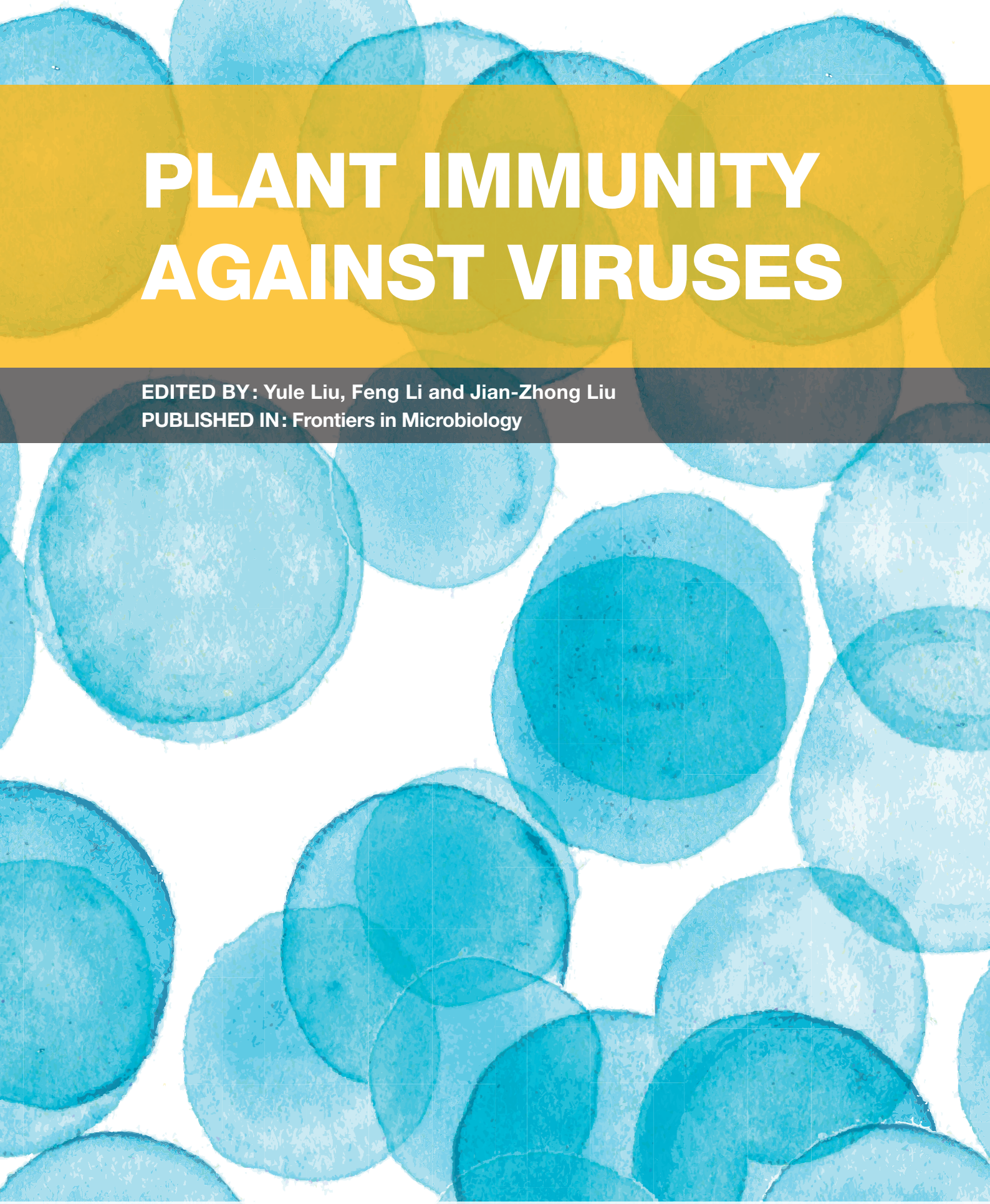
PLANT IMMUNITY AGAINST VIRUSES

EDITED BY: Yule Liu, Feng Li and Jian-Zhong Liu
PUBLISHED IN: Frontiers in Microbiology



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ISSN 1664-8714
ISBN 978-2-88945-269-9
DOI 10.3389/978-2-88945-269-9

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PLANT IMMUNITY AGAINST VIRUSES

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Plant viruses impose a serious threat on agriculture, which motivates extensive breeding efforts for viral resistant crops and inspires lasting interests on basic research to understand the mechanisms underlying plant immunity against viruses. Viruses are obligate intracellular parasites. Their genomes are usually small and only encode a few products that are essential to hijack host machinery for their nucleotide and protein biosynthesis, and that are necessary to suppress host immunity. Plants evolved multilayers of defense mechanisms to defeat viral infection.

