogens having *bona fide* RNA silencing machinery. *In planta* gene silencing of pathogen target genes, a mechanism known as HIGS, has also been reported (Nowara et al., 2010; Koch et al., 2013; Vega-Arreguin et al., 2014; Jahan et al., 2015; Zhu et al., 2017). Furthermore, for vegetatively propagated crops like grapevine, HIGS can be exploited to obtain RNAi-based rootstocks, which can produce sRNA able to move to a grafted untransformed scion and protect it from pathogen infection, as sRNAs have high mobility between shoot and root (Gouil and Lewsey, 2021; Li et al., 2021). In addition, *in planta* expressed RNAi sequences do not encode for protein products and are designed against specific genes of target pathogens or susceptibility factor without affecting other non-target organisms. All these features together could reduce data requirements for risk assessment of such RNAi-based plants (Limera et al., 2017; Arpaia et al., 2020).

In addition to the HIGS potential application, the results of this research confirm the potential of the gene silencing technology also to develop new RNAi-based fungicides, known as SIGS. To ensure sustainable food production, European Union and global sustainability policies emphasize the need to replace contentious pesticides with safe, efficient, and cost-effective alternatives (Taning et al., 2020). The high selectivity of RNAi-based products, due to sequence-specific modes of action, compared with other conventional pesticides, makes them a promising solution to substitute or reduce reliance on contentious pesticides. Yet there are still relevant aspects to be clarified, such as local and remote translocation and environmental stability of applied sRNAs, before pushing forward SIGS as an alternative solution to toxic pesticides. Despite many solutions reported to stabilize the RNA molecules and make their administration in the field easy and effective, more effort should be taken on the risk assessment studies in order to clarify the risks associated with the use of these molecule for the farmers, consumers, and environment and proceed with the necessary regulatory protocols in order for them to reach the market.

In this study, we demonstrated that dsRNA specifically designed to silence *PvDCL1* and *PvDCL2* genes efficiently controls downy mildew disease caused by *P. viticola* on grapevine, a disease that forces to consume significant amounts of pesticides that are applied every year on vineyards. Although the mechanism behind the uptake and transport of the externally applied dsRNA needs further studies, the presented data give important scientific information on such new-generation RNA-based fungicides, which are environmentally safe and sustainable. So far, externally applied RNAi-based disease suppression data are limited on plant pathogens from Ascomycetes, but with our findings, we extended the possibility of using externally applied dsRNA for managing devastating plant pathogen oomycetes like *Phytophthora* and *Pythium* species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

ZH made the experiments and wrote the manuscript. DG made the cloning and plasmid constructs together with LC. BMo and FN contributed to the experiment to confirm gene silencing. MC helped with infections. SS and BMe provided support with the RNAi experiment and participated actively in manuscript writing. EB provided funding and general supervision to the experiments and writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.667539/full#supplementary-material

REFERENCES

Armijo, G., Schlechter, R., Agurto, M., Muñoz, D., Nuñez, C., and Arce-Johnson, P. (2016). Grapevine pathogenic microorganisms: understanding infection strategies and host response scenarios. *Front. Plant Sci.* 7:382. doi: 10.3389/fpls.2016.00382

Arpaia, S., Christiaens, O., Giddings, K., Jones, H., Mezzetti, B., Moronta-Barrios, F., et al. (2020). Biosafety of GM crop plants expressing dsRNA: data requirements and EU regulatory considerations. *Front. Plant Sci.* 11:940. doi: 10.3389/fpls.2020.00940

Brilli, M., Asquini, E., Moser, M., Bianchedi, P. L., Perazzolli, M., and Si-Ammour, A. (2018). A multi-omics study of the grapevine-downy mildew (*Plasmoparaviticola*) pathosystem unveils a complex protein coding- and noncoding-based arms race during infection. *Sci. Rep.* 8:757. doi: 10.1038/s41598-018-19158-8

Cai, Q., Qiao, L., Wang, M., He, B., Lin, F. M., Palmquist, J., et al. (2018). Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. *Science* 360, 1126–1129. doi: 10.1126/science.aar4142

Castel, S. E., and Martienssen, R. A. (2013). RNA interference in the nucleus: roles for small RNAs in transcription, epi- genetics and beyond. *Nat. Rev. Genet.* 14, 100–112. doi: 10.1038/nrg3355

Eurostat. (2007). *The Use of Plant Protection Products in the European Union, Data 1992–2003*. Luxembourg: Office for Official Publications of the European Communities.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811.

Gambino, G., Perrone, I., and Gribaudo, I. (2008). A rapid and effective method for RNA extraction from different tissues of grapevine and other woody plants. *Phytochem. Anal.* 525, 520–525. doi: 10.1002/pca.1078

Gisi, U., and Sierotzki, H. (2008). Fungicide modes of action and resistance in downy mildews. *Eur. J. Plant Pathol.* 122, 157–167. doi: 10.1007/s10658-008-9290-5

Gouil, Q., and Lewsey, M. G. (2021). Small RNAs shoot for the root. *Nat. Plants* 7, 2–3. doi: 10.1038/s41477-020-00836-3

Hamilton, A. J., and Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950–952. doi: 10.1126/science.286.5441.950

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19. doi: 10.1186/gb-2007-8-2-r19

Jahan, S. N., Åsman, A. K. M., Corcoran, P., Fogelqvist, J., Vetukuri, R. R., and Dixelius, C. (2015). Plant-mediated gene silencing restricts growth of the potato late blight pathogen Phytophthora infestans. *J. Exp. Bot.* 66, 2785–2794. doi: 10.1093/jxb/erv094

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., et al. (2016). An RNAi-Based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathog.* 12:e1005901. doi: 10.1371/journal.ppat.1005901

Koch, A., Kumar, N., Weber, L., Keller, H., Imani, J., and Kogel, K. H. (2013). Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase-encoding genes confers strong resistance to Fusarium species. *Proc. Natl. Acad. Sci. U. S. A.* 110, 19324–19329. doi: 10.1073/pnas.1306373110

Li, S., Wang, X., Xu, W., Liu, T., Cai, Ch, Chen, L., et al. (2021). Unidirectional movement of small RNAs from shoots to roots in interspecific heterografts. *Nat. Plants* 7, 50–59. doi: 10.1038/s41477-020-00829-2

Limera, C., Sabbadini, S., Sweet, J. B., and Mezzetti, B. (2017). New biotechnological tools for the genetic improvement of major moody fruit species. *Front. Plant Sci.* 8:1418. doi: 10.3389/fpls.2017.01418

McLoughlin, A. G., Wytinck, N., Walker, P. L., Girard, I. J., Rashid, K. Y., de Kievit, T., et al. (2018). Identification and ap- plication of exogenous dsRNA confers plant protection against *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *Sci. Rep.* 8:7320. doi: 10.1038/s41598-018-25434-4

Nerva, L., Sandrini, M., Gambino, G., and Chittara, W. (2020). Double Stranded RNAs (dsRNAs) as a Sustainable Tool against Gray Mold (*Botrytis cinerea*) in Grapevine: effectiveness of different application methods in an open-air environment. *Biomolecules* 10:200. doi: 10.3390/biom10020200

Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., et al. (2010). HIGS: host-Induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell* 22, 3130–3141. doi: 10.1105/tpc.110.077040

Rieu, I., and Powers, S. J. (2009). Real-time quantitative RT-PCR: design, calculations, and statistics. *Plant Cell* 2, 1031–1033. doi: 10.1105/tpc.109.066001

Ruijter, J. M., Ramakers, C., Hoogaars, W. M., Karlen, Y., Bakker, O., van den Hoff, M. J., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37:e45. doi: 10.1093/nar/gkp045

Taning, C. N. T., Mezzetti, B., Kleter, G., Smagghe, G., and Baraldi, E. (2020). Does RNAi-Based Technology Fit within EU Sustainability Goals? *Trends Biotechnol.* doi: 10.1016/j.tibtech.2020.11.008 [Epub Online ahead of print].

Tomilov, A. A., Tomilova, N. B., Wroblewski, T., Michelmore, R., and Yoder, J. I. (2008). Trans-specific gene silencing between host and parasitic plants. *Plant J.* 56, 389–397. doi: 10.1111/j.1365-313X.2008.03613.x

Vaucheret, H., and Fagard, M. (2001). Transcriptional gene silencing in plants: targets, inducers and regulators. *Trends Genet.* 17, 29–35. doi: 10.1016/S0168-9525(00)02166-1

Vega-Arreguin, J. C., Jalloh, A., Bos, J. I., and Moffett, P. (2014). Recognition of an Avr3a homologue plays a major role in mediating nonhost resistance to *Phytophthora capsici* in *Nicotiana* species. *Mol. Plant Microbe Interact.* 27, 770–780. doi: 10.1094/MPMI-01-14-0014-R

Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P., Huang, H. D., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* 2:16151. doi: 10.1038/NPLANTS.2016.151

Weiberg, A., Bellinger, M., and Jin, H. (2015). Conversations between kingdoms: small RNAs. *Curr.Opin. Biotechnol.* 32, 207–215. doi: 10.1016/j.copbio.2014.12.025

Weiberg, A., Wang, M., Lin, F. M., Zhao, H., Zhang, Z., Kaloshian, I., et al. (2013). Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. *Science* 342, 118–123. doi: 10.1126/science.1239705

Zhu, X., Qi, T., Yang, Q., He, F., Tan, C., Ma, W., et al. (2017). Host-induced gene silencing of the *MAPKK* gene *PsFUZ7* confers stable resistance to wheat stripe rust. *Plant Physiol.* 175, 1853–1863. doi: 10.1104/pp.17.01223

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ORIGINAL RESEARCH
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RNAi of a Putative Grapevine Susceptibility Gene as a Possible Downy Mildew Control Strategy

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Downy mildew, caused by the oomycete *Plasmopara viticola*, is one of the diseases causing the most severe economic losses to grapevine (*Vitis vinifera*) production. To date, the application of fungicides is the most efficient method to control the pathogen and the implementation of novel and sustainable disease control methods is a major challenge. RNA interference (RNAi) represents a novel biotechnological tool with a great potential for controlling fungal pathogens. Recently, a candidate susceptibility gene (*VviLBDlf7*) to downy mildew has been identified in *V. vinifera*. In this work, the efficacy of RNAi triggered by exogenous double-stranded RNA (dsRNA) in controlling *P. viticola* infections has been assessed in a highly susceptible grapevine cultivar (Pinot noir) by knocking down *VviLBDlf7* gene. The effects of dsRNA treatment on this target gene were assessed by evaluating gene expression, disease severity, and development of vegetative and reproductive structures of *P. viticola* in the leaf tissues. Furthermore, the effects of dsRNA treatment on off-target (*EF1*α, *GAPDH*, *PEPC*, and *PEPCK*) and jasmonic acid metabolism (*COI1*) genes have been evaluated. Exogenous application of dsRNA led to significant reductions both in *VviLBDlf7* gene expression, 5 days after the treatment, and in the disease severity when artificial inoculation was carried out 7 days after dsRNA treatments. The pathogen showed clear alterations to both vegetative (hyphae and haustoria) and reproductive structures (sporangiophores) that resulted in stunted growth and reduced sporulation. Treatment with dsRNA showed signatures of systemic activity and no deleterious off-target effects. These results demonstrated the potential of RNAi for silencing susceptibility factors in grapevine as a sustainable strategy for pathogen control, underlying the possibility to adopt this promising biotechnological tool in disease management strategies.

Keywords: susceptibility gene, gene silencing, dsRNA, obligate parasite, disease resistance, *Vitis vinifera*

INTRODUCTION

Since the late 1800s, *Vitis vinifera* has suffered damage from downy mildew, a disease caused by the oomycete *Plasmopara viticola*, originating from Northern America. This biotrophic, obligate parasite can infect all the green parts of grapevine plants, causing quantitative and qualitative damage and leading to extensive yield losses (Yu et al., 2012). Non-*vinifera* species, such as those

with center of origin in North America and Asia, are resistant to *P. viticola* due to coevolution with the pathogen. This resistance is mediated by different mechanisms that at first detect the pathogen and then initiate a proper defense response (Gessler et al., 2011). The first level of defense is called Pathogen-Associated Molecular Pattern (PAMP)-Triggered Immunity (PTI), a plant basal immune response activated by the recognition of conserved molecules of the pathogen (Newman et al., 2013). A second, more selective, plant defense mechanism is called Effector-Triggered Immunity (ETI). ETI relies on a class of highly specific receptors, the resistance proteins (R-proteins), that recognize pathogen effectors. Activation of ETI leads to disease resistance and is often associated with localized apoptosis at the infection site (hypersensitive response) (Jones and Dangl, 2006).

Since effectors and receptors are codified by non-essential genes, resistance genes (R-genes) undergo rapid evolution, due to the strong selective pressure on both pathogen and plant (Buonassisi et al., 2017). This phenomenon implies a short duration of the resistance triggered by R-genes. For a durable resistance, targeting susceptibility genes (S-genes) can be a winning strategy in breeding (Zaidi et al., 2018). S-genes facilitate the compatibility between plant and pathogen and are essential for their interaction, especially for biotrophic pathogens. Therefore, mutation or loss of S-genes can limit pathogenicity toward the plant (Van Schie and Takken, 2014). *MLO* (Mildew Locus O) genes are a striking example of S-gene usefulness in breeding programs: their knockdown confers resistance to powdery mildew in grapevine, reducing the disease severity by up to 77% (Pessina et al., 2016). *MLO*-based resistance to powdery mildew is, indeed, widely employed in barley breeding since few decades even if the function encoded protein is not yet completely established (Kusch and Panstruga, 2017).

To date, downy mildew-resistant grapevine varieties are obtained by crossing *V. vinifera* cultivars with non-*vinifera* species or hybrids. Nevertheless, to reduce the background of non-*vinifera* species, several cycles of backcrossing with susceptible cultivars are needed, which makes the breeding process very lengthy. The identification of grapevine S-genes against *P. viticola* opens new possibilities to breed for downy mildew resistance because usually S-gene-mediated resistance is durable and broad spectrum. The research of S-genes in grapevine is still pioneering. Pessina et al. (2016) identified two *MLO* genes responsible for *V. vinifera* susceptibility to powdery mildew and some others were proposed for downy mildew (Toffolatti et al., 2020; Pirrello et al., 2021). Recently, resistance to *P. viticola* has been identified in Mgaloblishvili (Toffolatti et al., 2016), a *V. vinifera* cultivar from Georgia (Southern Caucasus). Studying its unique resistance mechanism, different R-genes and an interesting candidate S-gene have been identified (Toffolatti et al., 2018, 2020). The S-gene *VvLBDp1* [from here on called *VviLBDIf7* based on Grimplet et al. (2017) nomenclature], encoding for an LOB (LATERAL ORGAN BOUNDARIES) domain-containing (LBD) protein, belongs to plant LOB family of transcription factors. This family has been comprehensively analyzed in many species, such as *Arabidopsis thaliana* (Liu et al., 2005), *Malus domestica* (Wang et al., 2013), *Glycine max* (Yang et al., 2017), *Eucalyptus grandis* (Lu et al., 2018), *Brassica rapa* (Huang et al., 2018), *Camellia sinensis* (Teng et al., 2018), *Gossypium* spp. (Yu et al., 2020), and *Pyrus bretschneideri* (Song et al., 2020). LBD genes show a key role in the regulation of plant organ development and in the response to abiotic and biotic stresses (Xu et al., 2016). They are involved in the establishment of organ boundaries (Rast and Simon, 2012), leaf formation (Semiarti et al., 2001), pulvinus differentiation and petiole development (Chen et al., 2012), regulation of lateral root organogenesis (Okushima et al., 2007), root and stem development (Yu et al., 2020), development of sepal and petal primordia of flowers (Xu et al., 2008), pollen development (Kim et al., 2015), regulation of light/dark-dependent hypocotyl elongation (Mangeon et al., 2011), and secondary phloem growth (Yordanov et al., 2010). Moreover, LOB genes are also involved in pathogen and abiotic response (Thatcher et al., 2012; Grimplet et al., 2017; Yu et al., 2020), and they can act as repressor of anthocyanin biosynthesis and affect nitrogen response (Rubin et al., 2009). Biological processes where LBD genes are involved have been extensively reviewed by Zhang et al. (2020).

A genome-wide characterization has been performed in grapevine as well, and up to 50 LBD genes have been identified (Grimplet et al., 2017). Expression patterns across different tissues, including both mature/woody and vegetative/green tissues, indicate roles of LBD genes in organ differentiation, in berry development and ripening (Fasoli et al., 2012), and in response to abiotic (such as salt, cold and drought) and biotic stresses (such as *Botrytis cinerea* attack and Bois noir disease) (Albertazzi et al., 2009; Agudelo-Romero et al., 2015). The S-gene *VviLBDIf7* is the putative ortholog of an LBD transcriptional factor acting as repressor of jasmonate-mediated defense mechanisms during infection of *A. thaliana* roots with *Fusarium oxysporum*. In this system, LBD disruption resulted in an increased resistance to the pathogen (Thatcher et al., 2012).

Silencing plant S-genes represents a promising way to achieve disease resistance as an alternative or in addition to breeding for R-genes. However, gene silencing is usually obtained *via* stable plant transformation, and the use of genetically modified plants in Europe is strictly regulated, and in several countries, they are not authorized for cultivation. A novel emerging approach, which allows to overcome procedures for a stable genome modification, is represented by RNA interference (RNAi) triggered by the application of exogenous double-stranded RNA (dsRNA) molecules (Dubrovina et al., 2019). The effect of dsRNA treatment has been recently studied and proposed as a new environmentally-friendly crop protection tool from viruses, fungi, and insects (Konakalla et al., 2016; Luo et al., 2017; Wang et al., 2017; Cagliari et al., 2019; Dubrovina and Kiselev, 2019; Morozov et al., 2019; Vadlamudi et al., 2020). RNAi has emerged as a technique with the ability to selectively knock down target genes (Kerschen et al., 2004). RNAi is a natural mechanism used by various organisms, including plants, to regulate specific gene activities or to defend their genome from invasions of exogenous nucleic acids. In the first case, the plant specifically produces molecules called microRNAs (miRNAs) serving as guides to selectively degrade the mRNA of target genes. In the second case, the plant recognizes dsRNAs introduced into the cytoplasm and produces short interfering

RNA (siRNA) molecules that defend the plant from exogenous nucleic acids (Meister et al., 2004). As a consequence, RNAi mediated by post-transcriptional gene silencing can be stimulated also by the addition of *ad hoc* designed dsRNA molecules. This technique, although not yet regulated at European level, will likely fall outside the strict GMO regulation, being transgene-free. The exogenous application of polynucleotides that can affect mRNA levels of important virulence-related plants genes without modifying the host genome opens new opportunities for the development of new scientific techniques and crop improvement strategies (Dubrovina et al., 2019). This study evaluated the efficacy of a dsRNA treatment in silencing, through RNAi, *VviLBDIf7*, the candidate S-gene responsible for susceptibility toward *P. viticola* in *V. vinifera*. For this purpose, Pinot noir leaves were treated with synthetized dsRNA targeting *VviLBDIf7* gene and then inoculated with the pathogen, to evaluate (i) *VviLBDIf7* gene expression in the plant tissues after dsRNA treatment, (ii) disease severity and sporangia production on dsRNA-treated leaves inoculated with *P. viticola*, and (iii) the morphology of both vegetative and reproductive *P. viticola* structures in the dsRNA-treated leaf tissues. Finally, a preliminary investigation of the systemic effect of dsRNA and the evaluation of dsRNA treatment on off-target and jasmonic acid metabolism genes have been performed.

MATERIALS AND METHODS

Basal Expression of *VviLBDIf7*

The basal expression level of *VviLBDIf7* (LOC100246173), candidate S-gene, was evaluated on leaves collected from three 6-year-old *V. vinifera* L. cv. Pinot noir plants, grown in a greenhouse, as reported in Toffolatti et al. (2018), *via* RT-qPCR. The first two well-developed leaves were collected in June 2020 twice at 1-day intervals. Leaves were ground with liquid nitrogen into a fine powder using mortar and pestle. RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Germany) and then digested with Amplification Grade DNase I (Sigma-Aldrich), according to the manufacturer's instructions. Quantity and quality of RNA were checked by NanoDrop 1000 Spectrophotometer (Thermo Scientific, United Kingdom) and 1% agarose gel electrophoresis stained with Midori Green Advance® (Nippon Genetics, Japan). *VviLBDIf7* real-time PCR primers (VvLBD_F and VvLBD_R; **Table 1**) were designed on the available sequence [VIT_13s0019g03750 according to Canaguier et al. (2017)] using the Primer3 Plus software (Untergasser et al., 2007) and NCBI Primer-BLAST (Ye et al., 2012). To avoid amplification of eventual genomic DNA carried over, one of the primers was designed to span across the exon–exon junction of the gene. Ubiquitin (Fujita et al., 2007) and actin (Reid et al., 2006) genes were used as references for data normalization. Total RNA (500 ng) was reverse transcribed with SuperScript®IV Reverse Transcriptase (Invitrogen, United Kingdom), using a 1:1 mix of random primers and oligo(dT), following manufacturer's instructions. Real-time PCR reaction was carried out using 4 μl of cDNA diluted 1:10, 10 μl of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, United Kingdom), 500 nM of primer forward and reverse and water up to 20 μl. Each reaction was performed in triplicate on QuantStudio 3 Real-Time PCR Systems (Thermo Fisher, United Kingdom) using the following cycling conditions: 50°C for 2 min, 95°C for 10 min, 50 cycles at 95°C for 20 s, 60°C for 45 s, and 72°C for 30 s. Each thermal cycle was followed by a melting curve stage, with temperatures ranging from 60°C to 95°C. The *VviLBDIf7* gene expression at the two time points was calculated by comparing $2^{-\Delta\Delta Ct}$ (Ct = cycle threshold) values (Livak and Schmittgen, 2001). Geometric average of ubiquitin and actin was used to normalize the Ct values.

VviLBDIf7 dsRNA Design and Synthesis

In order to synthesize the dsRNA molecules, *VviLBDIf7* was partially amplified. To allow the synthesis of dsRNA template of proper dimension (400–500 bp) through PCR, new primers of *VviLBDIf7* (VvLBD_RNAi_F and VvLBD_RNAi_R; **Table 1**) were designed on the available sequence (LOC100246173) using the Primer3 Plus software and NCBI Primer-BLAST. In order to reduce the probability of off-target amplicons, the partial *VviLBDIf7* sequence was amplified starting from cDNA samples obtained in the *Basal expression of VviLBDIf7* subsection. PCR reaction was carried out using 4 μl of cDNA diluted 1:10, 5 μl of 5X Colorless GoTaq Reaction Buffer (Promega, Wisconsin, United States), PCR Nucleotide Mix 0.2 mM each dNTP (Promega), 0.6 μM of primer forward and reverse, 0.125 μl of GoTaq G2 (5 U/μl) (Promega), and water up to 25 μl. To ensure the production of enough DNA template for the next steps, the reaction was performed in five replicates using the following thermal cycling conditions: 95°C for 2 min, 35 cycles at 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a last step at 72°C for 5 min. PCR fragments were visualized on 1% agarose gel electrophoresis stained with Midori Green Advance. The amplification products were used as a template in another PCR reaction using RNAi primers 5′ attached with T7 RNA polymerase promoter sequences (VvLBD_RNAiT7_F and VvLBD_RNAiT7_R; **Table 1**), as requested by MEGAscript RNAi Kit protocol (Thermo Fisher Scientific, United Kingdom). PCR reaction was carried out using 1 μl of amplification product, 5 μl of 5X Colorless GoTaq Reaction Buffer, PCR Nucleotide Mix 0.2 mM each dNTP, 0.6 μM of primer forward and reverse, 0.125 μl of GoTaq G2 (5 U/μl), and water up to 25 μl. To guarantee a sufficient amount of dsRNA DNA template, the reaction was performed in 50 replicates using the following thermal cycling conditions: 94°C for 5 min, 5 cycles at 94°C for 45 s, 62°C for 1 min, 72°C for 1 min, 30 cycles at 94°C for 45 s, 65°C for 1 min, 72°C for 1 min, and a last step at 72°C for 5 min. PCR products were solved on 1% agarose gel electrophoresis stained with Midori Green Advance. Replicates of PCR product were pooled and purified through Wizard SV Gel and PCR Clean-Up System (Promega). Purified samples were quantified using Qubit™ 3.0 fluorometer (Thermo Fisher Scientific), using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific), and Sanger sequenced

TABLE 1 | List of primers used in this work to synthetize the dsRNA.

Name	Sequence (5′-3′)	Tm (°C)	GC %	Primer position (bp)
VvLBD_F	GCCTGCAAAATCCTTCGTCG	60.18	55.0	196–215
VvLBD_R	GACTCGGGAAGTTCCTGCAA	59.97	55.0	322–341
VvLBD_RNAi_F	TATGGTTGTGCTGGTGCAAT	60.00	45.0	406–425
VvLBD_RNAi_R	CACACGGCTCTCCTTTTTCT	59.50	50.0	798–817
VvLBD_RNAiT7_F	TAATACGACTCACTATAGGGAG ATATGGTTGTGCTGGTGCAAT	71.30	41.9	406–425
VvLBD_RNAiT7_R	TAATACGACTCACTATAGGGAG ACACACGGCTCTCCTTTTTCT	72.30	44.2	798–817
VvLBD_2_F	CTTGCAGGAACTTCCCGAGTC	60.94	57.0	321–341
VvLBD_2_R	GCCAAGAAGGCTCCAAGACGG	63.56	62.0	566–586

VvLBD_F and VvLBD_R primers were used to detect the basal gene expression of VvLBD in leaves of Pinot noir. VvLBD_RNAi_F and VvLBD_RNAi_R primers were used to amplify partially the target gene to synthetize the dsRNA. VvLBD_RNAiT7_F and VvLBD_RNAiT7_R were used to perform MEGAscript RNAi Kit protocol. VvLBD_2_F and VvLBD_2_R primers were used to evaluate the expression of VvLBD gene on dsRNA- and water-treated leaves. The primer position refers to the start of the sequence (LOC100246173) annotated in NCBI (https://www.ncbi.nlm.nih.gov/).

by Macrogen Europe B.V. in two replicates, using RNAiT7 primers. Sequences were aligned to reference sequence using ClustalW (Thompson et al., 1994). The obtained template was used in the dsRNA synthesis reaction using MEGAscript RNAi Kit, according to the manufacturer's instructions. Synthetized dsRNA was solved on 1% agarose gel electrophoresis stained with Midori Green Advance and quantified by NanoDrop 1000 Spectrophotometer.

VviLBDIf7 dsRNA Treatment

Treatments with *VviLBDIf7* dsRNA were carried out on 6-year-old Pinot noir plants grafted onto SO4, maintained in a glasshouse in 5-L pots filled with sand-peat mixture (7:3v/v), regularly watered *via* a drip system and fertilized twice a year with Osmocote Topdress fertilizer (ICL Specialty Fertilizers, Italy), as reported in Toffolatti et al. (2018). The plants, grown in a greenhouse, never came in contact with *P. viticola* structures and were regularly inspected for disease symptoms to be sure to work with healthy tissues. Three plants were treated with 100 μg/plant of dsRNA dissolved in 1 ml of sterilized water, and three were treated with only sterilized water (1 ml per plant), as suggested by Nerva et al. (2020). Treatments were performed with an airbrush on both sides of the first five fully developed leaves of a single vine shoot (from the second to the sixth leaf from the apex of the shoot) per plant (**Supplementary Figure 1**). One sprayed leaf per plant was randomly collected at 3, 5, 7, and 15 days after treatment (dat) (**Figure 1A**). To validate the dsRNA treatment data, the experiment was repeated, as previously described, on self-rooted cuttings of Pinot noir grown in the same conditions, to remove any possible effect of rootstock on scion behavior. In this second experiment, leaves were collected at 5 and 7 dat (**Figure 1B**), corresponding to the time points when, according to the first experiment, downregulation of S-gene and response to the pathogen occurred. In the second experiment, the possible systemic effect of dsRNA treatment was investigated on the untreated leaf immediately above the treated one at 7 dat (leaf S7, **Figure 1B**). From each leaf (biological replication), three 1.5-cm-diameter disks (technical replication) were excised and inoculated with the pathogen. The remaining leaf tissue was frozen in liquid nitrogen and stored at −80°C until gene expression analysis and assessment of *P. viticola* presence.

VviLBDIf7 Gene Expression Analysis on dsRNA- and Water-Treated Leaves

Expression of the candidate S-gene on dsRNA- and water-treated samples was evaluated through RT-qPCR. RNA extraction, RT-qPCR, and comparison of Ct values were carried out following the procedures described in the *Basal expression of VviLBDIf7* subsection, with some modifications: (i) to ensure the specific retrotranscription of only functional mRNAs, oligo(dT) primers were used in RT-PCR; (ii) qPCR was performed on 8 μl of cDNA; and (iii) to avoid possible amplification of incomplete cDNA sequences due to amplification of small RNAs resulting from degradation of the target gene mRNA following dsRNA treatment, qPCR primers (VvLBD_2_F and VvLBD_2_R; **Table 1**) were specifically designed, using Primer3 Plus software and NCBI Primer-BLAST. The forward primer was designed to match the target gene sequence in the middle of the region amplified by the first qPCR primer pair (see the *Basal expression of VviLBDIf7* subsection), and the reverse primer was designed to match the same sequence region targeted by the dsRNA fragment.

Inoculation of *Plasmopara viticola* on Treated Leaves and Phenotypic Characterization of the Plant–Pathogen Interaction

Experimental inoculation of the leaf disks with the pathogen were carried out by mixing *P. viticola* sporangia coming from Western (S. Maria della Versa, Pavia) and Eastern (Casarsa della Delizia, Pordenone) Italian field populations (Maddalena et al., 2020; Sargolzaei et al., 2020). To verify that the leaf tissues used for the experimental activities were not previously contaminated by *P. viticola*, a PCR assay with primers specific for the ITS region of *P. viticola* (Toffolatti et al., 2012) was carried out on all leaf samples. Leaf tissues were collected from each sample (dsRNA- and water-treated) at all-time points. DNA was extracted using the DNeasy Plant Mini kit (Qiagen Italia, Milano) and checked

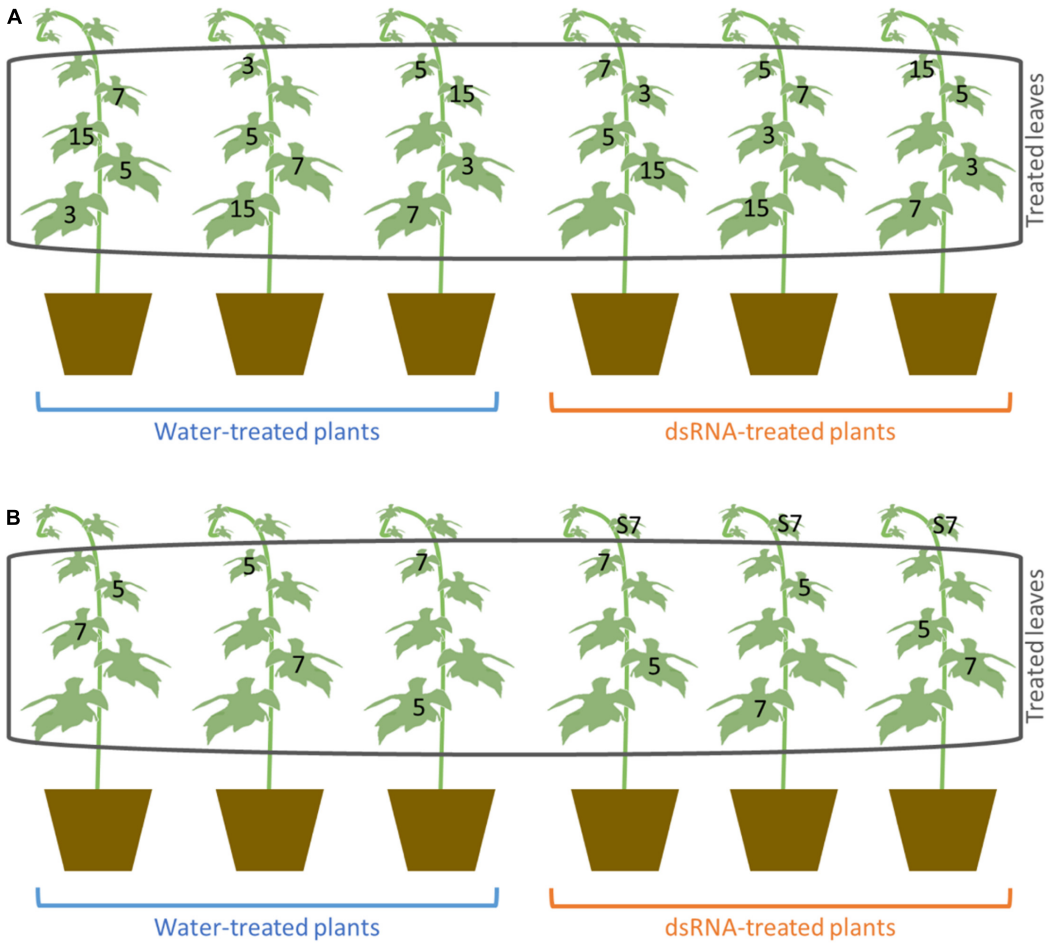

FIGURE 1 | Scheme of the treatment and sampling carried out in the first **(A)** and second experiment **(B)**. Numbers indicate the number of days after treatments (dat) at which the leaves were collected. S7 indicates the untreated leaves that were sampled at 7 dat for the evaluation of systemic activity of dsRNA in the second experiment.

by NanoDrop for quantity and quality. PCR reactions were performed using 2 µl of DNA template, 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific), 0.5 µM of forward (AF_2F: 5′-TCCTGCAATTCGCATTACGT-3′) and reverse (AF_2R: 5′-GGTTGCAGCTAATGGATTCCTA-3′) primers (Toffolatti et al., 2012), and water up to 25 µl. The thermal cycling conditions were as follows: 94°C for 3 min, 30 cycles at 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a last step at 72°C for 5 min. PCR products were solved on 2.5% agarose gel electrophoresis and stained with Realsafe nucleic acid staining solution (Real, Valencia). Positive control consisted of DNA extracted from sporangia and infected leaf disks and negative controls consisted of water and *B. cinerea* DNA.

Leaf disks, sampled at each time point, were placed, lower surface upward, in a Petri dish (9 cm diameter) containing moistened filter paper. The leaf disks were airbrushed with 0.2 ml of a sporangia suspension (5×10^4 sporangia ml^{-1}) obtained by collecting sporangia in sterile distilled water, and incubated in a growth chamber at 22°C with a 12-h photoperiod (Toffolatti et al., 2018). The effect of dsRNA treatment on the pathogen's ability to infect the leaf tissues was evaluated at 7 days after inoculation by combining quantitative (disease severity and the production of sporangia) and qualitative traits, related to the morphology of vegetative and reproductive structures of the pathogen. Disease severity was evaluated in both experiments, while sporangia production and microscopy were performed in the first experiment only. The disease severity of each biological replicate was estimated from the percentage of leaf disk area covered by sporulation (PSA) (Toffolatti et al., 2012). The number of sporangia produced by the pathogen per leaf unit (sporangia cm^{-2}) was determined as described by Toffolatti et al. (2016) by collecting the sporangia from each leaf disk in 1 ml of 20% glycerol:water (v:v) and counting them in Kova chambers. Microscopy observations were performed by aniline blue staining (Wick, 2009) on leaf disks fixed in absolute ethanol and cleared as described by Alexander et al. (2005) with some modifications: samples were boiled in 85% ethanol:water (v:v) for 10 min, and incubated in pre-warmed

lactic acid at 70°C for 30 min, following the procedure reported by Ricciardi et al. (2021). Reagents were purchased from Sigma-Aldrich. Samples were observed under an EasyLab CX40 (Olympus) bright-field optical microscope equipped with Primo Cam HD5 camera (Tiesselab, Milano, Italy). Pictures were taken as Z sections and overlapped by using ImageJ software[1].

Evaluation of dsRNA Treatment on Off-Target and Jasmonic Acid Metabolism Genes

In order to evaluate the effect of dsRNA treatment on off-target genes, the following genes were evaluated *via* RT-qPCR: *EF1α* (elongation factor 1α; EF1α_F: 5′-GAACTGGGTGCTTGATAGGC-3′; EF1α_R: 5′-ACCAAAATA TCCGGAGTAAAAGA-3′) and *GAPHD* (glyceraldehyde-3-phosphate dehydrogenase; GAPHD_F: 5′-TCAAGGTCAAGGA CTCTAACACC-3′; GAPHD_R: 5′-CCAACAACGAACATA GGAGCA-3′) (Figueiredo et al., 2015); *PEPC* (phosphoenolpyruvate carboxylases; PEPC_F: 5′-CATGAAGG GTATTGCTGCTG-3′; PEPC_R: 5′-AGAGGATTTGA TTTTGGTACGG-3′) and *PEPCK* (PEP carboxykinases; PEPCK_F: 5′-TGGCTGGTCAACACTGGTTG-3′; PEPCK_R: 5′-CTTCAGAAGGCTTCCAGAGTG) (Sweetman et al., 2012). Gene expression analysis was performed on dsRNA- and water-treated leaves at 3, 5, 7, and 15 dat (first experiment). On the same samples, the *COI1* (coronatine insensitive 1; COI1_F: 5′-ATGCCCATAGTATTCCCTTTT; COI1_R: 5′-GAACTTCTAATCCTCTGTCTC-3′) and *JAR1* (jasmonate-resistant 1; JAR1_F: 5′- GAGAATTGCGGATGGTGATA-3; JAR1_R: 5′-CTAAAGGCGAAAGAGGTT-3′) (Figueiredo et al., 2015) genes, involved in the jasmonic acid metabolism and modulated by *P. viticola* infection, were investigated *via* RT-qPCR. For more details about the reaction conditions, please refer to the *Basal expression of VviLBDIf7* subsection. Annealing temperature was set to 58°C.

Statistical Analysis

The $2^{-\Delta\Delta Ct}$ values were subjected to Levene's test to assess homogeneity of variance in R software. LSD (least significant difference) test was performed in R to evaluate (i) *VviLBDIf7* basal expression in leaves, (ii) differences among dsRNA- and water-treated leaves in *VviLBDIf7* gene expression values, and (iii) differences among dsRNA- and water-treated leaves in expression values of off-target and jasmonic acid metabolism genes.

ANOVA was carried out with IBM SPSS v.25 software on (i) transformed PSA values $[\text{asin}(\frac{\sqrt{PSA}}{100})]$ to establish the existence of significant differences among dsRNA- and water-treated samples at each sampling time, and (ii) sporangia cm^{-2} values to establish the existence of significant differences between dsRNA- and water-treated samples at each dat.

All the results were plotted in bar plots generated by SPSS v.25 software.

[1]https://imagej.nih.gov/ij/index.html

RESULTS

dsRNA Treatment Decreases *VviLBDIf7* Gene Expression

To assess the basal expression level of *VviLBDIf7* gene, RT-qPCR was performed on Pinot noir leaves collected at 1-day intervals. Results highlighted that the *VviLBDIf7* gene is constitutively expressed in leaves of Pinot noir plants grown in the glasshouse: there was no significant variation in basal expression level in the examined time points (0.45 ± 0.11 *versus* 0.83 ± 0.23, for first and second sampling, respectively, p value > 0.05), suggesting a relatively constant expression of the candidate S-gene. A dsRNA 412-bp long has been synthesized on the *VviLBDIf7* gene sequence of Pinot noir to be applied in the RNAi experiment (**Supplementary file 1**). Pinot noir plants grafted onto SO4 were treated with the dsRNA and the knocking down of the target gene was assessed at 3, 5, 7, and 15 days after dsRNA treatment (dat). A decrease in *VviLBDIf7* expression, even if not statistically significant, was observed between dsRNA- and water-treated leaves at 3 dat, and a statistically significant expression reduction was observed at 5 dat (**Figure 2A**). At 7 and 15 dat, no significant differences were detected between samples, indicating a progressive end of the transient dsRNA effect (**Figure 2A**). The experiment was repeated treating self-rooted cuttings of Pinot noir and collecting leaves at 5 and 7 dat. Statistically significant differences in the expression levels of *VviLBDIf7* gene were observed between dsRNA- and water-treated leaves at 5 dat also in the second trial (**Figure 2B**). No significant difference was observed at 7 dat (**Figure 2B**).

The dsRNA treatment induced no visible negative effects on grapevine plants, which kept their normal phenotypic traits and vigor.

dsRNA Treatment Reduces Pathogen Infection and Sporulation

The complete absence of pathogen DNA was observed in the analysis of the leaves used for the experimental inoculations with *P. viticola*, confirming that the samples were healthy before inoculation. An example of the results obtained is reported in **Supplementary Figure 2**. To investigate whether the dsRNA treatment induced changes in the downy mildew disease extent, dsRNA- and water-treated leaf tissues were experimentally inoculated with *P. viticola* to evaluate phenotypic traits such as disease severity, estimated through PSA, sporangia production, and morphology of pathogen structures. In the first experiment, phenotyping was carried out at 3, 5, 7, and 15 dat; in the second, at 5 and 7 dat.

Percentage of leaf disk area covered by sporulation values of water-treated samples ranged from 41 to 52% (**Figure 3A**) and from 70 to 73% (**Figure 3B**) in the first and second experiment, respectively. No significant differences ($0.8 < F < 2.5$; df = 1–4; $p > 0.22$) were found among PSA values of dsRNA- and water-treated samples inoculated at 3, 5, and 15 dat (**Figure 3A**). Instead, the PSA values of the water-treated leaves inoculated at 7 dat were four times higher, with statistical significance ($9.7 < F < 29.6$; df = 1–4; $p < 0.036$), than those recorded on

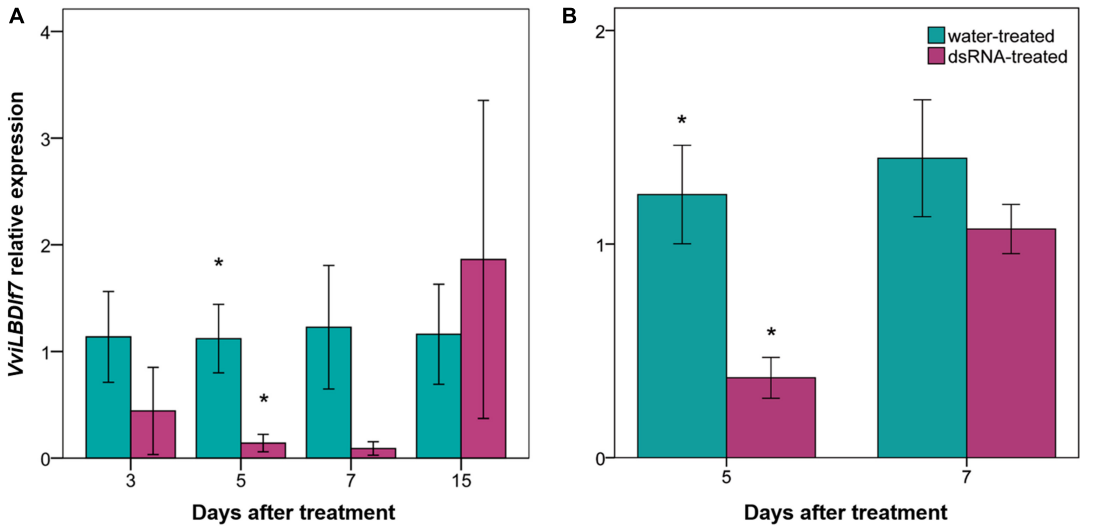

FIGURE 2 | Effect of dsRNA treatment on *VviLBDlf7* gene expression. *VviLBDlf7* gene expression analysis determined based on the $2^{-\Delta\Delta Ct}$ method at 3, 5, 7, and 15 days after treatment (dat) on Pinot noir plants grafted onto SO4 **(A)**, and at 5 and 7 dat on self-rooted Pinot noir plants **(B)**. Bars represent standard errors. Asterisks indicate statistically significant differences among the dsRNA- and water-treated conditions at each time point (*p value = 0.05).

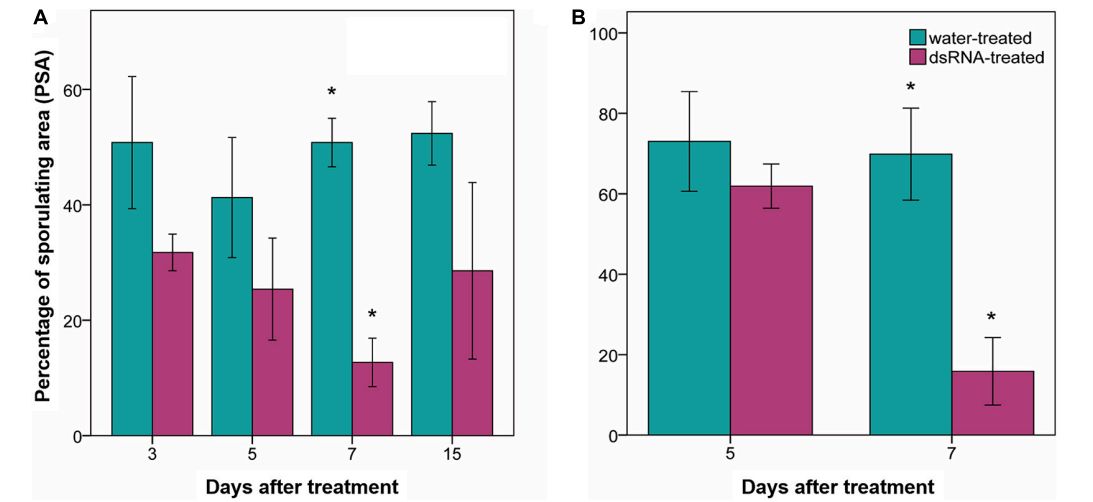

FIGURE 3 | Effect of dsRNA treatment on percentage of sporulating area (PSA) evaluated 7 days after *Plasmopara viticola* inoculation. PSA evaluated on Pinot noir plants grafted onto SO4 at 3, 5, 7, and 15 days after treatment (dat) **(A)**, and on self-rooted Pinot noir plants at 5 and 7 dat **(B)**. Bars represent standard error. Asterisks indicate statistically significant differences among the dsRNA- and water-treated conditions at each time point (*p value < 0.036).

dsRNA-treated samples inoculated at 7 dat in both experiments (**Figures 3A,B**).

Sporangia production in the water-treated leaf disks ranged from 13,683 ± 2959 (average ± standard error) to 36,801 ± 17,849 sporangia cm^{-2} and did not significantly differ from those of the dsRNA-treated samples at any time from treatment ($F < 4.9$; df = 1–4; $p > 0.09$) apart from 7 dat ($F = 31$; df = 1–4; $p = 0.005$), when sporangia production was significantly reduced to 1,486 ± 316 sporangia cm^{-2} in the dsRNA-treated samples (**Figure 4**).

The development of vegetative and reproductive structures of *P. viticola* in the leaf tissues was observed at the microscope by using a staining procedure with aniline blue dye, which allowed us to observe the pathogen structures in bright field. Hyphae with numerous haustoria, also clearly visible at low magnification (**Figures 5A,B**), regularly developed in the intercellular spaces of the lacunose space of the water-treated leaf tissues (**Figures 5A–C**). Sporangiophores emerged from the stomata (**Figure 5C**) and numerous sporangia could be observed on the leaf surface (**Figure 5A**). Alterations in the pathogen structures were clearly visible in the dsRNA-treated leaves only at 7 dat (**Figures 5D–G**): hyphae had a reduced diffusion (**Figure 5G**) and appeared lightly colored and highly vacuolated (**Figure 5D**); haustoria were lightly colored and visible only at high magnification (**Figure 5F**);

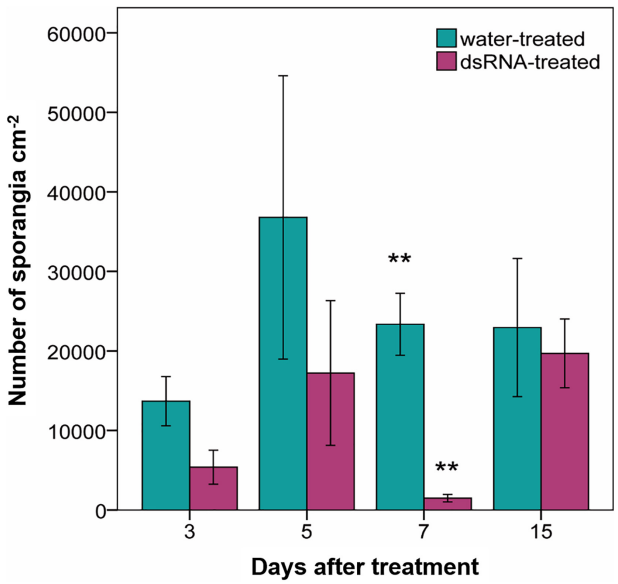

FIGURE 4 | Production of sporangia (number of sporangia cm^{-2}) evaluated 7 days after *Plasmopara viticola* inoculation on dsRNA- and water-treated leaves at 3, 5, 7, and 15 days after treatment of Pinot noir plants grafted onto SO4. Bars represent standard error. Asterisks indicate statistically significant differences among the dsRNA- and water-treated conditions at each time point (**p value 0.005).

short, hyperbranched, and sterile sporangiophores emerged from the stomata (**Figure 5E**). The same alterations were seen in all the points where the pathogen attempted penetration, indicating a uniform response of the leaf tissues to dsRNA treatment and pathogen inoculation.

The PSA values of the untreated leaves sampled immediately above the dsRNA-treated leaves (leaf sample S7; **Figure 1B**) at 7 dat in the second experiment (PSA = 21.4% ± 7.1 SD) did not significantly differ (F = 5.3; df = 1–4; P = 0.08) from the PSA values recorded in the dsRNA-treated leaves (PSA = 14.6% ± 8.4 SD) and significantly differed (F = 15.8; df = 1–4; P = 0.016) from the PSA values of the water-treated leaves (PSA = 69.8% ± 11.4 SD) sampled at the same time point.

Treatment With dsRNA Does Not Reduce Expression of Off-Target Genes

The effect of dsRNA treatment has been evaluated in the gene expression of some off-target genes: *EF1α*, *GAPHD*, *PEPC*, and *PEPCK* (**Figure 6**). Relative gene expression of *PEPC* and *PEPCK* genes did not show statistical significant differences between dsRNA- and water-treated leaves at any time points (**Figures 6C,D**). Statistical significant differences between dsRNA- and water-treated leaves have been detected at 3 and 7 dat for *EF1α* gene (**Figure 6A**) and at 5 and 7 dat for *GAPHD* gene (**Figure 6B**), with expression values of dsRNA-treated leaves higher than the water-treated ones.

Regarding the effect of dsRNA treatment on jasmonic acid metabolism genes, the relative gene expression of two genes (*COI1* and *JAR1*) has been investigated. *JAR1* primers did not amplify any fragments at our amplification conditions (annealing temperature = 58°C), while aspecific fragments have been amplified at lower annealing temperature (data not shown). Amplification of *COI1* gene showed statistically significant differences between dsRNA- and water-treated leaves only at 5 dat, with expression values of dsRNA-treated leaves lower than the water-treated ones (**Figure 6E**).

DISCUSSION

Foliar-Applied dsRNA Reduces Expression of *VviLBDIf7* Gene

All plant genes involved in facilitating pathogen infection and supporting a compatible plant–pathogen interaction are considered S-genes (Van Schie and Takken, 2014); therefore, silenced S-genes no longer support a compatible plant–pathogen interaction and can cause pathogen-specific resistance (Pavan et al., 2010; Thatcher et al., 2012).

RNAi is a post-transcriptional gene silencing mechanism triggered by dsRNA molecules to prevent the expression of target genes (Kim and Rossi, 2007). The exogenous application of dsRNAs targeting essential interaction genes in plants (S-genes) or plant pathogens and pests has been successfully used to both control diseases and induce gene silencing as a valid alternative to genetic transformation (Mitter et al., 2017; Ghosh et al., 2018; Rosa et al., 2018; Dubrovina and Kiselev, 2019; Gu et al., 2019; Dalakouras et al., 2020). In this work, the efficacy of RNAi approach in knocking down the putative grapevine S-gene (*VviLBDIf7*) identified by Toffolatti et al. (2020) in grapevine–*P. viticola* interaction has been investigated. A prerequisite for setting up a successful foliar RNAi experiment to knock down the target gene is the expression of the target gene in leaves at any stages, regardless of environmental conditions. Therefore, the first step of this study was the evaluation of S-gene basal expression. RT-qPCR data showed that the expression of *VviLBDIf7* in Pinot noir leaves did not change significantly over time in the absence of perturbing conditions, confirming that S-genes can show a constitutive expression (Eichmann et al., 2004).

Foliar-applied dsRNA molecules targeting plant *VviLBDIf7* gene proved to knock down *VviLBDIf7* gene expression in Pinot noir, a grapevine cultivar susceptible to *P. viticola* infection, at 5 dat. The RNAi triggered by exogenous dsRNA is known to be transient, lasting from a few days up to a couple of weeks (Dalakouras et al., 2016, 2020; Niehl et al., 2018; Dubrovina and Kiselev, 2019; Nerva et al., 2020). In this interval, the efficiency peak of the dsRNA treatment is affected by several factors, determining the absorption rate of the exogenous molecules by plant cells, including dsRNA concentration, dose and length, application method, delivery technique, plant organ-specific activities, and stability of the molecule under unfitting environmental conditions (Das and Sherif, 2020). In our experimental conditions, evaluations performed at 7 (both experiments) and 15 (first experiment) dat indicated a progressive reduction of the transient dsRNA effect in knocking down the expression of *VviLBDIf7* gene. Instead, knockdown

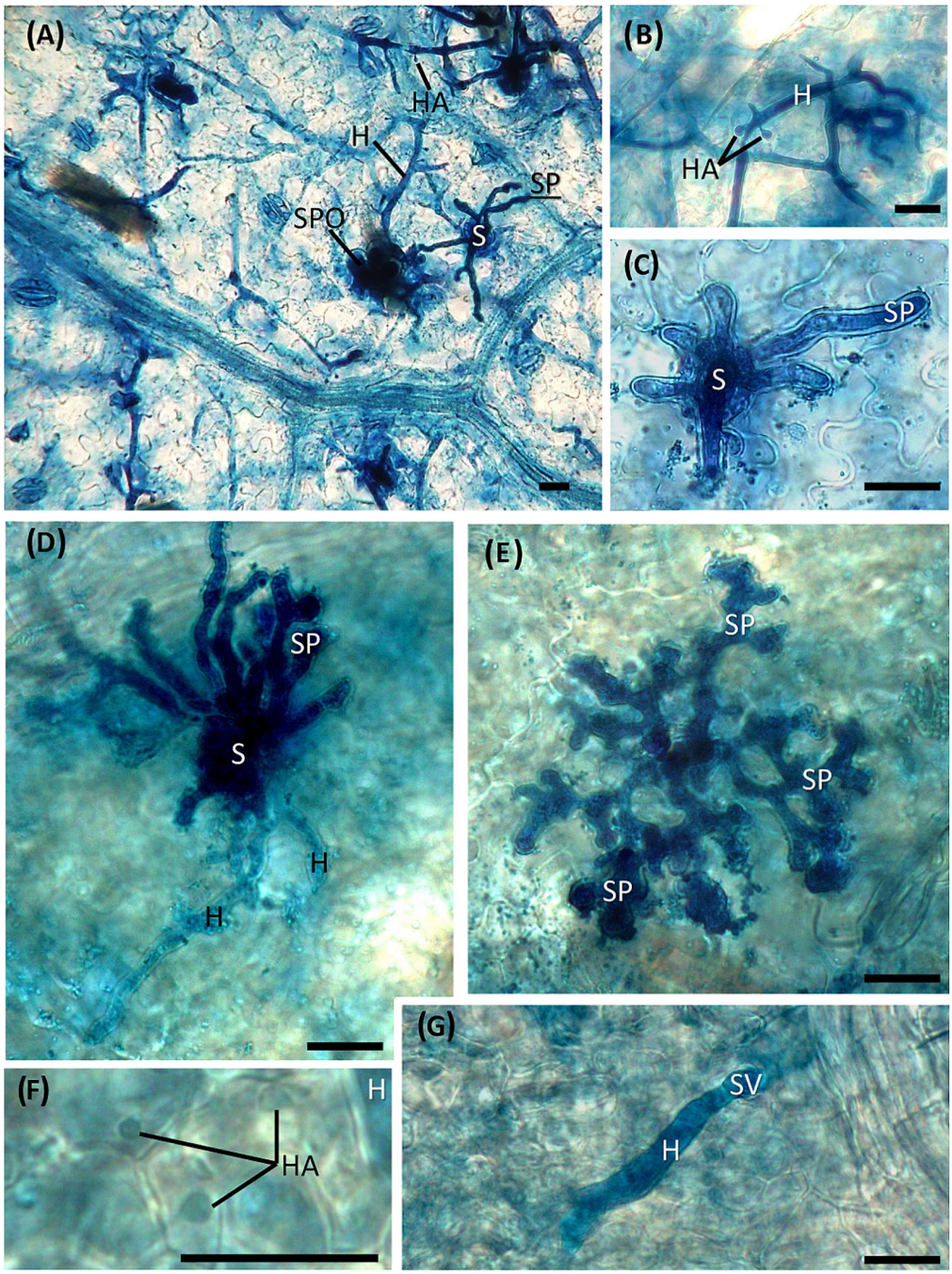

FIGURE 5 | Development of *Plasmopara viticola* structures inside untreated **(A–C)** and treated **(D–G)** leaf tissues inoculated at 7 dat. **(A)** Hyphae with haustoria developing in the mesophyll cells and sporangiophores emerging from the stomata. **(B)** Mycelium with haustoria; **(C)** detail of sporangiophores emerging from a stoma; **(D)** degenerating hyphae, slightly colored and vacuolized, with no visible haustoria, and hyperbranched sporangiophores emerging from the stoma; **(E)** short, hyperbranched and sterile sporangiophores; **(F)** light-colored haustoria; **(G)** hypha developing from the substomatal vesicle with no visible haustoria. S = stoma; H = hypha; HA = haustorium; SP = sporangiophore; SPO = sporangium; SV = substomatal vesicle. Scale bar, 20 μm.

at 5 dat has been confirmed by the second experiment as well, when self-rooted plants were used. This second approach was used to validate the results, avoiding any rootstock interference. Rootstock can affect the scion behavior at different levels, such as plant development, biomass accumulation and phenology (Ollat et al., 2015). These results demonstrated that the transient effect of dsRNA was not affected by rootstock and that in our conditions, the RNAi is terminated by 15 dat.

Fifty LBD genes have been identified in grapevine genome (Grimplet et al., 2017), expressed in different tissues (such as

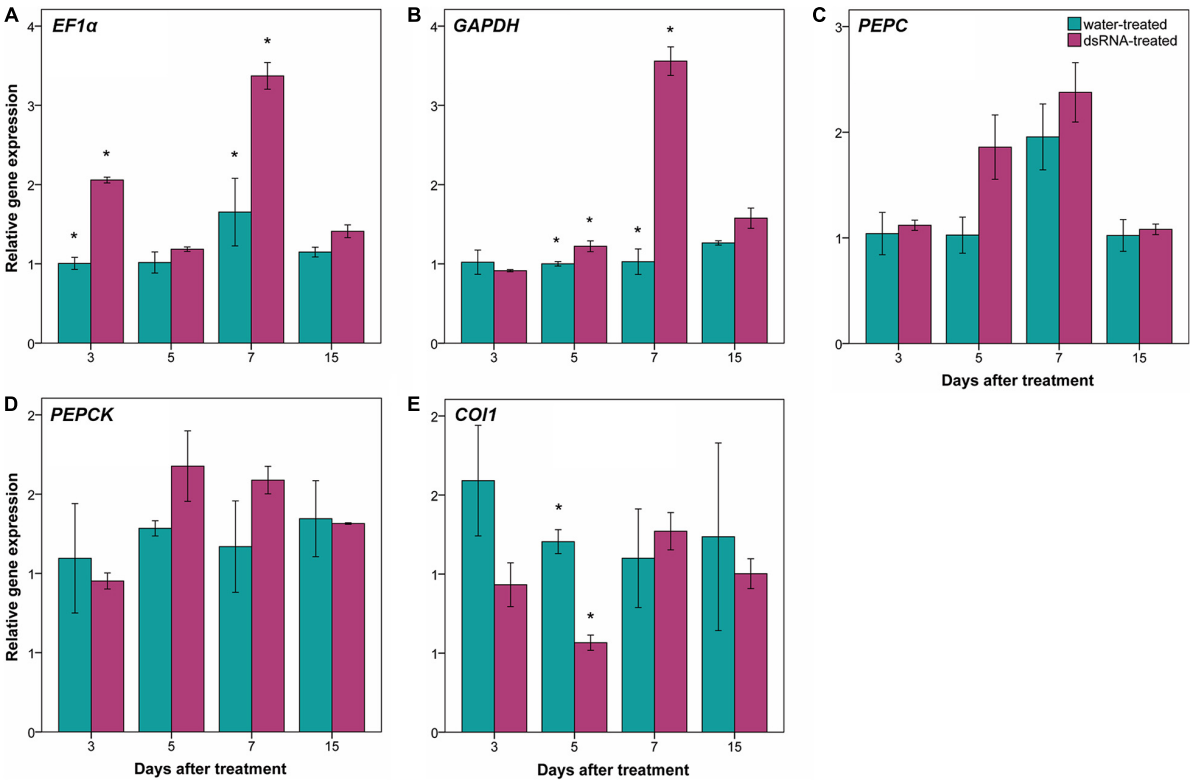

FIGURE 6 | Effect of dsRNA treatment on *EF1α* [elongation factor 1α; **(A)**], *GAPHD* (glyceraldehyde-3-phosphate dehydrogenase; B), *PEPC* [phosphoenolpyruvate carboxylases; **(C)**], *PEPCK* [PEP carboxykinases; **(D)**], and *COI1* [coronatine insensitive 1; **(E)**] gene expression at 3, 5, 7, and 15 days after treatment (dat) on Pinot noir plants grafted onto SO4. Gene expression analysis determined based on the $2^{-\Delta\Delta Ct}$ method. Bars represent standard errors. Asterisks indicate statistically significant differences among the dsRNA- and water-treated conditions at each time point (*p value = 0.05).

young leaves, developed tendril, and inflorescences), in berry development and ripening and in response to abiotic and biotic stresses (Albertazzi et al., 2009; Fasoli et al., 2012; Agudelo-Romero et al., 2015). *VviLBDIf7* seems to be expressed at low levels in all mature/woody and vegetative/green tissues, while it appears to be up-regulated in berries upon *B. cinerea* attack (Agudelo-Romero et al., 2015). In our experimental conditions, no negative effects could be observed in grapevine plants treated with dsRNA: this could be due to the transient effect of RNAi. The presence of pleiotropic effects on biological processes and in response to abiotic and biotic stresses (not yet investigated) associated with *VviLBDIf7* silencing should be better investigated in future studies on stable grapevine transformants.

Reduction of *VviLBDIf7* Expression Is Followed by Reduced Susceptibility to *Plasmopara viticola*

The developed staining protocol has two main advantages: firstly, the staining is stable and does not fade away rapidly, as normally occurs when using aniline blue staining techniques for fluorescence microscopy (Díez-Navajas et al., 2007), allowing the operator to perform a more thorough investigation of the pathogen structures; secondly, the observations can be performed with a microscope that is normally present in a basic mycology laboratory, without the need for a fluorescence microscope. Aniline blue binds to β-glucans (β-1,3-glucans in particular) located in the *P. viticola* cell wall, staining the pathogen structures in blue. However, also plant cell walls can be stained, especially callose depositions (Hood and Shew, 1996) that are frequently found as a defense reaction to the invading pathogen (Trouvelot et al., 2008), causing some faint blue staining also in plant tissues. No differences could be observed between treatments in pathogen growth and sporulation at all time point except at 7 dat (2 days after downregulation of *VviLBDIf7*), when Pinot noir leaves treated with dsRNA showed a significant reduction in disease severity as well as impairment in *P. viticola* growth and sporulation, compared to the water-treated leaves. In the dsRNA-treated samples, the alterations in the vegetative structures of the pathogen were evident. The hyphae did not freely diffuse inside the leaf tissues but developed only in the area immediately surrounding the infection point and led to the differentiation of hyperbranched, partly sterile, sporangiophores. Analogous alterations were observed as a consequence of resistance response in Mgaloblishvili (Toffolatti et al., 2018) and in response to environmental (Rumbolz et al., 2002) and chemical (Ricciardi et al., 2021) stresses. The vacuolation of the hyphae underneath the sporangiophore could be associated with the necessity of the mycelium to provide material to support sporulation, as occurs in true fungi (Thrane et al., 2004), or to

a degradation of the mycelium. Abnormal hyphal vacuolation has been observed in hyphae of the oomycete *Saprolegnia ferax* following the application of growth inhibitors (Bachewich and Heath, 1999). Moreover, the vegetative structures of the pathogen in the dsRNA-treated samples appeared more lightly colored compared to the water-treated samples. Since aniline blue binds to β-glucans, this discoloration could indicate an alteration in the cell wall composition. While this altered mycelium was clearly visible following aniline blue staining, haustoria were less colored and hardly recognizable. This could indicate that the dsRNA treatment primarily affected the haustoria, which are the only structures of the pathogen that actively interact with the host cell (Toffolatti et al., 2011). *P. viticola* is an obligate parasite of grapevine and does not directly damage the host cell, the activity of which must be preserved to allow the absorption of nutrients. Alterations at haustoria level can induce a reduced pathogen growth not only because they have a role in nutrient uptake, but also because they represent a specialized interface for delivering effectors to plants that are associated with resistance (Judelson and Ah-Fong, 2019). More accurate investigation on the haustoria ultrastructure is needed to confirm these preliminary results. The alteration in the differentiation of sporangiophores was associated with a significant reduction in the sporangia production.

The analogous disease severity of the pathogen observed in the second experiment both on the leaves that were directly treated with dsRNA and those directly above the treated leaves (systemic diffusion), which was significantly lower than that achieved in the water-treated samples, indicates the presence of a systemic effect of the treatment that should be more deeply investigated in future studies. Systemic RNAi has been observed in other plants, such as *A. thaliana* (Melnyk et al., 2011), *Hordeum vulgare* (Biedenkopf et al., 2020), and *Nicotiana benthamiana* (Chen et al., 2018), but to the best of our knowledge, no information is reported for grapevine.

VviLBDIf7 Is a Candidate Gene to Be Silenced to Reduce Downy Mildew Susceptibility

A potential limitation of RNAi approach can be the possible effect on off-target genes. Off-target effects occur when a siRNA down-regulates unintended targets. For this reason, it is useful to assess off-target potential in order to avoid undesirable phenotypes (Rosa et al., 2018). In this work, dsRNA treatment did not reduce the expression of *EF1α*, *GAPHD*, *PEPC*, and *PEPCK* off-target genes. The up-regulation of *EF1α* and *GAPHD* genes in dsRNA-treated leaves at some time points can be a consequence of the knockdown of the *VviLBDIf7* gene (the plant reacts to the gene silencing by improving its basal metabolism).

In *A. thaliana*, the *lbd20* mutant showed a reduced disease severity after *F. oxysporum* infection and a modulation of jasmonic acid-mediated defense genes (Thatcher et al., 2012). In grapevine, some evidences of jasmonic acid involvement in resistant cultivar in response to *P. viticola* infection have been described (Figueiredo et al., 2015; Li et al., 2015). After *P. viticola* inoculation, Figueiredo et al. (2015) observed an up-regulation of jasmonic acid biosynthesis-related genes (*LOXO*, *AOS*, *AOC*, and *OPR3*) at 6 and 12 hpi (hours post-infection), and an up-regulation of genes involved in the jasmonic acid activation and signaling (*JAR1* and *COI1*, respectively) at 18 and 24 hpi. In this work, due to the sampling times (3, 5, 7, and 15 dat), only the gene expression profile of *JAR1* and *COI1* genes has been investigated. Although we have to refer to only *COI1* gene expression data (no amplification fragments were detected for *JAR1* gene), the jasmonic acid pathway appears to be down-regulated, as *COI1* gene expression values at 5 dat in dsRNA-treated leaves were lower than in the water-treated ones. These data confirm what has been observed for *LBD20* gene in *A. thaliana*, where *coi1* mutants did not induce *LBD20* gene expression, and support the role of LBD genes in jasmonic acid signaling (Thatcher et al., 2012).

CONCLUSION

The results reported in this work highlight the great potential of RNAi-based strategies in sustainable defense management. In the current scenario, the treatment with fungicides still represents the most effective agronomical practice to defend vineyards by *P. viticola* attack. Nevertheless, Directive 2009/128/EC and Regulation (EC) No. 1107/2009 of the European Parliament and of the Council concerning the placing and use of plant protection products on the market impose to the farmers the reduction of fungicide applications, due to their negative impact on human health and environment. In this view, the development of novel and sustainable disease management strategies is essential. Numerous efforts are ongoing with the aim to obtain resistant varieties, exploiting alternative strategies to classical biotechnological tools, such as GMOs (genetically modified organisms), which are currently subjected to strict regulation (Capriotti et al., 2020). For this purpose, the use of RNAi for silencing plant susceptibility genes, which facilitate infection and support compatibility (Van Schie and Takken, 2014), represents a promising alternative to traditional means, such as fungicides, for disease control. Indeed, the phenotypic characterization of quantitative and qualitative *P. viticola* traits allowed us to establish the efficacy of exogenous dsRNA application in silencing the *VviLBDIf7* gene, which led to a reduced pathogen growth and sporulation rate in Pinot noir, a cultivar that is normally highly susceptible to the pathogen. Based on gene expression and PSA values, the RNAi effect is concluded by 15 dat indicating that further treatments are needed for the subsequent disease control. The signatures of systemic activity shown in the present study suggest that the dsRNA treatment could also reach untreated parts of the plant, a feature that is highly desirable since grapevine has an important growth in open field and systemic properties of RNAi could allow the protection of the newly formed vegetation not covered by the treatment. Further investigation is, however needed to clarify the movement inside the plant as well as the duration and the efficacy of dsRNA on untreated parts of the plant. Moreover, considering the high heterozygosity and the varietal rigidity imposed by registered designations of origin that affect development and cultivation of resistant varieties obtained

by crossing non-*vinifera* species with *V. vinifera*, the use of RNAi approach in grapevine could represent a valid tool for specifically targeting a known gene. Further implementation of the method is, however, needed to improve the delivery of the dsRNA and achieve a more rapid silencing of the gene that could be compatible with a field use, although an appropriated regulation of topical RNAi applications is still missing (Mezzetti et al., 2020). For an effective field application of dsRNAs as a disease management tool, the following aspects need to be optimized: (i) the concentration and length of dsRNA molecules, (ii) the formulation that should prevent dsRNA degradation (encapsulation could solve this issue) and allow the uptake of dsRNAs into cells, (iii) the delivery strategy (high-pressure spraying or brush-mediated leaf applications), and (iv) the efficacy of dsRNA recognition by the RNAi pathway of the target organism. Furthermore, this approach that uses dsRNA to silence the susceptibility gene of the plant instead of targeting an essential gene of the pathogen should reduce the possible cross-species effect of the dsRNA (e.g., on beneficial microorganisms) and the development of resistances in the pathogen population. Overall, RNAi-mediated silencing could make a great contribution toward integrated pest management, which, taking a holistic approach that exploits all the available disease management tools (i.e., resistant varieties, agronomic practices, and chemical and non-chemical pathogen control), represents the most effective way to manage diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

GDL and SLT conceived the study. GDL, SLT, AP, and PC set up the experiment. VR, AP, and GDL performed molecular biology analysis. SLT, DM, and EMF performed the phenotypic characterization of *P. viticola*. GDL, SLT, and GM wrote the manuscript. AP, PC, PAB, and OF critically revised the manuscript. All the authors reviewed and approved the manuscript before submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.667319/full#supplementary-material

REFERENCES

Agudelo-Romero, P., Erban, A., Rego, C., Carbonell-Bejerano, P., Nascimento, T., Sousa, L., et al. (2015). Transcriptome and metabolome reprogramming in Vitis vinifera cv. Trincadeira berries upon infection with Botrytis cinerea. *J. Exp. Bot.* 66, 1769–1785. doi: 10.1093/jxb/eru517

Albertazzi, G., Milc, J., Caffagni, A., Francia, E., Roncaglia, E., Ferrari, F., et al. (2009). Gene expression in grapevine cultivars in response to bois noir phytoplasma infection. *Plant Sci.* 176, 792–804. doi: 10.1021/jf104297n

Alexander, D. L., Mellor, E. A., and Langdale, J. A. (2005). CORKSCREW1 defines a novel mechanism of domain specification in the maize shoot. *Plant Physiol.* 138, 1396–1408. doi: 10.1104/pp.105.063909

Bachewich, C., and Heath, I. B. (1999). Cytoplasmic migrations and vacuolation are associated with growth recovery in hyphae of Saprolegnia, and are dependent on the cytoskeleton. *Mycol. Res.* 103, 849–858.

Biedenkopf, D., Will, T., Knauer, T., Jelonek, L., Furch, A. C. U., Busche, T., et al. (2020). Systemic spreading of exogenous applied RNA biopesticides in the crop plant Hordeum vulgare. *ExRNA* 2:12.

Buonassisi, D., Colombo, M., Migliaro, D., Dolzani, C., Peressotti, E., Mizzotti, C., et al. (2017). Breeding for grapevine downy mildew resistance: a review of "omics" approaches. *Euphytica* 213, 1–21. doi: 10.1007/s10681-017-1882-8

Cagliari, D., Avila dos Santos, E., Dias, N., Smagghe, G., and Zotti, M. (2019). "Nontransformative strategies for RNAi in crop protection," in *Modulating Gene Expression - Abridging the RNAi and CRISPR-Cas9 Technologies*, eds A. Singh and M. W. Khan (IntechOpen).

Canaguier, A., Grimplet, J., Di Gaspero, G., Scalabrin, S., Duchêne, E., Choisne, N., et al. (2017). A new version of the grapevine reference genome assembly (12X.v2) and of its annotation (VCost.v3). *Genomics Data* 14, 56–62. doi: 10.1016/j.gdata.2017.09.002

Capriotti, L., Baraldi, E., Mezzetti, B., Limera, C., and Sabbadini, S. (2020). Biotechnological approaches: gene overexpression, gene silencing, and genome editing to control fungal and oomycete diseases in grapevine. *Int. J. Mol. Sci.* 21:5701. doi: 10.3390/ijms21165701

Chen, J., Moreau, C., Liu, Y., Kawaguchi, M., Hofer, J., Ellis, N., et al. (2012). Conserved genetic determinant of motor organ identity in medicago truncatula and related legumes. *Proc. Natl. Acad. Sci.U.S.A.* 109, 11723–11728. doi: 10.1073/pnas.1204566109

Chen, W., Zhang, X., Fan, Y., Li, B., Ryabov, E., Shi, N., et al. (2018). A genetic network for systemic RNA silencing in plants. *Plant Physiol.* 176, 2700–2719.

Dalakouras, A., Wassenegger, M., Dadami, E., Ganopoulos, I., Pappas, M. L., and Papadopoulou, K. (2020). Genetically modified organism-free RNA interference: exogenous application of RNA molecules in plants. *Plant Physiol.* 182, 38–50. doi: 10.1104/pp.19.00570

Dalakouras, A., Wassenegger, M., McMillan, J. N., Cardoza, V., Maegele, I., Dadami, E., et al. (2016). Induction of silencing in plants by high-pressure spraying of in vitro-synthesized small RNAs. *Front. Plant Sci.* 7:1327. doi: 10.3389/fpls.2016.01327

Das, P. R., and Sherif, S. M. (2020). Application of exogenous dsRNAs-induced RNAi in agriculture: challenges and triumphs. *Front. Plant Sci.* 11:946. doi: 10.3389/fpls.2020.00946

Díez-Navajas, A. M., Greif, C., Poutaraud, A., and Merdinoglu, D. (2007). Two simplified fluorescent staining techniques to observe infection structures of the oomycete Plasmopara viticola in grapevine leaf tissues. *Micron* 38, 680–683. doi: 10.1016/j.micron.2006.09.009

Dubrovina, A. S., Aleynova, O. A., Kalachev, A. V., Suprun, A. R., Ogneva, Z. V., and Kiselev, K. V. (2019). Induction of transgene suppression in plants via external application of synthetic dsRNA. *Int. J. Mol. Sci.* 20:1585. doi: 10.3390/ijms20071585

Dubrovina, A. S., and Kiselev, K. V. (2019). Exogenous RNAs for gene regulation and plant resistance. *Int. J. Mol. Sci.* 20:2282.

Eichmann, R., Schultheiss, H., Kogel, K.-H., and Hückelhoven, R. (2004). the barley apoptosis suppressor homologue bax inhibitor-1 compromises nonhost penetration resistance of barley to the inappropriate pathogen blumeria graminis f. sp. tritici. *Mol. Plant-Microbe Interact.* 17, 484–490. doi: 10.1094/MPMI.2004.17.5.484

Fasoli, M., Dal Santo, S., Zenoni, S., Tornielli, G. B., Farina, L., Zamboni, A., et al. (2012). The grapevine expression atlas reveals a deep transcriptome shift driving the entire plant into a maturation program. *Plant Cell* 24, 3489–3505. doi: 10.1105/tpc.112.100230

Figueiredo, A., Monteiro, F., and Sebastiana, M. (2015). First clues on a jasmonic acid role in grapevine resistance against the biotrophic fungus Plasmopara viticola. *Eur. J. Plant Pathol.* 142, 645–652.

Fujita, A., Soma, N., Goto-yamamoto, N., Mizuno, A., Kiso, K., and Hashizume, K. (2007). Effect of shading on proanthocyanidin biosynthesis in the grape berry. *J. Japanese Soc. Hortic. Sci.* 76, 112–119.

Gessler, C., Pertot, I., and Perazzolli, M. (2011). *Plasmopara viticola*: a review of knowledge on downy mildew of grapevine and effective disease management. *Phytopathol. Mediterr.* 50, 3–44. doi: 10.14601/Phytopathol_Mediterr-9360

Ghosh, S. K. B., Hunter, W. B., Park, A. L., and Gundersen-Rindal, D. E. (2018). Double-stranded RNA oral delivery methods to induce RNA interference in phloem and plant-sap-feeding hemipteran insects. *J. Vis. Exp.* 135:57390. doi: 10.3791/57390

Grimplet, J., Pimentel, D., Agudelo-Romero, P., Martinez-Zapater, J. M., and Fortes, A. M. (2017). The lateral organ boundaries domain gene family in grapevine: genome-wide characterization and expression analyses during developmental processes and stress responses. *Sci. Rep.* 7: 15968. doi: 10.1038/s41598-017-16240-5

Gu, K.-X., Song, X.-S., Xiao, X.-M., Duan, X.-X., Wang, J.-X., Duan, Y.-B., et al. (2019). A β-tubulin dsRNA derived from Fusarium asiaticum confers plant resistance to multiple phytopathogens and reduces fungicide resistance. *Pestic. Biochem. Physiol.* 153, 36–46. doi: 10.1016/j.pestbp.2018.10.005

Hood, M., and Shew, H. (1996). Applications of KOH-aniline blue fluorescence in the study of plant-fungal interactions. *Phytopathology* 86:704.

Huang, X., Liu, G., and Zhang, W. (2018). Genome-wide analysis of LBD (lateral organ boundaries domain) gene family in brassica rapa. *Brazilian Arch. Biol. Technol.* 61:e18180049.

Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286

Judelson, H. S., and Ah-Fong, A. M. V. (2019). Exchanges at the plant-oomycete interface that influence disease. *Plant Physiol.* 179, 1198–1211. doi: 10.1104/pp.18.00979

Kerschen, A., Napoli, C. A., Jorgensen, R. A., and Müller, A. E. (2004). Effectiveness of RNA interference in transgenic plants. *FEBS Lett.* 566, 223–228.

Kim, D. H., and Rossi, J. J. (2007). Strategies for silencing human disease using RNA interference. *Nat. Rev. Genet.* 8, 173–184.

Kim, M.-J., Kim, M., Lee, M. R., Park, S. K., and Kim, J. (2015). Lateral organ boundaries domain (LBD)10 interacts with SIDECAR POLLEN / LBD27 to control pollen development in Arabidopsis. *Plant J.* 81, 794–809. doi: 10.1111/tpj.12767

Konakalla, N. C., Kaldis, A., Berbati, M., Masarapu, H., and Voloudakis, A. E. (2016). Exogenous application of double-stranded RNA molecules from TMV p126 and CP genes confers resistance against TMV in tobacco. *Planta* 244, 961–969. doi: 10.1007/s00425-016-2567-6

Kusch, S., and Panstruga, R. (2017). mlo -based resistance: an apparently universal "weapon" to defeat powdery mildew disease. *Mol. Plant-Microbe Interact.* 30, 179–189. doi: 10.1094/MPMI-12-16-0255-CR

Li, X., Wu, J., Yin, L., Zhang, Y., Qu, J., and Lu, J. (2015). Comparative transcriptome analysis reveals defense-related genes and pathways against downy mildew in Vitis amurensis grapevine. *Plant Physiol. Biochem.* 95, 1–14. doi: 10.1016/j.plaphy.2015.06.016

Liu, H., Wang, S., Yu, X., Yu, J., He, X., Zhang, S., et al. (2005). ARL1, a LOB-domain protein required for adventitious root formation in rice. *Plant J.* 43, 47–56. doi: 10.1111/j.1365-313X.2005.02434.x

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lu, Q., Shao, F., Macmillan, C., Wilson, I. W., van der Merwe, K., Hussey, S. G., et al. (2018). Genomewide analysis of the lateral organ boundaries domain gene family in Eucalyptus grandis reveals members that differentially impact secondary growth. *Plant Biotechnol. J.* 16, 124–136. doi: 10.1111/pbi.12754

Luo, Y., Chen, Q., Luan, J., Chung, S. H., Van Eck, J., Turgeon, R., et al. (2017). Towards an understanding of the molecular basis of effective RNAi against a global insect pest, the whitefly Bemisia tabaci. *Insect Biochem. Mol. Biol.* 88, 21–29. doi: 10.1016/j.ibmb.2017.07.005

Maddalena, G., Delmotte, F., Bianco, P. A., De Lorenzis, G., and Toffolatti, S. L. (2020). Genetic structure of Italian population of the grapevine downy mildew agent, plasmopara viticola. *Ann. Appl. Biol.* 176, 257–267. doi: 10.1111/aab.12567

Mangeon, A., Bell, E. M., Lin, W., Jablonska, B., and Springer, P. S. (2011). Misregulation of the LOB domain gene DDA1 suggests possible functions in auxin signalling and photomorphogenesis. *J. Exp. Bot.* 62, 221–233. doi: 10.1093/jxb/erq259

Meister, G., Landthaler, M., Dorsett, Y., and Tuschl, T. (2004). Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* 10, 544–550. doi: 10.1261/rna.5235104

Melnyk, C. W., Molnar, A., and Baulcombe, D. C. (2011). Intercellular and systemic movement of RNA silencing signals. *EMBO J.* 30, 3553–3563.

Mezzetti, B., Smagghe, G., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., et al. (2020). RNAi: What is its position in agriculture? *J. Pest Sci. (2004).* 93, 1125–1130.

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., et al. (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* 3:16207. doi: 10.1038/nplants.2016.207

Morozov, S. Y., Solovyev, A. G., Kalinina, N. O., and Taliansky, M. E. (2019). Double-stranded RNAs in plant protection against pathogenic organisms and viruses in agriculture. *Acta Naturae* 11, 13–21.

Nerva, L., Sandrini, M., Gambino, G., and Chitarra, W. (2020). Double-stranded rnas (Dsrnas) as a sustainable tool against gray mold (botrytis cinerea) in grapevine: Effectiveness of different application methods in an open-air environment. *Biomolecules* 10:200. doi: 10.3390/biom10020200

Newman, M. A., Sundelin, T., Nielsen, J. T., and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139. doi: 10.3389/fpls.2013.00139

Niehl, A., Soininen, M., Poranen, M. M., and Heinlein, M. (2018). Synthetic biology approach for plant protection using dsRNA. *Plant Biotechnol. J.* 16, 1679–1687. doi: 10.1111/pbi.12904

Okushima, Y., Fukaki, H., Onoda, M., Theologis, A., and Tasaka, M. (2007). ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in arabidopsis. *Plant Cell* 19, 118–130. doi: 10.1105/tpc.106.047761

Ollat, N., Peccoux, A., Papura, D., Esmenjaud, D., Marguerit, E., Tandonnet, J. P., et al. (2015). "Rootstocks as a component of adaptation to environment," in *Grapevine in a Changing Environment: A Molecular and Ecophysiological Perspective*, eds H. V. Gerós, M. M. Chaves, H. M. Gil, and S. Delrot (Chichester, UK), 68–94. doi: 10.1002/9781118735985.ch4

Pavan, S., Jacobsen, E., Visser, R. G. F., and Bai, Y. (2010). Loss of susceptibility as a novel breeding strategy for durable and broad-spectrum resistance. *Mol. Breed.* 25, 1–12. doi: 10.1007/s11032-009-9323-6

Pessina, S., Lenzi, L., Perazzolli, M., Campa, M., Dalla Costa, L., Urso, S., et al. (2016). Knockdown of MLO genes reduces susceptibility to powdery mildew in grapevine. *Hortic. Res.* 3:16016. doi: 10.1038/hortres.2016.16

Pirrello, C., Zeilmaker, T., Bianco, L., Giacomelli, L., Moser, C., and Vezzulli, S. (2021). Mining grapevine downy mildew susceptibility genes: a resource for genomics-based breeding and tailored gene editing. *Biomolecules* 11, 1–23. doi: 10.3390/biom11020181

Rast, M. I., and Simon, R. (2012). Arabidopsis jagged lateral organs acts with asymmetric leaves2 to coordinate knox and pin expression in shoot and root meristems. *Plant Cell* 24, 2917–2933. doi: 10.1105/tpc.112.099978

Reid, K. E., Olsson, N., Schlosser, J., Peng, F., and Lund, S. T. (2006). An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biol.* 6:27. doi: 10.1186/1471-2229-6-27

Ricciardi, V., Marcianò, D., Sargolzaei, M., Maddalena, G., Maghradze, D., Tirelli, A., et al. (2021). From plant resistance response to the discovery of antimicrobial compounds: the role of volatile organic compounds (VOCs) in grapevine downy mildew infection. *Plant Physiol. Biochem.* 160, 294–305. doi: 10.1016/j.plaphy.2021.01.035

Rosa, C., Kuo, Y.-W., Wuriyanghan, H., and Falk, B. W. (2018). RNA interference mechanisms and applications in plant pathology. *Annu. Rev. Phytopathol.* 56, 581–610. doi: 10.1146/annurev-phyto-080417-050044

Rubin, G., Tohge, T., Matsuda, F., Saito, K., and Scheible, W.-R. (2009). Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in arabidopsis. *Plant Cell* 21, 3567–3584. doi: 10.1105/tpc.109.067041

Rumbolz, J., Wirtz, S., Kassemeyer, H.-H., Guggenheim, R., Schäfer, E., and Büche, C. (2002). Sporulation of plasmopara viticola: differentiation and light regulation. *Plant Biol.* 4, 413–422. doi: 10.1093/jxb/erv137

Sargolzaei, M., Maddalena, G., Bitsadze, N., Maghradze, D., Bianco, P. A., Failla, O., et al. (2020). Rpv29, Rpv30 and Rpv31: three novel genomic loci associated with resistance to plasmopara viticola in vitis vinifera. *Front. Plant Sci.* 11:1537. doi: 10.3389/fpls.2020.562432

Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C., and Machida, Y. (2001). The ASYMMETRIC LEAVES2 gene of Arabidopsis thaliana regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* 128, 1771–1783.

Song, B., Tang, Z., Li, X., Li, J., Zhang, M., Zhao, K., et al. (2020). Mining and evolution analysis of lateral organ boundaries domain (LBD) genes in Chinese white pear (Pyrus bretschneideri). *BMC Genomics* 21:644. doi: 10.1186/s12864-020-06999-9

Sweetman, C., Wong, D. C., Ford, C. M., and Drew, D. P. (2012). Transcriptome analysis at four developmental stages of grape berry (Vitis vinifera cv. Shiraz) provides insights into regulated and coordinated gene expression. *BMC Genomics* 13:691. doi: 10.1186/1471-2164-13-691

Teng, R.-M., Wang, Y.-X., Wang, W.-L., Li, H., Shen, W., and Zhuang, J. (2018). Genome-wide identification, classification and expression pattern of LBD gene family in Camellia sinensis. *Biotechnol. Biotechnol. Equip.* 32, 1387–1397.

Thatcher, L. F., Powell, J. J., Aitken, E. A. B., Kazan, K., and Manners, J. M. (2012). The lateral organ boundaries domain transcription factor LBD20 functions in Fusarium wilt susceptibility and jasmonate signaling in Arabidopsis. *Plant Physiol.* 160, 407–418. doi: 10.1104/pp.112.199067

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Thrane, C., Kaufmann, U., Stummann, B., and Olsson, S. (2004). Activation of caspase-like activity and poly (ADP-ribose) polymerase degradation during sporulation in Aspergillus nidulans. *Fungal Genet. Biol.* 41, 361–368. doi: 10.1016/j.fgb.2003.11.003

Toffolatti, S. L., De Lorenzis, G., Brilli, M., Moser, M., Shariati, V., Tavakol, E., et al. (2020). Novel aspects on the interaction between grapevine and plasmopara viticola: dual-RNA-Seq analysis highlights gene expression dynamics in the pathogen and the plant during the battle for infection. *Genes (Basel).* 11:261. doi: 10.3390/genes11030261

Toffolatti, S. L., De Lorenzis, G., Costa, A., Maddalena, G., Passera, A., Bonza, M. C., et al. (2018). Unique resistance traits against downy mildew from the center of origin of grapevine (Vitis vinifera). *Sci. Rep.* 8:12523. doi: 10.1038/s41598-018-30413-w

Toffolatti, S. L., Maddalena, G., Salomoni, D., Maghradze, D., Bianco, P. A., and Failla, O. (2016). Evidence of resistance to the downy mildew agent Plasmopara viticola in the Georgian Vitis vinifera germplasm. *Vitis - J. Grapevine Res.* 55, 121–128. doi: 10.5073/vitis.2016.55.121-128

Toffolatti, S. L., Maffi, D., Serrati, L., and Vercesi, A. (2011). Histological and ultrastructural studies on the curative effects of mandipropamid on plasmopara viticola. *J. Phytopathol.* 159, 201–207.

Toffolatti, S. L., Venturini, G., Maffi, D., and Vercesi, A. (2012). Phenotypic and histochemical traits of the interaction between Plasmopara viticolaand resistant or susceptible grapevine varieties. *BMC Plant Biol.* 12:124. doi: 10.1186/1471-2229-12-124

Trouvelot, S., Varnier, A.-L., Allègre, M., Mercier, L., Baillieul, F., Arnould, C., et al. (2008). A β-1,3 glucan sulfate induces resistance in grapevine against plasmopara viticola through priming of defense responses, including HR-like cell death. *Mol. Plant-Microbe Interact.* 21, 232–243. doi: 10.1094/MPMI-21-2-0232

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J. A. M. (2007). Primer3Plus, an enhanced web interface to primer3. *Nucleic Acids Res.* 35, W71–W74. doi: 10.1093/nar/gkm306

Vadlamudi, T., Patil, B. L., Kaldis, A., Sai Gopal, D. V. R., Mishra, R., Berbati, M., et al. (2020). DsRNA-mediated protection against two isolates of Papaya ringspot virus through topical application of dsRNA in papaya. *J. Virol. Methods* 275:113750. doi: 10.1016/j.jviromet.2019.113750

Van Schie, C. C. N., and Takken, F. L. W. (2014). Susceptibility genes 101: how to be a good host. *Annu. Rev. Phytopathol.* 52, 551–581. doi: 10.1146/annurev-phyto-102313-045854

Wang, M., Thomas, N., and Jin, H. (2017). Cross-kingdom RNA trafficking and environmental RNAi for powerful innovative pre- and post-harvest plant protection. *Curr. Opin. Plant Biol.* 38, 133–141. doi: 10.1016/j.pbi.2017.05.003

Wang, X., Zhang, S., Su, L., Liu, X., and Hao, Y. (2013). A genome-wide analysis of the lbd (lateral organ boundaries domain) gene family in malus domestica with a functional characterization of MdLBD11. *PLoS One* 8:e57044. doi: 10.1371/journal.pone.0057044

Wick, R. (2009). Diagnostic tip of the month: clearing and staining roots and other plant tissues. *Natl. Plant Diagnostic Netw. Natl. Newsl.* 4, 3–5.

Xu, B., Li, Z., Zhu, Y., Wang, H., Ma, H., Dong, A., et al. (2008). Arabidopsis genes AS1, AS2, and JAG negatively regulate boundary-specifying genes to promote sepal and petal development. *Plant Physiol.* 146, 566–575. doi: 10.1104/pp.107.113787

Xu, C., Luo, F., and Hochholdinger, F. (2016). lob domain proteins: beyond lateral organ boundaries. *Trends Plant Sci.* 21, 159–167.

Yang, H., Shi, G., Du, H., Wang, H., Zhang, Z., Hu, D., et al. (2017). Genome−wide analysis of soybean lateral organ boundaries domain −containing genes: a functional investigation of GmLBD12. *Plant Genome* 10, 1–19. doi: 10.3835/plantgenome2016.07.0058

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., and Madden, T. L. (2012). Primer-blast: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134. doi: 10.1186/1471-2105-13-134

Yordanov, Y. S., Regan, S., and Busov, V. (2010). Members of the lateral organ boundaries domain transcription factor family are involved in the regulation of secondary growth in populus. *Plant Cell* 22, 3662–3677. doi: 10.1105/tpc.110.078634

Yu, J., Xie, Q., Li, C., Dong, Y., Zhu, S., and Chen, J. (2020). Comprehensive characterization and gene expression patterns of LBD gene family in Gossypium. *Planta* 251:81. doi: 10.1007/s00425-020-03364-8

Yu, Y., Zhang, Y., Yin, L., and Lu, J. (2012). The mode of host resistance to Plasmopara viticola infection of grapevines. *Phytopathology* 102, 1094–1101. doi: 10.1094/PHYTO-02-12-0028-R

Zaidi, S. S.-A., Mukhtar, M. S., and Mansoor, S. (2018). Genome editing: targeting susceptibility genes for plant disease resistance. *Trends Biotechnol.* 36, 898–906.

Zhang, Y., Li, Z., Ma, B., Hou, Q., and Wan, X. (2020). Phylogeny and functions of lob domain proteins in plants. *Int. J. Mol. Sci.* 21:2278. doi: 10.3390/ijms21072278

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REVIEW
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Considerations and Regulatory Approaches in the USA and in the EU for dsRNA-Based Externally Applied Pesticides for Plant Protection

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Increasing pest and pathogen challenges as well as having fewer conventional pesticides to employ require innovative and sustainable solutions for plant protection. One group of pesticides that is in the pipeline and is expected to be subject to regulation and risk assessment procedures in the near future, is based on the natural gene silencing mechanism RNA interference (RNAi). These dsRNA-based products can be highly specific for a target organism due to the sequence-specific interaction between effective small interfering RNAs (siRNAs) and a complementary target RNA. General regulatory frameworks for pesticide authorization in the U.S. and in the EU are presented. In addition, production and application procedures and specific characteristics of dsRNA-based pesticides relevant for risk assessment and regulation are considered.

Keywords: plant protection, pesticide, dsRNA, SIGS, pesticide regulation

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INTRODUCTION

Increasing food demands of a growing world population in combination with new plant pests and diseases due to climate change (Chakraborty and Newton, 2011) call for improved solutions for crop protection. The future potential of current pest and pathogen control in crops based on chemical pesticides is limited because fewer approved pesticides are available, especially in Europe. Actually, it is expected that the "Green Deal" recently initiated in the European Union (EU) will result in a further reduction of active pesticidal substances for the control of insect pests and fungal pathogens in Europe. Therefore, innovative and sustainable approaches are required to combat current and future pest problems.

One possible solution is the cultivation of genetically modified (GM) crops with built-in plant protection. Examples are GM crops expressing toxins from the soil bacterium *Bacillus thuringiensis* (Bt) that specifically kill certain pest insects (e.g., Perlak et al., 1990) and GM maize expressing double-stranded (ds) RNA resulting in the sequence specific degradation of messenger RNA (mRNA) coding for an essential insect protein by an RNA interference (RNAi) mechanism (Bachman et al., 2016). GM crops with such plant-incorporated protectants (PIP) have been authorized for cultivation in the USA several years ago (Mendelsohn et al., 2003; Head et al., 2017). In the EU, however, there is only cultivation of one insect resistant Bt maize line on small hectarages in Spain and Portugal (Gómez-Barbero et al., 2008; ISAAA, 2019).

RNAi, which is a natural gene silencing mechanism in plants and animals (Fire et al., 1998; Baulcombe, 2004) can not only be used for plant-incorporated protection in GM crops by means of host-induced gene silencing (HIGS), but can also serve as a new mode of action for exogenously applied pesticides. There have been various reports of RNAi molecules as plant protection compounds (San Miguel and Scott, 2016; Worrall et al., 2019) since the first successful foliar applications of dsRNAs for plant virus control (Tenllado and Diaz-Ruiz, 2001). Fungal pathogens as well as different insect pests are susceptible to RNAi and can be controlled by topical dsRNA applications (Fletcher et al., 2020; Taning et al., 2020). RNAi is a fast-growing technology with increasing commercial interest, which is reflected by global research trends and the patent situation (Jalaluddin et al., 2019). It is expected that RNAi-based plant protection products, which are much more specific than conventional pesticides and less persistent in the environment, will be available in the near future for practical uses and will need to undergo appropriate safety evaluation and authorization procedures.

UNITED STATES (U.S.) REGULATORY FRAMEWORK FOR PESTICIDES

In the U.S., a pesticide is defined as: (1) any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest; (2) any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant; and (3) any nitrogen stabilizer. Pesticide products must obtain a U.S. Environmental Protection Agency (EPA) registration before manufacture, transport, and sale (Leahy et al., 2014). Field testing prior to a registration requires an Experimental Use Permit. EPA assesses pesticide end-use and manufacturing-use products for registrations and evaluates the active ingredient as well as the actual products. Additionally, states or territories within the U.S. require pesticide product registration as well. While most states or territories rely on the review of data and safety findings of the U.S. federal government, there are several states, e.g., California, that also require data to be submitted to them, which they review and also do their own assessment.

EPA primarily regulates the use of pesticides under the legal basis of two federal statutes enacted by the U.S. Congress: the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). Other statutes that play roles in the regulation of pesticides in the U.S. include the Food Quality Protection Act of 1996 (FQPA), the Pesticide Registration Improvement Act (PRIA), the Endangered Species Act (ESA), the Migratory Bird Treaty Act, and the Clean Water Act. FIFRA is the pesticide licensing law and provides the basis for registration, sale, distribution, and use of pesticides in the U.S. FIFRA authorizes EPA to assess and register pesticides for specific uses. EPA also has the authority to suspend or cancel the registration of a pesticide if subsequent information shows that continued use would pose unreasonable risks. Pesticide registration is based on a risk/benefit standard. Through the registration process, EPA regulates pesticide use through labeling, packaging, composition, and disposal.

EPA must make a finding of no unreasonable adverse effects to man and the environment from use of the pesticide in order to support its registration decision under FIFRA. The Federal Food, Drug, and Cosmetic Act (FFDCA) authorizes EPA to set maximum residue levels, or tolerances, for pesticides used in or on foods or animal feed. Under FFDCA and amendments to both FFDCA and FIFRA under the FQPA, EPA must make a similar finding of a reasonable certainty of no harm if the use of such agents results in residues in food or feed. If the submitted information supports this safety finding, EPA may establish a numerical tolerance or an exemption from the requirement of a tolerance regarding those residues.

Data requirements for assessing pesticide products are laid down in the regulations enacted by EPA in Title 40 of the Code of Federal Regulations (CFR) Part 158. These data requirements support evaluation of both the pesticide active ingredients and products. In addition to data requirements for conventional chemical pesticides, 40 CFR Part 158 lists requirements for biochemical and microbial pesticides which are usually inherently less toxic than conventional chemical pesticides. Biochemical pesticides are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides include substances that interfere with mating, such as insect sex pheromones, as well as various scented plant extracts that attract insect pests to traps. Microbial pesticides consist of a microorganism (e.g., a bacterium, fungus, virus, or protozoan) as the active ingredient. Guidance for conducting the studies listed exist in EPA's Test Guidelines for Pesticides and Toxic Substances[1] (Leahy et al., 2014).

EUROPEAN UNION (EU) REGULATORY FRAMEWORK FOR PLANT PROTECTION PRODUCTS

The authorization process of plant protection products is already outlined in Schenkel and Gathmann (2021). The general principles of the European authorization process are again described hereafter to ease the comparison between the U.S. and the EU in this paper.

In the EU, in general, any plant protection product (PPP)—i.e., a pesticide that protects crops or other useful plants—placed on the market needs an authorization. The legal framework is defined in the Regulation (EC) No 1107/2009 (EC, 2009). The authorization process is divided in approval of the active substance and in the authorization of the PPP. A precondition for the authorization of a PPP is an approval of the active substance (a.s.) by the EU Commission based on a risk assessment led by the European Food Safety Authority (EFSA). A PPP containing an approved active substance is assessed and authorized by the Member States (MS). To enhance the efficiency of the authorization process, the EU is divided in three zones, the

[1] https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/final-test-guidelines-pesticides-and-toxic.

Northern, Central, and Southern zone (EC, 2009). The risk assessment of a PPP is conducted by one Member State [zonal rapporteur Member State (zRMS)] for the whole zone. In principle, MS of the same zone are forced to take over the results of the assessment and the decision of the zRMS. However, MS can make claims on national ecological or agricultural specificities to decide on dividing risk management options for their country.

Data requirements for assessing active substances are laid down in Regulation (EC) No 283/2013 (EC, 2013a) and for PPPs in Regulation (EC) No 284/2013 (EC, 2013b), respectively. Uniform principles for evaluation and authorization of plant protection products are laid down in the implementing Regulation (EU) No 546/2011 (EC, 2011a). The goal of the regulations is to achieve a high level of protection of human and animal health as well as the environment in all Member States. The regulations are complemented by guidance documents produced by OECD, EPPO, and EFSA to describe the methodological requirements for the risk assessment of active substances and pesticide products.

In principle the Regulation (EC) No 1107/2009 (EC, 2009) differentiates between PPPs based on chemicals and microorganisms, where microorganism means any microbiological entity, including lower fungi and viruses, cellular or non-cellular, capable of replication or of transferring genetic material. Specific data requirements for the assessment of microorganisms are laid down for active substances in Part B of Regulation (EC) No 283/2013 (EC, 2013a) and for PPP in Part B of Regulation (EC) No 284/2013 (EC, 2013b), respectively. Additionally, further categories of PPPs are defined in the Regulation (EC) No 1107/2009 (EC, 2009). PPPs classified as low-risk products have to fulfill strict criteria in particular low toxicological potential (and low persistence in the environment (for details see Article 22 Annex II of EC, 2009). Furthermore, no specific risk mitigation measures should be required following the risk assessment for low-risk PPPs and they have to be sufficiently effective. In practice only a few a.s. are authorized in the EU as low-risk PPPs (see EU, 2021). Another category of PPPs are basic substances. A basic substance is not predominantly used for plant protection purpose but nevertheless it is useful in plant protection either directly or in a product consisting of the substance and a simple diluent. It does not have any inherent capacity to cause endocrine disrupting, neurotoxic or immunotoxic effects and it is not a substance of concern (Article 23, EC, 2009), which means it has no capacity to cause an adverse effect on humans (Article 3, EC, 2009). Currently 23 basic substances are approved in the EU for example beer, *Allium cepa* extraxt or L-cysteine (EU, 2021). A category "biopestides" with divergent data requirements as in the U.S. does not exist in the EU. PPPs based on dsRNA do not fit in any of these categories.

The initial focus of the regulations was set on chemicals as active substances. However, Article 77 of the Regulation (EC) No 1107/2019 (EC, 2009) offers the possibility that "the Commission may … adopt or amend technical and other guidance documents such as explanatory notes or guidance documents on the content of the application concerning microorganisms, pheromones and biological products, for the implementation of this regulation. The Commission may ask the Authority to prepare or to contribute to such guidance documents." The rationale behind Article 77 might be that data requirements for these product classes deviate from those for chemicals. Such specific adaptions on the data requirement are already implemented for microorganisms, pheromones and botanicals (EC, 2011b, 2014a,b; EC, 2016). However, no specific guidance documents defining the data requirements for the authorization of dsRNA-based PPPs are in sight, but first considerations and recommendations were presented by OECD (2020).

In summary PPPs based on dsRNA currently have to be considered as chemical PPPs.

PRODUCTION AND APPLICATION METHODS OF dsRNA-BASED PESTICIDES

For successful commercial application of RNAi for crop protection, it is necessary that products are cost-efficient, safe and can be reliably delivered to the site of action in the target organism. There are several ways of production for sequence specific dsRNA. While chemical synthesis as well as *in vitro* transcription is rather costly, dsRNA production in microorganisms is much less expensive (Dalakouras et al., 2020; Taning et al., 2020). However, considerations for microbial production include possible by-products from fermentation and the added issue of genetically modified organisms (GMO). In addition to the usual quality control to ensure dsRNA purity, bacterial production systems require particular care in order to exclude potential contaminants and living GMOs. Recently cell-free platforms for dsRNA synthesis have been established which enable the inexpensive GMO-free production of large dsRNA amounts sufficient for usage in agricultural applications (Taning et al., 2020).

It has been found repeatedly that naked dsRNA is not stable long enough after foliar or soil application to ensure long-lasting plant protection (Mitter et al., 2017; Qiao et al., 2021), especially under field conditions where UV light, rain-fall and microbial degradation account for its rapid degradation and inactivation (Parker et al., 2019; Bachman et al., 2020). Therefore, stabilizing formulations may be necessary for successful usage of dsRNA in topical spray-induced gene silencing (SIGS) applications. Mitter et al. (2017) used positively charged layered double hydroxyde (LDH) clay nanosheets as dsRNA carrier (BioClay) in order to protect dsRNA from degradation after foliar application. Other potential dsRNA carriers that were found to increase the persistence of dsRNA in soil are cationic polymers (Whitfield et al., 2018).