In this research topic, we gathered 13 papers covering recent advances in different aspects of plant immunity against viruses, including reviews on RNA silencing and R gene based immunity and their application, translational initiation factor mediated recessive resistance, genome editing based viral immunity, role of chloroplast in plant-virus interaction, and research articles providing new mechanistic insights on plant-virus interactions. We hope that this Research Topic helps readers to have a better understanding of the progresses that have been made recently in plant immunity against viruses. A deeper understanding of plant antiviral immunity will facilitate the development of innovative approaches for crop protections and improvements.

Citation: Liu, Y., Li, F., Liu, J-Z., eds. (2017). Plant Immunity against Viruses. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-269-9

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frontiers in Microbiology

EDITORIAL
published: 29 March 2017
doi: 10.3389/fmicb.2017.00520

Editorial: Plant Immunity against Viruses

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Keywords: immunity, plant viruses, gene silencing, disease resistance gene, genome editing

Editorial on the Research Topic

Plant Immunity against Viruses

Plant viruses, the simple obligate intracellular parasites with small genomes, rely entirely on host machineries for their life cycle including replication, intracellular (cell-to-cell) and systemic movement (Nelson and Citovsky, 2005). Virus infections pose serious threats to agriculture and cause huge economic losses. Despite encoding only a limited number of proteins, numerous interactions of viral RNAs/proteins with host factors have puzzled the plant virologists for over a century and the complexity of these interactions is just becoming understood.

Plants have developed two major strategies to counteract virus infections: resistance (*R*) gene-mediated, and RNA silencing-based defenses. In addition, the mutation in essential genes for viral infection also causes plant resistance against viruses, called recessive gene-mediated resistance. These approaches have been used in crop protections and have shown significant economic impact (Abel et al., 1986; Whitham et al., 1996; Baulcombe, 2004; Kang et al., 2005; Wang and Krishnaswamy, 2012).

This Research Topic combines 13 publications, including 9 review articles and 4 research articles, covering almost every aspect of plant-virus interactions. The featured in-depth topic reviews in various sub-fields provide readers a convenient way to understand the current status of the related sub-fields and the featured research articles expand the current knowledge in related sub-fields.

Not unexpectedly, vast majority of the papers in this Research Topic are related to gene silencing but with totally distinct emphasis. Khalid et al. summarizes the applications of various small RNA based genetic engineering (SRGE) in crop protection, focusing on the technology evolution and successful cases in different crops. Andika et al. reviews the current information on the molecular aspects of antiviral RNA silencing in roots, with emphasis on the interactions between host antiviral defense and soil-borne viruses. The distinctive characteristic features of RNA silencing in roots relative to shoots are summarized. Moon and Park review how the RNA silencing pathway cross-talks with the resistance (*R*) gene-mediated defense. Several components involved in host RNA silencing mechanisms have recently been shown to be required for *R* gene-mediated defense. It seems that it is a common phenomenon that miRNAs or siRNAs regulate *R*-gene mediated resistance through targeting *R* genes for cleavage in plants (Moon and Park). It is plausible that the cross-talk between these two defense pathways is to maximize the efficiency of defense responses against viral infections (Nakahara and Masuta, 2014). Huang et al. summarize the various scenarios of host- and pathogen-derived sRNAs or pathogen-induced host sRNAs in regulating host resistance/susceptibility or pathogen virulence/pathogenicity.

The zigzag model (Jones and Dangl, 2006) presents a classic view of the interactions between plants and non-viral pathogens. Ding (2010) considered dsRNA as the Pathogen-associated Molecular Patterns (PAMPs) of viral pathogen and RNA silencing (or RNAi) as a form of PTI

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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *03 March 2017*
Accepted: *13 March 2017*
Published: *29 March 2017*

Citation:
Liu JZ, Li F and Liu Y (2017) Editorial: Plant Immunity against Viruses. Front. Microbiol. 8:520. doi: 10.3389/fmicb.2017.00520

against viruses. Recently, Mandadi and Scholthof (2013) further integrated these concepts into current plant-virus interaction models. In this model, dsRNAs produced during virus infection are regarded as viral PAMP; RNAi-mediated antiviral defense is analogous to PAMP-triggered immunity (PTI); viral suppressors of RNAi (VSR) such as coat proteins (CPs), movement proteins (MPs), and replicase are regarded as avirulent (Avr) factors or effectors; *R* gene-mediated viral resistance is considered as viral effector-triggered immunity (ETI). Moon and Park and Gouveia et al. review how this model is shaped in details. In addition, Gouveia et al. reviewed the recent progresses in antiviral immune receptors and co-receptors involved in antiviral innate immunity in plants and describe the NIK1-mediated antiviral signaling, which is specific to plant DNA viruses and relies on transmembrane receptor-mediated translational suppression for defense. It remains to be seen whether this is an exception or a common viral defense mechanism used by plants.

Recessive resistance is conferred either by a recessive gene mutation that encodes a host factor critical for viral infection or by a mutation in a negative regulator of plant defense responses, possibly due to the autoactivation of defense signaling. Eukaryotic translation initiation factor (eIF) 4E and eIF4G and their isoforms are the most widely exploited recessive resistance genes in several crop species (Kang et al., 2005). Hashimoto et al. thoroughly review the recent advances in recessive resistance studies not just limited to eIF4E and eIF4G.

The disturbance of chloroplast components and functions is largely responsible for the chlorosis symptoms that are associated with virus infection. Chloroplast is not only the organelle that conducts photosynthesis but also the site for the biosynthesis of SA and JA, two major phytohormones that play roles in disease and resistance. Zhao et al. review the different aspects of chloroplast during plant-virus interactions, particularly focusing on the interactions between chloroplast and viral proteins that underlie the interplay between chloroplast and virus. Liu et al. review the major advances that have been made recently in identifying both the virulence/avirulence factors of *Soybean mosaic virus* (SMV) and mapping of SMV resistant genes in soybean. A special focus is given to the progress made in dissecting the SMV resistant signaling pathways using virus-induced gene silencing (VIGS). Romay and Bragard review the latest progress in plant antiviral defenses mediated by genome editing systems (GES). TALEN and CRISPR-Cas9 have been applied to generate resistance against plant viruses in the families of *Geminiviridae* and *Potyviridae*. Interestingly, the newly developed CRISPR-Cas systems using new versions of Cas9 proteins, capable to cleave ssRNA molecules, can be applied to target RNA viruses (Sampson et al., 2013; Abudayyeh et al., 2016). One advantage of genome editing is that the transgenes can be removed via segregation after editing. Some genome-edited crops have already been made available without being restricted by the US Department of Agriculture (Waltz, 2016). The other advantage of genome editing is that multiple alleles or genes can be edited simultaneously.

Application of next generation sequencing (NGS) technology in small RNA profiling has significantly impact on the studies of plant-virus interactions. Li et al. investigate profiles of the Cucumber green mottle mosaic virus (CGMMV)-derived siRNAs (vsiRNA) in infected leaves and fruits of *L. siceraria* using NGS. The vsiRNA patterns of abundance, origination and polarity, hotspot distribution, GC content and 5′ terminal nucleotide are compared between the infected leaves and fruits. The similarities and distinct differences are revealed by this analysis. Co-infection of none-coding satellite RNAs (sat-RNAs) usually inhibits replication and attenuates disease symptoms of helper viruses. However, Xu et al. reveal that co-infection of none-coding satellite RNAs (sat-RNAs) of Beet black scorch virus (BBSV) enhances the replication and the symptoms of BBSV on *N. benthamiana* possibly through competitively occupying or saturating host silencing machinery. Fang et al. narrow down the functional domains of 2b protein of Cucumber mosaic virus (CMV) for dsRNA binding and Argonaute (AGO) interaction. Their findings demonstrate that the dsRNA-binding activity of the 2b is essential for virulence, whereas the 2b-AGO interaction is necessary for interference with RDR1/6-dependent antiviral silencing in Arabidopsis. Alpha-momorcharin (α-MMC) is a type-I ribosome inactivating protein (RIP) in *Momordica charantia*. Yang et al. provide evidence that α-MMC plays a positive role in the resistance against CMV in *M. charantia* and the antiviral activities of α-MMC may be achieved through up-regulating JA and ROS signaling pathway.