Depending on the target pest or pathogen there are different ways of exogenous application of dsRNA (Dalakouras et al., 2020). Most straightforward is SIGS that was shown to be effective for the control of leaf-feeding insects and against certain phytopathogenic fungi (Koch et al., 2016; San Miguel and Scott, 2016; Wang et al., 2016), when dsRNA uptake into plant cells is not required. If xylem sap-feeding insects or xylem-residing pathogens are the target, more laborious application techniques are required. Dalakouras et al. (2018) demonstrated that double-stranded hairpin (hp) RNA could be delivered into the xylem

of woody plants by trunk injection, where it was systemically transported. With conventional SIGS and trunk injection, dsRNA is not taken up by plant cells and thus is not processed into effective small interfering RNAs (siRNAs) within the plant. For the control of plant pathogenic viruses, however, dsRNA or effector siRNAs need to be delivered into plant cells in order to enter the plant RNA silencing machinery. Entry into plant cells is usually not easily achieved due to the cuticule and the plant cell wall, which act as physical barriers for nucleic acids. For successful delivery of RNAi inducers, mechanical wounding of plant tissues is required. This can be achieved by high-pressure spraying, as was shown by Dalakouras et al. (2016) or by special carrier molecules facilitating cellular uptake. Examples for carrier molecules successfully used for plant cell delivery are positively charged peptides (Numata et al., 2014), cationic fluorescent nanoparticles (Jiang et al., 2014), DNA nanostructures (Zhang et al., 2019), and carbon nanotubes (Demirer et al., 2020). These carriers also provide some protection against RNase degradation inside the plant.

Besides carrier molecules or formulations for the enhancement of environmental stability and plant cellular uptake, additional RNA modifications and/or carrier compounds like chitosan, carbon quantum dots (CQD), or silica nanoparticles may be used to enhance cellular intake by insects (Das et al., 2015). Thereby the range of RNAi-based pesticide control may be extended to pest organisms that are less susceptible to RNAi.

SPECIFIC CHARACTERISTICS OF dsRNA-BASED PESTICIDES

There are several factors to consider regarding the data needed to support dsRNA-based pesticides/PPPs due to their mode of action. In this section we highlight some aspects which might alter data needs for the risk assessment and decision making. A detailed overview about the state of the art is given in the OECD Working paper "Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides" (OECD, 2020).

Due to the sequence-specific interaction of siRNAs with complementary target RNA in RNAi (Fire et al., 1998; Baulcombe, 2004), this mode of action can be highly specific for a target organism or a group of target organisms. Yet, potential effects on non-target organisms (NTOs) due to partial sequence homologies of dsRNA and transcription products in NTOs have to be considered (Christiaens et al., 2018). There may be differences between organisms in terms of how the RNAi machinery functions in relation to base pair mismatches and there are scientific uncertainties about the rules determining interactions between siRNA and mRNA. However, current research on RNAi mechanisms, generation of genomic data libraries for relevant species, and design of algorithms to make reliable predictions are expected to increase the usability of bioinformatics data in the prediction of potential effects on NTOs. Under these conditions, bioinformatics models, and programmes that can access a database with a large selection of key sequences can help in identifying off-target sequences to minimize the risk of potential off-target hits. However, bioinformatics can only be one component of the consideration. Risk assessment is also strongly influenced by biological assay data and an overall understanding of the biological and ecological systems in which the dsRNA will be used (Fletcher et al., 2020). Sequence information and bioinformatics can inform the selection and prioritization of non-target species for toxicity and effects testing. Bioinformatics should be augmented by an empirical approach—to introduce dsRNA (that is perfectly complementary to the selected gene in a target organism) to a range of other organisms, starting with close relatives and then moving outwards, to see how more phylogenetically-distant organisms respond (Bachman et al., 2013; Romeis and Widmer, 2020).

Non-Target organism toxicity study protocols for addressing risk with dsRNA-based products require some revisions compared to how they are carried out for biochemical pesticides because dsRNA-based pesticides often take longer to display efficacy. Any evaluation needs to account for this time lag by extending the study observation period. For organisms that have been demonstrated to be responsive to environmental RNA, consideration of life cycle studies (growth, development, and reproduction) and studies on other non-lethal effects should be considered. The importance of sub-lethal endpoints in addition to mortality has been stressed by Romeis and Widmer (2020), who suggest an approach on how to conduct non-target studies for dsRNA-based plant protection products.

In the evaluation of environmental risks from the application of dsRNA-based pesticides, the distribution, fate, stability, and persistence of the dsRNA following product application in the environment will be important (OECD, 2020). The study by Dubelman et al. (2014) shows a rapid degradation of the DvSnf/dsRNA transcript derived from a genetically modified maize variety in different agricultural soils. Bachman et al. (2020) report a rapid decline in the concentration of foliar applied dsRNA under field conditions with 95% reduction after 3 days. In a recent study, Parker et al. (2019) found that decrease of dsRNA in soil was due to both adsorption to soil and to chemical and microbial degradation. However, the data regarding the environmental fate of dsRNA are still somewhat limited. According to USEPA (2013) the distribution and fate of dsRNA in the environment will depend on the following factors: application rate of active ingredient, application timing, application method, number of applications, off-site movement of applied dsRNA, and the stability and persistence of exogenously applied dsRNA following application.

Some insects, especially of the order Coleoptera (beetles), have been shown to be very sensitive to dsRNA (Baum and Roberts, 2014). Therefore, even small amounts of ingested dsRNA with target sequence homology can induce RNAi which may cause insect mortality. However, there are large differences in the response of insects to ingested dsRNA. Insects of the order Diptera (flies and mosquitoes), Hemiptera (aphids, hoppers, stinkbugs), and Lepidoptera (moths and butterflies), respond to dsRNA with greater variability compared to beetles (Cooper et al., 2019). Additionally, differences in the response can also

be found within the same order between species, life stages, genes, and tissues (reviewed in Christiaens et al., 2020). The current knowledge suggests that dsRNA based products might be very specific for an insect genus or species, respectively. The mechanisms triggering the sensitivity of different species to RNAi are not fully understood yet. The potential use of RNAi technology for pest and plant pathogen control is influenced by the cellular uptake of dsRNA (Cooper et al., 2019; Wytinck et al., 2020). Starting with the uptake process and followed by the behavior of the dsRNA in the organism there are many steps that will affect RNAi efficiency. Cooper et al. (2019) collected different hypotheses which were proposed to explain the observed differences in RNAi efficiency among insects. These factors are instability of dsRNA, incomplete dsRNA internalization, deficient core RNAi machinery, impaired systemic spreading of the RNAi signal and refractory target genes.

The problems identified for insects can be similarly transferred to other fields of dsRNA-based PPP applications. While it has been well-known for some time that certain fungi like *Ustilago maydis* and *Saccharomyces cerevisiae* lack core RNAi components (Drinnenberg et al., 2011), recent findings by Qiao et al. (2021) suggest large differences in dsRNA uptake between fungal pathogens. To counter some of the issues, producers are likely to use carriers and formulations containing synergists or co-formulants that stabilize dsRNA in the environment and enhance transport into the cells of target pests. Special attention should be paid to how barriers to uptake are proposed to be overcome for the target organism. Information and/or studies on the impact of carriers and formulations on uptake and environmental persistence are important to characterize exposure to the dsRNA (OECD, 2020).

Due to their relevance for gene regulation and virus defense in plants and animals, dsRNAs as well as their processing products are natural components of food and feed (Dávalos et al., 2019). Therefore, dsRNAs have a long history of safe consumption by humans and other vertebrates (OECD, 2020). Exposition of humans and farm animals after oral uptake of dsRNA or siRNA has been reported to be negligible because of degradation and multiple barriers in the gastrointestinal tract of mammals (O'Neill et al., 2011; Petrick et al., 2013). As is stated in the OECD Working paper (2020), the common occurrence of effective physiological and biochemical barriers among mammals and probably among other vertebrates is likely a consequence of the widespread presence of RNAs in the environment.

CLASSIFICATION AND AUTHORIZATION PROCEDURE OF dsRNA-BASED PESTICIDES FOR PLANT PROTECTION ACCORDING TO U.S. AND EU REGULATIONS

While data requirements specific to sprayed or externally applied dsRNA-based pesticides have not been enacted in the U.S., EPA bases its requirements for these pesticides on the biochemical pesticide requirements, outlined in Subpart U—Biochemical Pesticides of 40 CFR Part 158[2]. Often, the technical grade material of the pesticide is tested in mammalian and non-target organism toxicity studies along with product formulations for acute mammalian toxicity and irritation studies. Some of this data may be appropriate to waive according to 40 CFR Part 158.45[3]. Further, data not listed in Subpart U for Biochemical Pesticides that is more specific to sprayed or externally applied dsRNA-based pesticides may be required according to 40 CFR Part 158.75[4]. Where specific product formulations impact barriers to and uptake of the dsRNA, such additional data might include product-specific formulation non-target organism toxicology testing to better characterize the potential for hazard.

If a living genetically engineered organism is used in the manufacturing process to produce a sprayed or externally applied dsRNA-based pesticide, but is not viable in the final product, it would be considered a pesticide intermediate in the US under the Toxic Substances Control Act (TSCA). Pesticide intermediates are subject to oversight under TSCA and would likely require submission of a Microbial Commercial Activity Notice (MCAN) before initiating manufacture (Wozniak et al., 2013).

As outlined above, it can be expected that dsRNA-based PPPs will have different properties than the chemicals mostly used as active substances in PPPs until now. Adaptations of the EU data requirements for the risk assessment might be reasonable for different reasons (see previous chapter). In the EU applications of PPPs with dsRNA as active substance are expected in the coming years. Therefore, the Competent Authorities in the EU should start intensive discussion how the risk of such products will be adequately assessed in the future. One cannot expect that specific guidance documents will be in place when first applications are submitted in the EU. Experiences show that the development and implementation of such guidelines take several years. Until then, the same data requirements are requested as for chemical PPPs. Possible waivers or adjustments of the risk assessment for specific areas of concern might be decided on case-by-case basis.

In the EU additional regulatory requirements might be taken into account depending on the used production system. In the case of production systems based on GM microorganisms an authorization according to Directive (EC) 2001/18 is needed if it cannot be guaranteed that the GM microorganisms are completely inactivated (EC, 2001). It is important to note that GMO-derived products which do not contain a living organism and are not to be used as food or feed, like topically applied PPPs, are not regulated under GMO regulations within the EU (see also Schenkel and Gathmann, 2021).

CONCLUSIONS

The thoughts we highlight in this paper are pertinent for assessing the risk of sprayed or externally applied dsRNA-based pesticides for plant protection. Further discussion of

[2]https://www.ecfr.gov/cgi-bin/text-idx?SID=021c1f5a27d7da2ce852a0f903c6709b&mc=true&node=sp40.26.158.u&rgn=div6.

[3]https://www.ecfr.gov/cgi-bin/text-idx?SID=021c1f5a27d7da2ce852a0f903c6709b&mc=true&node=se40.26.158_145&rgn=div8.

[4]https://www.ecfr.gov/cgi-bin/text-idx?SID=021c1f5a27d7da2ce852a0f903c6709b&mc=true&node=se40.26.158_175&rgn=div8.

these regulatory and biosafety issues would help better clarify the risk assessment and risk management framework for these new and innovative products. Sprayed or externally applied dsRNA-based pesticides are in the pipeline and the regulatory authorities will likely be having to assess their risk in the near future.

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REFERENCES

Bachman, P., Bolognesi, R., Moar, W. J., Mueller, G. M., Paradise, M. S., Ramaseshadri, P., et al. (2013). Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). *Transgenic Res*. 22, 1207–1222. doi: 10.1007/s11248-013-9716-5

Bachman, P., Fischer, J., Song, Z., Urbanczyk-Wochniak, E., and Watson, G. (2020). Environmental fate and dissipation of applied dsRNA in soil, aquatic systems and plants. *Front. Plant Sci*. 11:21. doi: 10.3389/fpls.2020.00021

Bachman, P. M., Huizinga, K. M., Jensen, P. D., Mueller, G., Tan, J., Uffman, J. P., et al. (2016). Ecological risk assessment for DvSnf7 RNA: a plant-incorporated protectant with targeted activity against western corn rootworm. *Regul. Toxicol. Pharmacol*. 81, 77–88. doi: 10.1016/j.yrtph.2016.08.001

Baulcombe, D. (2004). RNA silencing in plants. *Nature* 431, 356–363. doi: 10.1038/nature02874

Baum, J. A., and Roberts, J. K. (2014). Progress Towards RNAi-mediated insect pest management. *Adv. Insect Physiol*. 47, 249–295. doi: 10.1016/B978-0-12-800197-4.00005-1

Chakraborty, S., and Newton, A. C. (2011). Climate change, plant diseases and food security: an overview. *Plant Pathol*. 60, 2–14. doi: 10.1111/j.1365-3059.2010.02411.x

Christiaens, O., Dzhambazova, T., Kostov, K., Arpaia, S., Joga, M. R., Urru, I., et al. (2018). Literature review of baseline information on RNAi to support the environmental risk assessment of RNAi-based GM plants. *EFSA* 15:173. doi: 10.2903/sp.efsa.2018.EN-1424

Christiaens, O., Whyard, S., Vélez, A. M., and Smagghe, G. (2020). Double-stranded RNA technology to control insect pests: current status and challenges. *Front. Plant Sci*. 11:451. doi: 10.3389/fpls.2020.00451

Cooper, A. M. W., Silver, K., Zhang, J., Park, Y., and Zhu, K. Y. (2019). Molecular mechanisms influencing efficiency of RNA interference in insects. *Pest Manag. Sci*. 75, 18–28. doi: 10.1002/ps.5126

Dalakouras, A., Jarausch, W., Buchholz, G., Bassler, A., Braun, M., Manthey, T., et al. (2018). Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. *Front. Plant Sci*. 9:1253. doi: 10.3389/fpls.2018.01253

Dalakouras, A., Wassenegger, M., Dadami, E., Ganopoulos, I., Pappas, M. L., and Papadopoulou, K. (2020). Genetically modified organism-free RNA interference: exogenous application of RNA molecules in plants. *Plant Physiol*. 182, 38–50. doi: 10.1104/pp.19.00570

Dalakouras, A., Wassenegger, M., McMillan, J. N., Cardoza, V., Maegele, I., Dadami, E., et al. (2016). Induction of silencing in plants by high-pressure spraying of in vitro-synthesized small RNAs. *Front. Plant Sci*. 7:1327. doi: 10.3389/fpls.2016.01327

Das, S., Debnath, N., Cui, Y., Unrine, J., and Palli, S. R. (2015). Chitosan, carbon quantum dot, and silica nanoparticle mediated dsRNA delivery for gene silencing in Aedes aegypti: a comparative analysis. *ACS Appl. Mater. Interfaces* 7, 19530–19535. doi: 10.1021/acsami.5b05232

Dávalos, A., Henriques, R., Latasa, M. J., Laparra, M., and Coca, M. (2019). Literature review of baseline information on non-coding RNA (ncRNA) to support the risk assessment of ncRNA-based genetically modified plants for food and feed. *EFSA* 16:220. doi: 10.2903/sp.efsa.2019.EN-1688

Demirer, G. S., Zhang, H., Goh, N. S., Pinals, R. L., Chang, R., and Landry, M. P. (2020). Carbon nanocarriers deliver siRNA into intact plant cells for efficient gene knockdown. *Sci. Adv*. 6:eaaz0495. doi: 10.1126/sciadv.aaz0495

Drinnenberg, I. A., Fink, G. R., and Bartel, D. P. (2011). Compatibility with killer explains the rise of RNAi-deficient fungi. *Science* 333:1592. doi: 10.1126/science.1209575

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., et al. (2014). Environmental fate of double-stranded RNA in agricultural soils. *PLoS ONE* 9:e93155. doi: 10.1371/journal.pone.0093155

EC (2001). Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC - Commission Declaration. *OJ L* 106, 1–39.

EC (2009). Regulation (EC) no 1107/2009 of the European parliament and of the council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. *OJ L* 309, 1–50.

EC (2011a). Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products. *OJ L* 155, 127–175.

EC (2011b). *Guidance Document on the Assessment of New Substances Falling Into the Group of Straight Chain Lepidopteran Pheromones (SCLPs) Included in Annex I of Council Directive 91/414/EEC (SANCO/5272/2009 rev. 3 28 October 2011)*.

EC (2013a). Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. *OJ L* 93, 1–84.

EC (2013b). Commission Regulation (EU) No 284/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. *OJ L* 93, 85–152.

EC (2014a). *Guidance Document for the Assessment of the Equivalence of Technical Grade Active Ingredients for Identical Microbial Strains or Isolates Approved Under Regulation (EC) no 1107/2009 (SANCO/12823/2012 –rev. 4,12 December 2014)*.

EC (2014b). *Guidance Document on Botanical Active Substances Used in Plant Protection Products (SANCO/11470/2012– rev. 8 20 March 2014)*.

EC (2016). *Guidance Document on Semiochemical Active Substances and Plant Protection Products (SANTE/12815/2014 rev. 5.2 May 2016)*.

EU (2021). *EU Pesticide Data Base*. Available online at: https://ec.europa.eu/food/plant/pesticides/eu/pesticides/database/active/substances/?event=search.as (accessed May 11, 2021).

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi: 10.1038/35888

Fletcher, S. J., Reeves, P. T., Hoang, B. T., and Mitter, N. (2020). A perspective on RNAi-Based biopesticides. *Front. Plant Sci.* 11:51. doi: 10.3389/fpls.2020.00051

Gómez-Barbero, M., Berbel, J., and Rodriguez-Cerezo, E. (2008). Bt corn in Spain – the performance of the EU's first GM crop. *Nat. Biotechnol.* 26, 384–386. doi: 10.1038/nbt0408-384

Head, G. P., Carroll, M. W., Evans, S. P., Rule, D. M., Willse, A. R., Clark, T. L., et al. (2017). Evaluation of SmartStax and SmartStax PRO maize against western corn rootworm and northern corn rootworm: efficacy and resistance management. *Pest Manag. Sci.* 73, 1883–1899. doi: 10.1002/ps.4554

ISAAA (2019). *Global Status of Commercialized Biotech/GM crops in 2019: Biotech crops Drive Socio-Economic Development and Sustainable Environment in the New Frontier*. Ithaca, NY: ISAAA.

Jalaluddin, N. S. M., Othman, R. Y., and Harikrishna, J. A. (2019). Global trends in research and commercialization of exogenous and endogenous RNAi technologies for crops. *Crit. Rev. Biotechnol.* 39, 67–78. doi: 10.1080/07388551.2018.1496064

Jiang, L., Ding, L., He, B., Shen, J., Xu, Z., Yin, M., et al. (2014). Systemic gene silencing in plants triggered by fluorescent nanoparticle-delivered double-stranded RNA. *Nanoscale* 6, 9965–9969. doi: 10.1039/C4NR03481C

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., et al. (2016). An RNAi-based control of Fusarium graminearum infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathogens* 12:e1005901. doi: 10.1371/journal.ppat.1005901

Leahy, J., Mendelsohn, M., Kough, J., and Jones, R., Berckes, N. (2014). "Biopesticide oversight and registration at the U.S. Environmental Protection Agency in Biopesticides: state of the art and future opportunities," in *ACS Symposium Series*, eds Coats et al. (Washington, DC: American Chemical Society). doi: 10.1021/bk-2014-1172.ch001

Mendelsohn, M., Kough, J., Vaituzis, Z., and Matthews, K. (2003). Are Bt crops safe? *Nat. Biotechnol.* 21, 1003–1009. doi: 10.1038/nbt0903-1003

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., et al. (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* 3:16207. doi: 10.1038/nplants.2016.207

Numata, K., Ohtani, M., Yoshizumi, T., Demura, T., and Kodama, Y. (2014). Local gene silencing in plants via synthetic dsRNA and carrier peptide. *Plant Biotechnol. J.* 12, 1027–1034. doi: 10.1111/pbi.12208

OECD (2020). *Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied dsRNA-based Pesticides*. Series on Pesticides No. 104, ENV/JM/MONO (2020) 26 (Paris).

O'Neill, M. J., Bourre, L., Melgar, S., and O'Driscoll, C. M. (2011). Intestinal delivery of non-viral gene therapeutics: physiological barriers and preclinical models. *Drug Discovery Today* 16, 203–218. doi: 10.1016/j.drudis.2011.01.003

Parker, K. M., Barragán Borrero, V., Van Leeuwen, D. M., Lever, M. A., Mateescu, B., and Sander, M. (2019). Environmental fate of RNA interference pesticides: adsorption and degradation of double-stranded RNA molecules in agricultural soils. *Environ. Sci. Technol.* 53, 3027–3026. doi: 10.1021/acs.est.8b05576

Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T., et al. (1990). Insect resistant cotton plants. *Bio/Technology* 8, 939–949. doi: 10.1038/nbt1090-939

Petrick, J. S., Brower-Toland, B., Jackson, A. L., and Kier, L. D. (2013). Safety assessment of food and feed from biotechnology-derived crops employing RNA-mediated gene regulation to achieve desired traits: a scientific review. *Reg. Toxicol. Pharm.* 66, 157–176. doi: 10.1016/j.yrtph.2013.03.008

Qiao, L., Lan, C., Capriotti, L., Ah-Fong, A., Sanchez, J. N., Hamby, R., et al. (2021). Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake. *bioRxiv [Preprint]*. doi: 10.1101/2021.02.01.429265

Romeis, J., and Widmer, F. (2020). Assessing the risk of topically applied dsRNA-based products to non-target arthropods. *Front. Plant Sci.* 11:679. doi: 10.3389/fpls.2020.00679

San Miguel, K., and Scott, J. G. (2016). The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.* 72, 801–809. doi: 10.1002/ps.4056

Schenkel, W., and Gathmann, A. (2021). "Regulatory aspects of RNAi in plant production," in *RNAi for Improved Crop Performance Plant Improvement and Protection,* eds. B. Mezzetti, J. B. Sweet, and L. Burgos (Oxfordshire: CABI), 154–168. doi: 10.1079/9781789248890.0154

Taning, C. N. T., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., Mezzetti, B., et al. (2020). RNA-based biocontrol compounds: current status and perspectives to reach the market. *Pest Manag. Sci.* 76, 841–845. doi: 10.1002/ps.5686

Tenllado, F., and Diaz-Ruiz, J. R. (2001). Double-stranded RNA-mediated interference with plant virus infection. *J. Virol.* 75, 12288–12297. doi: 10.1128/JVI.75.24.12288-12297.2001

USEPA (2013). *White Paper on RNAi Technology as a Pesticide: Problem Formulation for Human Health and Ecological Risk Assessment*. (Submitted to the FIFRA Science Advisory Panel for review on comment. October 29, 2013). Available online at: https://www.regulations.gov/document/EPA-HQ-OPP-2013-0485-0011 (accessed May 11, 2021).

Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P., Huang, H. D., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* 2:16151. doi: 10.1038/nplants.2016.151

Whitfield, R., Anastasaki, A., Truong, N. P., Cook, A. B., Omedes-Pujol, M., Loczwenski Rose, V., et al. (2018). Efficient binding, protection, and self-relaese of dsRNA in soil by linear and star cationic polymers. *ACS Macro Lett.* 7, 909–915. doi: 10.1021/acsmacrolett.8b00420

Worrall, E. A., Bravo-Cazar, A., Nilon, A. T., Fletcher, S. J., Robinson, K. E., Carr, J. P., et al. (2019). Exogenous applicaton of RNAi-inducing double-stranded RNA inhibits aphid-mediated transmission of a plant virus. *Front. Plant Sci.* 10:265. doi: 10.3389/fpls.2019.00265

Wozniak, C., McClung, G., Gagliardi, J., Segal, M., and Matthews, K. (2013). "Regulation of genetically engineered microorganisms under FIFRA, FFDCA and TSCA," in *Regulation of Agricultural Biotechnology: The United States and Canada*, eds C. A. Wozniak and A. McHughen (Dordrecht: Springer), 57–94. doi: 10.1007/978-94-007-2156-2_4

Wytinck, N., Manchur, C. L., Li, V. H., Whyard, S., and Belmonte, M. F. (2020). dsRNA uptake in plant pests and pathogens: insights into RNAi-based insect and fungal control technology. *Plants* 9:1780. doi: 10.3390/plants9121780

Zhang, H., Demirer, G. S., Zhang, H., Ye, T., Goh, N. S., Aditham, A. J., et al. (2019). DNA nanostructures coordinate gene silencing in mature plants. *Proc. Natl. Acad. Sci U.S.A.* 116, 7543–7548. doi: 10.1073/pnas.1818290116

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REVIEW
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RNA Interference-Based Forest Protection Products (FPPs) Against Wood-Boring Coleopterans: Hope or Hype?

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Forest insects are emerging in large extension in response to ongoing climatic changes, penetrating geographic barriers, utilizing novel hosts, and influencing many hectares of conifer forests worldwide. Current management strategies have been unable to keep pace with forest insect population outbreaks, and therefore novel and aggressive management strategies are urgently required to manage forest insects. RNA interference (RNAi), a Noble Prize-winning discovery, is an emerging approach that can be used for forest protection. The RNAi pathway is triggered by dsRNA molecules, which, in turn, silences genes and disrupts protein function, ultimately causing the death of the targeted insect. RNAi is very effective against pest insects; however, its proficiency varies significantly among insect species, tissues, and genes. The coleopteran forest insects are susceptible to RNAi and can be the initial target, but we lack practical means of delivery, particularly in systems with long-lived, endophagous insects such as the Emerald ash borer, Asian longhorn beetles, and bark beetles. The widespread use of RNAi in forest pest management has major challenges, including its efficiency, target gene selection, dsRNA design, lack of reliable dsRNA delivery methods, non-target and off-target effects, and potential resistance development in wood-boring pest populations. This review focuses on recent innovations in RNAi delivery that can be deployed against forest pests, such as cationic liposome-assisted (lipids), nanoparticle-enabled (polymers or peptides), symbiont-mediated (fungi, bacteria, and viruses), and plant-mediated deliveries (trunk injection, root absorption). Our findings guide future risk analysis of dsRNA-based forest protection products (FPPs) and risk assessment frameworks incorporating sequence complementarity-based analysis for off-target predictions. This review also points out barriers to further developing RNAi for forest pest management and suggests future directions of research that will build the future use of RNAi against wood-boring coleopterans.

Keywords: RNA interference, forest pests, double-stranded RNA delivery methods, enhancing RNAi efficiency, wood-boring coleopterans, symbiont mediated RNAi (SMR), forest protection products (FPPs)

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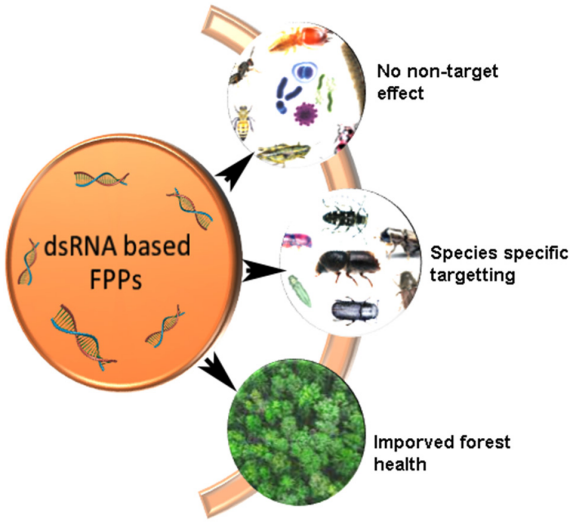

GRAPHICAL ABSTRACT | Three-tier impact of forest protection products (FPPs).

INTRODUCTION

Forests are of immense importance due to their socio-economic and ecosystem services (Pan et al., 2013). However, a decline in conifer forests is ongoing worldwide at an unprecedented rate due to a rise in temperature, drought stress, windthrows, and pest infestation (Safranyik et al., 2010; Hlásny et al., 2019). Most of the insect pest population outbreaks are temperature-dependent, and climate-driven intensification in the frequency, severity and cyclicity of forest pest outbreaks is already well documented (Bentz et al., 2010, 2019; Cudmore et al., 2010; de la Giroday et al., 2012; Haynes et al., 2014; Bentz and Jönsson, 2015). Besides, forest insects have expanded their geographic range by exploiting native hosts previously unexplored due to low temperature (Williams and Liebhold, 2002; Cipollini and Rigsby, 2015; Ramsfield et al., 2016). Such range expansion also causes widespread tree mortality, decreasing forest productivity and carbon storage and substantially enhancing discharge from the decay of dead tree woods (Kurz et al., 2008). Thus, severe depletion of trees due to pest outbreaks or range expansion may cause trajectories outside the resilience limits of forest ecosystems resulting in irreversible ecosystem regime shifts (Dhar et al., 2016).

Coleopteran forest pests such as Emerald ash borer (*Agrilus planipennis*, EAB), Asian longhorn beetles (*Anoplophora glabripennis*, ALB) and bark beetles took advantage of ongoing climate change and cause severe damage to the forests worldwide (Aukema et al., 2011; Meng et al., 2015; Hlásny et al., 2019). For instance, EAB is a devastating, tree-killing phloem-feeding beetle from northeastern Asia recently invaded North America through solid wood packaging material (Poland and McCullough, 2006). EAB has already killed millions of North American Ash (*Fraxinus* sp.) and became one of the costliest insect pest invaders in American history (Aukema et al., 2011). EAB larvae disrupt the translocation of essential nutrients and water in the infested plants while feeding on phloem tissues, leading to the death of the Ash trees within 3–4 years of infestation (Haack et al., 2002). Management of these notorious tree killers is a daunting task, and superior methods can bring hope. ALB, similar to EAB, is native to China and Korea and is a globally recognized invader with a history of attacking more than 100 different species of trees (Haack et al., 2010; Meng et al., 2015). Estimation of loss due to ALB infestation would be a staggering $889 billion if the ALB population left uncontrolled (McKenna et al., 2016). Interestingly, trunk injection of systemic insecticides such as imidacloprid was documented effective against ALB infestation. However, the cost and environmental impact of deploying chemical pesticides jeopardize such strategies and call for better alternatives for ALB management.

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are the most severe and destructive pests of conifer forests worldwide (Fairweather, 2006; Keeling et al., 2016; Hlásny et al., 2019). Since abiotic factors are primary drivers for bark beetle population growth (Biedermann et al., 2019), outbreaks of these aggressive forest pests are expected to increase frequency and severity due to ongoing climate change (Kurz et al., 2008). Warming temperatures promote bark beetle population growth due to reduced winter mortality and development time by allowing additional generations per year. A recent study featuring a tree-ring iso-demographic approach further supports the notion that temperature is more critical than drought for amplifying the eruptive bark beetle outbreaks (Pettit et al., 2020). Most bark beetle species breed on weak and dead trees during an endemic stage serving a crucial function in the forest ecosystem by recycling the nutrients from the dead plant tissues. However, once the bark beetle population increases to an epidemic level, they start attacking the healthy trees leading to an outbreak (Fairweather, 2006; Boone et al., 2011; Hlásny et al., 2019). Currently, frequent outbreaks have been a major disturbing factor for conifer forests in Europe and North America (Bentz et al., 2010; Meddens et al., 2012; Hicke et al., 2016; Hlásny et al., 2019; Lubojacký, 2019) that affects forest ecosystem functioning (Grégoire et al., 2015; Thom and Seidl, 2016), climate and carbon loss mitigation, water retention (Grégoire et al., 2015) and country economy via losses in timber and tourism revenue (Holmes, 1991; SFA, 2010; Pye et al., 2011; Arnberger et al., 2018; Cahyanto et al., 2018). Some aggressive bark beetles, such as the southern pine beetle (*Dendroctonus frontalis*) and the mountain pine beetle (*D. ponderosae*), undergo a substantial range expansion in the US due to favorable warmer climate and cause frequent outbreaks leading to catastrophic tree loss (Regniere, 2003; Chen and Goodwin, 2011; Lesk et al., 2017).

Several conventional approaches such as sanitation felling (Wermelinger, 2004; Seidl et al., 2016), removal of wind felled trees (Leverkus et al., 2018), and deployment of pheromone-baited and poisoned log tripod traps (Wermelinger, 2004) is used for the last few decades to manage the bark beetle population levels in endemic phase. However, the success of all these approaches is questionable in managing the recent bark beetle outbreaks (Billings, 2011; Hlásny et al., 2019). Furthermore, similar to other wood-boring forest insects,

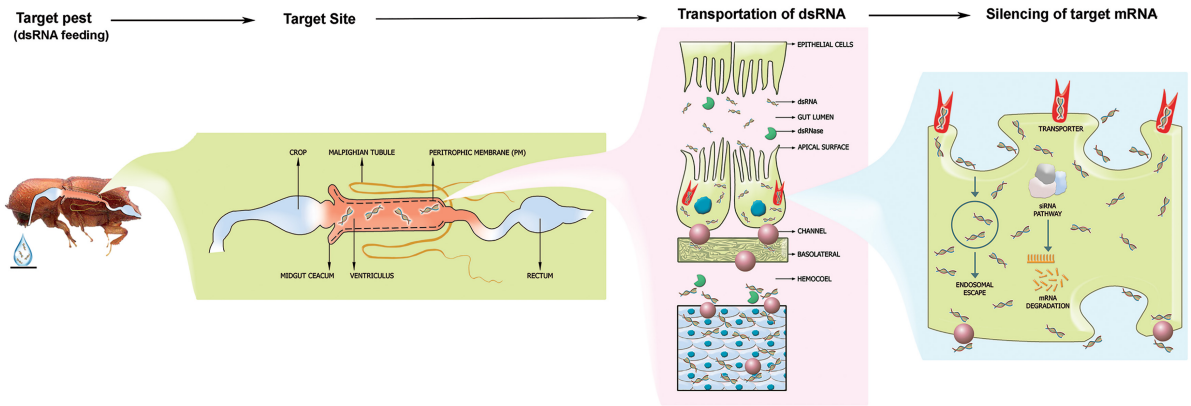

FIGURE 1 | The fundamental mechanism of RNAi from a coleopteran pest control standpoint. The target pest is feeding on the species-specific, gene-specific dsRNA droplet. After ingestion, the dsRNA will reach the midgut and passes through the peritrophic membrane. Subsequently, the dsRNA will be up-taken by the gut epithelial cells through the scavenger receptor-mediated endocytosis pathway and the transmembrane Sid-1 channel protein-mediated pathway. Further, the dsRNA is exported to hemocoel and fat bodies connected all over the body. The siRNA mechanism will be activated and triggers gene silencing upon dsRNA uptake by cells. Endosomal escape of the silencing signal resulting in RNAi efficiency within coleopteran pest induces RNAi effect.

several synthetic pesticides have also been used to suppress bark beetles over the past years (Williamson and Vité, 1971). However, many of these compounds caused other problems such as environmental pollution, detrimental effects on non-target organisms, and widespread pesticide resistance (Feder, 1979; Baum et al., 2007; Billings, 2011). Therefore, questions have been raised about the feasibility, effectiveness, and purpose of conventional phytosanitary measures. Hence, novel and aggressive management of these devastating coleopteran wood-boring forest pests is the highest priority in the Anthropocene.

RNA interference (RNAi) is an evolutionarily conserved post-transcriptional gene silencing mechanism, which is triggered by exogenous double-stranded RNA (dsRNA) (Fire et al., 1998; Zhu and Palli, 2020). Thus, RNAi becomes a promising tool for forest pest management in this era of genomics (Baum et al., 2007). Recent advancements in sequencing technology and platforms lead to higher availability of coleopteran forest pest genomes and transcriptomes that can serve as valuable resources for species-specific dsRNA design (Keeling et al., 2012, 2013; Scully et al., 2013; McKenna et al., 2016; Powell et al., 2020). It was pretty well known that coleopteran insects are usually susceptible to RNAi (**Figure 1**; Baum et al., 2007; Zhu et al., 2011; Palli, 2014; Prentice et al., 2015; Ulrich et al., 2015; Fishilevich et al., 2016; Li et al., 2018a; Bramlett et al., 2020; Mehlhorn et al., 2021; Willow et al., 2021). Recently, Yoon et al. (2018) reported the underlying cause of coleopteran insect susceptibility toward RNAi. With intriguing evidence of RNAi susceptibility in coleopterans, researchers started exploring the potential of RNAi in managing coleopteran forest pests (**Table 1**). Recent high-quality publications demonstrated the entomotoxicity of RNAi against wood-boring coleopteran forest pests such as southern pine beetle, mountain pine beetles, emerald ash borer, Asian longhorn beetles, and Chinese White pine beetle (Rodrigues et al., 2017a,b; Rodrigues et al., 2018; Kyre et al., 2019; Dhandapani et al., 2020a,b; Kyre et al., 2020). However, the potential of RNAi in coleopteran forest pest management is not yet comprehensively summarized elsewhere. Hence, it is essential to capture all aspects of such studies together and critically evaluate the future potential of RNAi against wood-boring coleopteran pest management. The current review focuses on synthesizing key challenges for RNAi-mediated forest pest management (**Table 2**). It can also serve as a valuable source of information for general foresters, private forest owners, forest managers, and researchers worldwide who are currently using RNAi or planning to use RNAi as a tool against coleopteran pests inside the forests.

RNAi MECHANISM IN INSECT PESTS: AN OVERVIEW

RNAi refers to a post-transcriptional gene silencing mechanism prohibiting protein formation by introducing environmental RNA (Fire et al., 1998). Three RNAi pathways have been characterized so far. These are common in insects but not in plants or other animals and include the small interfering RNA (siRNA) pathways, microRNA (miRNA) pathway, and piwiRNA (piRNA) pathway. However, siRNAs are highly sequence-specific to target transcripts, and miRNAs are partial complementarity to target transcripts (Lam et al., 2015; Zhu and Palli, 2020). In contrast to siRNAs and miRNAs, the piRNAs pathway is likely less understood (Farazi et al., 2008).

The RNAi technology application depends on introducing dsRNA into the insect pest body to silence a target gene, subsequently activating the siRNA pathway. Briefly, upon entry of exogenous dsRNA into the cell, the dsRNA is processed into siRNAs by an enzyme ribonuclease III, called Dicer-2. These siRNAs (21–24 nucleotide duplexes) are incorporated in the silencing complex, called the RNA-induced silencing complex (RISC), where the siRNA duplex is unwound. Subsequently, a protein called Argonaute2 (AGO2) cleaves the sense (passenger) strand, and the antisense (guide) strand remains connected with

TABLE 1 | List of RNAi experiments for wood-boring coleopteran pests.

S. No.	Author	Test organism	Common name	Target gene	Accession no.	Molecule (dsRNA/siRNA)	Size (bp)	Life stage tested	Mode of delivery	Concentration (μg/μL)	Measurement endpoints
1	Rodrigues et al., 2017b	*Agrilus planipennis*	Emerald ash borer	COP	Not informed	dsRNA	247	Larvae	Feeding	3 μg	24% mortality
				IAP	Not informed	dsRNA	272	Larvae	Feeding	3 μg	33% mortality
				IAP		dsRNA	272	Larvae	Feeding	10 μg	78% mortality
				IAP		dsRNA	272	Larvae	Feeding	1 μg	30% mortality
				IAP		dsRNA	272	Larvae	Feeding	6 μg	35% mortality
2	Rodrigues et al., 2018	*Agrilus planipennis*	Emerald ash borer	HSP	XM_018474521.1	dsRNA	468	Larvae	Feeding	10 μg	90% mortality
				HSP		dsRNA	468	Larvae	Feeding	1 μg	67% mortality
				HSP		dsRNA	468	Adults	Feeding	10 μg	40% mortality
				Shi	XM_018465318.1	dsRNA	483	Larvae	Feeding	10 μg	90% mortality
				Shi		dsRNA	483	Larvae	Feeding	1 μg	∼40% mortality
				Shi		dsRNA	483	Adults	Feeding	10 μg	30% mortality
				Shi + HSP	XM_018465318.1 + XM_018474521.1	dsRNA	483 + 468	Adults	Feeding	1 μg (500 ng/ μl each)	90% mortality
3	Zhao et al., 2015	*Agrilus planipennis*	Emerald ash borer	AplaScrB-2	KJ634683	dsRNA	475	Adults	Injection	200 ng	The expression of AplaScrB-2 was 73% repressed on day 3 and 90% repressed on day 6
4	Leelesh and Rieske, 2020	*Agrilus planipennis*	Emerald ash borer	Shi	XM_018465318.1	dsRNA	483	Larvae	Feeding (recombinant bacteria expressing dsRNA)	3 μL of bacterial suspension	69.44% mortality
				HSP	XM_018474521.1	dsRNA	468	Larvae			46.66% mortality
5	Dhandapani et al., 2020a	*Anoplophora glabripennis*	Asian longhorned beetle	IAP	XM_018711271.2	dsRNA	386	Larvae	Injection	10 μg	100% mortality
				Prosβ5	XM_018713596.1	dsRNA	377	Larvae	Injection	10 μg	80% mortality
				RpL6	XM_018709657.1	dsRNA	433	Larvae	Injection	10 μg	60% mortality
				Cas	XM_018707893.1	dsRNA	429	Larvae	Injection	10 μg	60% mortality
				Surf4	XM_018716988	dsRNA	357	Larvae	Injection	10 μg	60% mortality
				Ebony	XM_018713129	dsRNA	413	Larvae	Injection	10 μg	60% mortality
				Actin	XM_018721905.1	dsRNA	436	Larvae	Injection	10 μg	50% mortality
				SNF7	XM_018722997.1	dsRNA	342	Larvae	Injection	10 μg	50% mortality
				Dre4	XM_018708786.1	dsRNA	314	Larvae	Injection	10 μg	50% mortality
				Prosα6	XM_018714266.1	dsRNA	374	Larvae	Injection	10 μg	50% mortality
				Sec61α	XM_018707923.1	dsRNA	443	Larvae	Injection	10 μg	50% mortality
				VhaSFD	XM_018721020.1	dsRNA	410	Larvae	Injection	10 μg	50% mortality
				Unc-104	XM_018711981.1	dsRNA	406	Larvae	Injection	10 μg	40% mortality
				Rpn11	XM_018719490.1	dsRNA	441	Larvae	Injection	10 μg	40% mortality
				Sam-S	XM_018717099.1	dsRNA	425	Larvae	Injection	10 μg	40% mortality
				SSK	XM_018724815.1	dsRNA	326	Larvae	Injection	10 μg	40% mortality

(Continued)

TABLE 1 | Continued

S. No.	Author	Test organism	Common name	Target gene	Accession no.	Molecule (dsRNA/siRNA)	Size (bp)	Life stage tested	Mode of delivery	Concentration (μg/μL)	Measurement endpoints
				MESH	XM_018707459.2	dsRNA	341	Larvae	Injection	10 μg	40% mortality
				GW	XM_023456527.1	dsRNA	482	Larvae	Injection	10 μg	40% mortality
				IAP	XM_018711271.2	dsRNA	386	Adults	Injection	10 μg	100% mortality
				SNF7	XM_018722997.1	dsRNA	342	Adults	Injection	10 μg	100% mortality
				Shi	XM_018714700.1	dsRNA	427	Adults	Injection	10 μg	100% mortality
				Dre4	XM_018708786.1	dsRNA	314	Adults	Injection	10 μg	100% mortality
				Prosβ5	XM_018713596.1	dsRNA	377	Adults	Injection	10 μg	100% mortality
				Sec61α	XM_018707923.1	dsRNA	443	Adults	Injection	10 μg	100% mortality
				Sar1	XM_018718347.1	dsRNA	421	Adults	Injection	10 μg	∼80% mortality
				SSK	XM_018724815.1	dsRNA	326	Adults	Injection	10 μg	∼80% mortality
				Prosα6	XM_018714266.1	dsRNA	374	Adults	Injection	10 μg	∼80% mortality
				IAP	XM_018711271.2	dsRNA	386	Larvae and adults	Feeding	12 μg	No significant mortality in larvae and adults were observed and also did not notice knockdown of the IAP gene after feeding dsRNA
6	Dhandapani et al., 2020a,b	*Anoplophora glabripennis*	Asian longhorned beetle	IAP	XM_018711271.2	dsRNA	386	Larvae	Feeding	2 μg, 5 μg, 10 μg/day for 3 days	17%, 67% and 90% mortality
				SNF7	XM_018722997.1	dsRNA	342	Larvae	Feeding	2 μg, 5 μg, 10 μg/day for 3 days	25%, 50% and 75% mortality
				SSK	XM_018724815.1	dsRNA	326	Larvae	Feeding	2 μg, 5 μg, 10 μg/day for 3 days	17%, 67% and 80% mortality
7	Kyre et al., 2019	*Dendroctonus frontalis*	Southern pine beetle	HSP	XM_019906798.1	dsRNA	315	Adults	Feeding	10 μg	100% mortality
				Shi	XM_019900326.1	dsRNA	342	Adults	Feeding	10 μg	86.67% mortality
				IAP	XM_019910372.1	dsRNA	341	Adults	Feeding	10 μg	20% mortality
8	Kyre et al., 2020	*Dendroctonus ponderosae*	Mountain pine beetle	HSP	Not informed	dsRNA	351	Adults	Feeding	2.5 μg	∼85% mortality
				Shi	Not informed	dsRNA	379	Adults	Feeding	2.5 μg	∼80% mortality
				IAP	Not informed	dsRNA	370	Adults	Feeding	2.5 μg	∼75% mortality
9	Li et al., 2018b	*Dendroctonus armandi*	Chinese white pine beetle	CSP2	AGI05172.1	dsRNA	Not informed	Adults	Injection	200 ng	Antennal EAG activity reduced in response to host volatiles [(+)-α-pinene, (+)-β-pinene, (−)-β-pinene, (+)-camphene, (+)-3-carene, and myrcene]
10	Fu et al., 2019	*Dendroctonus armandi*	Chinese white pine beetle	DaAqp12L	XP_018562473.1	dsRNA	Not informed	Larvae	Injection	200 ng	Mortality of larvae were higher after cold stress

TABLE 2 | Challenges and putative mitigation strategies for the deployment of dsRNA-based forest protection products (FPPs).

Challenges	Putative mitigation
Lack of thorough knowledge of the mechanism of action.	It is essential to know the mechanism of action of dsRNA in target pests to measure the sustainability of the putative control measure. Therefore, dedicated studies on the mechanism of RNAi in forest pests are required. The recent study by Yoon et al. (2018) shows the way forward.
Limited number of forest pest genomes	Sequencing of more forest pest genomes will pave the way for highly efficient and species-specific FPPs.
Low efficiency in key pest species	Appropriate target genes need to be identified for control. Therefore, more studies are required to evaluate potential target genes in forest pest species, and their geographic variability in expression needs to be assessed.
Lack of much information on off-target, non-target effects of dsRNA	The unintended effects caused by dsRNA include silencing of target gene homologs in non-target organisms, off-target silencing of the gene in the target, non-target insects, a saturation of RNAi machinery, and stimulation of immune response. All these effects could influence the performance of natural control agents such as predators, parasites, etc. Hence, the persistence of dsRNA in the forest and the effect of dsRNAs on non-target organisms (NTOs) in the forest ecosystem need to be investigated thoroughly before applying dsRNA inside the forest for pest management.
dsRNA stability	Often low dsRNA stability becomes the major issue. Nanoparticle-based delivery methods can be tested for increasing the efficacy of the dsRNA.
Method of delivery	Considering the vast target area (forest) for application, it is crucial to formulate a cost-effective way to deliver the target dsRNA. Using symbiotic microorganisms such as bacteria or fungi as a potential carrier for dsRNA can be a promising approach.
Higher production and formulation cost	Methods for economical production and formulation of dsRNA need to be developed to compete well with the other commercial insecticides. Chemical synthesis and production of dsRNA in bacteria can pave the way for low-cost production.
The unknown potential for resistance development	Dedicated studies are required to assess the potential for resistance development in target insects as they can achieve resistance to dsRNA by a single mutation, for example, a mutation in the gene coding for proteins involved in dsRNA transport.
Public awareness	The public, forest authorities, and other individual forest owners need to be educated about RNAi technology by organizing public outreach events.

the RISC. Afterward, the antisense strand of the siRNA guides the RISC and allows base pairing to the complementary target mRNA. Subsequently, AGO2 protein degrades or cleaves the target mRNA, and specific post-transcriptional gene silencing occurs (Agrawal et al., 2003; Pecot et al., 2011).

Variable RNAi Efficiency: What Matters?

A significant degree of variability in RNAi efficiency has been observed between insects and between different orders and between members of the same insect order (Singh et al., 2017; Cooper et al., 2019). In addition, RNAi efficiency can vary among the same transcripts and different areas of the same transcripts, among different transcripts, genotypes, and tissues of the same transcripts (Baum et al., 2007; Zhu et al., 2011; Luo et al., 2013; Zhang et al., 2013; Camargo et al., 2015; Ulrich et al., 2015). For example, coleopterans are more susceptible to RNAi than other insect orders (Terenius et al., 2011; Singh et al., 2017; Cooper et al., 2019; Santos et al., 2021). In Coleoptera, several insects, including the Colorado potato beetle [*Leptinotarsa decemlineata*, CPB] (Zhu et al., 2011; Palli, 2014; Mehlhorn et al., 2021), Western corn rootworm [*Diabrotica virgifera virgifera*, WCR] (Baum et al., 2007; Li et al., 2015b; Fishilevich et al., 2016; Li et al., 2018a), EAB (Rodrigues et al., 2018; Leelesh and Rieske, 2020; Pampolini and Rieske, 2020), and ALB (Rodrigues et al., 2017b; Dhandapani et al., 2020a) have shown a remarkably high sensitivity toward RNAi. In contrast, some other Coleopterans, such as the model insect *Tribolium castaneum* and the African sweet potato weevil [*Cylas puncticollis*], seem less sensitive to RNAi when the dsRNA is administered orally (Prentice et al., 2017). A review by Joga et al. (2016) has discussed several factors contributing to this variability in RNAi sensitivity. These include cellular uptake from the gut environment and dsRNA stability in the digestive system due to enzymatic degradation and a high pH. These factors need to be considered for each target forest pests to ensure the higher efficacy of RNAi.

Systemic Properties and dsRNA Uptake

Two types of RNAi are categorized: cell-autonomous RNAi (within a cell) and non-cell-autonomous RNAi (one cell to another and one tissue to another). Environmental RNAi and systemic RNAi together are named non-cell-autonomous RNAi. Environmental RNAi refers to the uptake of exogenous dsRNA by cells in which gene silencing will take place. Whereas systemic RNAi refers to the spread of RNAi signal from one cell to another cell or tissues in the body of an organism (Jose and Hunter, 2007; Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). For the success of RNAi in any organism, including wood-boring coleopteran forest pests, both environmental RNAi and systemic RNAi should be present and robust.

Two different dsRNA uptake pathways have been illustrated in insects so far. They are the scavenger receptor-mediated endocytosis pathway and the transmembrane Sid-1 channel protein-mediated pathway (Winston et al., 2002; Ulvila et al., 2006; Shih and Hunter, 2011; Wynant et al., 2014a; Cappelle et al., 2016). Several studies have shown that Sid-1 like channel proteins are involved in dsRNA uptake in most insect species, such as the red flour beetle *Tribolium castaneum* (Tomoyasu et al., 2008), the brown planthopper, *Nilaparvata lugens* (Xu et al., 2013), and CPB (Cappelle et al., 2016). In contrast, in dipteran such as the common fruit fly *Drosophila melanogaster*, the dsRNA uptake relies on receptor-mediated endocytosis as this insect lacks *Sid-1* like genes in its genome (Ulvila et al., 2006). Moreover, the number of *Sid-1*-like genes found in the genome of insects varies between insects belonging to different species and orders (Joga et al., 2016). Insects that possess both the transmembrane Sid-1 channel protein-mediated

pathway have shown robust environmental RNAi and systemic RNA, for example, *L. decemlineata* (Cappelle et al., 2016). For that reason, CPB is very sensitive to RNAi. However, it was reported that a dsRNA binding protein called Staufen C plays a crucial role in processing the silencing signal and RNAi initiation (Yoon et al., 2018). The lack of Staufen C protein makes lepidopterans less efficient to RNAi.

An enzyme called RNA-dependent RNA polymerase (RdRP) produces the secondary siRNAs by a primer-independent mechanism, amplifying and extending the silencing effect (Schiebel et al., 1993). RdRPs are present in plants and certain eukaryotes, for example, nematodes (Cogoni and Macino, 1999; Mourrain et al., 2000; Smardon et al., 2000; Vaistij et al., 2002). In contrast, no clear RdRP homologs have been found in any insect pest genome so far. It does not mean that insects do not possess a silencing amplification system. RNAi sensitive insects, for instance, coleopterans, have a strong RNAi effect, and the silencing signal lasts for a more extended period. On the other hand, RNAi recalcitrant insects, such as lepidopterans, have moderate or minimal RNAi effect, and the silencing signal is often concise. It indicates that insects have a silencing amplification system, but its mechanism depends on another enzyme with a much similar mechanism as RdRP or depending on another mechanism that has to be discovered (Joga et al., 2016).

The dsRNA uptake by the epithelial cells from the insect gut of the perimicrovillar membrane (PM) is crucial for the success of RNAi as the PM allows the uptake of vitamins, minerals, and insecticidal molecules. However, it is not clear to what extent the PM in the midgut of insect pest functions as a physical barrier to the delivery of dsRNA (Lehane, 1997; Silva et al., 2004; Hegedus et al., 2009; Walski et al., 2014; Liu et al., 2019).

Nucleases, Gut pH, and Viruses

Generally, the dsRNA is more stable than the single-stranded RNA, and it should be uptaken by the midgut epithelial cells where the RNAi mechanism will be activated (Katoch and Thakur, 2012). However, salivary nucleases and gut nucleases degrade the dsRNA, limiting the RNAi efficiency (Thompson et al., 2012; Christensen et al., 2013). Christiaens et al. (2014) reported that the ingested dsRNA is degraded rapidly by dsRNases in the salivary secretions and hemolymph of the pea aphid, *Acyrthosiphon pisum*. In addition, Wynant et al. (2014b) reported that the dsRNA degraded in the midgut juices of the pest desert locust, *Schistocerca gregaria*. Similar observations were done by Taning et al. (2016) with the Asian fruitfly *Drosophila suzukii* and southern green stinkbug *Nezara viridula* by Sharma et al. (2021). Fortunately, RNAi susceptible insects often showed less degradation of dsRNA by dsRNAses.

The pH in the gut of insects has been found to vary between insects belonging to different orders, for example, high acidic in Coleopterans and high alkaline in few Lepidopterans. The alkaline nature of the midgut plays an important barrier for the delivery of dsRNA in Lepidopteran species and provides an unfavorable environment for ingested dsRNA (Dow, 1992; Kolliopoulou et al., 2017). Furthermore, the existence of viruses may also play a critical obstacle for RNAi efficiency as the viruses can saturate RNAi core machinery (Kanasty et al., 2012) and development of RNAi-blocking proteins called viral suppressors of RNA silencing (VSRs) (Haasnoot et al., 2007). Hence, all such possibilities need to be evaluated in the target forest pests.

Length and Concentration of dsRNA

The length and the concentration of exogenous dsRNAs are significant for the success of RNAi. However, the requisite length of dsRNA varies among insect pests (Bolognesi et al., 2012). For example, Miller et al. (2012) reported that 60 base pairs length of dsRNA induced 70% gene knockdown and 30 base pairs length of dsRNA induced 30% gene knockdown in *T. castaneum*. This study clearly explains that long dsRNAs are required for effective RNAi in insects. However, many studies reported that effective RNAi was seen when 140 to 500 -bp dsRNAs were used (Huvenne and Smagghe, 2010). Whereas the short length dsRNAs are preferred to induce the target-specific gene silencing to minimize the off-target and non-target effects.

For effective RNAi, optimal concentration has to be determined for every target gene in the forest pests. For example, Baum et al. (2007) demonstrated that dsRNA targeting the V-ATPase gene caused silencing in the WCR in a concentration-dependent manner. However, achieving higher silencing by exceeding optimal concentration is not true (Meyering-Vos and Müller, 2007; Shakesby et al., 2009). Moreover, introducing multiple dsRNAs of different target genes into the insect body may lead to poor RNAi efficiency due to competition among introduced dsRNAs during cellular uptake (Parrish et al., 2000; Barik, 2006; Miller et al., 2012).

PROSPECTS AND CHALLENGES FOR RNAi AGAINST FOREST PESTS

Wood-boring insects thrive inside the bark, trunk on a nutritionally limiting diet. They tunnel in the inner layer where water and nutrients are available. They attack either healthy or weakened and dead trees based on their statuses like primary invader or secondary invader. It is worth mentioning that primary invaders (i.e., *Ips typographus*, Eurasian spruce bark beetle) mostly kill the infested tree. However, most often, the damage caused by an infestation of wood borers remains unnoticed until the tree showed visible symptoms or external signs of damage, such as the entry hole of a wood borer or sawdust (Hlásny et al., 2019). This hidden lifestyle of wood borers, contrary to most other agricultural pests, causes considerable impediments for control measures, even for RNAi-based FPPs. Perhaps choosing a suitable strategy to deliver dsRNA is a big challenge in RNAi-based forest protection methods (**Table 2**). Several possible dsRNA application strategies are available that can be deployed against forest pests. They are transiently transformative (recombinant symbiont or virus) and non-transformative (nanoparticles, trunk injections and spraying, root soaking, and soil drench) methods (**Figure 2**). Due to the transient feature of the molecules used in non-transformative delivery methods, target pests have limited exposure to the dsRNA molecules, delaying the resistance

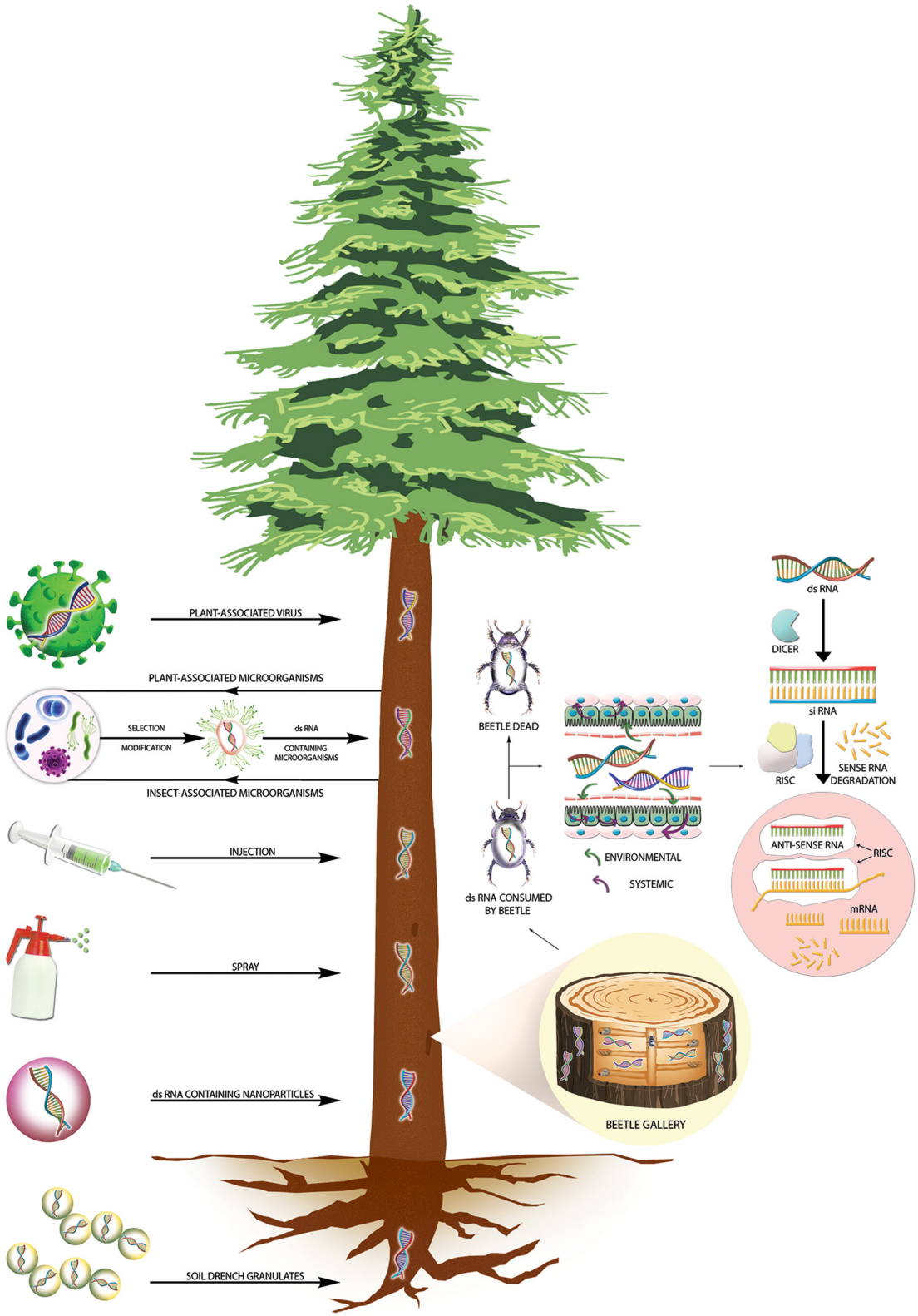

FIGURE 2 | RNAi-mediated plant protection from insect pests at the tree level. The left panel shows different transformative and non-transformative dsRNA delivery strategies to control wood-boring insect pests at the tree level. A section magnified in the right panel shows dsRNA in both vessel systems (xylem and phloem), allowing dsRNA to move up and down and reach the insect galleries. Upon ingestion of dsRNA by wood-boring coleopterans, the dsRNA will reach the gut epithelial cells, where the cellular siRNA mechanism of gene silencing will be initiated, which will lead to target insect death.

development. Deploying the RNAi based Plant-incorporated protectants (RNAi-PIPs) via transgenic trees (transformative approach) seems to be a less viable solution against wood-boring forest pests due to public and scientific concern (i.e., gene flow), lack of suitable tree transformation protocols, high development time and cost, and extensive regulatory processes (Cagliari et al., 2019). Hence, in the present review, we omitted the discussion on the transformative approaches. However, it is worth mentioning here that researchers already developed efficient and stable plastid transformation protocols for poplar, which can be considered for developing RNAi-PIPs against pests infesting the green tissues of poplar (Wu et al., 2019).

Selection of Target Gene

The success or failure of RNAi technology experiments mainly depends on the selection of the target gene. For successful gene silencing, selection of target genes and target regions within genes, its expression pattern (developmental and tissue-specific), the insect species and/or population, and the structure and sequence of the dsRNA are crucial. The second-generation sequencing, genome-wide screens, existing data from closely related species, tissue, and developmental stage-specific expression profiles, and gene ontology are valuable tools that can provide information on target gene selection and screening but require more extensive resources to perform (Wang et al., 2011; Knorr et al., 2018). The ideal target gene should have a high transcription rate and produce a protein with a low half-life, and transcription reduction of the intended target gene must cause mortality in the pest insect (Scott et al., 2013; Cooper et al., 2020). After candidate gene selection, dsRNA-induced mortality screening in multiple life stages is required to assess the desired phenotype induced by specific dsRNA. Often it is worth starting screening multiple genes for putative candidate selection for RNAi application. Moreover, the stage and tissue-specific expression levels of core genes in the RNAi machinery must be evaluated to achieve the optimum window for RNAi application against target forest pests (You et al., 2020). It will be optimal to follow up the RNAi studies via RNAiSeq studies to evaluate the changes in the expression of other genes impacted by the RNAi treatment (Oppert and Perkin, 2019; Xu et al., 2021). Furthermore, based on the recent report, it will be optimal to evaluate any synergistic engagement of the resident gut microbiome in the dsRNA-induced mortality of target insects (Xu et al., 2021). Such approaches will lead to a better understanding of the mechanism of action of RNAi-based FPPs and facilitate the enhancement of their sustainability.

RNAi for Disrupting Pest Communication

The main objective of RNAi-based FPPs is to reduce the forest pest population level below the epidemic level. Pheromone is a species-specific chemical substance for insect communication. The RNAi can be deployed to disturb the pest's reproductive behavior by silencing genes involved in producing sex pheromones. For instance, *Helicoverpa armigera* could not find the female moths when two pheromone-binding proteins were silenced by RNAi, which decreased mating behavior (Dong et al., 2017). Similarly, genes involved in pheromone production in bark beetles can be targeted via RNAi to disrupt communication, such as aggregation pheromone signal for a mass attack in *Ips typographus*. The delivery of dsRNA through trunk injection and/or soil drench molecules may move through the phloem, and the pheromone-binding proteins will be silenced in the beetle upon phloem-feeding (**Figure 1**).

Design of Species-Specific dsRNA

The optimal length of dsRNA uptake varies from insect to insect, and previous reports showed that this optimum lies between 200 and 520 bp (Huvenne and Smagghe, 2010; Bolognesi et al., 2012). Recent mutagenesis analysis revealed dsRNA with more than 80% sequence complementarity with the target gene substantially triggers the RNAi effect (Chen et al., 2021). A dsRNA containing $\geq$ 16 bp regions of perfect complementarity or >26 bp regions of nearly matched sequence with one or two mismatches barely distributed (i.e., single discrepancy placed between $\geq$ 5 bp complementary region or incompatible couplets inserted between $\geq$ 8 bp complementary regions) also trigger RNAi mediated gene silencing (Chen et al., 2021). A similar finding was also documented about RNAi against plant sap-feeding hemipteran pests indicating maximum dsRNA sequence complementarity was crucial for RNAi-based gene silencing in related hemipteran species (Arora et al., 2021). Additionally, most of the genes are not stably expressed during the life cycle of the insect, and the dosage of dsRNA concentration should be adjusted according to the abundance of target mRNA. The concentration should be species-specific as the RNAi efficiency is less where dsRNAses are more (i.e., insect gut), and in such cases, an overdose of dsRNA may be required to induce the desired knockdown of target genes (Scott et al., 2013). Considering such parameters to estimate off-target effect in the available target and non-target organism genome using bioinformatics tools (i.e., genome-wide blast analysis), dsRNAs can be designed with higher efficiency and species specificity. For instance, targeting segments (i.e., >100 bp with no contiguous stretches of sequence identity more than 20 bp) from orthologous genes with high divergence regions (HDRs) or homologous genes with HDRs or genes that are lost in all closely related species can be a good starting point for securing species-specific targets for RNAi (**Figure 3A**). Advanced bioinformatics pipelines can be prepared to find such targets in the forest pest genomes for screening. Hence, having high-quality forest pest genomes and transcriptomes (**Table 3**) is also critical in designing species-specific dsRNA (**Table 1**).

Evaluation of Off-Target and Non-target Effects

To minimize the off-target effects and avoid cross silencing, the dsRNA constructs should be chosen in non-conserved regions of other species or isoforms of the target gene (**Figure 3B**). The previous studies have shown that the well-designed dsRNA

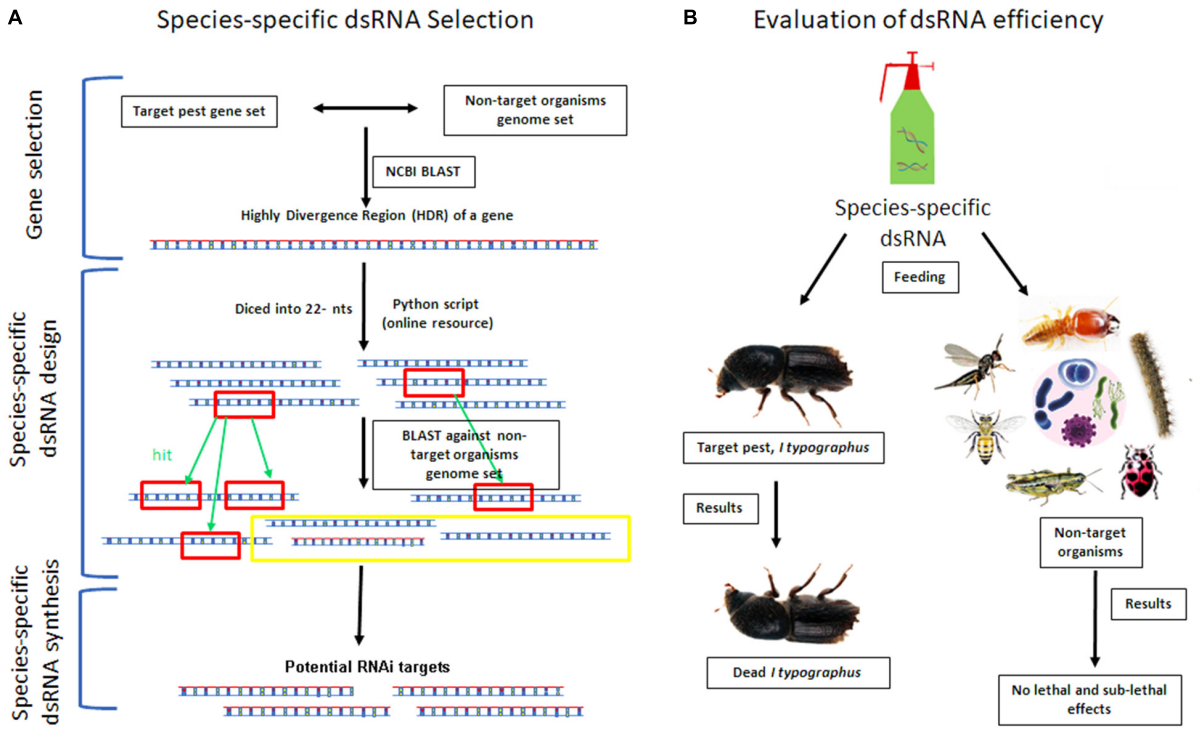

FIGURE 3 | Mitigation strategies of RNAi-based pesticides to overcome non-target effects. Schematic illustration of *in silico* analysis of species-specific dsRNA selection **(A)** and evaluation of dsRNA efficiency against the target pest and non-target organisms **(B)**. The selection of a target gene is a crucial step for the success of RNAi. For that, the Highly Divergence Region (HDR) of a target gene will be identified by doing NCBI BLAST of target pest gene set against the closely related non-target organism's genome set. Subsequently, the HDR of a gene will be diced into all possible 22-nt stretches by using a python script (Online resource) (Taning et al., 2021). Afterwards, these generated 22-nt sequence stretches will be used as a query in a BLAST search against the non-target organism's genome set for complementary sequences (hits). Finally, the sequence-specific dsRNA synthesis will be done for the identified distinct sequence region from the target gene **(A)**. Then, the sequence-specific dsRNA will be exposed to the target forest pest (i.e., *Ips typographus*) and other non-target organisms (i.e., honeybees, wasps, butterflies, earthworms; termites, and plant-growth-promoting and soil organisms). We hypothesize that only the target pest will be affected upon feeding with no lethal and sub-lethal effects on non-target organisms **(B)**. The species-specific dsRNA product can be commercially available for forest application after evaluation of all biosafety measures.

construct can be highly species-specific. For instance, the 3′-UTR region targeted γ-Tubulin transcript showed species-specific knockdown in four closely related *Drosophila* species (Whyard et al., 2009). Similarly, Kumar et al. (2012) used three highly similar CYP genes and showed particular transcript reductions in the Tobacco hornworm, *Manduca sexta*. Very recently, Pampolini and Rieske (2020) had evaluated the effect of EAB-dsRNA targeting heat shock 70-kDa protein (*hsp),* shibire *(shi), and* U1 small nuclear ribonucleoprotein *(sn-rnp)* against non-target organisms (NTOs) such as CPB (Coleoptera: Chrysomelidae); the spotted lady beetle *Coleomegilla maculata* (Coleoptera: Coccinellidae); the eastern subterranean termite, *Reticulitermes flavipes* (Blattodea: Rhinotermitidae); honeybee, *Apis mellifera* (Hymenoptera: Apidae); *Tetrastichus planipennisi* adult (Hymenoptera: Eulophidae), and *Spathius galinae* (Hymenoptera: Braconidae). Results did not suggest any adverse effect on the NTOs challenged against EAB-dsRNAs. However, such studies evaluating the RNAi against NTOs are scarce in the field of forestry and need to be incorporated regularly in the experimental plan for developing RNAi-based forest protection products (FPPs).

Enhancing the Stability of dsRNA for Environmental Application

Before RNAi-based non-transformative products are applied against forest pests, the obstacles of insufficient RNAi sensitivity (if present) or quick environmental degradation possibility (i.e., after tropical application, trunk injection, and root absorption) must be resolved. An elegant solution could be dsRNA packaging that protects dsRNA from degradation and facilitates selective uptake in the target tissue. It may be achieved via developing novel delivery systems. However, the specificity of these systems has to be prudently evaluated. Therefore, the effect of all proposed delivery strategies on NTOs needs to be thoroughly evaluated before applying FPPs.

NOVEL DELIVERY METHODS RELEVANT TO FOREST PEST MANAGEMENT

Nanoparticles as Protectors of dsRNA

The nanoparticles, typically one to a few hundred nanometers in size, are formed by encapsulating dsRNA with organic

TABLE 3 | List of genome and transcriptomics studies done with wood-boring coleopterans.

S.No.	Species	Common name	Genome		Transcriptome		References/source
			Available	Accession No.	Available	Accession No.	
1	*Ips typographus*	European spruce bark beetle	✓	PRJNA671615	✓	PRJNA702426; PRJNA679450; and PRJNA178930	Andersson et al., 2013; Powell et al., 2020
2	*Ips pini*	Pine engraver beetle	×		✓	PRJNA90755; PRJNA87977; and CB407466–CB409136	Eigenheer et al., 2003; Keeling et al., 2006
3	*Dendroctonus ponderosae*	Mountain pine beetle	✓	PRJNA360270; PRJNA162621 and PRJNA179493	✓	PRJNA37293; PRJNA189792; PRJNA189795; PRJNA269763; PRJNA203305; and PRJNA317010	Aw et al., 2010; Keeling et al., 2012, 2013, 2016; Robert et al., 2013; Nadeau et al., 2017
4	*Dendroctonus frontalis*	Southern pine beetle	×		✓	PRJNA79903	DOE Joint Genome Institute Dendroctonus frontalis EST project
5	*Dendroctonus armandi*	Chinese white pine beetle	✓	PRJNA530572	×		Godefroid et al., 2019
6	*Dendroctonus valens*	Red turpentine beetle	×		✓	PRJNA656966	Zhao et al., 2021
7	*Trypodendron signatum*	Ambrosia beetle	✓	PRJNA418542	×		Trypodendron signatum RefSeq Genome
8	*Tomicus yunnanensis*	Yunnan pine shoot beetle	×		✓	PRJNA362869; PRJDB2098; PRJDB746; PRJNA396694; and PRJNA175397	Zhu et al., 2012a,b
9	*Agrilus planipennis*	Emerald ash borer	✓	PRJNA230921; and PRJNA343475	✓	PRJNA271706; PRJNA263193; PRJNA222581; PRJNA79619; PRJNA173782; and PRJNA508756	Lowe and Eddy, 1997; Lord et al., 2016
10	*Anoplophora glabripennis*	Asian longhorned beetle	✓	PRJNA348318; PRJNA167479; PRJEB3278; and PRJNA167479	✓	PRJNA196436; PRJNA274806; PRJNA299040; PRJNA395783; PRJNA613658; and PRJNA691113	Lowe and Eddy, 1997
11	*Euwallacea fornicatus*	Polyphagous shot-hole borer	✓	MT897842	✓	PRJNA260703	Wang et al., 2020a

and inorganic materials. These nano-complexes have shown significant promise to overcome the obstacles for enhancing RNAi efficiency in many insect species by improving dsRNA absorbance, stability, protecting dsRNA from salivary and gut nucleases, and improving cellular uptake efficiency (Christiaens et al., 2020b; Yan et al., 2020b, 2021; Zhu and Palli, 2020). The chitosan-derived nanoparticles are widely used, biodegradable and non-toxic (Dass and Choong, 2008; Gurusamy et al., 2020a; Lichtenberg et al., 2020). Chitosan nanoparticle binds the silencing signal dsRNA/siRNA through electrostatic interaction (Zhang et al., 2010). Christiaens et al. (2018) reported that *chitin synthase B* targeting dsRNA delivered through guanylate polymers given protection to silencing signals from the gut nucleases, which led to increased mortality in the RNAi recalcitrant pest such as beet armyworm (*Spodoptera exigua*). However, nanoparticles with higher molecular weight may give better binding efficiency to dsRNAs/siRNAs, but their solubility may decrease, giving poor cellular uptake (Baigude and Rana, 2009). Nanoparticle encapsulation is proven highly effective in controlling many insects and may be used against forest pests via GMO-free approaches such as trunk injection or root absorption methods (**Figure 2**). Recent discoveries also indicated possibilities for a nanocarrier-mediated transdermal dsRNA delivery system to enhance RNAi efficiency after spraying (Zheng et al., 2019; Yan et al., 2020a).

Liposomes and Lipid-Based Transfection Reagents Enhancing Cellular Uptake

The delivery of dsRNA through liposomes would be a promising strategy as liposomes are made up of natural lipids, non-toxic and biodegradable (Van Rooijen and van Nieuwmegen, 1980). The dsRNA-encapsulated liposomes get into the cell's cytoplasm both by endocytosis and fusion with the plasma membrane. As already discussed in the above text that some insects

possess dsRNA uptake through receptor-mediated endocytosis only, and it is a prolonged process, and transfection reagent is required for the efficient cellular uptake of dsRNA (Saleh et al., 2006; Ulvila et al., 2006; Whyard et al., 2009). Taning et al. (2016) reported that effective RNAi was seen in the spotted-wing *Drosophila* (*Drosophila suzukii*) when the specific gene targeting dsRNA is delivered through liposomes. The transfection reagents are lipid-based products, and the dsRNA-transfection reagent complexes enhance the RNAi efficiency in insects by protecting the dsRNAs from endonucleases, aiding the dsRNA uptake into the insect cells, and escape of dsRNAs from endosomal compartments within the cells (Cooper et al., 2019; Christiaens et al., 2020b; Gurusamy et al., 2020b). The previous studies used different transfection reagents on insect species from different orders, including Diptera (*Drosophila* spp., *Ae. aegypti*), Hemiptera (Neotropical brown stinkbug, *Euschistus heros*), Blattodea (German cockroach, *Blattella germanica*), and Lepidoptera (fall armyworm, *Spodoptera frugiperda*) (Christiaens et al., 2020b). Karny et al. (2018) observed the translocation of liposome nanoparticles from leaves to roots. They used liposomes containing a fluorescent tracer (fluorescein, green) and applied foliar on cherry tomato plants. After 72 and 96 h post application, secondary and tertiary roots were collected and imaged using confocal microscopy. For 72 h, the particles gradually accumulated in individual root cells and after 96 h, the liposomes disintegrate and release their cargo into the cytoplasm. The light, pH, temperature, enzymatic condition, and oxygen mainly affect the stability of liposomes (Wang et al., 2020b). In our lab, for *in vitro* studies, we used liposomes or proteinaceous carriers for protecting the dsRNA from the enzymatic degradation and enhances the dsRNA uptake by cells and these results seem very promising. Although there are no reports on the delivery of dsRNA through liposomes and transfection reagents on the crop to suppress insect pests so far, they can be considered carriers of RNAi against forest pests.

Proteinaceous dsRNA Carriers Facilitating the Uptake

The negatively charged plasma membrane acts as the main barrier to the uptake of negatively charged dsRNAs. Cell-penetrating peptides (CPPs) transports the silencing signal dsRNAs/siRNAs and facilitates the uptake of the silencing signal into the gut epithelial cells (Milletti, 2012). Chen et al. (2012) and Zhou et al. (2015) reported that CPPs were successfully internalized hormones and plasmid DNA in insect cells. Gillet et al. (2017) reported that the gene *chitin synthase II* was successfully knockdown in the cotton boll weevil (*Anthonomus grandis*) when this pest fed on dsRNA pairing with the peptide-transduction domain (PTD) and a dsRNA-binding domain (dsRBD). Peptide-dsRNA complexes fed with early instar larvae of red flour beetle, *Tribolium castaneum* showed mortality (Avila et al., 2018). Additionally, feeding fluorescently tagged dsRNA peptide capsules were distributed throughout the red flour beetle, *Tribolium castaneum* tissues but fluorescently tagged dsRNA alone did not show widespread dispersal. In plant cells, delivery of dsRNAs has to face two barriers, i.e., cell wall and cell membrane. Several studies used polymer-based carriers to deliver plasmid DNA and proteins into intact plant cells and suggested that these systems deliver interfering RNAs (Martin-Ortigosa et al., 2012; Chang et al., 2013; Hussain et al., 2013; Demirer et al., 2019). However, few studies reported that nanoparticles were used for delivering RNAi molecules into the plant cells (Demirer and Landry, 2017; Mitter et al., 2017). The dsRNA conjugated with layered double hydroxide clay nanosheets (BioClay) was sprayed on *Nicotiana tabacum* and detected the dsRNA up to 30 days (Mitter et al., 2017). Demirer et al. (2019) used single-walled carbon nanotubes to improve the cellular delivery of siRNAs into *Nicotiana benthamiana* plants and observed that the polymeric carrier protects the siRNA against degradation RNaseA. The peptide-based carrier systems were used for rapid and efficient RNAi-mediated gene silencing in diverse plant species (*Arabidopsis thaliana*, *Nicotiana benthamiana*, *Solanum lycopersicum*, and poplar), *N. tabacum* suspension-cell cultures, and rice callus tissue (Unnamalai et al., 2004; Numata et al., 2014, 2018). More recently, Martinez et al. (2021) reported designing a lectin-based dsRNA delivery system by fusing a dsRNA-binding domain (dsRBD) to the GNA lectin domain. This GNA lectin-dsRBD fusion protein improved the cellular uptake of dsRNA in a midgut cells line and increased insect mortality for dsRNA-v-ATPase-A. Hence, De Schutter et al. (2021) reviewed the boosting of dsRNA delivery in plant cells with peptide and polymer-based carriers for an increased crossing of the plant cell wall, allowing efficient environmental RNAi in plants and improving the RNAi response in pest control. Such observations proved that CPPs or aliphatic peptide capsules could enhance RNAi efficiency and be considered against forest pests showing less sensitivity to RNAi treatments.

Root Absorption and Trunk Injection for Big Trees

It is known that phloem transports food, organic material, and the silencing signal, whereas the xylem is considered the channel for minerals and water movement (Buhtz et al., 2008). The dsRNAs or siRNAs are stable in the phloem where the environment is RNase-free (Doering-Saad et al., 2002). Once the dsRNA reaches the tissue, the silencing signal can spread to the adjacent cells (Melnyk et al., 2011). However, persistency and continuous supply of dsRNA are needed for the success of RNAi. Therefore, the dsRNA delivery through irrigation and trunk injection could be a more promising strategy than the foliar spray to control wood-boring insect pests (Wise et al., 2014; Ghosh et al., 2018; Berger and Laurent, 2019). With these strategies, suppressing all kinds of insects, like, chewing, piercing-sucking, root grubs, and wood-boring forest pests, would be possible as the exogenous dsRNA will reach every part of the plant (Andrade and Hunter, 2016). For example, Hunter et al. (2012) reported that the dsRNA is stable in the plants for 57 days, and siRNAs are detected in plants for almost 4 months when the citrus plants are drenched in dsRNA solution.

Moreover, the dsRNA is stable only for 5–8 days in leafhoppers and psyllids when fed on dsRNA-treated citrus plants. Additionally, Li et al. (2015a) also reported high mortality of the Asian corn borer, *Ostrinia furnacalis,* when dsRNA targeting Kunitz-type trypsin inhibitors (dsKTI) irrigated to maize seedlings. Finally, a recent confocal microscopy study provided evidence of EAB-dsRNA absorption via plant tissue, indicating the feasibility of dsRNA delivery against forest pests such as EAB (Pampolini et al., 2020).

Alternatively, by trunk injections, dsRNAs can deliver directly into a tree's phloem where the companion cells do not have a nucleus, and the silencing signal rapidly spreads toward the shoots and roots; and the silencing signal will last for a more extended period. Already several companies manufactured injectors such as Arborjet® (Joga et al., 2016) to deliver dsRNAs through trunk injections into trees. Dalakouras et al. (2018) showed that hairpin RNAs (hpRNAs) against *M. domestica* injected in *V. vinifera* plants via trunk injection was efficiently translocated and restricted to the xylem vessels and apoplasts, so the plant dicer-like (DCL) endonucleases were unable to process the hpRNAs and injected RNA molecules were stable for at least 10 days after post-application. These innovative methods may have a significant impact on RNAi-mediated forest protection. However, injecting each tree in the forest is not a sustainable solution.

Microorganisms as Carriers: Potential for Forestry Application

Bacteria for Minimizing Production Cost and Delivering dsRNA

A persistent and large amount of dsRNAs is required for effective RNAi in forestry applications. The commonly used and recently identified *Escherichia coli* strains, i.e., *HT115*(DE3) and pET28-*BL21*(DE3), contains the deletion of the RNase III gene (*rnc*) with a T7 expression vector that can be used to produce higher concentrations of dsRNA with less cost (Timmons et al., 2001; Ma et al., 2020). Apse RNA Containers^{TM} (ARCs) technology developed by a biotechnology company allows the production of dsRNAs in large volumes using bacteria. Plasmids coding for proteins such as capsids is co-transformed with another plasmid coding for dsRNA sequences plus a "packing site." While bacteria grow in culture, they make protein subunits self-assembled around RNA encompassing the packing site sequences. After purification of the engineered bacteria, the resulting RNA is environmentally stable and a ready-to-spray product (Joga et al., 2016). The production of bacterial-expressed dsRNA is inexpensive compared with *in vitro* production (Huvenne and Smagghe, 2010).

The delivery of dsRNA through bacteria is more beneficial than dsRNA delivery through spray and trunk injections. Dhandapani et al. (2020b) reported that when specific gene targeting dsRNA delivered through bacteria to the Asian long-horned beetle (*A. glabripennis*) has seen high efficiency of RNAi. The dsIAP and dsActin were combinedly expressed in the *HT115*(DE3) strain, and the heat-killed bacteria were sprayed on potato plants, which protected the plants from CPB damage (Máximo et al., 2020). The *HT115*(DE3) was used to express dsRNAs targeting the arginine kinase gene in South American tomato pinworm, *Tuta absoluta*, and the dsRNA expression expressed that heat-killed bacteria were spread on artificial media caused 70% larval mortality (Bento et al., 2020). The dsRNAs are expressed in heat-killed bacteria specific to the target genes SRP54 and actin fed to *Plagiodera versicolora* (Coleoptera: Chrysomelidae) caused significant mortality (Zhang et al., 2019). The dsHvSnf7 was expressed in heat-killed bacteria and sprayed on detached leaves, and plants showed significant mortality in *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae) larvae (Lü et al., 2020). More recently, researchers used endogenous symbionts like *Rhodococcus rhodnii* for expressing the dsRNAs, specifically targeting many different genes and observed phenotypes in insect species (Whitten et al., 2016). Researchers identified symbionts from both kissing bug (*Rhodnius prolixus*) and western flower thrips (*Frankliniella occidentalis*) that can be engineered to deliver dsRNA. Expressed dsRNAs symbionts administered orally, resulting in the suppression of target genes in insect species. The above research suggests that the symbiont mediated dsRNA delivery method may be viable in specific cases where symbionts can be transferred between individuals, making it less costly and efficient for forest pest management. Furthermore, symbiont mediated RNAi (SMR) gives two levels of specificity through carrying species-specific dsRNA by the species-specific symbionts.

Viruses Inducing and Delivering dsRNA

Plant infecting viruses move through the phloem systematically. Therefore, controlling insect pests through recombinant viruses would be a promising strategy. Virus-induced gene silencing (VIGS) is a method for enhancing RNAi efficiency in insects in two ways, i.e., either adding or replacing a gene in the virus and the target dsRNA is enclosed in viral capsid proteins called virus-like particles so that the recombinant virus will produce the desired dsRNA during replication specific to the target pests (Kolliopoulou et al., 2017, 2020). Insects viruses are species-specific, for example, baculoviruses, and these viruses are could be engineered to express specific target gene dsRNAs, subsequently delivered to the field to control insect pests (Swevers et al., 2013). *Nicotiana benthamiana* plants infected with recombinant tobacco mosaic virus expressed dsRNAs specific to the chitinase 1 or 2 genes of oriental armyworm, *M. separata*, and the larvae showed suppression in chitinase gene expression within gut tissues and reduced body weight (Bao et al., 2016). Wuriyanghan and Falk (2013) reported that the potato psyllid, *Bactericera cockerelli* successfully controlled by delivering dsRNA against *actin* and *V-ATPase* through the recombinant *Tobacco mosaic virus* (TMV). For that reason, this technique could be useful to control forest pests, including bark beetles. Perhaps more research is needed for the application of VIGS against coleopteran wood-boring forest pests. The VIGS mediated RNAi technology is still restricted to the lab experiments and no reports so far for forest insect pest management under field conditions. However, in a forest, the infested trees will be identified and subsequently applied

VIGS through trunk injection. However, it is very difficult to apply VIGS in the environment due to the strict regulations and environmentalists, and it seems very difficult after the Covid pandemic.

Fungi as dsRNA Carrier

Fungal-induced gene silencing (FIGS) technology also we can use to enhance RNAi in forest pest management. Generally, the FIGS system can develop in two main ways, i.e., dsRNA can express in common fungi (*Saccharomyces cerevisiae* or *Pichia pastoris*) and entomopathogenic fungi that will enhance RNAi efficiency in insects (Van Ekert et al., 2014; Chen et al., 2015; Hu and Wu, 2016; Hu and Xia, 2019; Mysore et al., 2019). Yeasts mediated dsRNA delivery caused significant mortality and delayed larval development in dipteran insects like *Ae. aegypti* (Van Ekert et al., 2014; Mysore et al., 2019). Likewise, the spotted-wing drosophila, *Drosophila suzukii* larval survival rate, and the egg-production rate were decreased drastically when dsRNA targeting *y-tubulin 23C* (*yTub23C*) was administered orally through the yeast *Saccharomyces cerevisiae* (Murphy et al., 2016). The dsTLR7 expressed entomopathogenic fungi, *Isaria fumosorosea*; consumption caused mortality up to 40% in second-instar *B. tabaci* nymphs (Chen et al., 2015). Similarly, the entomopathogenic fungi *Metarhizium acridum* were used to express dsRNAs targeting the α and/or β subunit genes of the locust F1F0-ATP synthase caused mortality in *L. migratoria* larvae (Hu and Xia, 2019). Recently, colleagues from the United States have started transforming bark beetle-associated yeast *Ogataea pini* to produce target dsRNA against bark beetles *Ips calligraphus* (source: personal communication). Hence, the FIGS technology is already under consideration for forest protection, but further optimization is required.

Microalgae or Lichens

Symbiotic interaction sustains expanded consideration among all parts of science because it helps to build the unifying themes across ecological, evolutionary, developmental, semiochemical, and pest management hypotheses. For example, Klepzig et al. (2009) reviewed the symbiotic relationship of bark beetles with fungus, bacteria, viruses, protozoa, and algae. Lichens are symbiotic organisms made up of a fungus and green algae or cyanobacterium growing jointly on the trees with multiple forms and colors. They provide food, cover, and nesting materials for a variety of insects like bristletails, barklice, katydids, grasshoppers, webspinners, butterflies, moths, moth larvae, lacewing larvae, mites, spiders, and many beetles (Speer and Waggoner, 1997; Edgerly and Rooks, 2004). Although commonly lichens are grouped into fruticose (branched or tubed), foliose (flattened or leafy), and crustose (crusty), most are pale green, brownish-green, orange, and yellow. In 2012, the University of Wisconsin reported that the lichen moth larvae (*Hypoprepia* sp.) eat lichens and blue-green algae that they find growing on tree trunks. In England, psocid species eat *Lecanora conizaeoide* lichens on larch trees randomly or only eat apothecia. For example, the *Campecopea hirsuta* feeds only algal cells on *Lichina pygmaea* (Wieser, 1963). In Australia, the web-spinner insects, i.e., *Notoligotoma hardyi,* preferred lichens as a food (Gressitt et al., 1965). Mites have a wide range of feeding habits, i.e., feed on dead plant materials, algae, and lichens (Walter and Proctor, 1999). A recent review revealed the potential use of lichen solvent extracts and metabolites as an insecticidal agent against various pests causing damage to plants, especially coleopterans (e.g., Sitophilus and Leptinotarsa) and insect vectors transmit dreadful diseases to humans such as Aedes, Anopheles, and Culex (reviewed by Sachin et al., 2018).

The marine algae (*S. wightii* and *P. pavonica*) extracts are used as an eco-friendly nymphicide or biopesticide for controlling the *Dysdercus cingulatus* (Fab.) (Hemiptera: Pyrrhocoridae) in cotton pest management (Asharaja and Sahayaraj, 2013). The mosquito larvae are aquatic and eat microalgae via filter feeding because microalgae grow within the larval habitat, and it is an excellent choice to provide direct exposure to dsRNA by engineered microalgae. Based on this idea, researchers developed engineered microalgae (*Chlamydomonas reinhardtii*) that express dsRNA specific to the enzyme 3-hydroxykynurenine transaminase fed to the *Anopheles stephensi* mosquito larvae showed 53% mortality (Kumar et al., 2013). Therefore, further research is needed for microalgae-mediated RNAi in forest insects, i.e., identifying and selecting suitable symbiont microalgae for forest insects, expressing desired dsRNAs for suitable target genes, and enhancing RNAi efficiency in forest insects.

BIOSAFETY CONSIDERATIONS FOR FPPs

RNAi has proved the potential to suppress pests and save beneficial insects from diseases and parasites. For example, a non-target organism screening with the dsRNA-based biocontrol product targeting *L. decemlineata* revealed the selectivity and safety of the dsRNA sequence even for closely related species and beneficial insects (Bramlett et al., 2020). Similarly, a genome-wide off-target screen in important bumblebee pollinators of *Bombus terrestris* with dsRNA targeting pollen beetle αCOP revealed no reduction in the transcript level for all putative off-targets, including an off-target with a 20-continuous-nucleotide match (Taning et al., 2021). Also, for a set of potential targets in the EAB, off-target effects were screened. After confirming the dsRNA's specificity, they are qualified as potential targets to suppress EAB populations (Rodrigues et al., 2018). However, deploying RNAi-based FPPs presents unique challenges for ecological, environmental, and human risk assessments.

In our opinion, for forestry application, safety assessments should include evaluating environmental safety for the NTOs (**Figure 3B**). Due to its rapid environmental degradation, the trees treated with the exogenous applications of dsRNAs to control forest pests will not be considered genetically modified organisms (GMOs) (Shew et al., 2017; Arpaia et al., 2021). However, it is significant to follow biosafety assessments for FPPs before deployment. The noticeable effects on non-target organisms exposed to RNAi will provide significant evidence for ensuring safety. Bioinformatics tools perform an essential role in the development of species-specific targets (**Figure 3A**). However, the availability of a limited number of genome

sequences from forest pests limits risk estimates. Furthermore, the effects of dsRNA on soil and other plant-associated beneficial microorganisms need to be evaluated to measure the effect of dsRNA in forest ecosystems accurately (**Figure 3B**).

The deployment of genetically engineered bacteria, fungi, or viruses capable of delivering the RNAi-based FPPs in the forest needs to be under some regulatory framework as they will also be considered GM products. Unfortunately, there is no risk assessment protocol for genetically engineered microorganisms delivering dsRNA (in short, RNAi-microbes) so far as RNAi-based GM crops (Papadopoulou et al., 2020). One putative reason is that the deployment of RNAi-microbes is an intriguing idea (i.e., SMR) that has just begun to blossom (Zhang et al., 2019). Regulatory agencies worldwide need to devise the environmental risk assessment protocol dedicated to deploying RNAi-microbes in the forest. In our opinion, RNAi-microbes can be evaluated for their mechanism of action, specificity (including the designing the dsRNA), active ingredient, environmental fate, ecotoxicology [impact on humans, other NTOs (including soil organisms), other microbes in the microhabitat, soil and water], Toxicity-Exposure-Ratio (TER), off-site movement, the requirement of the periodic application, immune response and resistance management in target pest while formulating the legal framework relating to the risk of dsRNA-based FPPs. Bioinformatics tools can assist in the selection of surrogate species for tiered toxicology testing. Nevertheless, RNAi pesticides occur naturally inside target organisms and thus a potentially safer alternative to synthetic pesticides.

CONCLUSION AND FUTURE PERSPECTIVES

RNAi is a robust technology that can bring a new paradigm in forest pest management, but some obstacles yet limit its implementation. Researchers are developing a variety of methods for boosting RNAi in wood-boring coleopterans, i.e., selection of appropriate target genes by using sequencing data, genetic modification of microbes and plants, identifying the components of extracellular vesicles, dsRNA complexation/encapsulation with nanomaterials (Sinisterra-Hunter and Hunter, 2018; Cooper et al., 2019; Christiaens et al., 2020a). Furthermore, forest protection with RNAi-based pesticides would be a novel integrated pest management strategy (IPM) due to its high sequence-dependent specificity and better safety than conventional pesticides (Wang et al., 2016; Mitter et al., 2017; Christiaens et al., 2020a; Taning C.N. et al., 2020). Hence, the researchers are focusing on controlling forest pests by using this technology, and its potential to control several coleopteran wood-boring forest pests such as bark beetles, EAB, ALB is already experimentally proven (Rodrigues et al., 2017a,b, 2018; Kyre et al., 2019, 2020; Dhandapani et al., 2020a).

However, the dsRNA production cost is still remarkably higher, although dsRNA production costs were lowered to 2 USD per gram in 2017 from 12,500 USD per gram back in 2008 (Zotti et al., 2018). The current dsRNA production capacity may be increased dramatically soon to produce vaccines against the COVID-19 (SARS-CoV2) pandemic to the global community. Furthermore, the same vaccine production platforms could be converted to produce large-scale RNAi-based pesticides, enabling dsRNA-based pesticides to be much cheaper (Taning C.N.T. et al., 2020).

It is a well-known phenomenon that the symbiont, blue-stain fungi pave the way for the successful colonization of coniferous bark beetles by acting as a source of bark beetles semiochemicals (Kandasamy et al., 2016), depletes spruce defense chemicals (Lahr and Krokene, 2013), and provide nutrient supplements (Bentz and Six, 2006; Six and Wingfield, 2011; Davis et al., 2019; Six, 2020; Six and Elser, 2020). Likewise, fungi also benefit from bark beetles getting inoculated into the phloem as they cannot penetrate bark alone (Franceschi et al., 2000). Thus, it concludes that the conifer bark beetles and their symbiont collectively causing to extensive Norway spruce forest mortality. For that reason, the researchers should work simultaneously on controlling symbionts and bark beetles by using the RNAi tool. Most interestingly, in our current research, we have seen that the RNAi is functional and highly efficient against the European spruce bark beetle, *Ips typographus,* in laboratory conditions (unpublished results).

Foliar application of RNAi-based pesticides may not be applicable for controlling the wood-boring insect pests due to the size of the trees and the presence of thick outer bark. In comparison, the delivery of dsRNA pesticides through the host such as trunk injection, soil drench, symbiotic microorganisms of plant and target pest, and viruses may promise the long-lasting protection of trees from insect pests and pathogens.

Restricting the off-target and non-target effects would be challenging with RNAi-based FPPs. However, species-specific and target-specific RNAi targets have to be identified for the effectiveness of this technology (Christiaens et al., 2018). A bioinformatics pipeline can be helpful here in finding HDRs in the target pest. However, the genome sequences of forest pests will be prerequisites for such strategies. Hence, research endeavors toward more forest insect genomes and tissue-specific transcriptomes are necessary for the future to obtain superior species-specific targets for dsRNA applications.

Lastly, the resistance against dsRNA-based FPPs would be a critical barrier for the deployment of this strategy. For example, Khajuria et al. (2018) reported that WCR got resistant upon exposure of several generations to *DvSnf7* dsRNA. Insect pests could evolve resistance in different possible ways. For instance, mutations of target and RNAi core machinery genes decreased dsRNA uptake and increased dsRNA degradation (Zhu and Palli, 2020). However, changes in the target gene selection could help delay the resistance, and delivery of dsRNA through nanoparticles and liposomes could improve the efficacy, stability, and dsRNA uptake mechanism by gut epithelial cells (Wytinck et al., 2020). The careful optimization of target gene selection, dsRNA design, synthesis and delivery, nanocarriers or symbiotic microbes, or virus-induced gene silencing can be highly effective for suppressing and causing mortality in forest insect pest species RNAi. However, field tests, environmental safety, and non-target effects are lacking for these efforts, and optimization is necessary for

producing various RNAi-based FPPs. In addition, researchers must take the initiative to create a perception of RNAi-based FPPs to state forest agencies, forest owners, and general foresters to facilitate its deployment for long-term forest protection. Nevertheless, RNAi-based FPPs in conjugation with existing forest pest management practices (i.e., silvicultural, biological) can aid a multi-faceted management approach that keeps the tree-killing forest pest populations in the endemic stage while conserving the beneficial species.

AUTHOR CONTRIBUTIONS

AR conceptualized the manuscript structure. MJ, KM, and AR wrote the first draft and did the revision. GS commented on the initial draft and revised the manuscript. All the authors approved the final version of the manuscript.

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REFERENCES

Agrawal, N., Dasaradhi, P., Mohmmed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K. (2003). RNA interference: biology, mechanism, and applications. *Microbiol. Mole. Biol. Rev.* 67, 657–685.

Andersson, M. N., Grosse-Wilde, E., Keeling, C. I., Bengtsson, J. M., Yuen, M. M., Li, M., et al. (2013). Antennal transcriptome analysis of the chemosensory gene families in the tree killing bark beetles, Ips typographus and Dendroctonus ponderosae (Coleoptera: Curculionidae: Scolytinae). *BMC Genom.* 14, 1–16.

Andrade, E. D., and Hunter, W. B. (2016). "RNA interference–natural gene-based technology for highly specific pest control (HiSPeC)," in *RNA Interference*, ed. I. Y. Abdurakhmonov (Croatia: InTech), 391–409.

Arnberger, A., Ebenberger, M., Schneider, I. E., Cottrell, S., Schlueter, A. C., von Ruschkowski, E., et al. (2018). Visitor preferences for visual changes in bark beetle-impacted forest recreation settings in the United States and Germany. *Env. Manag.* 61, 209–223. doi: 10.1007/s00267-017-0975-4

Arora, A. K., Chung, S. H., and Douglas, A. E. (2021). Non-Target Effects of dsRNA Molecules in Hemipteran Insects. *Genes* 12:407. doi: 10.3390/genes12030407

Arpaia, S., Smagghe, G., and Sweet, J. B. (2021). Biosafety of bee pollinators in genetically modified agro-ecosystems: Current approach and further development in the EU. *Pest Manag. Sci.* 77, 2659–2666. doi: 10.1002/ps.6287

Asharaja, A., and Sahayaraj, K. (2013). Screening of insecticidal activity of brown macroalgal extracts against Dysdercus cingulatus (Fab.)(Hemiptera: Pyrrhocoridae). *J. Biopest.* 6, 193–203.

Aukema, J. E., Leung, B., Kovacs, K., Chivers, C., Britton, K. O., Englin, J., et al. (2011). Economic impacts of non-native forest insects in the continental United States. *PLoS One* 6:e24587. doi: 10.1371/journal.pone.0024587

Avila, L., Chandrasekar, R., Wilkinson, K., Balthazor, J., Heerman, M., Bechard, J., et al. (2018). Delivery of lethal dsRNAs in insect diets by branched amphiphilic peptide capsules. *J. Contr Release* 273, 139–146. doi: 10.1016/j.jconrel.2018.01.010

Aw, T., Schlauch, K., Keeling, C. I., Young, S., Bearfield, J. C., Blomquist, G. J., et al. (2010). Functional genomics of mountain pine beetle (Dendroctonus ponderosae) midguts and fat bodies. *BMC Genom.* 11, 1–12.

Baigude, H., and Rana, T. M. (2009). Delivery of therapeutic RNAi by nanovehicles. *ChemBioChem* 10, 2449–2454. doi: 10.1002/cbic.200900252

Bao, W., Cao, B., Zhang, Y., and Wuriyanghan, H. (2016). Silencing of Mythimna separata chitinase genes via oral delivery of in planta-expressed RNAi effectors from a recombinant plant virus. *Biotechnol. Lett.* 38, 1961–1966. doi: 10.1007/s10529-016-2186-0

Barik, S. (2006). RNAi in moderation. *Nat. Biotechnol.* 24, 796–797. doi: 10.1038/nbt0706-796

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–1326. doi: 10.1038/nbt1359

Bento, F. M., Marques, R. N., Campana, F. B., Demétrio, C. G., Leandro, R. A., Parra, J. R. P., et al. (2020). Gene silencing by RNAi via oral delivery of dsRNA by bacteria in the South American tomato pinworm. *Tuta absoluta. Pest Manag. Sci.* 76, 287–295. doi: 10.1002/ps.5513

Bentz, B. J., and Jönsson, A. M. (2015). "Modeling bark beetle responses to climate change," in *Bark Beetles Biology and Ecology of Native and Invasive Species*, eds F. E. Vega and R. W. Hofstetter (London: Elsevier), 533–553. doi: 10.1016/b978-0-12-417156-5.00013-7

Bentz, B. J., Jönsson, A. M., Schroeder, M., Weed, A., Wilcke, R. A. I., and Larsson, K. (2019). Ips typographus and Dendroctonus ponderosae models project thermal suitability for intra-and inter-continental establishment in a changing climate. *Front. Forests Glob. Change* 2:1. doi: 10.3389/ffgc.2019.00001

Bentz, B. J., Régnière, J., Fettig, C. J., Hansen, E. M., Hayes, J. L., Hicke, J. A., et al. (2010). Climate change and bark beetles of the western United States and Canada: direct and indirect effects. *BioScience* 60, 602–613. doi: 10.1525/bio.2010.60.8.6

Bentz, B. J., and Six, D. L. (2006). Ergosterol content of fungi associated with Dendroctonus ponderosae and Dendroctonus rufipennis (Coleoptera: Curculionidae, Scolytinae). *Ann. Entomolog. Soc. Am.* 99, 189–194. doi: 10.1603/0013-8746(2006)099[0189:ecofaw]2.0.co;2

Berger, C., and Laurent, F. (2019). Trunk injection of plant protection products to protect trees from pests and diseases. *Crop Protect.* 124:104831. doi: 10.1016/j.cropro.2019.05.025

Biedermann, P. H., Müller, J., Grégoire, J.-C., Gruppe, A., Hagge, J., Hammerbacher, A., et al. (2019). Bark beetle population dynamics in the Anthropocene: challenges and solutions. *Trends Ecol. Evol.* 34, 914–924. doi: 10.1016/j.tree.2019.06.002

Billings, R. F. (2011). *Use of chemicals for prevention and control of southern pine beetle infestations. In: Coulson, RN; Klepzig, KD 2011. Southern Pine Beetle II. Gen. Tech. Rep. SRS-140*, Vol. 140. Asheville, NC: US Department of Agriculture Forest Service, Southern Research Station, 367–379.

Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flannagan, R., et al. (2012). Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (Diabrotica virgifera virgifera LeConte). *PLoS One* 7:e47534. doi: 10.1371/journal.pone.0047534

Boone, C. K., Aukema, B. H., Bohlmann, J., Carroll, A. L., and Raffa, K. F. (2011). Efficacy of tree defense physiology varies with bark beetle population density: a basis for positive feedback in eruptive species. *Can. J. Forest Res.* 41, 1174–1188. doi: 10.1139/x11-041

Bramlett, M., Plaetinck, G., and Maienfisch, P. (2020). RNA-based biocontrols—a new paradigm in crop protection. *Engineering* 6, 522–527. doi: 10.1016/j.eng.2019.09.008

Buhtz, A., Springer, F., Chappell, L., Baulcombe, D. C., and Kehr, J. (2008). Identification and characterization of small RNAs from the phloem of Brassica napus. *Plant J.* 53, 739–749. doi: 10.1111/j.1365-313x.2007.03368.x

Cagliari, D., Dias, N. P., Galdeano, D. M., Dos Santos, E. Á, Smagghe, G., and Zotti, M. J. (2019). Management of pest insects and plant diseases by non-transformative RNAi. *Front. Plant Sci.* 2019:10.

Cahyanto, I. P., Pennington-Gray, L., and Wehrung, J. (2018). The Mountain Pine Beetle: A Study of Tourism Businesses' Perceptions of the Risk of Ecological Disturbances. *J. Park Recreat. Administr.* 36:4.

Camargo, R. D. A., Herai, R. H., Santos, L. N., Bento, F. M., Lima, J. E., Marques-Souza, H., et al. (2015). De novo transcriptome assembly and analysis to identify potential gene targets for RNAi-mediated control of the tomato leafminer (Tuta absoluta). *BMC Genom.* 16, 1–17.

Cappelle, K., de Oliveira, C. F. R., Van Eynde, B., Christiaens, O., and Smagghe, G. (2016). The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. *Insect Mole. Biol.* 25, 315–323. doi: 10.1111/imb.12222

Chang, F.-P., Kuang, L.-Y., Huang, C.-A., Jane, W.-N., Hung, Y., Yue-ie, C. H., et al. (2013). A simple plant gene delivery system using mesoporous silica nanoparticles as carriers. *J. Mater. Chem. B* 1, 5279–5287. doi: 10.1039/c3tb20529k

Chen, J., Peng, Y., Zhang, H., Wang, K., Zhao, C., Zhu, G., et al. (2021). Off-target effects of RNAi correlate with the mismatch rate between dsRNA and non-target mRNA. *RNA Biol.* 2021, 1–13. doi: 10.1080/15476286.2020.1868680

Chen, X., and Goodwin, B. K. (2011). "Spatio-Temporal Modeling of Southern Pine Beetle Outbreaks with a Block Bootstrapping Approach," in *In 2011 Annual Meeting, July 24-26, 2011, Pittsburgh, Pennsylvania (No. 103668)*, (Pennsylvania: Agricultural and Applied Economics Association).

Chen, X., Li, L., Hu, Q., Zhang, B., Wu, W., Jin, F., et al. (2015). Expression of dsRNA in recombinant Isaria fumosorosea strain targets the TLR7 gene in Bemisia tabaci. *BMC Biotechnol.* 15, 1–8.

Chen, Y.-J., Liu, B. R., Dai, Y.-H., Lee, C.-Y., Chan, M.-H., Chen, H.-H., et al. (2012). A gene delivery system for insect cells mediated by arginine-rich cell-penetrating peptides. *Gene* 493, 201–210. doi: 10.1016/j.gene.2011.11.060

Christensen, J., Litherland, K., Faller, T., Van de Kerkhof, E., Natt, F., Hunziker, J., et al. (2013). Metabolism studies of unformulated internally [3H]-labeled short interfering RNAs in mice. *Drug Metab. Disposit.* 41, 1211–1219. doi: 10.1124/dmd.112.050666

Christiaens, O., Petek, M., Smagghe, G., and Taning, C. N. T. (2020a). "The use of nanocarriers to improve the efficiency of RNAi-based pesticides in agriculture," in *Nanopesticides - (From research and development to mechanisms of action and sustainable use in agriculture*, eds L. F. Fraceto, V. L. S. S. de Castro, R. Grillo, D. Ávila, H. C. Oliveira, and R. Lima (Switzerland: Springer Nature), 49–68. doi: 10.1007/978-3-030-44873-8_3

Christiaens, O., Swevers, L., and Smagghe, G. (2014). DsRNA degradation in the pea aphid (Acyrthosiphon pisum) associated with lack of response in RNAi feeding and injection assay. *Peptides* 53, 307–314. doi: 10.1016/j.peptides.2013.12.014

Christiaens, O., Tardajos, M. G., Martinez Reyna, Z. L., Dash, M., Dubruel, P., and Smagghe, G. (2018). Increased RNAi efficacy in Spodoptera exigua via the formulation of dsRNA with guanylated polymers. *Front. Physiol.* 9:316.

Christiaens, O., Whyard, S., Vélez, A. M., and Smagghe, G. (2020b). Double-stranded RNA technology to control insect pests: current status and challenges. *Front. Plant Sci.* 11:451.

Cipollini, D., and Rigsby, C. M. (2015). Incidence of infestation and larval success of emerald ash borer (Agrilus planipennis) on white fringetree (Chionanthus virginicus), Chinese fringetree (Chionanthus retusus), and devilwood (Osmanthus americanus). *Env. Entomol.* 44, 1375–1383. doi: 10.1093/ee/nvv112

Cogoni, C., and Macino, G. (1999). Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. *Nature* 399, 166–169. doi: 10.1038/20215

Cooper, A. M., Silver, K., Zhang, J., Park, Y., and Zhu, K. Y. (2019). Molecular mechanisms influencing efficiency of RNA interference in insects. *Pest Manag Sci.* 75, 18–28. doi: 10.1002/ps.5126

Cooper, A. M., Yu, Z., Biondi, M., Song, H., Silver, K., Zhang, J., et al. (2020). Stability of double-stranded RNA in gut contents and hemolymph of Ostrinia nubilalis larvae. *Pesticide Biochem. Physiol.* 169:104672. doi: 10.1016/j.pestbp.2020.104672

Cudmore, T. J., Björklund, N., Carroll, A. L., and Staffan Lindgren, B. (2010). Climate change and range expansion of an aggressive bark beetle: evidence of higher beetle reproduction in naïve host tree populations. *J. Appl. Ecol.* 47, 1036–1043. doi: 10.1111/j.1365-2664.2010.01848.x

Dalakouras, A., Jarausch, W., Buchholz, G., Bassler, A., Braun, M., Manthey, T., et al. (2018). Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. *Front. Plant Sci.* 9:1253.

Dass, C. R., and Choong, P. F. (2008). Chitosan-mediated orally delivered nucleic acids: a gutful of gene therapy. *J. Drug Target.* 16, 257–261. doi: 10.1080/10611860801900801

Davis, T. S., Stewart, J. E., Mann, A., Bradley, C., and Hofstetter, R. W. (2019). Evidence for multiple ecological roles of Leptographium abietinum, a symbiotic fungus associated with the North American spruce beetle. *Fungal Ecol.* 38, 62–70. doi: 10.1016/j.funeco.2018.04.008

de la Giroday, H. M. C., Carroll, A. L., and Aukema, B. H. (2012). Breach of the northern Rocky Mountain geoclimatic barrier: initiation of range expansion by the mountain pine beetle. *J. Biogeogr.* 39, 1112–1123. doi: 10.1111/j.1365-2699.2011.02673.x

De Schutter, K., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2021). Boosting dsRNA Delivery in Plant and Insect Cells with Peptide-and Polymer-based Carriers: Case-based Current Status and Future Perspectives. *RNAi Plant Imp. Protect.* 2021:11. doi: 10.1079/9781789248890.0011

Demirer, G. S., and Landry, M. P. (2017). Delivering genes to plants. *Chem. Eng. Prog.* 113, 40–45.

Demirer, G. S., Zhang, H., Goh, N. S., Pinals, R. L., Chang, R., and Landry, M. P. (2019). Carbon nanocarriers deliver siRNA to intact plant cells for efficient gene knockdown. *Sci. Adv.* 6:seaaz0495. doi: 10.1126/sciadv.aaz0495

Dhandapani, R. K., Duan, J. J., and Palli, S. R. (2020a). Orally delivered dsRNA induces knockdown of target genes and mortality in the Asian long-horned beetle, Anoplophora glabripennis. *Archiv. Insect Biochem. Physiol.* 104: e21679.

Dhandapani, R. K., Gurusamy, D., Duan, J. J., and Palli, S. R. (2020b). RNAi for management of Asian long-horned beetle, Anoplophora glabripennis: identification of target genes. *J. Pest Sci.* 93, 823–832. doi: 10.1007/s10340-020-01197-8

Dhar, A., Parrott, L., and Hawkins, C. D. (2016). Aftermath of mountain pine beetle outbreak in British Columbia: Stand dynamics, management response and ecosystem resilience. *Forests* 7:171. doi: 10.3390/f7080171

Doering-Saad, C., Newbury, H. J., Bale, J. S., and Pritchard, J. (2002). Use of aphid stylectomy and RT-PCR for the detection of transporter mRNAs in sieve elements. *J. Exp. Bot.* 53, 631–637. doi: 10.1093/jexbot/53.369.631

Dong, K., Sun, L., Liu, J.-T., Gu, S.-H., Zhou, J.-J., Yang, R.-N., et al. (2017). RNAi-induced electrophysiological and behavioral changes reveal two pheromone binding proteins of Helicoverpa armigera involved in the perception of the main sex pheromone component Z11–16: Ald. *J. Chem. Ecol.* 43, 207–214. doi: 10.1007/s10886-016-0816-6

Dow, J. (1992). pH gradients in lepidopteran midgut. *J. Exp. Biol.* 172, 355–375. doi: 10.1242/jeb.172.1.355

Edgerly, J. S., and Rooks, E. C. (2004). Lichens, sun, and fire: a search for an embiid-environment connection in Australia (Order Embiidina: Australembiidae and Notoligotomidae). *Env. Entomol.* 33, 907–920. doi: 10.1603/0046-225x-33.4.907

Eigenheer, A. L., Keeling, C. I., Young, S., and Tittiger, C. (2003). Comparison of gene representation in midguts from two phytophagous insects, Bombyx mori and Ips pini, using expressed sequence tags. *Gene* 316, 127–136. doi: 10.1016/s0378-1119(03)00749-2

Fairweather, M. L. (2006). *Field guide to insects and diseases of Arizona and New Mexico forests*. Southwestern Region: USDA Forest Service, 271.

Farazi, T. A., Juranek, S. A., and Tuschl, T. (2008). The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. *Development* 135, 1201–1214. doi: 10.1242/dev.005629

Feder, G. (1979). Pesticides, information, and pest management under uncertainty. *Am. J. Agr. Econ.* 61, 97–103. doi: 10.2307/1239507

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391, 806–811. doi: 10.1038/35888

Fishilevich, E., Vélez, A. M., Storer, N. P., Li, H., Bowling, A. J., Rangasamy, M., et al. (2016). RNAi as a management tool for the western corn rootworm, Diabrotica virgifera virgifera. *Pest Manag. Sci.* 72, 1652–1663. doi: 10.1002/ps.4324

Franceschi, V. R., Krokene, P., Krekling, T., and Christiansen, E. (2000). Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). *Am. J. Bot.* 87, 314–326. doi: 10.2307/2656627

Fu, D., Dai, L., Gao, H., Sun, Y., Liu, B., and Chen, H. (2019). Identification, expression patterns and RNA interference of aquaporins in Dendroctonus armandi (Coleoptera: Scolytinae) larvae during overwintering. *Front. Physiol.* 10:967.

Ghosh, S. K. B., Hunter, W. B., Park, A. L., and Gundersen-Rindal, D. E. (2018). Double-stranded RNA oral delivery methods to induce RNA interference in phloem and plant-sap-feeding hemipteran insects. *JoVE* 135:e57390.

Gillet, F.-X., Garcia, R. A., Macedo, L. L., Albuquerque, E. V., Silva, M., and Grossi-de-Sa, M. F. (2017). Investigating engineered ribonucleoprotein particles to improve oral RNAi delivery in crop insect pests. *Front. Phys.* 8:256.

Godefroid, M., Meseguer, A. S., Saune, L., Genson, G., Streito, J.-C., Rossi, J.-P., et al. (2019). Restriction-site associated DNA markers provide new insights into the evolutionary history of the bark beetle genus Dendroctonus. *Mole. Phylogenet. Evol.* 139:106528. doi: 10.1016/j.ympev.2019.106528

Grégoire, J.-C., Raffa, K. F., and Lindgren, B. S. (2015). "Economics and politics of bark beetles," in *Bark Beetles Biology and Ecology of Native and Invasive Species*, eds F. E. Vega and R. W. Hofstetter (London: Elsevier), 585–613. doi: 10.1016/b978-0-12-417156-5.00015-0

Gressitt, J. L., Sedlacek, J., and Szent-Ivany, J. (1965). Flora and fauna on backs of large Papuan moss-forest weevils. *Science* 150, 1833–1835. doi: 10.1126/science.150.3705.1833

Gurusamy, D., Mogilicherla, K., and Palli, S. R. (2020a). Chitosan nanoparticles help double-stranded RNA escape from endosomes and improve RNA interference in the fall armyworm, Spodoptera frugiperda. *Arch. Insect Biochem. Phys.* 104:e21677.

Gurusamy, D., Mogilicherla, K., Shukla, J. N., and Palli, S. R. (2020b). Lipids help double-stranded RNA in endosomal escape and improve RNA interference in the fall armyworm, Spodoptera frugiperda. *Arch. Insect Biochem. Physiol.* 104:e21678.

Haack, R. A., Hérard, F., Sun, J., and Turgeon, J. J. (2010). Managing invasive populations of Asian longhorned beetle and citrus longhorned beetle: a worldwide perspective. *Ann. Rev. Entomol.* 55, 521–546. doi: 10.1146/annurev-ento-112408-085427

Haack, R. A., Jendak, E., Houping, L., Marchant, K. R., Petrice, T. R., Poland, T. M., et al. (2002). The emerald ash borer: a new exotic pest in North America. *Newslett. Michigan Entomol. Soc.* 47, 1–5.

Haasnoot, J., Westerhout, E. M., and Berkhout, B. (2007). RNA interference against viruses: strike and counterstrike. *Nat. Biotechnol.* 25, 1435–1443. doi: 10.1038/nbt1369

Haynes, K. J., Allstadt, A. J., and Klimetzek, D. (2014). Forest defoliator outbreaks under climate change: effects on the frequency and severity of outbreaks of five pine insect pests. *Glob. Chang. Biol.* 20, 2004–2018. doi: 10.1111/gcb.12506

Hegedus, D., Erlandson, M., Gillott, C., and Toprak, U. (2009). New insights into peritrophic matrix synthesis, architecture, and function. *Ann. Rev. Entomol.* 54, 285–302. doi: 10.1146/annurev.ento.54.110807.090559

Hicke, J. A., Meddens, A. J., and Kolden, C. A. (2016). Recent tree mortality in the western United States from bark beetles and forest fires. *Forest Sci.* 62, 141–153.

Hlásny, T., Krokene, P., Liebhold, A., Montagné-Huck, C., Müller, J., Qin, H., et al. (2019). *Living with bark beetles: impacts, outlook and management options.* From Science to Policy 8. Finland: European Forest Institute.

Holmes, T. P. (1991). Price and welfare effects of catastrophic forest damage from southern pine beetle epidemics. *Forest Sci.* 37, 500–516.

Hu, J., and Xia, Y. (2019). Increased virulence in the locust-specific fungal pathogen Metarhizium acridum expressing dsRNAs targeting the host F1F0-ATPase subunit genes. *Pest Manag. Sci.* 75, 180–186. doi: 10.1002/ps.5085

Hu, Q., and Wu, W. (2016). Recombinant fungal entomopathogen RNAi target insect gene. *Bioengineered* 7, 504–507. doi: 10.1080/21655979.2016.1146833

Hunter, W. B., Glick, E., Paldi, N., and Bextine, B. R. (2012). Advances in RNA interference: dsRNA treatment in trees and grapevines for insect pest suppression. *Southwest. Entomol.* 37, 85–87. doi: 10.3958/059.037.0110

Hussain, H. I., Yi, Z., Rookes, J. E., Kong, L. X., and Cahill, D. M. (2013). Mesoporous silica nanoparticles as a biomolecule delivery vehicle in plants. *J. Nanoparticle Res.* 15, 1–15.

Huvenne, H., and Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J. Insect Physiol.* 56, 227–235. doi: 10.1016/j.jinsphys.2009.10.004

Joga, M. R., Zotti, M. J., Smagghe, G., and Christiaens, O. (2016). RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. *Front. Physiol.* 7:553.

Jose, A. M., and Hunter, C. P. (2007). Transport of sequence-specific RNA interference information between cells. *Annu. Rev. Genet.* 41, 305–330. doi: 10.1146/annurev.genet.41.110306.130216

Kanasty, R. L., Whitehead, K. A., Vegas, A. J., and Anderson, D. G. (2012). Action and reaction: the biological response to siRNA and its delivery vehicles. *Mole. Ther.* 20, 513–524. doi: 10.1038/mt.2011.294

Kandasamy, D., Gershenzon, J., and Hammerbacher, A. (2016). Volatile organic compounds emitted by fungal associates of conifer bark beetles and their potential in bark beetle control. *J. Chem. Ecol.* 42, 952–969. doi: 10.1007/s10886-016-0768-x

Karny, A., Zinger, A., Kajal, A., Shainsky-Roitman, J., and Schroeder, A. (2018). Therapeutic nanoparticles penetrate leaves and deliver nutrients to agricultural crops. *Sci. Rep.* 8, 1–10.

Katoch, R., and Thakur, N. (2012). Insect gut nucleases: a challenge for RNA interference mediated insect control strategies. *Int. J. Biochem. Biotechnol.* 1, 198–203.

Keeling, C., Bearfield, J., Young, S., Blomquist, G., and Tittiger, C. (2006). Effects of juvenile hormone on gene expression in the pheromone-producing midgut of the pine engraver beetle, Ips pini. *Insect Mole. Biol.* 15, 207–216. doi: 10.1111/j.1365-2583.2006.00629.x

Keeling, C. I., Henderson, H., Li, M., Yuen, M., Clark, E. L., Fraser, J. D., et al. (2012). Transcriptome and full-length cDNA resources for the mountain pine beetle, Dendroctonus ponderosae Hopkins, a major insect pest of pine forests. *Insect Biochem. Mol. Biol.* 42, 525–536. doi: 10.1016/j.ibmb.2012.03.010

Keeling, C. I., Li, M., Sandhu, H. K., Henderson, H., Saint Yuen, M. M., and Bohlmann, J. (2016). Quantitative metabolome, proteome and transcriptome analysis of midgut and fat body tissues in the mountain pine beetle, Dendroctonus ponderosae Hopkins, and insights into pheromone biosynthesis. *Insect Biochem. Mol. Biol.* 70, 170–183. doi: 10.1016/j.ibmb.2016.01.002

Keeling, C. I., Yuen, M. M., Liao, N. Y., Docking, T. R., Chan, S. K., Taylor, G. A., et al. (2013). Draft genome of the mountain pine beetle, Dendroctonus ponderosae Hopkins, a major forest pest. *Genome Biol.* 14, 1–20.

Khajuria, C., Ivashuta, S., Wiggins, E., Flagel, L., Moar, W., Pleau, M., et al. (2018). Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, Diabrotica virgifera virgifera LeConte. *PLoS One* 13:e0197059. doi: 10.1371/journal.pone.0197059

Klepzig, K., Adams, A., Handelsman, J., and Raffa, K. (2009). Symbioses: a key driver of insect physiological processes, ecological interactions, evolutionary diversification, and impacts on humans. *Env Entomol.* 38, 67–77. doi: 10.1603/022.038.0109

Knorr, E., Fishilevich, E., Tenbusch, L., Frey, M. L., Rangasamy, M., Billion, A., et al. (2018). Gene silencing in Tribolium castaneum as a tool for the targeted identification of candidate RNAi targets in crop pests. *Sci. Rep.* 8, 1–15. doi: 10.1111/j.1748-5967.2011.00437.x

Kolliopoulou, A., Kontogiannatos, D., and Swevers, L. (2020). The use of engineered plant viruses in a trans-kingdom silencing strategy against their insect vectors. *Front. Plant Sci.* 11:917.

Kolliopoulou, A., Taning, C. N., Smagghe, G., and Swevers, L. (2017). Viral delivery of dsRNA for control of insect agricultural pests and vectors of human disease: prospects and challenges. *Front. Physiol.* 8:399.

Kumar, A., Falcao, V. R., and Sayre, R. T. (2013). Evaluating nuclear transgene expression systems in Chlamydomonas reinhardtii. *Algal Res.* 2, 321–332. doi: 10.1016/j.algal.2013.09.002

Kumar, P., Pandit, S. S., and Baldwin, I. T. (2012). Tobacco Rattle Virus Vector: A Rapid and Transient Means of Silencing Manduca sexta Genes by Plant Mediated RNA Interference. *PLoS One* 7:e31347. doi: 10.1371/journal.pone.0031347

Kurz, W. A., Dymond, C., Stinson, G., Rampley, G., Neilson, E., Carroll, A., et al. (2008). Mountain pine beetle and forest carbon feedback to climate change. *Nature* 452, 987–990. doi: 10.1038/nature06777

Kyre, B. R., Bentz, B. J., and Rieske, L. K. (2020). Susceptibility of mountain pine beetle (Dendroctonus ponderosae Hopkins) to gene silencing through RNAi provides potential as a novel management tool. *Forest Ecol. Manag.* 473:8. doi: 10.1016/j.foreco.2020.118322

Kyre, B. R., Rodrigues, T. B., and Rieske, L. K. (2019). RNA interference and validation of reference genes for gene expression analyses using qPCR in southern pine beetle. Dendroctonus frontalis. *Sci. Rep.* 9, 1–8.

Lahr, E. C., and Krokene, P. (2013). Conifer stored resources and resistance to a fungus associated with the spruce bark beetle Ips typographus. *PLoS One* 8:e72405. doi: 10.1371/journal.pone.0072405

Lam, J. K., Chow, M. Y., Zhang, Y., and Leung, S. W. (2015). siRNA versus miRNA as therapeutics for gene silencing. *Mole. Ther. Nucleic Acids* 4:e252. doi: 10.1038/mtna.2015.23

Leelesh, R. S., and Rieske, L. K. (2020). Oral Ingestion of Bacterially Expressed dsRNA Can Silence Genes and Cause Mortality in a Highly Invasive, Tree-Killing Pest, the Emerald Ash Borer. *Insects* 11:440. doi: 10.3390/insects11070440

Lehane, M. (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol.* 42, 525–550. doi: 10.1146/annurev.ento.42.1.525

Lesk, C., Coffel, E., D'Amato, A. W., Dodds, K., and Horton, R. (2017). Threats to North American forests from southern pine beetle with warming winters. *Nat. Clim. Change* 7, 713–717. doi: 10.1038/nclimate3375

Leverkus, A. B., Rey Benayas, J. M., Castro, J., Boucher, D., Brewer, S., Collins, B. M., et al. (2018). Salvage logging effects on regulating and supporting ecosystem services—A systematic map. *Canadian J. Forest Res.* 48, 983–1000. doi: 10.1139/cjfr-2018-0114

Li, H., Bowling, A. J., Gandra, P., Rangasamy, M., Pence, H. E., McEwan, R. E., et al. (2018a). Systemic RNAi in western corn rootworm, Diabrotica virgifera virgifera, does not involve transitive pathways. *Insect Sci.* 25, 45–56. doi: 10.1111/1744-7917.12382

Li, H., Guan, R., Guo, H., and Miao, X. (2015a). New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests. *Plant Cell Env.* 38, 2277–2285. doi: 10.1111/pce.12546

Li, H., Khajuria, C., Rangasamy, M., Gandra, P., Fitter, M., Geng, C., et al. (2015b). Long ds RNA but not si RNA initiates RNA i in western corn rootworm larvae and adults. *J. Appl. Entomol.* 139, 432–445.

Li, Z., Dai, L., Chu, H., Fu, D., Sun, Y., and Chen, H. (2018b). Identification, expression patterns, and functional characterization of chemosensory proteins in Dendroctonus armandi (Coleoptera: Curculionidae: Scolytinae). *Front. Physiol.* 9:291.

Lichtenberg, S. S., Laisney, J., Elhaj Baddar, Z., Tsyusko, O. V., Palli, S. R., Levard, C., et al. (2020). Comparison of Nanomaterials for Delivery of Double-Stranded RNA in Caenorhabditis elegans. *J. Agricult. Food Chem.* 68, 7926–7934. doi: 10.1021/acs.jafc.0c02840

Liu, D., De Schutter, K., Smargiasso, N., De Pauw, E., Van Damme, E. J., and Smagghe, G. (2019). The N-glycan profile of the peritrophic membrane in the Colorado potato beetle larva (Leptinotarsa decemlineata). *J. Insect Physiol.* 115, 27–32. doi: 10.1016/j.jinsphys.2019.03.009

Lord, N. P., Plimpton, R. L., Sharkey, C. R., Suvorov, A., Lelito, J. P., Willardson, B. M., et al. (2016). A cure for the blues: opsin duplication and subfunctionalization for short-wavelength sensitivity in jewel beetles (Coleoptera: Buprestidae). *BMC Evol. Biol.* 16, 1–17.

Lowe, T. M., and Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* 25, 955–964. doi: 10.1093/nar/25.5.955

Lü, J., Liu, Z., Guo, W., Guo, M., Chen, S., Li, H., et al. (2020). Feeding delivery of dsHvSnf7 is a promising method for management of the pest Henosepilachna vigintioctopunctata (Coleoptera: Coccinellidae). *Insects* 11, 34. doi: 10.3390/insects11010034

Lubojacký, T. ČM. V. J. (2019). Analýza změn v produkci a zdravotním stavu smrkové tyčoviny v PLO 29, Nízký Jeseník. *Zprávy lesnického výzkumu* 64, 77–85.

Luo, Y., Wang, X., Wang, X., Yu, D., Chen, B., and Kang, L. (2013). Differential responses of migratory locusts to systemic RNA interference via double-stranded RNA injection and feeding. *Insect Mole. Biol.* 22, 574–583. doi: 10.1111/imb.12046

Ma, Z. Z., Zhou, H., Wei, Y. L., Yan, S., and Shen, J. (2020). A novel plasmid–*Escherichia coli* system produces large batch dsRNAs for insect gene silencing. *Pest Manag. Sci.* 76, 2505–2512. doi: 10.1002/ps.5792

Martinez, Z., De Schutter, K., Van Damme, E. J., Vogel, E., Wynant, N., Broeck, J. V., et al. (2021). Accelerated delivery of dsRNA in lepidopteran midgut cells by a Galanthus nivalis lectin (GNA)-dsRNA-binding domain fusion protein. *Pesticide Biochem. Physiol.* 175:104853. doi: 10.1016/j.pestbp.2021.104853

Martin-Ortigosa, S., Valenstein, J. S., Sun, W., Moeller, L., Fang, N., Trewyn, B. G., et al. (2012). Parameters affecting the efficient delivery of mesoporous silica nanoparticle materials and gold nanorods into plant tissues by the biolistic method. *Small* 8, 413–422. doi: 10.1002/smll.201101294

Máximo, W. P., Howell, J. L., Mogilicherla, K., Basij, M., Chereddy, S. C., and Palli, S. R. (2020). Inhibitor of apoptosis is an effective target gene for RNAi-mediated control of Colorado potato beetle, Leptinotarsa decemlineata. *Arch. Insect Biochem. Physiol.* 104:e21685.

McKenna, D. D., Scully, E. D., Pauchet, Y., Hoover, K., Kirsch, R., Geib, S. M., et al. (2016). Genome of the Asian longhorned beetle (Anoplophora glabripennis), a globally significant invasive species, reveals key functional and evolutionary innovations at the beetle–plant interface. *Genome Biol.* 17, 1–18. doi: 10.1673/031.009.2101

Meddens, A. J., Hicke, J. A., and Ferguson, C. A. (2012). Spatiotemporal patterns of observed bark beetle-caused tree mortality in British Columbia and the western United States. *Ecolog. Appl.* 22, 1876–1891. doi: 10.1890/11-1785.1

Mehlhorn, S., Ulrich, J., Baden, C. U., Buer, B., Maiwald, F., Lueke, B., et al. (2021). The mustard leaf beetle, Phaedon cochleariae, as a screening model for exogenous RNAi-based control of coleopteran pests. *Pesticide Biochem. Physiol.* 176:104870. doi: 10.1016/j.pestbp.2021.104870

Melnyk, C. W., Molnar, A., and Baulcombe, D. C. (2011). Intercellular and systemic movement of RNA silencing signals. *EMBO J.* 30, 3553–3563. doi: 10.1038/emboj.2011.274

Meng, P., Hoover, K., and Keena, M. (2015). Asian longhorned beetle (Coleoptera: Cerambycidae), an introduced pest of maple and other hardwood trees in North America and Europe. *J. Integr. Pest Manag.* 6:4.

Meyering-Vos, M., and Müller, A. (2007). RNA interference suggests sulfakinins as satiety effectors in the cricket Gryllus bimaculatus. *J. Insect Physiol.* 53, 840–848. doi: 10.1016/j.jinsphys.2007.04.003

Miller, S. C., Miyata, K., Brown, S. J., and Tomoyasu, Y. (2012). Dissecting systemic RNA interference in the red flour beetle Tribolium castaneum: parameters affecting the efficiency of RNAi. *PLoS One* 7:e47431. doi: 10.1371/journal.pone.0047431

Milletti, F. (2012). Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov. Today* 17, 850–860. doi: 10.1016/j.drudis.2012.03.002

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., et al. (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nature Plants* 3, 1–10. doi: 10.1079/9781789248890.0001

Mourrain, P., Béclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.-B., et al. (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 101, 533–542. doi: 10.1016/s0092-8674(00)80863-6

Murphy, K. A., Tabuloc, C. A., Cervantes, K. R., and Chiu, J. C. (2016). Ingestion of genetically modified yeast symbiont reduces fitness of an insect pest via RNA interference. *Sci. Rep.* 6, 1–13. doi: 10.1111/1744-7917.12006

Mysore, K., Li, P., Wang, C.-W., Hapairai, L. K., Scheel, N. D., Realey, J. S., et al. (2019). Characterization of a broad-based mosquito yeast interfering RNA larvicide with a conserved target site in mosquito semaphorin-1a genes. *Parasit. Vectors* 12, 1–15.

Nadeau, J. A., Petereit, J., Tillett, R., Jung, K., Fotoohi, M., MacLean, M., et al. (2017). Comparative transcriptomics of mountain pine beetle pheromone-biosynthetic tissues and functional analysis of CYP6DE3. *BMC Genom.* 18:1–15. doi: 10.1155/2012/491748

Numata, K., Horii, Y., Oikawa, K., Miyagi, Y., Demura, T., and Ohtani, M. (2018). Library screening of cell-penetrating peptide for BY-2 cells, leaves of Arabidopsis, tobacco, tomato, poplar, and rice callus. *Sci. Rep.* 8, 1–17.

Numata, K., Ohtani, M., Yoshizumi, T., Demura, T., and Kodama, Y. (2014). Local gene silencing in plants via synthetic ds RNA and carrier peptide. *Plant Biotechnol. J.* 12, 1027–1034. doi: 10.1111/pbi.12208

Oppert, B., and Perkin, L. (2019). RNAiSeq: How to see the big picture. *Front. Microbiol.* 10:2570.

Palli, S. R. (2014). RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. *Curr. Opinion Insect Sci.* 6, 1–8. doi: 10.1016/j.cois.2014.09.011

Pampolini, F., and Rieske, L. K. (2020). Emerald ash borer specific gene silencing has no effect on non-target organisms. *Front. Agron.* 2:29. doi: 10.3389/fagro.2020.608827

Pampolini, F., Rodrigues, T. B., Leelesh, R. S., Kawashima, T., and Rieske, L. K. (2020). Confocal microscopy provides visual evidence and confirms the feasibility of dsRNA delivery to emerald ash borer through plant tissues. *J. Pest Sci.* 93, 1143–1153. doi: 10.1007/s10340-020-01230-w

Pan, Y., Birdsey, R. A., Phillips, O. L., and Jackson, R. B. (2013). The structure, distribution, and biomass of the world's forests. *Ann. Rev. Ecol. Evol. Syst.* 44, 593–622.

Papadopoulou, N., Devos, Y., Álvarez-Alfageme, F., Lanzoni, A., and Waigmann, E. (2020). Risk assessment considerations for genetically modified RNAi plants: EFSA's activities and perspective. *Front. Plant Sci.* 11:445.

Parrish, S., Fleenor, J., Xu, S., Mello, C., and Fire, A. (2000). Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mole. Cell* 6, 1077–1087. doi: 10.1016/s1097-2765(00)00106-4

Pecot, C. V., Calin, G. A., Coleman, R. L., Lopez-Berestein, G., and Sood, A. K. (2011). RNA interference in the clinic: challenges and future directions. *Nat. Rev. Cancer* 11, 59–67.

Pettit, J. M., Voelker, S. L., DeRose, R. J., and Burton, J. I. (2020). Spruce beetle outbreak was not driven by drought stress: Evidence from a tree-ring iso-demographic approach indicates temperatures were more important. *Glob. Change Biol.* 26, 5829–5843. doi: 10.1111/gcb.15274

Poland, T. M., and McCullough, D. G. (2006). Emerald ash borer: invasion of the urban forest and the threat to North America's ash resource. *J. Forestry* 104, 118–124.

Powell, D., Großе-Wilde, E., Krokene, P., Roy, A., Chakraborty, A., Löfstedt, C., et al. (2020). A highly contiguous genome assembly of a major forest pest, the Eurasian spruce bark beetle Ips typographus. *bioRxiv* doi: 10.1101/2020.11.28.401976

Prentice, K., Christiaens, O., Pertry, I., Bailey, A., Niblett, C., Ghislain, M., et al. (2017). RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil Cylas puncticollis (Coleoptera: Brentidae). *Pest Manag. Sci.* 73, 44–52. doi: 10.1002/ps.4337

Prentice, K., Pertry, I., Christiaens, O., Bauters, L., Bailey, A., Niblett, C., et al. (2015). Transcriptome analysis and systemic RNAi response in the African sweetpotato weevil (Cylas puncticollis, Coleoptera, Brentidae). *PLoS One* 10:e0115336. doi: 10.1371/journal.pone.0115336

Pye, J. M., Holmes, T. P., Prestemon, J. P., and Wear, D. N. (2011). *Economic impacts of the southern pine beetle. In: Coulson, RN; Klepzig, KD 2011. Southern Pine Beetle II. Gen. Tech. Rep. SRS-140*. Asheville, NC: US Department of Agriculture Forest Service, Southern Research Station, 213–222.

Ramsfield, T., Bentz, B., Faccoli, M., Jactel, H., and Brockerhoff, E. (2016). Forest health in a changing world: effects of globalization and climate change on forest insect and pathogen impacts. *Forestry* 89, 245–252. doi: 10.1093/forestry/cpw018

Regniere, J. (2003). "Effect of climate change on range expansion by the mountain pine beetle in British Columbia," in *Mountain pine beetle symposium: challenges and solutions. Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Information Report BC-X-399*, eds T.L. Shore, J.E. Brooks and J.E. Stone (Victoria: British Columbia, Canada), 223–232.

Robert, J. A., Pitt, C., Bonnett, T. R., Yuen, M. M., Keeling, C. I., Bohlmann, J., et al. (2013). Disentangling detoxification: gene expression analysis of feeding mountain pine beetle illuminates molecular-level host chemical defense detoxification mechanisms. *PLoS One* 8:e77777. doi: 10.1371/journal.pone.0077777

Rodrigues, T. B., Dhandapani, R. K., Duan, J. J., and Palli, S. R. (2017a). RNA interference in the Asian longhorned beetle: identification of key RNAi genes and reference genes for RT-qPCR. *Sci. Rep.* 7, 1–10.

Rodrigues, T. B., Duan, J. J., Palli, S. R., and Rieske, L. K. (2018). Identification of highly effective target genes for RNAi-mediated control of emerald ash borer, Agrilus planipennis. *Sci. Rep.* 8, 1–9.

Rodrigues, T. B., Rieske, L. K., Duan, J. J., Mogilicherla, K., and Palli, S. R. (2017b). Development of RNAi method for screening candidate genes to control emerald ash borer, Agrilus planipennis. *Sci. Rep.* 7, 1–8.

Sachin, M., Mahalakshmi, S., and Kekuda, P. (2018). Insecticidal efficacy of lichens and their metabolites-A mini review. *J. Appl. Pharm. Sci.* 8, 159–164.

Safranyik, L., Carroll, A. L., Régnière, J., Langor, D., Riel, W., Shore, T., et al. (2010). Potential for range expansion of mountain pine beetle into the boreal forest of North America. *Can. Entomol.* 142, 415–442. doi: 10.4039/n08-cpa01

Saleh, M.-C., van Rij, R. P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P. H., et al. (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat. Cell Biol.* 8, 793–802. doi: 10.1038/ncb1439

Santos, D., Remans, S., Van den Brande, S., and Vanden Broeck, J. (2021). RNAs on the Go: Extracellular Transfer in Insects with Promising Prospects for Pest Management. *Plants* 10:484. doi: 10.3390/plants10030484

Schiebel, W., Haas, B., Marinković, S., Klanner, A., and Sänger, H. (1993). RNA-directed RNA polymerase from tomato leaves. II. Catalytic in vitro properties. *J. Biol. Chem.* 268, 11858–11867. doi: 10.1016/s0021-9258(19)50279-4

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., et al. (2013). Towards the elements of successful insect RNAi. *J. Insect Physiol.* 59, 1212–1221. doi: 10.1016/j.jinsphys.2013.08.014

Scully, E. D., Hoover, K., Carlson, J. E., Tien, M., and Geib, S. M. (2013). Midgut transcriptome profiling of Anoplophora glabripennis, a lignocellulose degrading cerambycid beetle. *BMC Genom.* 14, 1–26.

Seidl, R., Spies, T. A., Peterson, D. L., Stephens, S. L., and Hicke, J. A. (2016). Searching for resilience: addressing the impacts of changing disturbance regimes on forest ecosystem services. *J. Appl. Ecol.* 53, 120–129. doi: 10.1111/1365-2664.12511

SFA, S. (2010). *Statistical yearbook of forestry 2010. Official Statistics of Sweden*. Jönköping: Swedish Forest Agency, 337.

Shakesby, A., Wallace, I., Isaacs, H., Pritchard, J., Roberts, D., and Douglas, A. (2009). A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochem. Mol. Biol.* 39, 1–10. doi: 10.1016/j.ibmb.2008.08.008

Sharma, R., Taning, C. N. T., Smagghe, G., and Christiaens, O. (2021). Silencing of Double-Stranded Ribonuclease Improves Oral RNAi Efficacy in Southern Green Stinkbug Nezara viridula. *Insects* 12:115. doi: 10.3390/insects12020115

Shew, A. M., Danforth, D. M., Nalley, L. L., Nayga Jr., Tsiboe, F., and Dixon, B. L. (2017). New innovations in agricultural biotech: Consumer acceptance of topical RNAi in rice production. *Food Control* 81, 189–195. doi: 10.1016/j.foodcont.2017.05.047

Shih, J. D., and Hunter, C. P. (2011). SID-1 is a dsRNA-selective dsRNA-gated channel. *RNA* 17, 1057–1065. doi: 10.1261/rna.2596511

Silva, C. P., Silva, J. R., Vasconcelos, F. F., Petretski, M. D., DaMatta, R. A., Ribeiro, A. F., et al. (2004). Occurrence of midgut perimicrovillar membranes in paraneopteran insect orders with comments on their function and evolutionary significance. *Arthropod Struct. Dev.* 33, 139–148. doi: 10.1016/j.asd.2003.12.002

Singh, I. K., Singh, S., Mogilicherla, K., Shukla, J. N., and Palli, S. R. (2017). Comparative analysis of double-stranded RNA degradation and processing in insects. *Sci. Rep.* 7, 1–12. doi: 10.1002/9783527678679.dg03515

Sinisterra-Hunter, X., and Hunter, W. (2018). Towards a holistic integrated pest management: lessons learned from plant-insect mechanisms in the field. *Biol. Plant-Insect Interact.* 236, 204–226. doi: 10.1201/9781315119571-10

Six, D. L. (2020). A major symbiont shift supports a major niche shift in a clade of tree-killing bark beetles. *Ecol. Entomol.* 45, 190–201. doi: 10.1111/een.12786

Six, D. L., and Elser, J. J. (2020). Mutualism is not restricted to tree-killing bark beetles and fungi: the ecological stoichiometry of secondary bark beetles, fungi, and a scavenger. *Ecolog. Entomol.* 45, 1134–1145. doi: 10.1111/een.12897

Six, D. L., and Wingfield, M. J. (2011). The role of phytopathogenicity in bark beetle–fungus symbioses: a challenge to the classic paradigm. *Ann. Rev. Entomol.* 56, 255–272. doi: 10.1146/annurev-ento-120709-144839

Smardon, A., Spoerke, J. M., Stacey, S. C., Klein, M. E., Mackin, N., and Maine, E. M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans. *Curr. Biol.* 10, 169–178. doi: 10.1016/s0960-9822(00)00323-7

Speer, B. R., and Waggoner, B. (1997). Lichens: Life History & Ecology. *Univ. Calif. Museum Paleontol. Retr.* 28:2015.

Swevers, L., Vanden Broeck, J., and Smagghe, G. (2013). The possible impact of persistent virus infection on the function of the RNAi machinery in insects: a hypothesis. *Front. Physiol.* 4:319.

Taning, C. N., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., Mezzetti, B., et al. (2020). RNA-based biocontrol compounds: current status and perspectives to reach the market. *Pest Manag. Sci.* 76, 841–845. doi: 10.1002/ps.5686

Taning, C. N. T., Christiaens, O., Berkvens, N., Casteels, H., Maes, M., and Smagghe, G. (2016). Oral RNAi to control Drosophila suzukii: laboratory testing against larval and adult stages. *J. Pest Sci.* 89, 803–814. doi: 10.1007/s10340-016-0736-9

Taning, C. N. T., Gui, S., De Schutter, K., Jahani, M., Castellanos, N. L., Christiaens, O., et al. (2021). A sequence complementarity-based approach for evaluating off-target transcript knockdown in Bombus terrestris, following ingestion of pest-specific dsRNA. *J. Pest Sci.* 94, 487–503. doi: 10.1007/s10340-020-01273-z

Taning, C. N. T., Mezzetti, B., Kleter, G., Smagghe, G., and Baraldi, E. (2020). Does RNAi-Based Technology Fit within EU Sustainability Goals? *Trends Biotechnol.* 12:8. doi: 10.1016/j.tibtech.2020.11.008

Terenius, O., Papanicolaou, A., Garbutt, J. S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., et al. (2011). RNA interference in Lepidoptera: an overview of successful and unsuccessful studies and implications for experimental design. *J. Insect Physiol.* 57, 231–245.

Thom, D., and Seidl, R. (2016). Natural disturbance impacts on ecosystem services and biodiversity in temperate and boreal forests. *Biol. Rev.* 91, 760–781. doi: 10.1111/brv.12193

Thompson, J. D., Kornbrust, D. J., Foy, J. W., Solano, E. C., Schneider, D. J., Feinstein, E., et al. (2012). Toxicological and pharmacokinetic properties of chemically modified siRNAs targeting p53 RNA following intravenous administration. *Nucleic Acid Ther.* 22, 255–264. doi: 10.1089/nat.2012.0371

Timmons, L., Court, D. L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene* 263, 103–112. doi: 10.1016/s0378-1119(00)00579-5

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., and Bucher, G. (2008). Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. *Genome Biol.* 9, 1–22.

Ulrich, J., Majumdar, U., Schmitt-Engel, C., Schwirz, J., Schultheis, D., Ströhlein, N., et al. (2015). Large scale RNAi screen in Tribolium reveals novel target genes for pest control and the proteasome as prime target. *BMC Genom.* 16, 1–9.

Ulvila, J., Parikka, M., Kleino, A., Sormunen, R., Ezekowitz, R. A., Kocks, C., et al. (2006). Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in Drosophila S2 cells. *J. Biol. Chem.* 281, 14370–14375. doi: 10.1074/jbc.m513868200

Unnamalai, N., Kang, B. G., and Lee, W. S. (2004). Cationic oligopeptide-mediated delivery of dsRNA for post-transcriptional gene silencing in plant cells. *FEBS Lett.* 566, 307–310. doi: 10.1016/j.febslet.2004.04.018

Vaistij, F. E., Jones, L., and Baulcombe, D. C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* 14, 857–867. doi: 10.1105/tpc.010480

Van Ekert, E., Powell, C. A., Shatters Jr., and Borovsky, D. (2014). Control of larval and egg development in Aedes aegypti with RNA interference against juvenile hormone acid methyl transferase. *J. Insect Phys.* 70, 143–150. doi: 10.1016/j.jinsphys.2014.08.001

Van Rooijen, N., and van Nieuwmegen, R. (1980). Liposomes in immunology: multilamellar phosphatidylcholine liposomes as a simple, biodegradable and harmless adjuvant without any immunogenic activity of its own. *Immun. Comm.* 9, 243–256. doi: 10.3109/08820138009065997

Walski, T., Van Damme, E. J., and Smagghe, G. (2014). Penetration through the peritrophic matrix is a key to lectin toxicity against Tribolium castaneum. *J. Insect Phys.* 70, 94–101. doi: 10.1016/j.jinsphys.2014.09.004

Walter, D. E., and Proctor, H. C. (1999). *Mites: ecology, evolution, and behaviour*. Wallingford: CABI Publishing.

Wang, L.-J., Hsu, M.-H., Liu, T.-Y., Lin, M.-Y., and Sung, C.-H. (2020a). Characterization of the complete mitochondrial genome of Euwallacea fornicatus (Eichhoff, 1868)(Coleoptera: Curculionidae: Scolytinae) and its phylogenetic implications. *Mitochondrial DNA Part B* 5, 3502–3504. doi: 10.1080/23802359.2020.1827070

Wang, M., Weiberg, A., Lin, F.-M., Thomma, B. P., Huang, H.-D., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* 2, 1–10. doi: 10.1079/9781789248890.0001

Wang, X., Swing, C. J., Feng, T., Xia, S., Yu, J., and Zhang, X. (2020b). Effects of environmental pH and ionic strength on the physical stability of cinnamaldehyde-loaded liposomes. *J. Disp. Sci. Technol.* 41, 1568–1575. doi: 10.1080/01932691.2019.1627887

Wang, Y., Zhang, H., Li, H., and Miao, X. (2011). Second-generation sequencing supply an effective way to screen RNAi targets in large scale for potential application in pest insect control. *PLoS One* 6:e18644. doi: 10.1371/journal.pone.0018644

Wermelinger, B. (2004). Ecology and management of the spruce bark beetle Ips typographus—a review of recent research. *Forest Ecol. Manag.* 202, 67–82. doi: 10.1016/j.foreco.2004.07.018

Whangbo, J. S., and Hunter, C. P. (2008). Environmental RNA interference. *Trends Genet.* 24, 297–305.

Whitten, M. M., Facey, P. D., Del Sol, R., Fernández-Martínez, L. T., Evans, M. C., Mitchell, J. J., et al. (2016). Symbiont-mediated RNA interference in insects. *Proc. R. Soc. B: Biol. Sci.* 283:20160042. doi: 10.1098/rspb.2016.0042

Whyard, S., Singh, A. D., and Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* 39, 824–832. doi: 10.1016/j.ibmb.2009.09.007

Wieser, W. (1963). Adaptations of two intertidal isopods. II. Comparison between Campecopea hirsuta and Naesa bidentata (Sphaeromatidae). *J. Mar. Biol. Assoc. U K* 43, 97–112. doi: 10.1017/s0025315400005282

Williams, D. W., and Liebhold, A. M. (2002). Climate change and the outbreak ranges of two North American bark beetles. *Bark Beetles Fuels Fire Bibliogr.* 2002:34.

Williamson, D., and Vité, J. (1971). Impact of insecticidal control on the southern pine beetle population in east Texas. *J. Econ. Entomol.* 64, 1440–1444. doi: 10.1093/jee/64.6.1440

Willow, J., Sulg, S., Taning, C. N. T., Silva, A. I., Christiaens, O., Kaasik, R., et al. (2021). Targeting a coatomer protein complex-I gene via RNA interference results in effective lethality in the pollen beetle Brassicogethes aeneus. *J. Pest Sci.* 94, 703–712. doi: 10.1007/s10340-020-01288-6

Winston, W. M., Molodowitch, C., and Hunter, C. P. (2002). Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. *Science* 295, 2456–2459. doi: 10.1126/science.1068836

Wise, J., VanWoerkom, A., Acimovic, S., Sundin, G., Cregg, B., and Vandervoort, C. (2014). Trunk injection: a discriminating delivering system for horticulture crop IPM. *Entomol. Ornithol. Herpetol.* 3:1.

Wu, Y., Xu, L., Chang, L., Ma, M., You, L., Jiang, C., et al. (2019). Bacillus thuringiensis cry1C expression from the plastid genome of poplar leads to high mortality of leaf-eating caterpillars. *Tree Physiol.* 39, 1525–1532. doi: 10.1093/treephys/tpz073

Wuriyanghan, H., and Falk, B. W. (2013). RNA interference towards the potato psyllid, Bactericera cockerelli, is induced in plants infected with recombinant tobacco mosaic virus (TMV). *PLoS One* 8:e66050. doi: 10.1371/journal.pone.0066050

Wynant, N., Santos, D., Van Wielendaele, P., and Vanden Broeck, J. (2014a). Scavenger receptor-mediated endocytosis facilitates RNA interference in the desert locust. S chistocerca gregaria. *Insect Mole. Biol.* 23, 320–329.

Wynant, N., Santos, D., Verdonck, R., Spit, J., Van Wielendaele, P., and Broeck, J. V. (2014b). Identification, functional characterization and phylogenetic analysis of double stranded RNA degrading enzymes present in the gut of the desert locust, Schistocerca gregaria. *Insect Biochem. Mol. Biol.* 46, 1–8. doi: 10.1016/j.ibmb.2013.12.008

Wytinck, N., Manchur, C. L., Li, V. H., Whyard, S., and Belmonte, M. F. (2020). dsRNA Uptake in Plant Pests and Pathogens: Insights into RNAi-Based Insect and Fungal Control Technology. *Plants* 9:1780. doi: 10.3390/plants9121780

Xu, H. J., Chen, T., Ma, X. F., Xue, J., Pan, P. L., Zhang, X. C., et al. (2013). Genome-wide screening for components of small interfering RNA (siRNA) and

micro-RNA (miRNA) pathways in the brown planthopper, N ilaparvata lugens (H emiptera: D elphacidae). *Insect Mole. Biol.* 22, 635–647. doi: 10.1111/imb.12051

Xu, L., Xu, S., Sun, L., Zhang, Y., Luo, J., Bock, R., et al. (2021). Synergistic action of the gut microbiota in environmental RNA interference in a leaf beetle. *Microbiome* 9, 1–14.

Yan, S., Qian, J., Cai, C., Ma, Z., Li, J., Yin, M., et al. (2020a). Spray method application of transdermal dsRNA delivery system for efficient gene silencing and pest control on soybean aphid Aphis glycines. *J. Pest Sci.* 93, 449–459. doi: 10.1007/s10340-019-01157-x

Yan, S., Ren, B., Zeng, B., and Shen, J. (2020b). Improving RNAi efficiency for pest control in crop species. *BioTechniques* 68, 283–290. doi: 10.2144/btn-2019-0171

Yan, S., Ren, B. Y., and Shen, J. (2021). Nanoparticle-mediated double-stranded RNA delivery system: A promising approach for sustainable pest management. *Insect Sci.* 28, 21–34. doi: 10.1111/1744-7917.12822

Yoon, J.-S., Mogilicherla, K., Gurusamy, D., Chen, X., Chereddy, S. C., and Palli, S. R. (2018). Double-stranded RNA binding protein, Staufen, is required for the initiation of RNAi in coleopteran insects. *Proc Natl. Acad. Sci.* 115, 8334–8339. doi: 10.1073/pnas.1809381115

You, L., Zhang, F., Huang, S., Merchant, A., Zhou, X., and Li, Z. (2020). Over-expression of RNA interference (RNAi) core machinery improves susceptibility to RNAi in silkworm larvae. *Insect Mole Biol.* 29, 353–362. doi: 10.1111/imb.12639

Zhang, H., Li, H. C., and Miao, X. X. (2013). Feasibility, limitation and possible solutions of RNAi-based technology for insect pest control. *Insect Sci.* 20, 15–30. doi: 10.1111/j.1744-7917.2012.01513.x

Zhang, X., Zhang, J., and Zhu, K. (2010). Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (Anopheles gambiae). *Insect Mole. Biol.* 19, 683–693. doi: 10.1111/j.1365-2583.2010.01029.x

Zhang, Y., Xu, L., Li, S., and Zhang, J. (2019). Bacteria-mediated RNA interference for management of Plagiodera versicolora (Coleoptera: Chrysomelidae). *Insects* 10:415. doi: 10.3390/insects10120415

Zhao, C., Gonzales, M. A. A., Poland, T. M., and Mittapalli, O. (2015). Core RNAi machinery and gene knockdown in the emerald ash borer (Agrilus planipennis). *J. Insect Physiol.* 72, 70–78. doi: 10.1016/j.jinsphys.2014.12.002

Zhao, D., Zheng, C., Shi, F., Xu, Y., Zong, S., and Tao, J. (2021). Expression analysis of genes related to cold tolerance in Dendroctonus valens. *PeerJ* 9:e10864. doi: 10.7717/peerj.10864

Zheng, Y., Hu, Y., Yan, S., Zhou, H., Song, D., Yin, M., et al. (2019). A polymer/detergent formulation improves dsRNA penetration through the body wall and RNAi-induced mortality in the soybean aphid Aphis glycines. *Pest Manag. Sci.* 75, 1993–1999. doi: 10.1002/ps.5313

Zhou, Z., Li, Y., Yuan, C., Zhang, Y., and Qu, L. (2015). Oral administration of TAT-PTD–diapause hormone fusion protein interferes with Helicoverpa armigera (Lepidoptera: Noctuidae) development. *J. Insect Sci.* 15:1.

Zhu, F., Xu, J., Palli, R., Ferguson, J., and Palli, S. R. (2011). Ingested RNA interference for managing the populations of the Colorado potato beetle, Leptinotarsa decemlineata. *Pest Manag. Sci.* 67, 175–182. doi: 10.1002/ps.2048

Zhu, J.-Y., Zhao, N., and Yang, B. (2012a). Global transcriptional analysis of olfactory genes in the head of pine shoot beetle, Tomicus yunnanensis. *Comparative and functional genomics* 2012, 2012.

Zhu, J.-Y., Zhao, N., and Yang, B. (2012b). Global transcriptome profiling of the pine shoot beetle, Tomicus yunnanensis (Coleoptera: Scolytinae). *PLoS One* 7:e32291. doi: 10.1371/journal.pone.0032291

Zhu, K. Y., and Palli, S. R. (2020). Mechanisms, applications, and challenges of insect RNA interference. *Annu. Rev. Entomol.* 65, 293–311. doi: 10.1146/annurev-ento-011019-025224

Zotti, M., Dos Santos, E. A., Cagliari, D., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2018). RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Manag. Sci.* 74, 1239–1250. doi: 10.1002/ps.4813

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Exogenous Application of Non-mature miRNA-Encoded miPEP164c Inhibits Proanthocyanidin Synthesis and Stimulates Anthocyanin Accumulation in Grape Berry Cells

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Secondary metabolic pathways in grape berries are tightly regulated by an array of molecular mechanisms, including microRNA-mediated post-transcriptional regulation. As recently discovered, before being processed into mature microRNAs (miRNAs), the primary transcripts of miRNAs (pri-miRNAs) can encode for small miRNA-encoded peptides (micropeptides – miPEPs) that ultimately lead to an accentuated downregulation of the respective miRNA-targeted genes. Although few studies about miPEPs are available, the discovery of miPEPs reveals a new layer of gene regulation at the post-transcriptional level that opens the possibility to regulate plant metabolism without resorting to gene manipulation. Here, we identified a miPEP encoded in non-mature *miR164c* putatively targeting grapevine transcription factor VvMYBPA1 (miPEP164c/miPEP-MYBPA1), a positive regulator of key genes in the proanthocyanidin (PA)-biosynthetic pathway, a pathway that competes directly for substrate with the anthocyanin-biosynthetic pathway. Thus, the objective of this work was to test the hypothesis that the exogenous application of miPEP164c (miPEP-MYBPA1) can modulate the secondary metabolism of grape berry cells by inhibiting PA biosynthetic pathway while simultaneously stimulating anthocyanin synthesis. The exogenous application of miPEP164c to suspension-cultured cells from grape berry (cv. Gamay) enhanced the transcription of its corresponding non-mature *miR164c*, with a maximum effect at 1 μM and after a period of 10 days, thus leading to a more pronounced post-transcriptional silencing of its target VvMYBPA1. This led to a significant inhibition of the PA pathway, mostly *via* inhibition of leucoanthocyanidin reductase (LAR) and

anthocyanidin reductase (ANR) enzymatic activities and *VvLAR1* downregulation. In parallel, the anthocyanin-biosynthetic route was stimulated. Anthocyanin content was 31% higher in miPEP164c-treated cells, in agreement with the observed upregulation of *VvUFGT1* transcripts and UFGT enzyme activity levels.

Keywords: non-mature miRNA-encoded peptide, miRNA regulation, grape berry secondary metabolism, post-transcriptional regulation, anthocyanins

INTRODUCTION

Although grapevines are well adapted to semi-arid climate, the increasingly more frequent combined effect of drought, high air temperature and high evaporative demand has a negative impact in grapevine yield (Chaves et al., 2010) and, if severe, also in berry quality (Teixeira et al., 2013). Therefore, berry and wine quality depend strongly on the grapevine adaptability to drought, heat and light/UV intensity. This abiotic stressors particularly impact highly regulated molecular mechanisms underlying the synthesis of several quality-related compounds, such as anthocyanins, proanthocyanidins (PAs), flavanols, and flavonols (Downey et al., 2006; Teixeira et al., 2013).

Anthocyanin and PA (condensed tannins) biosynthetic pathways compete for two common precursors, leucoanthocyanidins and anthocyanidins (Li et al., 2016). Proanthocyanidins are composed of several monomers of (+)-catechin and (−)-epicatechin, both flavan-3-ols that originate in a branch deviation of the general flavonoid pathway. Catechin synthesis is catalyzed by leucoanthocyanidin reductase (LAR) that uses leucoanthocyanidins as substrate. However, leucoanthocyanidins can also be catalyzed by leucoanthocyanidin oxygenase (LDOX), continuing the flavonoid pathway and resulting in the formation of anthocyanidins, a substrate of both UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT), in the synthesis of anthocyanins, and anthocyanidin reductase (ANR), in the synthesis of epicatechin, another building block of PAs (Gagné et al., 2009).

In grapevine, many transcription factors belonging to the R2R3-MYB family are involved in the regulation of flavonoid synthesis by inducing or silencing key biosynthetic genes along the flavonoid pathway (Deluc, 2006; Matus et al., 2009). The transcription factors VvMYB5a and VvMYB5b are already described as positive regulators of the flavonoid pathway, inducing an upregulation of late-stage berry-associated genes such as *VvCHI* (chalcone isomerase), *VvF3′5* (flavonoid 3′,5′-hydroxylase), *VvDFR* (dihydroflavonol 4-reductase), *VvLDOX*, *VvANR*, and *VvLAR1* leading to the synthesis of flavonols, anthocyanidins, and PAs (Cavallini et al., 2014; Pérez-Díaz et al., 2016). *VvMYBPA1*, expressed during flowering and early berry development, is a positive regulator of PA synthesis, by upregulating *VvLDOX*, *VvANR*, and *VvLAR1* genes (Bogs et al., 2007; Cavallini et al., 2015), thus limiting the progress of the anthocyanin-biosynthetic route.

Regulation of the flavonoid pathway can also be coordinated at the post-transcriptional level by several microRNAs (miRNAs; Xie et al., 2010) that negatively regulate the expression of their target genes, either by promoting degradation of such target messenger RNAs (mRNAs) or by leading to inhibition of targeted mRNA translation (Pantaleo et al., 2010). MicroRNAs are initially transcribed as much larger primary transcripts (pri-miRNAs) and only become mature miRNA after several maturation processes (Xie et al., 2010). Like any other protein-coding gene, miRNAs are transcribed by RNA polymerase II originating the primary transcript of miRNA (pri-miRNA) that consists of a few hundred bases, a 5′cap and 3′ poly-A tail and the characteristic stem-loop structure where the miRNA sequence is inserted, and which is recognized by members of the Dicer-like1 family enzymes. This enzyme cleaves the 5′cap and 3′ poly-A tail of the primary transcript, transforming it in a precursor miRNA (pre-miRNA). DCL1 also carries out the subsequent cleavage of pre-miRNA to release the miRNA:miRNA* duplex which is then translocated to the nucleus by HASTY transporter where the correct miRNA strand is incorporated in a ribonuclear particle to form the RISC complex, the machinery that mediates miRNA-mediated gene silencing (Budak and Akpinar, 2015).

In a groundbreaking finding, it was discovered that, before being processed into mature miRNAs, some pri-miRNAs contain small open reading frames (ORF) that could encode for small regulatory peptides called miRNA-encoded peptides (miPEPs; Lauressergues et al., 2015). The mechanism of action of miPEPs is by enhancing the transcription and accumulation of the corresponding pri-miRNA, in a sort of positive feedback loop, that subsequently results in accentuated downregulation of the respective miRNA-targeted genes (Couzigou et al., 2015). For instance, the overexpression of miPEP171b in *Medicago truncatula* led to the increased accumulation of endogenous miR171b (involved in the formation of lateral roots), which resulted in significant changes in root development (Couzigou et al., 2016). Moreover, in soybean (*Glycine max*), it was demonstrated for the first time that the exogenous application of well-chosen, synthetic miPEP172c had a positive impact in nodule formation, by inducing the overexpression of *pri-miR172c*, whose correspondent miR172c accumulation results in an increase in nodule formation and consequent improvement of nitrogen fixation (Couzigou et al., 2016).

More recently, a miPEP in *Arabidopsis*, miPEP858, was reported by screening the 1,000 bp region upstream of pre-miR858 for small ORFs (Sharma et al., 2020) miPEP858 was able to modulate the expression of targets gene involved in plant growth and development and also on the phenylpropanoid pathway, by inducing the expression of *pri-miR858*.

In a very recent finding, using a fluorescein-labeled peptide, it was demonstrated that exogenously applied miPEP-156a could effectively penetrate plant seedlings through the root system and disperse systemically to the leaves of young seedlings of *Brassica*

rapa (Erokhina et al., 2021). The application of this miPEP exerted a moderate morphological effect consisting of a growth acceleration of the main root of the seedling, that was parallel to an increase in *pri-miR156a* expression. These morphological and molecular level effects were apparently related with the ability of the miPEP to rapidly transfer into the cell nuclei and bind to nuclear chromatin.

Screening for small ORFs, either in the precursor sequence of miRNA or in the region upstream of such precursor, is the mainstream method for finding putative miPEPs when a miRNA is available, or a targeted gene is in mind. Yet, alternative molecular approaches for screening for miPEPs have also been successful. These include homology based computational analysis using expressed sequence tags (ESTs) of a certain species genome by blasting it against miRNA sequences already described, to find homologous of miRNAs and then repeat the same methodologies of miRNA target prediction and screening for small ORFs in the pre-miRNA sequence (Ram et al., 2019).

Recently, a miPEP in grapevine was reported, miPEP171d1, originating from a non-mature miRNA conserved within different plant species and associated with root organ development. When exogenously applied, miPEP171d1 was able to promote adventitious root formation thus enabling to overcome challenges in clonal propagation (Chen et al., 2020).

Although few studies about miPEPs are available, the discovery of miPEPs reveals a new layer of gene regulation at the post-transcriptional level that opens the possibility to regulate plant metabolism without resorting to gene manipulation.

Taking these groundbreaking discoveries as basis, the objective of this work was to test the hypothesis that the exogenous application of a newly identified putative grapevine miPEP by our group (miPEP164c – miPEP-MYBPA1) can modulate the secondary metabolism of grape berry cells by inhibiting PA biosynthetic pathway while simultaneously stimulating anthocyanin synthesis. The micropeptide miPEP164c is putatively targeting MYBPA1, as predicted *in silico*, a gene encoding for a transcription factor that acts as a positive regulator of PA synthesis by activating the expression of *VvLAR* and *VvANR*, the genes encoding for the enzymes responsible for catechin and epicatechin synthesis (Bogs et al., 2007). For that, a wide array of molecular biology and classic biochemistry approaches were combined to better assess the impact of miPEP164c exogenous treatments on the transcription of key genes involved in secondary metabolic pathways, on the biochemical activity of the corresponding key enzymes, and on the final concentration of secondary metabolites such as anthocyanins and PAs.

MATERIALS AND METHODS

In silico Analyses

A series of *in silico* analyses to identify potential miPEPs in grapevine by combining several bioinformatic tools and databases such as the bioinformatic tool psRNATarget Finder (Dai et al., 2018), a plant small regulatory RNA target predictor, with the aid of GenBank, was used to retrieve information on which, how and where (in the target RNA) miRNAs putatively regulated key genes directly or indirectly involved in the flavonoid biosynthetic pathway. We identified *miR164c* as a putative negative regulator of transcription factor VvMYBPA1, involved in regulation of the PA pathway and proceeded to screen it in miRbase (microRNA database; Kozomara and Griffiths-Jones, 2014) for their stem-loop sequence or pri-miRNA sequence, the non-mature sequence of the regulatory miRNA possibly harboring small ORFs corresponding to regulatory miPEPs. Finally, the obtained stem-loop sequence was then ran in a bioinformatic ORF finder tool that recognizes in the introduced sequence all possible ORFs that could translate into a small peptide, by defining several parameters based on the few miPEPs so far identified in the literature (Lauressergues et al., 2015). This several step analysis led to the identification of one ORF with 48 bp, encoding a putative miPEP of 16 amino acids, that was designated as miPEP164c.

Solubilization of miRNA-Encoded Peptides

Following *in silico* identification, miPEP164c and a scrambled miPEP were ordered from Smart Bioscience as 1 mg aliquot. The scrambled miPEP aminoacidic sequence is the same as miPEP164c aminoacidic composition but in a random/scrambled order: MGTISKETCSQTNQCT. Solubilization of the micropeptides was conducted as recommended by Smart-Bioscience Peptide Solubility Guidelines.[1] Both miPEPs were solubilized in 200 μL of acetic acid (10%) and 800 μL of DMSO to a final concentration of 1 mg/mL. A solution of 200 μL of acetic acid (10%) and 800 μL of DMSO was used as control in the exogenous treatments.

Biological Material

Grape berry cell suspensions of the cultivar Gamay Freaux cv. were maintained in Gamborg B5 medium in 250 mL flasks at 25°C with constant agitation on a rotator shaker at 100 rpm and under 16 h/8 h photoperiod. The culture medium composition was as follows: 3 g/L Gamborg B5 salt mixture and vitamins; 30 g/L sucrose (3% m/v); 250 mg/L casein enzymatic hydrolysate; 0.1 mg/L α-naphthaleneacetic acid (NAA); 0.2 mg/L Kinetin, and a final pH of 5.7. The suspension-cultured cells were allowed to grow for 10-day, until the exponential phase, when they were subcultured by transferring 10 mL of cells to 30 mL of fresh medium.

Exogenous Addition of Micropeptides to Gamay Grape Cells

Immediately after sub-cultivation, concentrations ranging from 0.1 to 2 μM of miPEP164c were added to the cell cultures, in a volume that represented no more than 0.15% (v/v) of the total volume of the cell suspension. All cell suspensions, including control cells (treated with the same volume of control solution) and cells treated with the scrambled miPEP (a control miPEP with the same aminoacidic composition as miPEP164c

[1]https://www.smart-bioscience.com/support/solubility/

but with a scrambled amino acid sequence) were cultivated for 10-day with constant agitation and a 16 h/8 h photoperiod. A time-course analysis of 1 μM of miPEP164c with several time-points (1 h, 24 h, 3-, 5- and 10-day) was also conducted. Also, upon miPEP164c addition (t = 0 h), samples were taken for anthocyanin and PA base level quantifications. Cells were collected when necessary, filtered and immediately frozen with liquid nitrogen and stored at −80°C. A part of cells of each experimental condition was lyophilized.

Quantification of Anthocyanins

Anthocyanins were extracted from 100 mg of grape berry cells from each experimental condition. After adding 1 mL of 90% methanol and 10% deionized H_2O, the suspensions were vigorously shaken for 30 min, following centrifugation at 18000 × g for 20 min. The supernatants were collected and 200 μL of each supernatant was mixed with 1.8 mL of a solution of 25 mM KCl (pH = 1.0) and absorbance was measured at 520 and 700 nm. Total anthocyanin quantification was calculated in relation to cyanidin-3-glucoside (C-3-G) equivalents, as follows in the equation, and subsequently presented per dry weight (DW):

$$[\text{Total anthocyanins}]\left(\text{mg/L}\right) = \frac{(A_{520} - A_{700}) \times MW \times DF \times 1000}{\varepsilon \times 1}$$

where MW is the molecular weight of C-3-G (449.2 g mol^{-1}), DF is the dilution factor and ε is the molar extinction coefficient of C-3-G (26900 M^{-1} cm^{-1}).

Quantification of Proanthocyanidins

Proanthocyanidin content was determined using an adapted colorimetric vanillin-HCl assay described by Broadhurst and Jones (1978). To extract PAs, 1 mL of 100% methanol was added to 5 mg of lyophilized grape berry cells and vigorously shaken for 30 min followed by centrifugation at 18000 × g for 15 min. Supernatants were collected and diluted in a 1:1 ratio with methanol to final volume of 500 μL. The methanolic extracts were added to clean assay tubes wrapped with aluminum foil. Then, 3 mL of a solution of 4% (m/v) vanillic acid freshly prepared in methanol was added and mixed very gently. Finally, 1.5 mL of concentrated hydrochloric acid was added to each reaction tube and mixed very gently. The reactions were allowed to stand for 6 min and the absorbance of the samples was measured spectrophotometrically at 500 nm. To discard absorbance interference caused by anthocyanin presence in the samples, control reactions for each condition were prepared with 3 mL of methanol instead of vanillic acid and the absorbance measured at 500 nm was discounted from the absorbance of reaction mixtures with vanillic acid. An epigallocatechin gallate (EGCG) standard curve, with concentrations ranging from 10 to 200 μg employing the same method was always prepared for each quantification of PA content.

Protein Extraction

Protein extraction was conducted as described in Conde et al. (2016). Lyophilized grape berry cells were mixed with extraction buffer in approximately 1:1 (v/v) powder: buffer ratio. Protein extraction buffer contained 50 mM Tris–HCl, pH 8.9, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 5 mM DTT, and 0.1% (v/v) Triton X-100. Homogenates were thoroughly vortexed and centrifuged at 18000 × g for 15 min at 4°C. Supernatants were maintained on ice and used for all enzymatic assays. Total protein concentrations of the extracts were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Enzymatic Activity Assays

The biochemical activity of UFGT was determined as described by Lister et al. (1996) adapted by Conde et al. (2016). The assay mixture contained 385 μL of 100 mM Tris–HCl reaction buffer (pH 8), 100 μL of enzyme extract, 10 μL of 50 mM UDP-glucose and the reaction was initiated with 5 μL of 100 mM quercetin as substrate for the enzyme activity (saturating concentration) to a final reaction volume of 500 μL. Each mixture was incubated for 30 min in the dark with gentle shaking. After incubation, dilutions were prepared with 100 μL of each assay mixture and 900 μL of Tris–HCl reaction buffer and absorbance was read at 350 nm immediately after (t = 0) and 30 min later (t = 30) to follow the production of quercetin 3-glucoside (ε = 21877 M^{-1} cm^{-1}).

Leucoanthocyanidin reductase enzymatic activity was measured by spectrophotometrically monitoring the conversion of dihydroquercetin to (+)-catechin following the method of Gagné et al. (2009) with some adaptions. The assay mixture contained 1.7 mL of Tris–HCl buffer (0.1 M, pH 7.5), 300 μL of protein extract, 2 μL of NADPH (100 mM) and the reaction was initiated by adding 1 μL of dihydroquercetin (10 mg mL^{-1} in DMSO). The production of (+)-catechin (ε = 10233 M^{-1} cm^{-1}) was followed at 280 nm for 30 min.

The biochemical activity of ANR was determined as described by Zhang et al. (2012) with some adaptations. The assay mixture contained 1.5 mL of PBS buffer (0.1 M, pH 6.5), 60 μL of enzyme extract, 40 μL of ascorbic acid (20 mM), 50 μL of cyanidin chloride (2 mM) and the reaction was initiated by adding 75 μL of NADPH (20 mM) followed by a 1/10 dilution with PBS reaction buffer for proper absorbance measure. The enzyme activity was monitored by measuring the rate of NADPH (ε = 6.22 mM^{-1} cm^{-1}) oxidation at 340 nm for 20 min, at 45°C.

RNA Extraction and cDNA Synthesis

Total RNA extraction was performed according to Reid et al. (2006) in combination with purification steps from the GRS Total Plant RNA extraction kit. After treatment with DNase I (Qiagen), cDNA was synthesized from 1 μg of total RNA using Maxima first strand cDNA synthesis kit from Thermo Fisher Scientific, following the manufacturer's instructions. RNA concentration and purity were determined using Nanodrop and its integrity assessed in a 1% agarose gel stained with SYBR Safe (InvitrogenTM, Life Technologies).

Transcriptional Analyses by Real-Time qPCR

Quantitative real-time PCR was performed with QuantiTect SYBR Green PCR Kit (Qiagen) in a CFX96 Real-Time Detection System (Bio-Rad), using 1 μL of cDNA in a final reaction volume of 10 μL per well. Specific primer pairs used for each target gene are listed in **Supplementary Table 1**. Melting curve analysis was performed for specific gene amplification confirmation. As reference genes, *VvACT1* (actin) and *VvGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) were selected, as these genes were proven to be very stable and ideal for qPCR normalization purposes in grapevine (Reid et al., 2006). For all experimental conditions tested, three biological replicates were used with internal triplicates. The expression values were normalized by the average of the expression of the reference genes as described by Pfaffl (2001) and analyzed using the software Bio-Rad CFX Manager (Bio-Rad).

Statistical Analyses

The results were statistically analyzed by Student's *t*-test using Prism vs. 6 (GraphPad Software, Inc.). For each condition, statistical differences between the mean values of miPEP-treated cells and control cells are marked with asterisks. For every experimental approach, three biological replicates (constituted by three independent treatments and respective experiments) were used.

RESULTS

Identification and *in silico* Analysis of the Candidate Grapevine Micropeptide miPEP164c

An *in silico* analysis for micropeptide screening led to the selection of miPEP164c, a candidate miPEP with putative regulatory function in grape berry flavonoid biosynthesis metabolic pathway, particularly in the branch of PA synthesis. miR164c was predicted *in silico* to post-transcriptionally inhibit grapevine transcription factor *VvMYBPA1*, involved in the activation of flavonoid synthesis, specifically of PA synthesis (*via* LAR1, LAR2, and ANR activation). Relevant information obtained by the *in silico* analysis regarding the miPEP selected for this study, including its aminoacidic sequence, attributed name and respective mature miRNA name and miRbase accession number, as well as that of its precursor miRNA (pre-miRNA), is detailed in **Table 1**.

Effect of miPEP164c Exogenous Application on the Abundance of Pre-miR164c and Its Putative Target Transcription Factor VvMYBPA1

To confirm if miPEP164c exogenous application is indeed activating the accumulation of its *in silico* predicted miRNA (*miR164c*), gene expression analysis by real-time qPCR of the non-mature pre-miR164c was performed on cells treated with different concentrations of miPEP164c, ranging from 0.1 to 2 μM. As shown in **Figure 1A**, 10 days after treatment, the transcript levels of *pre-miR164c* were increasingly upregulated along miPEP concentration reaching a maximum effect at 1 μM (3.5-fold increase) that was approximately maintained at 2 μM of miPEP164c (3.2-fold increase). The exogenous application of a scrambled miPEP at 1 μM did not result in any changes in transcript levels of *pre-miR164c* after 10 days of treatment.

A time-course analysis of the transcript levels of *pre-miR164c* in cells treated with 1 μM of miPEP164c through a period of 10 days (1 h, 24 h, 3-, 5- and 10-day) confirmed, as shown in **Figure 2A**, that the expression of *pre-miR164c* was slightly stimulated at 24 h and 5-day of miPEP164c treatment, but had the highest increase (3.5 fold) after 10 days of treatment (**Figure 1B**).

No statistically significant change in the transcript levels of *VvMYBPA1* was observed after 10 days of miPEP164c treatment (**Figure 1C**).

Effect of miPEP164c Exogenous Application on Grape Berry Key Secondary Metabolites

Spectrophotometric quantifications of grape berry cells secondary metabolites revealed a significant increase in anthocyanin content at 0.5, 1, and 2 μM of miPEP164c after 10 days of treatment with the highest concentration of anthocyanins quantified in cells treated with 1 μM miPEP164c, with 7.4 mg of total anthocyanins per g of dry weight compared to only 5.6 mg per g of dry weight in control cells, which represented a 31% increase (**Figure 2A**).

In parallel, PAs concentration decreased significantly at those same concentrations of 0.5, 1, and 2 μM of miPEP164c with a more representative decrease (26%) from 54 mg of PAs per g of dry weight in control cells to 42.7 mg of PAs per g of dry weight after 10 days of treatment with 1 μM miPEP164c (**Figure 2B**). The exogenous application of 1 μM of the scrambled miPEP did not change the concentrations of both anthocyanins and PAs.

TABLE 1 | Detailed information about the micropeptide identified by an *in silico* analysis and selected for this study and its corresponding mature miRNA and mode of action.

miPEP	Amino acid sequence	miRNA	Stem-loop sequence	Mode of action	Predicted target
miPEP164c (miPEP-MYBPA1)	MEKQGTCITSSCTTNQ	miR164c (MIMAT0005660)*	MI006505*	Inhibition in translation	*VvMYBPA1*

**Acession code for miRBase (Kozomara and Griffiths-Jones, 2014).*

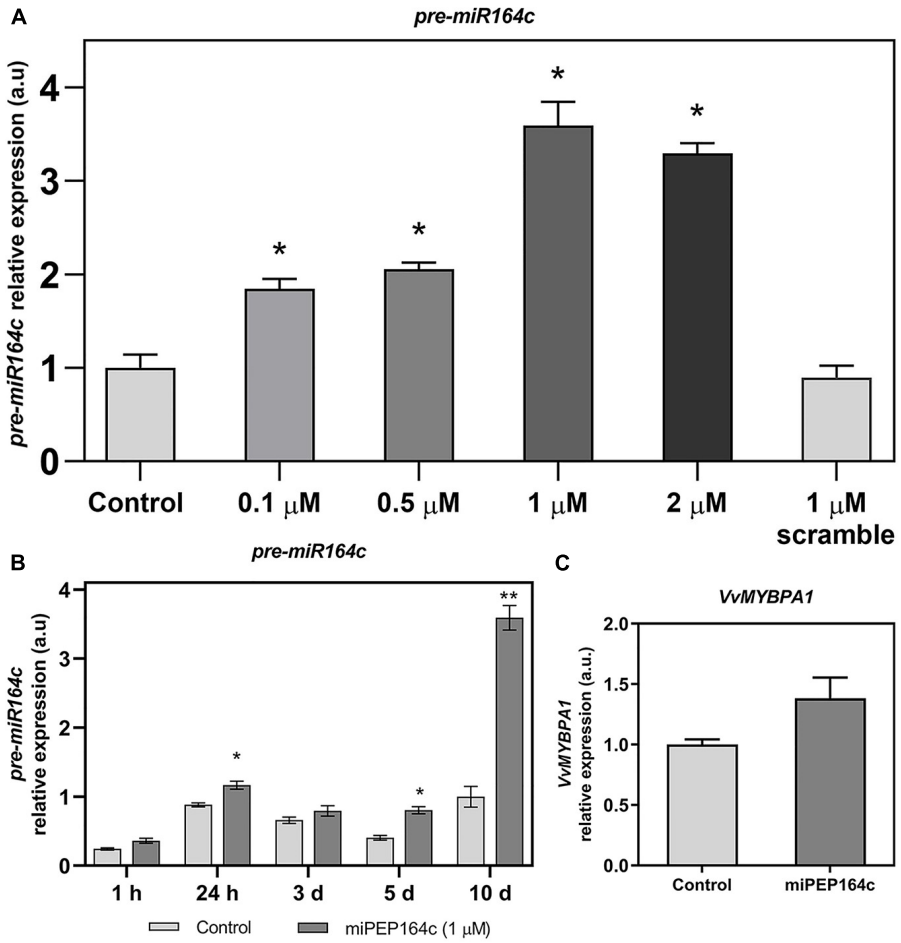

FIGURE 1 | Steady-state transcript levels of *pre-miR164c* in suspension-cultured grape berry cells (cv. Gamay) 10-day after elicitation with various concentrations of miPEP164c **(A)** in suspension-cultured grape berry cells (cv. Gamay) eliciated with 1 μM of miPEP164c throughout a period of 10-day **(B)** and the steady-state transcript levels of *VvMYBPA1* **(C)** in suspension-cultured grape berry cells (cv. Gamay) 10 days after elicitation with 1 μM miPEP164c. Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance in relation to the control (Student's *t*-test; $*P < 0.05$; $**P < 0.001$).

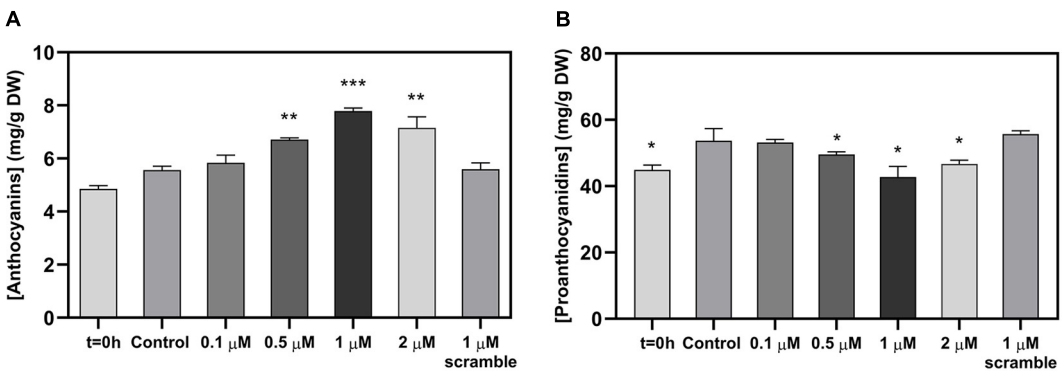

FIGURE 2 | Effect of the exogenous application of different concentrations of miPEP164c on total anthocyanin content **(A)** and on total proanthocyanidin (PA) content **(B)** in suspension-cultured grape berry cells (cv. Gamay) 10-day after elicitation with miPEP164c. Anthocyanin concentration is represented as mg of cyanidin-3-glucoside (C-3-G) equivalents per g of fresh weight (FW). Asterisks indicate statistical significance in relation to the control (Student's *t*-test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

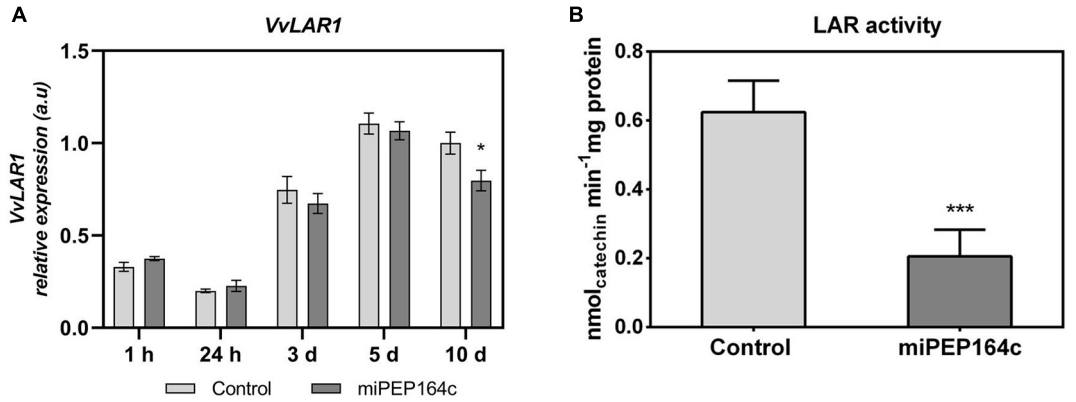

FIGURE 3 | Steady-state transcript levels of *VvLAR1* in suspension-cultured grape berry cells (cv. Gamay) 10-day after elicitation with various concentrations of miPEP164c **(A)** and the effect on the specific activity of leucoanthocyanidin reductase (LAR) **(B)** in suspension-cultured grape berry cells (cv. Gamay) after 10 days of elicitation with 1 μM miPEP164c. Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance in relation to the control (Student's *t*-test; $^{*}P < 0.05$). LAR biochemical activity represented as the V_{max} in grape berry cells under miPEP164c treatment. Values are the mean ± SEM. Asterisks indicates statistical significance (Student's *t*-test; $^{***}P < 0.001$).

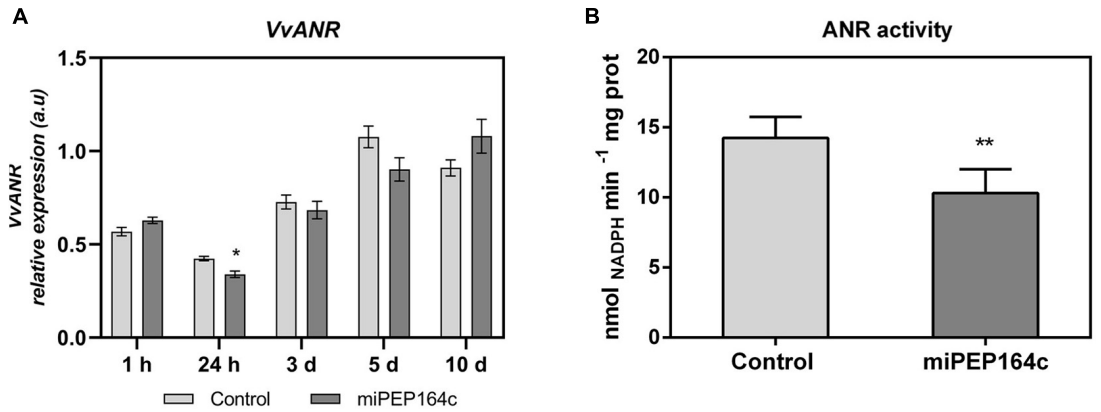

FIGURE 4 | Steady-state transcript levels of *VvANR* in suspension-cultured grape berry cells (cv. Gamay) 10-day after elicitation with various concentrations of miPEP164c **(A)** and the effect on the specific activity of anthocyanidin reductase (ANR) **(B)** in suspension-cultured grape berry cells (cv. Gamay) after 10 days of elicitation with 1 μM miPEP164c. Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance in relation to the control (Student's *t*-test; $^{*}P < 0.05$). ANR biochemical activity represented as the V_{max} in grape berry cells under miPEP164c treatment. Values are the mean ± SEM. Asterisks indicates statistical significance (Student's *t*-test; $^{**}P < 0.01$).

Transcriptional and Biochemical Changes Induced by miPEP164c on the Proanthocyanidin-Synthesizing Branch

A time-course analysis by real-time qPCR on *VvLAR1* expression in cells treated with 1 μM of miPEP164c showed a significant decrease of 20% after 10-day, while no statistically significant changes were observed on earlier time-points (**Figure 3A**). In agreement with the observed decrease in the *VvLAR1* transcripts, LAR enzyme activity was 3-fold reduced in Gamay cells elicited with 1 μM miPEP164c, decreasing from a V_{max} of 0.63 nmol min^{-1} mg $protein^{-1}$ in control cells to a V_{max} of 0.20 nmol min^{-1} mg $protein^{-1}$ in miPEP-treated cells (**Figure 3B**). Regarding *VvANR* transcript levels, although there was a slight decrease at 24 h of miPEP exposure (20%) when compared to control cells, no changes were observed at other time-points (**Figure 4A**). However, a significant decrease of 27% was observed in the enzyme activity of ANR in cells treated with 1 μM miPEP164c for 10-day, from a V_{max} of 14 nmol min^{-1} mg $protein^{-1}$ in control cells to 10.31 nmol min^{-1} mg $protein^{-1}$ in miPEP-treated cells (**Figure 4B**).

Transcriptional and Biochemical Changes Induced by miPEP164c on the Anthocyanin-Synthesizing Branch

The expression of *VvUFGT1* was strongly stimulated by miPEP164c application, reflected by a 4-fold increase in the expression levels in grape berry cells under this treatment (**Figure 5A**). The biochemical activity of UFGT was 3.2-fold higher in miPEP164c treated cells, in agreement with the transcriptional analysis, reaching a V_{max} of 3.4 μmol h^{-1} mg

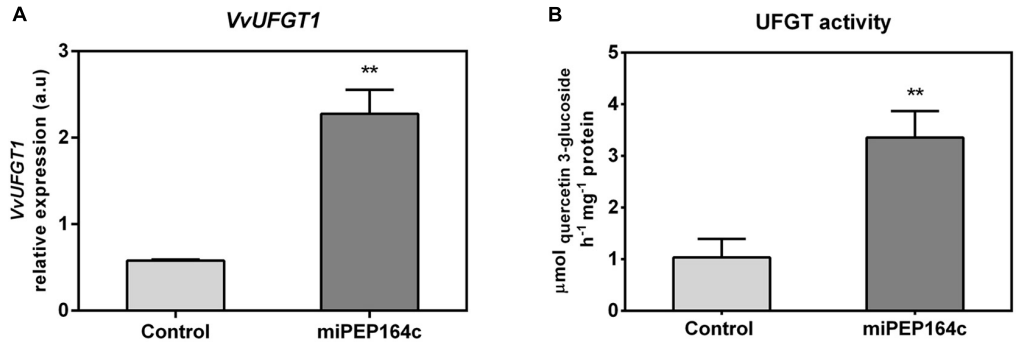

FIGURE 5 | Steady-state transcript levels of *VvUFGT1* **(A)** in suspension-cultured grape berry cells (cv. Gamay) 10-day after elicitation with 1 μM miPEP164c and the effect on the specific activity of UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT). **(B)** Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's *t*-test; $^{**}P < 0.01$). UFGT biochemical activity, represented as the V_{max} in grape berry cells under miPEP164c treatment. Values are the mean ± SEM. Asterisks indicates statistical significance (Student's *t*-test; $^{**}P < 0.01$).

protein^{-1} (**Figure 5B**), which corroborates with a significant increase in the total concentration of anthocyanins observed previously (**Figure 2A**).

The expression levels of *VvDFR* were also significantly stimulated, with a 2-fold increase in grape berry cells 10 days after miPEP164c treatment (**Figure 6A**). *VvLDOX* expression was also significantly stimulated by 42% when compared to control cells (**Figure 6B**).

Transcriptional analysis showed that the expression of *VvGST4* under miPEP164c treatment also increased 2-fold (**Figure 7A**). Similarly, the transcript levels of *VvMATE1* increased by 55% (**Figure 7B**), while the expression of *VvABCC1* seemed not to be affected by treatment with this micropeptide (**Figure 7C**). These genes encode transporters that accumulate anthocyanins in the vacuole.

DISCUSSION

Grape berry secondary metabolism generates, by a cascade of reactions scattered through different branches of the phenylpropanoid pathway, a wide range of bioactive compounds with key roles in plant defense responses and with several health-related benefits to humans, making them metabolites of interest for many industries (Teixeira et al., 2013). Therefore, the search for new strategies to modulate these complex pathways in the hopes of either minimizing the effects of several stress factors in the composition and quality of grape secondary metabolites or to increase the synthesis and accumulation of bioactive metabolites of interest such as antioxidant compounds like anthocyanins, is a research line of great importance, not only to the viticulture industry but also in several industries of health-promoting products (Zhang et al., 2015). In the present study, we sought to validate a new and promising strategy to modulate the secondary metabolism of Gamay grape berry cells by testing a synthetic miPEP, putatively enhancing the transcription and accumulation of *miR164c* and ultimately promoting a more pronounced silencing of its predicted target. Because this transcription factor is involved in the molecular activation of key genes in the PA pathway, ultimately, we wanted to evaluate if a miPEP-based treatment could regulate grape berry secondary metabolism, by activating miRNA-mediated post-transcription silencing mechanisms of specific targets. Results obtained were very promising as this treatment could represent an innovative and easy-to-apply strategy to modulate the synthesis of more quality-related compounds, resulting in crops with added-value characteristics, without the need for more drastic, time consuming and more expensive strategies, as genetic transformation of crops.

Elicitation of Gamay Cells With miPEP164c Induces Accumulation of miR164c and Consequent miR164c-Mediated Inhibition of Proanthocyanidin Biosynthetic Pathway

Overall, results confirmed that the exogenous application of miPEP164c is indeed enhancing the accumulation of *miR164c* which putatively resulted in a more pronounced post-transcriptional silencing of transcription factor VvMYBPA1 and consequently of MYBPA1-activated genes, here observed by a downregulation of *VvLAR1* expression and of LAR and ANR specific activity resulting in a significant decrease of 26% of total PA content in cells exposed to exogenously applied miPEP164c for 10-day. According to our results, the regulatory effect of miPEP164c, both at anthocyanin/PA concentrations and transcriptional levels, was most significant at a concentration of 1 μM and after a period of 10-day. This suggests that the regulatory effect of miPEP164c is cumulative along a period of exposure to the peptide, at least until 10-day, but not evident in a period of hours after the peptide treatment. It is possible that this cumulative effect after a longer period of exposure is due to the several molecular steps in the mechanistic underlying miPEP regulation intracellularly and the fact that miPEP164c does not

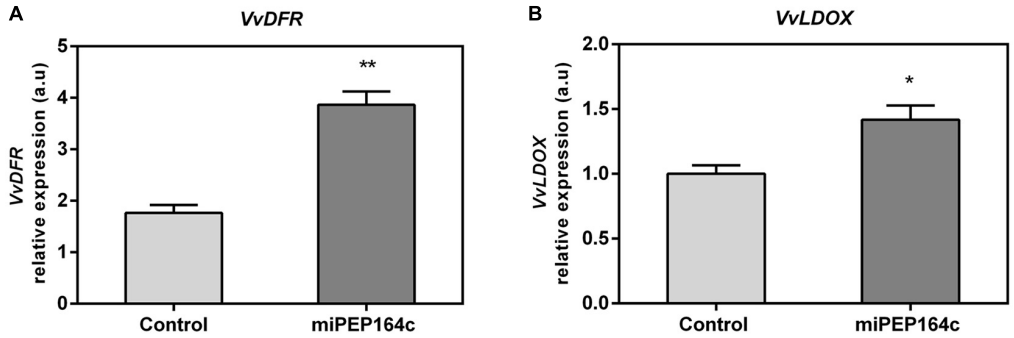

FIGURE 6 | Steady-state transcript levels of *VvDFR* **(A)** and *VvLDOX* **(B)** in suspension-cultured grape berry cells (cv. Gamay) 10-day after elicitation with 1 μM miPEP164c. Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$).

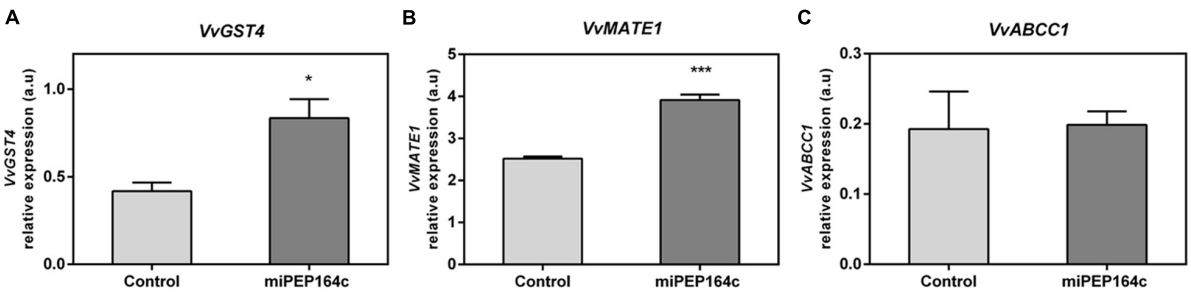

FIGURE 7 | Steady-state transcript levels of *VvGST4* **(A)**, *VvMATE1* **(B)** and *VvABCC1* **(C)** in suspension-cultured grape berry cells (cv. Gamay) 10-day after elicitation with 1 μM miPEP164c. Gene expression analysis, by real-time qPCR was normalized with the expression of reference gene *VvACT1* and *VvGAPDH*. Values are the mean ± SEM. Asterisks indicate statistical significance (Student's *t*-test; $^{*}P < 0.05$; $^{***}P < 0.001$).

directly regulate ultimate targets as LAR and ANR within PA-synthetic pathway, which probably results in more time needed to observe its regulatory effect.

Indeed, gene expression analysis by real-time qPCR confirmed that miPEP164c increased the expression levels of the *pre-miR164c* in Gamay cells along its concentration until 1 μM in an overwhelmingly more evident mode after 10-day. Thus, a positive loop was established, in which a consequent increased translation into miPEP164c, ultimately results in higher levels of mature miR164c and accentuated negative regulation of the target gene *VvMYBPA1*. *In silico* analyses suggests that the mode of action of *miR164c* is through inhibition of translation of *VvMYBPA1*, not by cleavage of the target messenger RNA, due to a lack of 100% complementarity between the guide miRNA and the target mRNA (Waterhouse and Hellens, 2015). This is in agreement with our results showing that the treatment with miPEP164c did not induce any significant changes in the expression levels of *VvMYBPA1*.

Evidence for the involvement of post-transcriptional silencing of *VvMYBPA1* mediated by miPEP164c was obtained when the MYBPA1-activated enzymes *VvLAR* and *VvANR* were clearly down-regulated. Both *VvANR* and *VvLAR1*, are key genes leading to the synthesis of PAs (Gagné et al., 2009). However, the expression of *VvANR*, encoding for the enzyme that synthesizes epicatechins from anthocyanidins, was not affected after 10-day of treatment, possibly to compensate the decreased activity of VvLAR, in order to ensure a certain amount of monomers for PAs biosynthesis. Also, *VvANR* expression may be regulated by several other regulatory proteins, such as *VvMYC1*, a bHLH transcription factor that physically interacts with MYB-like transcription factors like MYBPA1 and MYB5a/b to coordinate the regulation of *VvANR*, and therefore silencing of one transcription regulator may be overcome by another regulatory mechanism (Heppel, 2010).

Proanthocyanidin Synthesis Was Inhibited by miPEP164c While Anthocyanin Synthesis Was Simultaneously Increased

The observed significant increase in anthocyanin total content in Gamay cells mediated by the application of miPEP164c corroborates our hypothesis that a miPEP164c-mediated silencing of PA synthesis would divert the carbon flow to the anthocyanin branch, due to the constant competition of both pathways for the same substrates, as reported before (Liao et al., 2015). Gene expression analysis of *VvUFGT1*, that glycosylates anthocyanidins into anthocyanins, revealed a strong

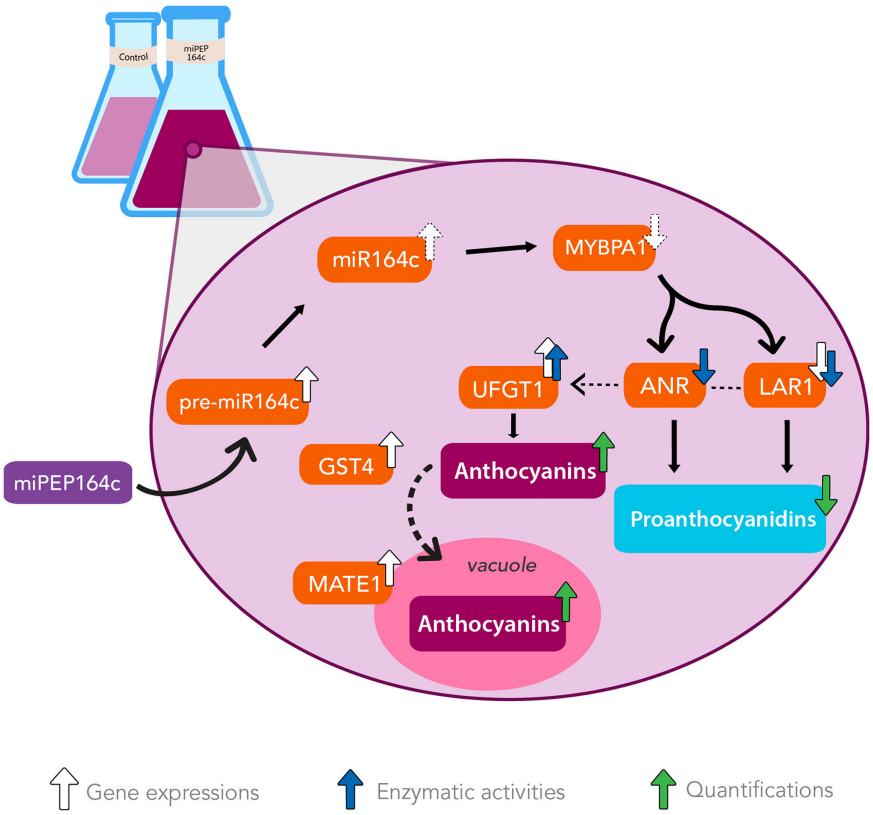

FIGURE 8 | The exogenous application of miPEP164c increases anthocyanin synthesis and accumulation while decreasing PA synthesis by enhancing *miR164c*-mediated downregulation of PA-synthetic pathway in Gamay grape berry cell suspensions. Addition of miPEP164c provoked an increase in the transcription of pre-miR164c and consequently of its mature form, miR164c, which putatively led to a decrease in the translation of transcription factor MYBPA1. This inhibition in MYBPA1 translation resulted in a downregulation of *VvLAR1* expression and LAR total biochemical activity, as well as ANR total biochemical activity, decreasing the intracellular concentration of PAs. This downregulation of the PA pathway indirectly led to the stimulation of the anthocyanin synthesis by increasing *VvUFGT1* expression and UFGT total biochemical activity, as well as vacuolar accumulation, as shown by *VvGST4* and *VvMATE1* overexpression.

upregulation of its expression levels in response to the elicitation with the micropeptide which goes in agreement with the observed increase of the UFGT specific activity that also increased.

In *Vitis vinifera* two types of anthocyanin tonoplast transporters that accumulate anthocyanins in the vacuole were identified: primary transporters from the ATP-binding cassette (ABC) family, such as the *VvABCC1* who requires the presence of reduced glutathione (GSH) to properly transport anthocyanins, through the tonoplast, into the vacuole (Jiang et al., 2019); and tonoplast secondary transporters like *VvMATE1* (anthoMATE) of the multidrug and toxic extrusion family that use the H^+ gradient to transport mostly acylated anthocyanins (Gomez et al., 2009). Also crucial for anthocyanin stabilization and transport are the glutathione S-transferases, as the paradigmatic case of grapevine's *VvGST4*, to promote anthocyanin S-conjugation with reduced glutathione for anthocyanin-stabilization purposes (Conn et al., 2008). Several studies on the role of GSTs in anthocyanin accumulation have described GSTs as escort/carrier proteins, binding anthocyanins to form a GST-anthocyanin complex, protecting them from oxidation and guiding anthocyanins from the cytosolic surface of the ER to the vacuole for proper storage mediated by tonoplast transporters such as *VvMATE1* and *VvABCC1* (Zhao and Dixon, 2010; Jiang et al., 2019). Our results strongly supported that anthocyanin transport capacity to the vacuole, where they are stored in grape berry cells, was also stimulated by miPEP164c application as the expression of the anthocyanin tonoplast transporter *VvMATE1* and anthocyanin carrier protein *VvGST4*, was upregulated by this micropeptide. It is not understood how plants choose between ATP-hydrolysis-dependent or H+/Na+-gradient dependent mechanisms for transport of native metabolites or xenobiotics. However, it is believed that the conjugation ligands, such as glucose or glutathione, play a key role in the determination of which transport mechanism will be used (Zhao and Dixon, 2010). However, the expression of *VvABCC1* was not affected by miPEP164c contrarily to what would be expected considering the upregulation of *VvGST4* expression. This could be due to the presence of other regulatory proteins affecting the expression of *VvABCC1*, other phenolic substrates that also need to be transported by this mechanism, the majority of anthocyanins might not be in the glycosylated form, which is the preferred form

of anthocyanins of this type of transporter, or simply because it is competing with the upregulated *VvMATE1* transporter for anthocyanins (Francisco et al., 2013).

CONCLUSION

In this study, recurring to a combination of molecular and biochemical approaches, we revealed that miPEP164c exogenous application induced a strong up-regulation of genes involved in anthocyanin synthesis, transport, and accumulation in the vacuole. Additionally, miPEP164c provoked a downregulation of PA synthesis (a pathway that directly competes with the anthocyanin-biosynthetic pathway), due to a decrease in *VvLAR1* expression levels with a corresponding very significant decrease in LAR total biochemical activity, accompanied also by a downregulated ANR biochemical activity.

This upregulation of the anthocyanin biosynthetic route seems to be an indirect effect of miPEP164c putatively inhibiting transcription factor MYBPA1, a known positive regulator of PA synthesis. Thus, these metabolic alterations triggered by miPEP164c clearly resulted in higher concentration of anthocyanins and lower concentration of PAs, due to miR164c-mediated negative regulation of PA-related transcription factor *VvMYBPA1* and, consequently, *VvLAR1* and *VvANR*, ultimately leading to PA synthesis inhibition and anthocyanin synthesis stimulation as these pathways directly compete for substrate, in a mechanism illustrated in **Figure 8**.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MV performed the experiments and wrote the manuscript. JR and HB performed the experiments. HG advised, wrote, and reviewed the manuscript. AC conceptualized the work, performed the experiments, and wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Bogs, J., Jaffe, F. W., Takos, A. M., Walker, A. R., and Robinson, S. P. (2007). The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. *Plant Physiol.* 143, 1347–1361. doi: 10.1104/pp.106.093203

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Broadhurst, R. B., and Jones, W. T. (1978). Analysis of condensed tannins using acidified vanillin. *J. Sci. Food Agric.* 29, 788–794. doi: 10.1002/jsfa.2740290908

Budak, H., and Akpinar, B. A. (2015). Plant miRNAs: biogenesis, organization and origins. *Funct. Integr. Genomics* 15, 523–531. doi: 10.1007/s10142-015-0451-2

Cavallini, E., Matus, J. T., Finezzo, L., Zenoni, S., Loyola, R., Guzzo, F., et al. (2015). The phenylpropanoid pathway is controlled at different branches by a set of R2R3-MYB C2 repressors in grapevine. *Plant Physiol.* 167, 1448–1470. doi: 10.1104/pp.114.256172

Cavallini, E., Zenoni, S., Finezzo, L., Guzzo, F., Zamboni, A., Avesani, L., et al. (2014). Functional diversification of grapevine MYB5a and MYB5b in the control of flavonoid biosynthesis in a petunia anthocyanin regulatory mutant. *Plant Cell Physiol.* 55, 517–534. doi: 10.1093/pcp/pct190

Chaves, M. M., Zarrouk, O., Francisco, R., Costa, J. M., Santos, T., Regalado, A. P., et al. (2010). Grapevine under deficit irrigation: hints from physiological and molecular data. *Ann. Bot.* 105, 661–676. doi: 10.1093/aob/mcq030

Chen, Q. J., Deng, B. H., Gao, J., Zhao, Z. Y., Chen, Z. L., Song, S. R., et al. (2020). A mirna-encoded small peptide, vvi-miPEP171d1, regulates adventitious root formation. *Plant Physiol.* 183, 656–670. doi: 10.1104/pp.20.00197

Conde, A., Pimentel, D., Neves, A., Dinis, L.-T., Bernardo, S., Correia, C. M., et al. (2016). Kaolin foliar application has a stimulatory effect on phenylpropanoid and flavonoid pathways in grape berries. *Front. Plant Sci.* 7:1150. doi: 10.3389/fpls.2016.01150

Conn, S., Curtin, C., Bézier, A., Franco, C., and Zhang, W. (2008). Purification, molecular cloning, and characterization of glutathione S-transferases (GSTs) from pigmented *Vitis vinifera* L. cell suspension cultures as putative anthocyanin transport proteins. *J. Exp. Bot.* 59, 3621–3634. doi: 10.1093/jxb/ern217

Couzigou, J., Lauressergues, D., and Combier, J. (2015). miRNA-encoded peptides (miPEPs): a new tool to analyze the roles of miRNAs in plant biology. *RNA Biol.* 12, 1178–1180.