We hope that this Research Topic helps readers to have a better understanding of the progresses that have been made recently in plant immunity against viruses. A deeper understanding of plant antiviral immunity will facilitate the development of innovative approaches for crop protections and improvements.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

The work in our labs is supported by grants from the National Science Foundation of China (31571423 and 31371401 to JL; 31272031 and 91440103 to FL; 31530059 to YL) and "Young 1000 talent program" to FL.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 04 October 2016
doi: 10.3389/fmicb.2016.01565

Chloroplast in Plant-Virus Interaction

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In plants, the chloroplast is the organelle that conducts photosynthesis. It has been known that chloroplast is involved in virus infection of plants for approximate 70 years. Recently, the subject of chloroplast-virus interplay is getting more and more attention. In this article we discuss the different aspects of chloroplast-virus interaction into three sections: the effect of virus infection on the structure and function of chloroplast, the role of chloroplast in virus infection cycle, and the function of chloroplast in host defense against viruses. In particular, we focus on the characterization of chloroplast protein-viral protein interactions that underlie the interplay between chloroplast and virus. It can be summarized that chloroplast is a common target of plant viruses for viral pathogenesis or propagation; and conversely, chloroplast and its components also can play active roles in plant defense against viruses. Chloroplast photosynthesis-related genes/proteins (CPRGs/CPRPs) are suggested to play a central role during the complex chloroplast-virus interaction.

Keywords: chloroplast, plant virus, protein interaction, virus infection, plant defense

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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *15 July 2016*
Accepted: *20 September 2016*
Published: *04 October 2016*

Citation:
Zhao J, Zhang X, Hong Y and Liu Y (2016) Chloroplast in Plant-Virus Interaction. Front. Microbiol. 7:1565. doi: 10.3389/fmicb.2016.01565

INTRODUCTION

Plant viruses, as obligate biotrophic pathogens, attack a broad range of plant species utilizing host plants' cellular apparatuses for protein synthesis, genome replication and intercellular and systemic movement in order to support their propagation and proliferation. Virus infection usually causes symptoms resulting in morphological and physiological alterations of the infected plant hosts, which always incurs inferior performance such as the decreased host biomass and crop yield loss.

Abbreviations: AbMV, Abutilon mosaic virus; AltMV, Alternanthera mosaic virus; AMV, Alfalfa mosaic virus; BaMV, Bamboo mosaic virus; BSMV, Barley stripe mosaic virus; CaMV, Cauliflower mosaic virus; CI protein, Cylindrical inclusion protein; CMV, Cucumber mosaic virus; CNV, Cucumber necrosis virus; CP, Coat protein, Capsid protein; CPRG/CPRP, chloroplast photosynthesis-related gene/protein; HC-Pro, Helper component protein proteinase; JA, Jasmonic acid; MDMV, Maize dwarf mosaic virus; MP, Movement protein; OEC, Oxygen evolving complex; OYDV, Onion yellow dwarf virus; PD, plasmodesmata; PMTV, Potato mop-top virus; PPV, Plum pox virus; PS II, photosystem II; PVX, Potato virus X; PVY, Potato virus Y; RCNMV, Red clover necrotic mosaic virus; RdRP, RNA-dependent RNA polymerase; *R* gene, *Resistance* gene; ROS, Reactive oxygen species; RSV, Rice stripe virus; RuBisCO, Ribulose-1,5-bisphosphate carboxylase/oxygenase; RYMV, Rice yellow mottle virus; SA, Salicylic acid; SCMV, Sugarcane mosaic virus; siRNA, small interfering RNA; SMV, Soybean mosaic virus; SNARE, soluble NSF attachment protein receptor; SYSV, Shallot yellow stripe virus; TBSV, Tomato bushy stunt virus; TEV, Tobacco etch virus; TGB proteins, Triple gene block proteins; TMV, Tobacco mosaic virus; TNV, Tobacco necrosis virus; ToMV, Tomato mosaic virus; TRSV, Tobacco ringspot virus; TuMV, Turnip mosaic virus; TVMV, Tobacco vein-mottling virus; TYMV, Turnip yellow mosaic virus; VRC, Viral replication complex; WMV, Watermelon mosaic virus.