Couzigou, J. M., André, O., Guillotin, B., Alexandre, M., and Combier, J. P. (2016). Use of microRNA-encoded peptide miPEP172c to stimulate nodulation in soybean. *New Phytol.* 211, 379–381. doi: 10.1111/nph.13991

Dai, X., Zhuang, Z., and Zhao, P. X. (2018). psRNATarget: a plant small RNA target analysis server (2017 release). *Nucleic Acids Res.* 46, W49–W54. doi: 10.1093/nar/gky316

Deluc, L. (2006). Characterization of a Grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. *Plant Physiol.* 140, 499–511. doi: 10.1104/pp.105.067231

Downey, M. O., Dokoozlian, N. K., and Krstic, M. P. (2006). Cultural practice and environmental impacts on the flavonoid composition of grapes and wine: a review of recent research. *Am. J. Enol. Vitic.* 57, 257–268.

Erokhina, T. N., Ryazantsev, D. Y., Samokhvalova, L. V., Mozhaev, A. A., Orsa, A. N., Zavriev, S. K., et al. (2021). Activity of chemically synthesized peptide encoded by the miR156A precursor and conserved in the Brassicaceae family plants. *Biochem. Moscow* 86, 551–562. doi: 10.1134/S0006297921050047

Francisco, R. M., Regalado, A., Ageorges, A., Burla, B. J., Bassin, B., Eisenach, C., et al. (2013). ABCC1, an ATP binding cassette protein from grape berry, transports anthocyanidin 3-O-glucosides. *Plant Cell* 25, 1840–1854. doi: 10.1105/tpc.112.102152

Gagné, S., Lacampagne, S., Claisse, O., and Gény, L. (2009). Leucoanthocyanidin reductase and anthocyanidin reductase gene expression and activity in flowers, young berries and skins of *Vitis vinifera* L. cv. Cabernet-Sauvignon during development. *Plant Physiol. Biochem.* 47, 282–290. doi: 10.1016/j.plaphy.2008.12.004

Gomez, C., Terrier, N., Torregrosa, L., Vialet, S., Fournier-level, A., Souquet, J., et al. (2009). Grapevine MATE-type proteins act as vacuolar H+-dependent acylated anthocyanin transporters. *Plant Physiol.* 150, 402–415. doi: 10.1104/pp.109.135624

Heppel, S. C. (2010). The basic helix-loop-helix transcription factor MYC1 is involved in the regulation of the flavonoid biosynthesis pathway in grapevine. *Mol. Plant.* 3, 509–523. doi: 10.1093/mp/ssp118

Jiang, S., Chen, M., He, N., Chen, X., Wang, N., Sun, Q., et al. (2019). MdGSTF6, activated by MdMYB1, plays an essential role in anthocyanin accumulation in apple. *Hortic. Res.* 6:40. doi: 10.1038/s41438-019-0118-6

Kozomara, A., and Griffiths-Jones, S. (2014). miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic Acids Res.* 42, 68–73. doi: 10.1093/nar/gkt1181

Lauressergues, D., Couzigou, J.-M., Clemente, H. S., Martinez, Y., Dunand, C., Bécard, G., et al. (2015). Primary transcripts of microRNAs encode regulatory peptides. *Nature* 520, 90–93. doi: 10.1038/nature14346

Li, P., Chen, B., Zhang, G., Chen, L., Dong, Q., Wen, J., et al. (2016). Regulation of anthocyanin and proanthocyanidin biosynthesis by *Medicago truncatula* bHLH transcription factor MtTT8. *New Phytol.* 210, 905–921. doi: 10.1111/nph.13816

Liao, L., Vimolmangkang, S., Wei, G., Zhou, H., Korban, S. S., and Han, Y. (2015). Molecular characterization of genes encoding leucoanthocyanidin reductase involved in proanthocyanidin biosynthesis in apple. *Front. Plant Sci.* 6:243. doi: 10.3389/fpls.2015.00243

Lister, C. E., Lancaster, J. E., and Walker, J. R. L. (1996). Developmental changes in enzymes of flavonoid biosynthesis in the skins of red and green apple cultivars. *J. Sci. Food Agric.* 71, 313–320. doi: 10.1002/(SICI)1097-0010(199607)71:3<313::AID-JSFA586<3.0.CO;2-N

Matus, J. T., Loyola, R., Vega, A., Peña-Neira, A., Bordeu, E., Arce-Johnson, P., et al. (2009). Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of *Vitis vinifera*. *J. Exp. Bot.* 60, 853–867. doi: 10.1093/jxb/ern336

Pantaleo, V., Szittya, G., Moxon, S., Miozzi, L., Moulton, V., Dalmay, T., et al. (2010). Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. *Plant J.* 62, 960–976. doi: 10.1111/j.0960-7412.2010.04208.x

Pérez-Díaz, J. R., Pérez-Díaz, J., Madrid-Espinoza, J., González-Villanueva, E., Moreno, Y., and Ruiz-Lara, S. (2016). New member of the R2R3-MYB transcription factors family in grapevine suppresses the anthocyanin accumulation in the flowers of transgenic tobacco. *Plant Mol. Biol.* 90, 63–76. doi: 10.1007/s11103-015-0394-y

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Ram, M. K., Mukherjee, K., and Pandey, D. M. (2019). Identification of miRNA, their targets and miPEPs in peanut (*Arachis hypogaea* L.). *Computat. Biol. Chem.* 83:107100. doi: 10.1016/j.compbiolchem.2019.107100

Reid, K. E., Olsson, N., Schlosser, J., Peng, F., and Lund, S. T. (2006). An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. *BMC Plant Biol.* 6:27. doi: 10.1186/1471-2229-6-27

Sharma, A., Badola, P. K., Bhatia, C., Sharma, D., and Trivedi, P. K. (2020). Primary transcript of miR858 encodes regulatory peptide and controls flavonoid biosynthesis and development in *Arabidopsis*. *Nat. Plants* 6, 1262–1274. doi: 10.1038/s41477-020-00769-x

Teixeira, A., Eiras-Dias, J., Castellarin, S. D., and Gerós, H. (2013). Berry phenolics of grapevine under challenging environments. *Int. J. Mol. Sci.* 14, 18711–18739. doi: 10.3390/ijms140918711

Vale, M., Rodrigues, J., Badim, H., Geros, H., and Conde, A. (2021). Exogenous application of non-mature miRNA-encoded miPEP164c inhibits proanthocyanidin synthesis and stimulates anthocyanin accumulation in grape berry cells. *bioRxiv* [Preprint]. doi: 10.1101/2021.03.04.433997

Waterhouse, P. M., and Hellens, R. P. (2015). Plant biology: coding in non-coding RNAs. *Nature* 520, 41–42. doi: 10.1038/nature14378

Xie, Z., Khanna, K., and Ruan, S. (2010). Expression of microRNAs and its regulation in plants. *Semin. Cell Dev. Biol.* 21, 790–797. doi: 10.1016/j.semcdb.2010.03.012

Zhang, X., Liu, Y., Gao, K., Zhao, L., Liu, L., Wang, Y., et al. (2012). Characterisation of anthocyanidin reductase from Shuchazao green tea. *J. Sci. Food Agric.* 92, 1533–1539. doi: 10.1002/jsfa.4739

Zhang, Y., Butelli, E., Alseekh, S., Tohge, T., Rallapalli, G., Luo, J., et al. (2015). Multi-level engineering facilitates the production of phenylpropanoid compounds in tomato. *Nat. Commun.* 6:8635. doi: 10.1038/ncomms9635

Zhao, J., and Dixon, R. A. (2010). The "ins" and "outs" of flavonoid transport. *Trends Plant Sci.* 15, 72–80. doi: 10.1016/j.tplants.2009.11.006

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Long-Term Efficacy and Safety of RNAi-Mediated Virus Resistance in 'HoneySweet' Plum

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Interfering RNA technology has been established as an effective strategy to protect plants against viral infection. Despite this success, interfering RNA (RNAi) has rarely been applied due to the regulatory barriers that confront genetically engineered plants and concerns over possible environmental and health risks posed by non-endogenous small RNAs. 'HoneySweet' was developed as a virus-resistant plum variety that is protected by an RNAi-mediated process against Sharka disease caused by the plum pox virus. 'HoneySweet' has been approved for cultivation in the United States but not in countries where the plum pox virus is endemic. In this study, we evaluated the long-term efficacy of virus resistance in 'HoneySweet,' the nature and stability of its sRNA profile, and the potential health risks of consuming 'HoneySweet' plums. Graft-challenged 'HoneySweet' trees carrying large non-transgenic infected limbs remained virus-free after more than 10 years in the field, and the viral sequences from the non-transgenic infected limbs showed no evidence of adaptation to the RNAi-based resistance. Small RNA profiling revealed that transgene-derived sRNA levels were stable across different environments and, on average, were more than 10 times lower than those present in symptom-less fruits from virus-infected trees. Comprehensive 90-day mouse feeding studies showed no adverse health impacts in mice, and there was no evidence for potential siRNA off-target pathologies predicted by comparisons of the most abundant transgene-derived sRNAs to the mouse genome. Collectively, the data confirmed that RNAi provides a highly effective, stable, and safe strategy to combat virus diseases in crop plants.

Keywords: transgene, feeding studies, GM, small RNAs, RNA expression

INTRODUCTION

The recent decision by the European Food Safety Authority (EFSA) to regulate gene-edited crops has sparked a re-evaluation of past and future advanced breeding strategies for crop improvement. Strategies to develop virus-resistant crop plants helped to bring about the dawn of genetic engineering. Virus-resistant papaya and squash were among the first commercialized products

derived from genetic engineering in the mid-1990s (Gottula and Fuchs, 2009). These first-generation crops were developed based on the concept of pathogen-derived resistance (PDR), which relied on the overexpression of viral coat protein genes in the plant (Beachy, 1997). It was not until the late 1990s that RNA interference (RNAi) mechanisms were discovered and later attributed to the resistance observed in some of these plants (Fire et al., 1998; Ruiz et al., 1998; Hamilton and Baulcombe, 1999). In recent years, new RNAi-based crops have been commercialized, including non-browning potatoes and apples, soybeans with improved oil content, alfalfa with reduced lignin, and low-acrylamide frying potatoes (Mat Jalaluddin et al., 2018). In addition, a slate of insect-resistant crops based on RNAi are now entering the markets (Zhang et al., 2017). This broader use of RNAi technology has prompted renewed concerns over its stability, long-term efficacy, and health and environmental risks.

The possible health effects caused by the consumption of small RNAs (sRNA) have been raised by the finding that insects are affected when consuming either plants producing RNAi targeted to the insect or consuming plants with RNAi applied ectopically (Zhang et al., 2017). For example, a modified bacterium living in the guts of bees produced an RNAi that killed the *Varroa* mites that consumed bee fat containing this RNAi (Leonard et al., 2020). These reports in insects demonstrate that it is possible to obtain RNAi-targeted effects through consumption, at least in some insects. A few publications have indicated that plant-derived sRNAs can downregulate genes in other animals and humans if sufficient homology exists and that these effects can lead to potential adverse health impacts (Zhang L. et al., 2012; Liu et al., 2017). Other researchers have refuted these findings as spurious (Dickinson et al., 2013; Tosar et al., 2014; Petrick et al., 2015; Chan and Snow, 2017). Studies such as these have fueled concerns that more complete information is necessary to confirm the safety of an RNAi approach (Heinemann et al., 2013).

Sharka is a serious disease of stone fruits that includes peaches, plums, apricots, and cherries. The disease, caused by the plum pox potyvirus (PPV), is marked by characteristic chlorotic rings on the leaves and fruits, premature fruit drop, and, in some cases, tree death. This disease was first recorded in Bulgaria in the early twentieth century and, since then, has spread throughout Europe and, more recently, to Asia, Africa, North, and South America, causing billions of dollars in losses to stone fruit industries worldwide (García et al., 2014). Natural resistance to the virus is very limited, with few reports of economically useful levels of resistance in the stone fruit germplasm (Vilanova et al., 2003; Hartmann and Neumüller, 2006; Zuriaga et al., 2013). Currently, PPV control efforts rely on intense monitoring, the destruction of infected trees, and the distribution of virus-free propagation materials, but these efforts have been insufficient to fully curb the spread of the disease (Rimbaud et al., 2015).

To combat PPV, the 'HoneySweet' plum (*Prunus domestica* L.) was developed in the early 1990s through a publicly funded US-French collaborative effort (Scorza et al., 1994). The original construct was designed to overexpress the PPV coat protein (CP) modeled after the papaya ringspot strategy that proved to be successful and is still in commercial use today (Gonsalves, 1998). Among more than 100 transgenic lines obtained, only a single PPV-resistant line (initially called C5) was identified and has since been released under the name 'HoneySweet' (**Supplementary Figure 1**) (Scorza et al., 2016). It was later discovered that recombination during the T-DNA insertion process resulted in a hairpin arrangement of the viral CP gene leading to RNAi-mediated virus resistance (Scorza et al., 2001). Over the past two decades, 'HoneySweet' trees have been evaluated in permitted field tests in the US, Spain, Poland, Romania, and the Czech Republic. 'HoneySweet' has proven to be a highly productive tree with excellent fruit quality and has been cleared for cultivation by the United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA). International efforts are currently underway to obtain Canadian and European Union approval for 'HoneySweet' import and cultivation (Scorza et al., 2016). To address the possible risks of sRNAs in RNAi crops, we tested the long-term efficacy of 'HoneySweet' resistance in field-grown 'HoneySweet' trees and used sRNA expression profiling on trees grown in different environments to assess the composition and stability of transgene sRNAs relative to virus-infected trees. We then used that data to predict the potential sRNA off-target effects in mice as part of comprehensive 90-day feeding trials to screen for potential toxicological effects.

MATERIALS AND METHODS

Germplasm and Locations

Graft-challenged trees: The plant material was collected from an experimental orchard of 'HoneySweet' grafted onto 'St. Julian' rootstock and planted in 2002 at a site in Praha, Czech Republic. The 'HoneySweet' grown in the Czech Republic were grown under the Ministry of Environment of the Czech Republic GM planting No. The 881/OER/GMO/01, and the field trial was extended with Ministry Reference Number 41538/ENV/09 issued on September 18, 2009. Eight of these trees were graft-inoculated in 2002 using PPV strain REC (PPV-REC)-infected buds of 'Emma Leppermann.' Leaves from three differently treated trees were evaluated: (A) one 'HoneySweet' tree with no virus-infected bud grafted on, (B) the eight 'HoneySweet' trees inoculated by PPV-REC using an infected graft of the cultivar 'Emma Leppermann,' which was removed from four of those trees after 5 years (in 2011), and C) infected 'Emma Leppermann' that had been grafted to the eight 'HoneySweet' trees (**Supplementary Data sheet 2**). Leaves were collected from individual trees in each treatment and were frozen at −80°C unless used immediately.

Multiple location trees: leaves and ripe fruits were collected from 'HoneySweet' in 2011 from two locations in Europe and one in the US (**Supplementary Table 1**). Leaves and ripe fruits were collected from a PPV-susceptible comparator, 'Stanley,' from three locations in Europe and one in the USA (**Supplementary Table 1**). Fruit and leaf material were shipped from Europe under Animal and Plant Health Inspection Service (APHIS) permit number P526P-11-02618 to APHIS inspection facilities before being forwarded from APHIS to the USDA, Kearneysville, West Virginia (WV) where the tissue was frozen

in liquid N2, lyophilized, and stored at −20°C. Samples from field-grown trees at USDA Kearneysville were harvested and in the same manner, frozen, and stored.

All three plum cultivars named are available commercially, including 'HoneySweet' (https://shop.cumminsnursery.com/shop/plum-trees/honeysweet-plum). Currently, 'HoneySweet' has only been approved for growth and cultivation in the United States. Use in other countries will depend on the policies of that country.

Quantification Analysis Using qPCR

Total RNA was extracted from the leaves of 'HoneySweet' trees (2005–2017) using Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA) using the instructions of the manufacturer as previously described in Singh and Kundu (2017). Complementary DNA (cDNA) was synthesized using a Reverse Transcription System (Promega, Madison, WI, USA).

Quantitative PCR was performed using a real-time system LightCycler 480 (Roche, Basel, Switzerland). The titer of PPV-strain D (PPV-D) and PPV-REC was measured using specific primers for PPV-REC CP (REC-J-F–AATGATATTGATGATAGCCTTGAC, REC-J-R—AGCTGGTTGAGTTGTTGCCAC) (Jarošová et al., 2010) and PPV-D CP (PPV-FD—TCAACGACACCCGTACGGGC, PPV-RR—GGAATGTGGGTGATGATGG) (Jarošová et al., 2010) with a melting temperature at 94°C and an annealing temperature at 60°C for 35 cycles. The PCR products were visualized following electrophoresis on 1.5% agarose gel and staining by SYBR Safe (Invitrogen, Waltham, MA, USA). Standards for qPCR were prepared by cloning the target sequence into the vector pGEM®-T Easy Vector (Promega) as described earlier (Singh and Kundu, 2017).

RNA Extraction for Sequencing

The small RNA (siRNA) from leaves and fruit (20 ng of lyophilized material) was extracted using a Norgen plant microRNA purification kit (Norgen Biotek Corp., Thorold, Canada; https://norgenbiotek.com/) following the instructions of the manufacturer. The mRNA from the fruit and leaves was extracted in the same manner.

RNA Sequence Processing Graft-Challenged Trees

The production and sequencing of the sRNA libraries from the graft-challenged tree experiment were carried out by the Genomics Resources Core Facility (GRCF, Weill Cornell Medical College; http://corefacilities.weill.cornell.edu/). A total of 22 sRNA libraries were sequenced on an Illumina HiSeq 2500® (https://emea.illumina.com/) (Illumina, Inc., San Diego, CA, USA). The sRNA sequences were parsed from FASTAQ formatted files and adapter trimming, and cleaning of the reads was carried out using the CLC Genomic Workbench (Qiagen, Germantown, MD, USA; https://www.qiagen.com) and Cutadapt (https://cutadapt.readthedocs.io/en/stable/). Quality control of the reads was performed at each step using FASTQC implemented in the CLC Genomic Workbench. Peach genome v2.1 (chromosome 4; chr4: 25507706…258432937) was used to remove tRNA sequences from the sRNAs (The International Peach Genome Initiative, 2013). The viral genomic sequences were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Viral siRNA (vsRNA) reads were mapped to the viral genomic sequences utilizing the CLC Genomic Workbench. Viral siRNA expression and abundance were visualized in the form of heat maps as log2-transformed reads per kilobase million (RPKM) (for sequencing) and log2-transformed quantitative data (for qPCR). The data were normalized by calculating Z-scores using the R-statistical package (Singh et al., 2014). All sRNA sequences have been deposited in GenBank, SRA, with the BioProject ID PRJNA741990.

RNA Sequence Processing Multi-Location Trees

The mRNA and sRNA RNA-Seq libraries were constructed and sequenced by the David H. Murdock Research Institution, Kannapolis, North Carolina using an Illumina GAII sequencer and paired 75b reads. Sequences were cleaned by the trimming of adaptor sequences, Ns, and quality scores as determined by the CLC Genomic Workbench (Qiagen, USA; https://www.qiagen.com). The RNA reads were then filtered by the peach rRNA sequence, grape mitochondrial sequence, and peach chloroplast sequences with the remaining sequences mapped to the peach genome V1.0 (The International Peach Genome Initiative, 2013). The remaining sequences were then mapped to either the transgene sequences of 'HoneySweet' or a reconstructed PPV-D viral genome from the infected 'Stanley' in Spain or the reconstructed PPV-M viral genome from the infected 'Stanley' in Bulgaria. The reads relative to the total reads were based on the number of sequences that mapped to the peach genome after the filtering process. The mRNA sequences have been deposited in GenBank, SRA, with the BioProject ID PRJNA741796. The sRNA sequences have been deposited in GenBank, SRA, with the BioProject ID PRJNA742049.

Mouse-Feeding Study

All procedures associated with this study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Oklahoma State University (OSU). Four-week-old CD-1 female ($n = 75$) and male ($n = 75$) mice were purchased from Charles River Laboratory. Upon arrival, the mice were maintained in standard shoebox cages in the OSU environmentally controlled laboratory animal facility (12-h light/dark cycle) on a standard chow diet for 5–7 days before being randomly assigned to either the control or one of the dried plum treatment groups. The treatment groups were fed a control diet based on the American Institute of Nutrition (AIN)-93G diet (control), control-supplemented with 5 or 15% plum (w/w) from two different varieties, 'HoneySweet' (HS5% and HS15%) or 'Stanley' (ST5% and ST15%). The rationale for the doses selected is that we estimated that 5% would represent routine consumption consistent with ∼25 g/day for human consumption and that the 15% would be in line with heavy consumption or ∼75 g/day for humans. Dried plum is, on average, 9.5 g (https://fdc.nal.usda.gov/fdc-app.html#/food-details/168162/nutrients) so the 5% would be 2–3 dried plums and the 15% would be 7–8. The source of the 'HoneySweet'

and Stanley plums used in the study was the Kearneysville, WV orchard. The plums were lyophilized and pooled into a batch for 'HoneySweet' and a batch for 'Stanley.' Each batch was then pulverized into a powder to have a uniform sample and then shipped to the investigators at OSU labeled as test products A and B to be incorporated into diets. The diets were formulated to have the same carbohydrate, fat, protein, fiber, calcium, and phosphorus contents, respectively (Halloran et al., 2010). Throughout the study, the mice had free access to food and reverse osmosis (RO) water. Clinical checks were performed five times per week by study personnel and included an examination of general appearance, fur, eyes, and animal behavior or activity. Food intake was documented, and body weight was recorded weekly.

At the end of the study, the mice were anesthetized with a ketamine xylazine cocktail (60 mg of ketamine and 6 mg of xylazine per kg body weight) and blood samples were collected from the carotid artery, following a 3-h fast for clinical chemistries and total white blood cell (WBC) counts before a blood smear was made for the evaluation of WBC differentials. The clinical chemistry panel and assays included the serums alanine aminotransferase (ALT), albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), bilirubin (TBIL), blood urea nitrogen (BUN), cholesterol (CHOL), creatinine (CREAT), glucose (GLU), globulin (GLOB), total protein, (TP), triglycerides (TRIG), phosphorus (PHOS), calcium (Ca), sodium (Na), potassium (K), and chloride (Cl).

Organs and tissues were collected immediately after euthanasia and fixed in 10% neutral buffered formalin. A broad spectrum of tissues was examined, namely, the brain (representative regions: cerebrum and cerebellum), eyes, thyroid, thymus, stomach, small intestine (duodenum, jejunum, and ilium), pancreas, colon, rectum, liver, kidneys, adrenal glands, spleen, heart, aorta, trachea, lungs, gonads (testes and ovaries), uterus, vagina, female mammary glands, urinary bladder, lymph nodes, bone, and bone marrow. All tissues were processed by the OSU Pathology Laboratories in the College of Veterinary Medicine and scored based on a 0–3 scale with 0 = no apparent abnormalities, 1 = mild abnormality, 2 = moderate abnormalities, or 3 = severe abnormalities. The most common noted abnormalities were mild inflammation, which is not uncommon in mouse colonies.

The primary outcomes of this study focused on the gross and microscopic pathology of tissues, clinical chemistry, and WBC results. All statistical analyses performed on the data from the animal study utilized SAS Version 9.3 (SAS Institute, NC). For continuous variables such as body weight, blood clinical chemistries, and WBC, the effects of the treatments were compared using ANOVA and Fisher's least significant difference (LSD) *post-hoc* analyses. For categorical data such as pathology scoring and abnormal clinical chemistries, the differences in the frequency of scores were determined using the chi-square and $\alpha = 0.05$ for all statistical analyses. All the investigators associated with the animal study were blinded to the treatments throughout the course of the study and data analyses. Only after a final report was generated was the code for the two different cultivars of plum revealed.

sRNA Homologies to Mouse

The most abundant 2,834 sRNAs related to the transgenic CP sequence in 'HoneySweet' were used to find homologies to mouse, Mus musculus, transcripts using the Basic Local Alignment Search Tool (BLAST) and sequence database at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Matches with two or fewer mismatches and one or fewer gaps were considered significant matches.

RESULTS

Efficacy of RNAi Under Intensive Long-Term Infection Pressure

Graft-Challenged Trees

A field trial consisting of nine 'HoneySweet' trees that were planted in the Czech Republic (Polák et al., 2017) was used to evaluate RNAi efficacy under long-term continual infection pressure. Eight 'HoneySweet' trees were inoculated with the PPV-REC *via* bud grafts from a susceptible plum variety, 'Emma Leppermann,' in 2002. The PPV-infected, non-transgenic grafts were allowed to grow out, producing large infected and symptomatic limbs (**Supplementary Figure 2**). In 2011, the infected grafts were removed from four of the trees, and all the trees were monitored for PPV infection and symptoms until 2017. The PPV-REC levels of these eight trees and a control 'HoneySweet' tree (not graft-challenged) were assayed by qPCR annually for 10 years in leaf tissue from 'HoneySweet' and the infected 'Emma Leppermann' graft tissue (**Figure 1A**). As a result, PPV-REC was detected only in the grafted non-transgenic 'Emma Leppermann' that had been initially infected. The PPV-D CP transgene expression was detectable in all the 'HoneySweet' trees and none of the 'Emma Leppermann' grafts (**Figure 1B**). The results show that, even under intense infection pressure from grafted infected limbs, the 'HoneySweet' trees remained virus-free after over a decade of continuous virus exposure.

Naturally Infected Tree Multilocation

'HoneySweet' leaf and ripe fruit tissue samples were collected from mature trees in field test plots located in Spain, the Czech Republic, and from the United States where there was no possibility of PPV exposure. Samples of 'Stanley' were collected at the same or similar sites as a comparator. 'Stanley' is highly susceptible to PPV but is grown commercially in Europe as it displays few symptoms in infected fruit. Among the 10 'Stanley' trees sampled, 2 'Stanley' trees from Spain were found to be infected with the PPV-D strain while 2 'Stanley' trees from Bulgaria had been infected with the PPV-M strain based on positive ELISA results (data not shown). The reads quantified from RNA-Seq and sRNA-Seq were also found to be matching their respective PPV strain genomes (**Figure 1C**; **Supplementary Table 2**). After over 8 years of exposure to endemic PPV in Spain and the Czech Republic, none of the 'HoneySweet' trees had detectable PPV. The levels of virus and transgene RNA relative to total mRNAs are shown in **Figure 1C** and **Supplementary Table 2**. The amount of transgene CP mRNA was less than 1/100 of the amount of natural virus

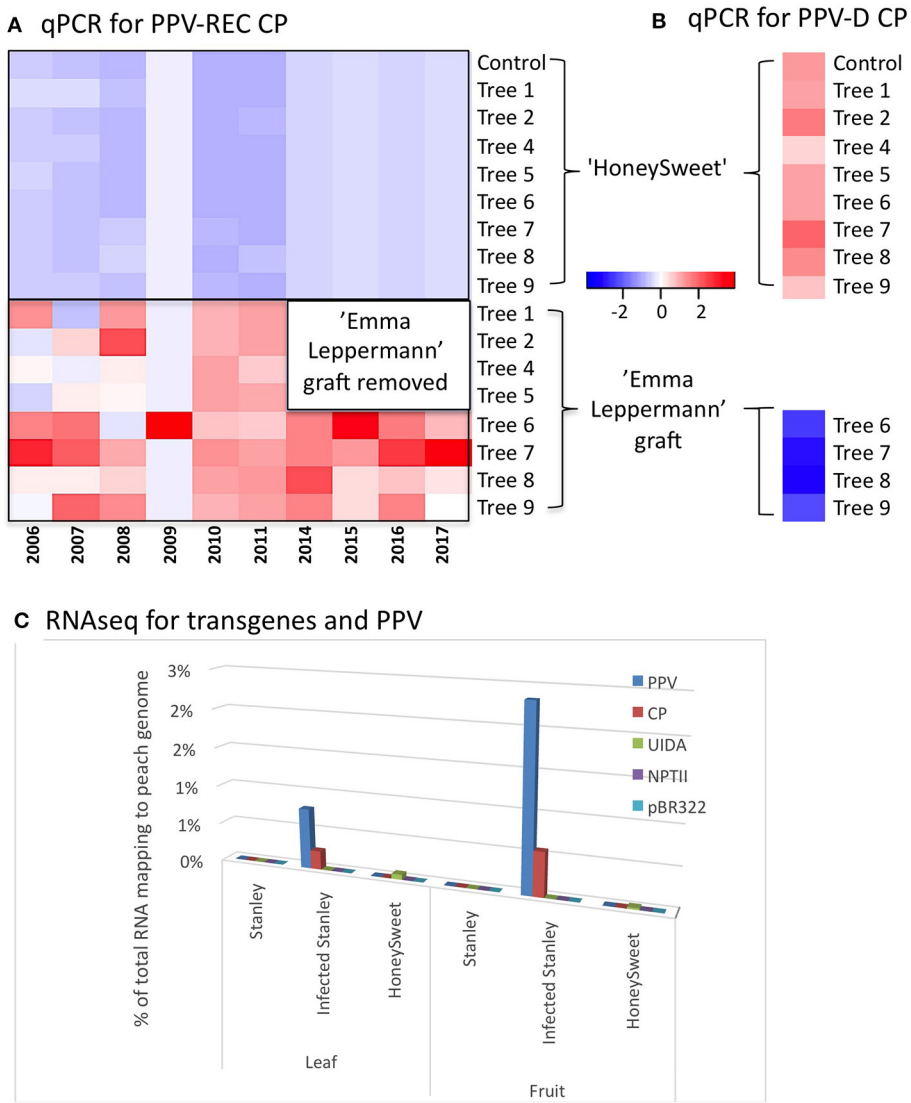

FIGURE 1 | The presence or absence of PPV and expression of the transgenes in 'HoneySweet' and comparators, PPV-REC-infected 'Emma Leppermann' and 'Stanley' infected or not was determined by qPCR analyses for the graft-challenged material and using RNA-Seq studies for the multi-location trial. Heatmaps **(A, B)** showing the relative abundance of CP RNA in 'HoneySweet' trees and grafted and artificially infected 'Emma Leppermann' limbs. 'HoneySweet' contains a CP transgene derived from the PPV-D serotype and 'Emma Lepperman' was infected with a PPV-REC serotype (originating from Bulgarian). **(A)** Leaves from eight 'HoneySweet' trees and their associated grafts (infected 'Emma Leppermann') were sampled together with a control 'HoneySweet' tree with no graft, for the presence of CP RNA from PPV-REC each year using qPCR with serotype-specific primers, REC-J-F and REC-J-R. **(B)** Transgene PPV-D was quantified in the same eight 'HoneySweet' trees and associated grafts in four of the trees and the control 'HoneySweet' with no graft, for 1 year utilizing serotype-specific primers, PPV-FD and PPV-RR. Heatmaps were drawn from log-transformed (log2) using the ggplots package of the R-statistical software. Color key represents the scale distribution of the expression/abundance. The PPV-REC CP RNA was only detected in 'Emma Leppermann' and the PPV-D CP RNA was only detected in 'HoneySweet' leaves. **(C)** Amount of RNA homologous to the transgenes contained in 'HoneySweet' and the PPV genome, as a percentage of the total reads that map to the peach genome with those related to rRNA, mitochondrial genome, and chloroplast genome. Only the infected 'Stanley' tissue has a significant % reads mapping to PPV with a larger proportion found in fruit tissue.

CP RNA in the infected 'Stanley' trees. Interestingly, the amount of viral RNA was ~two-fold higher in fruit than in the leaves of the naturally infected 'Stanley' trees even though leaves expressed symptoms of PPV infection while fruits generally did not. For 'HoneySweet,' there were approximately equal amounts of CP transgene RNA in leaves and fruit relative to total mRNA. The levels of sRNAs related to PPV and transgenes reflected the same profile trends as the mRNAs (**Supplementary Table 2**).

In summary, we found that the transmission of PPV to 'HoneySweet' was not detected after more than 8 years in the field, and the relative levels of transgene RNA were considerably less than that of viral CP RNA found in the infected, susceptible 'Stanley' trees.

sRNA Profiling of Transgene

Size Distribution of sRNA

Total sRNAs were sequenced for the graft-challenged 'HoneySweet,' the infected graft material 'Emma Leppermann,' and the multiple locations 'HoneySweet' and 'Stanley' samples. The RNAs that had homology to the CP transgene and the whole PPV sequence were sorted by size. The most striking differences were in the higher amount of 22 nt RNAs in infected 'Stanley' and the higher amount of 24 nt RNAs in 'HoneySweet.' All the 'HoneySweet' samples had similar proportions of sRNA by size, namely, 21 nt sRNAs (50–70% total), >22 nt sRNAs (20–30% total), >24 nt sRNAs (5–15% total), and the negligible detections of other sizes (**Supplementary Figure 3**). There were no differences between trees exposed and unexposed to PPV (**Supplementary Figure 3**). There was a small difference in the distribution between 'HoneySweet' leaves and fruits, in that fruit had an average of 23% of sRNAs in the 24-nt class while leaf had ∼9%. No significant differences were observed between the fruits and leaves in infected 'Stanley' trees. Likewise, the infected graft material, 'Emma Leppermann,' had similar amounts of 21 nt sRNAs, somewhat higher levels of 22 ng sRNAs, and negligible amounts of the other species including 24 nt sRNAs (**Supplementary Figure 3**).

Abundance of the Transgene- and Viral-Related sRNAs

To assess the potential risks posed by sRNAs, specific species of sRNAs that matched transgene sequences and viral sequences were quantified. In the graft-challenged 'HoneySweet,' the sRNAs in both the 'HoneySweet' and PPV-infected 'Emma Leppermann' were tabulated including the sRNA related to each transgene (*CP*, *UIDA*, *NPT*, and *BLA*) and each gene of PPV-REC (**Figure 2**). The levels of sRNA derived from the CP transgene in 'HoneySweet' were generally lower than those homologous to the CP from the PPV-REC in infected 'Emma Leppermann' leaves based on total read counts (**Figure 2**; **Supplementary Data Sheet 1**: Graft Challenged). In the case of the 'HoneySweet' and 'Stanley' from different locations, a similar pattern was observed in that viral RNAs were found in the infected 'Stanley' leaves and, to a greater extent, in fruits. Also, similar to the graft-challenged experiment, there were more CP-related sRNAs in trees that were exposed to PPV yet had no detectable virus RNA (**Figure 2**; **Supplementary Data Sheet 2**: Multi-location).

Consistency of sRNA Profiles

The eight individual trees that were graft-challenged in the Czech Republic along with the multiple-environment tree planting allowed us to look at the variation of CP sRNA in 'HoneySweet' trees under different cultivation practices, soils, and climates (**Supplementary Data Sheet 3**, ANOVA analyses). While there was variation among trees at the same location, there was only a statistically significant variation in the CP sRNA between fruit and leaf tissues and between 'HoneySweet' fruit grown in the US over different years and fruit grown at two locations in Europe for 1 year.

Relative Abundance of Transgene sRNAs

We noted that the relative abundance of individual sRNA species derived from the CP transgene in 'HoneySweet' was significantly lower than many of the endogenous sRNAs. When ranked by expression level, the most abundant CP-specific sRNA species ranked 11,007 out of all identified sRNAs with chloroplast-, ribosomal-, and miRNA-related species from 10 to 100-fold higher levels of expression (data not shown). This suggests that the 35S hairpin cassette present in the 'HoneySweet' genome does not produce high levels of sRNA in leaves or fruits relative to native sRNA species.

Movement of Transgene and Viral sRNAs Between 'HoneySweet' and Grafted PPV-Infected 'Emma Leppermann'

We were able to assess if the virus and/or virus sRNAs moved from the infected graft into 'HoneySweet' or if transgene-derived sRNAs moved into the infected graft because of the sequence differences between the transgenes and PPV-REC (**Supplementary Data Sheet 1**). Leaf samples were collected from 'HoneySweet' both close to the graft site (lower) and further from the graft site in the upper portion of the tree to consider potential limited distance mobility (**Supplementary Figure 2**). Samples were also collected from the grafted 'Emma Leppermann.' No statistically significant levels of transgene-specific sRNA were found in 'Emma Leppermann' and little to no viral sRNAs were found in 'HoneySweet.' Furthermore, no significant differences were detected between the samples taken below or above the graft union (**Figure 2**; **Supplementary Data Sheet 1**). Thus, no evidence of sRNA translocation from 'HoneySweet' into the infected 'Emma Leppermann' graft was found. The presence of low levels of viral sRNA in 'HoneySweet' could represent either translocated sRNAs or a consequence of the action of RNAi machinery on the infecting virus.

Lack of Transgene-Induced Selection Pressure on Virus Populations

We assessed whether there was any evidence of evolutionary pressure or adaptive evolution imposed by the 'HoneySweet' RNAi mechanism on PPV-REC present in the grafted 'Emma Leppermann' tissues. We reconstructed the PPV-REC genomic sequences from each infected tree by assembling the viral sRNA reads against the PPV-REC reference genome and identified the nucleotide variants present in each sample. The full list of synonymous and non-synonymous nucleotide variants is given in **Supplementary Data Sheet 4**. We observed a total of 131 unique single nucleotide polymorphisms (SNPs) in all samples (30–50 per sample) that were distributed throughout the PPV-REC genome (**Figure 3A**). The SNPs at 21 positions of the viral genome were present in more than one sample and, in all but two cases, the substitutions were the same. The SNPs showed a non-even distribution across the genome but relatively very few within the CP open reading frame (ORF), which is targeted by the 'HoneySweet' transgene.

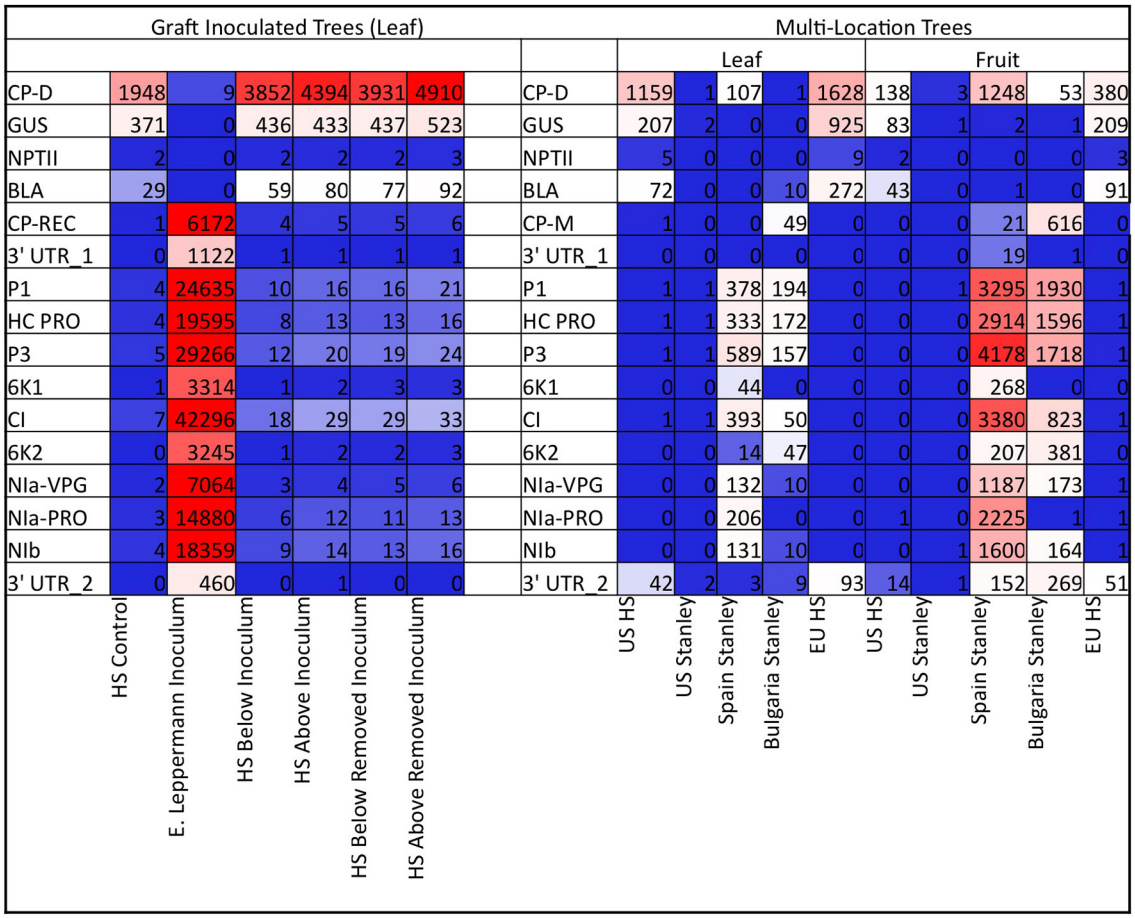

FIGURE 2 | Heatmap of the abundance of sRNAs with homologies to transgenes and PPV viral genes. The number of sRNA reads that matched transgenes (CP-D, GUS, NPTII, BLA) or PPV genes (CP-REC or CP-M, 3′UTR_1, P1, HC-PRO, P3, 6K1, CI, 6K2, NIa-VPG, NIa-PRO, NIb, 3′UTR_2) were tabulated and standardized by amounts per 1 million total sRNA reads. The higher the rate of reads, the more red, and fewer, including no reads, the more blue. The actual number of reads per million is included in each box. The values are an average of the trees under each category. The first set represents the graft-introduced PPV-REC. The six categories are HS control (uninfected 'HoneySweet,' one tree), E. Leppermann ('Emma Leppermann' infected grafts, four trees), HS below inoculum ('HoneySweet,' four trees where leaves were collected below the infected graft), HS above inoculum ('HoneySweet,' four trees where leaves were collected above the infected graft), HS below removed inoculum ('HoneySweet,' four trees where leaves were collected below where the infected graft had been), and HS above removed inoculum ('HoneySweet,' four trees where leaves were collected above where the infected graft had been). The second group is the multi-location 'HoneySweet' and 'Stanley' (infected and not). The 10 categories are US HS ('HoneySweet,' three trees with no PPV exposure), US Stanley ('Stanley,' 3 trees with no PPV exposure), Spain Stanley ('Stanley,' two trees, positive for PPV-D infection), Bulgaria Stanley ('Stanley,' two trees positive for PPV-M infection), and EU HS ('HoneySweet' from Spain and the Czech Republic, five trees). Both leaf material and fruit material were analyzed for the related species of sRNA. The map points out that in the graft-challenged experiment, basically only the initially infected 'Emma Leppermann' grafts contained sRNAs related to the virus and in the multi-location experiment, the infected 'Stanley,' both leaf and fruit were the only ones that had sRNAs related to the virus. In both experiments, 'HoneySweet' had sRNAs related to the transgenes, primarily CP and GUS.

Non-uniform Distribution of sRNA Produced From the Transgene

We examined the production of sRNA along with the CP in both 'HoneySweet' and virus-infected 'Stanley' and also found it to be non-uniform, consisting of "hotspots" where most of the identified sRNA species were derived. This was the case for US-grown 'HoneySweet,' European-grown 'HoneySweet,' and infected 'Stanley' (**Figure 3B**). There appeared to be islands of sequences that have different sized sRNAs overlapping in sequence. Interestingly, the sRNA species within the hotspots were highly variable among different samples, tissues, and virus strains, suggesting that they were not due to a sequence-specific sRNA ligation bias during the library preparation, but instead represent regions producing higher sRNA levels (**Figure 3B**, **Supplementary Table 3**).

Dose-Dependent Effects of 'HoneySweet' Plum Consumption and General Clinical Signs in Mice

Weight Gain in Response to Diets Containing Plum

To assess the potential health impacts of 'HoneySweet' sRNA consumption, we performed a comprehensive 90-day feeding study in outbred CD-1 mice. The experimental design included both male and female mice, two doses of 'HoneySweet' plum

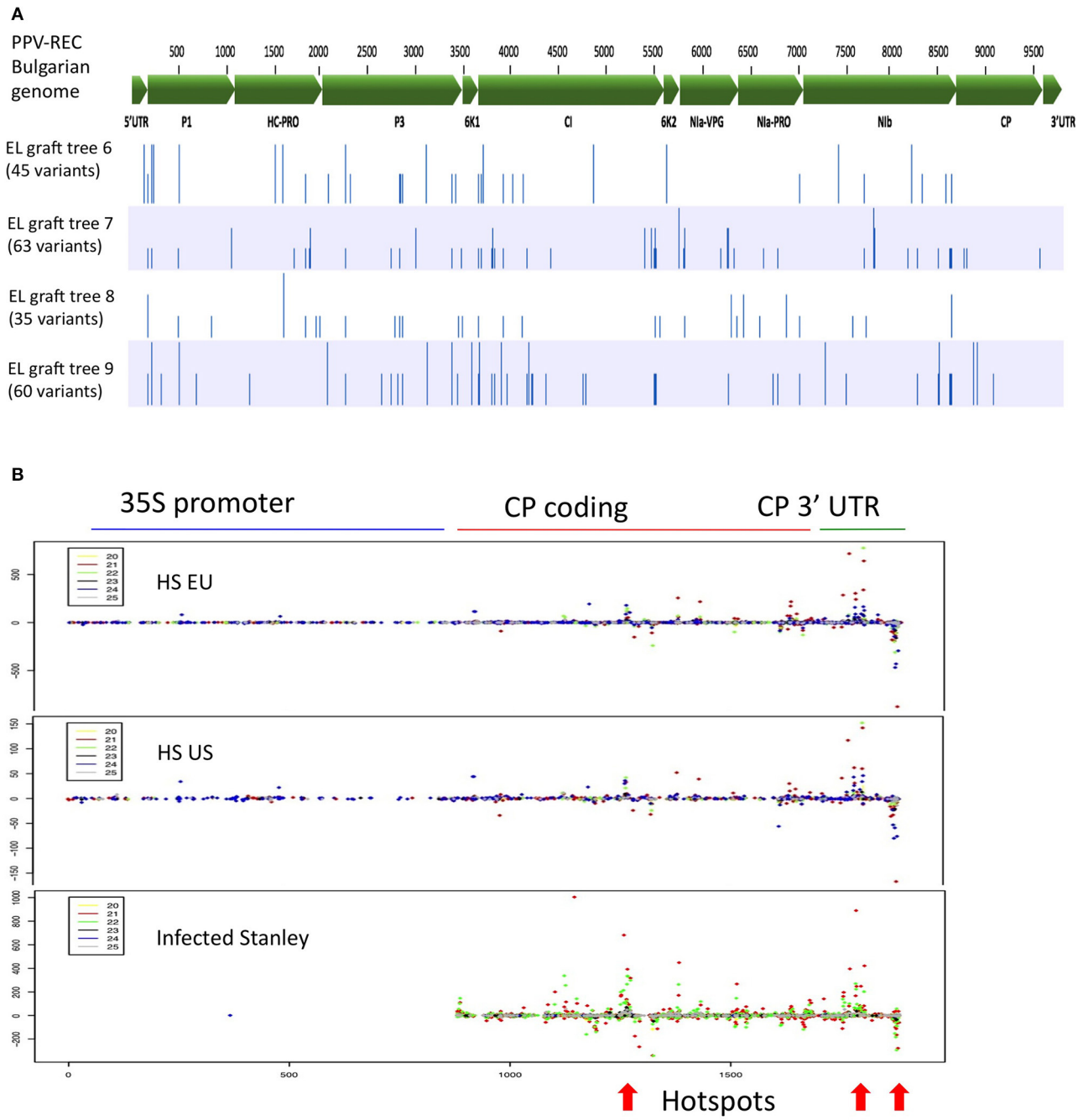

FIGURE 3 | Distribution of mutations in the PPV-REC genome after 10 years of exposure and the distribution of sRNAs related to the CP gene after 9 years of growth. **(A)** Sequence variation of the viral PPV-REC genome found in four different grafts of 'Emma Lepperman' on 'HoneySweet' for over 10 years. Variant analysis was performed by aligning viral sRNA reads (vsRNA) to the whole genome of PPV-REC strain of PPV including 5′ and 3′ UTRs. Genes and their positions on the genome are marked on the top with a green bar while variants are marked with blue bars for the individual tree. **(B)** Fruit sRNAs were mapped to the PPV-CP and promoter sequence of the transgene in 'HoneySweet' or the PPV CP in 'Stanley.' Each block represents the sRNAs from European-grown 'HoneySweet' (HS EU), United States-grown 'HoneySweet' (HS US), and 'Stanley' naturally infected by PPV. Each color represents a different length of sRNA (yellow-20 nt, red-21 nt, green-22 nt, black-23 nt, blue-24 nt, gray-25 nt). Dots above the bar represent the number of positive-strand sRNAs and those below represent the number of negative strand sRNAs. There are several regions of sequence that have larger numbers of related sRNAs, so-called hotspots. These can be seen in all three conditions, including the naturally infected 'Stanley,' and are indicated by an arrow at the bottom of the figure.

(HS5% or HS15% of diet) along with two doses of non-transgenic uninfected 'Stanley' fruit (ST5% or ST15%), and an AIN-93 control diet (CD) lacking plum. The body weights of all mice were recorded on a weekly basis, and no effects of either 'Stanley'

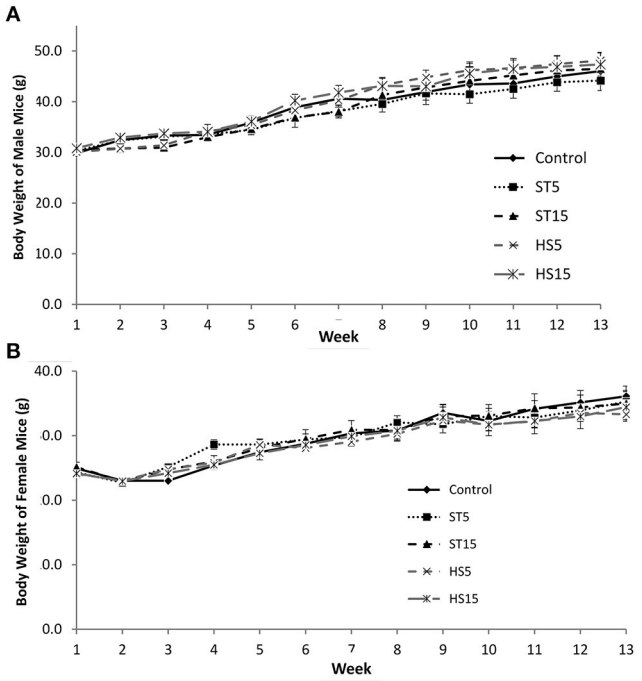

FIGURE 4 | Comparison of weight gain of mice fed either transgenic 'HoneySweet' dried fruit, 'Stanley' dried fruit or a control diet. **(A)** Body weight of male ($n = 75$), CD-1 mice. **(B)** Body weight of female ($n = 75$), CD-1 mice. No significant differences were observed in body weight across the 90-day study duration. Error bars reflect SE. Control = standard mouse diet; ST5, standard mouse diet with 5% by weight 'Stanley' dried plum included; ST15, standard mouse diet with 15% by weight 'Stanley' dried plum included; HS5, standard mouse diet with 5% by weight 'HoneySweet' dried plum included, and ST15, standard mouse diet with 15% by weight 'HoneySweet' dried plum included.

or 'HoneySweet' plum consumption at either dose were observed in either the male (**Figure 4A**) or female (**Figure 4B**) mice at any time point throughout the 90-day study period. Likewise, neither change in body weight nor percent change in body weight were affected by the sample type or the dose over the study period (data not shown). Food intake was recorded throughout the study, and no differences in food intake were documented between treatment groups in either gender nor were any noticeable clinical alterations in animal behavior observed in terms of grooming and physical activity (data not shown).

Effects of Consumption of Plum on White Blood Cells, Clinical Chemistries, and Pathology

To assess the general physiological functions in the mice, blood chemistries were performed on sera collected at the end of the study. Of the analytes assessed, only the triglycerides, total protein, and BUN revealed significant differences between treatment groups, but only in the male cohort (**Table 1**). The 'Stanley' plum 15% dose (ST15%) exhibited the greatest increase in serum triglycerides compared to the CD and ST5% groups. Triglycerides were also elevated in the HS15% and HS5% groups, but no dose-dependent effects were observed. Serum total protein was reduced in the HS5% group compared with the controls, but all values remained within the normal range. Likewise, differences existed between groups in terms of BUN, with the groups consuming ST5%, HS5%, and HS15% exhibiting a lower serum BUN compared with the CD group. However, all values were within the normal range. Within the female cohort of mice, no alterations in any blood chemistries were observed in response to the 'HoneySweet' plum or 'Stanley' plum and all values were within the normal limits (**Supplementary Table 4**).

Total WBC counts and differentials were assessed as general indicators of the immune response to plum. In the male animals, there were no differences in total WBC, mean percent or absolute neutrophils, monocytes, eosinophils, and basophils counts (**Supplementary Table 5**). Statistically significant differences were detected in the percent lymphocytes between the two cultivars of plum at the 5% dose (i.e., ST5% and HS5%) and the percent of lymphocytes in the HS5% was significantly higher than the CD group, but they were still within the normal range of values. Among the female cohort, the percent neutrophils were lower in the ST5% and ST15% treatment groups compared with the HS15% group, but again, total WBC and the percent and absolute differential counts were within the normal range (**Supplementary Table 6**). These findings indicate that no negative effects of plum on circulating leukocyte populations were detected.

An evaluation of the 38 different organs and tissues commonly evaluated in toxicology studies was performed representing all major physiological systems. The team of pathologists performing the evaluations were blinded to treatments throughout the course of reading and scoring the slides. In the male cohort, there were no differences between the groups in terms of the pathology scores for any of the organs and tissues assessed, indicating no negative effects of the 'HoneySweet' plum at either dose (**Table 2**). Mild salivary gland sialadenitis and the peripelvic nephritis of the kidney were noted in all groups including the CD mice, but there were no specific effects associated with 'HoneySweet' plum consumption. For the female cohort, similar observations were made with no apparent differences in pathology scores among any of the groups, including the HS5% and HS15% groups (**Supplementary Table 7**). The female animals exhibited a similar mild inflammation within the salivary glands and kidneys, but there was also a mild hepatic inflammation and vacuolation within the adrenal glands that was present to some degree in all groups. In summary, these data show that no detrimental effects of 'HoneySweet' plum consumption were detected.

Potential for Animal Off-Targeting by 'HoneySweet' sRNA Species

To predict the potential off-target effects of the most abundant sRNA species, we BLAST-searched individual sRNA sequences (most abundant 240 sRNAs) related to the transgenes in 'HoneySweet' against the mouse genome (**Table 3**; **Supplementary Data Sheet 5**). Among the mouse

TABLE 1 | 'HoneySweet' study blood chemistry for males.

Parameter	Control	ST 5%[1]	ST 15%[2]	HS 5%[3]	HS 15%[4]	*P* value	Normal range[5]
Chol[6] (mg/dL)	142.64 ± 9.53	147.09 ± 4.62	162.50 ± 7.34	149.83 ± 9.90	146.00 ± 8.12	0.4778	–
TRIG (mg/dL)	113.82 ± 9.24^{cd}	127.18 ± 10.42^{bd}	167.08 ± 14.13^{a}	154.83 ± 14.85^{ab}	150.25 ± 13.62^{ac}	**0.0351**	–
ALT (U/L)	27.42 ± 5.30	45.82 ± 13.46	74.67 ± 51.52	40.92 ± 6.13	38.50 ± 6.74	0.7177	17–77
AST (U/L)	86.75 ± 18.31	104.64 ± 21.63	103.08 ± 33.78	114.67 ± 23.15	86.75 ± 8.66	0.8813	54–298
GLU (mg/dL)	276.45 ± 24.24	228.45 ± 25.42	256.83 ± 30.71	194.33 ± 16.50	220.67 ± 30.93	0.2193	62–300
PHOS (mg/dL)	9.36 ± 0.39	9.94 ± 0.6	10.56 ± 1.02	10.82 ± 1.52	8.12 ± 0.25	0.2427	5.7–9.2
Ca (mg/dL)	9.08 ± 0.11	9.06 ± 0.12	9.31 ± 0.05	8.99 ± 0.11	8.97 ± 0.06	0.0893	7.1–10.1
TBIL (mg/dL)	0.24 ± 0.03	0.20 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.20 ± 0.02	0.2464	0–0.9
TP (g/dL)	4.60 ± 0.06^{abc}	4.58 ± 0.08^{bcd}	4.64 ± 0.03^{ac}	4.41 ± 0.05^{d}	4.43 ± 0.10^{de}	**0.0470**	3.5–7.2
ALB (g/dL)	2.57 ± 0.10	2.62 ± 0.07	2.69 ± 0.08	2.39 ± 0.08	2.48 ± 0.08	0.1080	2.5–3.0
GLOB (g/dL)	1.94 ± 0.05	1.97 ± 0.03	1.89 ± 0.03	1.90 ± 0.04	1.86 ± 0.05	0.3713	1.0–4.2
AG ratio	1.39 ± 0.04	1.37 ± 0.03	1.46 ± 0.04	1.33 ± 0.04	1.36 ± 0.04	0.2695	–
BUN (ng/dL)	26.64 ± 1.71^{a}	19.91 ± 1.01^{bc}	25.08 ± 0.65^{a}	17.25 ± 0.46^{c}	21.00 ± 1.40^{b}	**0.0001**	8–33
CREAT (mg/dL)	0.22 ± 0.01	0.21 ± 0.01	0.20 ± 0.00	0.20 ± 0.00	0.20 ± 0.00	0.2833	0.2–0.9
Na (mEq/L)	144.82 ± 0.48	145.90 ± 0.84	145.18 ± 1.08	144.10 ± 0.62	145.82 ± 0.92	0.5359	140–160
K (mEq/L)	5.22 ± 0.21	5.06 ± 0.12	5.05 ± 0.12	5.98 ± 1.04	4.67 ± 0.17	0.3836	5–7.5
CL (mEq/L)	110.27 ± 0.56	110.70 ± 0.94	110.09 ± 1.14	109.40 ± 1.18	110.91 ± 0.55	0.7986	88–110
Na/K	28.21 ± 1.13	28.00 ± 0.72	28.90 ± 0.78	27.47 ± 2.28	31.65 ± 1.27	0.2381	–

Treatments were compared using an ANOVA and Fisher's least significant difference (LSD).
Only three measurements had a p-value of 0.05 or lower and these are highlighted in bold.
Those values are then grouped as statistically different classes by the small superscript letters, abcde. Where classes overlap, they have two or more letters.
[1] 'Stanley' was added at 5% of the total weight of the diet.
[2] 'HoneySweet' was added at 5% of the total weight of the diet.
[3] 'Stanley' was added at 15% of the total weight of the diet.
[4] 'HoneySweet' was added at 5% of the total weight of the diet.
[5] Where known, the normal range of components found in the blood. Where blank, there is no consensus level.
[6] ALT, alanine aminotransferase; ALB, albumin; ALP, alkaline phosphatase; AST, aspartate aminotransferase; TBIL, bilirubin; BUN, blood urea nitrogen; CHOL, cholesterol; CREAT, creatinine; GLU, glucose; GLOB, globulin; TP, total protein; TRIG, triglycerides; PHOS, phosphorus; Ca, calcium; Na, sodium; K, potassium; Cl, chloride.

genes identified as potential matches were myoferlin (*Myof*), 5'nucleotidase ecto (*Nt5e*), and low-density lipoprotein receptor-related protein 6 (*Lrp6*). In particular, *Myof* encodes for a member of the ferlin family of proteins, which functions in muscle tubules and has been implicated in myoblast fusions within muscle tissue (Davis et al., 2002). Mice were deficient in this protein exhibit defects in muscle regeneration and angiogenesis. The *Nt5e* gene, also known as *CD73*, encodes for the membrane-bound nucleotidase, which hydrolyzes extracellular nucleoside monophosphates. Mice that lack this protein exhibit the calcification of joints and arteries, and alterations in the dendritic cell infiltration in tissues (Rashdan et al., 2016). Furthermore, *Lrp6* encoded protein functions as the receptor or in conjunction with *frizzled* as a co-receptor for *Wnt*, thereby activating the canonical *Wnt*/beta-catenin signaling pathway (He et al., 2004). Through the *Wnt*/beta-catenin signaling cascade, this gene is involved in the regulation of cell differentiation, proliferation, and migration. Mutations in *Lrp6* have been associated with diseases such as Alzheimer's, cancer, and osteoporosis (van Meurs et al., 2008; Liu et al., 2010, 2014). Potential pathologies associated with the altered expression of the off-target genes *MYOF*, *NT5E*, or *LRP6* were also assessed. No evidence of muscle dysfunction or histological anomalies was reported in the skeletal muscles of any of the animals in the study. Likewise, there were no alterations in lesions consistent with Alzheimer's in brain tissue sections, and no increased incidence of neoplasm or abnormal bone phenotypes were noted in any of the pathological reports.

DISCUSSION

The recent decision by the EU to regulate gene-edited crops has prompted a renewed urgency to provide sound evidence to support science-based regulatory decisions. Interfering RNA is one of the oldest biotechnology strategies in crops available and has a long track record of successful use. In this study, we carried out a comprehensive, field-based study to assess the stability, efficacy, and risks associated with RNAi technology.

Stable Resistance and sRNA Populations in 'HoneySweet'

'HoneySweet' trees subject to intense virus infection pressure *via* grafted limbs remained virus-free even after more than a decade of virus exposure (**Figure 1**). The sRNA profiles of both inoculated and uninoculated 'HoneySweet' trees were relatively constant even when the graft inoculum was removed (**Supplementary Data Sheet 1**: Individual Tree sRNAs Graft Challenged). While the levels of CP derived sRNA from 'HoneySweet' were only slightly lower than that derived from

TABLE 2 | The pathology report for male mice fed normal mouse chow or chow supplemented with either 'Stanley' plum at 5 and 15% or 'HoneySweet' plum at 5 and 15%.

Tissue	Control	ST[a]5%	ST15%	HS[b]5%	HS15%	Chi Sq (*P* value)
Heart	0/12[c] (0%)	0/12 (0%)	1/12 (8.3%)	0/12 (0%)	0/12 (0%)	0.397
Aorta	0/12 (0%)	0/11 (0/5)	1/12 (8.3%)	0/11 (0/5)	0/11 (0/5)	0.431
Lung	1/12 (8.3%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0.397
Trachea	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/8 (0%)	0/12 (0%)	–
Thyroid	0/7 (0%)	2/10 (20%)	0/10 (0%)	0/5 (0%)	0/8 (0%)	0.177
Thymus	0/11 (0/5)	0/12 (0%)	0/12 (0%)	0/11 (0/5)	0/10 (0%)	–
Lymph node	0/10 (0%)	0/7 (0%)	0/10 (0%)	0/9 (0%)	0/11 (0/5)	–
Bone	0/12 (0%)	1/12 (8.3%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0.397
Bone marrow	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Skeletal muscle	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Cerebrum-Gray	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Cerebrum-White	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Cerebellum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Meninges 1 and 2	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
spinal Cord	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Peripheral Nerve	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Eye-LENS	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/11 (0/5)	–
Eye-UVEA	0/12 (0%)	0/12 (0%)	0/11 (0/5)	0/12 (0%)	0/12 (0%)	–
Eye-Retina	0/12 (0%)	0/12 (0%)	0/10 (0%)	0/11 (0/5)	0/12 (0%)	–
Eye-Cornea	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Testis	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Acc. Sex Gland	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Skin	2/12 (16.7%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0.082
Liver	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Kidney[d]	3/12 (25%)	1/12 (8.3%)	2/12 (16.7%)	3/11 (27.3%)	3/12 (25%)	0.77
Urinary bladder	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Spleen	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Salivary glands[e]	3/12 (25%)	1/12 (8.3%)	3/12 (25%)	1/12 (8.3%)	2/12 (16.7%)	0.581
Esophagus	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Stomach	1/12 (8.3%)	1/12 (8.3%)	2/12 (16.7%)	1/12 (8.3%)	2/12 (16.7%)	0.914
Pancreas	0/12 (0%)	0/12 (0%)	0/12 (0%)	1/12 (8.3%)	1/12 (8.3%)	0.541
Duodenum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Jejunum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Ileum	0/12 (0%)	1/12 (8.3%)	1/12 (8.3%)	0/12 (0%)	0/12 (0%)	0.541
Cecum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Colon	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Rectum	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)	–
Adrenal glands	0/11 (0/5)	0/9 (0%)	0/12 (0%)	0/11 (0/5)	0/10 (0%)	–

There were no negative effects seen that could be attributed to the presence of the 'HoneySweet' plum.

[a] *ST, 'Stanley.'*

[b] *HS, 'HoneySweet.'*

[c] *Data presented as the number of animals with any abnormal pathology per tissue read.*

[d] *Salivary glands have mild sialadenitis.*

[e] *Kidneys have mild peripelvic nephritis.*

the CP of virus-infected leaf material in susceptible control trees, overall levels of viral sRNA were nearly 25-fold higher in PPV infected controls, thereby spanning the entire PPV genome (**Supplementary Data Sheet 2**: Individual sRNAs for Multi-location; **Supplementary Table 2**, **Figure 3B**). The composition of the sRNA derived from transgene CP and viral CP were similar with the exception of an increase in the 24-nt sRNA species in 'HoneySweet' and a higher proportion of 22-nt species in virus-infected controls (**Supplementary Figure 3**). Surprisingly, the overlapping regions of the CP ORF produced

TABLE 3 | PPV-CP sRNAs best match *Mus musculus*.

	Raw		Normalized TPM				Match		Bases				
	Rank[a]	Reads[b]	HSF[c]	STF[d]	HSL[e]	STL[f]	Length	Accession#	Matched	Gap	Start	Stop	Identity
AGAGGACACAGAGAGACACACC	17	1219	22.43	9.45	26.81	–	22	gi|315075272|	20	1	1	20	Low density lipoprotein receptor-related protein 6 Lrp6
AGAGGACACAGAGAGACACAC	27	799	17.15	–	18.47	–	21	ref|NM_008514.4| gi|315075272	20	1	1	20	Low density lipoprotein receptor-related protein 6 Lrp6
AGGGGAGTGTAGTGGTCTCGG	89	293	3.06	0.89	8.77	–	21	|ref|NM_008514.4| gi|153791795|	19	1	3	21	Myoferlin Myof
GGGGAGTGTAGTGGTCTCGGT	149	170	6.65	1.52	1.05	–	21	ref|NM_001099634.1| gi|153791795	20	1	2	21	Myoferlin Myof
AGGGGAGTGTAGTGGTCTCGGT	150	169	1.37	–	5.57	–	22	|ref|NM_001099634.1| gi|153791795|	20	1	3	22	Myoferlin Myof
GGTGTGTCTCTCTGTGTCCT	211	103	4.27	1.07	0.39	–	20	ref|NM_001099634.1| gi|284004882|	18	0	1	18	Tripartite motif-containing 66 Trim66
GGTGTGTCTCTCTGTGTCCT	211	103	4.27	1.07	0.39	–	20	ref|NM_001170913.1| gi|284004880	18	0	1	18	Tripartite motif-containing 66 Trim66 variant 1
GGTGTGTCTCTCTGTGTCCT	211	103	4.27	1.07	0.39	–	20	|ref|NM_001170912.1| gi|284004878|	18	0	1	18	Tripartite motif-containing 66 Trim66 variant 2
TAAAAATCAAAGGCATATCTG	139	187	0.63	1.96	5.96	–	21	ref|NM_181853.4| gi|118130656|ref|NM_029775.2|	19	0	3	21	DCN1 defective in cullin neddylation 1 domain containing 5 Dcun1d5
AGAGCTCCGCAGTCTTGTTT	184	129	–	5.08	2.81	–	20	gi|158341626|	18	1	1	18	Phospholipase B domain containing 2 (Plbd2)
AAATGACTTCAACGACACCCG	193	119	0.90	0.98	3.55	–	21	ref|NM_023625.4| gi|22208851|	19	1	1	19	Olfactory receptor142 OOlfr142
GCAACCTGACAGACTACAGCC	223	93	0.63	2.05	2.26	–	21	ref|NM_146984.1| gi|118150646| ref|NM_025852.3|	19	1	1	19	RNAexonuclease 1 homolog

(Continued)

TABLE 3 | Continued

	Raw		Normalized TPM				Match		Bases				
	Rank[a]	Reads[b]	HSF[c]	STF[d]	HSL[e]	STL[f]	Length	Accession#	Matched	Gap	Start	Stop	Identity
GTTCCATTCTCTATGCACCAA	114	227	0.42	–	8.53	–	21	gi|142349713|	19	1	2	20	Member RAS oncogene RAB9B
CTTTTAGACAAATTATGGCAC	155	161	–	2.85	5.03	–	21	ref|NM_176971.2| gi|256985127|	19	2	2	20	CCR-4-NOT transcription complex, subunit4 (Cnot4) variant 3
CTTTTAGACAAATTATGGCAC	155	161	–	2.85	5.03	–	21	ref|NM_001164411.1| gi|256985125|	19	2	2	20	CCR-4-NOT transcription complex, subunit4 (Cnot4) variant 2
CATTTCTCAATGCTGCTGCCT	225	92	–	–	3.58	–	21	ref|NM_016877.4| gi|291045287|	19	1	1	19	5′ nucleotidase ecto (Nt5e)
CATTTCTCAATGCTGCTGCCT	225	92	–	–	3.58	–	21	ref|NM_011851.4| gi|141802310| ref|NM_201362.2|	19	1	1	19	Coiled-coil domain containing 68 Ccdc68

The most abundant CP-specific sRNAs were matched to all cDNA sequences from the mice to determine if there were significant homologies.

[a] *Rank ordered by most abundant sRNA related to CP.*

[b] *Total reads for 16 'HoneySweet' libraries.*

[c] *'HoneySweet' fruit.*

[d] *'Stanley' fruit.*

[e] *'HoneySweet' leaf*

[f] *'Stanley' leaf.*

most of the sRNA in both the transgene and virus-infected conditions, although the specific sRNA species within the regions varied according to location, tissue, and sample (**Supplementary Table 3**). Collectively these data show that PPV infection pressure has little to no influence on the overall levels and composition of transgene-derived sRNA. And that hairpin transgene expression of CP sequence produces sRNAs that are lower in abundance than that found in virus-infected samples, but the sequences are consistent with that produced under natural infection except with respect to sRNA length – with 24-nt species being proportionally higher in 'HoneySweet' while 22-nt species were proportionally higher in virus-infected 'Stanley' trees.

No Long-Term Effect on Virus Populations

It is currently unknown if viruses exposed to RNAi-protected plants are subject to adaptive evolutionary pressures that could potentially break down virus resistance over time (Tepfer et al., 2015). To assess this possibility, virus-infected limbs grafted to 'HoneySweet' trees for over 10 years were analyzed for potential influences on viral populations. The viral CP, that is the target of 'HoneySweet' transgene-derived sRNA, displayed relatively few nucleotide changes (2-6), suggesting that there was little to no accumulation of variants that could potentially evade sRNA targeting by 'HoneySweet' (**Figure 3**, **Supplementary Data Sheet 4**: Base changes in PPV-REC viral genome). There were, likewise, no consistent impacts on the sequences of viral ORFs known to play a role in silencing suppression including HCPro and CI (Ivanov et al., 2016; Cheng and Wang, 2017; Rodamilans et al., 2018). The results show that long-term exposure to RNAi-expressing plants does not appear to impose any clear viral selection pressure. It is important to note that these findings do not exclude the potential mechanisms associated with the proliferation of naturally occuring viral strains or isolates that could potentially evade RNAi resistance due to naturally occurring low levels of target sequence similarity. To date, this is not known to occur for PPV strains that have highly conserved CP sequences.

Safe Consumption of 'HoneySweet'

Potential risks associated with the consumption of sRNA have been recently raised. A handful of studies have provided evidence that sRNAs may have off-target effects when consumed (Zhang Y. et al., 2012; Liu et al., 2017). These mechanisms presumably involve the silencing of genes that have sufficient levels of homology to plant-derived sRNA sequences. Here, we evaluated the health risks associated with 'HoneySweet' consumption and the potential for specific off-target effects in mice. First, we characterized the sRNA profiles of both leaves and ripe fruits and compared them to leaves and fruits of susceptible infected control plums (**Supplementary Table 2**). The results showed that the levels of viral CP-specific sRNA derived from transgene expression were lower than that produced by natural virus infection and were proportionally lower than many endogenous plant sRNAs. However, the overall levels of sRNA generated from PPV infection were significantly higher than 'HoneySweet' due to sRNA production across the entire length of the viral genome. In the ripe fruit of susceptible trees, the viral sRNA accounted for approximately 35% of all sRNA produced in the fruit (**Figure 2**; **Supplementary Table 2**). These data imply that the sRNA produced *via* the 'HoneySweet' transgene are proportionally lower than that produced by natural virus infection and make up a relatively low proportion of the total pool of sRNA in the plant. Next, we performed a comprehensive 90-day feeding study in mice that included physical and behavioral records, blood chemistries, and detailed pathological assessments of 38 different organs and tissues (**Figure 3**, **Tables 1**, **2**; **Supplementary Tables 4**–**7**). We found no evidence of any negative health impacts associated with 'HoneySweet' plum consumption in male or female mice. Likewise, we found no evidence for any pathologies associated with predicted off-target effects based on the similarity between the most abundant 'HoneySweet' sRNA species and the mouse genome (**Table 3**). Collectively, the data did not reveal any evidence of specific health or environmental risks and demonstrated the long-term efficacy of RNAi strategies to protect plants against virus infection.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/PRJNA741990; https://www.ncbi.nlm.nih.gov/genbank/PRJNA741796; https://www.ncbi.nlm.nih.gov/genbank/PRJNA742049.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Oklahoma State University.

AUTHOR CONTRIBUTIONS

AMC, BJS, RS, JP, JKK, and CD conceived and designed the project. KS, AMC, BJS, TM, JJ, and EB performed the experiments. KS, AMC, BJS, TM, RS, JJ, EB, and CD analyzed the data. JK and CD directed aspects of the project. KS, AMC, BJS, RS, JKK, and CD wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Beachy, R. N. (1997). Mechanisms and applications of pathogen-derived resistance in transgenic plants. *Curr. Opin. Biotech.* 8, 215–220. doi: 10.1016/S0958-1669(97)80105-X

Chan, S. Y., and Snow, J. W. (2017). Formidable challenges to the notion of biologically important roles for dietary small RNAs in ingesting mammals. *Genes Nutr.* 12:13. doi: 10.1186/s12263-017-0561-7

Cheng, X., and Wang, A. (2017). The potyvirus silencing suppressor protein VPg mediates degradation of SGS3 via ubiquitination and autophagy pathways. *J. Virol.* 91, e01478–e01416. doi: 10.1128/JVI.01478-16

Davis, D. B., Doherty, K. R., Delmonte, A. J., and McNally, E. M. (2002). Calcium-sensitive phospholipid binding properties of normal and mutant ferlin C2 domains. *J Biol. Chem.* 277, 22883–22888. doi: 10.1074/jbc.M201858200

Dickinson, B., Zhang, Y., Petrick, J. S., Heck, G., Ivashuta, S., and Marshall, W. S. (2013). Lack of detectable oral bioavailability of plant microRNAs after feeding in mice *Nat. Biotechnol.* 31:965. doi: 10.1038/nbt.2737

Fire, A., SiQun, X., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806. doi: 10.1038/35888

García, J. A., Glasa, M., Cambra, M., and Candresse, T. (2014). Plum pox virus and sharka: a model potyvirus and a major disease. *Mol. Plant Pathol.* 15, 226–241. doi: 10.1111/mpp.12083

Gonsalves, D. (1998). Control of papaya ringspot virus in papaya: a case study. *Annu. Rev. Phytopathol.* 36, 415–437. doi: 10.1146/annurev.phyto.36.1.415

Gottula, J., and Fuchs, M. (2009). Toward a quarter century of pathogen-derived resistance and practical approaches to plant virus disease control. *Adv. Virus Res.*75, 161–183. doi: 10.1016/S0065-3527(09)07505-8

Halloran, B. P., Wronski, T. J., VonHerzen, D. C., Chu, V., Xia, X., Pingel, J. E., et al. (2010). Dietary dried plum increases bone mass in adult and aged male mice. *J. Nutr.* 140, 1781–1787. doi: 10.3945/jn.110.124198

Hamilton, A. J., and Baulcombe, D. C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950–952. doi: 10.1126/science.286.5441.950

Hartmann, W., and Neumüller, M. (2006). Breeding for resistance: breeding for Plum pox virus resistant plums (*Prunus domestica* L.) in Germany. *Bull. OEPP* 36, 332–336. doi: 10.1111/j.1365-2338.2006.01010.x

He, X., Semenov, M., Tamai, K., and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/β-catenin signaling: arrows point the way. *Development* 131, 1663–1677. doi: 10.1242/dev.01117

Heinemann, J. A., Agapito-Tenfen, S. Z., and Carman, J. A. (2013). A comparative evaluation of the regulation of GM crops or products containing dsRNA and suggested improvements to risk assessments. *Environ. Int.* 55, 43–55. doi: 10.1016/j.envint.2013.02.010

Ivanov, K. I., Eskelin, K., Bašić, M., De, S., Lõhmus, A., Varjosalo, M., et al. (2016). Molecular insights into the function of the viral RNA silencing suppressor HCP ro. *Plant J.* 85, 30–45. doi: 10.1111/tpj.13088

Jarošová, J., Gadiou, S., Polák, J., Ravelonandro, M., Scorza, R., and Kumar, J. K. (2010). Evaluation of transgenic *Prunus domestica* L., clone C5 resistance to Plum pox virus. *Julius-Kühn-Archiv* 427:330.

Leonard, S. P., Powell, J. E., Perutka, J., Geng, P., Heckmann, L. C., Horak, R. D., et al. (2020). Engineered symbionts activate honey bee immunity and limit pathogens. *Science* 367, 573–576. doi: 10.1126/science.aax9039

Liu, C. C., Prior, J., Piwnica-Worms, D., and Bu, G., et al. (2010). LRP6 overexpression defines a class of breast cancer subtype and is a target for therapy. *Proc. Natl. Acad. Sci. USA.* 107, 5136–5141. doi: 10.1073/pnas.0911220107

Liu, C. C., Tsai, C. W., Deak, F., Rogers, J., Penuliar, M., Sung, Y. M., et al. (2014). Deficiency in LRP6-mediated Wnt signaling contributes to synaptic abnormalities and amyloid pathology in Alzheimer's disease. *Neuron* 84, 63–77. doi: 10.1016/j.neuron.2014.08.048

Liu, Y.-C., Chen, W. L., Kung, W.-H., and Huang, H.-D. (2017). Plant miRNAs found in human circulating system provide evidences of cross kingdom RNAi. *BMC Genomics* 18, 112. doi: 10.1186/s12864-017-3502-3

Mat Jalaluddin, N. S., Othman, R. Y., and Harikrishna, J. A. (2018). Global trends in research and commercialization of exogenous and endogenous RNAi technologies for crops. *Crit. Rev. Biotechnol.* 39, 1–12. doi: 10.1080/07388551.2018.1496064

Petrick, J. S., Moore, W. M., Heydens, W. F., Koch, M. S., Sherman, J. H., and Lemke, S. L. (2015). A 28-day oral toxicity evaluation of small interfering RNAs and a long double-stranded RNA targeting vacuolar ATPase in mice. *Regul. Toxicol. Pharmacol.* 71, 8–23. doi: 10.1016/j.yrtph.2014.10.016

Polák, J., Kundu, J. K., Krška, B., Beoni, E., Komínek, P., Pívalová, J., et al. (2017). Transgenic plum *Prunus domestica* L., clone C5 (cv. HoneySweet) for protection against sharka disease. *J. Integr. Agric.* 16, 516–522. doi: 10.1016/S2095-3119(16)61491-0

Rashdan, N. A., Rutsch, F., Kempf, H., Váradi, A., Lefthériotis, G., and MacRae, V. E. (2016). New perspectives on rare connective tissue calcifying diseases. *Curr. Opin. Pharmacol.* 28, 14–23. doi: 10.1016/j.coph.2016.02.002

Rimbaud, L., Dallot, S., Gottwald, T., Decroocq, V., Jacquot, E., Soubeyrand, S., et al. (2015). Sharka epidemiology and worldwide management strategies: learning lessons to optimize disease control in perennial plants. *Annu. Rev. Phytopathol.* 53, 357–378. doi: 10.1146/annurev-phyto-080614-120140

Rodamilans, B., Valli, A., Mingot, A., San León, D., López-Moya, J. J., and García, J. A. (2018). An atypical RNA silencing suppression strategy provides a snapshot of the evolution of sweet potato-infecting potyviruses. *Sci. Rep.* 8, 1–10. doi: 10.1038/s41598-018-34358-y

Ruiz, M. T., Voinnet, O., and Baulcombe, D. C. (1998). Initiation and maintenance of virus-induced gene silencing. *Plant Cell* 10, 937–946. doi: 10.1105/tpc.10.6.937

Scorza, R., Callahan, A., Levy, L., Damsteegt, V., Webb, K., and Ravelonandro, M. (2001). Post-transcriptional gene silencing in plum pox virus resistant transgenic European plum containing the Plum pox potyvirus coat protein gene. *Trans. Res.* 10, 201–209. doi: 10.1023/A:1016644823203

Scorza, R., Ravelonandro, M., Callahan, A., Zagrai, I., Polak, J., Malinowski, T., et al. (2016). 'HoneySweet'(C5), the first genetically engineered plum pox virus–resistant Plum (*Prunus domestica* L.) Cultivar. *HortScience* 51, 601–603. doi: 10.21273/HORTSCI.51.5.601

Scorza, R., Ravelonandro, M., Callahan, A. M., Cordts, J. M., Fuchs, M., Dunez, J., et al. (1994). Transgenic plums (*Prunus domestica* L.) express the plum pox virus coat protein gene. *Plant Cell Rep.* 14, 18–22. doi: 10.1007/BF00233291

Singh, K., and Kundu, J. K. (2017), Variation in coat protein sequence of Wheat streak mosaic virus among crop and no crop hosts. *Crop Pasture Sci.* 68, 328–336. doi: 10.1071/CP17025

Singh, K., Zouhar, M., Mazakova, J., and Rysanek, P. (2014), Genome Wide Identification of the Immunophilin Gene Family in *Leptosphaeria maculans*:

a causal agent of blackleg disease in oilseed rape (*Brassica napus*). *OMICS*. 18, 645–657. doi: 10.1089/omi.2014.0081

Tepfer, M., Jacquemond, M., and García-Arenal, F. (2015). A critical evaluation of whether recombination in virus-resistant transgenic plants will lead to the emergence of novel viral diseases. *New Phytol.* 207, 536–541. doi: 10.1111/nph.13358

The International Peach Genome Initiative (2013). The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. *Nat. Genet*. 45, 487–494. doi: 10.1038/ng.2586

Tosar, P., Rovira, C., Naya, H., and Cayota, A. (2014). Mining of public sequencing databases supports a non-dietary origin for putative foreign miRNAs: underestimated effects of contamination in NGS. *RNA* 20, 754–757. doi: 10.1261/rna.044263.114

van Meurs, J. B., Trikalinos, T. A., Ralston, S. H., Balcells, S., Brandi, M. L., Brixen, K., et al. (2008). Large-scale analysis of association between LRP5 and LRP6 variants and osteoporosis. *JAMA* 299, 1277–1290. doi: 10.1001/jama.299.11.1277

Vilanova, S., Romero, C., Abbott, A. G., Llacer, G., and Badenes, M. L. (2003). An apricot (*Prunus armeniaca* L.) F2 progeny linkage map based on SSR and AFLP markers, mapping plum pox virus resistance and self-incompatibility traits. *Theor Appl Genet* 107, 239–247. doi: 10.1007/s00122-003-1243-y

Zhang, J., Khan, S. A., Heckel, D. G., and Bock, R. (2017). Next-generation insect-resistant plants: RNAi-mediated crop protection. *Trends Biotechnol.* 35, pp.871–882. doi: 10.1016/j.tibtech.2017.04.009

Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., et al. (2012). Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Res.* 22, 107–126. doi: 10.1038/cr.2011.158

Zhang, Y., Wiggins, B. E., Lawrence, C., Petrick, J., Ivashuta, S., and Heck, G. (2012). Analysis of plant-derived miRNAs in animal small RNA datasets. *BMC Genomics* 13:381. doi: 10.1186/1471-2164-13-381

Zuriaga, E., Soriano, J. M., Zhebentyayeva, T., Romero, C., Dardick, C., Cañizares, J., et al. (2013). Genomic analysis reveals *MATH* gene(s) as candidate(s) for plum pox virus (PPV) resistance in apricot (*Prunus armeniaca* L.). *Mol. Plant Path*. 14, 663–677. doi: 10.1111/mpp.12037

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REVIEW
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Lab-to-Field Transition of RNA Spray Applications – How Far Are We?

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The drastic loss of biodiversity has alarmed the public and raised sociopolitical demand for chemical pesticide-free plant production, which is now treated by governments worldwide as a top priority. Given this global challenge, RNAi-based technologies are rapidly evolving as a promising substitute to conventional chemical pesticides. Primarily, genetically modified (GM) crops expressing double-stranded (ds)RNA-mediating gene silencing of foreign transcripts have been developed. However, since the cultivation of GM RNAi crops is viewed negatively in numerous countries, GM-free exogenous RNA spray applications attract tremendous scientific and political interest. The sudden rise in demand for pesticide alternatives has boosted research on sprayable RNA biopesticides, generating significant technological developments and advancing the potential for field applications in the near future. Here we review the latest advances that could pave the way for a quick lab-to-field transition for RNA sprays, which, as safe, selective, broadly applicable, and cost-effective biopesticides, represent an innovation in sustainable crop production. Given these latest advances, we further discuss technological limitations, knowledge gaps in the research, safety concerns and regulatory requirements that need to be considered and addressed before RNA sprays can become a reliable and realistic agricultural approach.

Keywords: RNAi-based plant protection, RNA biopesticides, dsRNA, siRNA, nanomaterial-based siRNA delivery, spray-induced gene silencing (SIGS)

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INTRODUCTION

Modern agriculture faces diverse challenges related to the fact that the world population is expected to rise by 26% by 2050. Given this projection, agriculturists must boost productivity to meet emerging food needs (United Nations Department of Economics and Social Affairs, 2019). Major challenges to these production levels arise due to agricultural pests. Pests contribute estimated losses of up to 40% of crops worldwide, with insects serving as causal agents of damage and disease transmission (Douglas, 2018). So far, plant protection primarily relies on the use of chemical pesticides. However, the use of conventional pesticides contributes to soil, water, and air pollution and is predicted to be a major driver of an alarming loss of biodiversity worldwide (Wagner et al., 2021). In addition, improper and controlled chemical treatments detrimentally affect the environment, consumers, and users. Moreover, perennial applications result in the development of pesticide resistance in pathogens and pests (Fotoukkiaii et al., 2021).

Intergovernmental initiatives such as the EU's farm-to-fork strategy (part of the European green deal) aim to rethink and redesign food systems, for example by developing frameworks for sustainable food production. A central goal is to reduce the overall use and risk of chemical

pesticides by 50% by 2030 (European Commission, 2021). Given the social-political demand for an ideally pesticide-free agriculture, safe "green" alternatives are urgently desired to facilitate sustainability in crop protection. Thus, RNAi-based technologies are discussed as low risk pesticides that may enable reaching pesticide reduction and sustainability goals (Taning et al., 2021).

A promising alternative to chemical pesticides relies on harnessing the mechanistic of RNA interference (RNAi). Briefly, RNAi is a conserved cellular mechanism that regulates and protects eukaryotic cells against harmful nucleic acids. In this natural phenomenon, double-stranded RNAs (dsRNAs) are processed by Dicer enzymes into 21–24 nucleotide (nt) small interfering RNAs (siRNAs). These siRNAs either mediate transcriptional gene silencing (TGS) by ensuring inhibitory DNA and histone modifications as well as chromatin remodeling, or they facilitate post-transcriptional gene silencing (PTGS) by preventing translation of targeted mRNA transcripts. Both TGS and PTGS served as blueprints for the development and continuous improvement of RNAi toolkits. In agriculture, RNAi has proven to be an effective strategy in controlling pathogens such as viruses, bacteria, and fungi, as well as insect pests, mites, and nematodes (Fire et al., 1998; Baum et al., 2007; Zotti et al., 2018; Mezzetti et al., 2020; Koch and Wassenegger, 2021).

A decade ago, studies focused on the transgene-based endogenous expression and formation of dsRNA in host plants, termed host-induced gene silencing (HIGS) (Nowara et al., 2010). Nowadays, transgenic approaches are clouded by some major drawbacks. First, these approaches are laborious, complicated, and time-consuming, and their applicability is restricted by the transformability of the host plant. They are also very expensive; for example, the commercialization of a transgenic crop is estimated to reach 140 million dollars (Rosa et al., 2018). Finally, they are weakly accepted, as public distrust of GM plants persists, with an ongoing upward trend (Herman et al., 2021). Additionally, orders and species present completely different and variable demands, raising concerns about whether this technology is effective under field conditions (Scott et al., 2013; Yu et al., 2016; Cooper et al., 2019). Given this assessment, there is not only a need for alternatives that avoid the use of chemical pesticides but also increasing interest in finding alternatives to GM-based measures (Dalakouras et al., 2020; Das and Sherif, 2020). A breakthrough was achieved by demonstrating that dsRNAs effectively induce PTGS and confer disease resistance upon foliar spray (Nature, 2016), termed spray-induced gene silencing (SIGS) (**Table 1**; Koch et al., 2016; Wang et al., 2016). Compared to HIGS, SIGS is much faster, cheaper and easier to handle, and it might reach a broader range of hosts. Interestingly, a direct comparison of HIGS and SIGS revealed SIGS to be more efficient under lab conditions (Koch et al., 2019; Höfle et al., 2020). However, the lab-to-field transition will require optimization and further development of SIGS technology to increase stability (UV, rain) and specificity (off-target risks) under environmental conditions. In this review, we will highlight the most promising attempts to further develop SIGS for its lab-to-field transition and discuss significant achievements in improving stability and reducing environmental risks. In addition, we provide an overview of RNA spray approaches and their particularities, including nanomaterial-based delivery, efficiencies and durability.

MAJOR CHALLENGES AND RECENT ACHIEVEMENTS IN TRANSFERRING RNA SPRAY TO FIELD ENVIRONMENTS

The immense potential of sprayable RNA biopesticides for meeting current agro-economic challenges has prompted the development of RNAi technology. Initial euphoria was a major driver leading to a rapid increase of reports, resulting in 54 studies over the last 3 years (PubMed, August 2021) demonstrating academic proof-of-concept for several pathosystems (**Table 1**). However, at the same time, it became obvious that the molecular mechanism for uptake, processing and transport of sprayed RNA biopesticides was inadequately understood.

Initially, only the GM-based HIGS approach was considered because transgene-derived dsRNA was thought to be robust and reliable in the delivery of PTGS inducers. Naked dsRNA showed effectiveness under lab conditions but had the potential for instability when sprayed in fields, where it could be degraded by UV radiation or washed off by rain. To circumvent this, several studies, inspired by human RNAi therapy research (Swaminathan et al., 2021), attempted to increase stability and durability by establishing protective envelops around dsRNAs and siRNAs (Pugsley et al., 2021; Yan et al., 2021). In mammals, the delivery of siRNA therapeutics is mediated by liposome encapsulation (Liu and Huang, 2021; Zabel et al., 2021). Nanoparticle- and other carrier-based delivery of dsRNA/siRNA has been widely used, with remarkable success, in start-up companies such as "RNAissance[1]" which focus on broad, cost-efficient topical RNAi uses in agriculture. Their final dsRNA products are advertised as safe, ready to use and stabler than naked dsRNAs. This advancement represents a step toward lowering the amount of applied RNA biopesticides since it is estimated that 2–10 g of dsRNA are required to protect 1 ha (Das and Sherif, 2020). However, the application amount and frequency depend on several largely unknown factors that determine dsRNA persistence, distribution and dilutional and degradational processes in host plants as well as target species. Moreover, it is indisputable that mechanistic insights, which determine strength and limitations in a pathosystem-specific manner, will be required to optimize and further develop RNA sprays, as well as to anticipate obstacles that will appear when transferring RNA sprays to field environments.

SIGNIFICANT ADVANCES OF RNA SPRAY CONTROLLING: VIRUSES

Given that the RNAi originally evolved as a primary antiviral defense in plants, it is not surprising that most studies have shown that RNAi is most effective at controlling viral

[1]https://www.rnaissanceag.net

TABLE 1 | Summary of RNA spray studies in plants.

	Target organism	Target gene (dsRNA length)	Plant species	Nanoformulation	Applied dsRNA (μ g)	Application method	Efficacies	Durability	References
Viruses	Sugarcane Mosaic Virus (SCMV)	Coat protein (~150 bp)	*Zea mays*	*Escherichia coli* HT115 (DE3)	3 μg/L	Spray of bacteria-produced dsRNA	Total inhibition of virus infection	30 days post inoculation (dpi)	Gan et al., 2010
	Pepper mild mottle virus (PMMoV) Cucumber mosaic virus (CMV)	PMMoV Replicase (977 bp) CMV Replicase (2b) (330 bp)	*Vigna unguiculata Nicotiana tabacum*	Layered double hydroxide (LDH) clay nanosheets	1.25 μg of dsRNA and/or 3.75 μg of LDH	Sprayed with an atomizer	Total inhibition of virus infection (systemic protection)	Up to 20 days after a single spray	Mitter et al., 2017
	Bean common mosaic virus (BCMV)	Nuclear inclusion b protein (480 bp) Coat protein (461 bp)	*Nicotiana benthamiana Vigna unguiculata*		100 μg	Mechanical inoculation with carborundum as an abrasive and sprayed with an atomizer	Reduction in infection to 45% and to 8.3%	Analyzed for up to 10 days	Worrall et al., 2019
	Tomato yellow leaf curl virus (TYLCV)	Coat protein (CP) (680–700 bp)	*Nicotiana benthamiana Solanum lycopersicum*		1.25 μg of pDNA, 3.75 μg of LDH	Sprayed using an atomizer at ~125 μl/cm^2	Delivered systemically. Lower incidence and severity. Symptom expression rate reduced to 41.7%	Up to 35 days	Liu et al., 2020
Insects	*Sitobeon avenae*	Salivary sheath protein (491bp)	*Hordeum vulgare*	Naked dsRNA	20 ng/μl	Foliar spray on leaves	Reduced transcript level of 60%	Monitored up to 5 days	Biedenkopf et al., 2020
	Leptinotarsa decemlineata	Inhibitor of apoptosis, Actin; HSP70; Dynamin (300–600 bp)	*Solanum tuberosum*	*Escherichia coli* HT115 (DE3)	15 μl of 0.85 μg/μl (larvae) and 2.5 μg/μl (adults) dsRNA spread on each disk surface. Expressed by bacteria heat−killed in 4ml sprayed	dsRNA spread on each disk surface and dried for 10 min	~100% larval mortality (dsIAP or dsActin); 80% adult mortality (dsActin) Feeding CPB with heat−killed bacteria induced significantly higher mortality (70–90%)	Mortality recorded until 11th day and up to 6th for the bacteria expressed assay	Máximo et al., 2020
		Actin (297bp)	*Solanum tuberosum*	naked dsRNA	12 μL (100, 30, 10, 3, 1, and 0.3 ng)	Purpose-built spraying device on potato leaf disks (Ø = 2 cm)	German strain and Spanish field strain E02 almost 100% mortality (30 ng dsActin). Spanish strain E01 showed only 30% mortality. By day four > 95% of the exposed larvae were dead	Monitored up 5 days	Mehlhorn et al., 2020

(Continued)

TABLE 1 | (Continued)

	Target organism	Target gene (dsRNA length)	Plant species	Nanoformulation	Applied dsRNA (μ g)	Application method	Efficacies	Durability	References
		Mesh gene (417 bp)	*Solanum tuberosum*	Naked dsRNA	10 μg/ml.	25 m^2 plots of potato plants were sprayed under field conditions	Observed field mortality was slightly lower compared to laboratory trials	Monitored up 13 day	Petek et al., 2020
	Phaedon cochleariae	Cactus, srp54k, rop, α-SNAP Shibire, PP-α, hsc70–3, rpn7, rpt3; (317 and 599 bp)	*Brassica oleracea*	Naked dsRNA	0.3 μg (9.6 g/ha), 1 μg (32 g/ha) to 3 μg (96 g/ha)	Custom-built spraying device, multi-well plate foliar RNAi screening procedure	Suppression of transcript level rpt3 (94.5%), srp54k (94.1%), rpn7 (93.9%), α-SNAP (84.9%), shibire (81.3%), PP-α (80.5%) hsc70–3 (75.9%)	Monitored over 10 days	Mehlhorn et al., 2021
	Helicoverpa armigera	Juvenile hormone methyltransferase, Acetylcholine esterase; (21 nt)	Chickpea	Chitosan nanoparticles (CNPs)	One milliliter of CNPs-dsRNA (200 μg:1,000 ng wt/wt)	Sprayed over the plant canopy with a hand-held mist sprayer	100% insect mortality	Monitored up to 5 days	Kolge et al., 2021
	Henosepilachna vigintioctopunctata	Ecdysone receptor (EcR)	*Solanum tuberosum*	*Escherichia coli* HT115 (DE3)	0.5 μg/mL	dsEcR-immersed foliage and dsEcR-*E. coli* directly sprayed to the foliage of greenhouse-growing potato plants	Only 40% of the treated larvae formed Pupae (6–8 days). Of them, 60% exhibited a defective phenotype	Monitored for up to 10 days	Wu et al., 2021
Fungi	*Fusarium graminearum*	Cytochrome P450 lanosterol C-14α-demethylases (CYP51A, CYP51B, CYP51C) (CYP3RNA) (791 nt; 21–24 nt)	*Hordeum vulgare*	Naked dsRNA	dsRNA was diluted in 500 μL water to a final concentration of 20 ng/μL	Detached barley leaves were sprayed using a spray flask	Reduction of transcript level: 72% (CYP51A), 90% (CYP51B), and 71% (CYP51C); inhibition of fungal growth	Analyzed for up to 8 days	Koch et al., 2016
	Botrytis cinerea Verticillium spp.	Dicer-like (DCL)1 DCL2 (315 bp)	*Arabidopsis thaliana Nicotiana tabacum Solanum lycopersicum Fragaria rosales Vitis vinifera Lactuta sativa Allium cepa Rosaceae*	naked dsRNA	20 ng/μL	dsRNA were dropped onto the surface of fruits, vegetables and rose petals and *B. cinerea* Inoculum was applied on the same spot	Reduced fungal growth	Analyzed for up to 8–10 days after	Wang et al., 2016

(Continued)

TABLE 1 | (Continued)

Target organism	Target gene (dsRNA length)	Plant species	Nanoformulation	Applied dsRNA (μ g)	Application method	Efficacies	Durability	References
Fusarium asiaticum	Myosin5 (Myo5) (~500 bp)	*Triticum aestivum*	Naked dsRNA	400 ng of fluorescein-Myo5-8 dsRNA	Sprayed using a spray flask	Inhibition of mycelial growth (31–70%), interference in life cycle and virulence, cell wall defects, life cycle disruption and virulence reduction	9 h unless the dsRNA was continuously supplied	Song et al., 2018
Fusarium graminearum	294 nt (FgCYP51A) 220 nt (FgCYP51B) 238 nt (FgCYP51C) 514 nt (FgCYP51A/CYP51B) 458 nt (FgCYP51B/CYP51C) 532 nt (FgCYP51A/CYP51C)	*Hordeum vulgare*	Naked dsRNA	dsRNA was diluted in 500 μL water to a final concentration of 20 ng/μL	Barley leaves were detached and sprayed using a spray flask	Reduced fungal growth and transcript level to less than 10%	Monitored up to 5 days	Koch et al., 2019
	DICER-like1 and 2; ARGONAUTE1 and 2; AGO-interacting protein FgQIP; RecQ Helicase;RNA-dependent RNA polymerases (~1,000 bp)					Central role in different steps of sexual and asexual reproduction, in fungal pathogenicity and DON production		Gaffar and Koch, 2019
	500–, 800–, 1,518 nt (FgCYP51A) 400–, 800–, 1,575 nt (FgCYP51B) 400–, 800–, 1,548 nt (FgCYP51C)					Inhibition of fungal infection symptoms up to 82%		Höfle et al., 2020
	365 nt, 1,529 nt (FgAGO1/AGO2) 355 nt, 1,570 nt (FgAGO1/DCL1) 366 nt, 1,528 nt (FgAGO1/DCL2) 374 nt, 1,783 nt (FgAGO2/DCL1) 1,741 nt (FgAGO2/DCL2) 1,782 nt (FgDCL1/DCL2)					Inhibition of fungal infection up to 60%; reduced transcript level up to 79%		Werner et al., 2020

(Continued)

TABLE 1 | (Continued)

	Target organism	Target gene (dsRNA length)	Plant species	Nanoformulation	Applied dsRNA (μ g)	Application method	Efficacies	Durability	References
	Phakopsora pachyrhizi	Acetyl-CoA acyltransferase 40S ribosomal protein S16, Glycine cleavage system H protein; (200–400 bp)	*Glycine max*	Diethyl-pyrocarbonate	1 ml of diluted dsRNA (20 μg dsRNA)	Each box (six detached individual leaflets) was evenly sprayed	Average of over 73% reduction of pustule numbers 75% reduction in biomass accumulation	Monitored up to 2 weeks	Hu et al., 2020
	Phytophthora infestans	Sorbitol dehydrogenase, Translation elongation factor 1-α, Phospholipase-D like 3, Glycosylphosphatidylinositol-anchored acidic serine-threonine rich HAM34-like protein, Heat shock protein-90; (402bp-536bp)	*Solanum tuberosum*	*Escherichia coli* HT115 (DE3) 0.5% Nanoclay solution (5, 10, and 20 ppm)	100–, 150–, 250– and 500 ng and 1 μg dsRNA	Sprayed with dsRNA-nano clay formulation using automizer	Reduction in growth, sporulation and symptom expression, 15 days, control collapsed and wilted while dsRNA nano clay sprayed plants were erect and healthy	15 days	Sundaresha et al., 2021
	Botryotinia fuckeliana	Chitin synthase class III and DCL1 and DCL2	*Fragaria ananassa*	*Escherichia coli*-derived anucleated minicells	125–1,000 ng/ml	Topical spray application	Selectively knocked-down the target genes and led to significant fungal growth inhibition *in vitro*. Compensatory relationship between DCL1 and DCL2 gene transcripts	12 days	Islam et al., 2021
	Fusarium oxysporum	CYP51, chitin synthase 1, Elongation factor 2 (732bp)	*Solanum lycopersicum*	Layered double hydroxide nanosheets	300 μg of dsRNAs in 3 mL of $_{dd}H_2O$ per plant	Spraying on plant leaves avoiding stems	Reduced fungal growth dsCYP51showed 93% reduction in transcript abundance	Monitored up to 8 days	Mohamed and Youssef, 2021
	Phytophthora infestans	Guanine-nucleotide binding protein β-subunit, haustorial membrane protein, cutinase, endo-1,3(4)-β-glucanase (436 bp)	*Solanum tuberosum*	Naked dsRNA	20 ng/μL	dsRNA sprayed on potato leaves in a detached leaf assay	Decreased disease progression, smaller and aberrant Mycelial phenotype	5 days	Kalyandurg et al., 2021
Plants	*Nicotiana benthamiana*	Green fluorescence protein (GFP), (21–, 22–, and 24 nt)	*Nicotiana benthamiana*	Naked dsRNA		High-pressure spraying procedure (HPSP)	Local and systemic silencing exception of siR24, delayed and weak local silencing (10 dpa), other GFP siRNAs induced local silencing 2 dpa	20 days post application	Dalakouras et al., 2016

(Continued)

TABLE 1 | (Continued)

Target organism	Target gene (dsRNA length)	Plant species	Nanoformulation	Applied dsRNA (μ g)	Application method	Efficacies	Durability	References
	GFP (322, 139 bp)			200 μl of dsRNA midGFP (10, 20, 200, and 240 ng/μl) 200 μl of dsRNA-5'GFP (24, 48, and 240 ng/μl)		None of the samples sprayed with dsRNA-midGFP (0/15), dsRNA-5'GFP (0/9) or water (0/9) showed silencing up to 3 weeks after spraying	–	Uslu et al., 2020
	(CaMV) 35S promotor (333bp)		Naked dsRNA	50 μg in 500 μl	High-pressure spraying (carborundum was added)	Methylation of the 35S promotor was observed 10 days after spraying	10 days post spraying	Dalakouras and Ganopoulos, 2021
	GFP (124 bp)		Carbon dots (CD) surfactant BreakThru S279 was added to the CD-dsRNA complexes at a final concentration of 0.4% (v/v)	12 ng/μL (3.8 μL/cm^2, 45 ng/cm^2)	Spray application was done with Iwata HP-M1 handheld airbrush sprayer with air pressure set to 82 kPA (∼12 PSI)	MgCheH transcript levels showed a 79% reduction in the phenotypic tissues at five days after treatment. Reduction of 88% in GFP protein levels was observed	Monitored up to 12 days	Schwartz et al., 2020
	GFP (22 nt)			60 μl of solution/plant (3-4-leaf transgenic seedling)		No difference was observed in the extent or frequency of systemic silencing comparing the events containing or not the partial transposase. Expression was reduced to 48 and 72%.	Up to 14 days	Hendrix et al., 2021
	GFP (siRNA loading)		DNA nanostructures	100 nM for both the nanostructure and the siRNA duplex	Infiltrating nanostructures with a 1-ml needleless syringe and without using any surfactant.	40–59% (varies with DNA structures) in both mRNA and protein level	Internalization into plant cells 12h post-infiltration; gene silencing disappears 7days post-infiltration	Zhang et al., 2020
			Gold nanoclusters (AuNCs)	25 ng siRNA per 1 μg AuNCs		32–35% reduction in GFP 3 days postinfiltration	Internalization into plant cells 0,5-1 h post-infiltration	Zhang et al., 2021
			Single-walled carbon nanotubes	100 nM siRNAs		95–92% (mRNA level) ∼40% (protein level)	Internalization into plant cells 4 h post-infiltration	Demirer et al., 2020

pathogens. For example, across 75 studies, HIGS-based RNAi showed an average viral resistance of 90% (Gaffar and Koch, 2019; Koch and Wassenegger, 2021). Notably, similar efficacies were demonstrated when dsRNAs were applied exogenously (**Table 1**). Conclusive RNAi-based effects were reported in 2016 by Konakalla et al., who demonstrated that the exogenous application of dsRNA targeting the viral silencing suppressor *p126* gene and the *CP* gene of TMV conferred virus resistance in tobacco (**Table 1**). The authors further showed systemic spreading of dsRNA from local to systemic tissues within an hour of dsRNA application using semi-quantitative RT-PCR (Konakalla et al., 2016). They found that p126 dsRNA levels continuously decreased in the local treated tissue, from 3 to 9 dpi, until dsRNA was no longer detectable. Another study supported these findings, showing that exogenously applied dsRNA derived from the *HC-Pro* and *CP* genes of ZYMV protects watermelon and cucumber against ZYMV and that it spread systemically over long distances in cucurbits (Kaldis et al., 2018). Further emphasizing the systemic spread of RNA biopesticides, the movement of sprayed dsRNA from barley leaves to stems and root tissues was demonstrated within 3 days after spray treatment (Biedenkopf et al., 2020). Systemic distribution is of key importance because it indicates that RNA biopesticides could be promising substitutes for systemic pesticides. Moreover, translocation from leaves (application sites) to roots suggests that foliar sprays may be able to target soil-borne infections, thus circumventing the need to develop soil-specific RNA treatments.

Interestingly, the induction of virus resistance by exogenous application had already been achieved 20 years ago (Tenllado and Díaz-Ruíz, 2001). However, "exogenous" does not necessarily imply a spray application. Thus, we must distinguish between different application strategies, as most of them work on a lab scale but are unsuitable and unpracticable for field applications (**Table 1**).

As already indicated, numerous exogenous application strategies rely on preparatory treatments, such as the mechanical inoculation of leaves, to guarantee efficient dsRNA uptake (**Table 1**). The rubbing or dusting of leaf surfaces with Carborundum (silicon carbide) as an abrasive is widely used as a pretreatment (Tenllado and Díaz-Ruíz, 2001; Tenllado et al., 2003; Yin et al., 2009; Konakalla et al., 2016; Necira et al., 2021). Given the unsuitability of such pretreatments on the field scale, subsequent research, inspired by human RNAi therapeutics where nanotechnology is unavoidable, has attempted to formulate dsRNA for efficient and targeted RNA delivery. Such formulations can help to increase cellular uptake, improve stability (and thus overcome environmental degradation by UV radiation or surface wash-off) and provide long-term protection against the targeted pathogen. Toward this, the first breakthrough was achieved by Mitter et al. (2017) by using positively charged layered double hydroxide (LDH) clay nanosheets as a dsRNA carrier (BioClay). This technology was originally developed for the delivery of siRNA therapeutics to mammalian cells (Ladewig et al., 2009, 2010). The authors found that loading dsRNA on LDH prolonged durability on the leaf surface for 30 days and increased stability through protection from nuclease degradation (Mitter et al., 2017). Moreover, they showed the uptake of dsRNA into plant cells and induction of endogenous RNA silencing, which mediated systemic protection against the targeted VSR *2b* gene of CMV on cowpea and tobacco. In field BioClay allows sustained release of dsRNA on the leaf surface under ambient conditions (Ram Reddy et al., 2006; Xu et al., 2006; Reddy et al., 2008; Mitter et al., 2017). Notably, this study proved that the LDH nanocarrier can be completely degraded over time, thus resulting in a slow and sustained release of dsRNA under environmental conditions (Mitter et al., 2017). After this pioneering work on the biodegradability of clay-based nanomaterials, dsRNA-BioClay has been used to target the bean common mosaic virus (BCMV) to protect *N. benthamiana* and *Vigna unguiculata* (Worrall et al., 2019).

While this study provides a significant step forward in making RNA spray an applicable and sustainable approach for pathogen and pest control in agriculture, questions remain about how to produce efficient amounts of dsRNA for spray applications in field trials. Initially, *Escherichia coli*-based dsRNA production was used (Tenllado and Díaz-Ruíz, 2001; Tenllado et al., 2003; Yin et al., 2009; Gan et al., 2010) due to financial constraints and to the poor availability of suitable dsRNA synthesis kits. Interestingly, the latest studies showed that *E. coli* cannot only be used as a dsRNA factory but also provides adequate properties for dsRNA encapsulation (Islam et al., 2021; Necira et al., 2021). The authors of these studies concluded that the effects of *E. coli*-encapsulated dsRNA did not differ from the topical application of naked dsRNAs (Necira et al., 2021; **Table 1**). *E. coli*-encapsulation may nevertheless prove superior to naked dsRNA, as it provides a protective envelope conferring higher stability under field conditions, though host species are limited.

Numerous studies have addressed the question of how to reduce the production costs of dsRNA. Some have synthesized dsRNA using *in vitro* transcription kits, suitable for lab experiments but inconceivable for large-scale applications given their bad price-performance ratio ($700/mg). Thus, subsequent attempts have focused on large-scale production and on the purification of sprayable RNAs to make them commercially competitive and economically achievable, with great success. For example, recent research efforts have rapidly produced cost-effective, large-scale microbial-based dsRNA production using bacteria, such as *E. coli* (Voloudakis et al., 2015; Ahn et al., 2019; Bento et al., 2020; Niño-Sánchez et al., 2021) and *Pseudomonas syringae* (Niehl et al., 2018) and the yeast *Yarrowia lipolytica* (Timmons et al., 2001; Palli, 2014). Given these significant achievements, dsRNA costs per gram have dropped from $12,500 in 2008 to $100 in 2016, $60 in 2020 and finally $2 in 2021 (de Andrade and Hunter, 2016; Zotti et al., 2018; Dalakouras et al., 2020). Recently, large-scale cell-free production has further lowered the price to less than $0.50 per gram, making RNAi competitive in the market.[2]

Based on the plethora of proof-of-concept studies and recent achievements in nanomaterial-based dsRNA and siRNA delivery, which allow for highly effective virus control even under field conditions, fundamental knowledge on the molecular mechanisms and factors that determine uptake (over

[2]http://www.globalengage.co.uk/pgc/docs/PosterMaxwell.pdf

cuticle and cellular barriers), processing and translocation has begun to emerge.

SIGNIFICANT ADVANCES OF RNA SPRAY CONTROLLING: FUNGI

Since 2010 the number of HIGS-based studies demonstrating fungal disease control has continued to increase (Nowara et al., 2010; Koch and Wassenegger, 2021). The first reports that showed that RNA spray can fight fungus were directed against two necrotrophic ascomycetes *Fusarium graminearum* (Koch et al., 2016) and *Botrytis cinerea* (Wang et al., 2016). These case studies further energized the debate on whether RNA spray is a realistic approach for future field applications. Beyond cost concerns, the obvious question was how to enhance uptake of RNA biopesticides by plants, thus avoiding degradation under environmental conditions. The uptake of dsRNA depends on leaf surface stability, and efficient and subsequent uptake can prevent premature degradation or wash-off by rain. Notably, sprayed RNAs must overcome several physical and cellular barriers to reach their cognate mRNA targets. First, they must overcome the "outside-inside" or cuticle barrier at the leaf surface, which is especially relevant when targeting pathogens that replicate and grow intra- or intercellularly, such as viruses and fungi, or sap-sucking insects like aphids or whiteflies. Like viral pathogens, fungi maintain an intimate relationship with their hosts, acquiring nutrients in a biotrophic, hemibiotrophic, or necrotrophic manner. Given their different lifestyles, fungi may take up sprayed RNAs from extra-, inter- as well as intracellular space. Passing through this barrier was assumed to be passively facilitated by stomata opening (Koch et al., 2016). Given this assumption, one could ask whether stomata density and leaf architecture represent limiting factors in RNA uptake. This would explain why SIGS works well for some plant species and not for others. Considering this, formulations that facilitate the opening of stomata or increase leaf permeability may help to increase dsRNA uptake from foliar sprays.

However, before sprayed dsRNA can enter plant cells for processing and translocation, they reach another barrier, the "apoplast-symplast" interface. Entering plant cells is necessary for processing into siRNAs by plant DCLs. However, in particular cases, the uptake of unprocessed dsRNA may also occur, depending on the lifestyle of the targeted pathogen. For example, SIGS of the necrotroph *F. graminearum* require the uptake of unprocessed dsRNA precursors and their subsequent processing by the fungal RNAi machinery (Koch et al., 2016; Gaffar et al., 2019). Based on these findings, it has been hypothesized that the uptake of long, unprocessed dsRNA and its processing into many different inhibitory siRNAs by the target organism might lead to higher gene silencing efficiencies and increased disease resistance (Koch et al., 2019). However, there is some controversy about the uptake of dsRNA versus siRNA, for instance in terms of the relative efficacies and off-target risks (Koch and Wassenegger, 2021). Interestingly, preliminary data suggest that if sprayed dsRNA is too long, the length might interfere with sufficient cellular uptake (Höfle et al., 2019). To prove whether uptake of unprocessed dsRNA really confers stronger effects, it will be helpful to compare SIGS efficacies of biotrophic and necrotrophic pathogens. So far, the disease resistance level achieved by SIGS has been comparable (Hu et al., 2020) or even superior (Höfle et al., 2019; Koch et al., 2019) to HIGS-based efficiencies under lab conditions.

Interestingly, a recent study that assessed the dsRNA uptake ability of different fungi revealed that dsRNA uptake efficiencies varied across the tested fungal and oomycete species (Qiao et al., 2021). For example, the authors demonstrated efficient dsRNA uptake for *Botrytis cinerea*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Aspergillus niger*, and *Verticillium dahliae*. They found no uptake for *Colleotrichium gloeosporioides*, modest uptake for the non-pathogenic fungus *Trichoderma virens* and limited uptake for the oomycete *Phytophthora infestans* (Qiao et al., 2021). This study is of great significance, as it confirms and extends previous findings (Koch et al., 2016; Wang et al., 2016; McLoughlin et al., 2018) and further supports the idea that necrotrophic fungi exhibit a stronger response to exogenous RNA applications. Whether this is because they can take up unprocessed dsRNA, which may lead to higher gene silencing efficiencies - based on the finding that *F. graminearum* DCLs are required for SIGS (Koch et al., 2016; Gaffar et al., 2019) – or whether the overall amount of dsRNA absorbed in less time correlates with their necrotrophic lifestyle needs further verification. Supporting this idea, Hailing Jin and her team observed dsRNA uptake as early as 6 h after YFP-dsRNA *in vitro* treatment of the necrotrophs *B. cinerea*, *S. sclerotinia*, and *R. solani*, suggesting faster dsRNA uptake compared to *A. niger* (10 hpt) and *V. dahliae* (12 hpt). In addition, they demonstrated the antifungal activity of topically applied dsRNAs targeting fungal vesicle pathway genes or *DCL* genes in *B. cinerea*, *S. sclerotinia*, *R. solani*, and *A. niger* (Qiao et al., 2021). However, as the dsRNAs were dropped onto the surface of each plant or fruit sample and the fungi were applied directly to the dsRNA-treated area, we do not know from this study whether the uptake of plant DCL-processed siRNA would have made a difference. In other words, the type of RNA offered for uptake was limited to dsRNA, bringing us back to the initial question of whether the uptake of unprocessed dsRNA is a crucial determinant of SIGS efficacy, and thus whether the uptake of dsRNA by the plant represents a limiting factor regarding the durability of protective RNA spray effects.

Regarding this question, we need to differentiate between sprayed RNAs that stick to the plant (or fruit or vegetable) surface and those that enter plant cells. We predict that surface interaction of dsRNA or siRNA with microbial targets will require intense stabilization formulas, with potential risks for environmental accumulation and pollution. Cellular uptake, on the other hand, may have a protective effect, but promoting fast processing or even bearing the risk of enzymatic and lytic degradation together with dilution over distance. Although we seek RNA-stabilizing formulas, at the same time we question RNA degradability as a selling point. Recently, dsRNA spray effects against *B. cinerea* infection were found to last for 7 days after spraying onto tomato plants in a greenhouse, indicating a quite rapid degradation. Notably, the authors of this study concluded that dsRNA gets degraded by the environment,

suggesting no cellular uptake by the plant (Qiao et al., 2021). Although short longevity can be determined by degradation, it remains unknown whether this degradation occurs outside or inside the sprayed plant.

So far, most SIGS studies demonstrating the control of fungal pathogens were conducted with naked, un-formulated dsRNA (**Table 1**). However, formulations that increase cellular uptake (especially for plants that showed no absorption of naked dsRNA from their surfaces) or surface stability (especially for post-harvest products such as fruits and vegetables) will boost broad applicability and the lab-to-field transition. A recent study showed that *E. coli*-derived anucleated minicells can be used as a cost-effective platform for dsRNA production and encapsulation, shielding dsRNA from RNase degradation (Islam et al., 2021). The authors demonstrated that the protection of strawberries from *Botryotinia fuckeliana* infection was prolonged to 12 days under greenhouse conditions, further emphasizing the added value of encapsulation for dsRNA stability. Although extending protection against the gray mold of tomatoes by 7 days (naked dsRNA) (Qiao et al., 2021) and strawberries by 12 days (Minicell-based) (Islam et al., 2021) already represent positive results, there is still room for further improvement. Thus, data on durability and persistence together with systemic distribution are highly desired for "lab-to-field" technology transfers. Moreover, we need to know which parameters promote or restrict dsRNA and siRNA uptake in a plant species-specific context at the "outside-inside" barrier and the "apoplast-symplast" interface, as well as in a pathosystem specific manner at the "plant-fungus" interface. However, the mechanism by which sprayed RNA overcomes the apoplast-symplast barrier is largely unknown. It is hypothesized that endocytosis and extracellular vesicles (EVs) play a crucial role in the uptake and translocation of HIGS- and SIGS-associated RNAs (Wytinck et al., 2020a; Koch and Wassenegger, 2021; Santos et al., 2021). Notably, recent data suggest that SIGS, in contrast to HIGS, might not involve EVs for siRNA delivery and uptake, at least in the *F. graminearum*-barley pathosystem (Schlemmer et al., 2021a,b). Based on insights into the mode of uptake, we have the chance to optimize and develop dsRNA and siRNA delivery and cellular uptake in the future.

SIGNIFICANT ADVANCES OF RNA SPRAY CONTROLLING: INSECTS

Host-induced gene silencing-mediated control of insect pests has been proven effective with an average of 50% conferred resistance (Koch and Wassenegger, 2021). Exogenous dsRNA and siRNA applications (e.g., feeding, injection, and oral delivery) to various insect pests and mites is routinely conducted to study gene function or identify RNAi targets (Lü et al., 2020; Mehlhorn et al., 2020, 2021; Máximo et al., 2020). In line with the above discussion about RNAs that remain on sprayed surfaces and those that enter plant cells, insect pests and mites can ingest dsRNA and siRNAs by chewing-biting or piercing-sucking feeding behaviors. In addition to the control of viruses and fungi, insect pests are also generally accessible to RNA sprays (**Table 1**). For example, a recent report demonstrated the efficiency of RNA sprays against *Henosepilachna vigintioctopunctata* (28-spotted ladybird) in the greenhouse (Wu et al., 2021). Spraying *E. coli*-expressed dsRNAs targeting the *ecdysone receptor* (*EcR*) gene onto the foliage of greenhouse-growing potato plants provoked the death of third and fourth instar larvae and reduced leaf damage (Wu et al., 2021). Confirming these results, another study showed exogenous dsRNA application as a promising alternative to chemical pesticides for controlling *H. vigintioctopunctata* (Lü et al., 2021). RNA sprays have also been shown to be effective in controlling the Colorado potato beetle, *Leptinotarsa decemlineata* (Mehlhorn et al., 2020). Even more importantly, the authors investigated geographical variation in RNAi sensitivity in the second instar larvae of 14 different European populations of field-collected *L. decemlineata* and found only minor variability in RNAi sensitivity between populations. This baseline study provides the first valuable insights on the broad applicability and transferability of RNA sprays over a geographic range in Europe (Mehlhorn et al., 2020). While the accessibility of phyllophagous pests by RNA sprays may seem trivial, a breakthrough was achieved by providing the first laboratory evidence that feeding dsRNA-coated oilseed rape buds to the pollen beetle *Brassicogethes aeneus* diminishes pollen beetle survival rate (Willow et al., 2021). This study is significant because many pathogens and pests that tremendously impact agriculture infect ears and buds rather than leaves.

Given the fact that we lack reliable results from field trials, or even from data generated under simulated field test conditions, lab-to-field transfer will require improvements of stability and adherence of dsRNA and siRNA to resist UV radiation and rain wash-off. For these purposes, encapsulation is pursued to guarantee and prolong the longevity of RNA sprays in the field. For example, *E. coli* is not only utilized for cost-effective dsRNA production but also represents a protective envelope for efficient dsRNA delivery (Lü et al., 2020; Máximo et al., 2020; Wang et al., 2021; Wu et al., 2021). Interestingly, encapsulation of dsRNA, if large enough, may prevent cellular uptake (plant intake) and thus shield dsRNA from plant DCL processing, as indicated by previous breakthroughs demonstrating that the expression of dsRNA in chloroplasts (DCL-free organelles) targeting the *ß-actin* gene of *L. decemlineata* (Colorado potato beetle) caused larval lethality (Zhang et al., 2015).

Targeting piercing-sucking insects by RNA spray requires cellular uptake and systemic distribution via the phloem. Thus, knowledge of the paths used by dsRNA and siRNA as SIGS inducers is a prerequisite for further developing and applying RNA sprays to the field. Given this assumption, previous reports revealed the systemic spread of sprayed RNAs (Koch et al., 2016; Konakalla et al., 2016; Kaldis et al., 2018; Biedenkopf et al., 2020). Spraying a fluorescent-labeled dsRNA onto barley leaves and subsequently examining longitudinal leaf sections revealed that the fluorescence was not confined to the apoplast but also was present in the symplast of phloem parenchyma cells, companion cells and mesophyll cells, as well as in trichomes and stomata (Koch et al., 2016). The finding that RNA biopesticides systemically spread through the phloem was confirmed by spraying fluorescent-labeled dsRNA followed by phloem sampling by stylectomy (excision of a stylet, typically

that of an aphid) of the distal, non-sprayed leaf parts. Using CLSM, a green fluorescent signal was detected after cutting off the stylet tip of feeding aphids (Biedenkopf et al., 2020). Even more importantly, it was shown that sprayed dsRNA moved from barley leaves over stems to the root tissue within 3 days of spray treatment (Biedenkopf et al., 2020). This finding is especially interesting for the control of root pathogens because so far dsRNA applied to soil has immediately degraded (Dubelman et al., 2014; Parker et al., 2019; Bachman et al., 2020; Qiao et al., 2021). However, systemic distribution may result in dilution of SIGS signals; thus, further research is needed to prove the activity of dsRNA and siRNA in systemic tissues and to address the question of how much or how often RNA biopesticides need to be applied to confer robust and durable SIGS-based disease resistance. Given these challenges, previous studies have proposed utilizing the symbiosis of plants with bacteria, for example by engineering symbionts that serve as dsRNA vectors to maintain long-lasting dsRNA production *in planta* (Whitten et al., 2016; Whitten and Dyson, 2017). However, emerging data suggest that pest control via RNA sprays does not necessarily require plant passage for uptake and SIGS induction (Thairu et al., 2017; Niu et al., 2019; Linyu et al., 2021). Due to the limited uptake of dsRNA in hemiptera insects (Niu et al., 2019) originally developed and described gene silencing induction in aphids upon foliar spray on pea aphids themselves to verify potent RNAi targets in aphids. More recently, another confirmatory report used nanocarrier SPc (star polycation) transdermal delivery systems to verify gene silencing efficiency of selected RNAi targets in *Aphis gossypii* (Linyu et al., 2021). However, both studies imply sensitivity of RNA aphid sprays, which may circumvent plant passage and uptake from the phloem when applied in the field. Moreover, the development of formulations that allow transdermal delivery in a species-specific context is very valuable, especially in light of risk assessment and regulations (avoiding off-target effects).

We predict that nanomaterial-based formulations can solve major challenges regarding lab-to-field transitions by increasing stability and warranting specificity and selectivity. Not only surface stability, but also rather the ability to resist enzymatic degradation by RNase or extreme pH-values, which are prominent in saliva and gut of insects (Christiaens et al., 2014; Peng et al., 2020a,b). Given these challenges, a recent breakthrough was achieved, demonstrating that chitosan nanoparticles-mediated dsRNA delivery in *Helicoverpa armigera* protects from degradation by nucleases and insect gut pH (Kolge et al., 2021). Beyond shielding dsRNA from RNase degradation, the silencing of a dsRNA ribonuclease improved oral RNAi efficacy in the southern green stinkbug (Sharma et al., 2021). This finding indicates that the simultaneous application of protective agents such as RNase inhibitors or the silencing of dsRNases might encourage targeted detoxification mediated by P450s, ABC transporters and others (Wang et al., 2021). Notably, a nanomaterial-based formulation may help to increase selectivity not only by shielding dsRNA from the environment but also by providing target-site-specific RNA release (e.g., pH-dependent release kinetics), or selectivity facilitated by attractants that are incorporated in the envelope of (nano)-capsules following the proven attract and kill principle.

SIGNIFICANT ADVANCES OF RNA SPRAY SILENCING PLANT GENES

Overcoming cuticle and cellular barriers is a prerequisite if RNA sprays are to substitute for conventional chemical herbicides or be used to study the gene function of plants. This is fundamentally different from RNA biopesticides that lay on plant, fruit or vegetable surfaces, in which cases sprays of naked dsRNA exhibit strong effects, for example, in controlling *B. cinerea* and *Botryotinia fuckeliana* (Wang et al., 2016; Islam et al., 2021; Qiao et al., 2021). However, previous attempts at spraying naked dsRNA to silence the expression of transgenes gave contrasting results, starting a controversial debate on the future herbicide uses of RNA sprays (Uslu and Wassenegger, 2020). For example, uptake of naked dsRNA and transgene silencing was demonstrated for the model plant *Arabidopsis thaliana* (Mitter et al., 2017; Dubrovina and Kiselev, 2019). However, attempts to silence transgene-expressed green fluorescence protein (GFP) in *Nicotiana benthamiana* by a high-pressure spray of naked dsRNA failed (Uslu et al., 2020). Notably, high-pressure sprays were specifically developed for RNAi applications and usage in *N. benthamiana*, resulting in transgene silencing (Dalakouras et al., 2016, 2018). Remarkably, based on their RNA-seq results the authors concluded that failure in transgene silencing correlates with the absence of dsRNA-derived specific siRNAs (Uslu et al., 2020). This finding raises the question of whether there was cellular uptake of high-pressure sprayed dsRNA, which is required for processing by plant DCLs. Notably, the spraying of 22 nt synthetic siRNA (matching the GFP sequence, position 164–187) as a positive control induced transgene silencing, as previously described (Dalakouras et al., 2016). Regarding this, another recent report showed induced methylation of the 35S promotor (which controls GFP transgene expression) upon 35S-dsRNA high pressure spray indicating cellular uptake of dsRNA (Dalakouras and Ganopoulos, 2021). Nevertheless, in contrast to siRNA spraying, cellular uptake of sprayed long dsRNAs seems to be less efficient, especially entering the nucleus to trigger RNA-directed DNA methylation is challenging. Moreover, another previous study demonstrated cellular uptake and plant DCL-mediated siRNA generation of a naked dsRNA sprayed onto barley leaves; however, this did not target a transgene (Koch et al., 2016). Nevertheless, these findings clearly illustrate how different plant species respond to RNA sprays, thus exemplifying the importance and urgency of research into the mechanistic basis (uptake, processing, and translocation) of RNA spray applications.

As much as spraying naked dsRNA highlights the simplicity of this technology, there are limits and restrictions that we have just begun to unravel. Given that sufficient RNA delivery is a continuing challenge in RNA spray applications, significant advances in nanomaterial-based formulations and the development of other smart delivery platforms have boosted technological development. Utilizing different biological,

physical, and chemical-assisted delivery methods such as Bacterium-mediated RNAi (Goodfellow et al., 2019), high-pressure sprays (Dalakouras et al., 2016, 2018; Uslu et al., 2020), lipid nanoparticles, cationic polymers, cell-penetrating peptides and clay nanosheets has been shown to provide protection from nucleases and pH and to improve cellular uptake increasing plant resistance to pathogens and pests (**Table 1**). Recent studies have demonstrated efficient siRNA delivery into intact plants facilitated by nanomaterials such as carbon dots (Schwartz et al., 2020), single-walled carbon nanotubes (Demirer et al., 2019, 2020), DNA nanostructure carriers (Zhang et al., 2019, 2020) and gold-nanoclusters (Zhang et al., 2021; **Table 1**). Based on their small size (3.9 nm) carbon dots can pass through the cell wall (size exclusion limit 3–10 nm) (Carpita et al., 1979) mediating siRNA delivery into plant cells (Schwartz et al., 2020). Notably, passing through a plant cell wall is not the only prerequisite for efficient silencing. As discussed above, RNAi effectors face several barriers in reaching their mRNA cognates. Thus, cellular uptake requires overcoming the apoplast-symplast barrier mediated by endocytosis through the plasma membrane and subsequent release from endomembrane vesicles (Wytinck et al., 2020b). Given these later barriers, the properties of carbon dots are found to be suboptimal (Schwartz et al., 2020). Although carbon-dots-mediated siRNA delivery needs further improvement for efficient endogenous gene silencing, the delivery of dsRNAs over plant cell walls that reach the apoplast might be sufficient for controlling pathogens that require the uptake of unprocessed dsRNAs for SIGS induction (Koch et al., 2016; Gaffar et al., 2019). Due to their ultrasmall size (2 nm) and easier and faster synthesis, gold-nanoclusters (AuNCs) have been adapted for the delivery of siRNAs in mature plants, inducing efficient transgene as well as endogenous gene silencing in *N. benthamiana* (Zhang et al., 2021).

However, as naked dsRNA/siRNA delivery is restricted by largely unknown parameters that prevent RNA sprays from reaching their full potential, usage of different delivery methods, as advantageous and promising they may be, have limitations, as indicated above (for a detailed comparison of delivery platforms, see also: Zhang et al., 2021). Regarding this, identification of chemicals (e.g., Sortin1) that increased RNAi potency by enhancing siRNA accumulation and loading into AGOs may boost the development of powerful RNA biopesticide formulations (Jay et al., 2019). Moreover, while all of these studies provided proof-of-concept, the suitability of nanomaterial-based RNAi effector delivery as well as application of chemical enhancers of PTGS needs to be approved under field conditions. In addition, toxicity and biodegradability, together with applicability to other plant species and transferability for delivering dsRNA targeting foreign genes, needs further research in order to be verified.

CONCLUSION AND FUTURE PERSPECTIVES

Despite the numerous proof-of-concept studies demonstrating the great potential underlying RNAi-based plant protection, especially GMO-free RNA sprays, we are still far from field applications or even product launches. Currently, we are facing complex and multi-layered challenges comprising technical-biological prospects (stability, selectivity, and broad applicability) as well as social-political demands (acceptance and regulations). To meet these challenges, significant advances in developing RNA sprays that guarantee stability in the field and minimize off-target risks for non-target organisms have been made, for example, by using nanomaterial-based formulations and the fusion of different technologies. However, we lack a sufficient data basis for understanding how nano-formulated RNA biopesticides will behave under field conditions. Moreover, we urgently require knowledge on durability, persistence and systemic effects of RNA sprays to develop recommendations for handling in the field. For example, when and how often do RNA biopesticides need to be applied or renewed to confer maximum protection? Based on the answer, we could calculate real costs for each pathosystem. To achieve broad applicability, which is often used as a selling point over GMO-based RNAi strategies (HIGS), we need to clarify the question of why RNA sprays work well for some pathosystems and not for others. If we know what determines SIGS efficiencies, we may have a starting point for developing solutions that demonstrate broad transferability. Elucidating the molecular mechanisms of uptake, processing and translocation of spray-delivered RNAs is crucial for making RNA sprays realistic and achievable in future field applications. In addition, emerging data that suggest extending RNA sprays to exploit the mechanisms of TGS that will allow epigenetic modifications of plant genes (Dalakouras and Vlachostergios, 2021) should be considered and discussed regarding the potential risk of introducing unintended off-target effects by modifying the plant's epigenome (Dalakouras and Papadopoulou, 2020).

In contrast to lab experiments, where dsRNA and siRNA effects are tested in isolation and on small scales, field environments are unpredictable and highly dynamic. For example, the occurrence of pathogens and pests, as well as their coincidences, represents a major challenge for determining application time and frequencies. Moreover, plants grown under environmental conditions develop differently from plants grown in greenhouses, especially in terms of cuticle properties, which may impact adherence, stability, and cellular uptake. Thus, we need smart application and formulation strategies, such as multi-targeting and site-directed targeting, to ensure high efficacies on the species level. For example, combining RNA biopesticides with distinct molecular targets may not only confer stronger effects but also allow high efficiencies on a species-specific scale. In other words, the majority of highly effective dsRNAs and siRNAs target highly conserved genes, which bear high off-target risks. They are suitable candidates when conducting proof-of-concept studies but are inappropriate for field trials. Thus, identifying molecular targets on species level and their simultaneous combinatory (multi-targeting) applications may exhibit strong effects and at the same time minimize off-target risks. However, lab-to-field transitions require further precision and accuracy in off-target predictions and their controllability for adequate risk assessment. Currently, data requirements specific for RNA spray-based plant

protection products are not yet in place under the regulatory frameworks for pesticides and plant protection products in the United States and EU (Dietz-Pfeilstetter et al., 2021). The OECD (Organization for Economic Cooperation and Development) organized a meeting on this question, which represented a major step forward in terms of testing regulation and research direction for future external dsRNA products (OECD, 2020). It is important to note that siRNAs display small gene regulatory units, which needs to be considered when developing directives that ensure appropriate risk assessment. Consistent with this, a recent report reviewed the evidence for and against the transfer of diet-derived miRNAs from plants, meat, milk and exosome and their putative molecular regulator roles as well as pharmacological opportunities for cross-kingdom regulation in the consuming organism (Mar-Aguilar et al., 2020; del Pozo-Acebo et al., 2021). The authors concluded that the transfer of miRNAs from the diet to the blood is still inconclusive and that the main source of controversy in plant studies is the lack of reproducibility of the findings. Currently, a risk assessment by the European Food Safety Authority (EFSA) classified RNA biopesticides as safe regarding low risks that sprayable RNAs pose for animals/humans (Olivier et al., 2018). The decisive argument was that oral uptake of RNAi products by consumers bears a low risk for interference with gene expression in humans, as too many biological and physical barriers have to be overcome (Schiemann et al., 2019; Kleter, 2020). However, so far, the available scientific data on environmental, consumer and user safety evaluations of RNA spray applications are still scarce. Preliminary data on the environmental fate (persistence and degradability) of sprayed dsRNA suggest a short shelf-life after application and absorption to soil (Parker et al., 2019; Bachman et al., 2020). However, ongoing nanomaterial-based attempts to increase stability may prolong environmental persistence and increase the risk to local ecosystems. In addition, it is still unclear whether and to what extent sprayed dsRNAs and siRNAs can accumulate along the food chain and how nanomaterials affect this accumulation. Thus, the environmental risk assessment of RNA biopesticides needs to be reinforced in order to facilitate their future placement on the market. Moreover, the length of the pesticide authorization process should be reduced to support a just transition. Given this, it is just a matter of time since first RNA biopesticides (e.g., Monsanto's/Bayer's BioDirect^{TM} technology) will access the market. Finally, we need to develop educational work and information campaigns for public outreach and transparency as early as possible.

AUTHOR CONTRIBUTIONS

Both authors contributed to wrote the manuscript and approved the submitted version.

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REFERENCES

Ahn, S.-J., Donahue, K., Koh, Y., Martin, R. R., and Choi, M.-Y. (2019). Microbial-Based Double-Stranded RNA Production to Develop Cost-Effective RNA Interference Application for Insect Pest Management. *Int. J. Insect Sci.* 11:117954331984032. doi: 10.1177/1179543319840323

Bachman, P., Fischer, J., Song, Z., Urbanczyk-Wochniak, E., and Watson, G. (2020). Environmental Fate and Dissipation of Applied dsRNA in Soil, Aquatic Systems, and Plants. *Front. Plant Sci.* 11:21. doi: 10.3389/fpls.2020.00021

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–1326. doi: 10.1038/nbt1359

Bento, F. M. M., Marques, R. N., Campana, F. B., Demétrio, C. G. B., Leandro, R. A., Parra, J. R. P., et al. (2020). Gene silencing by RNAi via oral delivery of dsRNA by bacteria in the South American tomato pinworm, *Tuta absoluta*. *Pest Manag. Sci.* 76, 287–295. doi: 10.1002/ps.5513

Biedenkopf, D., Will, T., Knauer, T., Jelonek, L., Furch, A. C. U., Busche, T., et al. (2020). Systemic spreading of exogenous applied RNA biopesticides in the crop plant *Hordeum vulgare*. *ExRNA* 2:12. doi: 10.1186/s41544-020-00052-3

Carpita, N., Sabularse, D., Montezinos, D., and Delmer, D. P. (1979). Determination of the pore size of cell walls of living plant cells. *Science* 205, 1144–1147. doi: 10.1126/science.205.4411.1144

Christiaens, O., Swevers, L., and Smagghe, G. (2014). DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. *Peptides* 53, 307–314. doi: 10.1016/j.peptides.2013.12.014

Cooper, A. M. W., Silver, K., Zhang, J., Park, Y., and Zhu, K. Y. (2019). Molecular mechanisms influencing efficiency of RNA interference in insects. *Pest Manag. Sci.* 75, 18–28. doi: 10.1002/ps.5126

Dalakouras, A., and Ganopoulos, I. (2021). Induction of Promoter DNA Methylation Upon High-Pressure Spraying of Double-Stranded RNA in Plants. *Agron* 11:789. doi: 10.3390/agronomy11040789

Dalakouras, A., and Papadopoulou, K. K. (2020). Epigenetic Modifications: an Unexplored Facet of Exogenous RNA Application in Plants. *Plants* 9:673. doi: 10.3390/plants9060673

Dalakouras, A., and Vlachostergios, D. (2021). Epigenetic approaches to crop breeding: current status and perspectives. *J. Exp. Bot.* 72, 5356–5371. doi: 10.1093/jxb/erab227

Dalakouras, A., Jarausch, W., Buchholz, G., Bassler, A., Braun, M., Manthey, T., et al. (2018). Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. *Front. Plant Sci.* 9:1253. doi: 10.3389/fpls.2018.01253

Dalakouras, A., Wassenegger, M., Dadami, E., Ganopoulos, I., Pappas, M. L., and Papadopoulou, K. (2020). Genetically modified organism-free RNA interference: exogenous application of RNA molecules in plants. *Plant Physiol.* 182, 38–50. doi: 10.1104/pp.19.00570

Dalakouras, A., Wassenegger, M., McMillan, J. N., Cardoza, V., Maegele, I., Dadami, E., et al. (2016). Induction of silencing in plants by high-pressure spraying of In vitro-synthesized small RNAs. *Front. Plant Sci.* 7:1327. doi: 10.3389/fpls.2016.01327

Das, P. R., and Sherif, S. M. (2020). Application of Exogenous dsRNAs-induced RNAi in Agriculture: challenges and Triumphs. *Front. Plant Sci.*11:946. doi: 10.3389/fpls.2020.00946

de Andrade, E. C., and Hunter, W. B. (2016). "RNA Interference – Natural Gene-Based Technology for Highly Specific Pest Control (HiSPeC)" in *RNA Interference*, ed. Y. A. Ibrokhim (InTechOpen). doi: 10.5772/61612

del Pozo-Acebo, L., López de las Hazas, M.-C., Margollés, A., Dávalos, A., and García-Ruiz, A. (2021). Eating microRNAs: pharmacological opportunities for cross-kingdom regulation and implications in host gene and gut microbiota modulation. *Br. J. Pharmacol.* 178, 2218–2245. doi: 10.1111/bph.15421

Demirer, G. S., Zhang, H., Goh, N. S., Pinals, R. L., Chang, R., and Landry, M. P. (2020). Carbon nanocarriers deliver siRNA to intact plant cells for efficient gene knockdown. *Sci. Adv.* 6:eaaz0495. doi: 10.1126/sciadv.aaz0495

Demirer, G. S., Zhang, H., Matos, J. L., Goh, N. S., Cunningham, F. J., Sung, Y., et al. (2019). High aspect ratio nanomaterials enable delivery of functional genetic material without DNA integration in mature plants. *Nat. Nanotechnol.* 14, 456–464. doi: 10.1038/s41565-019-0382-5

Dietz-Pfeilstetter, A., Mendelsohn, M., Gathmann, A., and Klinkenbuß, D. (2021). Considerations and Regulatory Approaches in the USA and in the EU for dsRNA-Based Externally Applied Pesticides for Plant Protection. *Front. Plant Sci.* 12:682387. doi: 10.3389/fpls.2021.682387

Douglas, A. E. (2018). Strategies for Enhanced Crop Resistance to Insect Pests. *Annu. Rev. Plant Biol.* 69, 637–660. doi: 10.1146/annurev-arplant-042817-040248

Dubelman, S., Fischer, J., Zapata, F., Huizinga, K., Jiang, C., Uffman, J., et al. (2014). Environmental fate of double-stranded RNA in agricultural soils. *PLoS One* 9:e93155. doi: 10.1371/journal.pone.0093155

Dubrovina, A. S., and Kiselev, K. V. (2019). Exogenous RNAs for gene regulation and plant resistance. *Int. J. Mol. Sci.* 20:ijms20092282. doi: 10.3390/ijms20092282

European Commission. (2021). *EU Biodiversity Strategy for 2030*. Available online at: https://op.europa.eu/en/publication-detail/-/publication/31e4609f-b91e-11eb-8aca-01aa75ed71a1 (accessed September 2021).

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi: 10.1038/35888

Fotoukkiaii, S. M., Wybouw, N., Kurlovs, A. H., Tsakireli, D., Pergantis, S. A., Clark, R. M., et al. (2021). High-resolution genetic mapping reveals cis-regulatory and copy number variation in loci associated with cytochrome P450-mediated detoxification in a generalist arthropod pest. *PLoS Genet.* 17:e1009422. doi: 10.1371/journal.pgen.1009422

Gaffar, F. Y., and Koch, A. (2019). Catch me if you can! RNA silencing-based improvement of antiviral plant immunity. *Viruses* 11:v11070673. doi: 10.3390/v11070673

Gaffar, F. Y., Imani, J., Karlovsky, P., Koch, A., and Kogel, K.-H. (2019). Different Components of the RNA Interference Machinery Are Required for Conidiation, Ascosporogenesis, Virulence, Deoxynivalenol Production, and Fungal Inhibition by Exogenous Double-Stranded RNA in the Head Blight Pathogen *Fusarium graminearum*. *Front. Microbiol.* 10:1662. doi: 10.3389/fmicb.2019.01662

Gan, D., Zhang, J., Jiang, H., Jiang, T., Zhu, S., and Cheng, B. (2010). Bacterially expressed dsRNA protects maize against SCMV infection. *Plant Cell Rep,* 29, 1261–1268. doi: 10.1007/s00299-010-0911-z

Goodfellow, S., Zhang, D., Wang, M. B., and Zhang, R. (2019). Bacterium-mediated RNA interference: potential application in plant protection. *Plants* 8, 1–18. doi: 10.3390/plants8120572

Hendrix, B., Hoffer, P., Sanders, R., Schwartz, S., Zheng, W., Eads, B., et al. (2021). Systemic gfp silencing is associated with high transgene expression in *Nicotiana benthamiana*. *PLoS One* 16:e0245422. doi: 10.1371/journal.pone.0245422

Herman, R. A., Storer, N. P., Anderson, J. A., Amijee, F., Cnudde, F., and Raybould, A. (2021). Transparency in risk-disproportionate regulation of modern crop-breeding techniques. *GM Crops Food* 12, 376–381. doi: 10.1080/21645698.2021.1934353

Höfle, L., Biedenkopf, D., Werner, B. T., Shrestha, A., Jelonek, L., and Koch, A. (2020). Study on the efficiency of dsRNAs with increasing length in RNA-based silencing of the Fusarium CYP51 genes. *RNA Biol.* 17, 463–473. doi: 10.1080/15476286.2019.1700033

Höfle, L., Shrestha, A., Werner, B., Jelonek, L., and Koch, A. (2019). Study on the efficacy of dsRNAs with increasing length in RNAi-based silencing of the Fusarium CYP51 genes. *Biorxiv* doi: 10.1101/824953

Hu, D., Chen, Z. Y., Zhang, C., and Ganiger, M. (2020). Reduction of *Phakopsora pachyrhizi* infection on soybean through host- and spray-induced gene silencing. *Mol. Plant Pathol.* 21, 794–807. doi: 10.1111/mpp.12931

Islam, M. T., Davis, Z., Chen, L., Englaender, J., Zomorodi, S., Frank, J., et al. (2021). Minicell-based fungal RNAi delivery for sustainable crop protection. *Microbial. Biotechnol.* 14, 1847–1856. doi: 10.1111/1751-7915.13699

Jay, F., Vitel, M., Brioudes, F., Louis, M., Knobloch, T., and Voinnet, O. (2019). Chemical enhancers of posttranscriptional gene silencing in Arabidopsis. *Rna* 25, 1078–1090. doi: 10.1261/rna.068627.118

Kaldis, A., Berbati, M., Melita, O., Reppa, C., Holeva, M., Otten, P., et al. (2018). Exogenously applied dsRNA molecules deriving from the Zucchini yellow mosaic virus (ZYMV) genome move systemically and protect cucurbits against ZYMV. *Mol. Plant Pathol.* 19, 883–895. doi: 10.1111/mpp.12572

Kalyandurg, P. B., Sundararajan, P., Dubey, M., Ghadamgahi, F., Zahid, M. A., Whisson, S., et al. (2021). Spray-induced gene silencing as a potential tool to control potato late blight disease. *Phytopathology* doi: 10.1094/PHYTO-02-21-0054-SC [Epub ahead of print].

Kleter, G. A. (2020). Food safety assessment of crops engineered with RNA interference and other methods to modulate expression of endogenous and plant pest genes. *Pest Manag. Sci.* 76, 3333–3339. doi: 10.1002/ps.5957

Koch, A., and Wassenegger, M. (2021). Host-induced gene silencing – mechanisms and applications. *New. Phytol.* 231, 54–59. doi: 10.1111/nph.17364

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., et al. (2016). An RNAi-Based Control of *Fusarium graminearum* Infections Through Spraying of Long dsRNAs Involves a Plant Passage and Is Controlled by the Fungal Silencing Machinery. *PLoS Pathog.* 12:e1005901. doi: 10.1371/journal.ppat.1005901

Koch, A., Höfle, L., Werner, B. T., Imani, J., Schmidt, A., Jelonek, L., et al. (2019). SIGS vs HIGS: a study on the efficacy of two dsRNA delivery strategies to silence Fusarium FgCYP51 genes in infected host and non-host plants. *Mol. Plant Pathol.* 20, 1636–1644. doi: 10.1111/mpp.12866

Kolge, H., Kadam, K., Galande, S., Lanjekar, V., and Ghormade, V. (2021). New Frontiers in Pest Control: chitosan Nanoparticles-Shielded dsRNA as an Effective Topical RNAi Spray for Gram Podborer Biocontrol. *ACS Appl. Bio Mater.* 4, 5145–5157. doi: 10.1021/acsabm.1c00349

Konakalla, N. C., Kaldis, A., Berbati, M., Masarapu, H., and Voloudakis, A. E. (2016). Exogenous application of double-stranded RNA molecules from TMV p126 and CP genes confers resistance against TMV in tobacco. *Planta* 244, 961–969. doi: 10.1007/s00425-016-2567-6

Ladewig, K., Niebert, M., Xu, Z. P., Gray, P. P., and Lu, G. Q. M. (2010). Efficient siRNA delivery to mammalian cells using layered double hydroxide nanoparticles. *Biomaterials* 31, 1821–1829. doi: 10.1016/j.biomaterials.2009.10.058

Ladewig, K., Zhi, P. X., and Gao, Q. L. (2009). Layered double hydroxide nanoparticles in gene and drug delivery. *Expert Opin. Drug Deliv.* 6, 907–922. doi: 10.1517/17425240903130585

Linyu, W., Lianjun, Z., Ning, L., Xiwu, G., and Xiaoning, L. (2021). Effect of RNAi targeting CYP6CY3 on the growth, development and insecticide susceptibility of *Aphis gossypii* by using nanocarrier-based transdermal dsRNA delivery system. *Pest. Biochem. Physiol.* 177:104878. doi: 10.1016/j.pestbp.2021.104878

Liu, Q., Li, Y., Xu, K., Li, D., Hu, H., Zhou, F., et al. (2020). Clay nanosheet-mediated delivery of recombinant plasmids expressing artificial miRNAs via leaf spray to prevent infection by plant DNA viruses. *Hortic. Res.* 7:179. doi: 10.1038/s41438-020-00400-2

Liu, Y., and Huang, L. (2021). "Preparation and Characterization of siRNA-Loaded Liposomes" in *Design and Delivery of SiRNA Therapeutics*. eds H. J. Ditzel, M. Tuttolomondo, and S. Kauppinen (New York, NY: Springer). 159–169. doi: 10.1007/978-1-0716-1298-9_10

Lü, J., Liu, Z., Guo, W., Guo, M., Chen, S., Li, H., et al. (2020). Feeding delivery of dsHvSnf7 is a promising method for management of the pest *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae). *Insects* 11:34. doi: 10.3390/insects11010034

Lü, J., Yang, C., Liu, Z., Vélez, A. M., Guo, M., Chen, S., et al. (2021). Dietary RNAi toxicity assay suggests α and γ subunits of HvCOPI as novel molecular targets for *Henosepilachna vigintioctopunctata*, an emerging coccinellid pest. *J. Pest Sci.* 94, 1–14. doi: 10.1007/s10340-021-01350-x

Mar-Aguilar, F., Arreola-Triana, A., Mata-Cardona, D., Gonzalez-Villasana, V., Rodríguez-Padilla, C., and Reséndez-Pérez, D. (2020). Evidence of transfer of miRNAs from the diet to the blood still inconclusive. *PeerJ*. 8:e9567. doi: 10.7717/peerj.9567

Máximo, W. P. F., Howell, J. L., Mogilicherla, K., Basij, M., Chereddy, S. C. R. R., and Palli, S. R. (2020). Inhibitor of apoptosis is an effective target gene for RNAi-mediated control of Colorado potato beetle, *Leptinotarsa decemlineata*. *Arch. Insect Biochem. Physiol.* 104:e21685. doi: 10.1002/arch.21685

McLoughlin, A. G., Wytinck, N., Walker, P. L., Girard, I. J., Rashid, K. Y., de Kievit, T., et al. (2018). Identification and application of exogenous dsRNA confers plant protection against *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *Sci. Rep.* 8:7320. doi: 10.1038/s41598-018-25434-4

Mehlhorn, S. G., Geibel, S., Bucher, G., and Nauen, R. (2020). Profiling of RNAi sensitivity after foliar dsRNA exposure in different European populations of Colorado potato beetle reveals a robust response with minor variability. *Pest. Biochem. Physiol.* 166:104569. doi: 10.1016/j.pestbp.2020.104569

Mehlhorn, S., Ulrich, J., Baden, C. U., Buer, B., Maiwald, F., Lueke, B., et al. (2021). The mustard leaf beetle, *Phaedon cochleariae*, as a screening model for exogenous RNAi-based control of coleopteran pests. *Pest. Biochem. Physiol.* 176:104870. doi: 10.1016/j.pestbp.2021.104870

Mezzetti, B., Smagghe, G., Arpaia, S., Christiaens, O., Dietz-Pfeilstetter, A., Jones, H., et al. (2020). RNAi: what is its position in agriculture? *J. Pest Sci.* 93, 1125–1130. doi: 10.1007/s10340-020-01238-2

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., et al. (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nature Plants* 3:1607. doi: 10.1038/nplants.2016.207

Mohamed, M. A., and Youssef, K. (2021). Topical delivery of host induced RNAi silencing by layered double hydroxide nanosheets: an efficient tool to decipher pathogenicity gene function of Fusarium crown and root rot in tomato. *Physiol. Mol. Plant Pathol.* 115:101684. doi: 10.1016/j.pmpp.2021.101684

Nature (2016). RNA spray fights fungus. *Nature* 538:293. doi: 10.1038/538293d

Necira, K., Makki, M., Sanz-García, E., Canto, T., Djilani-Khouadja, F., and Tenllado, F. (2021). Topical application of *Escherichia coli*-encapsulated dsRNA induces resistance in *Nicotiana benthamiana* to potato viruses and involves rdr6 and combined activities of dcl2 and dcl4. *Plants* 10:644. doi: 10.3390/plants10040644

Niehl, A., Soininen, M., Poranen, M. M., and Heinlein, M. (2018). Synthetic biology approach for plant protection using dsRNA. *Plant Biotechnol. J.* 16, 1679–1687. doi: 10.1111/pbi.12904

Niño-Sánchez, J., Chen, L. H., de Souza, J. T., Mosquera, S., and Stergiopoulos, I. (2021). Targeted delivery of gene silencing in fungi using genetically engineered bacteria. *J. Fungi* 7, 1–22. doi: 10.3390/jof7020125

Niu, J., Yang, W. J., Tian, Y., Fan, J. Y., Ye, C., Shang, F., et al. (2019). Topical dsRNA delivery induces gene silencing and mortality in the pea aphid. *Pest Manag. Sci.* 75, 2873–2881. doi: 10.1002/ps.5457

Nowara, D., Schweizer, P., Gay, A., Lacomme, C., Shaw, J., Ridout, C., et al. (2010). HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell* 22, 3130–3141. doi: 10.1105/tpc.110.077040

OECD (2020). *Considerations for the Environmental Risk Assessment of the Application of Sprayed or Externally Applied ds-RNA-Based Pesticides*. United Kingdom: Organisation for Economic Co-operation and Development.

Olivier. C., Teodora, D., Kaloyan, K., Salvatore, A., Mallikarjuna, R. J., Isabella, U. et al. (2018). *Literature Review of Baseline Information on RNAi to Support the Environmental Risk Assessment of RNAi-Based GM Plants*. Italy: European Food Safety Authority.

Palli, S. R. (2014). RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. *Curr. Opin. Insect Sci.* 6, 1–8. doi: 10.1016/j.cois.2014.09.011

Parker, K. M., Barragán Borrero, V., van Leeuwen, D. M., Lever, M. A., Mateescu, B., and Sander, M. (2019). Environmental Fate of RNA Interference Pesticides: adsorption and Degradation of Double-Stranded RNA Molecules in Agricultural Soils. *Environ. Scie. Technol.* 53, 3027–3036. doi: 10.1021/acs.est.8b05576

Peng, Y., Wang, K., Chen, J., Wang, J., Zhang, H., Ze, L., et al. (2020a). Identification of a double-stranded RNA-degrading nuclease influencing both ingestion and injection RNA interference efficiency in the red flour beetle *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* 125:103440. doi: 10.1016/j.ibmb.2020.103440

Peng, Y., Wang, K., Zhu, G., Han, Q., Chen, J., Elzaki, M. E. A., et al. (2020b). Identification and characterization of multiple dsRNases from a lepidopteran insect, the tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae). *Pest. Biochem. Physiol.* 162, 86–95. doi: 10.1016/j.pestbp.2019.09.011

Petek, M., Coll, A., Ferenc, R., Razinger, J., and Gruden, K. (2020). Validating the Potential of Double-Stranded RNA Targeting Colorado Potato Beetle Mesh Gene in Laboratory and Field Trials. *Front. Plant Sci.* 11:1250. doi: 10.3389/fpls.2020.01250

Pugsley, C. E., Isaac, R. E., Warren, N. J., and Cayre, O. J. (2021). Recent Advances in Engineered Nanoparticles for RNAi-Mediated Crop Protection Against Insect Pests. *Front. Agron.* 3:652981. doi: 10.3389/fagro.2021.652981

Qiao, L., Lan, C., Capriotti, L., Ah-Fong, A., Nino Sanchez, J., Hamby, R., et al. (2021). Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake. *Plant Biotechnol. J.* 19, 1756–1768. doi: 10.1111/pbi.13589

Ram Reddy, M. K., Xu, Z. P., Lu, G. Q., and da Costa, J. C. D. (2006). Layered double hydroxides for CO2 capture: structure evolution and regeneration. *Industr. Eng. Chem. Res.* 45, 7504–7509. doi: 10.1021/ie060757k

Reddy, M. K. R., Xu, Z. P., Lu, G. Q., and Diniz Da Costa, J. C. (2008). Effect of SOx adsorption on layered double hydroxides for CO2 capture. *Industr. Eng. Chem. Res.* 47, 7357–7360. doi: 10.1021/ie8004226

Rosa, C., Kuo, Y. W., Wuriyanghan, H., and Falk, B. W. (2018). RNA interference mechanisms and applications in plant pathology. *Annu. Rev. Phytopathol.* 56, 581–610. doi: 10.1146/annurev-phyto-080417-050044

Santos, D., Remans, S., van den Brande, S., and Broeck, J. V. (2021). RNAs on the go: extracellular transfer in insects with promising prospects for pest management. *Plants* 10, 1–22. doi: 10.3390/plants10030484

Schiemann, J., Dietz-Pfeilstetter, A., Hartung, F., Kohl, C., Romeis, J., and Sprink, T. (2019). Risk Assessment and Regulation of Plants Modified by Modern Biotechniques: current Status and Future Challenges. *Annu. Rev. Plant Biol.* 70, 699–726. doi: 10.1146/annurev-arplant-050718-100025

Schlemmer, T., Barth, P., Weipert, L., Preußer, C., Hardt, M., Möbus, A., et al. (2021a). Isolation and Characterization of Barley (*Hordeum vulgare*) Extracellular Vesicles to Assess Their Role in RNA Spray-Based Crop Protection. *Int. J. Mol. Sci.* 22:7212. doi: 10.3390/ijms22137212

Schlemmer, T., Weipert, L., Barth, P., Werner, B., Preußer, C., Hardt, M., et al. (2021b). Host-induced gene silencing involves Arabidopsis ESCRT-III pathway for the transfer of dsRNA-derived siRNA. *Biorxiv* 945154. doi: 10.1101/2020.02.12.945154

Schwartz, S. H., Hendrix, B., Hoffer, P., Sanders, R. A., and Zheng, W. (2020). Carbon dots for efficient small interfering RNA delivery and gene silencing in plants. *Plant Physiol.* 184, 647–657. doi: 10.1104/pp.20.00733

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., et al. (2013). Towards the elements of successful insect RNAi. *J. Insect Physiol.* 59, 1212–1221. doi: 10.1016/j.jinsphys.2013.08.014

Sharma, R., Taning, C. N. T., Smagghe, G., and Christiaens, O. (2021). Silencing of double-stranded ribonuclease improves oral RNAi efficacy in southern green stinkbug *Nezara viridula*. *Insects* 12, 1–16. doi: 10.3390/insects12020115

Song, X. S., Gu, K. X., Duan, X. X., Xiao, X. M., Hou, Y. P., Duan, Y. B., et al. (2018). Secondary amplification of siRNA machinery limits the application of spray-induced gene silencing. *Mol. Plant Pathol.* 19, 2543–2560. doi: 10.1111/mpp.12728

Sundaresha, S., Sharma, S., Bairwa, A., Tomar, M., Kumar, R., Bhardwaj, V., et al. (2021). Spraying of dsRNA molecules derived from *Phytophthora infestans*, as an effective plant protection strategies for the management of potato late blight. *Life Sci.* 2021:2021020280. doi: 10.20944/preprints202102.0280.v1

Swaminathan, G., Shigna, A., Kumar, A., Byroju, V. V., Durgempudi, V. R., and Dinesh Kumar, L. (2021). RNA Interference and Nanotechnology: a Promising Alliance for Next Generation Cancer Therapeutics. *Front. Nanotechnol.* 3:694838. doi: 10.3389/fnano.2021.694838

Taning, C. N. T., Mezzetti, B., Kleter, G., Smagghe, G., and Baraldi, E. (2021). Does RNAi-Based Technology Fit within EU Sustainability Goals? *Trends Biotechnol.* 39, 644–647. doi: 10.1016/j.tibtech.2020.11.008

Tenllado, F., and Díaz-Ruíz, J. R. (2001). Double-Stranded RNA-Mediated Interference with Plant Virus Infection. *J. Virol.* 75, 12288–12297. doi: 10.1128/JVI.75.24.12288-12297.2001

Tenllado, F., Martínez-García, B., Vargas, M., and Díaz-Ruíz, J. R. (2003). Crude extracts of bacterially expressed dsRNA can be used to protect plants against virus infections. *BMC Biotechnol.* 3:3. doi: 10.1186/1472-6750-3-3

Thairu, M. W., Skidmore, I. H., Bansal, R., Nováková, E., Hansen, T. E., Li-Byarlay, H., et al. (2017). Efficacy of RNA interference knockdown using aerosolized short interfering RNAs bound to nanoparticles in three diverse aphid species. *Insect Mol. Biol.* 26, 356–368. doi: 10.1111/imb.12301

Timmons, L., Court, D. L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263, 103–112. doi: 10.1016/S0378-1119(00)00579-5

United Nations Department of Economics and Social Affairs (2019). *World Population Prospects 2019*. Available online at: http://www.ncbi.nlm.nih.gov/pubmed/12283219 (accessed September 2021).

Uslu, V. V., and Wassenegger, M. (2020). Critical view on RNA silencing-mediated virus resistance using exogenously applied RNA. *Curr. Opin. Virol.* 42, 18–24. doi: 10.1016/j.coviro.2020.03.004

Uslu, V. V., Bassler, A., Krczal, G., and Wassenegger, M. (2020). High-Pressure-Sprayed Double Stranded RNA Does Not Induce RNA Interference of a Reporter Gene. *Front. Plant Sci.* 11:534391. doi: 10.3389/fpls.2020.534391

Voloudakis, A. E., Holeva, M. C., Sarin, L. P., Bamford, D. H., Vargas, M., Poranen, M. M., et al. (2015). Efficient double-stranded RNA production methods for utilization in plant virus control. *Methods Mol. Biol.* 1236, 255–274. doi: 10.1007/978-1-4939-1743-3_19

Wagner, D. L., Grames, E. M., Forister, M. L., Berenbaum, M. R., and Stopak, D. (2021). Insect decline in the Anthropocene: death by a thousand cuts. *Proc. Natl. Acad. Sci. U. S. A.* 118:e2023989118. doi: 10.1073/pnas.2023989118

Wang, K., Li, B., Yu, Y., Wei, J., Zhu, J., Wang, J., et al. (2021). Bacterial Rdl2 dsRNA increased the insecticidal activity of GABAR blockers and allosteric modulators against *Plutella xylostella*. *Biorxiv* doi: 10.1101/2021.03.24.436736

Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P. H. J., Huang, H. D., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* 2:16151. doi: 10.1038/nplants.2016.151

Werner, B. T., Gaffar, F. Y., Schuemann, J., Biedenkopf, D., and Koch, A. M. (2020). RNA-Spray-Mediated Silencing of *Fusarium graminearum* AGO and DCL Genes Improve Barley Disease Resistance. *Front. Plant Sci.* 11:476. doi: 10.3389/fpls.2020.00476

Whitten, M. M. A., Facey, P. D., del Sol, R., Fernández-Martínez, L. T., Evans, M. C., Mitchell, J. J., et al. (2016). Symbiont-mediated RNA interference in insects. *Proc. R. Soc. B Biol. Sci.* 283:20160042. doi: 10.1098/rspb.2016.0042

Whitten, M., and Dyson, P. (2017). Gene silencing in non-model insects: overcoming hurdles using symbiotic bacteria for trauma-free sustainable delivery of RNA interference: sustained RNA interference in insects mediated by symbiotic bacteria: applications as a genetic tool and as a biocid. *Bioessays* 39:1600247. doi: 10.1002/bies.201600247

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A. I., Taning, C. N. T., et al. (2021). RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. *Commun. Biol.* 4, 1–8. doi: 10.1038/s42003-021-01975-9

Worrall, E. A., Bravo-Cazar, A., Nilon, A. T., Fletcher, S. J., Robinson, K. E., Carr, J. P., et al. (2019). Exogenous application of RNAi-inducing double-stranded RNA inhibits aphid-mediated transmission of a plant virus. *Front. Plant Sci.* 10:265. doi: 10.3389/fpls.2019.00265

Wu, J. J., Mu, L. L., Kang, W. N., Ze, L. J., Shen, C. H., Jin, L., et al. (2021). RNA interference targeting ecdysone receptor blocks the larval–pupal transition in *Henosepilachna vigintioctopunctata*. *Insect Sci.* 28, 419–429. doi: 10.1111/1744-7917.12777

Wytinck, N., Manchur, C. L., Li, V. H., Whyard, S., and Belmonte, M. F. (2020a). DsRNA uptake in plant pests and pathogens: insights into RNAi-based insect and fungal control technology. *Plants* 9, 1–17. doi: 10.3390/plants9121780

Wytinck, N., Sullivan, D. S., Biggar, K. T., Crisostomo, L., Pelka, P., Belmonte, M. F., et al. (2020b). Clathrin mediated endocytosis is involved in the uptake of exogenous double-stranded RNA in the white mold phytopathogen *Sclerotinia sclerotiorum*. *Sci. Rep.* 10, 12773. doi: 10.1038/s41598-020-69771-9

Xu, Z. P., Stevenson, G., Lu, C. Q., and Lu, G. Q. (2006). Dispersion and size control of layered double hydroxide nanoparticles in aqueous solutions. *J. Phys. Chem. B* 110, 16923–16929. doi: 10.1021/jp062281o

Yan, S., Ren, B. Y., and Shen, J. (2021). Nanoparticle-mediated double-stranded RNA delivery system: a promising approach for sustainable pest management. *Insect Sci.* 28, 21–34. doi: 10.1111/1744-7917.12822

Yin, G., Sun, Z., Liu, N., Zhang, L., Song, Y., Zhu, C., et al. (2009). Production of double-stranded RNA for interference with TMV infection utilizing a bacterial prokaryotic expression system. *Appl. Microbiol. Biotechnol.* 84, 323–333. doi: 10.1007/s00253-009-1967-y

Yu, X. D., Liu, Z. C., Huang, S. L., Chen, Z. Q., Sun, Y. W., Duan, P. F., et al. (2016). RNAi-mediated plant protection against aphids. *Pest. Manag. Sci.* 72, 1090–1098. doi: 10.1002/ps.4258

Zabel, M. D., Mollnow, L., and Bender, H. (2021). "siRNA Therapeutics for Protein Misfolding Diseases of the Central Nervous System" in *Design and Delivery of SiRNA Therapeutics*, eds H. J. Ditzel, M. Tuttolomondo, and S. Kauppinen (New York, NY: Springer). 377–394. doi: 10.1007/978-1-0716-1298-9_20

Zhang, H., Cao, Y., Xu, D., Goh, N. S., Demirer, G. S., Cestellos-Blanco, S., et al. (2021). Gold-Nanocluster-Mediated Delivery of siRNA to Intact Plant Cells for Efficient Gene Knockdown. *Am. Chem. Soc.* 13, 5859–5866. doi: 10.1101/2021.03.17.435890

Zhang, H., Demirer, G. S., Zhang, H., Ye, T., Goh, N. S., Aditham, A. J., et al. (2019). DNA nanostructures coordinate gene silencing in mature plants. *Proc. Natl. Acad. Sci. U. S. A.* 116, 7543–7548. doi: 10.1073/pnas.1818290116

Zhang, H., Zhang, H., Demirer, G. S., González-Grandío, E., Fan, C., and Landry, M. P. (2020). Engineering DNA nanostructures for siRNA delivery in plants. *Nat. Protocols* 15, 3064–3087. doi: 10.1038/s41596-020-0370-0

Zhang, J., Khan, S. A., Hasse, C., Ruf, S., Heckel, D. G., and Bock, R. (2015). Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science* 347, 991L–994. doi: 10.1126/science.1261680

Zotti, M., dos Santos, E. A., Cagliari, D., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2018). RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest. Manag. Sci.* 74, 1239–1250. doi: 10.1002/ps.4813

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Spray-Induced Silencing of Pathogenicity Gene *MoDES1 via* Exogenous Double-Stranded RNA Can Confer Partial Resistance Against Fungal Blast in Rice

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Over the past years, RNA interference (RNAi) has been used as a promising combat strategy against a wide range of pests and pathogens in ensuring global food security. It involves the induction of highly specific posttranscriptional regulation of target essential genes from an organism, *via* the application of precursor long, non-coding double-stranded RNA (dsRNA) molecules that share sequence-complementarity with the mRNAs of the targets. Fungal blast disease caused by *Magnaporthe oryzae* is one of the most deadly diseases of rice and wheat incurring huge losses in global crop yield. To date, the host-induced gene silencing (HIGS) and virus-induced gene silencing (VIGS) aspects of RNAi have been successfully exploited in developing resistance against *M. oryzae* in rice. Spray-induced gene silencing (SIGS) is a current, potential, non-transformative, and environment-friendly pest and pathogen management strategy, where naked or nanomaterial-bound dsRNA are sprayed on leaves to cause selective knockdown of pathogenicity genes. Although it relies on the ability of fungal pathogens to uptake sprayed RNA, its efficiency varies largely across phytopathogens and their genes, targeted for silencing. Here, we report a transient dsRNA supplementation system for the targeted knockdown of *MoDES1*, a host-defense suppressor pathogenicity gene from *M. oryzae*. We validate the feasibility of *in vivo* SIGS and post-uptake transfer of RNA signals to distal plant parts in rice-*M. oryzae* pathosystem through a *GFP*-based reporter system. A protocol for efficient silencing *via* direct foliar spray of naked dsRNA was optimized. As proof-of-concept, we demonstrate the phenotypic impacts of *in vitro* ds*DES1* treatment on growth, conidiation, ROS-scavenging ability, and pathogenic potential of *M. oryzae*. Furthermore, our extrapolatory ds*DES1* spray experiments on wounded leaves and whole rice plants indicate resultant silencing of *MoDES1* that conferred significant resistance against the fungal blast disease. The evaluation of primary and secondary host defense responses provides evidence supporting the notion that spray of

sequence-specific dsRNA on wounded leaf tissue can cause systemic and sustained silencing of a *M. oryzae* target gene. For the first time, we establish a transgene-free SIGS approach as a promising crop protection strategy against the notorious rice-blast fungus.

Keywords: rice, fungal blast, *MoDES1*, double-stranded RNA (dsRNA), spray induced gene silencing (SIGS), disease resistance, RNA-based crop protection

INTRODUCTION

Plants, like all other organisms, have to survive in an environment where numerous microorganisms coexist. The interaction between plant and microbes is very specific when it comes to disease development. In order to evade pathogenic attacks, plants have developed an immune system that comprises a network of defense systems and molecules. The fundamental strategy to survive among a myriad of pathogens is the fast recognition of an invading pathogen, followed by the rapid induction of a multifaceted defense response. In plants, the first line of defense involves the perception of some signature molecular motifs specific to different classes of pathogens, which are known as pathogen-associated molecular patterns (PAMPs), recognized by some transmembrane pattern recognition receptor (PRR) proteins (Medzhitov and Janeway, 1997). Once the ligand-binding ecto-domain of PRRs get activated by other trans-membrane signaling adapters (Zipfel et al., 2006), the identification of the pathogen results in systemic acquired resistance (SAR) in plants (Mishina and Zeier, 2007). SAR is an integral part of PAMP-triggered immunity (PTI) and is known to confer durable resistance to the host against a broad spectrum of pathogens (Durrant and Dong, 2004). It induces physiological and molecular adaptations, namely, alteration in flux of ions such as Ca^{2+}, H^{+}, and Cl, across the plasma membrane (Garcia-Brugger et al., 2006), and the expression of WRKY transcription factors (Pandey and Somssich, 2009) that sometimes control reactive oxygen species (ROS) burst and salicylic acid (SA) production (Takabatake et al., 2007; Du et al., 2009). SA and other hormones such as jasmonic acid (JA) and ethylene (ET), are classically known to be involved in plant defense *via* the induction of pathogenesis-related (PR) genes against a wide range of pathogens (Bari and Jones, 2009). However, there is constant coevolution happening in the front of both pathogens and plants (Jones and Dangl, 2006). In order to overcome PTI successfully, some pathogens undergo modification in their genome and produce effectors. On recognition of such hypervariable molecules, effector-triggered immunity (ETI) develops in plants. Often, it encompasses specific "gene to gene" interaction between the effectors or avirulence (Avr) factors and R-proteins that the plants synthesize to counter such effector molecules (van der Biezen and Jones, 1998; Chisholm et al., 2006). ETI, which is characterized by cell death, lignification, ROS formation, and hypersensitive response (HR), is specific and robust (Nürnberger et al., 2004). Together, HR and the synthesis of toxic ROS are essential markers of plant immunity, as they influence other responses such as lignin and callose deposition, and phytoalexin synthesis (van Loon et al., 1998; Van Loon and Van Strien, 1999).

Rice *(Oryza sativa*) is a major agricultural crop consumed by over 50% of world population. Around 10–30% of its annual yields that could feed around 60 million people get destroyed by the fungus *M. oryzae* (Pennisi, 2010). Besides rice, this fungus also causes blast disease in another staple crop, wheat, where it may destroy the whole crop produce. Having made its position among the top 10 fungal diseases that infect plants (Dean et al., 2012), it continues to pose a major threat to global food production. Besides the use of fungicides and pesticides, developing genetic resistance against plant diseases by breeding-based crop improvement programs is one of the most popular and acceptable approaches to address food security. Although they are often achieved by traditional breeding, several other approaches involving genome editing and genetic modification have been in practice for imparting more durable resistance against the ever-evolving pathogens. The overexpression of defense-inducing factors such as PRRs, R genes, and even effectors (Wang et al., 2019), or the knockout of susceptibility (S) genes (Santillán Martínez et al., 2020) in host plants can provide a strong armament for arms race against its pathogen.

Highly unstable ROS-like peroxides and superoxides are known to be versatile players in disease development (Torres and Dangl, 2005; Shetty et al., 2007; Kou et al., 2019). In the case of the fungal blast disease, while *Magnaporthe*-NADPH oxidase-mediated ROS generation helps it in causing disease (Egan et al., 2007), ROS burst by NADPH-oxidase of invaded rice cells can prevent fungal colonization and induce resistance *via* callose formation and the expression of defense-related genes (Torres et al., 2006). *M. oryzae* penetrates rice cells *via* a special melanized appressorium that is formed at the tip of a germinated asexual spore or conidium. The appressoria form a penetration peg that differentiates inside the primary host cell to form invasive hyphae (Kankanala et al., 2007). One of the major pathways activated in rice cell in this stage in order to counter against fungal ROS is the *Os* chitin elicitor-binding protein (CEBIP)/*Os* chitin elicitor receptor kinase 1 (CERK1)-*Os* guanine nucleotide exchange factor(RACGEF1)-*Os*RAC1 module that is a pivotal component

Abbreviations: PAMP, Pathogen-associated molecular patterns; PRR, PAMP recognition receptor; ROS, Reactive oxygen species; HR, Hypersensitive response; PTI, PAMP-triggered immunity; ETI, Effector-triggered immunity; GFP, Green fluorescent protein; *GFP,* Gene-encoding green fluorescent protein; PR, Pathogenesis-related; *Os*CEBIP, *Oryza sativa* chitin elicitor-binding protein; *Os*CERK1, *Oryza sativa* chitin elicitor receptor kinase 1; HIGS, Host-induced gene silencing; SIGS, Spray-induced gene silencing; DCLs, Dicer like proteins; DES1, Defense suppressor 1 protein; *MoDES1,* Defense suppressor 1 gene; ds*GFP*, dsRNA specific to *GFP;* ds*DES1*, dsRNA specific to *MoDES1;* DAB, 3,3′-Diaminobenzidine; DIC, Differential interface contrast; FITC, Fluorescein isothiocyanate.

of the defensome involved specifically in chitin-mediated PTI (Akamatsu et al., 2013). *Os*CEBIP is a rice receptor-like protein without an intracellular kinase domain that recognizes fungal chitin. Upon binding to chitin directly, it dimerizes, and the CEBIP dimer interacts with the PRR *Os*CERK1 (Kaku et al., 2006; Shimizu et al., 2010). The receptor complex then activates *Os*RACGEF1 by phosphorylation. Therefore, in response to chitin, *Os*RACGEF1 activates *OsRAC1*, which switches on rice immunity by transducing signals to various downstream signaling components: NADPH oxidase (Rboh) for ROS production, lignin synthesis (Kawasaki et al., 2006), induction of cell death, PR-genes, and phytoalexins (Dang et al., 2013).

One of the ways by which *M. oryzae* secures its successful pathogenesis is by scavenging these host NADPH oxidase-generated ROS. Enzymes, such as peroxidases (Mir et al., 2015), redox-sensitive transcription factors (Guo et al., 2011; Qi et al., 2012), and pathogenicity factors that influence extracellular enzyme activity *via* pleiotropic changes, are central to the fungal defense against exogenous or host-derived ROS. Being a hemibiotroph, in a susceptible plant, *M. oryzae* suppresses the metabolic pathways associated with ROS formation after incipient ROS-burst *via* effectors and defense suppressors (Samalova et al., 2014). *MoDES1* is one such innate defense suppressor of rice that is important in detoxification of plant-driven ROS, and crucial for pathogenesis. It gets triggered in response to initial PTI-mediated ROS burst and is the encounter strategy of the pathogen against its host to suppress downstream defense-associated signaling, such as further ROS generation, HR, and SAR (Chi et al., 2009). *MoDES1* mutants showed difficulty in fungal penetration and colonization, and ROS-sensitivity because of reduction in extracellular detoxifying enzyme activity (Chi et al., 2009; Kou et al., 2019).

Apart from traditional crop breeding approaches, one of the key strategies of developing durable resistance against a pathogen on field, is the targeted *in planta* knockdown of one or more pathogenicity factor(s). RNA interference (RNAi) technology can specifically silence a target gene *via* a dsRNA (double-stranded RNA) molecule sharing sequence complementarity with its mRNA (Fire et al., 1998; Wilson and Doudna, 2013). The RNAi tool has been used successfully in developing resistance against pests, pathogens, and viruses (Zotti et al., 2017). Nowadays, two major aspects of using RNAi in crop improvement are transgene-mediated HIGS and exogenous dsRNA spray-induced gene silencing (SIGS), with the central principle being the same. The already proven efficient HIGS (Baulcombe, 2015) is based on the ability of filamentous fungi to take RNA species (double-stranded and siRNAs) from host plants while drawing nutrition through haustoria. A successful demonstration of effective assimilation and processing of dsRNAs has been reported in several plant pathogenic fungi such as *Botrytis cinerea* (Wang et al., 2016), *Fusarium graminerium* (Koch et al., 2016; Baldwin et al., 2018), *Fusarium asiaticum* and *Fusarium* sp. (Song et al., 2018), *Magnaporthe oryzae* (Zhu et al., 2017; Guo et al., 2019), and *Puccinia striiformis* fsp. *tritici* (Qi et al., 2018), *via* their transformed hairpin-loop-expressing host. On the contrary, SIGS is a relatively new and emerging "non-transgenic" crop-protection strategy that looks promising. The spray application of dsRNA on plants has been able to suppress endogenous genes and transgenes in a target plant (Dubrovina and Kiselev, 2019), and has efficiently silenced genes among several insects, pests, and pathogens (Morozov et al., 2019). The efficacy of SIGS in suppressing specific genes in a pathogen lies not only on the machinery of RNAi but also on the cross-kingdom movement and exchange of RNAs between the plant and the interacting pathogen (Wang and Dean, 2020). The extracellular vesicles of a plant play a crucial role in transporting sRNA cargo across the plant-fungus interface, after plant dicers process the exogenously sprayed dsRNA, thereby leading to the homology-based degradation of fungal target transcripts (Cai et al., 2021). Since the response of different host-pathosystems toward the phenomenon may vary, it is essential to assess the efficiency of SIGS in curbing a particular disease. In *M, oryzae*, while a few reports have been published about HIGS, the SIGS approach is relatively less explored. The silencing of a specific target gene, *MoAP1,* achieved by the spraying of artificial siRNAs (asiRNAs) on rice leaves has been reported to reduce disease symptoms caused by *M, oryzae*. Although that was a proof-of-concept experiment for HIGS (Guo et al., 2019), no dedicated study on SIGS has been conducted on the fungal blast-rice pathosystem to date.

In this study, we tried to address the possibility of utilizing SIGS on *M. oryzae* to reduce knowledge gap and generate more insights into the process. Considering recent reports on different pathosystems into consideration, we hypothesized that SIGS might act as a promising transgene-free alternative to HIGS in controlling fungal blast disease. Unlike HIGS, which involves the stable expression of a hairpin construct in the host, SIGS can be a relatively simpler, less time-consuming, and robust strategy for selectively targeting pathogenicity factors, thereby conferring disease resistance. However, the efficiency of eukaryotic pathogens to uptake externally applied dsRNA generally varies, and the assertion of SIGS largely depends on that factor (Qiao et al., 2021). Therefore, to prove our hypothesis, SIGS was first demonstrated in the *M. oryzae*-rice pathosystem using a *green fluorescent protein* (*GFP*) reporter system. dsRNA uptake-efficiency and specificity, and its *in planta* impact were assessed in dsRNA-sprayed rice plants. The impact of spraying dsRNAs on both intact and wounded leaves was evaluated to better understand the sustenance of gene silencing through RNA signals. Furthermore, SIGS was performed to selectively target *MoDES1* in rice, and the status of rice defense response was analyzed. The results generated from this study indicate that SIGS can efficiently target a pathogenicity gene and render this pathogen less-suited for pathogenesis, and experimentally established that *MoDES1* could be a target candidate gene for the development of resistance against *M. oryzae* in other hosts. Our optimizations will also help in the potential utilization of this gene knockdown strategy more efficiently in the rice-*M. oryzae* pathosystem. To our knowledge, this will be the first study dedicated to *in planta* SIGS contributing to disease resistance in this model pathosystem. We hope that these findings will open leads toward transient RNAi-based research on this destructive pathogen and further exploration of the functional and mechanistic aspects of the lesser-known SIGS technology.

MATERIALS AND METHODS

Fungal Strains, Rice Varieties, and Vector, and Their Growth Conditions

In this study, an Indian strain of *M. oryzae*, i.e., B157 (international race IC9) isolated from Hyderabad, and its cytoplasmic GFP-expressing transformant (*M. oryzaeGFP*) were grown on yeast extract glucose (YEG) media (Saha et al., 2020). The blast-susceptible indica rice cultivar- C0-43, was grown at 27°C under a 16:8 light/dark photoperiod. The binary vector pCAMBIA1302 was streaked onto Luria Bertani (Himedia, Mumbai, India) agar plates supplemented with 50 mg/L Kanamycin (Himedia, Mumbai, India) and incubated overnight at 37°C. Fungal growth assays were performed on a complete medium (CM) supplemented with or without dsRNA, at 28°C for 7 days. For conidiation, the fungi were grown in dark on YEG plates for 8–10 days. The fungal biomass was then scraped on 10 days after inoculation and homogenized into uniform slurry by vortexing. Spores were separated from the mycelial debris by filtering the slurry through sterile MiraCloth (Calbiochem, Darmstadt, Germany), and then centrifuged at 7,000 rpm for 7 min. The conidia were visualized and counted under a microscope using a hemocytometer (Neubauer, Marienfeld, Germany). The length and breadth of conidia were measured keeping a 20-μm scale reference using an built-in microscope camera. Microscopic examination of at least 50 conidia per replicate was done in at least three independent experiments.

Isolation of Fungal Genomic DNA and Polymerase Chain Reaction

The fungal culture was grown in YEG broth by the inoculation of solid culture agar blocks. Fungal balls were filtered out of the 4-day-old culture, and mycelia were harvested, dried, and weighed before genomic DNA isolation was carried out (Dellaporta et al., 1983). Plasmid DNA was isolated using alkaline lysis method (Sambrook et al., 1989) from the fully grown culture. Each 20 μl PCR reaction was set up for 100 ng pure genomic DNA or plasmid DNA containing forward and reverse primers, deoxynucleotide triphosphates (dNTPs), 10 × reaction buffer, and 1.5 units of Taq DNA Polymerase (NEB, Ipswich, Massachusetts, United States). The PCR was carried out in a thermal cycler (Genetix, New Delhi, India), with standard PCR conditions, whereby the annealing temperatures were maintained at 52 (for *GFP*) and 54°C (for *MoDES1*) for 30 s per cycle. Resultant PCR products were used for *in vitro* transcription, as will be described later. The primers used are listed in **Supplementary Table 2**.

Design and Synthesis of Double-Stranded RNA

The sequence information of *GFP* [obtained from pCAMBIA-1302 (GenBank: AF234298.1)] and *MoDES1* (MGG_04163) was obtained from NCBI.[1] Target regions were selected from their exonic regions, as indicated in **Supplementary Figure 1**. A target sequence homology of more than 19-mer with any other gene can render non-specificity to the precursor dsRNA. Hence, the target sequences were checked for homology across the whole genome assembly of the *M. oryzae*, *O. sativa* subsp. *indica*, *O. sativa* subsp. *japonica*, and *O. sativa* subsp. *javanica* groups using NCBI BLASTn. Homology was similarly checked between *MoDES1* target sequence and its counterparts coding for its homologs known from other closely related Ascomycetous fungi (Chi et al., 2009). Only members that showed a significant percentage sequence similarity of greater than 40% were considered for BLAST analysis with the *MoDES1* dsRNA sequence. After ensuring that there was no significant similarity among the interrogated sequences, the target regions (**Supplementary Figure 1**) were PCR-amplified from the *M. oryzaeGFP* genomic DNA using sense and anti-sense primers linked with a T7 promoter sequence at their 5′ ends (**Supplementary Table 2**). While the sense forward primer (FP) and the anti-sense reverse primer (RP) had a T7 promoter sequence attached to them, the sense RP and anti-sense FP did not have it. The PCR conditions have been discussed above, in the "genomic DNA isolation and PCR" section of "Materials and methods." The amplicons were checked for band specificity, purified using Purelink PCR Purification Kit (Invitrogen, Waltham, MA, United States), and a further 1 μg each of individual amplicons were used as templates for T7 DNA-dependent RNA polymerase-based *in vitro* transcription. For this, TranscriptAid T7 High-Yield Transcription Kit (Invitrogen, Waltham, MA, United States) was used following the instructions of the manufacturer. The sense and anti-sense transcripts for each target sequence were incubated with RNase-free DN*aseI* (NEB, Ipswich, Massachusetts, United States) for 1 h at 37°C to remove residual template DNA. DN*aseI* digestion was stopped with0.5 M EDTA at 65°C for 10 min, followed by the phenol:chloroform-based extraction and ethanol-based precipitation of RNA transcripts. The single-stranded RNAs (ssRNAs), being self-complementary, were annealed in equimolar concentrations to generate respective ds*GFP* and ds*DES1* by initially incubating at 75°C for 10 min, and gradually cooling them down at room temperature. The dsRNAs were further run on 2% agarose gel with their corresponding ssRNA transcripts for integrity analysis and successful annealing. To prevent the formation of secondary structures, the ssRNAs were mixed with an RNA-loading dye, and denatured and chilled before loading into the wells. Next, ds*GFP* and ds*DES1* were purified using.a volume of 3 M sodium acetate (pH 5.2) and three volumes of chilled absolute ethanol. After overnight incubation at –20°C, the dsRNA was centrifuged in a Sigma 2–16 KL cooling-centrifuge at 13,000 rpm for 30 min. The precipitated pellets were washed twice with 70% ethanol, air-dried, and quantified with a UV spectrophotometer, and $A_{260/280}$ ratio was measured (**Supplementary Figure 2**). A schematic representation of the synthesis of dsRNAs and *in vitro* and *in vivo* proof-of-concept experiments is provided in **Figure 1**.

In vitro Feeding of Double-Stranded RNA to *Magnaporthe oryzae*

The *in vitro* treatment involved the co-incubation of wild type *M. oryzae* along with the purified dsRNA in both solid and liquid media. The fungus was grown on CM plates supplemented with

[1] https://www.ncbi.nlm.nih.gov/nucleotide/

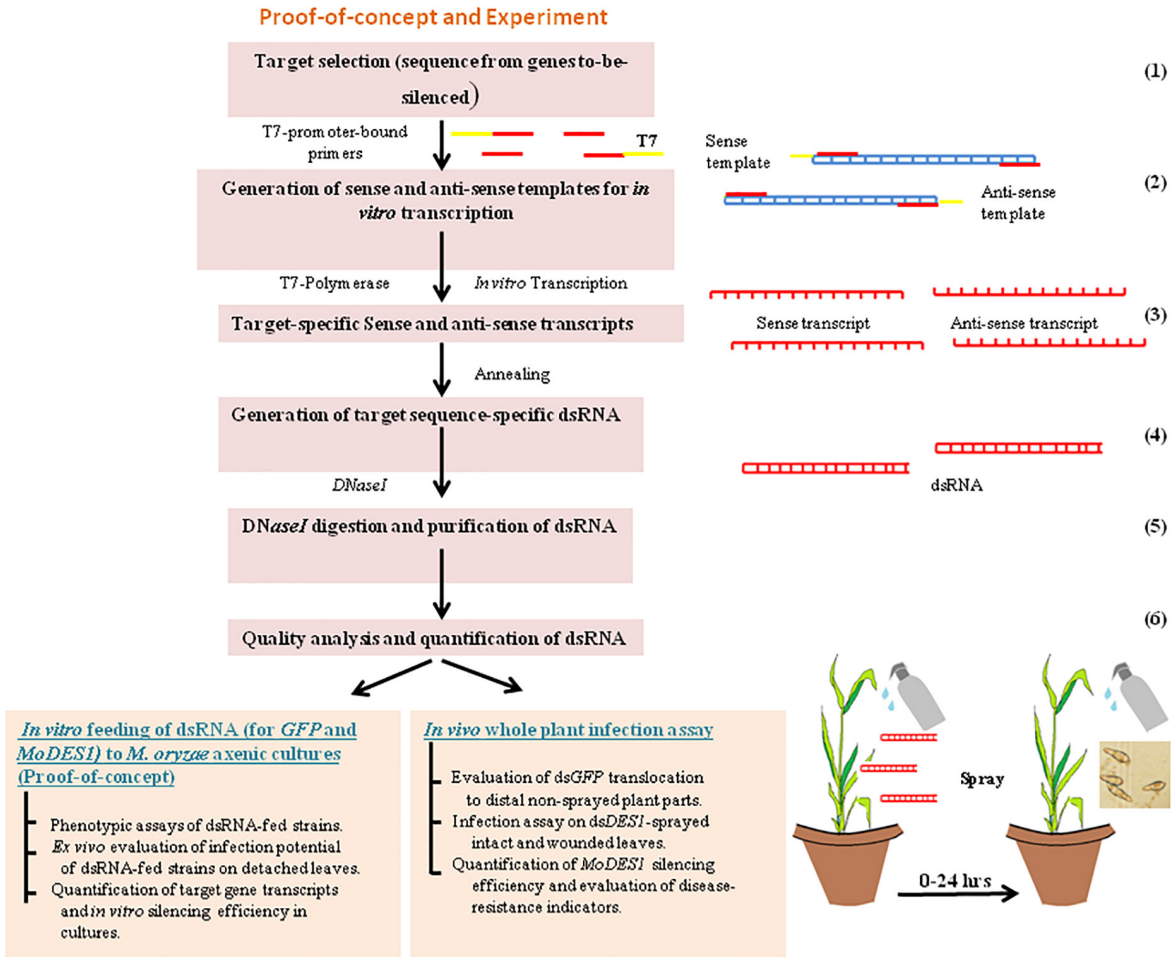

FIGURE 1 | Schematic flow of work. Schematic flow diagram for *in vitro* synthesis of dsRNA, and proof-of-concept representing its *in vitro* feeding into *Magnaporthe oryzae* axenic cultures, *in vivo* spray-application on rice plant, and infection assay. Numbers on the right hand side indicate the steps involved in the process.

50 nM of ds*DES1* by inoculating hyphal block and incubating it for 9 days, Spores were isolated from the culture. Then, the spore suspension was diluted and spreaded on ds*DES1*-supplemented CM plates, following the procedure as published (Guo et al., 2019). An individual colony was further sub-cultured onto a 50 nM ds*DES1*-amended CM plate and allowed to grow at 28°C. This dsRNA-fed strain was used for phenotypic assays and transcript analyses of *MoDES1*, to study the effects of ds*DES1* on the wild type upon its uptake. The GFP fluorescence of *M. oryzaeGFP* was visualized by growing it both on sterile slides layered with YEG agar media, and on 24-well culture plates containing YEG broth. YEG agar was aliquoted, 2 ml each in a sterile centrifuge tube, cooled down, and supplemented with 5 nM ds*GFP*. The dsRNA-mixed YEG medium was layered onto sterile slides and allowed to solidify. A 10 µl drop of a 1×10^5 spores/ml suspension was smeared along the length of the slide and incubated in the dark at 28°C for 2 days. In the liquid medium, a 10-µl drop of a 1×10^5 spores/ml suspension was added to each well containing 1 ml of YEG broth and 1 ml of 5 nM ds*GFP* [dissolved in diethyl pyrocarbonate (DEPC)-water]. The culture plate was allowed to rotate at 45 rpm for mycelia formation and dsRNA-uptake. Conditions for the microscopic visualization of GFP fluorescence will be described later in the "Histochemical staining and microscopy" section. In order to mimic the oxidative stress in the environment, 1 mM H_2O_2 was added to the culture media, 30 min before RNA extraction from the dsRNA-fed and untreated axenic cultures. This was performed to elicit the transcription of *MoDES1*, which expresses in response to oxidative stress. The RNA was then used for the quantification of *MoDES1* transcripts in the treated and untreated fungi.

Appressorium Penetration, Host Cell Colonization, and Infection Assay

An infection assay was performed on onion-peel, detached rice leaves and intact and abraded rice leaves, and 20 µl of a spore solution containing an equal number of spores (1×10^5 spores/ml) was drop-inoculated on the sterile hydrophobic surface of the onion epidermis. Epidermal peels were placed on clean glass slides, kept inside moist petriplates, and incubated

for 24 h at 28°C. Appressorium count was observed using a hemocytometer, and appressorium penetration was assessed following a published procedure (Chida and Sisler, 1987). The ease of cell-to-cell movement of invasive hyphae was determined by counting the infection units on onion epidermal cells and scoring them based on the penetration and branching pattern of invasive hyphae (IH) during *in planta* biotrophic growth. The percentage fraction of infection units showing 0, 1, and > 2-cell penetration was categorized considering 50 infection units in each case, with the experiment having three replicates; 10 μl drops of the 1×10^5 spores/ml suspension [dissolved in 0.02% V/V Tween 20 (Merck, Bangalore, India) and 0.25% W/V gelatin] were spot-inoculated on the adaxial surface of detached leaves (Jia et al., 2003) and rice sheath (Kankanala et al., 2007) of 21 day-old plants. Mean lesion length and area were measured using the Image J software, whereas infection severity in terms of lesion densities per 5 cm^2 leaf area (Liu et al., 2010) was evaluated by spray-inoculating 3-week-old plants (Akagi et al., 2015). Among other whole-plant infections, the leaves were either abraded and punch-inoculated (Park et al., 2016), or spot-inoculated. In both cases, 50 μl spore drops were inoculated on 4-week-old plant leaves, and drops were secured to leaf surface with adhesive tapes. The plants were maintained in a growth chamber with 27°C temperature and 90% relative humidity settings.

Reactive Oxygen Species Sensitivity Test, Extracellular Enzyme Assay, and Protoplast Release Assay

Hyphal blocks of uniform size, from the 7-day old fungal culture were sub-cultured onto CM plates amended with 0, 3, and 7 mM H_2O_2, and incubated for 10 days at 28°C. ROS sensitivity was determined from the mean diameter of the growing culture at 5 and 10 dpi, and in all cases three biological replicates were maintained. Extracellular laccase activity was measured in solid CM media, supplemented with a 0.2 mM 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate, while peroxidase activity was measured from 200 μg/ml Congo red (CR)-amended CM (Guo et al., 2019). The mean diameter of degradation halos was measured on 5 dpi and in triplicates. The laccase and peroxidase activity of the fungal culture filtrates was measured from the absorbance at 420 nm using 10 mM ABTS and 3 mM H_2O_2 (Chi et al., 2009). For the protoplast release assay, which is an indicator of cell wall integrity, mycelia were grown in CM, and protoplasts were isolated from 3-day-old culture (Chen et al., 2017). Observations were conducted after 90 min of lysis with a lysing enzyme, and the experiments were repeated thrice.

Ex vivo and *in vivo* Treatment and Spraying of Double-Stranded RNA on Rice Leaves

For initial optimization, the detached leaves were sprayed locally with 50, 150, and 300 nM of ds*GFP* 24 h prior to *M. oryzaeGFP* inoculation. Later, for checking of systemic nature, 300 nM of ds*GFP* was sprayed in local and distal regions of sandpaper-abraded leaves, as described (Koch et al., 2016). The first and second (lower) leaves sprayed directly with dsRNA were considered as local regions, while the third and fourth leaves (upper) were considered as distal unsprayed parts. In case of infection on the punch-abraded leaves, 20 μl drops of 300 nM ds*DES1* were spotted on abraded areas of the leaves and were secured with adhesive tapes. The same abraded regions, 24 h later, were inoculated with 20-μl drops of the spore suspension *via* syringes, without disturbing the tapes. Similarly, dsRNA treatments were performed for the intact leaves in whole plants, but without abrasion. For the whole plant leaf infection assays in the experimental sets, sandpaper-abraded leaves were sprayed with purified 300 nM ds*DES1*, 0, 12, and 24 h before fungal spray inoculation. While in set I he dsRNA and spore suspension were mixed together and sprayed simultaneously (0 h), in set II and III, dsRNA was sprayed 12 and 24 h, respectively, prior to infection.

Histochemical Staining and Microscopy

Fungal infection units were observed by staining the infected onion epidermal cells with a ready-to-use lactophenol blue (Sigma Aldrich, MI, United States) solution. The epidermal peels infected with conidia were stained with one drop of lactophenol blue for 15 min, destained with lactophenol until the excess stain got removed, and finally mounted with 60% glycerol before visualization. The infected rice sheaths were excised, and the epidermal layers of mid-vein were lactophenol-fixed and stained with 0.01% Aniline Blue (Chi et al., 2009) for the observation of callose plugs and secondary wall deposition in and the around primary infected cells. *In planta* ROS generation was visualized by staining the infected sheath epidermal cells at room temperature with a 1 mg/ml 3,3′-diaminobenzidine (DAB) (BioSB, CA, United States) staining solution prepared as per the instructions of the manufacturer. The sheaths were destained with a clearing solution (ethanol:acetic acid = 94:4 V/V) for 1 h and visualized under a differential interference contrast (DIC) filter. The infected cells and their neighboring cells were color-coded based on staining intensity, representing the level of H_2O_2 generation. The cell death assay involved overnight staining of the leaf sheaths with 0.01% trypan blue (HiMedia) at room temperature, followed by destaining in chloral hydrate for 24 h. The stained epidermal tissues were visualized in a bright field. All DIC and fluorescence images were taken using an Axio mRm camera coupled with a Zeiss Axio Imager A1 fluorescence microscope (Carl Zeiss, Göttingen, Germany). Fungal GFP fluorescence in the leaf lesions was observed under a fluorescein isothiocyanate (FITC) filter, and all the images were processed using the Zen Light Blue software. The mean intensity of GFP expressed by *M. oryzaeGFP* in the dsRNA- sprayed and unsprayed sets were documented considering leaves from at least three plants from each set in three independent experiments.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from both the dsRNA-fed fungal strain and DEPC water-treated wild type fungus [4 days post treatment (dpt)], and dsRNA-sprayed and unsprayed infected rice leaf-lesions 3, 4, 5, and 7 dpt. The fungus and infected leaf biomass were crushed using liquid nitrogen and taken forward for RNA extraction using RNeasy Plant Mini Kit (Qiagen, Germany), as recommended by the manufacturers. The RNA was treated with

RN*ase*-free DN*aseI* to remove traces of genomic DNA, assessed for quality and integrity, and further quantified by spectroscopy; 100 ng of RNA from local, distal-sprayed and unsprayed leaves was resolved on a 6% polyacrylamide gel (PAGE), and *in vitro*-transcribed ds*GFP* was kept as positive control. After the electro-transfer of the RNA onto a Ambion BrightStar positively charged nylon membrane, the membrane was hybridized with 150 ng of a non-radioactively labeled *GFP* probe (303 bp gel-purified PCR product). The primers used for PCR amplification of the *GFP* probe are listed in **Supplementary Table 2**. The labeling and detection of signals were performed using direct labeling reagents and CDP-Star (Amersham; GE Biosciences, United Kingdom) as per given instructions.

Reverse Transcriptase-PCR and qRT-PCR

First-strand cDNA synthesis was performed by reverse transcribing a 1 μg template RNA with RevertAid H Minus M-MuLV Reverse Transcriptase (NEB, Ipswich, Massachusetts, United States). Reactions, 50 μl, were set up in a thermal cycler programmed at 42°C for 1 h, followed by termination of the reverse transcription at 70°C for 5 min. For transcript quantification by qRT-PCR, 2 μl of cDNA was taken as template in Power SYBR Green PCR Master Mix (Applied Biosystems, United Kingdom) consisting of gene-specific forward and reverse RT primers (**Supplementary Table 2**). Each reaction was carried out in triplicates, and the C_t values were normalized against the internal housekeeping control *MoACTIN* of the wild-type B157 strain. In the case of RT-PCR, 50 μl reactions were set up, each containing forward and reverse RT primers, deoxynucleotide triphosphates (dNTPs), 10 × reaction buffer, and 2.5 units of Taq DNA Polymerase (NEB, Ipswich, MA, United States). For molecular the analysis of fungal growth, the cDNA synthesized from the RNA extracted from infected leaf lesions were first diluted to different concentrations and calibrated using the rice housekeeping *OsACTIN*. Then, the amounts of cDNA corresponding to equal band intensity were used for the RT-PCR analysis of both the rice 25S rRNA and the fungal 28S rRNA. This was used to ensure that comparison of fungal growth was done in equal amounts of infected leaf tissue, from the experimental and control sets of rice plants. Standard PCR settings were maintained.

Statistical Analysis

Each experiment was repeated at least three times independently. The statistical analyses were performed with the GraphPad Prism 8 (GraphPad, San Diego, CA, United States) software. Student's *t*-test and pairwise comparison were performed by Tukey's test with Bonferroni correction for the determination of significant outcomes. The alpha level was set at 0.05 in all cases.

Biosafety Measures

All the relevant experiments from this study that involved the use of the phytopathogenic strain of *M. oryzae*, foliar spray of dsRNA, and infection assays of the rice plants were conducted following the biosafety norms as per Institutional Biosafety Committee (IBSC). To prevent environmental contamination through the dissemination of any of the above-mentioned agents, the experimental materials were handled under confined environment and laboratory conditions, restricted to growth chambers and the green house. Experimental wastes and unused materials were autoclaved and discarded in the end.

RESULTS

BLAST Analysis of Sequences for Target Specificity Determination

Double-stranded RNA-mediated gene silencing is a target mRNA sequence-specific phenomenon. Hence, the *MoDES1* and *GFP* dsRNA sequences chosen as target were analyzed for selective specificity toward only the target gene and target organism. BLASTn analysis was done for the 303 bp and 300 bp target regions (**Supplementary Figure 1**) against *M. oryzae* and *O. sativa* genomic sequences. The *GFP* sequence showed significant similarity with neither the fungus nor the plant host. It only showed a 100% similarity with the *GFP* sequence from pCAMBIA1302. Similarly, the *MoDES1* target did not show any significant similarity with the *indica*, *japonica*, and *javanica* groups of rice. The BLAST analysis of *MoDES1* target across *M. oryzae* genome showed 100% sequence identity with only MGG_04163, which was the intended gene target. Three other *M. oryzae* strains, MZ5-1-6, LpKY97, and B71, showed 99.64% similarity with the query but had primary hosts other than rice (**Supplementary Table 1**). However, the chosen sequence for dsRNA generation would not lead to unspecific silencing in the B157 and 70–15 strains that are known to infect rice primarily. Ascomycetous fungi, such as *Chaetomium globosum*, *Podospora anserina*, *Neurospora crassa*, *Fusarium* sp., and *Botrytis cinerea*, have DES1 homologs and share a sequence similarity of greater than 44% with *MoDES1* (Chi et al., 2009). Sequences for the homologs were obtained from the Locus information and compared with the *MoDES1* target, and no significant similarity was observed (**Supplementary Table 1**). To this end, the target regions chosen from *MoDES1* and *GFP* were found to be specific only toward the intended *GFP* gene and MGG_04163, with no predictable off-targets in rice and *M. oryzae* used in the experiments.

Double-Stranded RNA Synthesis and Dosage Optimization

A proof-of-concept experiment was performed with an *M. oryzae-GFP* reporter system for the demonstration of SIGS in rice. Similar to the previous reports, out of the many parameters that determine the efficacy of RNAi phenotype, the optimum dosage of dsRNA or asiRNA, abundance of the target gene, size of the target dsRNA, and sequence complementarity of the designed dsRNA and its delivery method are the most crucial (Bennett et al., 2020). The 303 bp and 300 bp target regions (**Supplementary Figure 1**) chosen from *GFP* and *MoDES1* were used, respectively, as templates for ds*GFP* and ds*DES1* generation. In each case, an average concentration of 30 μg/μl

of purified dsRNA was obtained from one set of transcription and annealing reaction (**Supplementary Figure 2**). It was demonstrated that 50 nM of asiRNAs, when supplemented in media, could induce silencing of the target *M. oryzae* gene (Guo et al., 2019). Hence the efficiency of the chosen target ds*GFP* was assessed by *in vitro* treatment of *M. oryzaeGFP* spores with 50 nM of ds*GFP*. The microscopic evaluation of the mycelia (**Figure 2A**) revealed a 76% reduction in the fluorescence intensity of the dsRNA-treated mycelia compared with the DEPC water-treated controls 3 dpt. The specificity of the observed *GFP*-silencing phenotype was further confirmed using a positive control, where ds*DES1* was used for the *in vitro* treatment of *M. oryzaeGFP*. There was no observable reduction in fluorescence in this set, thereby attributing the formerly observed silencing effect to sequence-specific *GFP* knockdown *via* ds*GFP*. Since the effective dosage of dsRNA can often vary across organisms and delivery methods, optimization was performed by spraying 50, 150, and 300 nM of *in vitro* synthesized *GFP*-specific dsRNA (ds*GFP*) onto rice leaves, followed by *M. oryzae GFP* inoculation after 24 h. The silencing effect, in all the cases, was observed by measuring the mean intensity of GFP fluorescence in the inoculated *GFP*-transformant to optimize maximum silencing effect. It was observed that although the lower concentrations were able to induce silencing, the effect did not last beyond 36–48 h post treatment (data not shown). On the contrary, the effect of 300 nM ds*GFP* was perceived even after 4 days of application. During a 0–5 days time frame, the minimum intensity of GFP was observed 96 h post dsRNA treatment (hpt) or 72 h post inoculation (hpi) (**Figure 2B**), after which the effect of gene silencing started to reduce. Since 300 nM of the selected ds*GFP* was able to not only induce but sustain silencing for a longer period of time on the treated rice leaves,

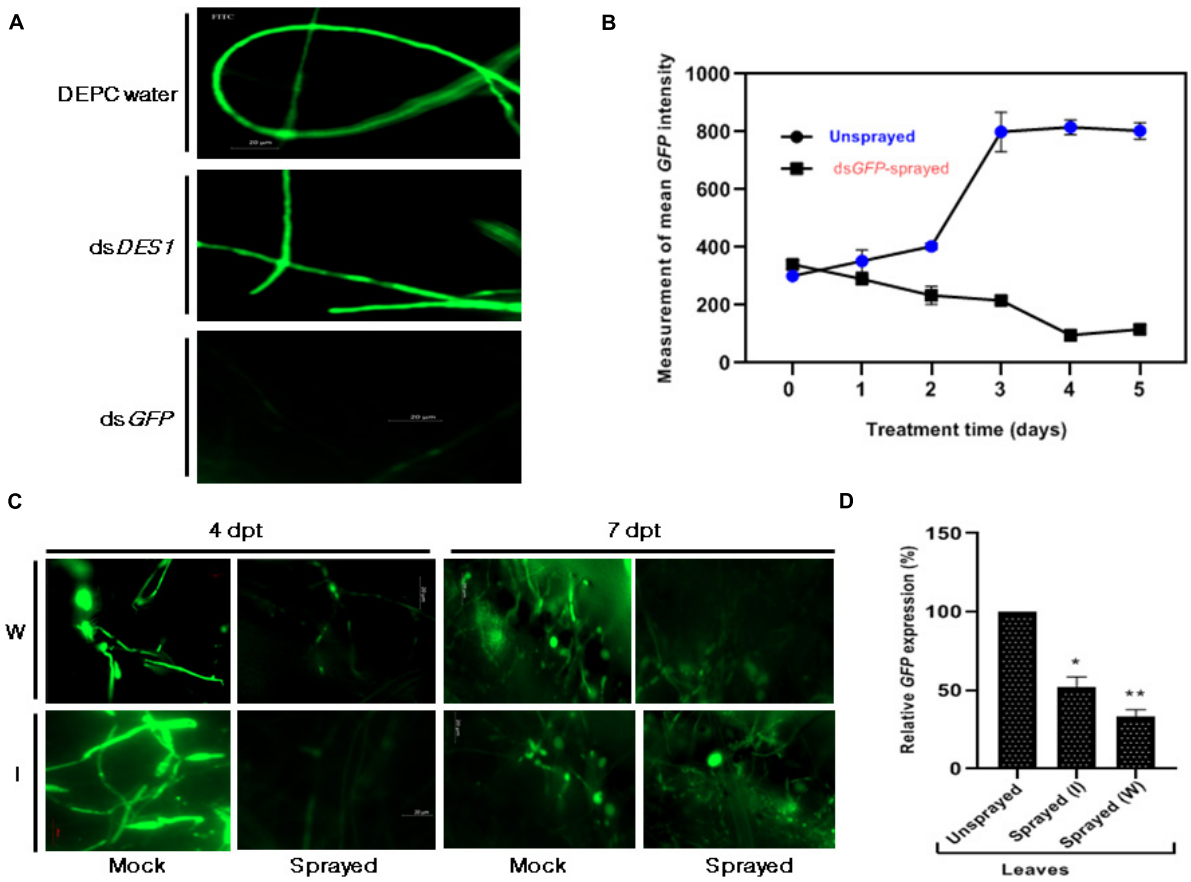

FIGURE 2 | Effect of ds*GFP* on *M. oryzae GFP*. **(A)** Microscopic assessment of GFP intensity 3 dpt, among axenic cultures of *M. oryzae GFP* fed with and without ds*GFP*. While the culture that was not fed with any dsRNA (DEPC water- treated) was kept as the negative control, the culture that was fed with ds*DES1* (non-specific for *GFP*) served as the positive control. **(B)** Time-dependent fluorescence trend of *M. oryzaeGFP* grown on 300 nM ds*GFP*-sprayed leaves with respect to the unsprayed set, across 0–5 days. **(C)** Fluorescence of *M. oryzaeGFP* on wounded (W) and intact (I) rice leaves sprayed with ds*GFP*, 24 h prior to infection. GFP fluorescence was observed under an FITC filter of AxioCam mRm 0 [4 h post treatment (hpt)], 4, and 7 dpt, and fluorescence emitted by *M. oryzaeGFP* on Tris EDTA (TE)-sprayed leaves were kept as mock. TE was sprayed 24 h prior to infection, as replacement for ds*GFP*. Comparisons were made between the sets, at similar time points, with observations made from at least 10 leaves for each set. The experiment was repeated thrice, and the results were compared independently. **(D)** Relative abundance of *GFP* transcripts in dsRNA-sprayed wounded and intact leaves, with respect to TE-sprayed leaves, at 4 dpt. *OsACTIN* was used as internal control for normalization. The experiments were repeated thrice, each time with three biological replicates. Error bars represent standard deviation ($n = 3$), where * and ** denote statistical significance at *the* $P \leq 0.05$ and $P \leq 0.01$ levels, respectively.

and considering that the abundance of our target pathogenicity gene *MoDES1* is unknown, the 300 nM dsRNA concentration was used for spraying and infection-related experiments to ensure effective RNAi.