The most common viral symptom is leaf chlorosis, reflecting altered pigmentation and structural change of chloroplasts. Viral influence on chloroplast structures and functions usually leads to depleted photosynthetic activity. Since the first half of the twentieth century, an increasing number of reports on a broad range of plant-virus combinations have revealed that virus infection inhibits host photosynthesis, which is usually associated with viral symptoms (Kupeevicz, 1947; Owen, 1957a,b, 1958; Hall and Loomis, 1972; Mandahar and Garg, 1972; Reinero and Beachy, 1989; Balachandran et al., 1994b; Herbers et al., 2000; Rahoutei et al., 2000; Guo et al., 2005; Christov et al., 2007; Kyseláková et al., 2011). It is suggested that modification of photosynthesis is a common and conserved strategy for virus pathogenesis to facilitate infection and to establish an optimal niche (Gunasinghe and Berger, 1991). The disturbance of chloroplast components and functions may be responsible for the production of chlorosis symptoms that are associated with virus infection (Manfre et al., 2011).

A series of typical changes followed by chlorotic symptoms imply the occurrence of chloroplast-virus interactions. These changes include (1) fluctuation of chlorophyll fluorescence and reduced chlorophyll pigmentation (Balachandran et al., 1994a), (2) inhibited photosystem efficiency (Lehto et al., 2003), (3) imbalanced accumulation of photoassimilates (Lucas et al., 1993; Olesinski et al., 1995, 1996; Almon et al., 1997), (4) changes in chloroplast structures and functions (Bhat et al., 2013; Otulak et al., 2015), and (5) repressed expression of nuclear-encoded chloroplast and photosynthesis-related genes (CPRGs) (Dardick, 2007; Mochizuki et al., 2014a), (6) direct binding of viral components with chloroplast factors (Shi et al., 2007; Bhat et al., 2013; Zhao et al., 2013).

In fact, the chloroplast itself is a chimera of components of various origins coming from its bacterial ancestors, viruses and host plants. For example, chloroplast contains the nuclear-encoded phage T3/T7-like RNA polymerase (Hedtke et al., 1997; Kobayashi et al., 2001; Filée and Forterre, 2005). It is not surprising that chloroplast has an important role in plant-virus interactions. Indeed, more and more chloroplast factors have been identified to interact with viral components (**Table 1**). These factors are involved in virus replication, movement, symptoms or plant defense, suggesting that viruses have evolved to interact with chloroplast.

In this review, we focus on the topic of how chloroplast factors and viral components interact with each other and how these interactions contribute to viral pathogenesis and symptom development, especially in virus-susceptible hosts.

CHLOROPLAST IS INVOLVED IN VIRAL SYMPTOM PRODUCTION

Although the development of viral symptoms can be traced back to different causes, the disruption of normal chloroplast function has been suggested to cause typical photosynthesis-related symptoms, such as chlorosis and mosaic (Rahoutei et al., 2000). Chloroplast has been implicated as a common target of plant viruses for a long time. For instance, the severe chlorosis on systemic leaves infected by CMV in *Nicotiana tabacum* cv. Xanthi nc is associated with size-reduced chloroplasts containing fewer grana (Roberts and Wood, 1982). A second example shows that the leaf mosaic pattern caused by virus infection can be due to the layout of clustered mesophyll cells in which chloroplasts were damaged to various degrees (Almási et al., 2001). A third example shows that symptom caused by PVY infection is often associated with decrease in the number and size of host plant chloroplasts as well as inhibited photosynthesis (Pompe-Novak et al., 2001). Based on the current studies, the ultrastructural alteration of chloroplast and the reduced abundance of proteins involved in photosynthesis are the two main causes of virus induced chloroplast symptomatology (see below).