SIGS Is More Efficient and Sustained on Wounded Leaves

Ribonucleic acid molecules, being relatively more unstable than DNA or other biomolecules, have a limited longevity on plant surface. It has been shown that leaf cuticle can act as a barrier for uptake, hence, high-pressure spraying or abrading can ensure robust silencing (Dalakouras et al., 2016). Thus, we further wanted to check if removing physical barriers by sandpaper-mediated abrasion (Bennett et al., 2020), followed by spraying, would affect the longevity and efficiency of gene knockdown. Based on our previous observations on the detached leaf (**Figure 2B**), the 96 hpt time point was fixed for assessment and quantification of the silencing effect of ds*GFP* sprayed on both intact and wounded rice leaves. The microscopic observations revealed that the reduction in fluorescence beyond 4 dpt was more pronounced and sustained in the wounded ds*GFP*-sprayed sets (**Figure 2C**). **Figure 2D** further revealed a higher silencing efficiency in the wounded, dsRNA-sprayed leaves. The quantitative analyses showed that the relative abundance of the transcripts was relatively lower in the intact leaves, with 48% silencing, in contrast to the 67% knockdown observed in the case of the wounded leaves.

Sprayed dsRNA Can Translocate and Assert Silencing in Distal Plant Parts

The efficiency of SIGS largely relies on its systemic nature, i.e., the ability of RNA signals to travel from the sprayed region (local) to the unsprayed (distal) parts of the plant. To determine if the dsRNA can traverse to unsprayed regions, the detached leaves of 2-week-old rice seedlings were sprayed locally and distally with 300 nM of ds*GFP* or Tris-EDTA buffer (mock) and given a lag period of 24 h to facilitate the uptake of dsRNA by the leaf tissue. The leaves were then inoculated with 5×10^5 spores/ml of *M. oryzaeGFP*. The microscope images showed relatively more intense fluorescence in the mock-treated infected leaves (**Figure 3A**) compared with the directly and distally ds*GFP*-treated leaves. Based on this finding and some previous reports on *Fusarium graminearum* (Koch et al., 2016) indicating silencing in distal parts of the leaves, whole plant infection experiments were conducted to explore the effectiveness of SIGS in *M. oryzae*. DsRNA specific to *GFP* were sprayed

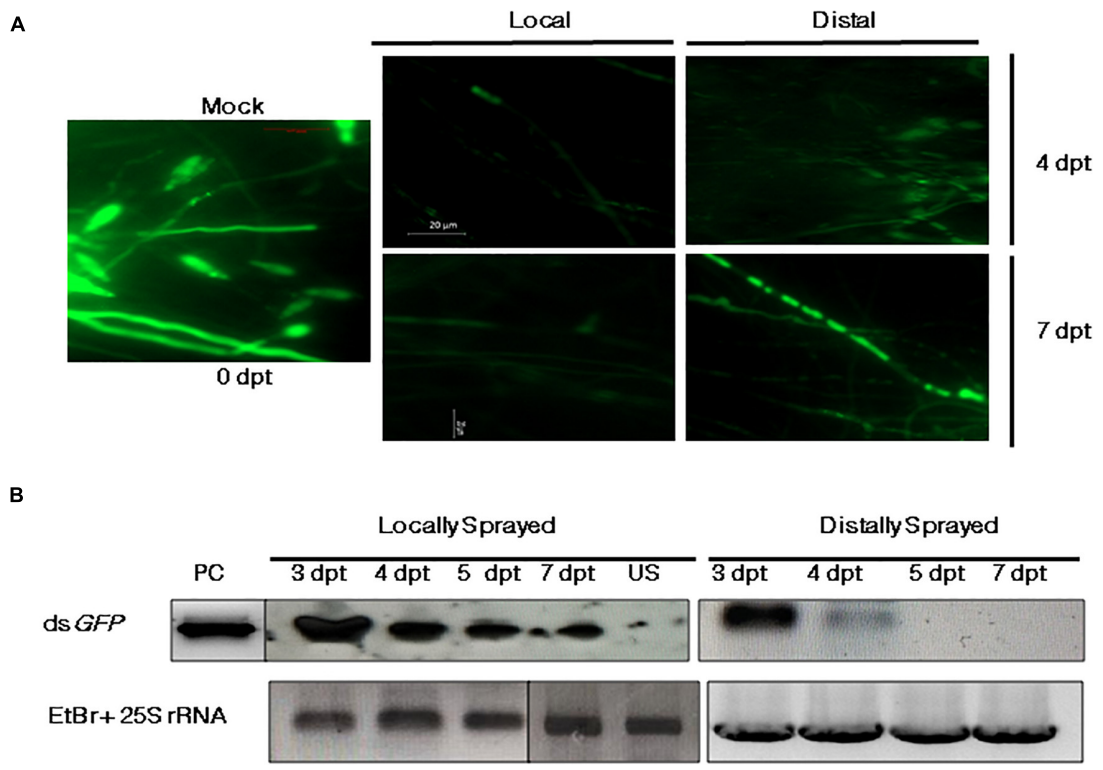

FIGURE 3 | Systemic silencing activity of dsRNA and its accumulation in the local (sprayed) and distal (unsprayed) leaves. **(A)** Fluorescence microscopy of wounded *M. oryzaeGFP*-inoculated leaves that were either sprayed with either TE (mock) or ds*GFP* in local and distal regions. Mean fluorescence intensity of GFP from approximately five plants (10 lesions) of each set was monitored 4 and 7 dpt. **(B)** Northern blot profile for *GFP*-specific dsRNA. The longevity and accumulation of RNA signals were assessed 3, 4, 5, and 7 dpt on locally and distally sprayed leaves. The total RNA isolated from unsprayed (US) leaves was run as negative control. *In vitro*-synthesized and purified ds*GFP* was used as the positive control (PC), and rice 25S rRNA was kept as the loading control. Sense *GFP* probe with the same sequence as the parent ds*GFP* and PCR-purified rice 25S rRNA (primers listed in **Supplementary Table 2**) were used for hybridization.

onto the wounded leaves to ensure effective silencing. The RNA extracted 4 dpt from the direct and distally sprayed leaves was assessed for the presence of spray-supplied 300 bp ds*GFP* by northern blotting. The results suggested the presence of ds*GFP* in both locally and distally treated regions. While in the directly sprayed parts dsRNA was detectable up to 7 dpt, in the unsprayed distal regions, dsRNA was detected up to 4 dpt. The accumulation pattern of ds*GFP* in the local and distal leaves of the dsRNA-sprayed plants was aligned with the time dependent reduction of silencing phenotype in the sprayed leaves, as shown in **Figure 3A**.

Conidiation and ROS-Sensitivity Got Negatively Affected in ds*DES1*-Treated *M. oryzae*

MoDES1 is a novel gene encoding a hypothetical protein that was found to be involved in suppression of PTI in rice *via* host-derived ROS-scavenging activity. In a previous report, it has been functionally characterized and found to have homologs in other members of ascomycetes (Chi et al., 2009). Targeted gene silencing needs to be highly specific; hence, the selected target region of 300 bp is aligned with the genomic sequences of both rice and fungal members that possess a DES1 (**Supplementary Table 1**) homolog. The BLASTn and BLASTp results showed no significant homology, with no prediction of off-target effects. The *in vitro*-synthesized dsRNA was fed to the wild-type fungus, which after two generations of dsRNA treatment, showed a slight reduction in growth diameter, as compared with the untreated fungus (**Figures 4A,E**). The axenic cultures of the phenotypically distinguishable dsRNA-fed strain demonstrated an approximately 86% reduction in the *MoDES1* transcripts (**Figure 4B**). Although conidiation rate was significantly higher, the treated strain produced a majority of short and broad deformed conidia (**Figures 4C,E**). Besides, an impediment was noted in appressorial penetration with respect to the untreated strain, although the rate of appressorium production was comparable in both strains (**Figures 4D,E**). In order to check ROS-scavenging activity, the treated and untreated strains were grown in media supplemented with ≥ 2 mM H_2O_2. The dsRNA-fed strain showed a marked reduction in culture diameter in all the concentrations 5 and 10 dpi (**Figure 4A** and **Supplementary Figure 3**), depicting the apparent reduction in ROS scavenging potential. As per previous studies conducted on the *MoDES1* mutant, ROS sensitivity was due to impaired extra-cellular enzyme activity. Hence, enzymatic assays for laccases and peroxidases that are known to neutralize some free radicals were conducted. The dsRNA-fed strain that previously showed significant silencing of *MoDES1* was observed to demonstrate a pronounced reduction in the aforementioned enzymatic activities in both the solid and liquid media (**Figures 5A,B**). This corresponded with the reduced ROS-scavenging activity that has been seen previously. The cell-wall integrity test, in terms of protoplast release assay, did not show any significant defects in cell wall integrity as a result of *MoDES1* silencing (**Supplementary Figure 4**).

Ds*DES1*-Feeding of *M. oryzae* Impaired Its Biotrophic Growth and Virulence

Based on previous studies, *M. oryzae* has been reported to use *DES1* as a suppressor of basal defense for survival within rice. Also, it has an active role in the extension of the IH inside host cell and its colonization, which is essential for pathogenicity. As a proof-of-concept for the *in vivo* whole plant spray experiments, the *in vitro* impacts of ds*DES1* on the *M. oryzae* wild-type strain were analyzed. Microscopy results of the onion peel assay showed that after 24 h, germination and IH development was defective in the ds*DES1*-fed strain as compared with the wild type. Most of the IH were thick, stout, and short, and confined to the primary host cell (**Figures 5C,D**), indicating impairment in the colonization of epidermal cells similar to the *DES1* mutant (Chi et al., 2009). Our *in vitro* dsRNA feeding experiment indicated that the strain of *M. oryzae* that was fed with ds*DES1* for two consecutive generations showed significant downregulation of *MoDES1*. Therefore, we further wanted to check if its virulence got affected because of dsRNA feeding. The detached leaves of 2-week-old rice seedlings were spot-inoculated with both the untreated and dsRNA-fed strains, and were observed for symptoms 5 dpi. While average lesion length in the case of the wild type was 12 mm, it was only 4.6 mm in the case of the leaves inoculated with the dsRNA-fed strain. Therefore, on an account of the silencing of *MoDES1*, there was almost a 62% reduction in lesion length (**Figures 5E,F**). As suggested by our previous results, this reduction in virulence could be attributed to the defects in IH and host colonization.

Fungal ds*DES1* Uptake *via* Plant Cells Imparted Higher Disease Resistance

The dsRNA sprayed on the plant can be uptaken either directly by the fungus on the surface *or* via the plant cell post penetration. Since our results indicated that the silencing effect shows greater longevity in the wounded plants, it was assessed further if the fungus got silenced better *via* the plant cell. Four sets were kept, whereby no lag period was given in set I, and both the dsRNA and fungal spores were mixed together and sprayed. In set II, ds*DES1* was sprayed on rice leaves 12 h prior to spray inoculation with the fungal spores; in set III, dsRNA was sprayed 24 h before the fungal inoculation to facilitate dsRNA uptake *via* the plant cells. Set IV was the control group where no ds*DES1* was sprayed on the leaves before fungal infection. It was observed that in the plants where the dsRNA was sprayed 24 h before wild-type spray inoculation (set III), disease severity was around 60% less as compared with the set IV that was inoculated with wild type spores without any dsRNA treatment (set IV). On the contrary, when the wild-type spores were mixed together with dsRNA before spray-inoculation (set I), there was only a 25% reduction in symptoms, which was not significantly less than the untreated control (IV). The symptoms were milder in set II (where dsRNA and spores were sprayed 12 h apart) than in set I (**Figure 6A**). As studied by DAB staining of sheaths, the generation of *in planta* ROS indicated that the maximum number of heavily stained cells were observed in the plants that were

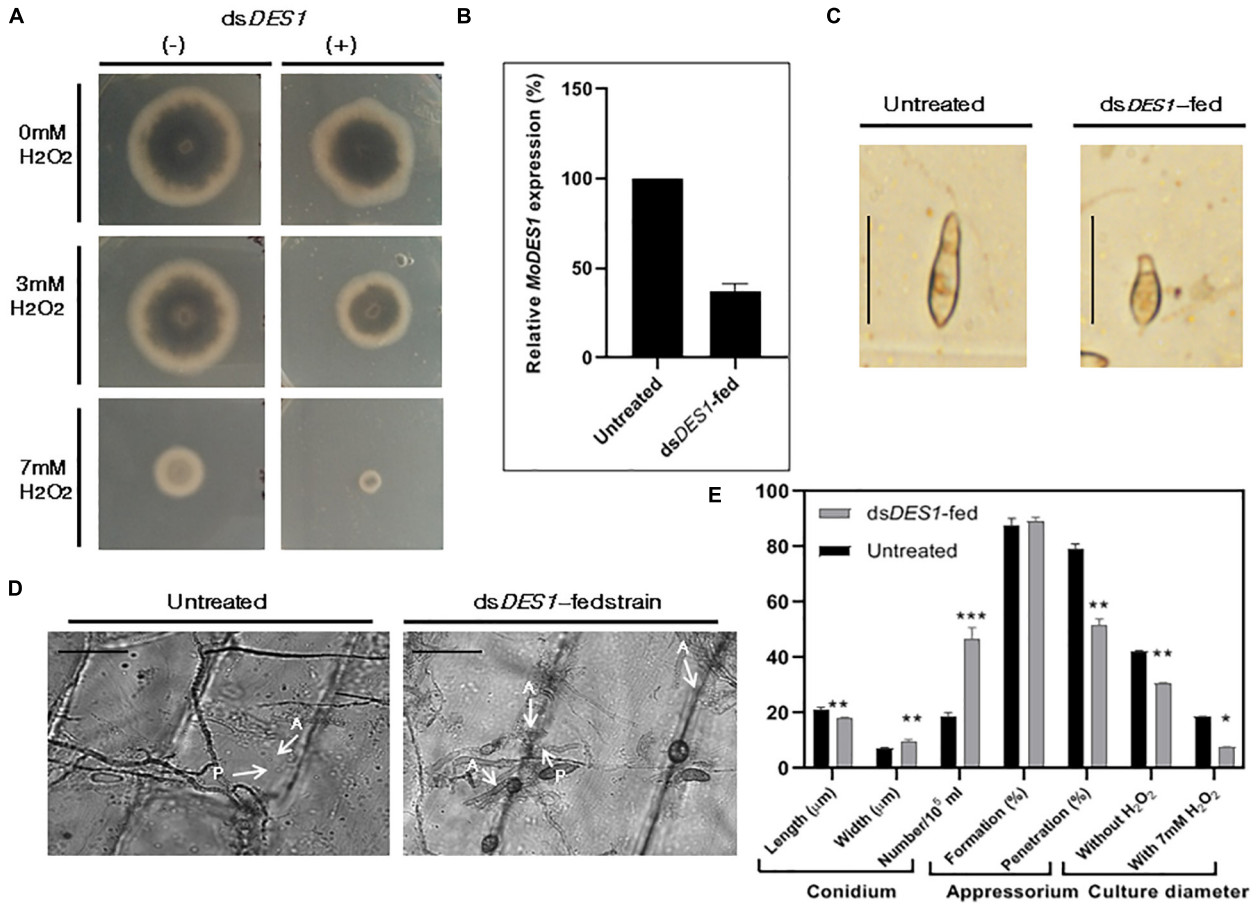

FIGURE 4 | Assessment of ROS-scavenging activity, morphological traits, and target transcripts in the *in vitro* ds*DES1*- fed strain. **(A)** Reactive oxygen species (ROS) sensitivity assay of ds*DES1*-fed and untreated strains of wild-type B157. The ds*DES1*-fed and ds*DES1*-untreated strains were grown on CM plates with 0, 3, and 7 mM H_2O_2, and average growth diameter of the cultures was recorded from 5- to 10-day-old plates in triplicates. The experiment was repeated twice. **(B)** Relative *MoDES1* expression in the ds*DES1*-fed strain with respect to the untreated strain 72 hpt. The reactions were set up in triplicates, and *MoACTIN* was used as internal control. **(C)** The dominant morphological deformity in a major fraction of conidia isolated from the ds*DES1*-fed strain. For size reference, a 20-μm scale has been used. **(D)** Penetration potential of germinated appressoria among the untreated and ds*DES1*-fed strain, on onion epidermal peel. A 50-μm scale has been used for size- reference. Three replicates were used, and at least 50 conidia were assessed in each category. **(E)** Comparison of phenotypic parameters between untreated and ds*DES1*-fed strain. Error bars represent standard deviation, and *, **, *** denote statistical significance at the $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ level, respectively.

sprayed with dsRNA first and then inoculated with fungal spores. In the plants where dsRNA and spores were mixed together and sprayed, the maximum number of cells showed medium staining or no staining, with lesser number of heavily stained cells (**Figure 6B**). This indicated lower levels of ROS-mediated resistance in the later. While in the first case, a lag period was given to ensure that the dsRNA would be uptaken by the host cell first, in the second case, the fungal spores were directly mixed with the dsRNA to ensure it takes up the dsRNA first. As our results indicated, the inhibitory effect of ds*DES1* was more pronounced, leading to higher disease resistance, when the dsRNA was sprayed prior to fungal infection, allowing for a lag period of at least 12 h. Taken together with the finding from wounded leaves, it can be assumed that processing of sprayed dsRNA by the plant might have an important role in amplifying the sRNA signals.

In planta SIGS of *MoDES1* Confers Resistance Against *M. oryzae* via PTI Responses

After assessing the functionality of SIGS both *in vitro* and *in vivo*, we wanted to explore the efficiency of spray-induced downregulation of *MoDES1* in developing resistance against fungal blast disease in whole plants. For this, Leaves of the susceptible CO-43 rice variety were sprayed with ds*DES1* and inoculated with a 1×10^5 spores/ml solution 24 h later (**Figure 6C**). The total RNA isolated 3 dpi from the dsRNA-sprayed leaves was analyzed for *MoDES1* transcripts, and it was found to have only 35% of relative abundance of what was observed in unsprayed plants. The ds*DES1*-sprayed leaves that were spray-inoculated showed a relatively lesser number of lesions/cm^2 of infected leaves as compared with the control

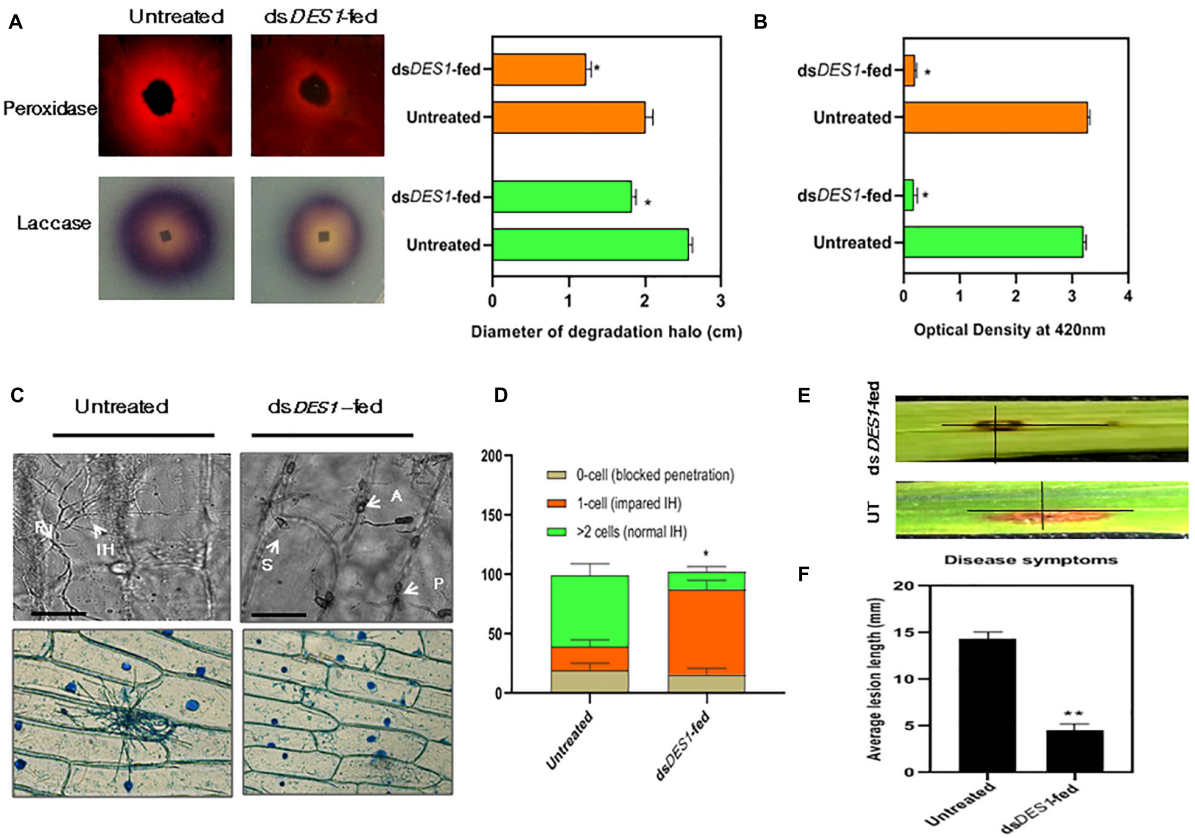

FIGURE 5 | Extracellular enzyme activity and virulence potential of ds*DES1*-fed strain. **(A)** The extracellular laccase and peroxidase activity of the *in vitro*-ds*DES1*-fed strain on CM plates supplemented with ABTS and Congo red (CR), respectively. The average diameter of halo or discoloration indicator of substrate breakdown was measured in triplicates from 5-day-old plates. **(B)** Activities of laccases (green) and peroxidases (orange) in culture filtrates of the untreated and ds*DES1*-fed strain. **(C)** The upper panel indicates the 40 × -magnified differential interference contrast (DIC) image of onion peel infected with untreated and ds*DES1*-fed strain. The white arrows indicate the spore, S; appressoria, A; point of penetration, P; invasive hyphal, (IH) growth. The lower panel shows a 10 × -magnified bright field image of Lactophenol blue-stained fungal infection units grown on onion epidermal cells. This is indicative of the cell-to-cell movement of IH and host colonization. **(D)** Graphical representation of the fraction of infection units that showed differential ease of host-colonization, beyond the primary site of penetration. The potential for cell-to-cell movement of IH was color-coded based on the number of cells they penetrated (0– > 2). At least 50 different infection units were observed for each set, and the experiment was repeated thrice. **(E,F)** Spot inoculation of detached rice leaves with spores isolated from the *in vitro* ds*DES1*-fed strain and untreated wild-type strain. Average length of lesion was measured for each set with approximately 50 leaves (≈ 100 lesions). The error bars represent ± SD, and * and ** denote statistical significance at the $P \leq 0.05$ and $P \leq 0.01$ levels.

Tris-EDTA-treated (mock), spore-inoculated leaves. Similar results were obtained when the dsRNA-pre-treated leaves were punch- inoculated, whereby the area of lesions got drastically reduced (**Figures 6D,E**), with a notably reduced load of fungal biomass (**Figure 7A**). The fungal biomass was semi-quantitatively measured from the fungal specific 28S rRNA, with respect to the rice 25S rRNA, in both the sprayed and unsprayed infected leaves. When analyzed from the same amount of infected leaf tissue, the relative expression of 28S rRNA, both 6 and 8 dpi, was found to be lower in the sprayed set (**Figure 7B**). Since *MoDES1* is known to suppress PTI, we wanted to check if the resistance developed *via* sprayed ds*DES1* was due to innate host defense responses. The histochemical DAB and aniline blue staining of infected sheath sections showed that the host-derived ROS generation (**Figure 7C**), lignin deposition, and callose plug formation (**Figures 7D,E**) were more in the case of the dsRNA-sprayed sheaths. The microscopic analysis of dsRNA-sprayed infected sheaths showed that cell death response was at par with the resistance phenotype, as depicted by the trypan blue staining (**Figure 7F**). This corroborated with previous observations, as cell death is often a manifestation of mild HR, which is part of PTI in its mild form. The reduction in disease severity in the ds*DES1* spray-treated set could, hence, be attributed to the reduced fungal load observed in the infected leaves.

In planta Silencing of *MoDES1* Led to Pathogenesis-Related Gene Induction in Double-Stranded RNA Treated Plants

The phytohormone salicylic acid-mediated pathway in the hemibiotrophic rice-blast pathosystem is central to the defense armor of the plant. Apart from early PTI responses, such as ROS-burst and callose deposition, the induction of defense-related PR genes on account of increased endogenous SA levels

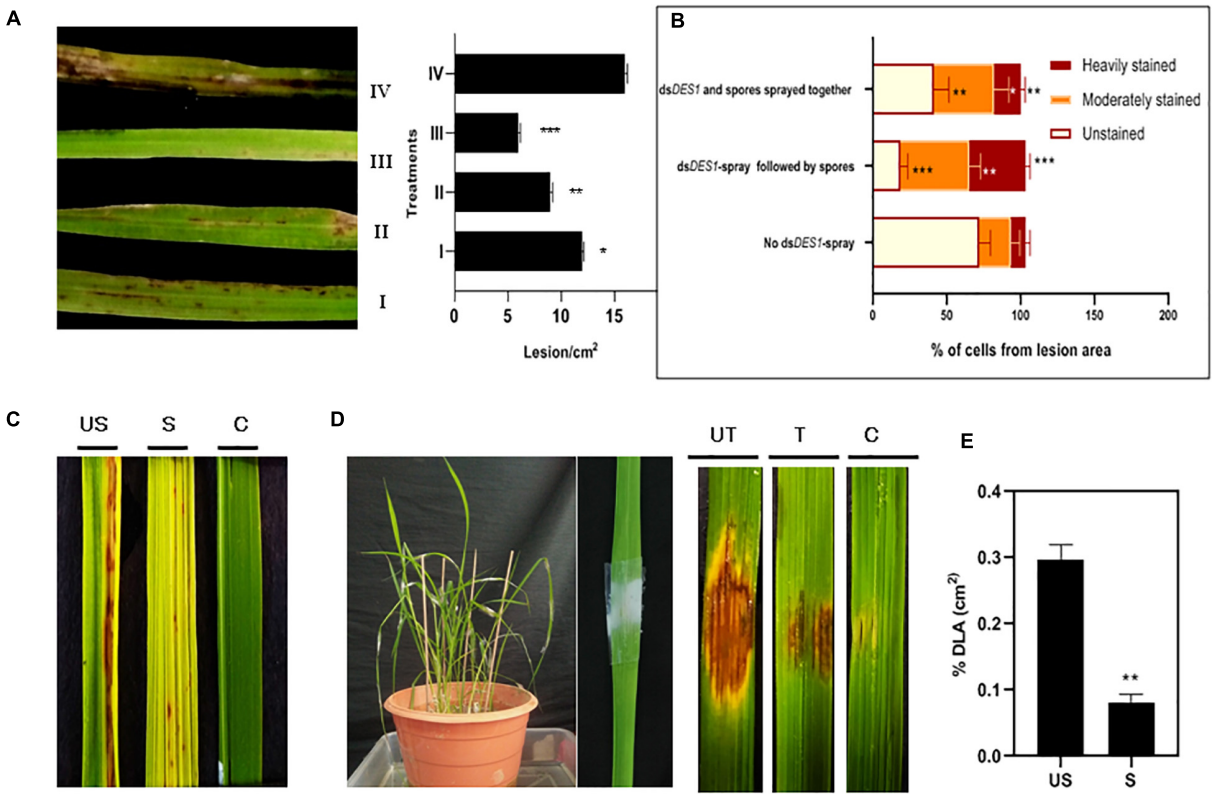

FIGURE 6 | *In vivo* infection assay of ds*DES1*-sprayed plants. **(A)** The left panel shows spray-infected rice leaves that were sandpaper-abraded (to reduce the mechanical barrier) and sprayed with 300 nM ds*DES1* (III) 24 h prior, (II) 12 h prior, or (I) 0 h prior (spores and ds*DES1* were mixed together before spraying) to fungal inoculation. The unsprayed but fungus-inoculated set (IV) was used as control. The right panel indicates the relative infection severity for each treatment based on lesions/cm^2. **(B)** DAB staining of lesions isolated from treatment sets I, III, and IV, representative of counter-infection host-derived ROS generation assay. The level of ROS generation was color-coded, and the fraction of cells representing each color was categorized. **(C)** Spray infection assay of ds*DES1*-sprayed (S) and unsprayed (US) leaves. Leaves that were neither dsRNA-sprayed nor spore-inoculated, were used as control **(C)**. **(D)** Punch inoculation-mediated infection assay for ds*DES1* drop-treated (24 h prior) rice leaves. Leaves that were simply punched and inoculated with Tween 20-Gelatin mixture without spores or ds*DES1* were kept as control. **(E)** Disease severity in ds*DES1*-sprayed and unsprayed leaves expressed in terms of% diseased leaf Area (DLA). For infection related experiments, observations were performed on at least 50 leaves, and% DLA was assessed using the Image J software. Error bars represent standard deviation ($n = 50$); where *, **, and *** denote statistical significance (Student's *t*-test) at the $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$ levels, respectively.

is a crucial indicator of a robust SAR response. *OsPR1a* is one such marker gene that has generally been used to assess defense responses in rice. Hence, its expression levels were checked in the dsRNA-treated and untreated infected (punch-inoculated) plants 2, 3, and 4 dpi. The semi-quantitative qPCR (**Figure 8A**) showed that 3 dpi, the ds*DES1*-treated leaves showed a greater accumulation of *OsPR1a* transcripts. The quantitative real-time PCR exhibited that, in comparison with the internal housekeeping *OsACTIN*, 2 dpi, the untreated plants showed a 2.5-fold change in the *OsPR1a* transcript, while there was a 64.8-fold change in the treated plants. While 3 dpi, the *OsPR1a* mRNA had twofold upregulation in the ds*DES1*-treated plants, 4 dpi, the relative expression of *PR1a* was found to be almost threefold higher in the sprayed set with respect to their corresponding untreated sets (**Figure 8B**). These observations were in support of the argument that the mild necrotic responses in the sprayed plants were a result of SAR, which was a manifestation of the sustained upregulation of PR gene.

DISCUSSION

The prerequisite for the induction of RNAi is a dsRNA that bears complementarity with the gene targeted for silencing. Besides this, the efficiency of gene knockdown depends on additional factors such as size of the target region, basal expression of the gene, method of dsRNA delivery, and the system in which the induction is intended. Often, the dosage of dsRNA and its treatment technique for effective silencing vary among target plants, pathogens, and genes (based on their secondary siRNA signal amplification efficiency and target transcript abundance) (Das and Sherif, 2020). Our optimization studies, using a GFP-reporter system, revealed that the *in vitro* feeding of *M. oryzaeGFP* with 50 nM naked dsRNA through culture media could successfully induce the silencing of the target *GFP* transgene in the fungal mycelia (**Figure 2A**). We also found that the silencing effect of sprayed ds*GFP* on rice leaves could be more pronounced and lasted up to more than 4 dpt when the amount of dsRNA sprayed was increased to

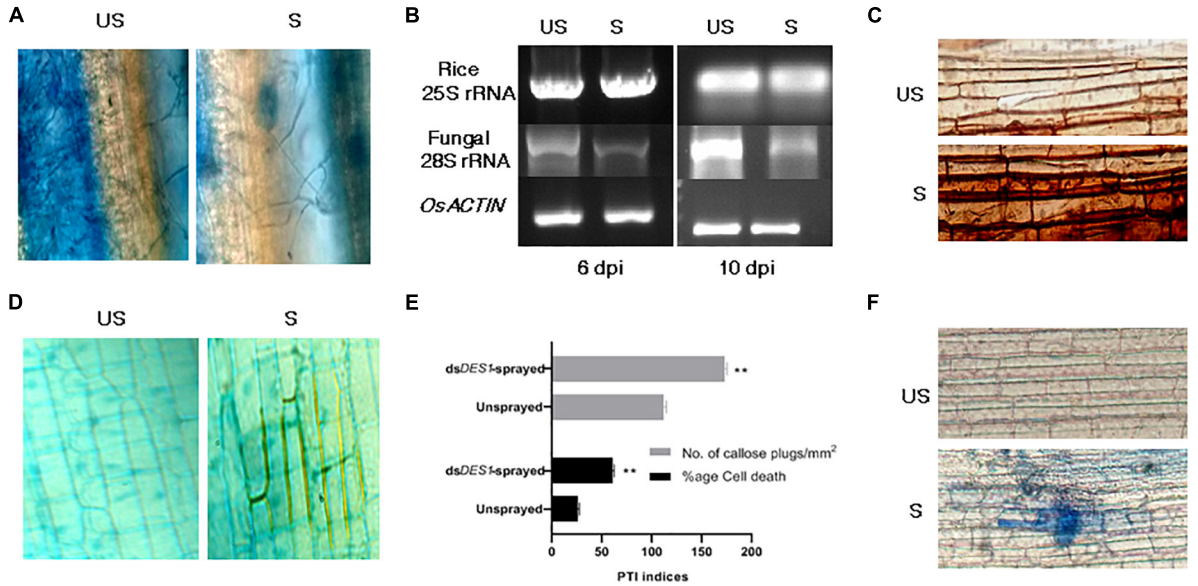

FIGURE 7 | Evaluation of fungal growth and defense parameters of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). **(A)** Qualitative assessment of fungal growth. Lactophenol blue staining of fungal mycelia observed in infection lesions from ds*DES1*-sprayed and unsprayed leaves. **(B)** Semi-quantitative reverse transcriptase PCR for *Mo*28S rRNA that is representative of fungal biomass in sprayed and unsprayed infected leaves. While *Os*25S rRNA was the growth control from rice that was used for comparison, *MoACTIN* served as the internal control. **(C)** DAB assay. Host-derived ROS generation in infected cells from ds*DES1*-sprayed and unsprayed leaf-sheaths was visualized by DAB staining. **(D)** Secondary wall deposition around primary infected cells and their neighbors to curb disease progression *via* aniline blue staining. **(E)** Callose plug formation (aniline blue staining) and percentage of cell death (trypan blue staining) in ds*DES1*-sprayed and unsprayed infected rice cells. **(F)** Trypan blue staining of sprayed and unsprayed sheath cells undergoing necrosis. The error bars represent standard deviation, and ** denotes statistical significance at the $P \leq 0.01$ level.

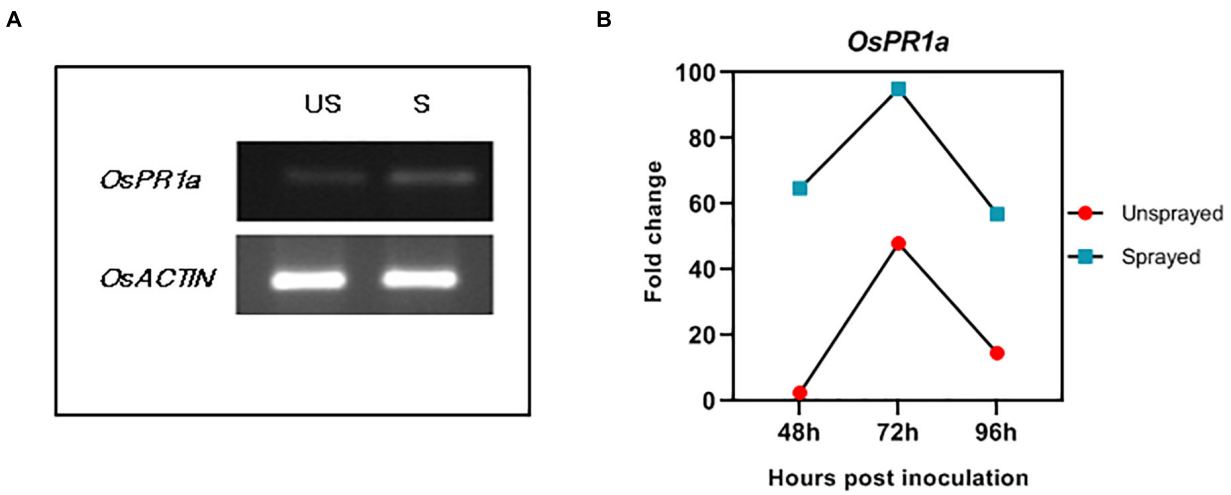

FIGURE 8 | Expression of *OsPR1a*. **(A)** Semi-quantitative Reverse transcriptase PCR for rice defense- related *PR1a* in ds*DES1*-sprayed and unsprayed plants 72 hpi. **(B)** The relative fold change for *OsPR1a* expression in sprayed and unsprayed plants 48, 72, and 96 hpi, normalized against the house-keeping control *OsACTIN*. The reactions were set up in triplicates.

300 nM (**Figure 2B**). This is in accordance with other reports where naked dsRNAs were sprayed against fungal pathogens to treat *Hordeum vulgare* (Koch et al., 2016), *Glycine max*, and *Triticum aestivum* (Gu et al., 2019). The uptake of the sprayed dsRNA by the plant tissues is crucial to assert gene silencing. However, there has been evidence in tobacco (Dalakouras et al., 2016) and Amaranthus leaves (Bennett et al., 2020) that the cuticle of leaves can act as a significant barrier against the uptake because of surface hydrophobicity. Hence, foliar spray experiments were carried out on both intact and sandpaper-abraded adaxial leaf surfaces (Huang et al., 2018; Guo et al., 2019). Our microscopic observations indicated a higher and sustained

incidence of silencing in the wounded plants (**Figure 2C**), where a 67% reduction in *GFP* transcripts was seen as compared with 48% silencing in the intact set 4 dpt (**Figure 2D**). This could suggest that the silencing efficiency is more sustained in the abraded leaves because of the proper entry of the naked dsRNA and plausible replenishment of secondary siRNAs. On the contrary, the low silencing efficiency in the case of the intact leaves could either be due to a fraction of dsRNAs getting degraded by UV and environmental nucleases before reaching into the plant cell (Cagliari et al., 2018) nor lack of the efficient regeneration of sRNA signals, as found in *Fusarium asiaticum* (Song et al., 2018). Once sprayed on the leaf surface, the longevity and efficiency of functional naked dsRNAs can vary from plant to plant. While naked dsRNAs have shown silencing activity for only up to 7 days on tobacco leaves (Konakalla et al., 2016), a relatively longer period of stability of up to 28 days was observed in potato leaves (San Miguel and Scott, 2016). In the wounded and sprayed rice leaves, the reduced fluorescence phenotype of the *M. oryzaeGFP* was conspicuous even 7 dpt. Some reports have shown that topically applied dsRNA can move systemically across plant tissues to prevent pathogen colonization even in the unsprayed regions (Koch et al., 2016; Mitter et al., 2017; Song et al., 2018). Systemic silencing was also demonstrated (Hendrix et al., 2021), where it was hypothesized that 22-bp siRNAs could induce silencing in systemic regions of *Nicotiana benthamiana* by secondary siRNA generation. The key to a prolonged and robust silencing induced by sprayed dsRNA can be attributed to their systemic nature. When dsRNA was sprayed both distally and locally on detached leaves and whole plants, our microscopic results (**Figure 3A**) demonstrated systemic silencing, as reported previously in other pathosystems. The analyses of total RNA revealed the accumulation of ds*GFP* in both local (up to 7 dpt) and unsprayed regions (4 dpt) (**Figure 3B**). This supported the fact that the silencing of *GFP,* as visualized under the microscope, was specifically induced by the sprayed ds*GFP*, which got processed into siRNAs by dicer-like proteins. The higher relative abundance of dsRNA in both sprayed and unsprayed plant parts indicated that spraying of dsRNA can give a lasting effect to SIGS. With this, it can be concluded that the silencing effect in distal unsprayed areas can be attributed to the systemic nature of the dsRNA. This also corroborated the findings of Koch et al. (2016) in barley leaves and Konakalla et al. (2016) in tobacco leaves.

The efficiency of gene knockdown may depend on the length and region of the target sequence selected for dsRNA. Thus, having validated the functionality of SIGS in the rice-*M. oryzae* pathosystem, an *in vitro* proof-of-concept experiment was carried out to check the efficacy of the 300 bp target region selected for silencing *MoDES1*. It has been demonstrated previously (Guo et al., 2019) that *M. oryzae* is capable of uptaking artificial siRNAs (asiRNA) from media. Upon feeding the wild-type strain with ds*DES1* for two fungal generations, observable phenotypic changes were documented with a significant reduction in *MoDES1* transcripts. As revealed by the phenotypic and ROS sensitivity assays (**Figure 4**), the observations made in the dsRNA-fed strain were at par with the findings reported in *MoDES1* mutants (Chi et al., 2009). A majority of the conidia showing morphological deformity corroborated the partial loss of *MoDES1 via* knockdown. The role of ROS is instrumental in the progression of pathogen within its host, and in determining the fate of such biotic interactions (Shetty et al., 2007). Several reports including Samalova et al. (2014) claimed the quintessential role of host-derived ROS in defending *M. oryzae*, and the importance of ROS scavenging for successful virulence has been proved in *M. oryzae* and other fungi like *Ustilago maydis* (Molina and Kahmann, 2007). In *M. oryzae, MoDES1* is known to be involved in ROS scavenging, and the ds*DES1*-fed strains showed sensitivity toward media-derived H_2O_2 in a dose-dependent manner (**Figure 4A**). The role of pathogen-derived extracellular enzymes is critical to the dismutation of ROS generated during PTI (Apostol et al., 1989; Tanabe et al., 2011). Our results revealed that the laccase and peroxidase activities of the dsRNA-fed strain were significantly reduced (**Figures 5A,B**), and that these phenotypes were similar to that of *MoDES1* mutants. ROS generation in a host was correlated to disease progression in hemibiotrophic interaction based on the structure and dynamics of invasive hyphae (Tanabe et al., 2011). The ds*DES1*-fed strain showed a clear impediment in host cell penetration and cell-to-cell progression (**Figures 5C,D**), as attributed by their short and less profusely branched biotrophic growth. Additionally, it revealed an almost one-third reduction in the length of lesions produced by them with respect to the untreated wild type (**Figures 5E,F**). Hence, for the ds*DES1*-fed strain, the inability to detoxify host-derived ROS had obvious consequences on host cell colonization and virulence potential.

In SIGS, dsRNA can have two outcomes after being sprayed. It can either be uptaken by the fungus from the surface of the plant, or, it can be uptaken by the plant, processed into siRNAs, and then transferred to the interacting fungus *via* cross-kingdom RNA exchange (Song et al., 2018; Wang and Dean, 2020). In the intact leaves, the fungus takes time to penetrate into the host cell, and a larger fraction of RNA gets absorbed by the fungus readily (Koch et al., 2016) before making its way into the plant tissues. However, under such circumstances, a fraction of dsRNA also gets dried up and degraded on the surface itself, and only some of it gets taken up by the fungus. Primarily, better uptake of dsRNA and longevity of silencing efficiency were demonstrated when it was sprayed on abrading surface. Since the silencing phenotype was more in wounded or abraded plants, it led us to hypothesize that the sprayed dsRNA can assert RNAi more efficiently in the fungus if it gets maximally uptaken *via* the plant tissues. This hypothesis was later proved in spray-infection experiments (**Figures 6A,B**), where the disease resistance phenotype and *in planta* ROS generation, associated with the silencing of pathogenicity gene *MoDES1*, corroborated our assertion mentioned above. It was observed that relative disease resistance, correlated with the percentage silencing of *MoDES1,* was highest when the dsRNA was sprayed 24 h before the fungal inoculation. This indicated that SIGS was more efficient when the majority of dsRNA was uptaken by the fungus *via* the plant tissues. Studies on *Fusarium asiaticum* (Song et al., 2018) showed that the effect of the dsRNA uptaken by the fungus directly, is short lived, and that the intended silencing effect almost disappears after 9 h. This is attributed to the lack of secondary amplification of siRNAs in the fungus.

Based on existing reports on the cross-kingdom transfer of dsRNA and siRNAs (Wang et al., 2016; Dubrovina and Kiselev, 2019), it might also be possible that there is a cross-talk happening between rice and fungal systems. However, further research is required to elucidate this mechanism and the extent of involvement of rice and *M. oryzae*-silencing players. However, having evaluated the impacts of ds*DES1* on the wild type fungus *in vitro* and in detached rice leaves, the application of SIGS was extended to whole plants. As optimized previously, ds*DES1* was sprayed onto abraded leaves of whole rice plants, and 65% *in planta* silencing of *MoDES1*was achieved. Our final aim was to develop a PTI response robust enough for *M. oryzae* to succumb to the rice defense system, and the sprayed plants indeed showed stronger resistance against the pathogen (**Figures 6C–E**). Disease payoff in the form of size and density of lesions directly correlates with fungal growth and plant defense cascade. The leaves treated and sprayed with ds*DES1* manifested notably less severity (**Figures 6E**, **7A,B**), and the histological assays of the leaf sheaths in the early biotrophic phase revealed the infection and defense-related structures at the *M. oryzae*-rice interface (**Figures 7C–F**). Higher percentage of host cells across infection units showed enhanced PTI responses through generation of ROS, callose deposition, and cell death. The attenuated virulence in the ds*DES1*-sprayed plants could be correlated with the reduced fungal biomass as indicated by the semi-quantitative expression of the fungal 28S rRNA with respect to the rice 25S rRNA (**Figure 7B**). As a part of PTI cascade, infection-related cell death and necrosis are generally associated with the relay of defense signals, leading to an eventual SAR in hemi-biotrophic interactions (Bari and Jones, 2009). In a resistant cultivar, prolonged induction of PR genes and generation of phytoalexins are a hallmark of active defense. *PR1* is a commonly used marker for the host defense response triggered, and its quantitative transcript analyses (**Figures 8A,B**) depicted that the dsRNA sprayed plants showed a higher level of PR gene expression, even 4 dpi, than the unsprayed controls.

Spray-induced gene silencing is a promising method of tackling the ever-evolving plant disease-causing fungi, exploiting the mechanisms of very fundamental processes, such as RNAi and cross-kingdom transfer of silencing RNA species. The topical application of naked dsRNA and nanoparticle-bound RNA-based pest and pathogen-control compounds backed up by sufficient biosafety evaluation is cost-effective and the environment-compatible future of RNAi technology for crop protection (Gebremichael et al., 2021; Taning et al., 2021). Besides, sprayed dsRNA can assert the knockdown of endogenous genes in plants, whereby physiological conditions and means of dsRNA application influence the efficacy of the silencing treatment (Kiselev et al., 2021). Based on the findings from this study and the reported mechanism of RNA exchange across plant and fungal cells, a possible mode of inductive action of dsRNA with reference to *MoDES1* in silencing-mediated defense elicitation in rice has been diagrammatically represented (**Figure 9**). To this end, our study validates that SIGS is an efficient strategy for targeted gene silencing and conferring disease resistance in the rice-blast pathosystem. Besides, this investigation sheds

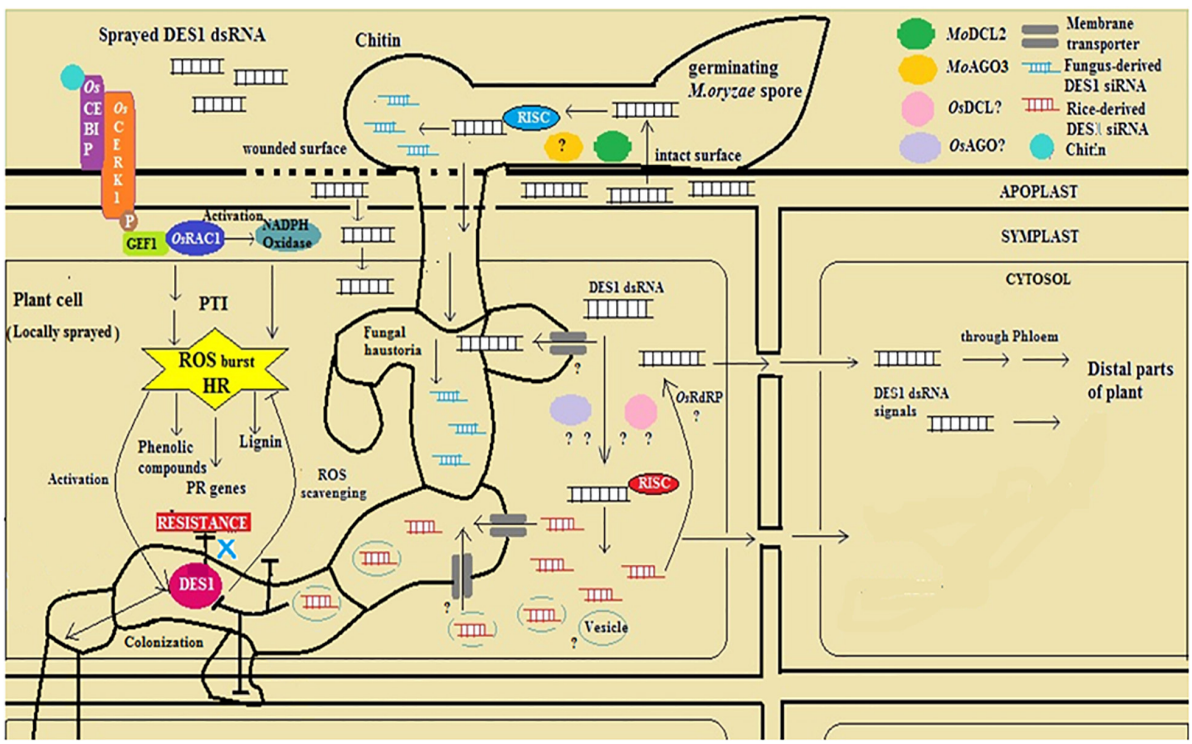

FIGURE 9 | Diagrammatic representation of mode-of-action of ds*DES1* in conferring spray-induced gene silencing (SIGS)-mediated fungal blast resistance in rice.

light on ways to optimally exploit SIGS as a crop-protection strategy and alternative to genetic modification. However, our investigation unravels possibilities for further research on the interaction between silencing players of host and pathogen, the extent of their involvement in secondary siRNA generation and silencing signal amplification, with reference to the rice-blast blast pathosystem, This relatively new and lesser-known dsRNA-based method can be better understood and exploited as an armament against several fungal phytopathogens in safeguarding their economically important host crops by means of addressing such general questions pertaining to its mechanistic details.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AS and SR-B conceived and designed the experiments, analyzed the data, and wrote the manuscript. AS performed the experiments. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.733129/full#supplementary-material

REFERENCES

Akagi, A., Jiang, C.-J., and Takatsuji, H. (2015). *Magnaporthe oryzae* inoculation of rice seedlings by spraying with a spore suspension. *Bioprotocol* 5:e1486.

Akamatsu, A., Wong, H. L., Fujiwara, M., Okuda, J., Nishide, K., and Uno, K. (2013). An OsCEBiP/OsCERK1–OsRacGEF1–OsRac1 module is an essential early component of chitin-induced rice immunity. *Cell Host Microbe* 13, 465–476. doi: 10.1016/j.chom.2013.03.007

Apostol, I., Heinstein, P. F., and Low, P. S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: role in defense and signal transduction. *Plant Physiol.* 90, 109–116.

Baldwin, T., Islamovic, E., Klos, K., Schwartz, P., Gillespie, J., Hunter, S., et al. (2018). Silencing efficiency of dsRNA fragments targeting *Fusarium graminearum* TRI6 and patterns of small interfering RNA associated with reduced virulence and mycotoxin production. *PLoS One* 13:e0202798. doi: 10.1371/journal.pone.0202798

Bari, R., and Jones, J. D. (2009). Role of plant hormones in plant defence responses. *Plant Mol. Biol.* 69, 473–488. doi: 10.1007/s11103-008-9435-0

Baulcombe, D. C. (2015). VIGS, HIGS and FIGS: small RNA silencing in the interactions of viruses or filamentous organisms with their plant hosts. *Curr. Opin. Plant Biol.* 26, 141–146. doi: 10.1016/j.pbi.2015.06.007

Bennett, M., Deikman, J., Hendrix, B., and Iandolino, A. (2020). Barriers to efficient foliar uptake of dsRNA and molecular barriers to dsRNA activity in plant cells. *Front. Plant Sci.* 11:816. doi: 10.3389/fpls.2020.00816

Cagliari, D., Santos, E. A., Dias, N., Smagghe, G., and Zotti, M. (2018). "Modulating gene expression–abridging the RNAi and CRISPR-Cas9 technologies," in *Nontransformative Strategies for RNAi in Crop Protection*, eds A. Singh and M. W. Khan (London: IntechOpen), 1–18.

Cai, Q., He, B., Wang, S., Fletcher, S., Niu, D., Mitter, N., et al. (2021). Message in a bubble: shuttling small RNAs and proteins between cells and interacting organisms using extracellular vesicles. *Annu. Rev. Plant Biol.* 72, 497–524. doi: 10.1146/annurev-arplant-081720-010616

Chen, Y., Le, X., Sun, Y., Li, M., Zhang, H., Tan, X., et al. (2017). MoYcp4 is required for growth, conidiogenesis and pathogenicity in *Magnaporthe oryzae*. *Mol. Plant Pathol.* 18, 1001–1011. doi: 10.1111/mpp.12455

Chi, M. H., Park, S. Y., Kim, S., and Lee, Y. H. (2009). A novel pathogenicity gene is required in the rice blast fungus to suppress the basal defenses of the host. *PLoS Pathog.* 5:e1000401. doi: 10.1371/journal.ppat.1000401

Chida, T., and Sisler, H. D. (1987). Effect of inhibitors of melanin biosynthesis on appressoria1 penetration and reductive reac- tions in *Pyricularia oryzae* and *Pyricularia grisea*. *Pestic. Biochem. Physiol.* 29, 244–245. doi: 10.1016/0048-3575(87)90154-4

Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124, 803–814.

Dalakouras, A., Wassenegger, M., McMillan, J. N., Cardoza, V., Maegele, I., Dadami, E., et al. (2016). Induction of silencing in plants by high-pressure spraying of in vitro-synthesized small RNAs. *Front. Plant Sci.* 7:1327. doi: 10.3389/fpls.2016.01327

Dang, T. T., Shimatani, Z., Kawano, Y., Terada, R., and Shimamoto, K. (2013). Gene editing a constitutively active OsRac1 by homologous recombinationbased gene targeting induces immune responses in rice. *Plant Cell Physiol.* 54, 2058–2070. doi: 10.1093/pcp/pct147

Das, P. R., and Sherif, S. M. (2020). Application of exogenous dsRNAs-induced RNAi in agriculture: challenges and triumphs. *Front. Plant Sci.* 11:946. doi: 10.3389/fpls.2020.00946

Dean, R., Van Kan, J. A., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., et al. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* 13, 414–430.

Dellaporta, S. L., Wood, J., and Hicks, J. B. (1983). A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* 1, 19–21. doi: 10.1007/bf02712670

Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S., et al. (2009). Ca2+/calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* 457, 1154–1158. doi: 10.1038/nature07612

Dubrovina, A. S., and Kiselev, K. V. (2019). Exogenous RNAs for gene regulation and plant resistance. *Int. J. Mol. Sci.* 20:2282. doi: 10.3390/ijms20092282

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annu. Rev. Phytopathol.* 42, 185–209. doi: 10.1146/annurev.phyto.42.040803.140421

Egan, M. J., Wang, Z.-Y., Jones, M. A., Smirnoff, N., and Talbot, N. J. (2007). Generation of reactive oxygen species by fungal NADPH oxidases is required

for rice blast disease. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11772–11777. doi: 10.1073/pnas.0700574104

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi: 10.1038/35888

Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S., Lecourieux, D., Poinssot, B., et al. (2006). Early signaling events induced by elicitors of plant defenses. *Mol. Plant Microbe Interact.* 19, 711–724.

Gebremichael, D. E., Haile, Z. M., Negrini, F., Sabbadini, S., Capriotti, L., Mezzetti, B., et al. (2021). RNA interference strategies for future management of plant pathogenic fungi: prospects and challenges. *Plants* 10:650. doi: 10.3390/plants10040650

Gu, K. X., Song, X. S., Xiao, X. M., Duan, X. X., Wang, J. X., Duan, Y. B., et al. (2019). A β 2 -tubulin dsRNA derived from *Fusarium asiaticum* confers plant resistance to multiple phytopathogens and reduces fungicide resistance. *Pestic. Biochem. Physiol.* 153, 36–46. doi: 10.1016/j.pestbp.2018.10.005

Guo, M., Chen, Y., Du, Y., Dong, Y., Guo, W., Zhai, S., et al. (2011). The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog.* 7:e1001302. doi: 10.1371/journal.ppat.1001302

Guo, X.-Y., Li, Y., Fan, J., Xiong, H., Xu, F.-X., Shi, J., et al. (2019). Host-induced gene silencing of MoAP1 confers broad-spectrum resistance to *Magnaporthe oryzae*. *Front. Plant Sci.* 10:433. doi: 10.3389/fpls.2019.00433

Hendrix, B., Zheng, W., Bauer, M. J., Havecker, E. R., Mai, J. T., Hoffer, P. H., et al. (2021). Topically delivered 22 nt siRNAs enhance RNAi silencing of endogenous genes in two species. *Planta* 254:60. doi: 10.1007/s00425-021-03708-y

Huang, S., Iandolino, A., and Peel, G. (2018). *Methods and Compositions for Introducing Nucleic Acids into Plants.* U.S. Patent No. 20180163219A1. Washington, DC: Patent and Trademark Office.

Jia, Y., Valent, B., and Lee, F. N. (2003). Determination of host responses to *Magnaporthe grisea* on detached rice leaves using a spot inoculation method. *Plant Dis.* 87, 129–133. doi: 10.1094/PDIS.2003.87.2.129

Jones, J. D., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329.

Kaku, H., Nishizawa, Y., Ishll-Minami, N., Akimoto-Tomlyama, C., Dohmae, N., Taklo, K., et al. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11086–11091. doi: 10.1073/pnas.0508882103

Kankanala, P., Czymmek, K., and Valent, B. (2007). Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* 19, 706–724. doi: 10.1105/tpc.106.046300

Kawasaki, T., Koita, H., Nakatsubo, T., Hasegawa, K., Wakabayashi, K., Takahashi, H., et al. (2006). Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. *Proc. Natl. Acad. Sci. U.S.A.* 103, 230–235. doi: 10.1073/pnas.0509875103

Kiselev, K. V., Suprun, A. R., Aleynova, O. A., Ogneva, Z. V., and Dubrovina, A. S. (2021). Physiological conditions and sRNA application approaches for exogenously induced RNA interference in *Arabidopsis thaliana*. *Plants* 10:264. doi: 10.3390/plants10020264

Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., et al. (2016). An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathog.* 12:e1005901. doi: 10.1371/journal.ppat.1005901

Konakalla, N. C., Kaldis, A., Berbati, M., Masarapu, H., and Voloudakis, A. E. (2016). Exogenous application of double-stranded RNA molecules from TMV p126 and CP genes confers resistance against TMV in tobacco. *Planta* 244, 961–969. doi: 10.1007/s00425-016-2567-6

Kou, Y., Qiu, J., and Tao, Z. (2019). Every coin has two sides: reactive oxygen species during rice-*Magnaporthe oryzae* interaction. *Int. J. Mol. Sci.* 20:1191. doi: 10.3390/ijms20051191

Liu, W., Xie, S., Zhao, X., Chen, X., Zheng, W., Lu, G., et al. (2010). A homeobox gene is essential for conidiogenesis of the rice blast fungus *Magnaporthe oryzae*. *Mol. Plant Microbe Interact.* 23, 366–375. doi: 10.1094/MPMI-23-4-0366

Medzhitov, R. Jr., and Janeway, C. A. (1997). Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* 9, 4–9. doi: 10.1016/s0952-7915(97)80152-5

Mir, A. A., Park, S.-Y., Sadat, M. A., Kim, S., Choi, J., Jeon, J., et al. (2015). Systematic characterization of the peroxidase gene family provides new insights into fungal pathogenicity in *Magnaporthe oryzae*. *Sci. Rep.* 5:11831. doi: 10.1038/srep11831

Mishina, T. E., and Zeier, J. (2007). Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in *Arabidopsis*. *Plant J.* 50, 500–513. doi: 10.1111/j.1365-313X.2007.03067.x

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R. G., Taochy, C., et al. (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants.* 3:16207. doi: 10.1038/nplants.2016.207

Molina, L., and Kahmann, R. (2007). An *Ustilago maydis* gene involved in H_2O_2 detoxification is required for virulence. *Plant Cell* 7, 2293–2309.

Morozov, S. Y., Solovyev, A. G., Kalinina, N. O., and Taliansky, M. E. (2019). Double-stranded RNAs in plant protection against pathogenic organisms and viruses in agriculture. *Acta Nat.* 11, 13–21. doi: 10.32607/20758251-2019-11-4-13-21

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunol. Rev.* 198, 249–266.

Pandey, S. P., and Somssich, I. E. (2009). The role of WRKY transcription factors in plant immunity. *Plant Physiol.* 150, 1648–1655. doi: 10.1104/pp.109.138990

Park, C. H., Shirsekar, G., Bellizzi, M., Chen, S., Songkumarn, P., Xie, X., et al. (2016). The E3 ligase APIP10 connects the effector AvrPiz-t to the NLR receptor Piz-t in rice. *PLoS Pathog.* 12:e1005529. doi: 10.1371/journal.ppat.1005529

Pennisi, E. (2010). Armed and dangerous. *Science* 327, 804–805. doi: 10.1126/science.327.5967.804

Qi, T., Zhu, X., Tan, C., Liu, P., Guo, J., Kang, Z., et al. (2018). Host-induced gene silencing of an important pathogenicity factor PsCPK1 in *Puccinia striiformis* f. sp. *tritici* enhances resistance of wheat to stripe rust. *Plant Biotechnol. J.* 16, 797–807. doi: 10.1111/pbi.12829

Qi, Z. Q., Wang, Q., Dou, X. Y., Wang, W., Zhao, Q., Lv, R. L., et al. (2012). MoSwi6, an APSES family transcription factor, interacts with MoMps1 and is required for hyphal and conidial morphogenesis, appressorial function and pathogenicity of *Magnaporthe oryzae*. *Mol. Plant Pathol.* 13, 677–689. doi: 10.1111/j.1364-3703.2011.00779.x

Qiao, L., Lan, C., Capriotti, L., Ah-Fong, A., Nino Sanchez, J., Hamby, R., et al. (2021). Spray-induced gene silencing for disease control is dependent on the efficiency of pathogen RNA uptake. *Plant Biotechnol. J.* 19, 1756–1768. doi: 10.1111/pbi.13589

Saha, P., Ghosh, S., and Roy-Barman, S. (2020). MoLAEA regulates secondary metabolism in *Magnaporthe oryzae*. *mSphere* 5:e00936–19. doi: 10.1128/mSphere.00936-19

Samalova, M., Meyer, A. J., Gurr, S. J., and Fricker, M. D. (2014). Robust anti-oxidant defences in the rice blast fungus *Magnaporthe oryzae* confer tolerance to the host oxidative burst. *New Phytol.* 201, 556–573. doi: 10.1111/nph.12530

Sambrook, J., Fritsch, E. R., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

San Miguel, K., and Scott, J. G. (2016). The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest. Manag. Sci.* 72, 801–809. doi: 10.1002/ps.4056

Santillán Martínez, M. I., Bracuto, V., Koseoglou, E., Appiano, M., Jacobsen, E., Visser, R. G. F., et al. (2020). CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene PMR4 for resistance against powdery mildew. *BMC Plant Biol.* 20:284. doi: 10.1186/s12870-020-02497-y

Shetty, N. P., Mehrabi, R., Lütken, H., Haldrup, A., Kema, G. H., Collinge, D. B., et al. (2007). Role of hydrogen peroxide during the interaction between the hemibiotrophic fungal pathogen *Septoria tritici* and wheat. *New Phytol.* 174, 637–647. doi: 10.1111/j.1469-8137.2007.02026.x

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., et al. (2010). Two Lys M receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64, 204–214. doi: 10.1111/j.1365-313X.2010.04324.x

Song, X. S., Gu, K. X., Duan, X. X., Xiao, X. M., Hou, Y. P., Duan, Y. B., et al. (2018). A myosin5 dsRNA that reduces the fungicide resistance and pathogenicity of *Fusarium asiaticum*. *Pestic. Biochem. Physiol.* 150, 1–9. doi: 10.1016/j.pestbp.2018.07.004

Takabatake, R., Karita, E., Seo, S., Mitsuhara, I., Kuchitsu, K., and Ohashi, Y. (2007). Pathogen-induced calmodulin isoforms in basal resistance against bacterial and fungal pathogens in tobacco. *Plant Cell Physiol.* 48, 414–423. doi: 10.1093/pcp/pcm011

Tanabe, S., Ishii-Minami, N., Saitoh, K. I., Otake, Y., Kaku, H., Shibuya, N., et al. (2011). The role of catalase-peroxidase secreted by*Magnaporthe oryza*eduring early infection of rice cells. *Mol. Plant Microbe Interact.* 24, 163–171. doi: 10.1094/MPMI-07-10-0175

Taning, C. N. T., Mezzetti, B., Kleter, G., Smagghe, G., and Baraldi, E. (2021). Does RNAi-based technology fit within EU sustainability goals? *Trends Biotechnol.* 39, 644–647. doi: 10.1016/j.tibtech.2020.11.008

Torres, M. A., and Dangl, J. L. (2005). Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* 8, 397–403. doi: 10.1016/j.pbi.2005.05.014

Torres, M. A., Jones, J. D., and Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* 141, 373–378.

van der Biezen, E. A., and Jones, J. D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci.* 23, 454–456.

van Loon, L. C., Bakker, P. A., and Pieterse, C. M. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu. Rev. Phytopathol.* 36, 453–483. doi: 10.1146/annurev.phyto.36.1.453

Van Loon, L. C., and Van Strien, E. A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* 55, 85–97. doi: 10.1006/pmpp.1999.0213

Wang, M., and Dean, R. A. (2020). Movement of small RNAs in and between plants and fungi. *Mol. Plant Pathol.* 21, 589–601.

Wang, M., Weiberg, A., Lin, F. M., Thomma, B. P. H. J., Huang, H.-D., and Jin, H. (2016). Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nat. Plants* 2:16151. doi: 10.1038/nplants.2016.151

Wang, X., Zhang, Y., Zhang, R., and Zhang, J. (2019). The diversity of pattern recognition receptors (PRRs) involved with insect defense against pathogens. *Curr. Opin. Insect Sci.* 33, 105–110. doi: 10.1016/j.cois.2019.05.004

Wilson, R. C., and Doudna, J. A. (2013). Molecular mechanisms of RNA interference. *Annu. Rev. Biophys.* 42, 217–239. doi: 10.1146/annurev-biophys-083012-130404

Zhu, L., Zhu, J., Liu, Z., Wang, Z., Zhou, C., Wang, H., et al. (2017). Host-induced gene silencing of rice blast fungus *Magnaporthe oryzae* pathogenicity genes mediated by the brome mosaic virus. *Genes* 8, 241–256. doi: 10.3390/genes8100241

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D. G., Boller, T., et al. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125, 749–760. doi: 10.1016/j.cell.2006.03.037

Zotti, M., dos Santos, E. A., Cagliari, D., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2017). RNAi technology in crop protection against arthropod pests, pathogens and nematodes. *Pest. Manag. Sci.* 74, 1239–1250. doi: 10.1002/ps.4813

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Highly Variable Dietary RNAi Sensitivity Among Coleoptera

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Many herbivorous beetles (Order Coleoptera) contribute to serious losses in crop yields and forest trees, and plant biotechnology solutions are being developed with the hope of limiting these losses. Due to the unprecedented target-specificity of double-stranded RNA (dsRNA), and its utility in inducing RNA interference (RNAi) when consumed by target pest species, dsRNA-based plant biotechnology approaches represent the cutting edge of current pesticide research and development. We review dietary RNAi studies in coleopterans and discuss prospects and future directions regarding RNAi-based management of coleopteran plant pests. Herein, we also provide a balanced overview of existing studies in order to provide an accurate re-assessment of dietary RNAi sensitivity in coleopterans, despite the limitations to the existing body of scientific literature. We further discuss impediments to our understanding of RNAi sensitivity in this important insect order and identify critical future directions for research in this area, with an emphasis on using plant biotechnology approaches.

Keywords: RNA interference, dsRNA, plant biotechnology, beetle, biological variability, biopesticide, insecticide, dietary RNAi

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INTRODUCTION

Of all arthropod orders, the insect order Coleoptera (beetles) contains the greatest number of described species (Stork, 2018). While many beetle species perform valuable agroecological services, such as biological control and pollination (Rader et al., 2016), others can be highly damaging pests of crops and forest trees. The most common solution to managing important coleopteran plant pests globally is the use of synthetic broad-spectrum insecticides. In addition, organic and other ecologically intensified farming systems use environmentally sound management techniques, such as enhancing conservation biological control, creating refuge areas, mixed cropping systems, and the use of cover crops. Plant protection biotechnologies are increasingly considered for combating coleopteran pest pressure, and such technologies include the use of *Bacillus thuringiensis* toxins and double-stranded RNA (dsRNA).

dsRNA currently represents the most species-specific approach to managing plant pests, given its nucleotide sequence-specific mode of action that relies on base-pairing between applied exogenous dsRNA (processed *in vivo* into segments 20–24 nucleotides in length) and complementary endogenous messenger RNA (mRNA). Generally, the complementarity of 21 consecutive nucleotides initiates the cleaving of the base-paired mRNA region, preventing translation of the targeted mRNA, a process known as RNA interference (RNAi). This unprecedented

target-specificity is the basis for RNAi's promise for biosafety to non-target organisms, and in turn the increasing interest in developing RNA-based biotechnology solutions to address plant protection. RNAi-based plant protection biotechnologies that have made significant progress include host-induced gene silencing (HIGS) technology *via* the use of transgenic cultivars containing RNAi traits, and spray-induced gene silencing (SIGS) technology *via* exogenous application of dsRNA to plants. Open-field RNAi-based management of western corn rootworm (*Diabrotica virgifera*) will commence in the United States in 2022 (followed by Canada in 2023), *via* the HIGS approach of consuming the tissues of the maize cultivar SmartStax PRO (Bayer, Germany), containing an RNAi-inducing trait. The biotechnology company GreenLight Biosciences (United States) is currently developing a SIGS approach against this pest Colorado potato beetle (*Leptinotarsa decemlineata*).

Investigations into RNAi-based management of coleopteran plant pests began with Baum et al. (2007), who demonstrated high dietary RNAi sensitivity (e.g., achieving target phenotype post-consumption of low amounts of dsRNA) in both *D. virgifera* and *L. decemlineata*. Since then, there has been an ongoing narrative, in RNAi-based insect management literature, that most coleopterans are sensitive to dietary RNAi. While this sensitivity has been clearly demonstrated in some coleopteran plant pests, many coleopteran species that have been examined show little to moderate sensitivity to dietary RNAi, and the vast majority of Order Coleoptera has not been examined. For the coleopteran species that have been examined, no overview of RNAi efficacy between and within coleopteran taxa has yet been presented to the scientific community.

Our timely review provides a balanced overview of dietary RNAi studies conducted on coleopteran species and discusses impediments to our knowledge regarding the overall prospects for RNAi-based biotechnology solution in the management of coleopteran plant pests. Given the upcoming introduction of insect RNAi technology to the American plant protection marketplace *via* the cultivation of transgenic maize containing an RNAi trait against *D. virgifera*, together with the role of coleopteran herbivores in causing significant damage globally to crops and forest trees, this represents a timely and critical re-assessment of our current state of knowledge. Our assessment is standardized to examine studies using non-formulated dsRNA (e.g., without the use of nanoparticles that enhance cellular uptake and systemic RNAi) to provide a reasonable comparison within and between coleopterans thus far studied. Biotechnologies considered in the present review include transgenic RNAi plants, "naked" dsRNA applications and bacterially expressed dsRNA.

OBSERVED RNAi SENSITIVITIES IN COLEOPTERA

Family Chrysomelidae

Leaf beetles (Family Chrysomelidae) feed on a variety of plant tissues, and all chrysomelid species are fully herbivorous, many being serious crop pests. Several studies have shown that dietary RNAi sensitivity is quite high in the two chrysomelids *D. virgifera* (Baum et al., 2007; Bolognesi et al., 2012; Bachman et al., 2013; Miyata et al., 2014; Niu et al., 2017; Vélez et al., 2020) and *L. decemlineata* (Baum et al., 2007; Miguel and Scott, 2015; Cappelle et al., 2016; Spit et al., 2017; Máximo et al., 2020; Petek et al., 2020; Ren et al., 2021). Notably, Petek et al. (2020) demonstrated, in a small field study in Slovenia, that treating plants with water containing 0.01 µg dsRNA/µl significantly reduced *L. decemlineata* infestation in the field. This study was a landmark, as it suggests potential for dsRNA spray-based management of a major agricultural pest in open-field conditions, by mixing dsRNA with water alone.

Another chrysomelid, the mustard leaf beetle (*Phaedon cochleariae*), was recently examined for its potential as a screening model for coleopteran pests, and this species also demonstrated high RNAi sensitivity, with a strong lethal phenotype observed *via* consumption of leaf disks treated with water containing 0.025 µg dsRNA/µl (Mehlhorn et al., 2021). Zhang et al. (2019) demonstrated that painting a thin layer of water, containing a diluted suspension of dsRNA-expressing bacteria, on fresh willow leaves resulted in approximately 40 and 80% mortality of *Plagiodera versicolora* larvae after 2 and 7 days, respectively. More recently, Xu et al. (2021) painted poplar leaves with dsRNA to a concentration of 8 ng/cm^2 and demonstrated 100% mortality in *P. versicolora* larvae within 5–7 days, depending on target gene. Notably, the authors observed over 60% mortality after 3 days of feeding on dsRNA targeting *actin* (see Supplementary Material in Xu et al., 2021). These studies with *P. versicolora*, together with that of Máximo et al. (2020), who observed effective *L. decemlineata* mortality after 2 days, suggest that RNAi can work in a timely manner in some coleopterans, perhaps in particular chrysomelids of the subfamily Chrysomelinae. Furthermore, studies performed in chrysomelids collectively suggest high dietary RNAi efficacy *via* various sources of dsRNA consumption (e.g., RNAi cultivar., naked dsRNA, and bacterially expressed dsRNA).

Family Tenebrionidae

Darkling beetles (Family Tenebrionidae), while occupying a diverse array of ecological niches, consist of several important plant product pests, particularly in cereal and flour silos, as well as other stored food products. To our knowledge, the only tenebrionid species examined using dietary RNAi is the red flour beetle (*Tribolium castaneum*), a pest of global importance, and the existing data on dietary RNAi efficacy in *T. castaneum* are variable. Whyard et al. (2009) reported, after 7 days of dietary exposure, an LC_{50} of 0.0025 µg dsRNA/mg diet, and Laudani et al. (2017) reported approximately 30% mortality after 3 days of feeding on 0.015 µg dsRNA/mg diet, both studies being in contrast to Cao et al. (2018) who reported far more delayed RNAi in *T. castaneum via* feeding on 17- and 33-fold higher concentrations than those used by Laudani et al. (2017). Showing intermediate efficacy relative to the abovementioned studies, Abd El Halim et al. (2016) demonstrated 50% mortality after 6 days of feeding on 0.1 µg dsRNA/mg diet. As each study, with the exception of Abd El Halim et al. (2016), targeted *vATPase* components, these contrasting results clearly indicate a need for more investigations

in order to solidify any conclusions regarding dietary RNAi efficacy in *T. castaneum*. Furthermore, expanding dietary RNAi studies to consider other important tenebrionid pests represent a critical route toward future biotechnological solutions to protect plant-based foods globally.

Family Coccinellidae

Lady beetles (Family Coccinellidae) are an important group of coleopterans, regarding plant protection, due to different species' roles as predators of crop pests (e.g., *Hippodamia convergens*, *Coleomegilla maculata*, and *Coccinella septempunctata*), invasive pests that outcompete beneficial lady beetles (e.g., *Harmonia axyridis*), and herbivorous pests that can be highly damaging to crop yields (e.g., *Henosepilachna vigintioctopunctata*). Targeting the same gene in four lady beetle species (*H. convergens*, *H. axyridis*, *C. maculata,* and *C. septempunctata*), Pan et al. (2020) observed approximately 80, 5, 60, and 70% mortality, respectively. These results indicate that dietary RNAi efficacy may be highly variable between coccinellid species. Furthermore, the concentration of dsRNA in the diet provided to coccinellids during the abovementioned study (4 μg dsRNA/μl) was rather high in relation to what would be realistically applicable in RNAi-based plant protection, suggesting the need to re-evaluate the dietary RNAi susceptibility of some of these more RNAi-sensitive species, using more economically feasible dsRNA concentrations.

Lü et al. (2020a,b, 2021a,b) and Guo et al. (2021) examined RNAi efficacy *via* oral delivery of dsRNAs targeting various genes in the 28-spotted potato ladybird (*H. vigintioctopunctata*). Lü et al. (2020b) observed approximately 60% mortality (8 days, target gene *Snf7*) and 70–85% mortality (10 days, targeting eight different coatomer components) *via* feeding on leaves treated with dsRNA at a concentration of 0.005 μg dsRNA/μl. Guo et al. (2021) observed nearly complete mortality after 7 days (target gene *vATPase E*) of feeding on leaves treated with dsRNA at a concentration of 0.01 μg dsRNA/μl. These studies by Lü et al. (2020a,b, 2021a,b) and Guo et al. (2021) additionally examined dietary RNAi efficacy in *H. vigintioctopunctata* first- and third-instar larvae and adults, using bacterially expressed dsRNA and demonstrated higher and faster RNAi efficacy in larvae compared to adults. Finally, Ren et al. (2021) observed significant growth retardation in *H. vigintioctopunctata via* consumption of transgenic potato plants. While sufficient data now exist for *H. vigintioctopunctata*, indicating its sensitivity to dietary RNAi, especially when fed leaves treated with low dsRNA concentrations (both naked and bacterially expressed), more data are required with regard to other coccinellid species, both pests and beneficials.

Family Curculionidae

True weevils (Family Curculionidae) make up the largest coleopteran family. Many species are plant pests, and several have been studied with regard to their sensitivity to dietary RNAi. Christiaens et al. (2016) demonstrated, in the sweetpotato weevil *Cylas brunneus*, 46–49% mortality (depending on target gene) after 7 days of feeding on artificial diet containing dsRNA at a concentration of 0.01 μg dsRNA/μl. *C. brunneus* mortality reached 85–89% after 14 days. Prentice et al. (2019) demonstrated, in the sweetpotato weevil *Cylas puncticollis*, that 7 days of feeding on artificial diet treated with dsRNA (target gene *Snf7*) at a concentration of 0.01 μg dsRNA/μl resulted in 36% mortality after 14 days. Wu et al. (2019) observed no effect on survival of pepper weevil (*Anthonomus eugenii*) after dietary exposure to pepper coated with dsRNA (target genes *Snf7* and *vATPase A*) at a concentration of 0.3 μg dsRNA/μl. Pinheiro et al. (2020) observed between 43 and 93% mortality in the Sri Lanka weevil (*Myllocerus undecimpustulatus*), depending on the target gene. However, as mortality was only monitored after 26 days (dsRNA treatments were administered for 20 days), there remain critical unknowns with regard to timeliness of RNAi-induced mortality. Salvador et al. (2021) reported that 14 days of dietary exposure, using artificial diet overlaid with treatment solutions containing 0.2 μg dsRNA/μl, resulted in 40–60% mortality in cotton boll weevil (*Anthonomus grandis*) larvae, and 10–30% mortality in adults, percent mortality depending on target gene in both life stages.

Finally, Kyre et al. (2019) provided a sucrose-based liquid diet containing 10 μg dsRNA/μl to southern pine beetle (*Dendroctonus frontalis*) adults. While this dsRNA concentration is unrealistically high in relation to what is feasible for RNAi-based plant protection, they nevertheless provided a proof-of-concept that this forest pest species is susceptible to dietary RNAi. Here, RNAi-induced mortality ranged from completely ineffective to 100% mortality, depending on target gene. Taking these data on *D. frontalis*, and further examining this species' RNAi sensitivity when exposed to field-realistic concentrations of dietary dsRNA, represents a vital step toward understanding the potential for controlling this forest pest. More broadly, curculionids clearly show variable susceptibility to dietary RNAi. Curculionidae being the largest family in Order Coleoptera, with many curculionid species being detrimental to economically important plant species globally, much more research is needed in order to conclude any patterns regarding RNAi sensitivity in this family. Further studies with curculionids would benefit from including multiple sources of dsRNA consumption (e.g., RNAi cultivar., bacterially expressed dsRNA), as thus far studies in weevils have only examined naked dsRNA in artificial diet.

Family Nitidulidae

Sap beetles (Family Nitidulidae) represent an important family containing several very important plant pest species. The first nitidulid to be examined for sensitivity to dietary RNAi was the small hive beetle (*Aethina tumida*), a facilitative pest of flowering plant species due to its status as a major pest of honey bees (honey bees being important for sexual reproduction of many flowering plant species). Powell et al. (2017) reported 73–93% adult *A. tumida* emergence (depending on target gene), compared 100% adult emergence in control group, after 6 days of dietary exposure to 33 μg dsRNA/g artificial diet.

The only other nitidulid to be examined thus far *via* dietary RNAi is the pollen beetle *Brassicogethes aeneus*. Knorr et al. (2018) observed at least 90% adult *B. aeneus* mortality after 14 days, in all four target gene treatments examined, after dietary

exposure to water containing 1 µg dsRNA/µl followed by continuous uptake of dsRNA in artificial diet containing 500 ng/cm^2. Willow et al. (2020, 2021a,b,c) examined RNAi sensitivity in *B. aeneus* *via* multiple field-relevant routes of exposure, and in both adults and larvae. A SIGS approach to achieving RNAi-induced mortality in *B. aeneus*, *via* feeding on dsRNA-treated oilseed rape buds and anthers, leads to variable success in achieving a lethal phenotype in this pest species. Most notably, high concentrations of 2.5–5 µg dsRNA/µl proved effective after 3 days of feeding on dsRNA-treated anthers, while these concentrations and a lower concentration of 0.5 µg dsRNA/µl showed significantly greater efficacy when dsRNA-treated anthers were provided for 17 days (Willow et al., 2021b). This is an important finding due to constant development and senescence of oilseed rape flowers, making successive sprays over the growing season a potential necessity for implementing a SIGS approach. In contrast, 3 days of feeding on treated oilseed rape buds, even when using a dsRNA concentration as high as 5 µg dsRNA/µl, showed negligible effect on *B. aeneus* adult survival (Willow et al., 2020).

The abovementioned studies indicate that both *A. tumida* and *B. aeneus* demonstrate little to moderate susceptibility to dietary RNAi. Further studies examining field-relevant dsRNA concentrations in a SIGS approach may provide evidence necessary to make conclusions regarding RNAi susceptibility in these two important pest species. The significantly enhanced RNAi efficacy from chronic dsRNA feeding, in *B. aeneus* (Willow et al., 2021b), also suggests potentially important benefits of developing RNAi cultivars to managing *B. aeneus* or other pests showing similar differences in RNAi efficacy between short-term and chronic dsRNA feeding. Furthermore, other economically important nitidulids (e.g., *Stelidota geminata* and *Carpophilus* spp.) should be examined, in order for us to understand more about the potential for implementing RNAi to manage nitidulid pests.

Families Buprestidae and Cerambycidae

Jewel beetles (Family Buprestidae) and longhorn beetles (Family Cerambycidae) consist of herbivorous species that feed on the tissues on roots, stems, and leaves of various herbaceous and woody plants. Some species are wood-borers that are highly damaging to forest trees. The only buprestid to be examined for dietary RNAi susceptibility is emerald ash borer (*Agrilus planipennis*). Rodrigues et al. (2017) exposed *A. planipennis* larvae to dietary dsRNA (*in vitro* synthesized) in sucrose solution for 4 days, and mortality of *A. planipennis* was monitored at 10 days. The authors observed 30, 35, and 78% larval mortality associated with 1, 6, and 10 µg dsRNA/µl treatments, respectively. More recently, Leelesh and Rieske (2020) demonstrated 47–69% mortality (depending on target gene) in *A. planipennis* larvae fed bacterially expressed dsRNA (at approximately 100 bacterial cells/nl).

The only cerambycid to be examined for dietary RNAi susceptibility is Asain long-horned beetle (*Anoplophora glabripennis*). Dhandapani et al. (2020a) exposed *A. glabripennis* larvae to dietary dsRNA (*in vitro* synthesized) in sucrose solution for 3 days, and mortality was monitored up to 10 days. The authors observed (depending on target gene) 17–25%, 50–67%, and 75–90% larval mortality associated with force-fed doses of 2, 5, and 10 µg dsRNA/day, respectively. Another study reported approximately 20% mortality, after 20 days, in both larval and adult *A. glabripennis* that were fed 15 µg dsRNA/day for 3 days (see Supplementary Material in Dhandapani et al., 2020b).

As the concentrations/doses used in the abovementioned buprestid and cerambycid studies are all above what is realistic for pest management, together with the fact that these wood-boring species would not be exposed to dsRNA through sucrose solutions or dsRNA-expressing bacteria, more studies should be conducted using field-realistic concentrations, and *via* field-relevant exposure routes (e.g., vascular cambium and wood). Furthermore, studies examining dietary RNAi susceptibility in wood-boring beetles may benefit greatly from the development of RNAi cultivars of the relevant tree species, for use in experiments; this is important, as a HIGS approach may represent the most economic and effective RNAi technique for managing forest tree pests.

CONCLUSION AND FUTURE DIRECTIONS

Few families in the order Coleoptera have been examined for dietary RNAi efficacy. Furthermore, within each family examined, few species are represented in the existing dietary RNAi literature. Surprisingly, our findings from this review indicate that less than half of the coleopteran species thus far examined for dietary RNAi efficacy are sensitive to dietary RNAi. While methods and concentrations used are variable within chrysomelid studies, all chrysomelid species thus far examined seem to show high sensitivity to dietary RNAi, a possible reason for the ongoing narrative that most coleopterans have high sensitivity to dietary RNAi. The species considered here to be insensitive to dietary RNAi have either demonstrated very little RNAi-induced mortality, or effective lethality of dsRNA has been observed for these species only when using unrealistically high concentrations of dsRNA. Considering that these studies are conducted after dsRNA-microinjection bioassays have already taken place, indicating what would theoretically be the most appropriate gene to target for inducing a lethal phenotype upon dsRNA ingestion, the dietary RNAi-insensitivity of more than half of all coleopterans examined is alarming. As some studies have indicated the role of dsRNases in lowering, limiting, or preventing dietary RNAi in certain coleopteran species (Almeida Garcia et al., 2017; Spit et al., 2017; Prentice et al., 2019; Peng et al., 2020), this potential limiting factor should be investigated in other coleopteran species for which dietary RNAi sensitivity is observed to be low. Furthermore, numerous studies have only examined routes of exposure that are not applicable to real-world pest management.

Source of dsRNA consumption represents an important variable when determining dietary RNAi sensitivity in any organism. Other than the three dsRNA-sensitive species *D. virgifera* (Baum et al., 2007; Vélez et al., 2020), *L. decemlineata* (Ren et al., 2021), and *H. vigintioctopunctata* (Ren et al., 2021), no other coleopteran has been examined for RNAi cultivar-based management efficacy. Such studies would contribute much to forwarding our knowledge regarding the prospects for using transgenic approaches to RNAi-based management of coleopteran

plant pests. Most studies in coleopterans examine potential for RNAi-based management *via* the use of naked dsRNA in artificial diets. This has allowed us to make general comparisons, regarding dietary RNAi efficacy, across and between taxa. The use of naked dsRNA also enables accurate reporting of dsRNA doses/concentrations examined. Some studies reported in our review examine dietary RNAi efficacy *via* the use of bacterially expressed dsRNA. In the case of the lady beetle *H. vigintioctopunctata*, dietary RNAi efficacies are similar whether using naked dsRNA or bacterially expressed dsRNA.

Other than the need to examine a greater diversity of pest species within the coleopteran families already discussed in the present review, there are numerous other important coleopteran taxa deserving of attention with regard to potential susceptibility to dietary RNAi. These include not only pest species, but also species that are important for directly providing ecosystem services (e.g., biological control, soil decomposition, and pollination), or species that play more facilitative roles in maintaining ecosystem biodiversity (e.g., those which are important food sources for higher trophic levels). Understanding dietary RNAi sensitivity in these beneficial coleopterans should be of great interest due to the concern for safeguarding non-target organisms. Furthermore, as co-formulating dsRNA with various types of nanoparticles can enhance dsRNA stability and RNAi efficacy in various insects (Pugsley et al., 2021), thereby making such formulations vital for SIGS approaches to plant protection, the use of different dsRNA−nanoparticle complexes continues to represent a critical avenue of investigation in both pest and beneficial coleopterans.

Finally, dsRNA resistance has been demonstrated *in vivo via* selective breeding in both *D. virgifera* (Khajuria et al., 2018) and *L. decemlineata* (Mishra et al., 2021), which is of great concern RNAi-based management of coleopteran pests. Yoon et al. (2018) observed that the dsRNA binding protein Staufen isoform C (*stauC*) is present in 24 of the 32 beetle genomes/transcriptomes they analyzed. The authors subsequently demonstrated *stauC* mRNA was significantly less expressed in an RNAi-resistant *L. decemlineata* cell line, and that in these cells, processing of dsRNA into small interfering RNAs was reduced by more than 80% compared to a susceptible *L. decemlineata* cell line. While the abovementioned study also suggests *stauC* downregulation as a catalyst for RNAi resistance in *D. virgifera* and *T. castaneum*, *stauC*'s presence in only three-fourths of the coleopteran species bioinformatically analyzed in the study suggests a diversity of RNAi resistance potential and/or mechanisms within Coleoptera. RNAi being a promising and biosafe technology for plant protection, these findings on RNAi resistance support the need for investigations into precise RNAi resistance mechanisms among different coleopteran taxa.

RNAi technology will likely play a major role in plant protection against coleopteran insects, given the current momentum in both research and development for the use of this biotechnology to protect both crop yields and forest trees. For RNAi-sensitive chrysomelid species, both HIGS and SIGS approaches are in development for the plant biotechnology marketplace. HIGS, through the development and use of RNAi cultivars that constantly produce target-specific dsRNA, may be the most appropriate method of dsRNA delivery when dealing with plant pests that only show effective dietary RNAi when chronically exposed to dsRNA. This transgenic approach is also likely an appropriate direction for managing tree pests, as successful RNAi-based management of these pests seems more likely if the target-specific dsRNA is constantly produced in the tree tissues, as opposed to forest management's reliance on a future of accurately timed trunk injections. That being said, it remains critical to examine possibilities for using both transgenic and non-transgenic approaches to managing coleopteran plant pest species.

AUTHOR CONTRIBUTIONS

JW conceived the manuscript and wrote the original draft. All authors made comments or suggestions toward revising the original draft and approved the final version of the manuscript.

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REFERENCES

Abd El Halim, H. M., Alshukri, B. M. H., Ahmad, M. S., Nakasu, E. Y. T., Awwad, M. H., Salama, E. M., et al. (2016). RNAi-mediated knockdown of the voltage gated sodium ion channel TcNa$_v$ causes mortality in *Tribolium castaneum*. *Sci. Rep.* 6:29301. doi: 10.1038/srep29301

Almeida Garcia, R., Lima Pepino Macedo, L., Cabral do Nascimento, D., Gillet, F.-X., Moreira-Pinto, C. E., Faheem, M., et al. (2017). Nucleases as a barrier to gene silencing in the cotton boll weevil, *Anthonomus grandis*. *PLoS One* 12:e0189600. doi: 10.1371/journal.pone.0189600

Bachman, P. M., Bolognesi, R., Moar, W. J., Mueller, G. M., Paradise, M. S., Ramaseshadri, P., et al. (2013). Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *Trans. Res.* 22, 1207–1222. doi: 10.1007/s11248-013-9716-5

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–1326. doi: 10.1038/nbt1359

Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flannagan, R., et al. (2012). Characterizing the mechanism of action of doubloe-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *PLoS One* 7:e47534. doi: 10.1371/journal.pone.0047534

Cao, M., Gatehouse, J. A., and Fitches, E. C. (2018). A systematic study of RNAi effects and dsRNA stability in *Tribolium castaneum* and *Acyrthosiphon pisum*, following injection and ingestion of analogous dsRNAs. *Int. J. Mol. Sci.* 19:1079. doi: 10.3390/ijms19041079

Cappelle, K., de Oliveira, C. F. R., Van Eynde, B., Christiaens, O., and Smagghe, G. (2016). The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. *Insect Mol. Biol.* 25, 315–323. doi: 10.1111/imb.12222

Christiaens, O., Prentice, K., Pertry, I., Ghislain, M., Bailey, A., Niblett, C., et al. (2016). RNA interference: a promising biopesticide strategy against the African sweetpotato weevil *Cylas brunneus*. *Sci. Rep.* 6:38836. doi: 10.1038/srep38836

Dhandapani, R. K., Duan, J. J., and Palli, S. R. (2020a). Orally delivered dsRNA induces knockdown of target genes and mortality in the Asian long-horned beetle, *Anoplophora glabripennis*. *Arch. Insect Biochem. Physiol.* 104:e21679. doi: 10.1002/arch.21679

Dhandapani, R. K., Gurusamy, D., Duan, J. J., and Palli, S. R. (2020b). RNAi for management of Asian long-horned beetle, *Anoplophora glabripennis*: identification of target genes. *J. Pest. Sci.* 93, 823–832. doi: 10.1007/s10340-020-01197-8

Guo, W., Guo, M., Yang, C., Liu, Z., Chen, S., Lü, J., et al. (2021). RNA interference-mediated silencing of vATPase subunits A and E affect survival and development of the 28-spotted ladybeetle, *Henosepilachna vigintioctopunctata*. *Insect Sci.* [Epub ahead of print]. doi: 10.1111/1744-7917.12899

Khajuria, C., Ivashuta, S., Wiggins, E., Flagel, L., Moar, W., Pleau, M., et al. (2018). Development and characterization of the first dsRNA-resistant insect population from western corn rootworm, *Diabrotica virgifera virgifera* LeConte. *PLOS One* 13:e0197059. doi: 10.1371/journal.pone.0197059

Knorr, E., Fishilevich, E., Tenbusch, L., Frey, M. L. F., Rangasamy, M., Billion, A., et al. (2018). Gene silencing in *Tribolium castaneum* as a tool for the targeted identification of candidate RNAi targets in crop pests. *Sci. Rep.* 8, 2061–2015. doi: 10.1038/s41598-018-20416-y

Kyre, B. R., Rodrigues, T. B., and Rieske, L. K. (2019). RNA interference and validation of reference genes for gene expression analyses using qPCR in southern pine beetle, *Dendroctonus frontalis*. *Sci. Rep.* 9:5640. doi: 10.1038/s41598-019-42072-6

Laudani, F., Strano, C. P., Edwards, M. G., Malacrinò, A., Campolo, O., Halim, H. M. A. E., et al. (2017). RNAi-mediated gene silencing in *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae). *Open Life Sci.* 12, 214–222. doi: 10.1515/biol-2017-0025

Leelesh, R. S., and Rieske, L. K. (2020). Oral ingestion of bacterially expressed dsRNA can silence genes and cause mortality in a highly invasive, tree-killing pest, the emerald ash borer. *Insects* 11:440. doi: 10.3390/insects11070440

Lü, J., Guo, W., Chen, S., Guo, M., Qiu, B., Yang, C., et al. (2020b). Feeding delivery of dsHvSnf7 is a promising method for management of the pest *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae). *Insects* 11:34. doi: 10.3390/insects11010034

Lü, J., Guo, M., Chen, S., Noland, J. E., Guo, W., Sang, W., et al. (2020a). Double-stranded RNA targeting vATPase B reveals a potential target for pest management of *Henosepilachna vigintioctopunctata*. *Pestic. Biochem. Physiol.* 165:104555. doi: 10.1016/j.pestbp.2020.104555

Lü, J., Liu, Z.-Q., Guo, W., Guo, M.-J., Chen, S.-M., Yang, C.-X., et al. (2021a). Oral delivery of dsHvlwr is a feasible method for managing the pest *Henosepilachna vigintioctopunctata* (Coleoptera: Coccinellidae). *Insect Sci.* 28, 509–520. doi: 10.1111/1744-7917.12784

Lü, J., Yang, C., Liu, Z., Vélez, A. M., Guo, M., Chen, S., et al. (2021b). Dietary RNAi toxicity assay suggests α and γ subunits of HvCOPI as novel molecular targets for *Henosepilachna vigintioctopunctata*, an emerging coccinellid pest. *J. Pest. Sci.* 94, 1473–1486. doi: 10.1007/s10340-021-01350-x

Máximo, W. P. F., Howell, J. L., Mogilicherla, K., Basij, M., Chereddy, S. C. R. R., and Palli, S. R. (2020). Inhibitor of apoptosis is an effective target gene for RNAi-mediated control of Colorado potato beetle, *Leptinotarsa decemlineata*. *Arch. Insect Biochem. Physiol.* 104:e21685. doi: 10.1002/arch.21685

Mehlhorn, S., Ulrich, J., Baden, C. U., Buer, B., Maiwald, F., Lueke, B., et al. (2021). The mustard leaf beetle, *Phaedon cochleariae*, as a screening model for exogenous RNAi-based control of coleopteran pests. *Pestic. Biochem. Phys.* 176:104870. doi: 10.1016/j.pestbp.2021.104870

Miguel, K. S., and Scott, J. G. (2015). The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Manag. Sci.* 72, 801–809. doi: 10.1002/ps.4056

Mishra, S., Dee, J., Moar, W., Dufner-Beattie, J., Baum, J., Dias, N. P., et al. (2021). Selection for high levels of resistance to double-stranded RNA (dsRNA) in Colorado potato beetle (*Leptinotarsa decemlineata* say) using non-transgenic foliar delivery. *Sci. Rep.* 11:6523. doi: 10.1038/s41598-021-85876-1

Miyata, K., Ramaseshadri, P., Zhang, Y., Segers, G., Bolognesi, R., Tomoyasu, Y., et al. (2014). Establishing an *in vivo* assay system to identify components involved in environmental RNA interference in the western corn rootworm. *PLoS One* 9:e101661. doi: 10.1371/journal.pone.0101661

Niu, X., Kassa, A., Hu, X., Robeson, J., McMahon, M., Richtman, N. M., et al. (2017). Control of Western corn rootworm (*Diabrotica virgifera virgifera*) reproduction through plant-mediated RNA interference. *Sci. Rep.* 7:12591. doi: 10.1038/s41598-017-12638-3

Pan, H., Yang, X., Romeis, J., Siegfried, B. D., and Zhou, X. (2020). Dietary RNAi toxicity assay exhibits differential responses to ingested dsRNAs among lady beetles. *Pest Manag. Sci.* 76, 3606–3614. doi: 10.1002/ps.5894

Peng, Y., Wang, K., Chen, J., Wang, J., Zhang, H., Ze, L., et al. (2020). Identification of a double-stranded RNA-degrading nuclease influencing both ingestion and injection RNA interference efficiency in the red flour beetle *Tribolium castaneum*. *Insect Biochem. Mol. Biol.* 125:103440. doi: 10.1016/j.ibmb.2020.103440

Petek, M., Coll, A., Ferenc, R., Razinger, J., and Gruden, K. (2020). Validating the potential of double-stranded RNA targeting Colorado potato beetle mesh gene in laboratory and field trials. *Front. Plant Sci.* 11:1250. doi: 10.3389/fpls.2020.01250

Pinheiro, D. H., Taylor, C. E., Wu, K., and Siegfried, B. D. (2020). Delivery of gene-specific dsRNA by microinjection and feeding induces RNAi response in Sri Lanka weevil, *Myllocerus undecimpustulatus* undatus Marshall. *Pest Manag. Sci.* 76, 936–943. doi: 10.1002/ps.5601

Powell, M. E., Bradish, H. M., Gatehouse, J. A., and Fitches, E. C. (2017). Systemic RNAi in the small hive beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae), a serious pest of the European honey bee *Apis mellifera*. *Pest Manag. Sci.* 73, 53–63. doi: 10.1002/ps.4365

Prentice, K., Smagghe, G., Gheysen, G., and Christiaens, O. (2019). Nuclease activity decreases the RNAi response in the sweetpotato weevil *Cylas puncticollis*. *Insect Biochem. Mol. Biol.* 110, 80–89. doi: 10.1016/j.ibmb.2019.04.001

Pugsley, C. E., Isaac, R. E., Warren, N. J., and Cayre, O. J. (2021). Recent advances in engineered nanoparticles for RNAi-mediated crop protection against insect pests. *Front. Agron.* 3:652981. doi: 10.3389/fagro.2021.652981

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P. D., Howlett, B. G., Winfree, R., et al. (2016). Non-bee insects are important contributors to global crop pollination. *Proc. Natl. Acad. Sci. U. S. A.* 113, 146–151. doi: 10.1073/pnas.1517092112

Ren, B., Cao, J., He, Y., Yang, S., and Zhang, J. (2021). Assessment on effects of transplastomic potato plants expressing Colorado potato beetle *β-actin* double-stranded RNAs for three non-target pests. *Pestic. Biochem. Phys.* 178:104909. doi: 10.1016/j.pestbp.2021.104909

Rodrigues, T. B., Rieske, L. K., Duan, J., and J., Mogilicherla, K., and Palli, S. R., (2017). Development of RNAi method for screening candidate genes to control emerald ash borer, *Agrilus planipennis*. *Sci. Rep.* 7:7379. doi: 10.1038/s41598-017-07605-x

Salvador, R., Niz, J. M., Nakaya, P. A., Pedarros, A., and Hopp, H. E. (2021). Midgut genes knockdown by oral dsRNA administration produces a lethal effect on cotton boll weevil. *Neotrop. Entomol.* 50, 121–128. doi: 10.1007/s13744-020-00819-1

Spit, J., Philips, A., Wynant, N., Santos, D., Plaetinck, G., and Vanden Broeck, J. (2017). Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* 81, 103–116. doi: 10.1016/j.ibmb.2017.01.004

Stork, N. E. (2018). How many species of insects and other terrestrial arthropods are there on earth? *Annu. Rev. Entomol.* 63, 31–45. doi: 10.1146/annurev-ento-020117-043348

Vélez, A. M., Fishilevich, E., Rangasamy, M., Khajuria, C., McCaskill, D. G., Pereira, A. E., et al. (2020). Control of western corn rootworm *via* RNAi traits in maize: lethal and sublethal effects of Sec23 dsRNA. *Pest Manag. Sci.* 76, 1500–1512. doi: 10.1002/ps.5666

Whyard, S., Singh, A. D., and Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* 39, 824–832. doi: 10.1016/j.ibmb.2009.09.007

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A. I., Taning, C. N. T., et al. (2020). First evidence of bud feeding-induced RNAi in a crop pest *via* exogenous application of dsRNA. *Insects* 11:769. doi: 10.3390/insects11110769

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A. I., Taning, C. N. T., et al. (2021a). Anther-feeding-induced RNAi in *Brassicogethes aeneus* larvae. *Front. Agron.* 3:633120. doi: 10.3389/fagro.2021.633120

Willow, J., Soonvald, L., Sulg, S., Kaasik, R., Silva, A. I., Taning, C. N. T., et al. (2021b). RNAi efficacy is enhanced by chronic dsRNA feeding in pollen beetle. *Commun. Biol.* 4:444. doi: 10.1038/s42003-021-01975-9

Willow, J., Sulg, S., Taning, C. N. T., Silva, A. I., Christiaens, O., Kaasik, R., et al. (2021c). Targeting a coatomer protein complex-I gene *via* RNA interference results in effective lethality in the pollen beetle *Brassicogethes aeneus*. *J. Pest. Sci.* 94, 703–712. doi: 10.1007/s10340-020-01288-6

Wu, K., Taylor, C. E., Pinheiro, D. H., Skelley, L. H., McAuslane, H. J., and Siegfried, B. D. (2019). Lethal RNA interference response in the pepper weevil. *J. Appl. Entomol.* 143, 699–705. doi: 10.1111/jen.12644

Xu, L., Xu, S., Sun, L., Zhang, Y., Luo, J., Bock, R., et al. (2021). Synergistic action of the gut microbiota in environmental RNA interference in a leaf beetle. *Microbiome* 9:98. doi: 10.1186/s40168-021-01066-1

Yoon, J.-S., Mogilicherla, K., Gurusamy, D., Chen, X., Chereddy, S. C. R. R., and Palli, S. R. (2018). Double-stranded RNA binding protein, Staufen, is required for the initiation of RNAi in coleopteran insects. *Proc. Natl. Acad. Sci. U. S. A.* 115, 8334–8339. doi: 10.1073/pnas.1809381115

Zhang, Y., Xu, L., Li, S., and Zhang, J. (2019). Bacteria-mediated RNA interference for management of *Plagiodera versicolora* (Coleoptera: Chrysomelidae). *Insects* 10:415. doi: 10.3390/insects10120415

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Direct Foliar Application of dsRNA Derived From the Full-Length Gene of *NSs* of Groundnut Bud Necrosis Virus Limits Virus Accumulation and Symptom Expression

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Groundnut bud necrosis virus (GBNV) is the most significant member of the genus *Orthotospovirus* occurring in the Indian subcontinent. There is hardly any effective measure to prevent GBNV in crop plants. In order to develop GBNV infection prevention procedure, we examined the effect of the direct foliar application of double-stranded RNA (dsRNA) derived from the full-length *NSs* gene (1,320 nucleotides) of GBNV. The bacterially expressed dsRNA to the non-structural (dsNSs) gene of GBNV was purified and delivered to plants as an aqueous suspension containing 0.01% Celite for evaluating its efficacy in preventing GBNV infection in systemic host, *Nicotiana benthamiana* as well as in local lesion and systemic host, cowpea cv. Pusa Komal (*Vigna unguiculata*). The dsNSs application and challenge-inoculation were conducted in three different combinations, where plants were challenge-inoculated with GBNV a day after, immediately, and a day before the application of dsNSs. *N. benthamiana* plants, which were not treated with dsRNA showed severe systemic wilting and death by 9–16 days post-inoculation (dpi). The non-treated cowpea plants exhibited many chlorotic and necrotic lesions on the cotyledonary leaves followed by systemic necrosis and death of the plants by 14–16 dpi. The dsNSs treated plants in all the combinations showed significant reduction of disease severity index in both *N. benthamiana* and cowpea. The treatment combination where the GBNV inoculation was conducted immediately after the dsNSs treatment was found to be the most effective treatment in preventing symptom expression. The viral RNA analysis by real time PCR also showed 20 and 12.5 fold reduction of GBNV in cowpea and *N. benthamiana*, respectively. Our results suggest that the foliar application of dsRNA derived from the full-length *NSs* gene of GBNV through Celite is successful in delivering long dsRNA leading to effective prevention of GBNV infection.