Effect of Virus Infection on Chloroplast Structure

Successions of analysis on the ultrastructural organization of plant cells infected with viruses have been performed with electron microscopy since the 1940s. There is a stunning convergence among different host-virus systems where significant alteration or rearrangement of the chloroplast ultrastructure is correlated with the symptom development (Bald, 1948; Arnott et al., 1969; Ushiyama and Matthews, 1970; Allen, 1972; Liu and Boyle, 1972; Mohamed, 1973; Moline, 1973; Appiano et al., 1978; Tomlinson and Webb, 1978; Schuchalter-Eicke and Jeske, 1983; Bassi et al., 1985; Choi, 1996; Mahgoub et al., 1997; Xu and Feng, 1998; Musetti et al., 2002; Zechmann et al., 2003; Guo et al., 2004; El Fattah et al., 2005; Schnablová et al., 2005; Li et al., 2006; Yan et al., 2008; Laliberté and Sanfaçon, 2010; Montasser and Al-Ajmy, 2015; Zarzyńska-Nowak et al., 2015; Zhao et al., 2016). The chloroplast malformations include (1) overall decrease of chloroplast numbers and chloroplast clustering; (2) atypical appearance of chloroplast, such as swollen or globule chloroplast, chloroplast with membrane-bound extrusions or amoeboid-shaped chloroplast, generation of stromule (a type of dynamic tubular extensions from chloroplast); (3) irregular out-membrane structures such as peripheral vesicle, cytoplasmic invagination, membrane proliferations and broken envelope; (4) changes of content inside the chloroplast such as small vesicles or vacuoles in stroma, large inter-membranous sac, numerous, and/or enlarged starch grains, increase in the number and size of electron-dense granules/plastoglobules/bodies; (5) unusual photosynthetic structures such as disappearance of grana stacks, distorted, loosen, or dilated thylakoid and the disappearance of stroma; and (6) completely destroyed chloroplasts and disorganized grana scattering into the cytoplasm. In these studies, the viruses are from 12 families and have either sense ssRNA, antisense ssRNA or ssDNA genomes, covering the majority of genera and including those responsible for devastating disease. This implies that chloroplast abnormality is a common event across diverse plant-virus interactions. The types of chloroplast abnormalities caused by virus infection are summarized in **Table 2** and schemed in **Figure 1**.

TABLE 1 | Chloroplast factors interacting with virus nucleic acids or proteins.

Plant Virus*	Virus components	Chloroplast factors	Subcellular localization	Biological process	References
ssRNA POSITIVE-STRAND VIRUSES					
Potexvirus/Alphaflexiviridae					
Alternanthera mosaic virus (AltMV)	TGB3	Chloroplast membrane	Chloroplast	Cell-to-cell movement, long-distance movement, symptom	Lim et al., 2010
		PsbO	Surrounding chloroplast	Symptom	Jang et al., 2013
Bamboo mosaic virus (BaMV)	RNA 3′ UTR	cPGK	Chloroplast Cytoplasm,	Replication	Cheng et al., 2013
Potato virus X (PVX)	CP	Plastocyanin	Chloroplast	Symptom	Qiao et al., 2009
Alfamovirus/Bromoviridae					
Alfalfa mosaic virus (AMV)	CP	PsbP	Cytoplasm	Replication	Balasubramaniam et al., 2014
Cucumovirus/Bromoviridae					
Cucumber mosaic virus (CMV)	1a, 2a	Tsip1	Cytoplasm	Replication	Huh et al., 2011
Cucumber mosaic virus Y strain satellite RNA (CMV-Y-sat)	22-nt vsiRNA**	*Chll mRNA*	Cytoplasm	Symptom	Shimura et al., 2011; Smith et al., 2011
Potyvirus/Potyviridae					
Potato virus Y (PVY)	CP	RbCL	–	Symptom	Feki et al., 2005
	HC-Pro	MinD	Cytoplasm	Symptom	Jin et al., 2007
		CF1β	Chloroplast	Symptom	
Onion yellow dwarf virus (OYDV)	P3	RbCL, RbCS	–	–	Lin et al., 2011
Plum pox virus (PPV)	CI	PsaK	–	Host defense	Jimenez et al., 2006
Sugarcane mosaic virus (SCMV)	HC-Pro	Fd V	Cytoplasm	Symptom	Cheng et al., 2008
Soybean mosaic virus (SMV)	P1	Rieske Fe/S	–	Symptom	Shi et al., 2007
	P3	RbCL, RbCS	–	–	Lin et al., 2011
Shallot yellow stripe virus (SYSV)	P3	RbCL, RbCS	–	–	Lin et al., 2011
Turnip mosaic virus (TuMV)	CP	37-kD protein	–	–	McClintock et al., 1998
	P3	RbCL, RbCS	–	–	Lin et al., 2011
Tobacco vein-mottling virus (TVMV)	CI	PsaK	–	Host defense	Jimenez et al., 2006
Dianthovirus/Tombusviridae					
Red clover necrotic mosaic virus (RCNMV)	MP	GAPDH-A	Chloroplast, Endoplasmic reticulum	Cell-to-cell movement	Kaido et al., 2014
Pomovirus/Virgaviridae					
Potato mop-top virus (PMTV)	TGB2	Chloroplast lipid	Chloroplast	Replication	Cowan et al., 2012
Tobamovirus/Virgaviridae					
Tobacco mosaic virus (TMV)	126 K replicase	PsbO	–	Host defense	Abbink et al., 2002
		NRIP	Cytoplasm, Nucleus	Host defense	Caplan et al., 2008
	126 K/183 K replicase	AtpC	VRCs	Host defense	Bhat et al., 2013
		RCA	VRCs		Host defense
	MP	RbCS	Cytoplasm	Cell-to-cell movement	Zhao et al., 2013
Tomato mosaic virus (ToMV)	CP	Fd I	Cytoplasm	Symptom	Sun et al., 2013; Ma et al., 2008
		IP-L	Thylakoid membrane	Long distance movement	Li et al., 2005; Zhang et al., 2008
	MP	RbCS	Cytoplasm	Cell-to-cell movement	Zhao et al., 2013
ssRNA NEGATIVE SENSE VIRUSES					
***Tenuivirus*/Unassigned**					
Rice stripe virus (RSV)	SP	PsbP	Cytoplasm	Symptom	Kong et al., 2014

(Continued)

TABLE 1 | Continued

Plant Virus*	Virus components	Chloroplast factors	Subcellular localization	Biological process	References
ssDNA VIRUSES					
Begomovirus/Geminiviridae					
Abutilon mosaic virus (AbMV)	MP	cpHSC70-1	Cell periphery, Chloroplast	Cell-to-cell movement	Krenz et al., 2010, 2012
dsDNA VIRUSES					
Caulimovirus/Caulimoviridae					
Cauliflower mosaic virus (CaMV)	P6	CHUP1	VRCs	Cell-to-cell movement	Angel et al., 2013

**Virus taxonomy is in format of Genus/Family. **Virus-derived small interfering RNA. – Not addressed. ssRNA, single-stranded RNA; ssDNA, single-stranded DNA.*

Viral Effectors Are Related to the Chloroplast Structural Changes

Recent reports have revealed that viral factors, especially coat proteins (CPs), affect chloroplast ultrastructure and symptom development (see below).

Viral coat proteins (CPs) have been demonstrated as determinants of symptom phenotypes for a much long period (Heaton et al., 1991; Neeleman et al., 1991). The earlier research showed that virion-like particles or virus inclusion in chloroplast are positively related to the development of mosaic symptom caused by TMV (Bald, 1948; Shalla, 1964). The more virion-like particles accumulated in chloroplast, the more severe morphological defects of chloroplast structure occurred (Matsushita, 1965; Shalla, 1968; Granett and Shalla, 1970; Betto et al., 1972). Later researches indicate that virion-like particles in chloroplast are pseudovirions, in which chloroplast transcripts are encapsidated by TMV CPs (Shalla et al., 1975; Rochon and Siegel, 1984; Atreya and Siegel, 1989), highlighting the involvement of CPs in the alteration of chloroplast ultrastructure. TMV CP does not possess a classical chloroplast transit peptide (TP) but can be imported into chloroplast effectively in a ATP-independent mode (Banerjee and Zaitlin, 1992). The majority of TMV CPs in chloroplasts are associated with the thylakoid membranes in systemically invaded *N. tabacum* leaves (Reinero and Beachy, 1986; Hodgson et al., 1989). Various natural TMV mutants, whose CPs excessively accumulate in chloroplast, always induce more severe symptoms and aggravated inhibition of the PS II activity (Regenmortel and Fraenkel-Conrat, 1986; Reinero and Beachy, 1986, 1989; Banerjee et al., 1995; Lehto et al., 2003), suggesting that chloroplast-targeted CPs act as the inducer of chloroplast ultrastructure rearrangements (**Figure 1**, **Table 2**). Tobamovirus CP can bind tobacco chloroplast Ferredoxin I (Fd I) (Sun et al., 2013, **Table 1**), while TMV infection reduces the protein level of Fd I in tobacco leaves (Ma et al., 2008). Silencing of *Fd1* in tobacco plants leads to symptomatic chlorosis phenotype and enhances CP accumulation in chloroplast as well as virus multiplication, suggesting that the CP-Fd I interaction may contribute to the development of chlorosis and mosaic symptoms.