Keywords: groundnut bud necrosis virus, GBNV, tospovirus, dsRNA mediated protection, topical application of dsRNA, *NSs* gene

INTRODUCTION

Negative-sense ssRNA plant viruses are classified within the four families, *Ophioviridae, Phenuiviridae, Tospoviridae*, and *Rhabdoviridae*. Members of family, *Tospoviridae* are highly significant viruses as they are known to infect >1,000 plant species and are distributed all over the world (Kormelink et al., 2011). The genome of orthotospoviruses contains three segments of ssRNA: large (L), medium (M), and small (S) (Pappu et al., 2009). The L RNA is of negative polarity and encodes for the RNA-dependent RNA polymerase. The M RNA contains two genes, one in positive sense orientation encoding the movement protein and the other in negative sense orientation encoding Gn and Gc proteins required for thrips transmission. Similarly, the S RNA also contains two genes, the sense oriented one encodes a non-structural (NSs) protein that plays the role of a suppressor of RNA silencing and symptom determinant, and the antisense-oriented gene encodes for the nucleoprotein (NP) (Prins and Goldbach, 1998; Akram et al., 2004). Orthotospoviruses are transmitted by thrips vectors in a persistent propagative manner and are serious viral pathogens of numerous plant species. There are 26 orthotospovirus species[1] known all over the world. Tomato-spotted wilt virus (TSWV) is the most widespread and most studied tospovirus species (Pappu et al., 2009), however, in the Indian subcontinent, groundnut bud necrosis virus (GBNV) is the most prevalent orthotospovirus species. The complete genome sequence of GBNV has been generated and limited gene function studies have been conducted (Basavaraj et al., 2017). The NSs protein of GBNV has been shown as a bifunctional enzyme having both ATPase and phosphatase activities (Lokesh et al., 2010). Subsequently, the NSs protein of GBNV was found to be the symptom determinant, suppressor of RNA silencing, and inducer of plant cell death (Goswami et al., 2012; Singh et al., 2017). GBNV infects a wide variety of important crops such as cowpea, mungbean, peanut, potato, tomato, soybean, and urdbean. The conventional management approaches with cultural practices and insecticide treatment are not much effective to manage GBNV due to its wide host ranges, the abundance of thrips vector, and the lack of resistance cultivars which lead to a frequent disease outbreak causing serious crop losses (70–90%) in India (Reddy et al., 1995; Mandal et al., 2012). A few studies have attempted to utilize the molecular approaches to prevent GBNV infections, where the sense or antisense, hairpin, and artificial micro RNA constructs derived from *NP* and *NSs* genes of GBNV were shown to reduce GBNV infection in the experimental transgenic plants like tobacco and cowpea (Venkatesan et al., 2009; Goswami et al., 2012; Babu et al., 2019).

RNA interference (RNAi) plays a significant role in the plant development and defense against invasive nucleic acid such as transposable elements, virus, and virus-like pathogens (Agrawal et al., 2003; Obbard et al., 2009). For silencing or knocking down the expression of specific viral genes, plants have developed a strategy known as post-transcriptional gene silencing (PTGS) that involves sequence-specific degradation of viral RNA conferring resistance to plants (Unver and Budak, 2009). The important feature of this mechanism is to process the double-stranded RNA (dsRNA) or partial overlapping transcripts of DNA viruses into small interfering RNA (siRNA) of approximately 21–24 nucleotides by the enzymatic activity of dicer like enzymes (Carthew and Sontheimer, 2009). The processed siRNA binds to argonaute (AGO) protein and then is incorporated into RNA-induced silencing complex, which ensures degradation of the target RNA or viral transcript sharing the sequence similarity with the siRNA (Bologna and Voinnet, 2014). Additionally, the complementary guide RNA can also serve as a primer for the RNA-dependent RNA (RDR) polymerase for the generation of secondary siRNA ensuring amplification of siRNA signal. The RNAi-based defense mechanism is being utilized as a powerful strategy to develop disease resistance in crop plants against pests and pathogens such as viruses (Sanan-Mishra et al., 2021).

The principle of RNAi has been extensively utilized for engineering transgenic resistance in plants against viruses through genetic modification of plants with a segment of nucleotide sequence from the viral genome. The transgenic technology has been successfully utilized to develop virus-resistant cultivars of different crop plants (Leibman et al., 2011; Zhao et al., 2019; Gaffar and Koch, 2019). However, the practical field application of transgenic technology is greatly limited by the stringent regulatory laws in the different countries. An alternative approach to the transgenic technology is to induce RNAi in plants against the viruses through external or topical application of dsRNA derived from the viral genome. Topical application of dsRNA is emerging as an appealing non-GMO approach for the effective management of plants against virus infection (Dubrovina and Kiselev, 2019; Taliansky et al., 2021). To date, the studies on the topical application of dsRNA have been conducted against at least 14 plant viruses having positive sense ssRNA as their genome (Taliansky et al., 2021). To some extent, the approach was also found to be effective against ssDNA virus (genus *Begomovirus*), however, not much information is available as only three studies are available that showed limited or no effect against *Begomovirus* (Namgiala et al., 2019; Rego-Machado et al., 2020; Melita et al., 2021). Recently, two studies had shown the efficacy of topically applied dsRNA against the type member of the genus *Orthotospovirus*, TSWV, a negative/ambisense ssRNA virus (Tabein et al., 2020; Konakalla et al., 2021). However, no study so far demonstrated the usefulness of the exogenous application of dsRNA against GBNV.

The objective of the present study was to examine the potentiality of foliar application of dsRNA derived from the full-length gene of *NSs* (dsNSs) of GBNV in the two hosts, *N. benthamiana*, a systemic host and *Vigna unguiculata* (cowpea), a local lesion and systemic host. Our study demonstrated that the foliar application of dsNSs significantly reduced symptom expression as well as the viral load in both systemic and local host plants indicating high potential for developing anti-tospoviral RNA-therapeutic.

[1]https://talk.ictvonline.org/taxonomy/

MATERIALS AND METHODS

Test Plant and Virus Isolate

Seedlings of *N. benthamiana* and *V. unguiculata* cv. Pusa Komal (cowpea) were raised in potting-mix in 10 cm pots in an environmentally controlled growth room with an average temperature of 24 ± 2°C, average humidity of 65% and 16/8 h of light and dark periods. GBNV was isolated from a tomato plant showing leaf and bud necrosis, which was confirmed by ELISA with the polyclonal antibody to GBNV and subsequently sequencing the *NP* gene. The virus culture was maintained in both cowpea and tomato host through sap inoculation.

Preparation of dsRNA

A full-length *NSs* gene was amplified by RT-PCR from the above GBNV culture and cloned into pGEM T-Easy vector. The sequence of NSs was submitted to the GenBank with the accession number, OK105104. The *NSs* gene was further sub-cloned in the L4440 vector[2] having a T7 promoter at both ends. The recombinant vector was then used to transform the *Escherichia coli* strain HT115 for the production of dsRNA. To optimize the conditions for dsRNA generation, experiments with the different concentrations of IPTG (0.01, 0.1, 0.4, and 1.0 mM), different induction period after IPTG treatment (30 min, 1 h, 4 h, and overnight) and with different enzyme treatments [RNase A alone in buffer containing 300 mM sodium acetate, 10 mM Tris–HCl pH 7.5 and 5 mM EDTA (Thermo Fisher Scientific, United States) or without buffer; DNase I alone in buffer containing 10 mM Tris–HCl pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ (New England Biolabs, United Kingdom); both RNase A and DNase I together and without any enzyme treatment] were evaluated and the best combination of these factors were utilized for the production of dsRNA.

Purification of dsRNA from the bacterial culture was followed with some modification of the protocol as described by Posiri et al., 2013. Briefly, the IPTG-induced bacterial cells from 100 ml culture were harvested and used for the purification of dsRNA. The cell pellet was re-suspended in 1X PBS containing 70% v/v ethanol and incubated at 4°C for 30 min. After incubation, the cell pellets were collected by centrifugation at 6,000 × *g* for 10 min at 4°C. Then, the bacterial cells were re-suspended in 2.0 ml of 150 mM NaCl and incubated at 4°C for 1 h and centrifuged at 6,000 × *g* for 10 min at 4°C. To the supernatant, four units of RNase free DNase I and 20 μg of RNase A were added at 37°C for 30 min to remove DNA and ssRNA. The dsRNA was then precipitated by adding 500 μl of absolute ethanol. The pellet was air-dried, dissolved in deionized water, and the quantity was estimated spectrophotometrically (NanoDrop, Thermo Fisher Scientific, United States). The dsRNA preparation was stored at −20°C for further use. The quality of the purified dsRNA was judged by visualizing the expected dsRNA band on agarose gel. To examine the double stranded nature of the purified preparation, RNase treatment was performed in high and low salt concentration (Libonati and Sorrentino, 1992). Further, to confirm that the obtained band was of dsRNA from the *NSs* gene, nucleic acid hybridization assay (Northern/dot blot) was conducted with a DIG-labeled probe to the full-length *NSs* gene, which was prepared using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, F. Hoffmann-La Roche Ltd., Switzerland).

Delivery of dsRNA to Plant

In order to know, the entry of dsRNA into the plant system, the dsRNA aqueous solution containing 0.01% Celite 545 (BDH, England) was applied at the rate of 5.0 μg of dsRNA/plant by gentle rubbing on the adaxial surface of cowpea and *N. benthamiana* leaves. The excess dsRNA present on the leaf surface was removed by washing 4–5 times with distilled water. To detect the dsRNA in the different washes, each washout-water was collected separately, lyophilized to approximately 20 μl volume, and loaded in 1.5% agarose gel. The dsRNA treated leaves, after thorough and repeated washing, were used to isolate RNA by using TRIzol (Thermo Fisher Scientific)[3]. To understand the stability of the entered dsNSs in the plant tissues, cDNA of *NSs* was prepared using the total RNA isolated from the treated leaves with the reverse primer of *NSs* (BM1251: 5′ataagcttttactctggcttcacaatga3′) using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States). The stability pattern of dsNSs in the plant tissue was analyzed in the local leaves (treated) of cowpea as well as both the local and systemic (non-treated) leaves of *N. benthamiana* by semi-quantitative reverse transcriptase PCR (semi-qRT-PCR) with the primer pair, BM1260: 5′gacagatgcagagggaaatg3′; BM1251: 5′ataagcttttactctggcttcacaatga3′ at 15, 28, and 35 PCR cycles. The PCR conditions were: initial denaturation at 95°C for 3 min, followed by each cycle of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s. The final extension at 72°C was allowed for 5 min. The *GAPDH* and *actin* genes (Reddy et al., 2016) were used as internal controls for cowpea and *N. benthamiana*, respectively.

Challenge-Inoculation With Groundnut Bud Necrosis Virus

The challenge-inoculation of dsNSs-treated plants with GBNV was performed as described by Rai et al., 2020. The GBNV inoculated *N. benthamiana* leaves showing initiation of mottling and bending symptoms were used to prepare inoculum in the extraction buffer at a ratio 1:6 (1.0 g of leaves in 6 ml of extraction buffer) in a pre-chilled mortar and pestle. The extraction buffer was composed of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.15% sodium sulfite, and 0.002% beta-mercaptoethanol. The test plants were pre-dusted lightly with Celite powder and then 100 μl of the inoculum was applied gently on the surface of each leaves. The inoculated plants were sprayed with distilled water and maintained in a controlled environment room for symptom expression.

[2]https://www.addgene.org/1654/

[3]https://www.thermofisher.com

Treatments for Assessing dsNSs Against Groundnut Bud Necrosis Virus

The effectiveness of dsNSs against GBNV was assayed in three different treatment combinations. (T1) dsNSs treatment followed by GBNV (dsNSs – GBNV): in this combination, 5.0 μg of dsNSs dissolved in deionized water containing 0.01% Celite was applied by gentle rubbing on the adaxial surface of leaves and 1-day post-application of dsRNA, plants were challenged with GBNV through mechanical sap inoculation by the method as described above. (T2) Succeeding application of dsNSs and GBNV (dsRNA + GBNV): in this combination, 5.0 μg of dsNSs solution containing 0.01% Celite was similarly applied on the leaves and allowed for 10–15 min and then plants were challenged by mechanical sap inoculation with GBNV. (T3) GBNV inoculation followed by dsNSs treatment (GBNV – dsNSs): in this combination, leaves were first sap inoculated with GBNV, and at 1-day post-inoculation (dpi), 5.0 μg of dsNSs solution containing 0.01% Celite was applied. For the above experiment, two fully expanded primary leaves of cowpea and two leaves of 1-month-old *N. benthamiana* plants were used for treating with dsRNA and GBNV inoculation. The experiment with these three treatment combinations was repeated three times on *N. benthamiana* and cowpea var. Pusa Komal. The experimental plants were maintained for further observations and the number of chlorotic and necrotic lesions in local and systemic leaves were counted at regular intervals in cowpea and the systemic disease progress was monitored for both *N. benthamiana* and cowpea.

Disease Severity Analysis

To assess the impact of dsNSs treatment on symptom expression by GBNV, disease rating scales were developed on the basis of symptom severity grades by assigning the numerical values of 0–4 and 0–8 for *N. benthamiana* and cowpea, respectively (**Table 1**). The disease severity index (DSI) was assessed based on the disease incidence, which was calculated as [Σ (Grade assigned × number of infected plants)/Total grade × total number of plants] × 100. The relative progression of the disease till the death of the dsNSs-untreated GBNV inoculated plants was recorded and calculated using the area under the disease severity curve (AUDSC), following the standard method (Campbell and Madden, 1990; Bag et al., 2014) with the formula $Y = \sum_{i=1}^{n-1}[(X_i + X_{i+1})/2](t_{i+1} - t_i)$ where Y is the AUDPC, X_i is the disease incidence of the i^{th} evaluation and X_{i+1} is the disease incidence of the subsequent evaluation; (t_{i+1}-t_i) is the number of days between two subsequent evaluations.

TABLE 1 | Disease grading scales of groundnut bud necrosis virus in cowpea and *Nicotiana benthamiana*.

Cowpea		*N. benthamiana*	
Symptom	**Grade**	**Symptom**	**Grade**
No symptom	0	No Symptom	0
Chlorotic patches	1	Chlorotic/necrotic lesions (1–5)	1
Bending of shoots	2	Chlorotic/necrotic lesions (5–15)	2
Wilting of leaves	3	Chlorotic/necrotic lesions (15–30)	3
Death of plant	4	Complete leaf necrosis	4
		Wilting and leaf fall	5
		Systemic necrosis on new leaves	6
		Petiole and stem necrosis	7
		Death of plant	8

Quantification of Viral Load in the dsNSs-Treated Plants

The viral load with or without dsNSs treatment was analyzed using semi qRT-PCR as well as qPCR. On the onset of disease symptoms at 9 dpi in *N. benthamiana* and at 6 dpi in cowpea, RNA was isolated from the fresh leaf samples. cDNA was prepared with 1.0 μg of RNA sample and random hexamers by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States). Semi qPCR was performed using 1.0 μl of cDNA as a template. For performing qPCR analysis, 1.0 μl of cDNA was used as template and a portion of *NP* gene of GBNV was amplified using specific primers (PF: 5′GACAGGTCTGGCACCAATTA3′ and PR: 5′GGCTACTTTGCAAACCTGTTC3′) with 1× KAPA SYBR® FAST qPCR Master Mix (Roche, F. Hoffmann-La Roche Ltd., Switzerland). The *actin* and *GAPDH* genes were used as an endogenous control gene for *N. benthamiana* and cowpea, respectively. The relative viral load in the dsNSs-treated plants was calculated using the double C_t method (Livak and Schmittgen, 2001) comparing with the viral load as 1 unit in the untreated plants (calibrator). Three technical replicates were used in all the qPCR analysis.

Statistical Analysis

For comparison of disease severity between the three combinations of dsRNA with GBNV treatments and the control (only GBNV inoculation), one-way ANOVA test was performed. Homogeneity of variance test (Levene's statistic) was performed using SPSS package 20.0 to check the equality of variance in three datasets as the experiments were executed independently. Differences in the variance of the treatments were analyzed by Brown–Forsythe statistics.

For qPCR analysis, all the treatment combinations were evaluated as independent experiments and the data were statistically analyzed by ANOVA considering p-value < 0.05 as significant. The significance of the data within the dataset is also analyzed by performing student's t-test at $p < 0.1$ and $p < 0.05$ in *N. benthamiana* and cowpea, respectively.

RESULTS

dsRNA Derived From the *NSs* Gene of Groundnut Bud Necrosis Virus

The clone of *NSs* gene contained 1,320 nucleotides and shared up to 97.5% sequence identity with that of other isolates of GBNV available in the GenBank. The clone carrying dsNSs in L4440 vector was subjected to the various parameters for the expression of dsRNA in the bacterial culture, which

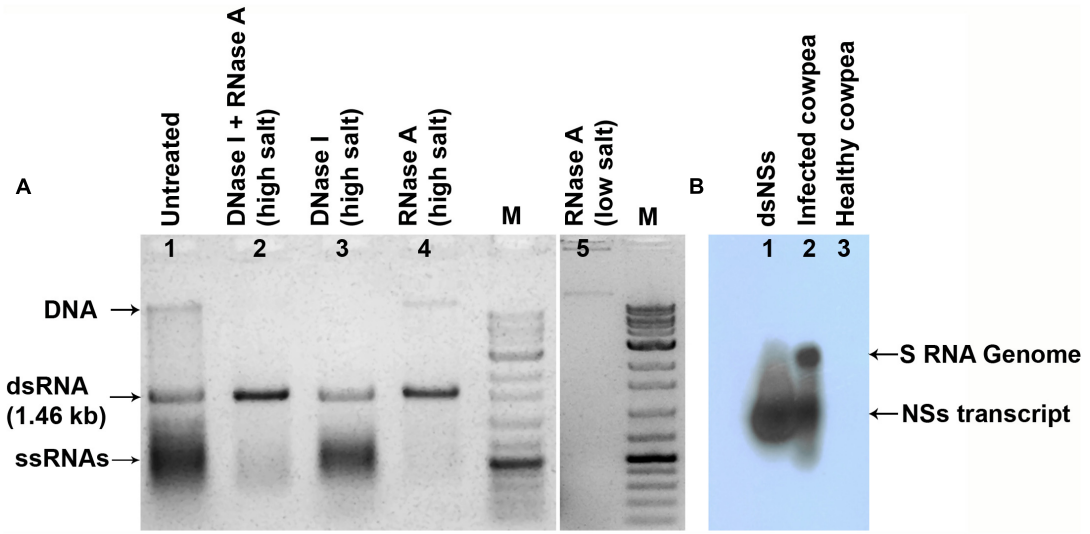

FIGURE 1 | The dsRNA specific to *NSs* gene of groundnut bud necrosis virus (GBNV) expressed in *E. coli* strain HT119. **(A)** Agarose gel showing nuclease digestion of the isolated nucleic acid from the bacteria transformed with pL4440, containing *NSs*. RNase A in high salt resisted digestion of 1.46 kb fragment (lane 4), but completely digested (lane 5) in low salt condition, indicating double stranded form. **(B)** Northern blot sowing hybridization of dsNSs isolated from bacteria (lane 1) and GBNV infected cowpea plant (lane 2) with the DIG-labeled probe to the PCR product of *NSs* gene of GBNV.

revealed incubation of bacterial culture with 0.4 mM IPTG at 37°C for 4 h induced maximum expression of the expected approximately 1.46 kb dsNSs. Purification of dsRNA from bacteria yielded approximately 1.5–2.0 mg of dsNSs per liter of bacterial culture. The dsRNA from the bacterial cells when was treated with RNase A in high salt, the approximately 1.46 kb band was not digested; however, it was digested with RNase A in low salt condition indicating double stranded nature of 1.46 kb band (**Figure 1A**). Further, the approximately 1.46 kb band was of *NSs* gene origin was confirmed through both Northern blot as well as dot blot analyses, which showed strong hybridization signal with the DIG-labeled probe to the NSs clone (**Figure 1B**).

Entry and Stability of dsNSs in Plants

To know the entry of dsRNA in the leaf tissues, the excess dsRNA present on the leaf surface was removed by repeated washing. It was found that after 4–5 washes, no dsRNA on the leaf surface could be detected in the agarose gel electrophoresis. Therefore, while detecting dsRNA in the plant tissue by semi qRT-PCR, the treated leaves were thoroughly washed five times before isolating the total RNA from the plant tissues. In *N. benthamiana* plants, dsNSs was tested by semi qRT-PCR with the NSs primers in the treated as well as in the newly developed leaves at 1, 3, and 7 days post-treatment. At the 15th cycle of semi qPCR, no detection of *NSs* gene was obtained; however, at 28th cycle of semi qRT-PCR, 118 nucleotide fragment of *NSs* was detected at all the three-time points in the treated leaves, interestingly, and NSs could be detected in the systemic leaves at 7 days post-treatment (**Figure 2B**). In cowpea, NSs was detected at the site of treatment till 5 days post-treatment but not at 6 or 7 days post-treatment at 28 cycles of semi qRT-PCR (**Figure 3B**).

Efficacy of dsNSs Against Groundnut Bud Necrosis Virus in *N. benthamiana*

The effectiveness of the topically applied dsNSs of GBNV for the prevention of GBNV infection was tested in 30 days old *N. benthamiana* plants containing 4–5 well-developed leaves ($n = 4$ plants in each of the three experiments). The dsNSs non-treated control plants of *N benthamiana* inoculated with GBNV showed no local lesion on the inoculated site; however, chlorotic patches were observed in these leaves at 9 dpi. Subsequently, systemic symptoms like veinal chlorosis, necrosis of newly developed leaves, and bending of the shoot with wilting of plants from the upper plant part were observed. Finally, the infected plant collapsed and died. To estimate the DSI each of the sequentially advancing symptoms like no symptom, chlorotic patches, bending of the shoot, wilting of leaves, and death of plants were graded as 0, 1, 2, 3, and 4, respectively (**Table 1**). The GBNV inoculated plants, where dsRNA was not applied, reached the severe disease grade of 3–4 by 9–16 days. Whereas the treatment combination-T2 (dsNSs + GBNV: where the dsRNA application was followed by the virus inoculation immediately), and the treatment combination-T3 (GBNV – dsNSs: where the dsRNA was applied at 1.0 dpi) showed a low disease severity grade of 0–2. The treatment combination-T1: dsNSs – GBNV, where the dsRNA was applied a day before GBNV inoculation, showed higher disease severity compared to that in the treatment combination-T2 and -T3 (**Figure 2A**). The DSI in the dsRNA-treated plants ranged between 50 and 66.7 indicating a reduction in the disease severity of about 43.3–50% in the dsRNA-treated plants compared to the non-treated controls. The AUDSC showed significantly less AUDSC values (669–924) in the treatment combination-T2 and -T3, where dsRNA treatment was conducted just before GBNV inoculation and a day post-GBNV

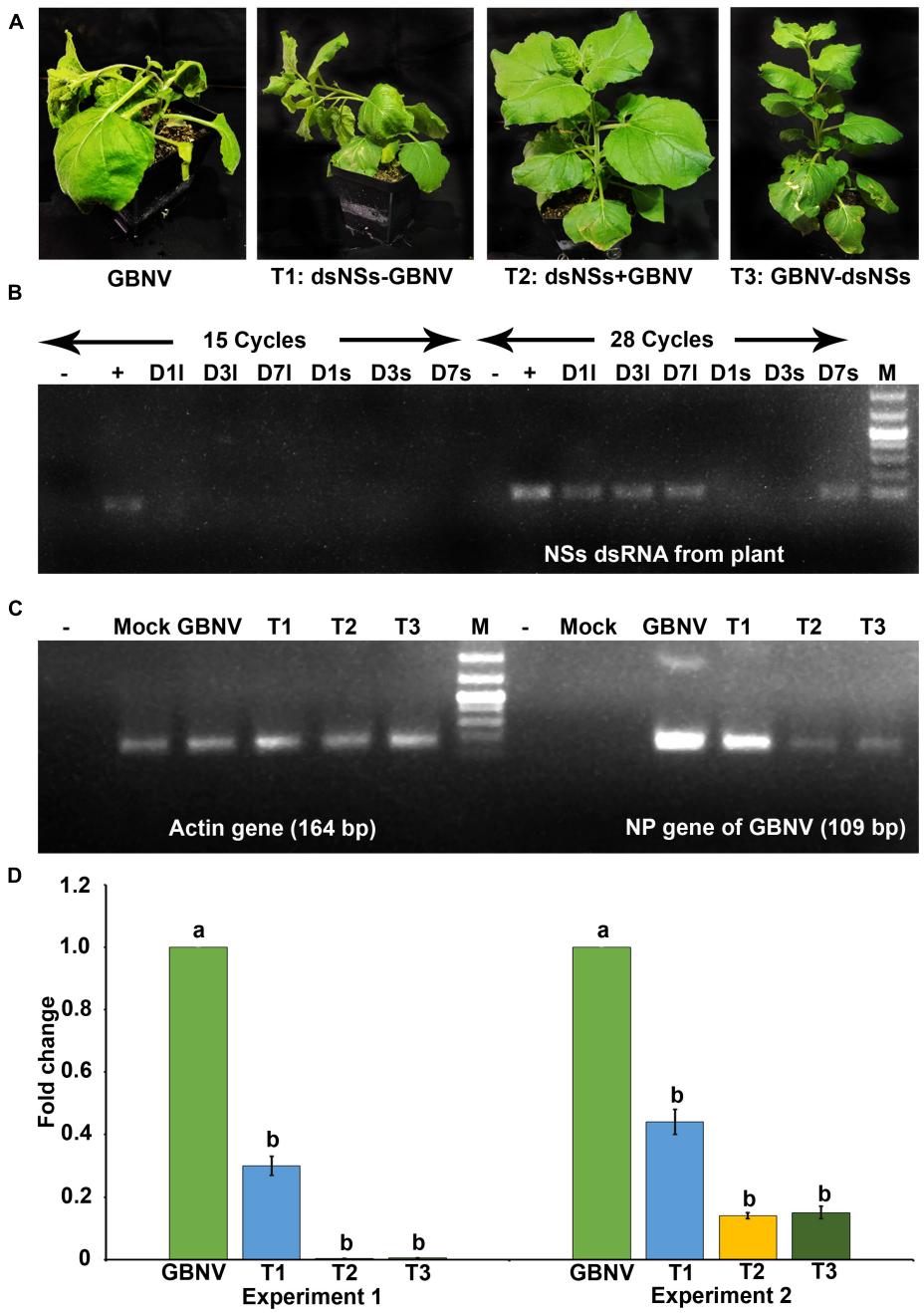

FIGURE 2 | Effect of topically applied dsNSs of GBNV in the *Nicotiana benthamiana* challenged with GBNV at 9 days post inoculation (dpi). **(A)** Treatment combinations: T2: dsNSs + GBNV (dsNSs treatment followed by virus inoculation) and T3: GBNV-dsNSs (dsNSs was applied a day post inoculation with GBNV) showed no necrosis and wilting while treatment combination T1: dsNSs – GBNV (dsNSs was applied a day before GBNV inoculation) as well as dsNSs untreated control plants exhibited those symptoms. **(B)** Stability analysis of dsNSs in the absence of GBNV in the local (l) and systemic (s) leaves during 1–7 dpi (D1–D7) by semiquantitative reverse transcriptase PCR (sqRT-PCR). **(C)** Detection of GBNV in the dsNSs-treated plant by sqRT-PCR of *NP* gene showing reduction of amplification of *NP* gene in different treatments compared to dsNSs untreated control plants. Actin was taken as internal control. **(D)** Quantitative reverse transcriptase PCR (qRT-PCR) showing significant reduction ($p \leq 0.05$) of GBNV titer in all the three treatment combinations. Different letters indicate statistically significant difference ($p \leq 0.05$). Two independent experiments were analyzed using Actin gene as endogenous reference control. –, reagent control; +, Plasmid of NSs clone (Positive control); Mock, buffer inoculated healthy plant; M, DNA mark 1 kb plus DNA ladder (G-Biosciences, United States).

inoculation, respectively, compared to the non-treated control which showed AUDSC value of 1378 (**Table 2**). Disease severity in GBNV treatment was significantly higher than the other three treatments ($p < 0.05$) indicated other three treatments were effective against viral infection. Although, homogeneity of variance test was significant ($p < 0.05$), but the differences in the

TABLE 2 | Effect of topically applied dsRNA of *NSs* gene of groundnut bud necrosis virus (GBNV) on the disease severity in *Nicotiana benthamiana* and cowpea cv. Pusa Komal.

	N. benthamiana		Cowpea	
Treatments[1]	**DSI[2]**	**AUDSC**	**DSI**	**AUDSC**
GBNV	100 ± 0^a	1378 ± 9^a	100 ± 0^a	544.1 ± 26^a
T1: dsNSs-GBNV	66.7 ± 8.4^b	994 ± 103.2	84.4 ± 32^b	427.4 ± 26^b
T2: dsNSs+GBNV	50 ± 16.7^b	669 ± 165^b	9.7 ± 2^b	86.6 ± 10^b
T3: GBNV-dsNSs	56 ± 15.4^b	924.7 ± 161^b	35 ± 7^b	188.9 ± 26^b

[1]GBNV, Only virus; T1: dsNSs-GBNV, dsNSs was applied on leaves and then a day after, plants were sap inoculated with GBNV; T2: dsNSs+GBNV, dsNSs was applied on leaves, then after 10–15 min, they were sap inoculated with GBNV; T3: GBNV-dsNSs, leaves were inoculated with GBNV and a day after, dsNSs was applied. 5.0 μg of dsNSs was dissolved in autoclaved distilled water containing 0.01% Celite was applied on each leaf.

[2]Disease Severity Index (DSI) was at 9 and 14 days post inoculation of Nicotiana benthamiana and cowpea, respectively, as on these days, death of plant started due to GBNV infection in the dsNSs untreated plants. AUDSC, Area under disease severity curve. AUDSC was calculated based on the progression of the disease till the death of the dsNSs untreated GBNV inoculated plants. Three independent experiments were conducted for each treatment. Homogeneity of variance of the experiments was tested by Levene's statistic using SPSS package 20.0. Differences in the variance of the treatments were analyzed by Brown-Forsythe statistics. Different letters within a column indicate statistically significant difference ($p \leq 0.05$).

treatments based on Brown–Forsythe statistics as the assumption of equal variances has been violated was found to be significant indicated differences really existed among the treatments.

In semi qRT-PCR, of all the three combinations, the combination-T2 and -T3 showed a marked reduction of *NP* gene amplification compared to the control or the combination-T1 (**Figure 2C**). The qRT-PCR results with *NP* gene primers showed a significant reduction in the viral load in plants treated with dsRNA compared to the non-treated plants (**Figure 2D**). The maximum reduction of viral load (12.5 fold) was found in the treatment combination-T2: dsNSs + GBNV followed by the treatment combination-T3 and i, which were 3.57 and 1.81 fold, respectively (**Figure 2D**). In the treatment combination-T2, which showed the maximum reduction of the viral load, the plants survived for a longer period of time compared to the non-treated ones (**Figure 4A**). In the three independent experiments of three application combinations of dsRNA, plants survived upto 23–25 dpi, indicating an 8–10 days delay in the death of the plants due to GBNV infection.

Efficiency of dsNSs Against Groundnut Bud Necrosis Virus in Cowpea

The efficacy of dsNSs was further examined in cowpea cv. Pusa Komal plants (n = 4, 10, and 3 plants in the three experiments). The primary leaves of cowpea cv. Pusa Komal inoculated with GBNV exhibited the chlorotic lesions at 4–6 dpi followed by necrotic lesions (**Figure 3A**), which coalesced, the inoculated leaves wilted and fell by 8–12 dpi. Subsequently, the plants developed systemic symptoms like necrotic lesions on newly developed leaves and necrosis on the petiole and stem. Eventually, the plants collapsed and died by 14–16 dpi. To determine the disease severity index, the level of the disease that is no-symptom to local necrosis and subsequent systemic necrosis followed by the death of plants were graded from 0 to 8 point scale (**Table 1**). The three treatment combinations of dsNSs and challenge with GBNV as carried out for *N. benthamiana* were also examined for cowpea. The dsNSs application on the two primary leaves of each seedling (5.0 μg/plant) in all the three treatments showed a significant reduction in the number of local lesions in cowpea seedlings (**Figure 3D**). The treatment combination-T2: dsNSs + GBNV showed a significant reduction of local lesions (1–2/seedling) compared to the non-treated plants (15–25 lesions/plant) (**Figure 3A**). The treatment combination T2 and T3 also resulted in a significant reduction of the number of lesions (3–5 lesions/plant) (**Figures 3A,D**). The dsRNA treatment combinations showed a significant effect on the reduction of local symptoms, which had a great implication on the restriction of *in planta* spread of the virus and survival of plant as when the non-treated plants died by 15–16 dpi due to the combined effect of local and systemic disease pressure, the dsRNA-treated plant survived with the development of new leaves (**Figure 4B**). The DSI, as well as AUDSC at 6 dpi in cowpea, were significantly low (DSI: 9.7–84.4 versus 100; AUDSC: 86.6–427.4 versus 544.1) in all the three combinations of dsRNA treatment as compared to the non-treated control (**Table 2**). The least DSI and AUDSC were obtained from the treatment combination-T2: dsNSs + GBNV. The presence of GBNV was detected by RT-PCR in all the dsRNA treatment combinations following the virus inoculation; however, the intensity of amplification was relatively less compared to the control especially in the case of the treatment combination T2 and T3 (**Figure 3C**). The GBNV load in the dsRNA-treated plants as judged by qRT-PCR was also significantly less compared to the non-treated control plants (**Figure 3E**).

DISCUSSION

In this study, we attempted to limit GBNV infection through the external application of dsRNA derived from the full-length gene of *NSs*. Our results indicate that the foliar treatment of plant with dsNSs significantly reduced the symptom expression as well as the viral load in the two experimental plant species, *N. benthamiana* and *V. unguiculata*.

RNA interference is an important mode of cellular immunity against viruses in the plant. The dsRNA plays a critical role in initiating RNAi in a cell. The preparation of dsRNA is an important step in the topical application approach of inducing RNAi. The dsRNA can be produced *in vivo* through bacterial

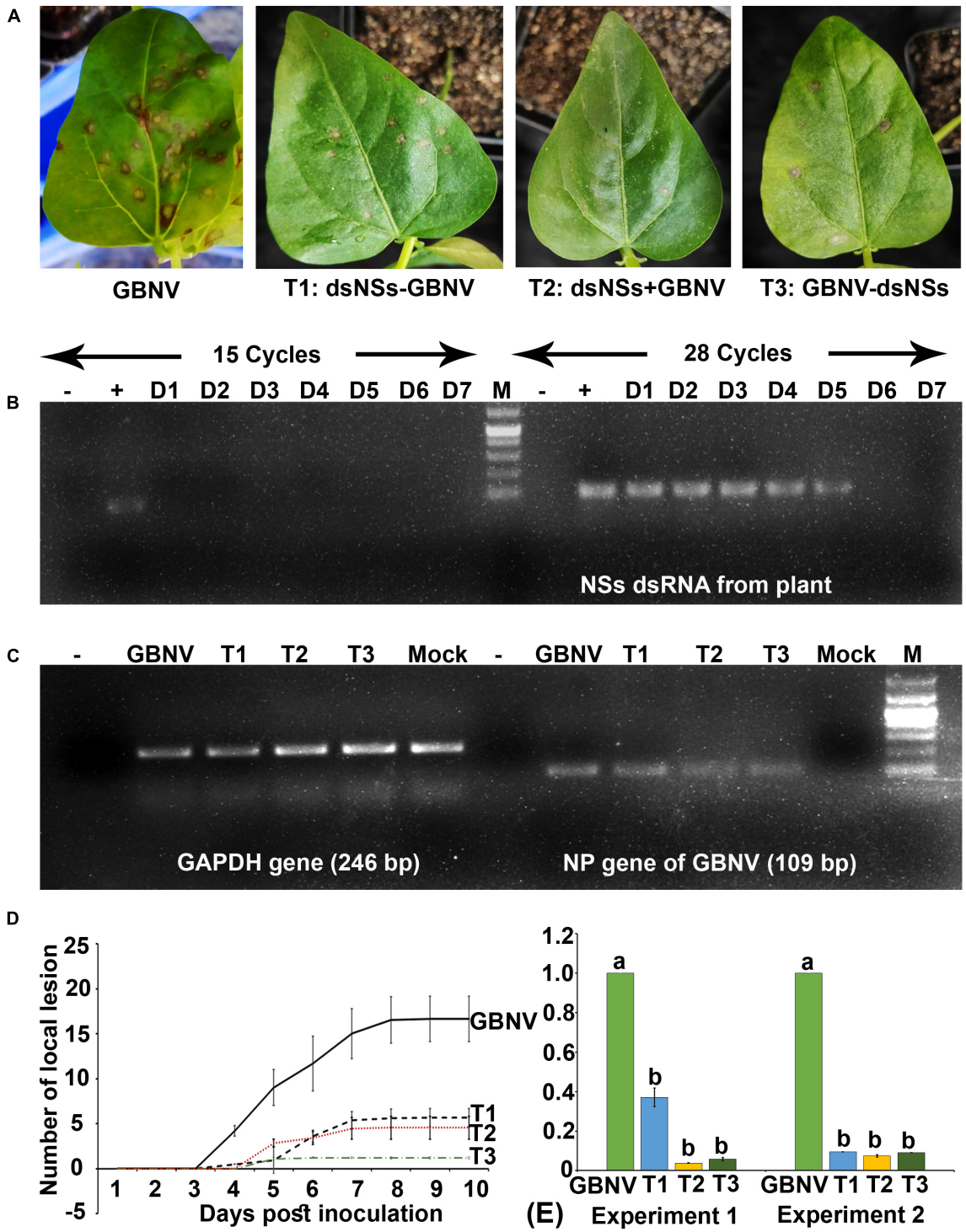

FIGURE 3 | Effect of topically applied dsNSs of GBNV in the cowpea cv. Pusa Komal challenged with GBNV at 6 days post inoculation (dpi). **(A)** The cowpea leaf showing reduced number of local lesions with T1, T2, and T3 treatment combinations compared to dsNSs untreated control plants. **(B)** Stability analysis of dsNSs in the absence of GBNV in the local leaves during 1–7 dpi (D1–D7) by sqRT-PCR. **(C)** Detection of GBNV in the dsNSs-treated plant by sqRT-PCR of *NP* gene. GAPDH was taken as internal control. **(D)** Reduction of the progression of local lesions in the three treatments (T1, T2, and T3) as compared to untreated GBNV inoculated control plants. **(E)** qRT-PCR showing significant reduction ($p \leq 0.05$) of GBNV titer in all the three treatment combinations. Different letters indicate statistically significant difference ($p \leq 0.05$). Two independent experiments were analyzed using GAPDH gene as endogenous reference control. –, reagent control; +, Plasmid of NSs clone (Positive control); Mock, buffer inoculated healthy plant; M, DNA mark 1 kb plus DNA ladder (G-Biosciences, United States).

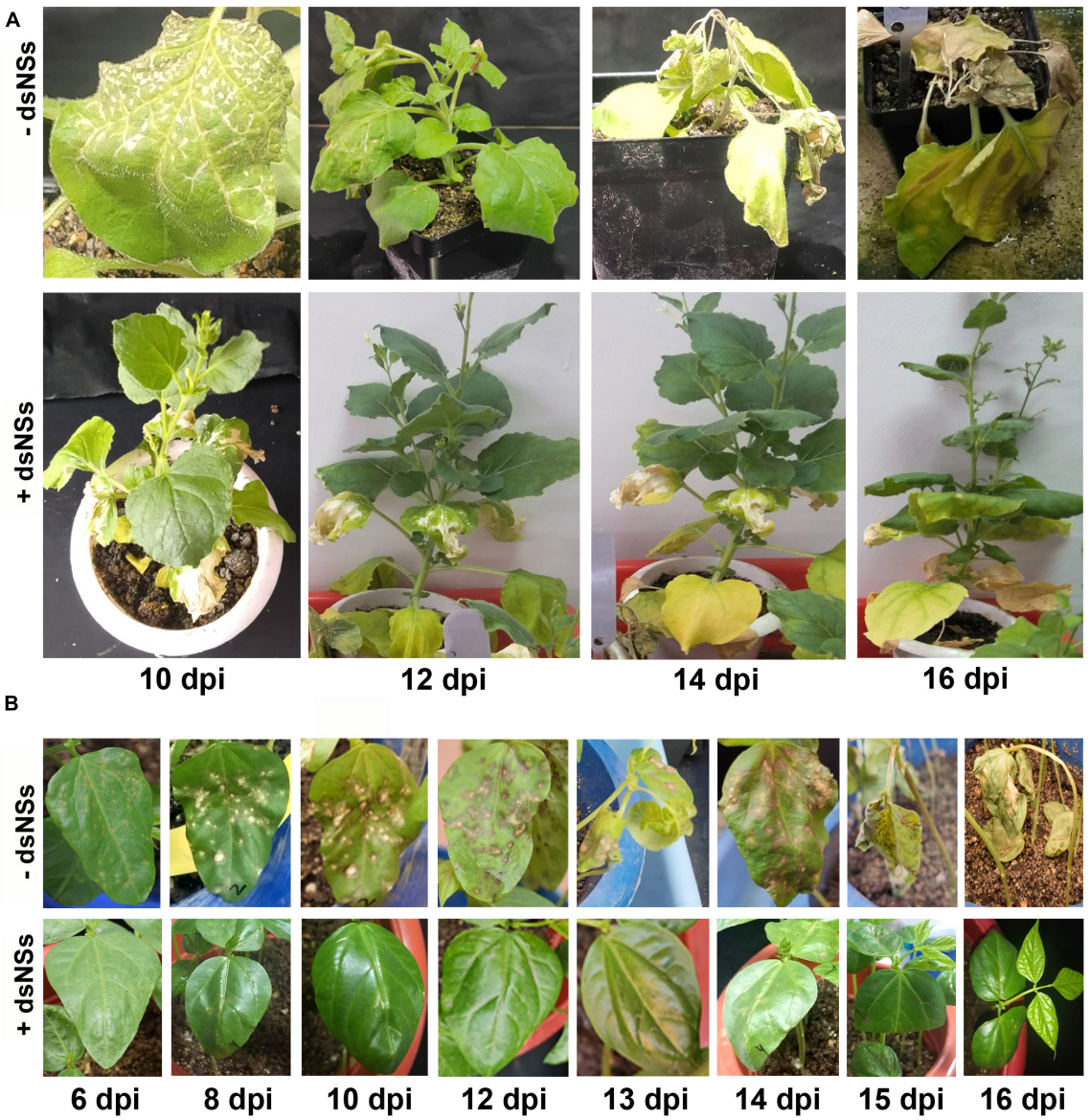

FIGURE 4 | The disease progress of GBNV in *Nicotiana benthamiana* **(A)** and cowpea cv. Pusa Komal **(B)** plants treated with dsNSs. *N. benthamiana* showed systemic symptoms of GBNV, whereas cowpea showed both local and systemic symptoms. Disease progress was observed at different days post inoculation (dpi) till the death of the inoculated plants. –dsNSs, no dsNSs treatment was given prior to GBNV inoculation; +dsNSs, an amount of 5.0 μg/plant of dsNSs in autoclaved distilled water containing 0.01% Celite was applied followed by GBNV inoculation after 10–15 min (T2 treatment combination).

expression or *in vitro* using RNA polymerase (Paula et al., 2012; Voloudakis et al., 2015; Papic et al., 2018; Li and Zamore, 2019). Different bacterial expression systems for obtaining dsRNA are known, which are more convenient and economic (Voloudakis et al., 2015). In the present study, we used the bacterial expression system to prepare dsRNA of *NSs* gene of GBNV, which is 1.3 kb long and is located toward the 5′ end of the S RNA genome segment of GBNV. Optimization of IPTG concentration, induction time, and different enzyme treatment resulted in a yield of about 1.5–2.0 mg dsNSs RNA per liter of bacterial culture. The yield of dsNSs was 50% less compared to that obtained in the published protocol (Posiri et al., 2013). This may be due to the difference in the length of dsRNA expressed in the two studies. Posiri et al., 2013 used dsRNA with a maximum size of 400 bp, whereas in the present study, the length of NSs dsRNA was more than three times longer.

In the external application of dsRNA, entry of dsRNA is imperative in the induction of RNAi. In plants, due to the presence of several physical barriers like wax layer, cuticle, cell wall, and cell membrane, delivery of dsRNA into the plant cell is a challenge (Bennett et al., 2020). Several methods like mechanical rubbing, pressure spray, infiltration, injection, root, or petiole absorption, nano-carrier conjugation of dsRNA

have been used to deliver dsRNA into the plant cells for silencing of endogenous genes or plant virus genes (Tenllado and Diaz-Ruiz, 2002; Numata et al., 2014; Mitter et al., 2017; Dalakouras et al., 2018; Dubrovina and Kiselev, 2019). In the present study, dsNSs RNA aqueous suspension containing Celite was applied on the adaxial surface of leaves through gentle rubbing. Celite is known as diatomaceous earth and is used as an abrasive for the mechanical sap transmission of plant viruses (Hull, 2013). Our results showed that Celite could also facilitate the successful entry of dsNSs in the plant tissues of both *N. benthamiana* and cowpea. The RT-PCR detection of dsRNA both in local as well as distal leaves indicated the systemic movement of dsRNA within the plant system.

Nicotiana benthamiana plant is a super susceptible host of GBNV (Mandal et al., 2012). The inoculated leaves of *N. benthamiana* did not produce any local lesions as found in cowpea, rather they showed mild yellow blotches followed by rapid and severe systemic disease response. Hence, cowpea and *N. benthamiana* plants provided both local lesion and systemic assay systems for the evaluation of the efficacy of externally applied dsRNA against important tospovirus like GBNV. In the natural conditions, virus infection can occur any time before, during or after the application of dsRNA. Considering these possibilities, in the present study, dsNSs was applied on the leaves a day before (T1), immediately (T2), and a day after (T3) the challenge-inoculation of plants with GBNV. The analysis of disease progress showed lower DSI and AUDPC in dsRNA-treated plants of both cowpea and *N. benthamiana* and a strong positive correlation was established between DSI and AUDPSC values, which indicated a good covenant between the two parameters of disease measurement (Roy et al., 2009 and Chattopadhyay et al., 2010).

All the three dsRNA treatment combinations not only showed a significant reduction of disease severity, but also reduced the GBNV titer in both *N. benthamiana* and cowpea. The treatment combinations-T2 and -T3 appeared to be more effective compared to T1. The possible hypothetical explanation of such differential treatment effect may be, in T1, when dsNSs was applied 1 day prior to challenge inoculation, the resultant siRNA after biogenesis, might not immediately form an activated RISC complex in the absence of corresponding target transcript of *NSs* from GBNV. Hence, some portion of siRNAs might have been lost due to nucleolytic degradation (Ji and Chen, 2012) that might resulted in less efficacy. On the contrary, in other two treatments (T2 and T3), the presence of GBNV during or prior to the application of dsNSs might have immediately activated the RISC complex, resulting in minimal or no loss of siRNA; hence, the viral titer in these two treatments was lower than the first treatment. The lower viral titer in treatment T2 and T3 compared to T1 presumably contributed less symptom severity. Furthermore, GBNV infection during or prior to the dsNSs application might have triggered biogenesis of GBNV genome-wide siRNA and that might have further accentuated by the dsNSs treatment. However, such hypotheses need to be examined.

Induction of resistance through foliar application of dsRNA has been studied recently for TSWV, the type member of the genus *Orthotospovirus* (Tabein et al., 2020; Konakalla et al., 2021). The dsRNAs in these studies were derived from the partial sequence of *N, NSs,* and *NSm* genes of TSWV. It was found that the dsRNA obtained from these genes had differential efficacy. *NSs* gene of TSWV is known to act as a PTGS suppressor and plays a vital role in the proliferation of virus by interfering with the RNAi mechanism of the host (Takeda et al., 2002). The dsRNA derived from the 646 nt sequence of *NSs* gene of TSWV showed a lower level of resistance compared to that of dsRNA from the 717 nt sequence of *N* gene (Konakalla et al., 2021). However, the efficacy of full-length *NSs* is not known. NSs protein of GBNV was shown as a pathogenic factor as it induced necrosis symptom and plant cell death (Goswami et al., 2012; Singh et al., 2017). In this study, *NSs* being a symptom determinant, the dsRNA derived from the full-length gene was highly effective in protecting from GBNV infection in cowpea as well as in *N. benthamiana* plants. We chose to utilize the full-length NSs dsRNA as it would include all the effective siRNA (Gago-Zachert et al., 2019). Furthermore, the use of full-length dsNSs RNA may result in silencing expansion beyond the 5′ and 3′ regions of the NSs open reading frame (ORF) as the NSs transcript is longer than its ORF. The use of longer dsRNA compared to the smaller one is supposed to include bigger pool of siRNAs even after the expansion of silencing. The protective efficacy of dsNSs was observed superior in the case of cowpea compared to *N. benthamiana*. This may be due to a higher level of vulnerability of *N. benthamiana* to viral infection as it lacks RNAi factors to resist virus infection. Previous studies have shown that *N. benthamiana* did not possess an active salicylic acid- and virus-inducible RDR and hence it was hyper susceptible to viruses (Yang et al., 2004). This was further supported by another study showing that the natural loss of variant of RDR1 in *N. benthamiana* resulted in the hypersensitivity of this plant to a large number of viruses (Ying et al., 2010).

Although there was a reduction of disease progression and severity, the RT-PCR test showed the presence of GBNV in each inoculated plants. However, the load of GBNV as judged by semi qRT-PCR and qRT-PCR showed a significant reduction in the dsRNA-treated plants of both *N. benthamiana* (12.5 folds reduction) and cowpea (20 folds reduction). Though the single application of dsRNA showed protection for a limited time in *N. benthamiana*, all the inoculated plants were affected and eventually died. However, the majority of cowpea plants that were treated with dsRNA survived with the single application of dsRNA. The disease response of GBNV in *N. benthamiana* is drastic compared to cowpea. In transgenic plants, dsRNA is abundant in the plant system as it is continuously and synchronously generated in all the cells (Smith et al., 2000). In contrast, exogenous application of dsRNA contributes only a limited amount of dsRNA in the plant system (Konakalla et al., 2016; Kaldis et al., 2018; Namgiala et al., 2019). Therefore, effective delivery system and continuous application of dsRNA

are required for the sustainable protection of plants from viral infection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BM, SM, and AR planned the experimental layout. DG and OS performed the experiments. DG and BM wrote the manuscript. BM, SM, AR, and YB helped the trouble shooting for technical problems and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Agrawal, N., Dasaradhi, P. V., Mohmmed, A., Malhotra, P., Bhatnagar, R. K., and Mukherjee, S. K. (2003). RNA interference: biology, mechanism, and applications. *Microbiol. Mol. Biol. Rev.* 67, 657–685. doi: 10.1128/mmbr.67.4.657-685.2003

Akram, M., Jain, R. K., Chaudhary, V., Ahlawat, Y. S., and Khurana, S. M. P. (2004). Comparison of groundnut bud necrosis virus isolates based on the movement protein (NSm) gene sequences. *Ann. Appl. Biol.* 145, 285–289. doi: 10.1111/j.1744-7348.2004.tb00385.x

Babu, K. P., Maligeppago, M., Asokan, R., and Reddy, M. (2019). Screening of a multi-virus resistant RNAi construct in cowpea though transient vacuum infiltration method. *VirusDisease* 30, 269–278. doi: 10.1007/s13337-018-0509-y

Bag, M. K., Gautam, N. K., Prasad, T. V., Pandey, S., Dutta, M., and Roy, A. (2014). Evaluation of an Indian collection of black gram germplasm and identification of resistance sources to Mungbean yellow mosaic virus. *Crop Protect.* 61, 92–101.

Basavaraj, Y. B., Mandal, B., Gawande, S., Renukadevi, P., Holkar, S., Krishnareddy, M., et al. (2017). "The occurrence, biology, serology and molecular biology of tospoviruses in Indian agriculture," in *A Century of Plant Virology in India*, eds B. Mandal, G. P. Rao, V. K. Baranwal, and R. K. Jain (Basingstoke: Springer Nature), 445–474.

Bennett, M., Deikman, J., Hendrix, B., and Iandolino, A. (2020). Barriers to efficient foliar uptake of dsRNA and molecular barriers to dsRNA Activity in plant cells. *Front. Plant Sci.* 11:816. doi: 10.3389/fpls.2020.00816

Bologna, N. G., and Voinnet, O. (2014). The diversity, biogenesis, and activities of endogenous silencing small RNAs in arabidopsis. *Ann. Rev. Plant Biol.* 65, 473–503. doi: 10.1146/annurev-arplant-050213-035728

Campbell, C. L., and Madden, L. V. (1990). *Introduction to Plant Disease Epidemiology*. New York, NY: Wiley.

Carthew, R. W., and Sontheimer, E. J. (2009). Origins and mechanisms of miRNAs and siRNAs. *Cell* 136, 642–655. doi: 10.1016/j.cell.2009.01.035

Chattopadhyay, S., Ali, K. A., Doss, S. G., Das, N. K., Aggarwal, R. K., Bandopadhyay, T. K., et al. (2010). Evaluation of mulberry germplasm for resistance to powdery mildew in the field and greenhouse. *J. Gen. Plant Pathol.* 76, 87–93. doi: 10.1007/s10327-010-0221-x

Dalakouras, A., Jarausch, W., Buchholz, G., Bassler, A., Braun, M., Manthey, T., et al. (2018). Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. *Front. Plant Sci.* 9:1253. doi: 10.3389/fpls.2018.01253

Dubrovina, A. S., and Kiselev, K. V. (2019). Exogenous RNAs for gene regulation and plant resistance. *Int. J. Mol. Sci.* 20:2282. doi: 10.3390/ijms20092282

Gaffar, F. Y., and Koch, A. (2019). Catch me if you can! RNA silencing-based improvement of antiviral plant immunity. *Viruses* 11:673. doi: 10.3390/v11070673

Gago-Zachert, S., Schuck, J., Weinholdt, C., Knoblich, M., Pantaleo, V., Grosse, I., et al. (2019). Highly efficacious antiviral protection of plants by small interfering RNAs identified in vitro. *Nucleic Acids Res.* 47, 9343–9357. doi: 10.1093/nar/gkz678

Goswami, S., Sahana, N., Pandey, V., Doblas, P., Jain, R. K., Palukaitis, P., et al. (2012). Interference in plant defense and development by non-structural protein NSs of Groundnut bud necrosis virus. *Virus Res.* 163, 368–373. doi: 10.1016/j.virusres.2011.08.016

Hull, R. (2013). *Plant Virology*, 5th Edn. Cambridge, MA: Academic Press.

Ji, L., and Chen, X. (2012). Regulation of small RNA stability: methylation and beyond. *Cell Res.* 22, 624–636. doi: 10.1038/cr.2012.36

Kaldis, A., Berbati, M., Melita, O., Reppa, C., Holeva, M., Otten, P., et al. (2018). Exogenously applied dsRNA molecules deriving from the Zucchini yellow mosaic virus (ZYMV) genome move systemically and protect cucurbits against ZYMV. *Mol. Plant Pathol.* 9, 883–895. doi: 10.1111/mpp.12572

Konakalla, N. C., Bag, S., Deraniyagala, A. S., Culbreath, A. K., and Pappu, H. R. (2021). Induction of plant resistance in tobacco (*Nicotiana tabacum*) against Tomato Spotted Wilt Orthotospovirus through foliar application of dsRNA. *Viruses* 13:662. doi: 10.3390/v13040662

Konakalla, N. C., Kaldis, A., Berbati, M., Masarapu, H., and Voloudakis, A. E. (2016). Exogenous application of double-stranded RNA molecules from TMV p126 and CP genes confers resistance against TMV in tobacco. *Planta* 244, 961–969. doi: 10.1007/s00425-016-2567-6

Kormelink, R., Garcia, M. L., Goodin, M., Sasaya, T., and Haenni, A. L. (2011). Negative-strand RNA viruses: the plant-infecting counterparts. *Virus Res.* 162, 184–202. doi: 10.1016/j.virusres.2011.09.028

Leibman, D., Wolf, D., Saharan, V., Zelcer, A., Arazi, T., Yoel, S., et al. (2011). A high level of transgenic viral small RNA is associated with broad potyvirus resistance in cucurbits. *Mol. Plant Microbe Interact.* 24, 1220–1238. doi: 10.1094/MPMI-05-11-0128

Li, C., and Zamore, P. D. (2019). RNAi in Drosophila S2 cells by dsRNA soaking. *Cold Spring Harb. Protoc.* 3, 207–208.

Libonati, M., and Sorrentino, S. (1992). Revisiting the action of bovine ribonuclease A and pancreatic-type ribonucleases on double-stranded RNA. *Mol. Cell. Biochem.* 117, 139–151. doi: 10.1007/BF00230753

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lokesh, B., Rashmi, P. R., Amruta, B. S., Srisathiyanarayanan, D., Murthy, M. R. N., and Savithri, H. S. (2010). NSs encoded by groundnut bud necrosis virus is a bifunctional enzyme. *PLoS One* 5:e9757. doi: 10.1371/journal.pone.0009757

Mandal, B., Jain, R. K., Krishnareddy, M., Kumar, N. K. K., Ravi, K. S., and Pappu, H. R. (2012). Emerging problems of tospoviruses (Bunyaviridae) and their management in the Indian subcontinent. *Plant Dis.* 96, 468–479. doi: 10.1094/PDIS-06-11-0520

Melita, O., Kaldis, A., Berbati, M., Reppa, C., Holeva, M., Lapidot, M., et al. (2021). Topical application of double-stranded RNA molecules deriving from Tomato

yellow leaf curl virus reduces cognate virus infection in tomato. *Biol. Plantarum* 65, 100–110. doi: 10.32615/bp.2020.172

Mitter, N., Worrall, E. A., Robinson, K. E., Li, P., Jain, R., Taochy, C., et al. (2017). Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants* 3:16207. doi: 10.1038/nplants.2016.207

Namgiala, T., Kaldisa, A., Chakraborty, S., and Voloudakis, A. (2019). Topical application of double-stranded RNA molecules containing sequences of Tomato leaf curl virus and Cucumber mosaic virus confers protection against the cognate viruses. *Physiol. Mol. Plant Pathol.* 108:101432. doi: 10.1016/j.pmpp.2019.101432

Numata, K., Ohtani, M., Yoshizumi, T., Demura, T., and Kodama, Y. (2014). Local gene silencing in pants via synthetic dsRNA and carrier peptide. *Plant Biotechnol. J.* 12, 1027–1034. doi: 10.1111/pbi.12208

Obbard, D. J., Gordon, K. H., Buck, A. H., and Jiggins, F. M. (2009). The evolution of RNAi as a defence against viruses and transposable elements. *Philosop. Trans. R. Soc. B.* 364, 99–115. doi: 10.1098/rstb.2008.0168

Papic, L., Rivas, J., Toledo, S., and Romero, J. (2018). Double-stranded RNA production and the kinetics of recombinant *Escherichia coli* HT115 in fed-batch culture. *Biotechnol. Rep.* 20:e00292. doi: 10.1016/j.btre.2018.e00292

Pappu, H. R., Jones, R. A. C., and Jain, R. K. (2009). Global status of tospovirus epidemics in diverse cropping systems: successes achieved and challenges ahead. *Virus Res.* 141, 219–236. doi: 10.1016/j.virusres.2009.01.009

Paula, L., Harris, J., and Cook, M. (2012). Exploring RNAi as a therapeutic strategy for controlling disease in aquaculture. *Fish Shellfish Immunol.* 34, 729–743. doi: 10.1016/j.fsi.2012.11.037

Posiri, P., Ongvarrasopone, C., and Panyim, S. (2013). A simple one-step method for producing dsRNA from E. coli to inhibit shrimp virus replication. *J. Virol Methods* 188, 64–69. doi: 10.1016/j.jviromet.2012.11.033

Prins, M., and Goldbach, R. (1998). The emerging problem of tospovirus infection and nonconventional methods of control. *Trends Microbiol.* 6, 31–35. doi: 10.1016/S0966-842X(97)01173-6

Rai, A. K., Basavaraj, Y. B., Sadashiva, A. T., Reddy, M. K., Ravishankar, K. V., Hussain, Z., et al. (2020). Evaluation of tomato genotypes for resistance to bud necrosis disease caused by groundnut bud necrosis virus (GBNV). *Crop Protect.* 131:105074. doi: 10.1016/j.cropro.2019.105074

Reddy, D. S., Bhatnagar-Mathur, P., Reddy, P. S., Sri-Cindhuri, K., Sivaji-Ganesh, A., and Sharma, K. K. (2016). Identification and validation of reference genes and their impact on normalized gene expression studies across cultivated and wild Cicer species. *PLoS One* 11:e0148451. doi: 10.1371/journal.pone.0148451

Reddy, D. V. R., Buiel, A. A. M., Satyanarayana, T., Dwivedi, S. L., Reddy, A. S., Ratna, A. S., et al. (1995). "Peanut bud necrosis disease: an overview," in *Recent studies on peanut bud necrosis disease: proceedings of a meeting. ICRISAT Asia Center, Hyderabad*, (Patancheruvu), 3–7.

Rego-Machado, C. M., Nakasu, E. Y. T., Silva, J. M. F., Lucinda, N., Nagata, T., and Inoue Nagata, A. K. (2020). siRNA biogenesis and advances in topically applied dsRNA for controlling virus infections in tomato plants. *Sci. Rep.* 10:22277. doi: 10.1038/s41598-020-79360-5

Roy, A., Acharya, S., Das, S., Ghosh, R., Paul, S., Srivastava, R. K., et al. (2009). Distribution, epidemiology and molecular variability of the begomovirus complexes associated with yellow vein mosaic disease of mesta in India. *Virus Res.* 141, 237–246. doi: 10.1016/j.virusres.2008.11.022

Sanan-Mishra, N., Jailani, A. A. K., Mandal, B., and Mukherjee, S. K. (2021). Secondary siRNAs in plants: biosynthesis, various functions, and applications in virology. *Front. Plant Sci.* 12:610283. doi: 10.3389/fpls.2021.610283

Singh, A., Permar, V., Jain, R. K., Goswami, S., Kumar, R. R., Canto, T., et al. (2017). Induction of cell death by tospoviral protein NSs and the motif critical for cell death does not control RNA silencing suppression activity. *Virology* 508, 108–117. doi: 10.1016/j.virol.2017.05.003

Smith, N. A., Singh, S. P., Wang, M. B., Stoutjesdijk, P. A., Green, A. G., and Waterhouse, P. M. (2000). Total silencing by intron-spliced hairpin RNAs. *Nature* 407, 319–320. doi: 10.1038/35030305

Tabein, S., Jansen, M., Noris, E., Vaira, A. M., Marian, D., Behjatnia, S. A. A., et al. (2020). The induction of an effective dsRNA-mediated resistance against tomato spotted wilt virus by exogenous application of double-stranded RNA largely depends on the selection of the viral RNA target region. *Front. Plant Sci.* 11:533338. doi: 10.3389/fpls.2020.533338

Takeda, A., Sugiyama, K., Nagano, H., Mori, M., Kaido, M., Mise, K., et al. (2002). Identification of a novel RNA silencing suppressor, NSs protein of tomato spotted wilt virus. *FEBS Lett.* 532, 75–79. doi: 10.1016/s0014-5793(02)03632-3

Taliansky, M., Samarskaya, V., Zavriev, S. K., Fesenko, I., Kalinina, N. O., and Love, A. J. (2021). RNA-based technologies for engineering plant virus resistance. *Plants* 10:82. doi: 10.3390/plants10010082

Tenllado, F., and Diaz-Ruiz, J. R. (2002). Double-stranded RNA-mediated interference with plant virus infection. *J. Virol.* 75, 12288–12297. doi: 10.1128/JVI.75.24.12288-12297.2001

Unver, T., and Budak, H. (2009). Virus-induced gene silencing, a post transcriptional gene silencing method. *Int. J. Plant Genomics* 2009:198680. doi: 10.1155/2009/198680

Venkatesan, S., Raja, J. A. J., Maruthasalam, S., Kumar, K. K., Ramanathan, A., Sudhakar, D., et al. (2009). Transgenic resistance by N gene of a *Peanut bud necrosis virus* isolate of characteristic phylogeny. *Virus Genes* 38, 445–454. doi: 10.1007/s11262-009-0342-5

Voloudakis, A., Holeva, M., Sarin, P., Bamford, D., Vargas, M., Poranen, M., et al. (2015). Efficient double-stranded RNA production methods for utilization in plant virus control. *Methods Mol. Biol.* 1236, 255–274. doi: 10.1007/978-1-4939-1743-3_19

Yang, S., Carter, S. A., Cole, A. B., Cheng, N., and Nelson, R. S. (2004). A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana*. *Proc. Natl. Acad. Sci. U.S.A* 101, 6297–6302. doi: 10.1073/pnas.0304346101

Ying, X. B., Dong, L., Zhu, H., Duan, C. G., Du, Q. S., Lv, D. Q., et al. (2010). RNA-dependent RNA polymerase from Nicotiana tabacum suppresses RNA silencing and enhances viral infection in *nicotiana benthamiana*. *Plant Cell* 22, 1358–1372. doi: 10.1105/tpc.109.072058

Zhao, Y., Yang, X., Zhou, G., and Zhang, T. (2019). Engineering plant virus resistance: from RNA silencing to genome editing strategies. *Plant Biotechnol. J.* 18, 328–336. doi: 10.1111/pbi.13278

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RNA Interference-Based Genetic Engineering Maize Resistant to *Apolygus lucorum* Does Not Manifest Unpredictable Unintended Effects Relative to Conventional Breeding: Short Interfering RNA, Transcriptome, and Metabolome Analysis

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The use of omics techniques to analyze the differences between genetic engineering organisms and their parents can identify unintended effects and explore whether such unintended effects will have negative consequences. In order to evaluate whether genetic engineering will cause changes in crops beyond the changes introduced by conventional plant breeding, we compared the extent of transcriptome and metabolome modification in the leaves of three lines developed by RNA interference (RNAi)-based genetic engineering and three lines developed by conventional breeding. The results showed that both types of plant breeding methods can manifest changes at the short interfering RNA (siRNA), transcriptomic, and metabolic levels. Relative expression analysis of potential off-target gene revealed that there was no broad gene decline in the three RNAi-based genetic engineering lines. We found that the number of DEGs and DAMs between RNAi-based genetic engineering lines and the parental line was less than that between conventional breeding lines. These unique DEGs and DAMs between RNAi-based genetic engineering lines and the parental lines were not enriched in detrimental metabolic pathways. The results suggest that RNAi-based genetic engineering do not cause unintended effects beyond those found in conventional breeding in maize.

Keywords: RNAi-based genetic engineering maize, unintended effect, insect-resistant, biosafety assessment, transcriptome, metabolome

Abbreviations: RNAi, RNA interference; dsRNA, double-stranded RNA; siRNAs, short interfering RNAs; HPLC-MS, high-performance liquid chromatography mass spectrometry; FPKM, fragments per Kb per million reads; FDR, false discovery rate; DEGs, differentially expressed genes; DAMs, differentially accumulated metabolites; OPLS-DA, orthogonal partial least squares discriminant analysis; VIP, variable importance for the projection; PCA, principal components analysis; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes.

INTRODUCTION

The application of genetic engineering (GE) technology to develop new crops with excellent biological characteristics was one of the strategies to ensure food security in the 21st century. To guarantee global food security, expert practitioners have applied biotechnology to crop breeding to obtain many GE crops with disease and insect resistance, abiotic (salt, drought, cold, and heat) resistance, and nutritional improvement, and some of these crops that have undergone rigorous biosafety assessment have been widely planted in some countries worldwide [Ricroch et al., 2011; Li et al., 2020; International Service for the Acquisition of Agri-biotech Applications [ISAAA], 2021]. RNA interference (RNAi)-based insect-resistant crops were transferred into exogenous fragments of vital pest genes to produce double-stranded RNA (dsRNA), which was then cut into approximately 21–24 nt short interfering RNAs (siRNAs) in the plant using Dicer or Dicer-like proteins. By means of base pairing, these siRNAs can target mRNA sequences in pests and subsequently degrade for pest control (Mamta and Rajam, 2017). RNAi can be used in the "in-species" mode of plant genomes to improve nutritional content by reducing antinutrients, allergens, and toxins while increasing the level of beneficial nutrients and inhibiting the growth of undesirable traits to improve productivity (Ramon et al., 2014). Similarly, RNAi can be used in plants to express dsRNA derived from genes outside the parental plant. Virus-resistant and insect-resistant crops obtained using RNAi have been approved for cultivation, including plums (*Prunus salicina*), soybeans (*Glycine max*), maize (*Zea mays*), Cassava (*Manihot esculenta*), and apples (*Malus pumila*).[1] The public's concerns mainly focus on potential risks to the environment and human health, contributing to delayed commercialization of GE products in many countries (Li et al., 2020). Therefore, before any new GE products have been allowed to enter the market, rigorous safety assessment research was crucial, the purpose of which was to identify and avoid risks.

The potential risks of GE crops have been separated into two broad categories: intended and unintended changes (Ladics et al., 2015). The assessment of intended changes uses measurement indicators such as molecular and biological characteristics and crop phenotypes, while there is no uniform standard for the measurement of unintended changes (Ladics et al., 2015). We can obtain specific biological traits or expected phenotypic traits by transferring specific exogenous genes into recipient crops using GE technology, however, we cannot guarantee that the exogenous gene will be integrated into the specific location of the recipient genome, which may lead to unintended integration, such as undesirable integration location, copy number, etc. This unintended integration often causes the final phenotypic change, but the trace compounds that caused the phenotypic change may not be detected downstream of the breeding process, which these trace amounts of compounds may affect nutrition and quality, even related to allergic and toxic effects (Zhao and Wolt, 2017). Based on this, two unintended risk assessment strategies have been suggested. The first strategy is to collect data on the phenotypic characteristics of GE crops including measuring the overall phenotype of crops and the composition of principal tissues, transcriptome, proteome, and metabolome (Ladics et al., 2015). Unintended changes are identified by comparing these data between GE crops and their counterparts (Bregitzer et al., 1998; Harrigan et al., 2010). The second strategy uses targeted sequencing and non-targeted omics data analysis of possible unintended omics data changes (Ladics et al., 2015). The latter approach considers risk assessment to be a hypothesis-driven test more comprehensively and scientifically reflecting differences between GE crops and their comparable counterparts (Ladics et al., 2015). Guided by relevant legislation and regulation (Evans et al., 2006), risk assessment experts determine the possible unintended effects and establish a series of scenarios that may be caused by specific events, excluding extreme and scientifically unreliable assumptions after the fact, determine the probability of these events and their frequency or magnitude, and collect data to test these hypotheses and characterize risks, including unintended effects (Ladics et al., 2015). The risk assessment based on tests and hypotheses can minimize unintended risks in GE crops (Ladics et al., 2015). Transcriptome, proteomics, and metabolomics have been used to evaluate the unintended effects of GE crop breeding at the mRNA, protein, and metabolite levels (Ricroch et al., 2011). Most such studies use one or two omics techniques to investigate the unintended effects, focusing on the comparison between GE lines and parental line, revealing some degree of variation. These studies cannot entirely and clearly establish whether the detected variation comes from insertion of exogenous gene or from the environment or genetic background or if they are attributed to normal variation in conventional breeding lines (Wang et al., 2019). At present, some have proposed that omics evaluation experiments on GE plants should not only establish parental control lines but also should use conventional breeding lines as controls for comparative analysis, which is a highly recognized approach (Klumper and Qaim, 2014).

Many things can induce unintended effects, which may emerge at any stage of the GE plant development process, including random mutations, somatic mutations, gene sequence insertions, positional effects, inductive effects, mutations in the tissue culture process, and pleiotropy (Weng et al., 2019). However, RNAi-based GE crops have remarkable molecular characteristics compared with conventional GE crops. If siRNAs were highly matched with non-target sequences to produce inhibitory effects, they may have unintended off-target effects although non-target species may include the GE plants themselves. The possible impact of siRNAs on plant genomes has not been clarified. The European Food Safety Agency's GE management team believes that the relevant safety assessment content provided by research and development (R&D) applicants must be as detailed as possible and emphasize the importance of bioinformatics in the process of off-target effect analysis (Papadopoulou et al., 2020).

Maize, one of the most important food, feed, and energy crops in the world, is damaged by pests, and diseases bring massive losses to farmers (Li et al., 2020). The data show that the four countries with the most GE crops have planted large

[1] https://www.isaaa.org/gmapprovaldatabase/default.asp

areas of GE maize (International Service for the Acquisition of Agri-biotech Applications [ISAAA], 2021). In the context of increased resistance to individual Bacillus thuringiensis (Bt) target pests, GE plants expressing dsRNA represent a new generation of GE plants, but there has been little research on safety assessments of the unintended effects of RNAi-based GE crops (Casacuberta et al., 2015; Mamta and Rajam, 2017). In this study, RNAi-based GE lines (DTS_108, DTS_123, DTS_127) resistant to *Apolygus lucorum* (Meyer-Dür) (Hemiptera: Miridae) (reference patent No. US9944948B2), the parental line (TJ806), and conventional breeding lines (AR02 and AR03) were used to compare and evaluate unintended effects. We compared biological variations among the six maize lines at the siRNA, mRNA, and metabolite levels (**Figure 1**). The research constructed the datasets using omics-based systems biology methods, including siRNA sequencing, transcriptomics using RNA-seq, and metabolomics using high-performance liquid chromatography mass spectrometry (HPLC-MS). Based on these results, the potential unintended effects caused by two different plant breeding methods were analyzed comparably.

MATERIALS AND METHODS

Materials

In this study, we used a total of six maize lines, including three RNAi-based GE lines (DTS_108, DTS_123, DTS_127), one parental line (TJ806), and two conventional breeding lines (AR02 and AR03). Three RNAi-based GE lines resistant to *A. lucorum* were different transformants containing the same exogenous inverted repeat sequences and transformed from the same parental line TJ806 (**Figures 1A,B** and **Supplementary Table 1**). Three RNAi-based GE lines have completed the intermediate test stage of biosafety evaluation and were about to enter the stage of environmental release. AR03 was a derivative line of AR02, among which AR02 was the hybrid parent of AR03. The genetic relationship between these two lines was similar, and both have been cultivated in China for several years. The parental line TJ806 has no genetic relationship with conventional breeding lines AR02 and AR03. We set up the comparison between the RNAi-based GE lines and parental line, including DTS_108/TJ806, DTS_123/TJ806, and DTS_127/TJ806, which belong to group 1. We also set up the comparisons between parental line and conventional breeding lines, including AR02/TJ806, AR03/TJ806, and AR02/AR03, which belong to group 2. The comparisons were also set up between GE lines, including DTS_108/DTS_123, DTS_108/DTS_127, and DTS_123/DTS_127, which belong to group 3. The comparisons between GE lines and conventional breeding lines, including DTS_108/AR02, DTS_123/AR02, DTS_127/AR02, DTS_108/AR03, DTS_123/AR03, and DTS_127/AR03, were set up, which belong to group 4. A total of 15 pariwise comparisons were set up as shown in **Figure 1B**. In order to make the analysis more clear, we divided the 15 pariwise comparisons into four groups, which represent the differences between RNAi-based GE lines and parental line (group 1), the differences between conventional breeding lines and parental line (group 2), the differences between GE lines (group 3), and the differences between GE lines and conventional breeding lines (group 4) (**Figures 1A,B** and **Supplementary Table 1**). All materials were contributed by Dabeinong Biotechnology Co., Ltd (Haidian, Beijing, China).

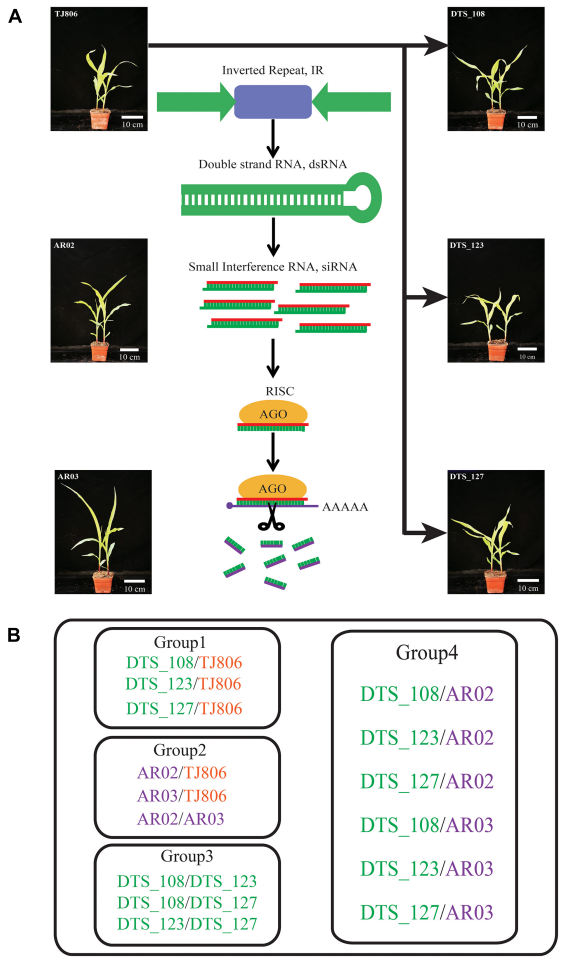

FIGURE 1 | Genetic relations among the studied maize lines and grouping comparison design for the analyses. **(A)** Genetic relations among the studied maize lines. **(B)** Experimental design for pairwise comparisons of short interfering RNA (siRNA) expression, gene expression, and metabolite accumulation between different maize lines. Group 1, comparisons between RNA interference (RNAi)-based genetic engineering (GE) lines and parental line. Group 2, comparisons between conventional breeding lines and the non-GE parental line. Group 3, comparisons between RNAi-based GE lines with the same parents. Group 4, comparisons between RNAi-based GE lines and conventional breeding lines. DTS_108, DTS_123, and DTS_127 were RNAi-based GE lines transformed using the conventionally bred maize line TJ806. Maize lines with green, orange, and purple colors represent the RNAi-based GE lines, parental line of RNAi-based GE lines, and conventional bred maize lines, respectively.

Plant Growth Conditions and Tissue Sampling

The surface-sterilized rice seeds were germinated on half-strength MS medium. After a week, we transplanted the seedlings into individual plastic pots and placed all potted plants in a cement pool maintained in a glasshouse (28 ± 2°C RT, 65 ± 10%

RH, 16 h light/8 h dark). After 5 weeks, from each plant, we sampled a leaf section (approximately 2 cm) from the middle part of the second leaf blade from the top. The samples from six plants were pooled as one biological replicate, and three replicates were collected for each maize line. The leaf samples were immediately frozen in liquid nitrogen and stored at −80°C.

Small RNA Extraction, Library Preparation, and Small RNA Sequencing

To construct libraries for small RNA sequencing, we extracted and purified small RNA from maize leaves using RNAi-so Plus system and BioAnalyser 2100. The cDNA synthesis was carried out using small RNA with linkers as a template, and the small RNA library was constructed by cDNA after 15 PCR cycles. Library sequencing is based on the method of sequencing by synthesis using Illumina HiSeq 2000. We generated the original fastq file data using primers and vector sequences, subjected them to quality inspection and length screening of the sequenced fragment bases, and finally selected reliable small RNA sequencing fragments with lengths of 18–30 nt.

Mock Library Construction, Sequence Alignment, and Verification of Gene Expression Level

The inverted repeat sequence with a length of 876 bp mainly derived from *A. lucorum* was used for library construction. We used Jellyfish (v.2.2.5) software to cut candidate sequences from different starting positions (first, second, etc.) of the dsRNA sequence, assembling a 21–24 bp kmer sequence library, and used Bowtie (v.1.1.0) software to compare it with maize transcripts to determine the start and end positions of each kmer. The mismatch parameter was set to within two bases.

First, we compared the siRNA in the mock library to the maize transcript (B73 RefGen_v4). The alignment setting allows two gaps to obtain specific maize transcripts (the potential off-target genes in maize). Second, we constructed the siRNA library by comparing actual small RNA sequencing to dsRNA sequence. To ensure that the analyzed siRNAs were all derived from the inverted repeat sequence inserted from an external source, we set the alignment threshold to 0 gaps. The results of the two comparisons can be combined to obtain the number of genes that siRNA has compared to the maize transcript. Base preference was profiled with WebLogo (Crooks et al., 2004). In addition, the distribution of siRNAs highly enriched on the dsRNA was determined with Grahprism7.0 software. These genes were used as potential off-target genes for further analysis, and their expression levels were measured using qRT-PCR calculated by $2^{-\Delta\Delta Ct}$ method. The experiment was designed in three parallels, and the significance between gene expression levels was tested using a student *t*-test ($p < 0.05$).

Total RNA Extraction, Library Preparation, and RNA Sequencing for Transcriptome

Total RNA per samples was extracted using NEBNext® UItra™ RNA Library Prep Kit for Illumina® (NEB, United States). In total, 18 cDNA libraries, which were sequenced using Illumina HiSeq 4000 platform, were constructed. The clean reads were aligned to the reference genome AGPv4 using Bowtie2 software.[2] The expression levels of all transcripts from the six leaves were quantified as the fragments per Kb per million reads (FPKM) (Trapnell et al., 2010) using the omicshare platform.[3] The DEGs were identified using Benjamini and Hochberg's approach with an adjusted *P*-value less than 0.05 and a fold change ≥2 or ≤0.5. The verification method of DEGs was same as described in Section "Mock Library Construction, Sequence Alignment, and Verification of Gene Expression Level." The heatmap of relative expression level of the DEGs was plotted using the omicshare platform. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses were performed using omicshare platform and MetaboAnalyst 4.0 (Chong et al., 2018) with a false discovery rate (FDR) adjusted *P*-value < 0.05 (hypergeometric test).

Metabolite Profiling

Metabolites Extraction Process

After grinding six maize samples with liquid nitrogen, we added 400 μl of precooled methanol/acetonitrile/water solution (4:4:2, v/v) to the samples (Mix, stand at −20°C for 60 min, centrifuged at 14,000*g* at 4°C for 20 min). We vacuum dried the supernatant, added 100 μL of acetonitrile aqueous solution (acetonitrile: water = 1:1, v/v) to reconstitute during mass spectrometry, vortexed, centrifuged at 14,000*g* at 4°C for 15 min, and took 2 μL of supernatant for sample analysis.

High-Performance Liquid Chromatography and Electrospray Ionization-Q trap-MS/MS Running Conditions

The samples were separated using an Agilent 1290 Infinity LC Ultra HPLC system (UHPLC) (HILIC column temperature 25°C, flow rate 0.3 mL/min, injection volume 2 μL) following the manufacturer's instructions. The sample was placed for the entire analysis process in the autosampler at 4°C. We adopted a random order for continuous analysis of the samples.

We used electrospray ionization (ESI) positive ion and negative ion modes for detection. We separated the samples using UHPLC and analyzed them with a Triple TOF 6600 mass spectrometer (AB SCIEX) following the manufacturer's instructions from Hoogen biotech Co., Ltd (Minhang, Shanghai, China).

Acquiring Metabolic Data

The original data to mzXML format using Proteo Wizard then used the XCMS program for peak alignment, retention time correction, and peak area extraction. The metabolite structure identification uses accurate mass matching (<25 ppm) and secondary spectrum matching methods and searches the laboratory's self-built database. For the data extracted by XCMS, ion peaks with missing values >50% were deleted in the group. The application software SIMCA-P 14 (Umetrics, Umea, Sweden)

[2]http://tophat.cbcb.umd.edu/

[3]https://www.omicshare.com/

was used for pattern recognition and the data were preprocessed by Pareto-scaling for multidimensional statistical analysis.

Data Analysis

According to the variable weight value, variable importance for the projection (VIP), obtained by the orthogonal partial least squares discriminant analysis (OPLS-DA) model, the influence intensity and explanatory power of the expression pattern for each metabolite can be measured. Those with fold change (FC) > 2.0 and *P*-value < 0.05 were used as differential cumulative metabolites. Using qualitatively significant differences in metabolite expression levels for each group of samples, the clustering (hierarchical clustering) helps us to accurately screen marker metabolites and use the KEGG public database to conduct pathway analysis of differential metabolites.

RESULTS

Evaluating Omic Data Derived From Leaves From Six Maize Lines

We conducted siRNA-seq, RNA-seq, and metabolite analysis to investigate unintended effects in RNAi-based maize. For siRNAs, we constructed a total of 18 small RNA libraries resulting in approximately 15.84–23.80 million raw reads per library and 10.34–11.98 million clean reads per library being cleated. The rate of clean reads ranged from 50.54 to 83.51%. The percentage of bases with a Phred value greater than 30 compared to total bases was between 95.73 and 97.50% (**Supplementary Table 2**). For transcriptome analysis, the 18 RNA-seq libraries were constructed, resulting in approximately 51–56 million clean reads per library being cleated. Using the *Zey_mays* AGPv4 as a reference genome, 92.05–96.75% of the clean reads were mapped. The percentage of bases with a Phred value greater than 30 compared to total bases was between 94.06 and 94.55% (**Supplementary Table 3**). We profiled metabolic changes in the six maize lines. We detected a total of 1,954 metabolites (**Supplementary Table 4**). The majority of these metabolites belonged to different ontologies, i.e., diterpenoids, phenolic glycosides, and alpha amino acids and derivatives (**Supplementary Table 4**). These results suggest that the datasets generated from these six maize lines were sufficient for further analyses.

Features of Short Interfering RNAs of Six Maize Lines

We analyzed the lengths of highly enriched siRNAs, base preferences, and their distributions on the dsRNA sequence (**Figure 2** and **Supplementary Tables 2–4**). The lengths of highly enriched siRNAs were concentrated at 21 nt in all maize lines (**Supplementary Table 5**). The siRNA size distribution shown in **Figure 2A** was comparable with previous observations in Arabidopsis that the 21 nt long siRNA is the predominant antiviral silencing component. Robust guanine and cytosine (GC) bias (52.75%) was observed for all siRNAs highly enriched in the maize transcriptome. The adenine or uracil (A/U) content of five bases at the 5′ end of siRNAs was higher than the A/U content of other positions (**Figure 2B** and **Supplementary Table 6**), related to the binding stability of the siRNA targeting mRNA and one of the conditions for effective siRNA silencing. We analyzed the distribution of siRNAs and noted the positions of their first bases in the dsRNA sequence, with specific distribution characteristics present in six maize lines, although the distribution trend of siRNAs was similar (**Figure 2C** and **Supplementary Table 7**). Notably, the number of reads of siRNAs in RNAi-based GE lines was approximately 20,000 times greater than in non-GE maize lines.

Verification of Potential Off-Target Gene Expression Levels Using RT-qPCR

We obtained 35 transcripts identified as potential off-target genes of maize. One gene (Zm00001d014294) was mapped to the transcriptome of maize when a 1 bp mismatch was set. Thirty-five genes were mapped to the transcriptome of maize when a 2 bp mismatch was set (**Supplementary Table 6**). Using *zssIIb* as an internal reference gene, RT-qPCR was used to analyze the relative expression of potential off-target genes, as shown in the heatmap (**Figure 2D**). The expression levels of the Zm00001d001906 and Zm00001d007394 genes in AR02 were significantly lower than those in DTS_127. The genes whose expression levels in AR02 were higher than those in AR03, DTS_123, TJ806, and DTS_127 were Zm00001d014451, Zm00001d015139, Zm00001d027839, and Zm00001d034588, respectively. The expression level of Zm00001d048703 in AR03 was significantly lower than in DTS_123. The expression patterns of these genes in specific strains were completely consistent with the expression patterns of DEGs in the transcriptome. In addition, compared with the parental line, the expression of genes Zm00001d000055 and Zm00001d000295 decreased in DTS_123. Although the expression levels of these potential off-target genes were lower than those in TJ806, this did not appear in all RNAi-based GE lines (**Figure 2D**).

Analyzing Gene Expression Through RNA-Sequencing

The RNA-seq dataset was normalized to FPKM values to quantify the levels of gene expression, including 28,852 genes (**Supplementary Table 8**). The principal component analysis (PCA) was performed on all 18 transcriptomic datasets. As shown in **Figure 3A**, the first two principal components (PCs) explain 53.8% (PC1) and 17.7% (PC2) of total variance. PC1 revealed a clear separation between conventional breeding lines and RNAi-based GE lines compared with the parental line. However, the first two PCs failed to separate the GE lines from the parental line. Consistently, RNAi-based GE lines and conventional breeding lines were hierarchically clustered in the respective classes. Three RNAi-based GE lines had a closer genetic relationship with parental line (**Figure 3B**).

Subsequently, differentially expressed genes (DEGs) of the six maize lines based on different grouping comparisons as described in **Figure 1** were screened, showing distinct differences in gene expression among the lines. A total of 8–4765 DEGs were detected among the 15 pairwise comparisons (**Figure 3C**

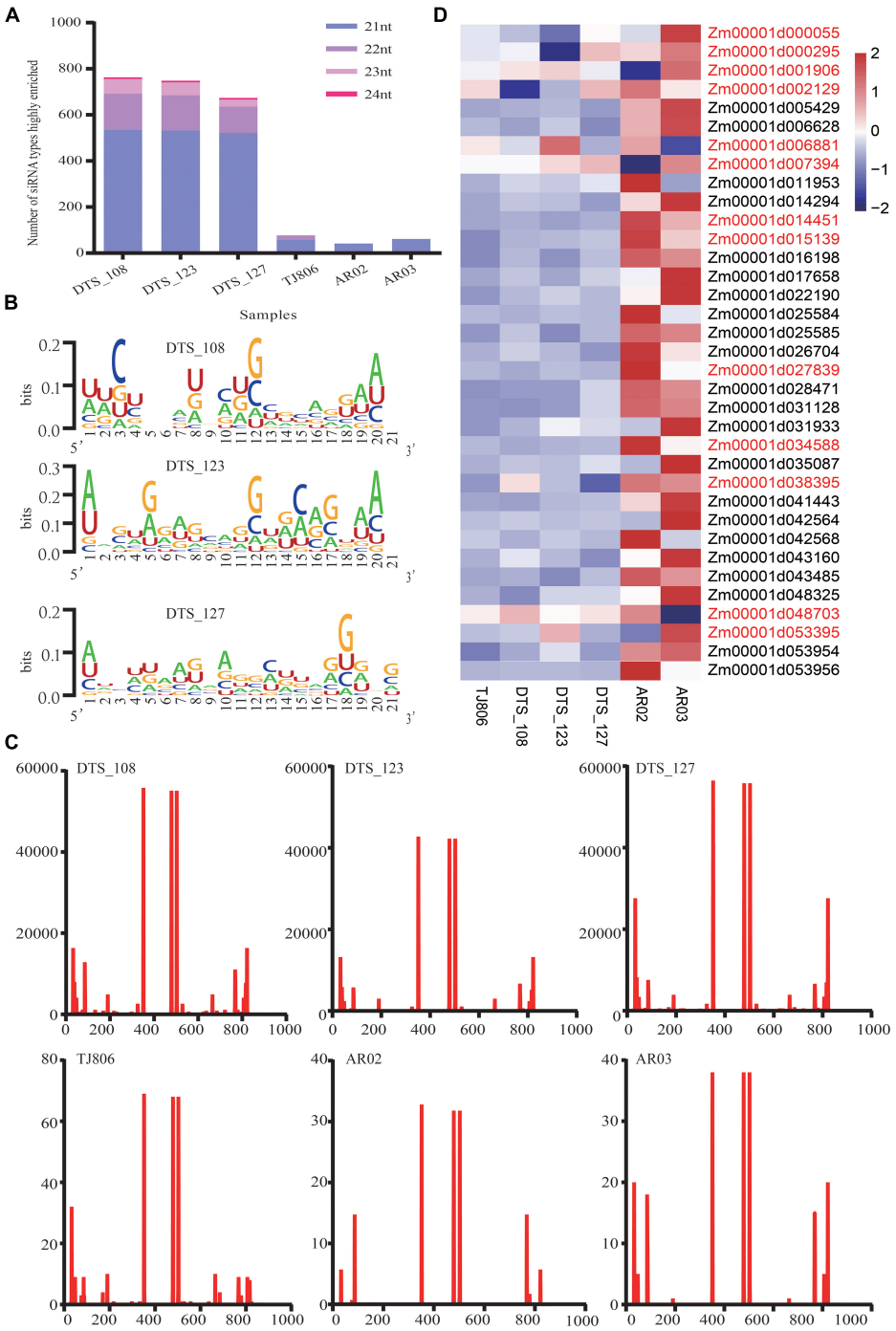

FIGURE 2 | The features of siRNAs highly enriched in maize and expression of potential off-target genes. **(A)** The number of siRNA types with lengths of 21–24 nt highly enriched in six maize lines. **(B)** The distribution of the first base of siRNAs in double-stranded RNA (dsRNA) sequences in six maize lines. The abscissa indicates the base position of the dsRNA sequence. The ordinate indicates that the number of siRNA reads was highly enriched in six maize lines. **(C)** The base preference of siRNAs with a length of 21 nt highly enriched in three RNAi-based GE lines. The abscissa indicates the base position of siRNAs with a length of 21 nt. The ordinate indicates the proportion of bases (A/U/G/C) in each base position. Larger bases represent a higher frequency of bases. **(D)** Heatmap of the expression levels of 35 potential off-target genes in six maize lines. Gene names colored red indicates that a gene was expressed differentially in maize lines (*t*-test, $p < 0.05$).

and **Supplementary Table 9**). The number of DEGs between RNAi-based GE lines and parental line was less than between conventionally breeding lines. The number of DEGs between RNAi-based GE lines and parental line was within the normal variation of gene expression changes in conventionally breeding lines. The number of DEGs between RNAi-based GE lines

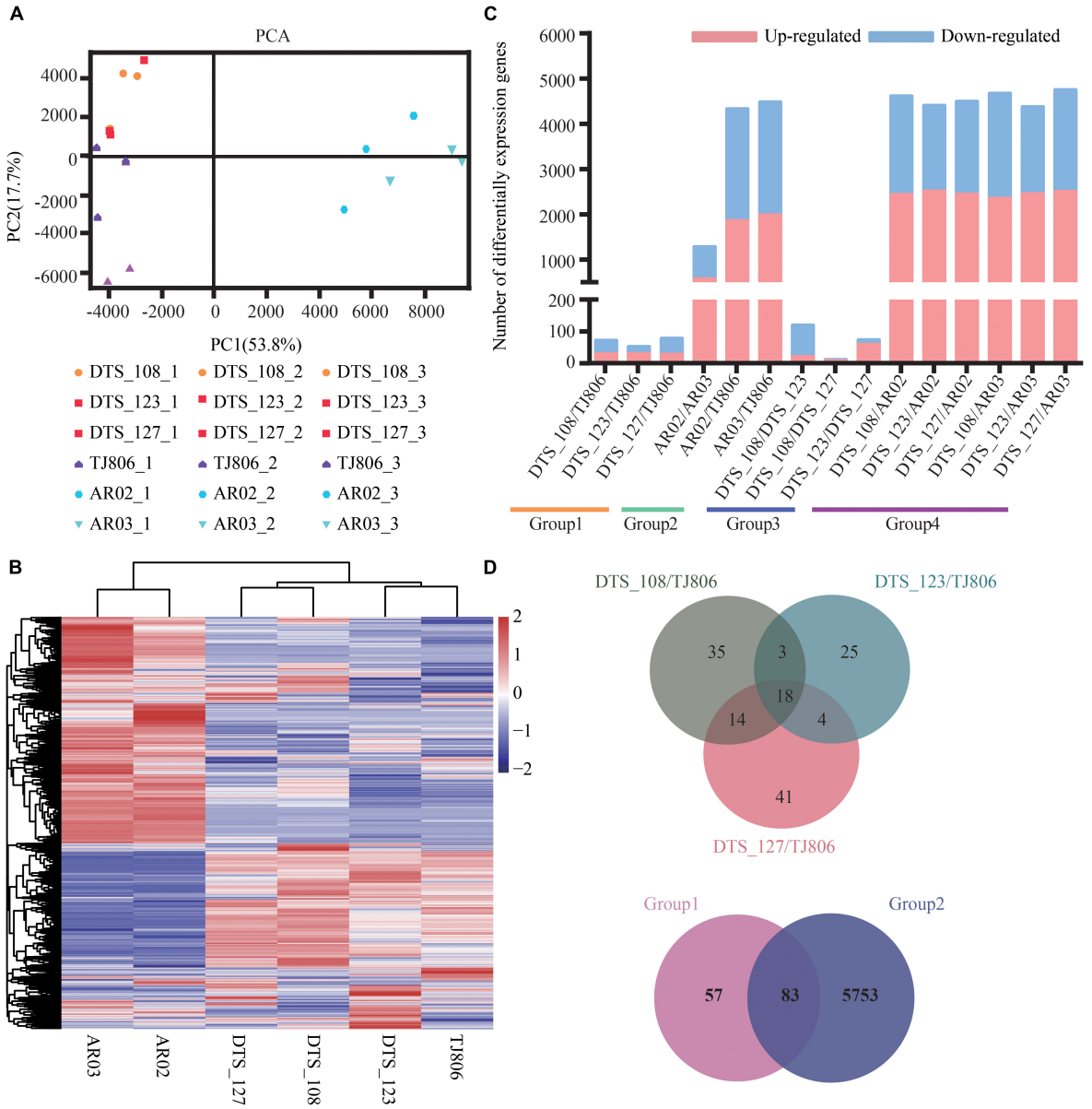

FIGURE 3 | Overall description of transcriptome data. **(A)** Principal component analysis (PCA) of gene expression levels in the leaves of six maize lines. Score plot of the first two principal components with the explained variance. **(B)** Hierarchical clustering of six maize lines using the total detected gene expression data. In the heatmap, each maize line is visualized in a single column and each gene is represented by a single row. Gene expression levels are shown in different colors, where red indicates high abundance and low relative expression is shown in blue (color key scale right of the heatmap). **(C)** Pairwise comparisons of DEGs between different maize lines. **(D)** Venn diagrams depicting the unique and shared DEGs between lines obtained by RNAi-based genetic modification and conventional breeding methods.

and conventionally breeding lines was similar to that between parental line and conventionally breeding lines except for AR02/AR03, which is far more than the number of DEGs between RNAi-based GE lines and parental line, suggesting that different genetic backgrounds may bring more changes in gene expression (**Figure 3C**).

We calculated the distribution of DEGs for each comparison and present them in Venn diagrams (**Figure 3D**). This distribution was genotype-specific. As shown in **Figure 3D**, we analyzed the distribution of DEGs between three GE lines and parental line. The results showed that there were 35, 25, and 41 unique DEGs in DTS_108, DTS_123, and DTS_127, respectively. At the same time, three GE lines shared 18 DEGs compared with parental line (**Figure 3D**). Although a large number of DEGs were detected in pairwise comparisons, they still shared 83 DEGs between group 1 and group 2. There were 57 and 5753 unique DEGs in group 1 and group 2 representing the different breeding lines, respectively (**Figure 3D**). The number of unique DEGs in

each RNAi-based GE line compared with group 2 was similar and was significantly less than that in group 2 (**Supplementary Figure 1**). These results suggest that both conventional and GE breeding methods can change the expression of non-target genes. We verified the expression levels of 20 DEGs from the shared and unique collection described above using RT-qPCR. As shown in the **Supplementary Figure 1**, which were RNA-seq and qPCR data, respectively. The results of heatmap showed that the expression trend of DEGs was consistent with the transcriptome sequencing data, indicating that the transcriptome sequencing results were accurate and credible.

Functional Enrichment Analysis of Differentially Expressed Genes

Gene ontology pathway enrichment analyses of the DEGs in a total of 15 pairwise group comparisons were conducted (**Supplementary Table 10**). No significantly enriched biological process GO terms were found in comparisons between RNAi-based GE lines and parental line (**Supplementary Table 10**). Different biological process terms were enriched in specific comparisons, with hydrolase activity acting on glycosyl bonds as the most popular pathway terms in the group 2, group 3, and group 4. We performed GO pathway enrichment analyses of the unique and shared DEGs of comparisons (not attached). Interestingly, there were zero and 43 significantly enriched GO terms of unique DEGs in group 1 and group 2, respectively. Only cytoskeletal terms were significantly enriched GO terms of unique DEGs in the comparisons of DTS-123/TJ806 when compared with group 2 and none of those in DTS-108/TJ806 and DTS-127/TJ806. The performances of the three RNAi-based maize lines were similar, with the number of enrichment pathways of unique DEGs far less than those in group 2 (not attached).

Similarly, the KEGG enrichment analyses indicated that there were no significantly enriched pathways for the comparisons between RNAi-based GE lines and parental line except for the eukaryotic ribosome biogenesis pathway in the comparison of DTS_123/TJ806. In addition, there were some pathways, such as ABC transporters, Diterpenoid biosynthesis, Flavonoid biosynthesis, Monoterpenoid biosynthesis, Phenylpropanoid biosynthesis, and Plant hormone signal transduction, which were significantly enriched in the comparisons between conventionally breeding lines with a high enrichment score. There were Photosynthesis, Flavonoid biosynthesis, Cyanoamino acid metabolism, Starch and sucrose metabolism, Ribosome biogenesis in eukaryotes, and Phenylpropanoid biosynthesis that were significantly enriched in the comparisons between the RNAi-based GE lines and conventionally breeding lines (**Table 1**).

Metabolomic Differences in Leaves Among Maize Lines

A PCA plot for metabolite accumulation was constructed and shown in **Figure 4A**, where the abscissa and the ordinate represent the scores of PC1 and PC2, respectively. The first two PCs explain 87.5 and 3.9% of the total variance, respectively. PC1 showed a clear separation between maize lines with different genetic backgrounds. For specific maize lines, the first two PCs could not separate the GE lines from the parental line. Consistently, clustering analysis of the metabolites from the six maize lines showed that the conventional breeding lines and RNAi-based GE lines were clustered into distinct groups (**Figure 4B**).

The differentially accumulated metabolites (DAMs) in leaves among different maize lines were identified. A total of 6–129 DAMs were identified, ranging from 0.31 to 6.60% of the total detected metabolites in each of the 15 comparisons (**Figure 4C** and **Supplementary Table 11**). In addition, the number of DAMs between RNAi-based GE lines and parental line were, in all cases, less than those between RNAi-based GE lines and conventional breeding lines (**Figure 4C** and **Supplementary Table 11**). We constructed a venn diagram for qualitative analysis of the metabolites. The number of unique DAMs in DTS_108, DTS_123, and DTS_127 were 8, 36, 16, respectively, when compared with parental line (**Figure 4D**). It was noticed that there was no shared DAMs in three RNAi-based GE lines compared with parental line. Group 1 and group 2 shared six metabolites, with 59 and 160 unique metabolites, respectively (**Figure 4D**). Similar to the transcriptome results, the numbers of unique DAMs in the three RNAi-based maize lines were similar and far less than those in group 2 (**Figure 4D**).

The KEGG pathway enrichment analysis showed that no pathways were significantly enriched in the DAMs of group 1 and group 3. In contrast, some pathways were significantly enriched in the DAMs of group 2, including glycerolipid metabolism, selenocompound metabolism, galactose metabolism, alanine, aspartate and glutamate metabolism, aminoacyl-tRNA biosynthesis, and vitamin B6 metabolism. We found vitamin B6 metabolism to be enriched in DAMs between conventionally bred lines and the parental line of group 4 (**Table 2**). We profiled KEGG pathway enrichment analysis of significantly enriched shared or specific DAMs of comparisons of group 1 and group 2. Five KEGG pathways were significantly enriched in the unique DAMs of group 2 when compared with group 1, including vitamin B6 metabolism, ubiquinone and other terpenoid-quinone biosynthesis, nicotinate and nicotinamide metabolism, pyrimidine metabolism, and tyrosine metabolism. There was no significant enrichment of metabolites specific to group 1 or any metabolites specific to RNAi-based GE maize.

DISCUSSION

In the 2000s, new methodologies were developed to allow, in theory, a holistic search for alterations in GE crops at different biological levels (transcripts, proteins, metabolites) (Ricroch et al., 2011). However, research on the assessment of unintended effects in RNAi-based GE crops, including off-target effect analysis, was little enough that their risk assessment has not been well-understood (Auer and Frederick, 2009). We attempted to determine the amount of variation among RNAi-based GE lines resistant to *A. lucorum* by establishing parental controls and conventional breeding lines using siRNA, mRNA, and metabolite data analysis.

TABLE 1 | Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of significantly DEGs.

Group	Comparisons	Id	Term	ListHits	ListTotal	PopHits	PopTotal	pval	padj	Enrichment_score
Group 1	DTS_108/TJ806	-								
	DTS_123/TJ806	path:zma03008	Ribosome biogenesis in eukaryotes	2	44	49	6694	0.0040	0.0287	6.2096
	DTS_127/TJ806	-								
Group 2	AR02/TJ806	path:zma00500	Starch and sucrose metabolism	26	771	112	6694	0.0001	0.0146	2.0155
		path:zma02010	ABC transporters	2	44	15	6694	0.0001	0.0033	20.2848
		path:zma00902	Monoterpenoid biosynthesis	4	771	7	6694	0.0003	0.0201	4.9613
	AR03/TJ806	path:zma00500	Starch and sucrose metabolism	26	771	112	6694	0.0001	0.0146	2.0155
		path:zma00941	Flavonoid biosynthesis	2	63	27	6694	0.0020	0.0098	7.8707
		path:zma00940	Phenylpropanoid biosynthesis	5	63	145	6694	0.0023	0.0098	3.6639
		path:zma00480	Glutathione metabolism	21	809	74	6694	0.0000	0.0042	2.3481
	AR02/AR03	path:zma00904	Diterpenoid biosynthesis	2	63	16	6694	0.0004	0.0058	13.2817
		path:zma04075	Plant hormone signal transduction	6	63	202	6694	0.0027	0.0098	3.1561
Group 3	DTS_108/DTS_123	-								
	DTS_108/DTS_127	-								
	DTS_123/DTS_127	-								
Group 4	DTS_108/AR02	path:zma00940	Phenylpropanoid biosynthesis	8	100	145	6694	0.0003	0.0050	3.6932
	DTS_123/AR02	path:zma00196	Photosynthesis - antenna proteins	3	100	24	6694	0.0004	0.0050	8.3675
	DTS_127/AR02	path:zma00460	Cyanoamino acid metabolism	3	100	34	6694	0.0015	0.0100	5.9065
		path:zma00941	Flavonoid biosynthesis	2	63	27	6694	0.0020	0.0098	7.8707
	DTS_108/AR03	path:zma00500	Starch and sucrose metabolism	5	63	112	6694	0.0006	0.0058	4.7435
	DTS_123/AR03	-								
	DTS_127/AR03	path:zma03008	Ribosome biogenesis in ryeukaotes	2	63	49	6694	0.0108	0.0251	4.3369
		path:zma00500	Starch and sucrose metabolism	7	100	112	6694	0.0002	0.0050	4.1838

Consistent with research on matching siRNA and target sequences, 21 nt siRNAs accounted for a large proportion of all siRNAs to be analyzed, indicating that among the 21–24 nt siRNAs, 21 nt siRNAs played a major role when siRNAs and non-target sequences were matched (Papadopoulou et al., 2020; **Figure 2A**). Interestingly, some potential off-target genes in RNAi-based GE lines with expression levels lower than those in the parental line were not common in all the three RNAi-based GE lines (**Figure 2D**). These results suggest that no off-target phenomenon was found by molecular experiments at the gene expression level. In fact, the gene expression levels are affected by many factors such as environment, weather, and varieties, possibly resulting in differences in gene expression and phenotypic changes (Zhang et al., 2017). The high abundance of siRNA in the maize genome did not possess an obvious inhibitory effect. This result is supported by previous studies showing that the off-target suppression effect does not solely depend on the abundance of siRNAs (Praveen et al., 2010; Papadopoulou et al., 2020). Whether off-target occurs other important factors must be considered such as the concentration of siRNAs in plant cells, the amount of siRNA loaded with AGO protein, and the binding energy between siRNA and its target mRNA. In the end, off-target effects should be verified by biological experiments (Ma et al., 2006; Papadopoulou et al., 2020). Therefore, the relationship among gene expression differences, phenotypic changes, and off-target effects should be explained carefully (Casacuberta et al., 2015; Ladics et al., 2015; Arpaia et al., 2020). Obviously, using bioinformatics to predict off-target genes is only a basic auxiliary approach, while the application of bioinformatics can provide a reference for off-target effects analysis (Ahmed et al., 2020; Papadopoulou et al., 2020).

Both PCA and hierarchical cluster analyses of the datasets showed a distinct separation between samples with different genetic backgrounds at both the transcriptome and metabolome levels. Specifically, there was a distinct separation between conventional breeding maize lines and RNAi-based GE lines (including parental line), but there was no separation

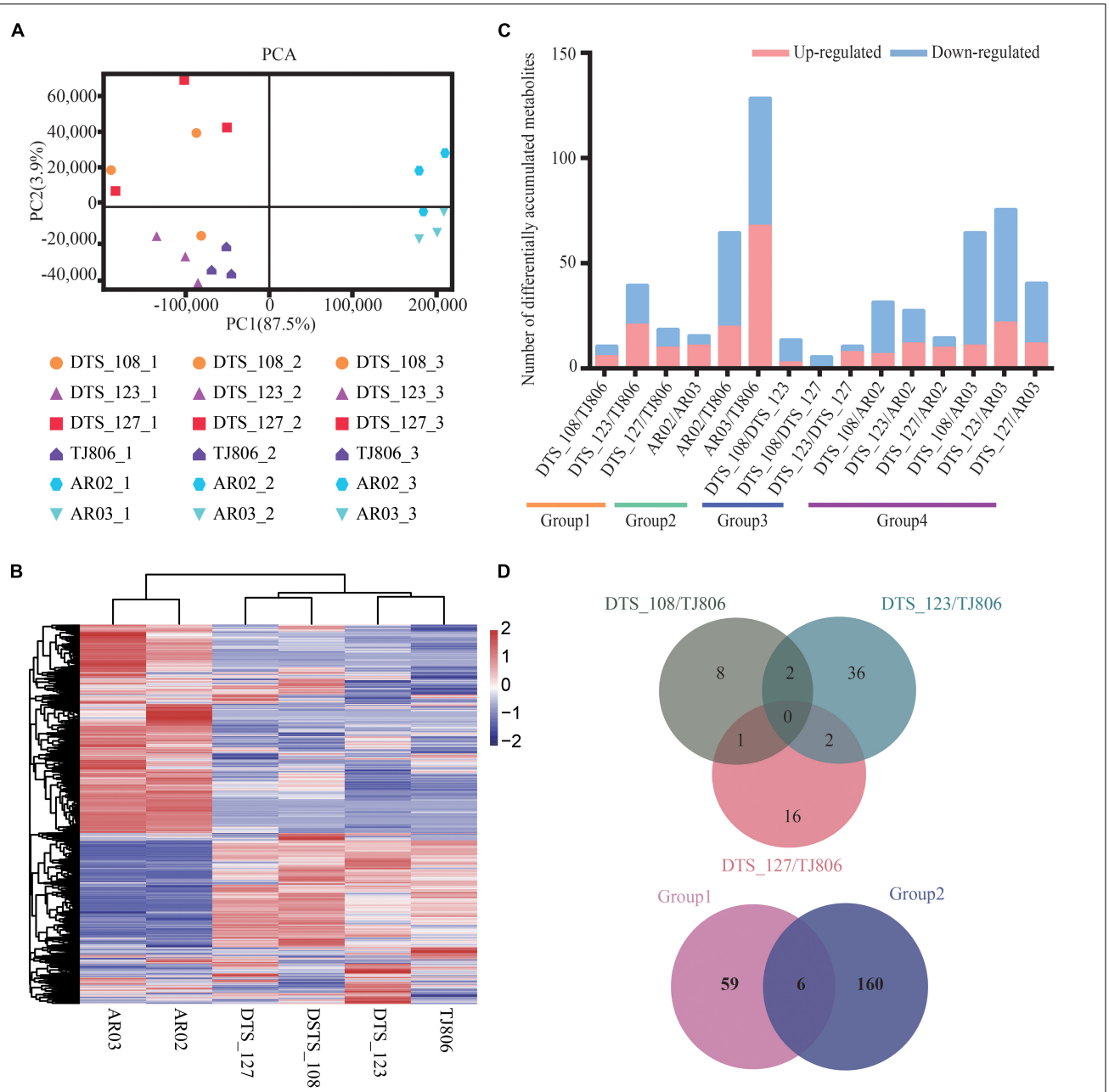

FIGURE 4 | Overall description of metabolome data. **(A)** Principal component analyses (PCA) of metabolite accumulation levels in leaves of six maize lines. Score plot of the first two principal components with the explained variance. **(B)** Hierarchical clustering of six maize lines using the total detected metabolite accumulation data. In the heatmap, each maize line is visualized in a single column and each metabolite is represented by a single row. Metabolite accumulation levels are shown in different colors, where red indicates high abundance and low relative expression is shown in blue (color key scale right of the heatmap). **(C)** Pairwise comparisons of DAMs between different maize lines. **(D)** Venn diagrams depicting the unique and shared DAMs between lines obtained by RNAi-based genetic modification and conventional breeding methods.

trend between RNAi-based maize lines and the parental line (**Figures 3A,B**, **4A,B**). This result was consistent with previous studies showing that different background varieties are clearly distinguishable but no distinction is seen between GE lines and parental line. Natural variation in plants is very common at the transcriptional and metabolic levels (Batista et al., 2008). These current results and previous studies suggest that intrinsic differences in genetic background bring much greater variation to the plant transcriptome and metabolome than the introduction of foreign genes by genetic manipulation or conventional breeding methods (Ladics et al., 2015; Wang et al., 2018).

As expected, pairwise comparisons reveal differences between the GE lines and parental line with respect to gene expression and metabolite accumulation, as reported previously for GE maize

TABLE 2 | Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis of significantly DAMs.

Group	Pairwise comparison	KEGG pathway	Total	Expected	Hits	Raw *p*
Group 1	DTS_108/TJ806	-				
	DTS_123/TJ806	-				
	DTS_127/TJ806	-				
Group 2	AR02/AR03	Glycerolipid metabolism	16	0.021	1	0.0209
		Selenocompound metabolism	20	0.0263	1	0.0261
		Galactose metabolism	27	0.0355	1	0.0352
	AR02/TJ806	Alanine, aspartate, and glutamate metabolism	28	0.0368	1	0.0365
		Ubiquinone and other terpenoid-quinone biosynthesis	9	0.0296	1	0.0293
	AR03/TJ806	Vitamin B6 metabolism	9	0.0946	2	0.00357
Group 3	DTS-108/DTS-123	-				
	DTS-108/DTS-127	-				
	DTS-123/DTS-127	-				
Group 4	DTS_108/AR02	Vitamin B6 metabolism	9	0.0237	1	0.0235
	DTS-123/AR02	Ubiquinone and other terpenoid-quinone biosynthesis	9	0.0296	1	0.0293
	DTS-127/AR02	-				
	DTS_108/AR03	Vitamin B6 metabolism	9	0.0591	2	0.00137
	DTS_123/AR03	-				
	DTS_127/AR03	Vitamin B6 metabolism	9	0.065	2	0.00166
		Thiamine metabolism	7	0.0506	1	0.0496

and soybeans (Liu et al., 2020). However, the number of DEGs and DAMs observed when comparing GE maize lines and the parental line were significantly less than those present when comparing conventional breeding lines and the parental line (**Figures 3C**, **4C**; Wang et al., 2018). However, the number of DEGs in the transcriptome was not always consistent with the number of DAMs in the metabolome (Wang et al., 2019; Liu et al., 2020). Specifically, the number of DEGs between the parental line and the conventional breeding lines was close to the number of DEGs between the RNAi-based GE lines and the conventional bred lines but the performance of DAMs was different, indicating that genetic changes commonly occur during the plant breeding process whether done by GE or by conventional breeding, and the extent of those changes seems not always relevant to the extent of metabolomic changes in maize (Wang et al., 2019). The detection of DEGs and DAMs in RNAi-based GE plants was carried out under specific developmental or environmental conditions, which could yield unintended effects upon analysis. Assessment of unintended effects during specific developmental periods and conditions often ignores other factors, especially because these differences can affect gene transcription in GE plants (Herman and Price, 2013; Olmos et al., 2019). Therefore, it has been recommended that the unintended effects evaluation of GE plants should include a combination of omics to provide a parameter platform easier to understand and analyze (Ricroch et al., 2011; Liu et al., 2020).

We found that the number of shared DAMs between three GE lines and parental line was zero when we contacted the DEGs and DAMs between three GE lines and parental line indicating that we could not analyze whether there were common changes between the GE lines and parental line from the transcriptome and metabolome level. We therefore have tried to perform an association analysis on the unique DEGs and DAMs in group 1 and group 2, such as looking for DEGs that participated in expression regulation and can produce specific DAMs that belong to group 1 or group 2. Unfortunately, our analysis did not yield meaningful data. We thus analyzed the possible reasons for this result as follows. First, the number of DEGs and DAMs obtained by analyzing were too relatively small to support association strategy. Second, we did find some DEGs and DAMs between transgenic plants and parental line at the transcriptome level and metabolome level, respectively. However, these differences cannot be correlated from the transcriptome to the metabolome, which showed that none of the pathway from gene expression to metabolic regulation significantly altered; this result was consistent with the published research results (Ricroch et al., 2011). In addition, we found that these unique DEGs and DAMs have no same KEGG enrichment pathway. We speculate that the possible reason is that it is difficult to identify a pathway that significantly changes from gene expression to metabolic regulation, because it requires a lot of work to verify the key genes and their functions involved in the relevant biological pathways (Ricroch et al., 2011).

We did find some unique DEGs and DAMs in RNAi-based GE lines, although the number was significantly less than those in conventional breeding lines compared with the parental line (**Figures 3D**, **4D** and **Supplementary Tables 9, 11**). This result implies that the process of GE may bring different stresses to the host genome relative to conventional breeding, indicating that the two plant breeding processes may result in variations in genes and metabolites at different levels (Hoekenga, 2008; Liu et al., 2020). Notably, there were more DEGs and DAMs in plants produced by conventional breeding than by genetic modification, possibly implying that the conventional breeding requires multiple crosses between two or more breeding lines, thus causing more variation at both genotypic and phenotypic

levels. We used the substantial equivalence standard to assess the safety of food or feed produced by GE (Schiemann et al., 2019). We found some DAMs in the comparisons between RNAi-based GE plants and their parental counterparts, and these DAMs are also found in conventional breeding lines. Since these conventional breeding lines are considered to have a long history of use and safety, a control consisting of conventional breeding lines should be implemented when evaluating unintended effects (Chen et al., 2014). The European Food Safety Agency's genetic modification management team has pointed out that the safety evaluation of GE plants includes GE near-isogenic control and reference commercial variety control (Eckerstorfer et al., 2019; Papadopoulou et al., 2020).

Based on the experimental data and results of siRNA, transcriptome, and metabolome, it can be concluded that RNAi-based GE maize is essentially equivalent to conventional breeding. The differences brought by GE breeding were not as obvious as those caused by conventional breeding, although conventional breeding also has some difference that cannot be explained clearly so far (Ricroch et al., 2011). It was important to keep in mind that the standard proposed by the OECD/Food and Agriculture Organization of the United Nations/WHO was substantial equivalence rather than total equivalence and that there was no specific statistical or biological basis to define "substantial" (Hoekenga, 2008). In other words, no "limits of concern" have been defined regarding differences. In order to make the conclusions more reliable, we considered the following points that may affect the data and even affect the risk assessment results of GE crops. One of the factors we need to pay attention to was the selection of experimental materials. We used leaves for testing mainly because RNAi-based GE maize was resistant to *A. lucorum*, which mainly damages leaves, that is, siRNA from *A. lucorum* plays a vital role in leaves. Meanwhile, the existing literatures have studied the unintended effects of GE crops using leaves (Wang et al., 2018; Liu et al., 2020). Furthermore, the gene expression and metabolism of plant leaves are active, which is very conducive to the collection of transcriptome and metabolome data. However, as grains of maize are the edible part, we should consider that the analysis of them may obtain more meaningful data. The leaves and grains or other reasonable research sample tissues should be considered in the future research. The second factor we considered was the number of samples. The prior probability refers to the probability obtained based on inference and observations when using omics technology to evaluate the unintended effects of GE crops. Increasing the number of test samples was a prerequisite for ensuring a higher prior probability (Ricroch et al., 2011). To obtain more accurate results, we set up three biological replicates of each of six maize lines to compare differences in gene expression and metabolite accumulation levels and each biological replicate is a mixture of 10 individual plant samples. We have chosen different omics methods to evaluate the unexpected effects of genetically modified crops such as siRNA, transcriptome, and metabolome since sample selection, sample numbers, and sampling location may affect the results of omics data. We found highly enriched siRNAs in the RNAi-based GE lines; however, we did not find that there were generally reduced potential off-target genes in all three RNAi-based GE lines during qPCR test. We note that not only eukaryotic ribosome biogenesis but also starch and sucrose metabolism, phenylpropanoid biosynthesis, and flavonoid biosynthesis were significantly enriched in the comparison of conventionally breeding maize lines and RNAi-based GE lines, indicating that the GE process resulted in DEGs and DAMs at the transcriptome and metabolome levels (Liu et al., 2020), but these pathways enriched in DEGs and DAMs were within the range of comparisons between conventional breeding lines and parental line (Wang et al., 2019; Liu et al., 2020). These results may thus suggest that the GE processes do not have unique effects on plant pathways compared with conventional breeding lines (**Tables 1**, **2** and **Supplementary Table 12**; Wang et al., 2019; Liu et al., 2020). If we can find some DEGs associated with DAMs in the transcriptome, the results will be easier to interpret, but this was not easy to achieve, although this was an ideal result. Thus, we propose a combination of multiple omics analysis, which can avoid the differences in the analysis of single omics data and explore as much as possible a metabolic pathway that regulated from gene expression to metabolites.

CONCLUSION

In conclusion, we successfully employed siRNA-seq, RNA-seq, and HPLS-MS technology to investigate the changes in siRNA and gene expression and metabolite accumulation in six maize lines developed by RNAi-based GE or conventional breeding. We did find that the inverted repeat gene sequence from *A. lucorum* produced highly enriched siRNAs in GE maize lines. However, qRT-PCR and transcriptome data analysis showed that the decline in gene expression levels of these potential off-target genes was not universal in the three transgenic lines, meaning that the siRNA targeted for *A. lucorum* did not occur detectable gene suppression in maize, indicating that bioinformatics analysis can be used to determine which genes in non-target organisms have a certain degree of sequence homology with target genes. The current results showed that both GE and conventional breeding method can result in potential changes at transcriptome and metabolome levels and the GE does not cause unintended effects that go beyond conventional breeding. There was no pathway that significantly altered from gene expression to metabolic regulation involved in the study, suggesting that a comprehensive and comparative multi-omics sharing platform should be established to improve the effective utilization of data when assessing the unintended effect of GE crops.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA725413.

AUTHOR CONTRIBUTIONS

CH, ZW, ZL, WF, and SZ conceived the idea. CH, ZW, PZ, and CGW designed the study. CH, CNW, and WX performed the experiments. ZW and WX analyzed the data. CH and ZW wrote the manuscript. All authors have read and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.745708/full#supplementary-material

Supplementary Figure 1 | Heatmap of verification results of DEGs. **(a)** Heatmap of verification results of DEGs for RNA-seq data. **(b)** Heatmap of verification results of DEGs for qPCR data.

Supplementary Figure 2 | Venn diagram analysis between three GE lines and group 2 at the transcriptome and metabolome level. **(a–c)** Venn diagram analysis of DEGs between three GE lines and group 2 at the transcriptome level. **(d–f)** Venn diagram analysis of DAMs between three GE lines and group 2 at the metabolome level.

REFERENCES

Ahmed, F., Senthil-Kumar, M., Dai, X., Ramu, V. S., Lee, S., Mysore, K. S., et al. (2020). pssRNAit: a Web Server for Designing Effective and Specific Plant siRNAs with Genome-Wide Off-Target Assessment. *Plant Physiol.* 184, 65–81. doi: 10.1104/pp.20.00293

Arpaia, S., Christiaens, O., Giddings, K., Jones, H., Mezzetti, B., Moronta-Barrios, F., et al. (2020). Biosafety of GM Crop Plants Expressing dsRNA: data Requirements and EU Regulatory Considerations. *Front. Plant Sci.* 11:940. doi: 10.3389/fpls.2020.00940

Auer, C., and Frederick, R. (2009). Crop improvement using small RNAs: applications and predictive ecological risk assessments. *Trends. Biotechnol.* 27, 644–651. doi: 10.1016/j.tibtech.2009.08.005

Batista, R., Saibo, N., Lourenco, T., and Oliveira, M. M. (2008). Microarray analyses reveal that plant mutagenesis may induce more transcriptomic changes than transgene insertion. *Proc. Natl. Acad. Sci. USA* 105, 3640–3645. doi: 10.1073/pnas.0707881105

Bregitzer, P., Halbert, S. E., and Lemaux, P. G. (1998). Somaclonal variation in the progeny of transgenic barley. *Theor. Appl. Genet.* 96, 421–425. doi: 10.1007/s001220050758

Casacuberta, J. M., Devos, Y., du Jardin, P., Ramon, M., Vaucheret, H., and Nogue, F. (2015). Biotechnological uses of RNAi in plants: risk assessment considerations. *Trends Biotechnol.* 33, 145–147. doi: 10.1016/j.tibtech.2014.12.003

Chen, W., Gao, Y., Xie, W., Gong, L., Lu, K., Wang, W., et al. (2014). Genome-wide association analyses provide genetic and biochemical insights into natural variation in rice metabolism. *Nat. Genet.* 46, 714–721. Epub 2014. doi: 10.1038/ng.3007

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., et al. (2018). MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucl. Acids Res.* 46, W486–W494. doi: 10.1093/nar/gky310

Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome Res.* 14, 1188–1190. doi: 10.1101/gr.849004

Eckerstorfer, M. F., Dolezel, M., Heissenberger, A., Miklau, M., Reichenbecher, W., Steinbrecher, R. A., et al. (2019). An EU Perspective on Biosafety Considerations for Plants Developed by Genome Editing and Other New Genetic Modification Techniques (nGMs). *Front. Bioeng. Biotechnol.* 7:31. doi: 10.3389/fbioe.2019.00031

Evans, J., Wood, G., and Miller, A. (2006). The risk assessment-policy gap: an example from the UK contaminated land regime. *Environ. Int.* 32, 1066–1071. doi: 10.1016/j.envint.2006.06.002

Harrigan, G. G., Lundry, D., Drury, S., Berman, K., Riordan, S. G., Nemeth, M. A., et al. (2010). Natural variation in crop composition and the impact of transgenesis. *Nat. Biotechnol.* 28, 402–404. doi: 10.1038/nbt0510-402

Herman, R. A., and Price, W. D. (2013). Unintended compositional changes in genetically modified (GM) crops: 20 years of research. *J. Agric. Food Chem.* 61, 11695–11701. doi: 10.1021/jf400135r

Hoekenga, O. A. (2008). Using metabolomics to estimate unintended effects in transgenic crop plants: problems, promises, and opportunities. *J. Biomol. Tech.* 19, 159–166.

International Service for the Acquisition of Agri-biotech Applications [ISAAA] (2021). Global status of commercialized biotech/gm crops in 2019. *China Biotechnol.* 41, 114–119.

Klumper, W., and Qaim, M. (2014). A meta-analysis of the impacts of genetically modified crops. *PLoS One* 9:e111629. doi: 10.1371/journal.pone.0111629

Ladics, G. S., Bartholomaeus, A., Bregitzer, P., Doerrer, N. G., Gray, A., Holzhauser, T., et al. (2015). Genetic basis and detection of unintended effects in genetically modified crop plants. *Transgenic. Res.* 24, 587–603. doi: 10.1007/s11248-015-9867-7

Li, Y., Hallerman, E. M., Wu, K., and Peng, Y. (2020). Insect-Resistant Genetically Engineered Crops in China: development, Application, and Prospects for Use. *Annu. Rev. Entomol.* 65, 273–292. doi: 10.1146/annurev-ento-011019-025039

Liu, Q., Yang, X., Tzin, V., Peng, Y., Romeis, J., and Li, Y. (2020). Plant breeding involving genetic engineering does not result in unacceptable unintended effects in rice relative to conventional cross-breeding. *Plant J.* 103, 2236–2249. doi: 10.1111/tpj.14895

Ma, Y., Creanga, A., Lum, L., and Beachy, P. A. (2006). Prevalence of off-target effects in Drosophila RNA interference screens. *Nature* 443, 359–363. doi: 10.1038/nature05179

Mamta, B., and Rajam, M. V. (2017). RNAi technology: a new platform for crop pest control. *Physiol. Mol. Biol. Plants* 23, 487–501. doi: 10.1007/s12298-017-0443-x

Olmos, V., Marro, M., Loza-Alvarez, P., Raldua, D., Prats, E., Pina, B., et al. (2019). Assessment of tissue-specific multifactor effects in environmental-omics studies of heterogeneous biological samples: combining hyperspectral image information and chemometrics. *Talanta* 194, 390–398. doi: 10.1016/j.talanta.2018.10.029

Papadopoulou, N., Devos, Y., Alvarez-Alfageme, F., Lanzoni, A., and Waigmann, E. (2020). Risk Assessment Considerations for Genetically Modified RNAi Plants: eFSA's Activities and Perspective. *Front. Plant Sci.* 11:445. doi: 10.3389/fpls.2020.00445

Praveen, S., Ramesh, S. V., Mishra, A. K., Koundal, V., and Palukaitis, P. (2010). Silencing potential of viral derived RNAi constructs in Tomato leaf curl virus-AC4 gene suppression in tomato. *Transgenic. Res.* 19, 45–55. doi: 10.1007/s11248-009-9291-y

Ramon, M., Devos, Y., Lanzoni, A., Liu, Y., Gomes, A., Gennaro, A., et al. (2014). RNAi-based GM plants: food for thought for risk assessors. *Plant Biotechnol. J.* 12, 1271–1273. doi: 10.1111/pbi.12305

Ricroch, A. E., Berge, J. B., and Kuntz, M. (2011). Evaluation of genetically engineered crops using transcriptomic, proteomic, and metabolomic profiling techniques. *Plant Physiol.* 155, 1752–1761. doi: 10.1104/pp.111.173609

Schiemann, J., Dietz-Pfeilstetter, A., Hartung, F., Kohl, C., Romeis, J., and Sprink, T. (2019). Risk Assessment and Regulation of Plants Modified by Modern Biotechniques: current Status and Future Challenges. *Annu. Rev. Plant Biol.* 70, 699–726. doi: 10.1146/annurev-arplant-050718-100025

Trapnell, C., William, B. A., and Pertea, G. (2010). Transcript assembly and quantification by RNA_Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* 28, 511–515. doi: 10.1038/nbt.1621

Wang, X., Zhang, X., Yang, J., Liu, X., Song, Y., and Wang, Z. (2019). Genetic variation assessment of stacked-trait transgenic maize via conventional breeding. *BMC Plant Biol.* 19:346. doi: 10.1186/s12870-019-1956-y

Wang, X. J., Zhang, X., Yang, J. T., and Wang, Z. X. (2018). Effect on transcriptome and metabolome of stacked transgenic maize containing insecticidal cry and glyphosate tolerance epsps genes. *Plant J.* 93, 1007–1016. doi: 10.1111/tpj.13825

Weng, M. L., Becker, C., Hildebrandt, J., Neumann, M., Rutter, M. T., Shaw, R. G., et al. (2019). Fine-Grained Analysis of Spontaneous Mutation Spectrum and Frequency in Arabidopsis thaliana. *Genetics* 211, 703–714. doi: 10.1534/genetics.118.301721

Zhang, J., Khan, S. A., Heckel, D. G., and Bock, R. (2017). Next-Generation Insect-Resistant Plants: RNAi-Mediated Crop Protection. *Trends Biotechnol.* 35, 871–882. doi: 10.1016/j.tibtech.2017.04.009

Zhao, H., and Wolt, J. D. (2017). Risk associated with off-target plant genome editing and methods for its limitation. *Emerg. Top Life Sci.* 1, 231–240. doi: 10.1042/ETLS20170037

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RNAi in *Piezodorus guildinii* (Hemiptera: Pentatomidae): Transcriptome Assembly for the Development of Pest Control Strategies

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Red-banded stink bug *Piezodorus guildinii* (*P. guildinii*) has been described as the most damaging stink bug regarding soybean crops, leading to seed injury, low germination percentages, and foliar retention, at low population densities. In recent years, RNA interference (RNAi), a conserved eukaryote silencing mechanism has been explored to develop species-selective pesticides. In this work, we evaluated RNAi in *P. guildinii* to develop new pest-control strategies. For this, we assembled and annotated a *P. guildinii* transcriptome from a pool of all developmental stages. Analysis of this transcriptome led to the identification of 56 genes related to the silencing process encompassing siRNA, miRNA, and piRNA pathways. To evaluate the functionality of RNAi machinery, *P. guildinii* adults were injected with 28 ng/mg of body weight of double stranded RNA (dsRNA) targeting *vATPase A*. A mortality of 35 and 51.6% was observed after 7 and 14 days, respectively, and a downregulation of *vATPase A* gene of 84% 72 h post-injection. In addition, *Dicer-2* and *Argonaute-2* genes, core RNAi proteins, were upregulated 1.8-fold 48 h after injection. These findings showed for the first time that RNAi is functional in *P. guildinii* and the silencing of essential genes has a significant effect in adult viability. Taken together, the work reported here shows that RNAi could be an interesting approach for the development of red-banded stink bug control strategies.

Keywords: stink bug, RNA-seq, dsRNA, RNAi, vATPase A, pest control

INTRODUCTION

Soybean (*Glycine max*) is one of the most extensively grown legume used for protein meal and vegetable oil, with an estimated 6% usage of the worlds' arable land (Hartman et al., 2011). Soybean cultivation in the United States covers large areas, being the largest producing countries Brazil, the United States, and Argentina, which have an adoption of biotech-enhanced soybean seedstock of

more than 90%.[1] Phytophagous stink bug complex (*Pentatomidae* family) is an important sanitary problem of the crop (Zerbino et al., 2016). In particular, red-banded stink bug or small-green stink bug *Piezodorus guildinii* (Westwood) (*P. guildinii*) has a Neotropical distribution, spanning from Argentina to the southern United States (Panizzi and Slansky, 1985). Economic damage, estimated at approximately 5% of the harvest, occurs when feeding, from pod formation to maturity (Bundy et al., 2019). The magnitude of the damage depends on the population reached, and the time of exposure to the infestation (Corrêa-Ferreira and De Azevedo, 2002). *P. guildinii* has been described as one of the most damaging stink bug regarding soybean, leading to reduced yield and quality, affecting seed weight and oil content, delaying crop maturity, and reducing the germination of the harvested seed (Depieri and Panizzi, 2011; Bundy et al., 2019). Current control strategies relay on the application of insecticides based on pyrethroids and neonicotinoids. Pyrethroids act on sucking and chewing insects with tumbling power, while neonicotinoids are highly residual systemic compounds used in sucking insects (Baur et al., 2010; Sosa-Gómez et al., 2020). It has been shown that these insecticides negatively impact natural enemies, are particularly harmful on bees, and have a detrimental effect on the environment (Giorio et al., 2021). Moreover, the lack of options for the control of these insects leads to the use of active principles in the same season and for several years with a similar mode of action, favoring the emergence of resistance (Temple et al., 2013).

RNA interference (RNAi) is a natural gene regulation mechanism present in eukaryotic cells (Svoboda, 2020). In this process, small RNA molecules (sRNA) associate with Argonaute proteins, forming the RNA-induced silencing complex (RISC), which uses complementary base pairing of the sRNA to identify target RNA molecules to be silenced (Ketting, 2011). RNAi pathways differ in the proteins involved, the origin and type of sRNA, the target RNA and, thus, the biological function. Biological roles of RNA silencing pathways include the regulation of endogenous gene expression (miRNA), antiviral immunity (siRNA), and genome protection against transposable elements (piRNA) (Ketting, 2011; Dowling et al., 2016). In siRNA, the basic mechanism consists of a long double stranded RNA (dsRNA) molecule (exogenous or endogenous) which is processed by ribonuclease III type Dicer-2 (Dcr-2) to a small duplex of 21–23 nucleotides. The resulting siRNAs are then loaded into RISC where the duplex is unbounded by the action of Ago-2, the sense strand is degraded, and the antisense strand guides the RISC to the target mRNA. Finally, Ago-2 provides the endonucleolytic activity, silencing gene expression (Mello and Conte, 2004; de Andrade and Hunter, 2016; Svoboda, 2020).

Given the growing need for alternatives to chemical pesticides, the use of RNAi emerges as a highly specific strategy, low environmental impact, and non-transgenic alternative (Christiaens et al., 2020). Reports of successful progress in pest control strategies in insects has been reviewed extensively (Christiaens et al., 2020; Zhu and Palli, 2020; Nitnavare et al., 2021). Nevertheless, the development of effective RNAi strategies in insects is a complex task and there is a great variability response in different insect orders. While coleopterans has shown to be highly susceptible (Baum et al., 2007; Baum and Roberts, 2014), insects from Lepidoptera (Terenius et al., 2011; Kolliopoulou and Swevers, 2014), Diptera (Maktura et al., 2021), and Hemiptera (Jain et al., 2021) respond with greater variability (Cooper et al., 2019). Several factors play a role in this observed differential RNAi efficiency: the presence of dsRNA degrading enzymes in the hemolymph and lumen (Christiaens and Smagghe, 2014; Song et al., 2017), inefficient intracellular transport of dsRNA including entrapment in the endosomes (Shukla et al., 2016; Yoon et al., 2017), or missing core components of the RNAi machinery (Arraes et al., 2021).

Several studies have shown that RNAi is efficient in stink bugs. In the brown marmorated stink bug *Halyomorpha halys*, administration of dsRNA by injection and feeding showed a reduction in the expression levels and mortality of up to 70% targeting essential genes (Mogilicherla et al., 2018). In the Neotropical brown stink bug *Euschistus heros*, RNAi core proteins were identified in the transcriptome, and adult injection of dsRNA targeting vATPase A caused a reduction in *vATPase A* gene expression and significant mortality (Cagliari et al., 2020). Moreover, the insecticidal effect with the same target in second instar nymphs reached 80% of mortality 14 days after injection. In feeding assays, the formulation with ethylenediaminetetraacetic acid (EDTA) or Liposome encapsulation enhanced the mortality up to 45 and 51%, respectively, compared with naked dsRNA against *vATPae A*, where no significant mortality was reported (Castellanos et al., 2019). In addition, a complex of a shRNA against troponin coupled to nanoparticles showed significant mortality by oral administration (Laisney et al., 2021). Studies in southern green stink bug *Nezara viridula* showed that injected dsRNA targeting essential genes could induce significant mortality (Riga et al., 2020; Gurusamy et al., 2021). Particularly, dsRNA targeting *vATPase A* achieved more than 80% mortality in injection assays, and 45% when feeding. This response was enhanced when specific *N. viridula* dsRNases were silenced by injection before oral administration in second instar nymphs targeting the α*cop* gene (Sharma et al., 2020, 2021). In the harlequin bug, *Murgantia histrionica* (Hahn), RNAi core proteins were identified and significant mortality was shown by injection and feeding targeting several genes (Howell et al., 2020). Finally, the brown-winged green stinkbug *Plautia stali* was very sensitive to vATPase E dsRNA injection, but a lower response was reported by oral feeding (Nishide et al., 2021).

In this work, we explored RNAi in *P. guildinii* towards the development of environmentally low impact pest control strategies. For this, we generated, to our knowledge, the first transcriptome of this stink bug species. Machinery RNAi genes were annotated, finding core RNAi genes, RISC associated genes, uptake, and intracellular transport related genes as well as RNAases. We demonstrated RNAi *in vivo* by dsRNA injection targeting *vATPase A*. Gene expression was significantly reduced in treated animals, and a 51.6% mortality rate was observed after 14 days. The gene expression of core proteins after injection in dsRNA treated animals was evaluated as well, with a significant overexpression of *Dcr-2* and *Ago-2* 48 h after injection. These

[1] http://soystats.com/international-adoption-of-biotech-enhanced-seedstock/

results showed that *P. guildinii* is susceptible to RNAi, and this approach could be exploited for the development of integrated pest control strategies.

MATERIALS AND METHODS

Insect Rearing

Adults *P. guildinii* were collected in INIA La Estanzuela, Colonia, Uruguay (S34° 20′W 57°41′) and maintained at 26 ± 1°C, 80 ± 10% RH, and a 16:8 light:dark cycle. Insects were fed *ad livitum* with green bean pods (*Phaseolus vulgaris*), dry soybean seeds (*G. max*), and raw shelled peanuts (*Arachis hypogaea*), distilled water was supplied every day by moistened cotton. Eggs were removed to a different plastic container and nymphs were checked daily until adult emergence.

cDNA Libraries and Sequencing

Total RNA was purified from all nymphal stages (1st–5th) and adults (male and female) using an RNeasy Mini Kit Qiagen (Hilden, Germany), according to manufacturer instructions. RNA integrity was determined with a Bioanalyzer 2100 Agilent Technologies (Santa Clara, United States). Equal RNA quantity of every stage was pooled to a 1 μg sample used for cDNA library preparation and Illumina (San Diego, United States) sequencing was conducted by Macrogen, Inc. (Seoul, South Korea). TruSeq stranded mRNA libraries were generated with a TruSeq Stranded mRNA LT Sample Prep Kit Ilumina (San Diego, United States), and sequencing was performed with Illumina HiSeq 2500 platform with a coverage of 75 G (1 lane), 150 bp paired ends reads.

Transcriptome Assembly

The quality of raw reads from the Illumina sequencing was analyzed by FastQC software[2] and filtered as follows: erroneous k-mers were eliminated with r-Corrector software (Song and Florea, 2015), adaptors and bases with a Phred score lower than 30 were trimmed with TrimmGalore.[3] Additionally, reads were mapped against SILVA (LSU/SSU) Database[4] using Bowtie2[5] to eliminate rRNA contaminant reads. The filtered reads were *de novo* assembled using Trinity software[6] with default parameters, using a de Bruijn graph algorithm and a k-mer length of 25. The quality of transcriptome assembly was evaluated by mapping the reads over the assembled contigs with bowtie2. Completeness was analyzed with Benchmarking Universal Single-Copy Orthologs (BUSCO) using the Arthropoda odb9 database,[7] and the number of fully reconstructed coding transcripts was evaluated by a BLASTx search against Swiss-Prot database with a cut off E-value ≤ 1e-20. All raw reads have been deposited in the sequence reads archive (SRA) at National Center for Biotechnology Information (NCBI), and may be accessed using the access code PRJNA772728.

Homology Search and Annotation

The generated contigs were analyzed by searching homology with a BLASTx tool against public databases, such as the non-redundant protein database (nr) filtered by Insecta Taxa (NCBI), Uni-Prot TrEMBL, and Swiss-Prot with a cut off E-value ≤ 1e-5. InterproScan available in OmicsBox software[8] was used for conserved domains search (Zdobnov and Apweiler, 2001). Gene ontology (GO) categories were assigned from pooled BLAST and InterPro hits according to the pipeline available in the Blast2Go software (Götz et al., 2008).

RNAi Related Genes

Genes related to RNAi machinery classified as core RNAi (**Table 1**), auxiliary RISC factors (**Table 2**), uptake, nucleases, antiviral, and intracellular transport proteins (**Table 3**) previously described were used as reference for the search in the *P. guildinii* transcriptome (Prentice et al., 2015; Taning et al., 2016; Cagliari et al., 2020). Homologous sequences for these proteins were obtained from NCBI and used as queries in a tBLASTn search with a cut off E-value < 1e-5. Hits with the lowest E-value were further analyzed to confirm their identity. An ORF Finder tool from NCBI[9] was used to predict Open Reading Frames and protein domains were predicted by the NCBI Conserved Domains Database.[10] A BLASTp search against non-redundant protein database at NCBI was performed as well. To provide additional confirmation on the identity of core RNAi proteins, a phylogenetic analysis was performed. Proteins sequences from different insect orders were aligned using MUSCLE program from MEGA 7.0.26, and neighbor-joining algorithm with 1,000 bootstrap replicates was used to predict phylogeny.

dsRNA Synthesis and Purification

Piezodorus guildinii transcriptome was screened for *vATPAase A* nucleotide sequence using as a query *H. halys* protein sequence XM_014417043.2 (**Table 3**). A 300 bp sequence showing low cross-reactivity to other organisms evaluated by BLASTp against nr-NCBI database was selected. A 496 bp green fluorescent protein (GFP) dsRNA was designed from pRFHUE-eGFP plasmid Addgene (Watertown, United States). Plasmid DNA was purified from transformed *Escherichia coli* DH5α cells according to Green and Sambrook (2016) protocol. Specific primers were designed to amplify DNA templates that included T7 promoter sequence placed at the 5′-end of both forward and reverse primer to enable *in vitro* transcription (**Table 4**). Further, 1 μg of total RNA from adults *P. guildinii* was used as template to synthesize cDNA and oligodT primer using SuperScript™ IV Reverse Transcriptase Thermo Fischer Scientific (Waltham, United States), according

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc

[3]https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

[4]https://www.arb-silva.de

[5]http://bowtie-bio.sourceforge.net/bowtie2/

[6]http://trinityrnaseq.sourceforge.net/

[7]https://busco.ezlab.org/

[8]https://www.biobam.com/omicsbox

[9]https://www.ncbi.nlm.nih.gov/orffinder/

[10]https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

TABLE 1 | Core RNAi related genes identified in *Piezodorus guildinii* transcriptome.

Gene ID	Homolog ID – species	*P. guildinii* ID	Comparison	% Identity
miRNA				
DCR-1	AVK59457.1 – *Nezara viridula*	TRINITY_DN8985_c0_g1_i5	E = 0.0 – bits = 1,321	93.56
AGO-1 isoform 1	AVK59466.1 – *Nezara viridula*	TRINITY_DN2687_c0_g1_i1	E = 0.0 – bits = 1,257	99,84
Loquacious	XP_014274312.1 – *Halyomorpha halys*	TRINITY_DN19426_c0_g1_i2	E = 0.0 – bits = 739	95.32
Drosha	XP_014278529.1 – *Halyomorpha halys*	TRINITY_DN28734_c0_g1_i1	E = 0.0 – bits = 1,442	92.75
Pasha/DGCR8	XP_014282581.1 – *Halyomorpha halys*	TRINITY_DN3240_c0_g1_i1	E = 0.0 – bits = 1,210	87.41
Exportin-5	XP_014280932.1 – *Halyomorpha halys*	TRINITY_DN9946_c0_g1_i3	E = 0.0 – bits = 2,405	85.60
siRNA				
DCR-2	XP_014275310.1 – *Halyomorpha halys*	TRINITY_DN9350_c0_g1_i2	E = 0.0 – bits = 2,808	83.81
Ago-02	AVK59468.1 – *Nezara viridula*	TRINITY_DN2417_c0_g1_i1	E = 0.0 – bits = 1,582	81.26
R2D2	XP_014288218.1 – *Halyomorpha halys*	TRINITY_DN2682_c1_g1_i1	E = 2.73e-170 – bits = 582	77.134
piRNA				
Ago-3	XP_014276831.1 – *Halyomorpha halys*	TRINITY_DN83747_c0_g1_i1	E = 0.0 – bits = 1,028	85.60
Aubergine (AUB)	XP_014275927.1 – *Halyomorpha halys*	TRINITY_DN5247_c0_g1_i2	E = 0.0 – bits = 1,195	65.38
Piwi	XP_014270559.1 – *Halyomorpha halys*	TRINITY_DN56355_c0_g1_i1	E = 0.0 – bits = 1,714	96.89
Zucchini (Zuc)	XP_014288409.1 – *Halyomorpha halys*	TRINITY_DN47842_c0_g2_i3	E = 3.52e-167 – bits = 468	92.41

TABLE 2 | RISC-related genes identified in *Piezodorus guildinii* transcriptome.

Gene ID	Homolog ID – species	*P. guildinii* ID	Comparison	% Identity
Tudor-SN	XP_014284230.1 – *Halyomorpha halys*	TRINITY_DN7506_c0_g1_i1	E = 0.0 – bits = 1,660	75.37
Translin	XP_014290495.1 – *Halyomorpha halys*	TRINITY_DN9072_c0_g1_i2	E = 8.5e-162 – bits = 513	86.40
Similar to translin associated factor-X (TRAX)	XP_014289754.1 – *Halyomorpha halys*	TRINITY_DN5790_c0_g1_i5	E = 1.50e-160 – bits = 613	87.60
Armitage	XP_014289817.1 – *Halyomorpha halys*	TRINITY_DN4232_c0_g1_i1	E = 0.0 – bits = 2,098	95.52
Homeless (spindle-E)	XP_014286769.1 – *Halyomorpha halys*	TRINITY_DN8326_c0_g1_i2	E = 0.0 – bits = 2,285	87.77
Maelstrom	XP_014290039.1 – *Halyomorpha halys*	TRINITY_DN4191_c0_g1_i1	E = 0.0 – bits = 449	82.56
HEN1	XP_014284423.1 – *Halyomorpha halys*	TRINITY_DN3220_c0_g1_i1	E = 0.0 – bits = 1,264	67.36
PRP16, mut6 homolog	XP_014279344.1 – *Halyomorpha halys*	TRINITY_DN6502_c0_g1_i1	E = 0.0 – bits = 2,428	98.81
Clp1 homolog (kinase)	XP_014275582.1 – *Halyomorpha halys*	TRINITY_DN2409_c0_g1_i16	E = 0.0 – bits = 2,821	96.43
Elp-1	XP_014290480.1 – *Halyomorpha halys*	TRINITY_DN1844_c0_g1_i1	E = 0.0 – bits = 2,104	85.16
GLD-1 homolog	XP_014290348.1 – *Halyomorpha halys*	TRINITY_DN6361_c0_g2_i1	E = 0.0 – bits = 1,058	85.96
ACO-1 homolog	XP_014275296.1 – *Halyomorpha halys*	TRINITY_DN320_c0_g1_i9	E = 0.0 – bits = 1,709	94.14
Vasa intronic gene (VIG)	XP_014292052.1 – *Halyomorpha halys*	TRINITY_DN5670_c0_g1_i1	E = 0.0 – bits = 782	96.57
Staufen	XP_014282526.1 – *Halyomorpha halys*	TRINITY_DN4585_c0_g1_i5	E = 0.0 – bits = 1,323	95.83
RNA helicase Belle	XP_014279436.1 – *Halyomorpha halys*	TRINITY_DN10378_c0_g3_i2	E = 0.0 – bits = 340	98.11
Protein arginine methyltransferase 7 (PRMT)	XP_014292128.1 – *Halyomorpha halys*	TRINITY_DN4183_c0_g1_i2	E = 0.0 – bits = 749	87.11
Gawky	XP_014288686.1 – *Halyomorpha halys*	TRINITY_DN12193_c0_g2_i6	E = 0.0 – bits = 2,830	98.59
Similar to fragile X mental retardation syndrome related protein 1 (FXMR1)	XP_969396 E – *Tribolium castaneum*	TRINITY_DN1626_c0_g1_i4	E = 0.0 – bits = 613	71.18
Gemin 3 homolog	EFA00789 – *Tribolium castaneum*	TRINITY_DN55620_c0_g1_i1	E = 0.0 – bits = 427	49.76
p68 Helicase	NP_001164095 – *Tribolium castaneum*	TRINITY_DN10378_c0_gi_i5	E = 0.0 bit = 688	73.85

to instructions from manufacturer. Templates were amplified from cDNA by PCR with 2.5 μl of cDNA template, 5 μl of 10× Buffer Thermo Scientific (Waltham, United States), 1.8 mM $MgCl_2$, 0.25 mM DNTPs, 0.25 mM of each primer, and 2.5 U of Taq Polymerase Thermo Scientific (Waltham, United States) in a 50 μl reaction. PCR cycle consisted of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 58°C, 45 s at 72°C, and a final extension of 10 min at 72°C. PCR products were purified by isopropanol precipitation, quantified by Nanodrop 8000 Thermo (Waltham, United States), and analyzed by agarose 1.5% gel electrophoresis. Sequences were confirmed by Sanger sequencing in Macrogen Inc. (Seoul, South Korea). DsRNAs were synthesized using a MEGAScript kit Thermo Fisher Scientific (Waltham, United States) from 1.2 μg of PCR product in a 16 h reaction and then purified by phenol:chloroform extraction following the manufacturer's instructions. Integrity was evaluated by agarose 1.5% gel electrophoresis, and, finally, concentration was determined in a

TABLE 3 | Uptake, nucleases, antiviral, and intracellular transport genes identified in *Piezodorus guildinii* transcriptome.

Gene ID	Homolog ID – species	*P. guildinii* ID	Comparison	% Identity
Uptake				
Scavenger	XP_024218066.1 – *Halyomorpha halys*	TRINITY_DN11492_c2_g2_i1	E = 0.0 – bits = 1,026	95.99
CG4966 = orthologous to the Hermansky-Pudlak Syndrome4 (HSP4)	XP_014288755.1 – *Halyomorpha halys*	TRINITY_DN9038_c0_g1_i1	E = 0.0 – bits = 897	89.00
F-box protein 11 (FBX011)	XP_014287303.1 – *Halyomorpha halys*	TRINITY_DN19510_c0_g1_i1	E = 0.0 – bits = 1,799	99.17
Clathrin heavy chain (Chc)	XP_014287090.1 – *Halyomorpha halys*	TRINITY_DN2469_c0_g1_i1	E = 0.0 – bits = 3,485	99.52
AP2u (Ap50)	NP_001280510.1 – *Tribolium castaneum*	TRINITY_DN5859_c0_g1_i3.	E = 0.0 bits = 866	94.33
ADP-ribosylation factor-like protein 1 (Arl1)	EFA02719.2 – *Tribolium castaneum*	TRINITY_DN8242_c0_g1_i1	E = 7.14e-115 bits = 323	85.55
Eater	XP_969372 – *Tribolium castaneum*	TRINITY_DN758_c1_g1_i2	E = 7.57e-47 – bits	36.79
Epsin 2 (Epn2)	XP_014270392.1 – *Halyomorpha halys*	TRINITY_DN2686_c2_g1_i4	E = 0.0 – bits = 1,026	91.21
Gap Junction protein (Innexin2)	XP_014292574.1 – *Halyomorpha halys*	TRINITY_DN2653_c0_g1_i1	E = 0.0 – bits = 1,026	99.44
Nucleases				
Exoribonuclease 1 (Eri1)	XP_014290344.1 – *Halyomorpha halys*	TRINITY_DN6568_c0_g1_i4	E = 0.0 – bits = 1,026	84.22
DNA/RNA non-specific endonuclease isoform 1	XP_024218583.1 – *Halyomorpha halys*	TRINITY_DN4766_c0_g1_i2	E = 1.03e-173 bits = 494	82.05
DNA/RNA non-specific endonuclease isoform 3	XP_014293261.1 – *Halyomorpha halys*	TRINITY_DN14109_c0_g1_i4	E = 0.0 – bits = 687	74.51
Small RNA degrading nuclease 1 (SDN1-like)	XP_014279339.1 – *Halyomorpha halys*	TRINITY_DN36580_c0_g1_i1	E = 0.0 – bits = 871	75.46
Exosome	XP_014288410.1 – *Halyomorpha halys*	TRINITY_DN30464_c0_g1_i1	E = 0.0 – bits = 1,845	94.64
PolyApolymerase	EFA00912 – *Tribolium castaneum*	TRINITY_DN5556_c0_g1_i1	E = 0.0 – bits = 1,026	65.37
Nibbler	XP_024216394.1 – *Halyomorpha halys*	TRINITY_DN76599_c0_g1_i1	E = 0.0 bit = 1,424	85.76%
Antiviral				
Ars2	XP_014277995.1 – *Halyomorpha halys*	TRINITY_DN4735_c0_g1_i1	E = 0.0 – bits = 1,507	94.90
NinaC	XP_014281724.1 – *Halyomorpha halys*	TRINITY_DN11848_c0_g1_i1	E = 0.0 – bits = 1,097	95.08
Egghead Beta 1,4-mannosyltransferase (egh)	XP_014283435.1 – *Halyomorpha halys*	TRINITY_DN10121_c0_g1_i1	E = 0.0 – bits = 918	96.94
CG4572	XP_014280828.1 – *Halyomorpha halys*	TRINITY_DN91529_c0_g1_i1	E = 0.0 – bits = 870	90.13
Intracellular transport				
Vacuolar H + ATPase sub unit A (vha68)	XP_014272529.1 – *Halyomorpha halys*	TRINITY_DN3993_c2_g1_i1.p1	E = 0.0 – bits = 1,256	99.35
Vacuolar H + ATPase sub unit C (vha16)	XP_014275063.1 – *Halyomorpha halys*	TRINITY_DN1028_c0_g3_i1.p1	1.13e-102 bits = 298	98.08
Small Rab GTPases	XP_014286452.1 – *Halyomorpha halys*	TRINITY_DN3137_c0_g1_i1.p1	4.24e-154 bits = 425	99.03

Nanodrop 8000 Thermo (Waltham, United States). Aliquots were stored at −80°C.

Adult dsRNA Injection

RNA interference in *P. guildinii* was evaluated by injection of 1.2 μg of dsRNA in adults (28 ng/mg of insect, average adult weight ∼45 mg) based on previous reports (Castellanos et al., 2019; Cagliari et al., 2020; Sharma et al., 2020). A 300 bp dsRNA targeting *vATPase A* was used to evaluate silencing and a 496 bp GFP dsRNA was used as control. Adults were anesthetized by incubation on ice for 5 min and then placed with the ventral side up under a binocular loupe (2×) (**Supplementary Figure 3**). A volume of 0.5 μl of dsRNA (2.4 μg/μl) was injected in the ventral septum between the thoracic and abdominal segments using a 25 μl Hamilton syringe with a 33G needle coupled to a PB600 repeater Hamilton (Reno, United States). Control group was injected with water. After injections, insects were placed in plastic plates containing green beans, peanut, and water *ad libitum*, and kept in the conditions previously described. For survival assays 30 adults were used per group, and mortality was evaluated every day for 14 days. Each assay was repeated two times. Survival curves were compared using the log-rank test ($p < 0.01$). For gene silence evaluation by real-time quantitative PCR (RT-qPCR), 27 adults were injected per group, and the pools of three individuals were processed at 24, 48, and 72 h post-injection by triplicate.

Real-Time Quantitative PCR

Total RNA was purified from insect samples corresponding to 24, 48, and 72 h post-injection using TRIzol Reagent Ambion (Austin, United States), following manufacturer's instructions. RNA was quantified using a Nanodrop 8000 (Thermo) and quality was evaluated by 1.5% agarose gel electrophoresis. Then, 1 μg of total RNA was treated with DNAase I NZY (Lisbon, Portugal) and used as template to synthesize cDNA, as described.

TABLE 4 | Primers used for dsRNA synthesis and RT-qPCR.

Gene name	Primer	Sequence 5′–3′	Amplicon (bp)	Amplification efficiency (%)
dsvATPase A	Fw	TAATACGACTCACTATAGGGAGATATCCAGCGACCCCTGAAG	300	–
	RV	TAATACGACTCACTATAGGGAGATTAGTTTTCTCACCATCAAACTCTG		
ds GFP	Fw	TAATACGACTCACTATAGGGAGAATGGTGAGCAAGGGCGAG	496	–
	RV	TAATACGACTCACTATAGGGAGATGTTCTGCTGGTAGTGGTCG		
18S	Fw	GTGCTTTGCAGTGGTTGTGT	107	99.3
	RV	TCGGGCCGTTCGACTTAATG		
60S	Fw	GCTCCCAAGATCGGTCCTCT	119	96.8
	RV	TGCCTGTTTTGAATAGTGAGGC		
vATPase A	Fw	AATTGTGCAGCTGGTCGGTA	127	99.6
	RV	TGGGCAGAACCGATCGTAAG		
Dcr-2	Fw	ACATTGCTGATGGAACGGGAT	84	104.9
	RV	AGGCTGTTTGGTCGACTTCC		
Ago-2	Fw	TACGGCAGAGACCTCCATCA	102	102.6
	RV	GAGGAGGTCCTCTTTGTGCC		

T7 Promoter sequence is underlined. Amplicon size is indicated, as well as primer efficiency when calculated.

Primers were designed using Primer-Blast from NCBI based on Primer3 software,[11] and were validated with a standard curve based on serial dilution of cDNA to determine amplification efficiency, calculated as E = [10^(−1/slope) − 1] × 100 (**Table 4**). A melting curve analysis with temperature range from 60 to 95°C and agarose gel electrophoresis confirmed primer specificity. RT-qPCRs were performed in a QuantStudioTM 3 Real-Time PCR System (Applied Biosystems) in 96 well plates. Each reaction included 2 μl of cDNA dilution, 5 μl iQ SYBR Green Supermix 2X Bio-Rad (Hercules, United States), and 0.25 mM of each primer in a 10 μl reaction. Amplification conditions were 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C, and a melting curve stage from 60 to 90°C. Data were analyzed using the Quant Studio design and analysis software v1.4.3. As endogenous control, *18S ribosomal protein* (*18S*) and *60S ribosomal protein* (*60S*) described previously for *H. halys* (Mogilicherla et al., 2018) were checked for stability with RefFinder software (Xie et al., 2012) and used for data normalization. Relative gene expression was calculated using the equation $2^{(-\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001). Normal distribution of the data was checked with Shapiro–Wilk test and Levene's test to compare variances. The statistical significance was analyzed by an un-paired *t*-test ($p < 0.05$, $n = 3$) using R-studio.

RESULTS

Piezodorus guildinii Transcriptome

Illumina sequencing led to 520,093,434 raw reads, corresponding to 260,046,717 unprocessed pairs with a GC content of 41.88%. Through the quality filtering process, 50,599,592 reads (19.4%) were eliminated (**Supplementary Table 1**). The *de novo* assembly of the *P. guildinii* transcriptome was performed with filtered reads resulting in 172,298 transcripts that correspond to 119,178 unigenes. The assembled transcripts' GC content was 35.57% and the average contig size was 750 bp. Assembly quality, as evaluated by mapping the filtered reads against the assembled transcripts, indicated that 97.49% of the paired reads were included in the assembly. Completeness of the transcriptome evaluated by BUSCO against an Arthropoda database showed that five out of 1,066 genes (0.5%) were not identified (**Supplementary Table 1**). Finally, the number of fully reconstructed transcripts was evaluated by means of a BLASTx search against the Swiss-Prot database, showing that 4,499 hits presented coverage greater than or equal to 90%.

The functional annotation of transcripts was performed by BLASTx, 55,706 hits (36.8%) were obtained against an Insecta subset of nr-NCBI protein database, 33,490 against Swiss-Prot (22%), while the BLASTx using insect TrEMBLE database yielded 52,721 matches (36.8%). Taking together, 58,922 hits were obtained, corresponding to 39% of the transcriptome (**Figure 1A**). Interestingly, when databases are compared, 53.60% of transcripts were identified by all three. Analysis of the sequences identified by nr-NCBI insect database showed that 76.8% of the hits corresponded to Hemiptera order as follows; Pentatomidae: 65% (*H. halys, N. viridula*), Aphidae: 4% (*Aphis craccivora*, *Aphis glycines*), Delphacidae: 2.16% (*Nilaparvata lugens*, *Laodelphax striatellus*), Miridae: 2.08% (*Apolygus lucorum*, *Nesidiocoris tenterauis*), Hemicidae: 1.79% (*Cimex lectularius*), and Psylliae: 0.8% (*Diaphorina citri*). The remaining hits were distributed among species of the orders Hymenoptera 3.9%, Coleoptera 2.4%, Lepidoptera 2.16%, and Diptera 1.19% (**Figure 1B** and **Supplementary Table 2**). It should be noted that BLAST analysis is based on sequence homology, which is dependent on the database used and does not imply phylogenetic relationships. Additionally, InterPro search identified 41,172 hits (27.2%). Sequences identified by both BLASTx and InterPro were used for the assignment of GO terms. A total of 42,316 terms were obtained, 54.5% from the Molecular Function category (MF), 30.5% from the Biological

[11] https://www.ncbi.nlm.nih.gov/tools/primer-blast/

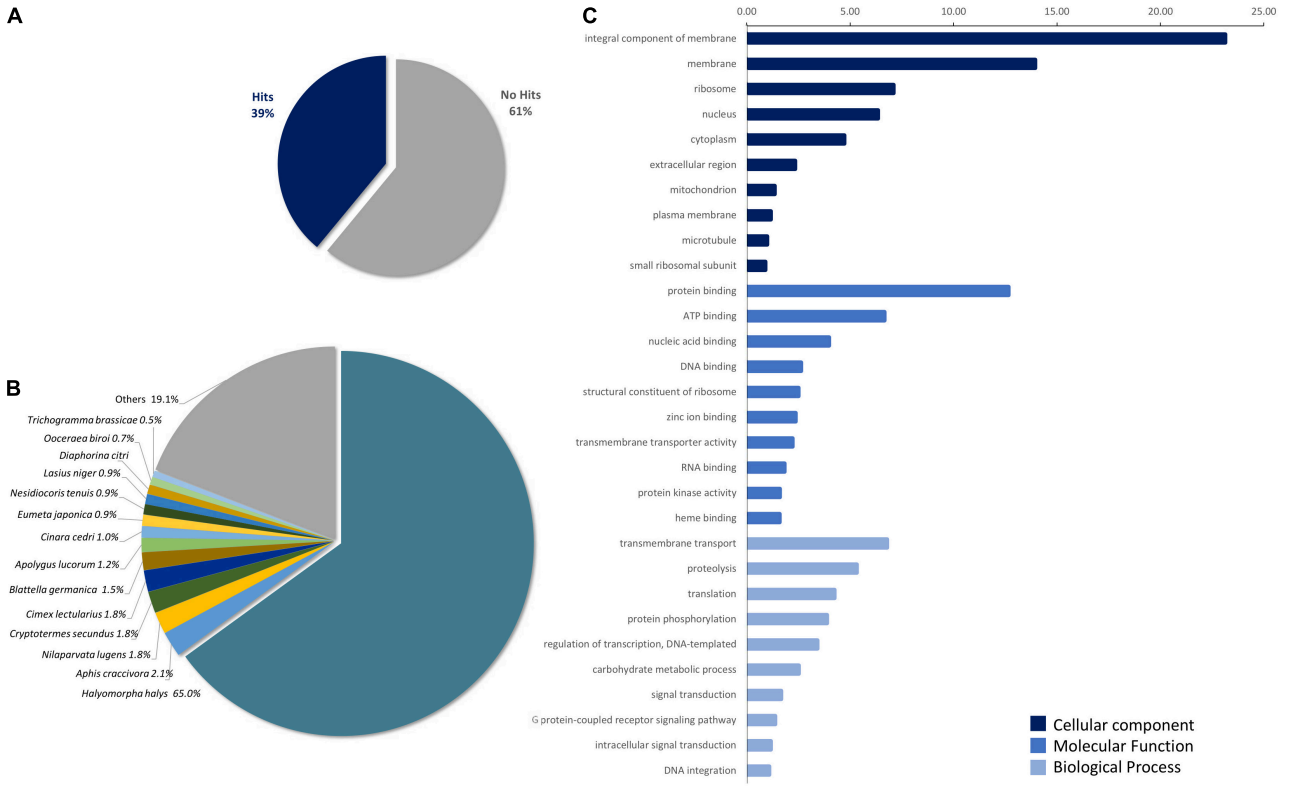

FIGURE 1 | *Piezodorus guildinii* transcriptome homology analysis. **(A)** Percentage of hits identified by BLASTx in *P. guildinii* transcriptome against nr-NCBI, Swiss-Prot, and TrEMBLE databases (cutoff E-Value < 10e-5). **(B)** Species distribution of transcripts with known function, hits from nr-NCBI database. **(C)** Gene Ontology (GO) terms in *P. guildini* transcriptome. Percentage distribution of 10 principal GO terms classified in Molecular Function (MF), Biological Process (BP), and Cellular Component (CC) assigned to *P. guildinii* contigs. Analysis was performed using hits from BLASTx and InterProScan using OmicsBox software.

Process category (BP), and 15.02% from the Cellular Component category (CC). The 10 main components from each category are detailed in **Figure 1C**.

Identification of RNAi-Related Genes

To establish RNAi strategies as a tool to control the red banded stink bug, the assembled transcriptome was used to annotate contigs related to RNAi machinery by tBLASTn. A total of 56 contigs were identified and are detailed in **Tables 1–3** and **Supplementary Material**.

Core RNAi machinery genes related to miRNA, siRNA, and piRNA pathways were predicted in the *P. guildinii* transcriptome (**Table 1** and **Supplementary Data 1**). In relation to Dicer proteins, in the miRNA pathway, only a partial sequence of *Dcr-1* was found, consisting in two RNAase III domains and a Double stranded RNA-binding domain (dsRBD). On the other hand, *Dcr-2* from siRNA pathways contained all the conserved domains (an *N*-terminal helicase domain, a PAZ domain, two RNAase III domains, and a dsRBD), while Drosha protein sequence was found with two Ribonuclease III family domain and a dsRBD. To further analyze the identified sequences, a phylogenetic analysis was performed with Dicer sequences from the different orders of insects (**Supplementary Figure 1**). Three main clades were inferred, one containing Dcr-1, another with Dcr-2, and the third one with Drosha sequences. In all cases, *P. guildinii* proteins were grouped with Hemiptera Pentatomidae sequences from *H. halys*, *N. viridula,* and *E. heros*. Within Dcr-1 subclade, *P. guildinii* Dcr-1 was grouped with *N. viridula*, while for Dcr-2, *P. guildinii* was grouped with *E. heros*. Drosha protein from *P. guildinii* was found in a clade with *E. heros* and *H. halys*. Co-factors Pasha, Loquacious, and R2D2 were identified with two dsRBD conserved domains. Regarding Argonaute, *Ago-1, Ago-2, Ago-3, Piwi*, and *Aubergine* were identified, all presented conserved domains PAZ and PIWI (**Supplementary Data 1**). Argonaute protein phylogeny showed three main clades, separating AGO subfamily (Ago-1 and Ago-2), Ago-3, and another clade with Aubergine and Piwi. In Ago subfamily, *P. guildinii* Ago-1 was grouped with *E. heros, H. halys,* and *N. viridula*, but in a different final branch, showing minor differences. Regarding Ago-2, *P. guildinii* protein was clustered with *N. viridula* and *H. halys*. In the PIWI subfamily, *P. guildinii* Ago-3 was clustered with *E. heros* and *H. halys*, while Piwi and Aubergine were clustered with *E. heros* (**Supplementary Figure 2**). In addition, the endoribonuclease Zucchini, essential for the primary piRNA biogenesis and Exportin5, a protein related to the nuclear export of pre-miRNAs was also identified.

The presence of auxiliary factors of the RISC complex was examined. Overall, 20 sequences were identified related to

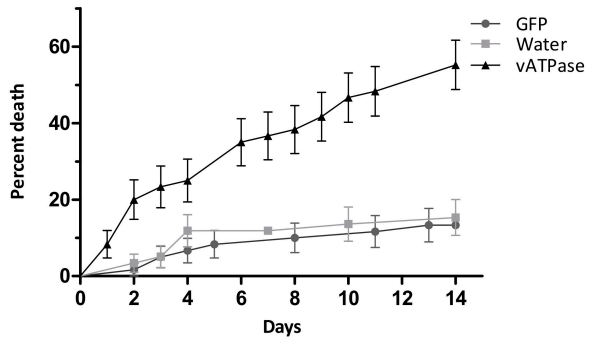

FIGURE 2 | dsRNA injection in *P. guildinii.* Cumulative mortality expressed as percentage in *P. guildinii* adults after injection with dsRNA (28 ng/μl per mg of body weight) targeting *vATPase A*, green fluorescent protein (GFP), or water. Error bars represent SE from two independent assays.

intracellular factors that are associated with the activity of the RISC complex (**Table 2**). Holo-RISC related proteins *Tudor-SN*, *Vasa-intronic gene*, and *fragile X related protein 1* were annotated with all conserved domains predicted. *Translin* and *Translin-associated factor X* (*TRAX*) components of C3P0 were also found. Additionally, nucleases involved in piRNA biogenesis, *Armitage, Homeless (splindle-E),* and *Maesltrom* were present as well. As detailed in **Table 2**, other contigs identified were *GLD-1* homolog*, Aco-1, Elp-1, RNA Belle helicase*, *Gawky, Staufen, helicase p68,* and *Clp-1.* Partial sequences were identified from *Hen-1 metyltransferase*, without a dsRBD2 nor methyltransferase conserved domains*, PRP16 helicase* sequence lacked one DEAH Helicase- domain, and *Arginine methyltransferase 7* did not present any SAM-dependent methyltransferase PRMT-type domain.

Nine proteins involved in dsRNA uptake were identified in *P. guildinii* transcriptome, as shown in **Table 3**. *Clathrin* heavy chain, *Clathrin adaptor AP50*, *Epsin-2,* and *HSP4* related to receptor mediated endocytosis were found. In addition, three proteins related to intracellular vesicle transport were also annotated, *Vacuolar H + ATPase subunit A (vha68*), *Vacuolar H + ATPase subunit C (vha16*), and *Small Rab GTPases*. Particularly, *Vacuolar H + ATPase subunit A (vATPase A)* was used as a target to evaluate RNAi response in *P. guildinii.* Putative dsRNA receptors Scavenger and Eater were described as well. Interestingly, transcripts coding for *sid-like* proteins were not found.

Regarding RNAi related nucleases, *Eri-1, SDN1-like,* and *Nibble* were found and all conserved domains were predicted. Two *DNA/RNA endonucleases* isoforms were found, but only isoform 1 contained a nuclease conserved domain (**Supplementary Data 1**). In addition, *Exosome complex exonuclease* and *Poly(A)polymerase* were described.

Antiviral RNAi related genes *Ars2* involved in RISC complex regulation, *Egghead*, a transmembrane-domain glycosyltransferase and *ninaC*, a protein related to vesicle transport, were identified as shown in **Table 3**.

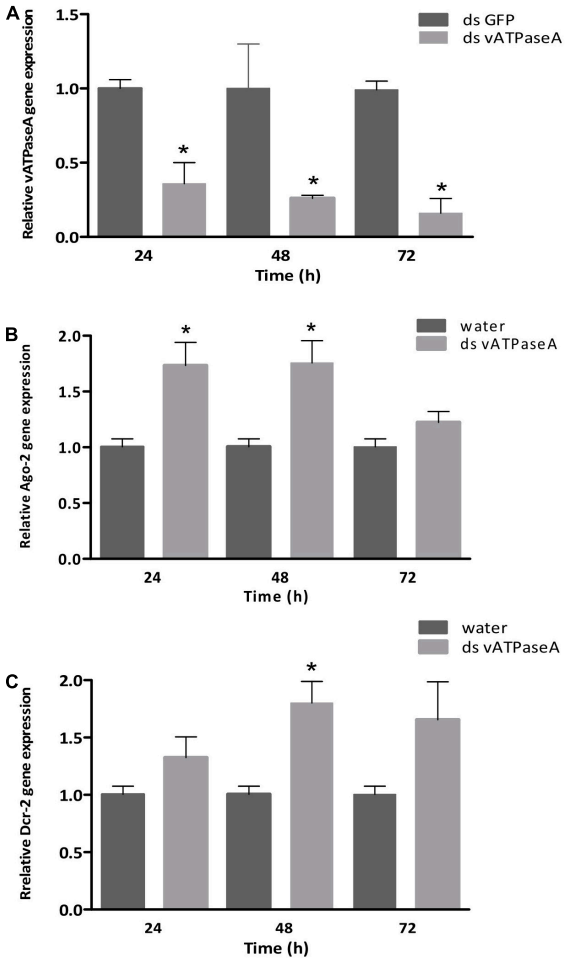

FIGURE 3 | Effect of dsRNA injection on gene expression by RT-qPCR in *P. guildinii*. Adults were injected with 28 ng/μl per mg of body weight with dsRNA targeting *vATPase A* or GFP used as a negative control. Adults were sampled at 24, 48, and 72 h post-injection in both treatments. As internal control, *18S* and *60S* genes were used. **(A)** *vATPase A* relative expression, GFP treated insects were used for normalization. **(B)** *Ago-2* relative gene expression in dsRNA treated insects in comparison with water-treated insects. **(C)** *Dcr-2* relative expression of insects treated with ds-vATPase A; water-treated insects were used as control. Bars represent mean and SEM based on three biological repeats consisting of a pool of three insects each. Statistical significance was calculated by an unpaired *t*-test. *$p \leq 0.05$.

dsRNA Injection in *Piezodorus guildinii*

The effect of dsRNA targeting *vATPase A* was studied by injection in *P. guildinii* adults. Significant differences were observed in the survival rates between insects treated with vATPase A, control GFP, or water assessed by log-rank test ($\chi^2 = 42.76$, df = 2 $p < 0.0001$). In vATPase A group, as shown in **Figure 2**, treated insects mortality was 35% after 7 days post-injection, reaching 51.6% after 14 days, while control groups behaved similarly. In GFP group, mortality ranged 10% on day 7 and 14% at the end of the assay; while in water injected insects, mortality was 11.5 and 15.5% after 7 and 14 days, respectively, as shown in **Figure 2**.

RT-qPCR in Injection Assays

To confirm *vATPase A* gene silencing after dsRNA injection, RT-qPCR analysis was performed using cDNA from treated insects. The *vATPase A* transcript level showed a significant reduction of 64.4% at 24 h after injection (p = 0.025), at 48 h, gene expression decreased 74% (p = 0.019), and 84% of reduction (p = 0.002) at 72 h compared with control samples injected with dsGFP (**Figure 3A**).

To assess the involvement of the siRNA machinery following dsRNA injection, the differential expression of the *Dcr-2* and *Ago-2* genes was analyzed by RT-qPCR. *Argonaute-2* showed a significant overexpression of 70 and 80% at 24 and 48 h, respectively, (p = 0.02); at 72 h, *Ago-2* expression was 1.3-fold, which was not statistically significant (**Figure 3B**). For *Dcr-2*, after 24 h a 30% overexpression was observed, but showed no statistical significance, while at 48 h, a 1.8-fold expression of *Dcr-2* was significantly different compared with control group (p = 0.04). A 1.6-fold expression was maintained at 72 h, even though this difference was not statistically significant (**Figure 3C**).

DISCUSSION

To evaluate the potential of RNAi as a tool in the control of the red-banded stink bug, we first generated a *P. guildinii* transcriptome, since little sequence information was available for this species. This approach allowed to get insight into the RNAi gene machinery and to gather information about possible target genes and housekeeping genes sequences. Recently, a complete genome assembly of *P. guildinii* has been reported (Perera, 2021-preprint). Taken together, these data will generate complementary information that will broaden the knowledge in the biology of this insect species. Homology analysis showed a 39% of contigs of known functions, revealing that *P. guildinii* transcriptome contains a high percentage of genes with non-assigned functions, a similar proportion of what was reported in *E. heros* transcriptome (Cagliari et al., 2020). The stink bug *H. halys* showed the highest homology from the identified transcripts, covering 65% of the hits; this could be explained by database coverage rather than phylogenetical homology (Ioannidis et al., 2014).

Differences in RNAi efficacy among insects can be partially explained by the diversity in the RNAi pathway genes present in different lineages (Dowling et al., 2016). To increase our understanding about *P. guildinii* RNAi response, we first analyzed the transcriptome for the annotation of genes related to this process. As shown in **Tables 1–3**, we were able to identify 56 sequences. Core components of the RNAi machinery with small RNA fragments are the main effectors in gene silencing. They can be divided into three pathways based on Dicers, Argonautes, and small RNAs involved. The siRNA pathway is activated by long exogenous or endogenous dsRNAs and involves Dicer-2 (Dcr-2), co-factor R2D2, and Argonaute-2 (Ago-2). The miRNA pathway involves miRNA from endogenous transcripts, nuclear Dicer, Drosha and co-factor Pasha, cytoplasmic Dicer-1 (Dcr-1), co-factor Loquatios, and Argonaute-1 (Ago-1). Finally, the piRNA pathway is germline-specific, derived from single-stranded RNA (ssRNA) and independent of Dicer proteins. It is characterized by endonuclease Zucchini (Zuc) and Argonaute proteins of the PIWI class: Aubergine, Piwi, and Argonaute-3 (Ago-3) (Ambrus and Frolov, 2009; Zhu and Palli, 2020). All the above-mentioned core proteins were identified in *P. guildinii* transcriptome (**Table 1** and **Supplementary Data 1**). Particularly, *Dicer-1* lacked the *N*-terminal helicase and PAZ domain. Functional Dcr-1 lacking the *N*-terminal helicase domain has been reported previously in *D. melanoganster* (Lee et al., 2004; Kavi et al., 2008). In *P. guildinii*, *Dicer-1* resembled nuclear RNAase III Drosha protein (two RNAse III domains and a carboxyterminal dsRBD). It would be interesting to further study if this Dicer-1 is functional in *P. guildinii* miRNA pathway. The phylogenetic analysis of these protein sequences (**Supplementary Figures 1**, **2**) further validated the identity of the annotated transcripts. *P. guildinii* proteins were grouped with Hempitera:Pentatomidae species *E. heros*, *N. viridula,* and *H. halys*. *Pasha*, *Loquacious,* and *R2D2* dsRNA binding proteins, co-factors of Drosha, Dicer-1, and Dicer-2, respectively, were also found with all their conserved domains. Although the structure of Ago proteins is highly conserved, the number of Ago proteins varies between different species from 1 in the fission yeast *Schizosaccharomyces pombe* to 27 in the nematode worm *Caenorhabditis elegans* (Höck and Meister, 2008; Meister, 2013). In insects, five Argonautes were reported for *D. melanoganster, T. castaneum, Cylas puncticollis*, and among others. Five members belonging to the Argonaute superfamily were identified in *P. guildinii* as well (**Table 1**). Argonaute subfamily: *Ago-1* and *Ago-2*; and PIWI subfamily: *Ago-3, Aubergine,* and *Piwi*. In all cases, conserved PAZ and Piwi domains were predicted. Interestingly, the phylogenetic analysis showed that identified sequences were grouped within Hemiptera:Pentatomidae species clades with a common ancestor (**Supplementary Figure 2**). In addition, the *Zucchini* endonuclease involved in piRNA maturation was also annotated. RdRP protein that can amplify synthesizing small RNAs or dsRNAs on targeted RNA templates was not found. This is consistent with previous reports that indicate that no homologs of this protein have been described in vertebrate or insect genomes (Maida and Masutomi, 2011).

We then analyzed auxiliary RISC factors associated with the activity of the RISC complex. In siRNA described in *D. melanoganster*, Holo-RISC or the mature form of RISC is composed by Dcr-2 and R2D2 (components of the RISC Loading Complex), proteins Ago-2, Tudor-SN (Gutierrez-Beltran et al., 2016), Vasa intronic gene, Fragile X related protein (FXMR1) (Caudy et al., 2002), and C3P0, an heterodimer of Translin and Trax proteins (Kavi et al., 2008; Liu et al., 2009). Sequences for all the proteins mentioned were identified in *P. guildinii* transcriptome with their conserved domains predicted as shown in **Table 2**. In addition, *Clp-1 kinase* related to siRNAs phosphorylation before RISC loading was also annotated (Davies and Samuels, 2010). Helicases play different functions in the silencing processes, from unwinding RNA duplexes to proper RNA loading to RISC (Ambrus and Frolov, 2009). Helicases *Belle*, *Armitage*, *p68 RNA helicase*, *Gemin 3,* and a partial sequence of *PRP16* were identified in *P. guildinii*. In addition, Piwi pathway related proteins *Spindle E*, *Maelstrom*, *Armitage*, *methyltransferases PRMT,* and a partial sequence of *Hen-1* were also found. Other auxiliary factors, such as *Elp-1*, a component of

pol II core elongation complex, *Staufen, GLD-1,* and *ACO-1* were present as well.

Internalization of dsRNA into cells is an essential step for the generation of RNAi. Two different pathways have been described for dsRNA uptake in insects, Sid-1 channel protein-mediated pathway and the endocytic pathway (Joga et al., 2016). The first pathway, described in *C. elegans*, involves transmembrane proteins SID-1 and SID-2 (Hunter et al., 2006). Orthologous proteins to *C. elegans* SID-1, called SID-like proteins (SIL-A, SIL-B, and SIL-C), have been identified in several insect species, although their direct involvement in dsRNA uptake is still not clear (Vélez and Fishilevich, 2018). SID-2 has not been reported in any insect order (Hinas et al., 2012). In *P. guildinii* transcriptome, *SilA, SilB,* and *SilC* homologous sequences were absent (**Table 3**), similarly in Diptera, where no *SID-like* genes have been reported, and more recently in the Hemiptera *E. heros* transcriptome (Tomoyasu et al., 2008; Huvenne and Smagghe, 2010; Cagliari et al., 2020). The other uptake pathway described is Clathrin-mediated endocytosis. The sequences identified in the present transcriptome encompassed the endocytic pathway (**Table 3** and **Supplementary Material**), from early vesicle formation, *Clathrin heavy chain (Chc), Clathrin adapter protein AP50*, to the late endosomal formation and release, *HPS4, Rab7*, endosome acidification proteins: *Vacuolar H + ATPase sub unit A (vha68)* and *Vacuolar H + ATPase sub unit C (vha16)*, as reported previously (Vélez and Fishilevich, 2018). These findings suggest that besides the absence of SID-like proteins, dsRNA uptake could be mediated by endocytosis in *P. guildinii*. In Drosophila S2 cells, scavenger receptors, SR-CI and Eater, account for more than 90% of dsRNA uptake (Saleh et al., 2006; Ulvila et al., 2006). A *scavenger receptor class B* and *Eater* sequence was annotated in *P. guildinii* (**Table 3** and **Supplementary Material**); further functional evaluation of these proteins would be needed to determine if they have an active role in dsRNA uptake.

A rapid degradation of dsRNA by dsRNases causes a reduction in RNAi response (Cooper et al., 2019). We described the presence of seven nucleases in the transcriptome of *P. guildinii* (**Table 3** and **Supplementary Material**): *Eri-1,* an evolutionarily conserved 3′–5′ exoribonuclease related in siRNA and miRNA pathways (Kennedy et al., 2004; Thomas et al., 2014), *Sdn1-like protein*, a 3′–5′ exonuclease related to the degradation of mature miRNA (Ramachandran and Chen, 2008). In addition, *Nibble*, a 3′–5′ exoribonuclease described in *Drosophila* to process 3′-end trimming in miRNA pathway (Han et al., 2011) and a *Poly(A)polymerase*, involved in the mRNA degradation (Yamanaka et al., 2013). Most insects possess 2–4 dsRNase genes, as revealed by genome-wide analysis (Cooper et al., 2019). In *P. guildinii* transcriptome, we found two sequences, but only isoform 1 showed a nuclease conserved domain (**Table 3** and **Supplementary Material**). *Exosome complex exonuclease*, a 3′—5′ exonuclease (Morlando et al., 2008) reported in *N. viridula* saliva, was also present (Lomate and Bonning, 2016). In addition, we report here the presence of anti-viral proteins (**Table 3** and **Supplementary Material**), such as *Ars2*, related to the regulation of the RISC complex, (Sabin et al., 2009), *Egghead* (*Egh)*, a seven transmembrane-domain glycosyltransferase, *nina C,* a protein involved in vesicle transport, and *C4572* protein, a carboxypeptidase with unknown function (Saleh et al., 2009).

Once we identified transcripts homologous to essential RNAi genes in *P. guilidinii* transcriptome, we evaluated *in vivo* whether the machinery was functional after an injection of a dsRNA targeting *vATPase A*. Vacuolar-type proton pumping ATPase (V-ATPase) is a ubiquitous enzyme responsible for proton (H^+) transport across membranes and the acidification of cellular compartments (Utai et al., 2019). Silencing and mortality after an administration of dsRNA vATPase A have been reported in different insect orders, such as Coleoptera (Baum et al., 2007; Whyard et al., 2009; Christiaens et al., 2016), Diptera (Whyard et al., 2009), Lepidoptera (Whyard et al., 2009; Camargo et al., 2016), Homoptera (Whyard et al., 2009), and Hemiptera (Singh et al., 2015; Basnet and Kamble, 2018). In *P. guildinii,* an injection of 28 ng/mg of body weight of dsRNA targeting vATPase A showed a mortality of 35% after 7 days, and of 51.6% after 14 days of injection. These results show that *P. guildinii* is susceptible to RNAi, with a significant mortality effect. In *E. heros*, an injection of dsRNA against *vATPase A* at the same concentration caused a mortality of 35% in adults 4 days after injection (Cagliari et al., 2020), while in second instar nymph a 50% mortality was shown after 7 days, reaching 80% after 14 days (Castellanos et al., 2019). A similar observation was reported in *N. viridula*, where second instar nymphs injected with 40 ng/mg of body weight of dsRNA against *vATPase A* showed 63 and over 80% mortality after 7 and 14 days, respectively (Sharma et al., 2020), while in adults, mortality rates were not significantly different to the control group (Gurusamy et al., 2021). These reports point out a differential response to RNAi regarding developmental stages in stink bugs. Further analysis in *P. guildinii* at different life stages could show higher mortality rates.

The effect of dsRNA injection was further assessed by RT-qPCR. The relative expression of *vATPase A* at 24, 48, and 72 h after injection was significantly reduced to 84% at 72 h, which is in accordance with a functional RNAi machinery. Moreover, *Dicer-2* and *Argonaute-2*, two fundamental core components of the process, were significantly upregulated at 48 h as well. Even though at 72 h, the levels of these proteins were not significantly different from control non-treated samples, the effect in the reduction of the expression of *vATPase A* was maintained. The upregulation of core siRNA enzymes in response to exogenous dsRNA occurs in many insect species, and can be related not only to the exposure to dsRNA, but also to environmental factors, and pathogens have an influence as well (Cooper et al., 2019).

The establishment of RNAi pest control strategies is a complex task affected by several variables raging from the presence and copy number of core RNAi machinery proteins, the selection of target genes and dsRNA design to delivery strategies that must overcome nuclease degradation, dsRNA cellular uptake, and systemic spreading (Jain et al., 2021). In this context, the present work generated a dataset that will be useful for the selection and design of new target genes to be further evaluated. In addition, the

direct effect of dsRNA on insect survival was evaluated bypassing the salivary system and the digestive tract, which may contain enzymes that degrade RNA and may reduce the effect observed, as reported previously in *E. heros* and *N. viridula* (Castellanos et al., 2019; Sharma et al., 2021). Delivery strategies to provide dsRNA protection to nuclease activity must be evaluated to achieve a robust *P. guildinii* control using RNAi.

CONCLUSION

In this work, we described the assemble and analysis of *P. guildinii* transcriptome focusing on RNAi machinery. In this sense, we described the annotation of RNAi machinery-related genes and showed that this process is functional in *P. guildinii* by injection of dsRNA into adults. The administration of dsRNA targeting *vATPase A* showed a reduction in the expression of this gene and it had significant effect on survival rates. Taken together, these results show that RNAi could be a potential tool for the development of new control strategies in *P. guildinii* in soybean crops.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository and accession number can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA772728.

AUTHOR CONTRIBUTIONS

MD-R, CS, and MM conceived and designed the experiments. CS and SM performed the experiments. CS and PF analyzed the data. CS and MD-R wrote the manuscript. All authors have discussed the findings, interpreted the results, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.804839/full#supplementary-material

REFERENCES

Ambrus, A. M., and Frolov, M. V. (2009). The diverse roles of RNA helicases in RNAi. *Cell Cycle* 8, 3500–3505. doi: 10.4161/cc.8.21.9887

Arraes, F. B. M., Martins-de-Sa, D., Noriega Vasquez, D. D., Melo, B. P., Faheem, M., de Macedo, L. L. P., et al. (2021). Dissecting protein domain variability in the core rna interference machinery of five insect orders. *RNA Biol.* 18, 1653–1681. doi: 10.1080/15476286.2020.1861816

Basnet, S., and Kamble, S. T. (2018). RNAi-Mediated Knockdown of vATPase Subunits Affects Survival and Reproduction of Bed Bugs (*Hemiptera*: *Cimicidae*). *J. Med. Entomol.* 55, 540–546. doi: 10.1093/jme/tjy001

Baum, J. A., and Roberts, J. K. (2014). *Progress Towards RNAi-Mediated Insect Pest Management. 1st ed*. London, United Kingdom: Elsevier Ltd, doi: 10.1016/B978-0-12-800197-4.00005-1

Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–1326. doi: 10.1038/nbt1359

Baur, M. E., Sosa-Gomez, D. R., Ottea, J., Leonard, B. R., Corso, I. C., Da Silva, J. J., et al. (2010). Susceptibility to insecticides used for control of piezodorus guildinii (Heteroptera: *Pentatomidae*) in the United States and Brazil. *J. Econ. Entomol.* 103, 869–876. doi: 10.1603/EC09364

Bundy, S., Esquivel, J., Panizzi, A., Eger, J., Davis, J., and Jones, W. (2019). "Piezodorus guildinii (Westwood)," in *Invasive Stink Bugs and Related Species (Pentatomoidea): biology, Higher Systematics, Semiochemistry, and Management*, ed. J. E. McPherson (Boca Raton: CRC Press), doi: 10.1201/9781315371221-16

Cagliari, D., Dias, N. P., dos Santos, E. Á, Rickes, L. N., Kremer, F. S., Farias, J. R., et al. (2020). First transcriptome of the Neotropical pest Euschistus heros (*Hemiptera*: *Pentatomidae*) with dissection of its siRNA machinery. *Sci. Rep.* 10, 1–16. doi: 10.1038/s41598-020-60078-3

Camargo, R. A., Barbosa, G. O., Possignolo, I. P., Peres, L. E. P., Lam, E., Lima, J. E., et al. (2016). RNA interference as a gene silencing tool to control Tuta absoluta in tomato (Solanum lycopersicum). *PeerJ* 4:e2673. doi: 10.7717/peerj.2673

Castellanos, N. L., Smagghe, G., Sharma, R., Oliveira, E. E., and Christiaens, O. (2019). Liposome encapsulation and EDTA formulation of dsRNA targeting essential genes increase oral RNAi-caused mortality in the Neotropical stink bug Euschistus heros. *Pest Manag. Sci.* 75, 537–548. doi: 10.1002/ps.5167

Caudy, A. A., Myers, M., Hannon, G. J., and Hammond, S. M. (2002). Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.* 16, 2491–2496. doi: 10.1101/gad.1025202

Christiaens, O., and Smagghe, G. (2014). The challenge of RNAi-mediated control of hemipterans. *Curr. Opin. Insect Sci.* 6, 15–21. doi: 10.1016/j.cois.2014.09.012

Christiaens, O., Prentice, K., Pertry, I., Ghislain, M., Bailey, A., Niblett, C., et al. (2016). RNA interference: a promising biopesticide strategy against the African Sweetpotato Weevil Cylas brunneus. *Sci. Rep.* 6, 1–11. doi: 10.1038/srep38836

Christiaens, O., Whyard, S., Vélez, A. M., and Smagghe, G. (2020). Double-Stranded RNA Technology to Control Insect Pests?: current Status and Challenges. *Front. Plant Sci.* 11:451. doi: 10.3389/fpls.2020.00451

Cooper, A. M. W., Silver, K., Zhang, J., Park, Y., and Zhu, K. Y. (2019). Molecular mechanisms influencing efficiency of RNA interference in insects. *Pest Manag. Sci.* 75, 18–28. doi: 10.1002/ps.5126

Corrêa-Ferreira, B. S., and De Azevedo, J. (2002). Soybean seed damage by different species of stink bugs. *Agric. For. Entomol.* 4, 145–150. doi: 10.1046/j.1461-9563.2002.00136.x

Davies, M. A., and Samuels, Y. (2010). Analysis of the genome to personalize therapy for melanoma. *Oncogene* 29, 5545–5555. doi: 10.1038/onc.2010.323

de Andrade, E. C., and Hunter, W. B. (2016). "RNA Interference – Natural Gene-Based Technology for Highly Specific Pest Control (HiSPeC)," in *RNA Interference*, Chap. 19, ed. I. Y. Abdurakhmonov (Rijeka: IntechOpen), doi: 10.5772/61612

Depieri, R., and Panizzi, A. (2011). Duration of feeding and superficial and in-depth damage to soybean seed by selected species of stink bugs (*Heteroptera*: *Pentatomidae*). *Neotrop. Entomol.* 40, 197–203. doi: 10.1590/S1519-566X2011000200007

Dowling, D., Pauli, T., Donath, A., Meusemann, K., Podsiadlowski, L., Petersen, M., et al. (2016). Phylogenetic origin and diversification of RNAi pathway genes in insects. *Genome Biol. Evol.* 8, 3784–3793. doi: 10.1093/gbe/evw281

Giorio, C., Safer, A., Sánchez-Bayo, F., Tapparo, A., Lentola, A., Girolami, V., et al. (2021). An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. *Environ. Sci. Pollut. Res.* 28, 11709–11715. doi: 10.1007/s11356-021-12853-6

Götz, S., García-Gómez, J. M., Terol, J., Williams, T. D., Nagaraj, S. H., Nueda, M. J., et al. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic Acids Res.* 36, 3420–3435. doi: 10.1093/nar/gkn176

Green, M. R., and Sambrook, J. (2016). Preparation of plasmid DNA by alkaline lysis with sodium dodecyl sulfate: minipreps. *Cold Spring Harb. Protoc.* 2016, 911–916. doi: 10.1101/pdb.prot093344

Gurusamy, D., Howell, J. L., Chereddy, S. C. R. R., Mogilicherla, K., and Palli, S. R. (2021). Improving RNA interference in the southern green stink bug, Nezara viridula. *J. Pest Sci.* 94, 1461–1472. doi: 10.1007/s10340-021-01358-3

Gutierrez-Beltran, E., Denisenko, T. V., Zhivotovsky, B., and Bozhkov, P. V. (2016). Tudor staphylococcal nuclease: biochemistry and functions. *Cell Death Differ.* 23, 1739–1748. doi: 10.1038/cdd.2016.93

Han, B. W., Hung, J. H., Weng, Z., Zamore, P. D., and Ameres, S. L. (2011). The 3'-to-5' exoribonuclease nibbler shapes the 3' ends of microRNAs bound to *drosophila* argonaute1. *Curr. Biol.* 21, 1878–1887. doi: 10.1016/j.cub.2011.09.034

Hartman, G. L., West, E. D., and Herman, T. K. (2011). Crops that feed the World 2. Soybean-worldwide production, use, and constraints caused by pathogens and pests. *Food Secur.* 3, 5–17. doi: 10.1007/s12571-010-0108-x

Hinas, A., Wright, A. J., and Hunter, C. P. (2012). SID-5 is an endosome-associated protein required for efficient systemic RNAi in C. elegans. *Curr. Biol.* 22, 1938–1943. doi: 10.1016/j.cub.2012.08.020

Höck, J., and Meister, G. (2008). The Argonaute protein family. *Genome Biol.* 9:210. doi: 10.1186/gb-2008-9-2-210

Howell, J. L., Mogilicherla, K., Gurusamy, D., and Palli, S. R. (2020). Development of RNAi methods to control the harlequin bug, Murgantia histrionica. *Arch. Insect Biochem. Physiol.* 104, 1–14. doi: 10.1002/arch.21690

Hunter, C. P., Winston, W. M., Molodowitch, C., Feinberg, E. H., Shih, J., Sutherlin, M., et al. (2006). Systemic RNAi in Caenorhabditis elegans. *Cold Spring Harb. Symp. Quant. Biol.* 71, 95–100. doi: 10.1101/sqb.2006.71.060

Huvenne, H., and Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. *J. Insect Physiol.* 56, 227–235. doi: 10.1016/j.jinsphys.2009.10.004

Ioannidis, P., Lu, Y., Kumar, N., Creasy, T., Daugherty, S., Chibucos, M. C., et al. (2014). Rapid transcriptome sequencing of an invasive pest, the brown marmorated stink bug Halyomorpha halys. *BMC Genomics* 15:738. doi: 10.1186/1471-2164-15-738

Jain, R. G., Robinson, K. E., Asgari, S., and Mitter, N. (2021). Current scenario of RNAi-based hemipteran control. *Pest Manag. Sci.* 77, 2188–2196. doi: 10.1002/ps.6153

Joga, M. R., Zotti, M. J., Smagghe, G., and Christiaens, O. (2016). RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. *Front. Physiol.* 7:553. doi: 10.3389/fphys.2016.00553

Kavi, H. H., Fernandez, H., Xie, W., and Birchler, J. A. (2008). Genetics and biochemistry of RNAi in *Drosophila*. *Curr. Top. Microbiol. Immunol.* 320, 37–75. doi: 10.1007/978-3-540-75157-1_3

Kennedy, S., Wang, D., and Ruvkun, G. (2004). A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. *Nature* 427, 640–645. doi: 10.1038/nature02320.1

Ketting, R. F. (2011). The Many Faces of RNAi. *Dev. Cell* 20, 148–161. doi: 10.1016/j.devcel.2011.01.012

Kolliopoulou, A., and Swevers, L. (2014). Recent progress in RNAi research in Lepidoptera: intracellular machinery, antiviral immune response and prospects for insect pest control. *Curr. Opin. Insect Sci.* 6, 28–34. doi: 10.1016/j.cois.2014.09.019

Laisney, J., Loczenski Rose, V., Watters, K., Donohue, K. V., and Unrine, J. M. (2021). Delivery of short hairpin RNA in the neotropical brown stink bug, Euschistus heros, using a composite nanomaterial. *Pestic. Biochem. Physiol.* 177:104906. doi: 10.1016/j.pestbp.2021.104906

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., et al. (2004). Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* 117, 69–81. doi: 10.1016/S0092-8674(04)00261-2

Liu, J., Yang, M., Wang, J., Xu, Y., Wang, Y., Shao, X., et al. (2009). Improvement of tumor targeting and antitumor activity by a disulphide bond stabilized diabody expressed in *Escherichia coli*. *Cancer Immunol. Immunother.* 58, 1761–1769. doi: 10.1007/s00262-009-0684-9

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real- Time Quantitative PCR and the $2^{(-\Delta\Delta Ct)}$ Method. *Methods* 408, 402–408. doi: 10.1006/meth.2001.1262

Lomate, P. R., and Bonning, B. C. (2016). Distinct properties of proteases and nucleases in the gut, salivary gland and saliva of southern green stink bug, Nezara viridula. *Sci. Rep.* 6, 1–10. doi: 10.1038/srep27587

Maida, Y., and Masutomi, K. (2011). RNA-dependent RNA polymerases in RNA silencing. *Biol. Chem.* 392, 299–304. doi: 10.1515/BC.2011.035

Maktura, C. G., Paranhos, J. B., and Marques-Souza, H. (2021). RNAi in fruit flies (*Diptera*: *Tephritidae*): successes and challenges. *J. Appl. Entomol.* 145, 740–756. doi: 10.1111/jen.12905

Meister, G. (2013). Argonaute proteins: functional insights and emerging roles. *Nat. Rev. Genet.* 14, 447–459. doi: 10.1038/nrg3462

Mello, C. C., and Conte, D. (2004). Revealing the world of RNA interference. *Nature* 431, 338–342. doi: 10.1038/nature02872

Mogilicherla, K., Howell, J. L., and Palli, S. R. (2018). Improving RNAi in the Brown Marmorated Stink Bug: identification of target genes and reference genes for RT-qPCR. *Sci. Rep.* 8, 1–9. doi: 10.1038/s41598-018-22035-z

Morlando, M., Ballarino, M., Gromak, N., Pagano, F., Bozzoni, I., and Proudfoot, N. J. (2008). Primary microRNA transcripts are processed co-transcriptionally. *Nat. Struct. Mol. Biol.* 15, 902–909. doi: 10.1038/nsmb.1475

Nishide, Y., Kageyama, D., Tanaka, Y., Yokoi, K., Jouraku, A., Futahashi, R., et al. (2021). Effectiveness of orally-delivered double-stranded RNA on gene silencing in the stinkbug Plautia stali. *PLoS One* 16:e0245081. doi: 10.1371/journal.pone.0245081

Nitnavare, R. B., Bhattacharya, J., Singh, S., Kour, A., Hawkesford, M. J., and Arora, N. (2021). Next Generation dsRNA-Based Insect Control: success So Far and Challenges. *Front. Plant Sci.* 12:673576. doi: 10.3389/fpls.2021.673576

Panizzi, A. R., and Slansky, F. (1985). Review of Phytophagous Pentatomids (*Hemiptera*: *Pentatomidae*) Associated with Soybean in the Americas. *Fla. Entomol.* 68, 184–214. doi: 10.2307/3494344

Perera, O. P. (2021). Chromosome length genome assembly of the redbanded stink bug, Piezodorus guildinii. *Preprint*. doi: 10.21203/rs.3.rs-892524/v1

Prentice, K., Pertry, I., Christiaens, O., Bauters, L., Bailey, A., Niblett, C., et al. (2015). Transcriptome analysis and systemic RNAi response in the African sweetpotato weevil (Cylas puncticollis, *Coleoptera*, *Brentidae*). *PLoS One* 10:e0115336. doi: 10.1371/journal.pone.0115336

Ramachandran, V., and Chen, X. (2008). Degradation of microRNAs by a family of exoribonucleases in *Arabidopsis*. *Science* 321, 1490–1492. doi: 10.1126/science.1163728

Riga, M., Denecke, S., Livadaras, I., Geibel, S., Nauen, R., and Vontas, J. (2020). Development of efficient RNAi in Nezara viridula for use in insecticide target discovery. *Arch. Insect Biochem. Physiol.* 103, 1–9. doi: 10.1002/arch.21650

Sabin, L. R., Zhou, R., Gruber, J. J., Lukinova, N., Bambina, S., Lau, C., et al. (2009). Ars2 regulates both miRNA- and siRNA-dependent silencing and suppresses RNA virus infection in *Drosophila*. *Cell* 138, 340–351. doi: 10.1016/j.cell.2009.04.045.Ars2

Saleh, M. C., Tassetto, M., van Rij, R. P., Goic, B., Gausson, V., Berry, B., et al. (2009). Antiviral immunity in *Drosophila* requires systemic RNAi spread. *Nature* 458, 346–350. doi: 10.1038/nature07712.Antiviral

Saleh, M. C., van Rij, R. P., Hekele, A., Gillis, A., Foley, E., O'Farrell, P. H., et al. (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat. Cell Biol.* 8, 793–802. doi: 10.1038/ncb1439

Sharma, R., Christiaens, O., Taning, C. N. T., and Smagghe, G. (2020). RNAi-mediated mortality in southern green stinkbug Nezara viridula by oral delivery of dsRNA. *Pest Manag. Sci.* 77, 77–84. doi: 10.1002/ps.6017

Sharma, R., Taning, C. N. T., Smagghe, G., and Christiaens, O. (2021). Silencing of double-stranded ribonuclease improves oral RNAi efficacy in southern

green stinkbug nezara viridula. *Insects* 12, 1–16. doi: 10.3390/insects12020115

Shukla, J. N., Kalsi, M., Sethi, A., Narva, K. E., Fishilevich, E., Singh, S., et al. (2016). Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. *RNA Biol.* 13, 656–669. doi: 10.1080/15476286.2016.1191728

Singh, A., Upadhyay, V., and Panda, A. (2015). "Solubilization and Refolding of Inclusion Body Proteins," in *Insoluble Proteins SE - 15 Methods in Molecular Biology*, ed. E. García-Fruitós (New York: Springer), 283–291. doi: 10.1007/978-1-4939-2205-5_15

Song, H., Zhang, J., Li, D., Cooper, A. M. W., Silver, K., Li, T., et al. (2017). A double-stranded RNA degrading enzyme reduces the efficiency of oral RNA interference in migratory locust. *Insect Biochem. Mol. Biol.* 86, 68–80. doi: 10.1016/j.ibmb.2017.05.008

Song, L., and Florea, L. (2015). Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. *Gigascience* 4:48. doi: 10.1186/s13742-015-0089-y

Sosa-Gómez, D. R., Corrêa-Ferreira, B. S., Kraemer, B., Pasini, A., Husch, P. E., Delfino Vieira, C. E., et al. (2020). Prevalence, damage, management and insecticide resistance of stink bug populations (*Hemiptera*: *Pentatomidae*) in commodity crops. *Agric. For. Entomol.* 22, 99–118. doi: 10.1111/afe.12366

Svoboda, P. (2020). Key Mechanistic Principles and Considerations Concerning RNA Interference. *Front. Plant Sci.* 11:1237. doi: 10.3389/fpls.2020.01237

Taning, C. N. T., Andrade, E. C., Hunter, W. B., Christiaens, O., and Smagghe, G. (2016). Asian Citrus Psyllid RNAi Pathway-RNAi evidence. *Sci. Rep.* 6, 1–10. doi: 10.1038/srep38082

Temple, J. H., Davis, J. A., Hardke, J., Moore, J., and Leonard, B. R. (2013). Susceptibility of southern green stink bug and redbanded stink bug to insecticides in soybean field experiments and laboratory bioassays. *Southwest. Entomol.* 38, 393–406. doi: 10.3958/059.038.0304

Terenius, O., Papanicolaou, A., Garbutt, J. S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., et al. (2011). RNA interference in Lepidoptera?: an overview of successful and unsuccessful studies and implications for experimental design. *J. Insect Physiol.* 57, 231–245. doi: 10.1016/j.jinsphys.2010.11.006

Thomas, M. F., L'Etoile, N. D., and Ansel, K. M. (2014). Eri1: a conserved enzyme at the crossroads of multiple RNA-processing pathways. *Trends Genet.* 30, 298–307. doi: 10.1016/j.tig.2014.05.003

Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., and Bucher, G. (2008). Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. *Genome Biol.* 9, 1–22. doi: 10.1186/gb-2008-9-1-r10

Ulvila, J., Parikka, M., Kleino, A., Sormunen, R., Ezekowitz, R. A., Kocks, C., et al. (2006). Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in *Drosophila* S2 cells. *J. Biol. Chem.* 281, 14370–14375. doi: 10.1074/jbc.M513868200

Utai, B. M. F., Ada, G. S. U. N., and Ada, Y. W. (2019). Review Vacuolar-type ATPase?: a proton pump to lysosomal trafficking. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci.* 95, 261–277. doi: 10.2183/pjab.95.018

Vélez, A. M., and Fishilevich, E. (2018). The mysteries of insect RNAi: a focus on dsRNA uptake and transport. *Pestic. Biochem. Physiol.* 151, 25–31. doi: 10.1016/j.pestbp.2018.08.005

Whyard, S., Singh, A. D., and Wong, S. (2009). Ingested double-stranded RNAs can act as species-specific insecticides. *Insect Biochem. Mol. Biol.* 39, 824–832. doi: 10.1016/j.ibmb.2009.09.007

Xie, F., Xiao, P., Chen, D., Xu, L., and Zhang, B. (2012). miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Mol. Biol.* 80, 75–84. doi: 10.1007/s11103-012-9885-2

Yamanaka, S., Mehta, S., Reyes-Turcu, F. E., Zhuang, F., Fuchs, R. T., Rong, Y., et al. (2013). RNAi triggered by specialized machinery silences developmental genes and retrotransposons. *Nature* 493, 557–560. doi: 10.1038/nature11716

Yoon, J. S., Gurusamy, D., and Palli, S. R. (2017). Accumulation of dsRNA in endosomes contributes to inefficient RNA interference in the fall armyworm, Spodoptera frugiperda. *Insect Biochem. Mol. Biol.* 90, 53–60. doi: 10.1016/j.ibmb.2017.09.011

Zdobnov, E. M., and Apweiler, R. (2001). InterProScan – an integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 17, 847–848. doi: 10.1093/bioinformatics/17.9.847

Zerbino, M. S., Altier, N. A., and Panizzi, A. R. (2016). Performance of Nymph and Adult of Piezodorus guildinii (Westwood) (*Hemiptera*: *Pentatomidae*) Feeding on Cultivated Legumes. *Neotrop. Entomol.* 45, 114–122. doi: 10.1007/s13744-015-0345-y

Zhu, K. Y., and Palli, S. R. (2020). Mechanisms, applications, and challenges of insect RNA interference. *Annu. Rev. Entomol.* 65, 293–311. doi: 10.1146/annurev-ento-011019-025224

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