PVX CP and viral particles can also be detected in chloroplast of the infected plants, causing structural alteration of chloroplast membranes and grana stacks (Kozar and Sheludko, 1969; Qiao et al., 2009). PVX CP interacts with the chloroplast TP of plastocyanin (**Table 1**), and silencing of plastocyanin in *N. benthamiana* reduces viral symptom severity. In plastocyanin silenced plants, the accumulation of CP in chloroplasts was also reduced although total CP amount in infected cells did not change (Qiao et al., 2009), suggesting that the CP-plastocyanin interaction positively contributes to viral symptom-associated chloroplast abnormality (**Figure 1**, **Table 2**).

PVY CP is preferentially associated with the thylakoid membranes (Gunasinghe and Berger, 1991). PVY CP interacts with the large subunits of RuBisCO (RbCL) (**Table 1**) and this interaction may be involved in the production of mosaic and chlorosis symptoms (Feki et al., 2005). Further research indicates that chloroplast-targeted, but not cytosol-localized CP induces virus-like symptom (Naderi and Berger, 1997a,b). These observations suggest an intimate relationship between chloroplasts and PVY CP during the process of inhibiting PS II in viral pathogenesis.

CMV infection causes symptoms associated with chloroplast ultrastructure changes (Roberts and Wood, 1982; Shintaku et al., 1992; Mazidah et al., 2012). CMV CP can be transported into intact chloroplast promptly in a ATP-independent mode and the amount of CP into chloroplast correlated with the severity of mosaic symptoms (Liang et al., 1998). The single amino acid substitution at residue 129 in CP of CMV pepo strain is found to induce chloroplast abnormalities (**Figure 1**, **Table 2**) associated with the alteration of chlorosis severity (Shintaku et al., 1992; Suzuki et al., 1995; Mochizuki and Ohki, 2011; Mochizuki et al., 2014b), suggesting that CMV CP alone possess the virulence to induce chlorosis and chloroplast abnormalities in CMV-infected tobacco plants (Mochizuki and Ohki, 2011; Mochizuki et al., 2014b).

Viral CPs could also impose virulent effects from outside of the chloroplasts. A series of CP deletion mutants of TMV (Lindbeck et al., 1991) and ToMV spontaneous mutant ToMV-L_{11}Y (Ohnishi et al., 2009) causes severe chlorosis associated with severe deformation and disruption of chloroplasts and the mutant CPs are shown to contribute to this severe chlorosis (Lindbeck et al., 1991; Ohnishi et al., 2009). Because the mutant CPs aggregate outside of chloroplasts, they may subvert the chloroplast development and cause the degradation of

TABLE 2 | Structural changes of chloroplasts induced by virus infection.

Plant Virus*	Chloroplast Abnormality	Plant Host	Virus Factor	References
ssRNA POSITIVE-STRAND VIRUSES				
Potexvirus/Alphaflexiviridae				
Potato virus X (PVX)	Invaginations of cytoplasm into chloroplast	*Datura stramonium*, *Solanum tuberosum*	Virus particle, Virus inclusion	Kozar and Sheludko, 1969
	Dilated granal lamella, enlarged stromal areas, thylakoid vesicles	*Nicotiana benthamiana*	CP	Qiao et al., 2009
Alternanthera mosaic virus (AltMV)	Vesicular invaginations	*Nicotiana benthamiana*	Viral RNA, TGB3	Lim et al., 2010
Carlavirus/Betaflexiviridae				
Potato virus S (PVS)	Cytoplasm invagination	*Chenopodium quinoa*	Virion	Garg and Hegde, 2000
Cucumovirus/Bromoviridae				
Cucumber mosaic virus isolate 16 (CMV-16)	Reduction in chloroplast number and size, completely destroyed chloroplasts and disorganized grana scattering into the cytoplasm	*Lycopersicon esculentum*	–	Montasser and Al-Ajmy, 2015
CMV P6 strain (CMV-P6)	Tiny chloroplast with fewer grana, myelin-like chloroplast-related structures	*Nicotiana tabacum*	–	Roberts and Wood, 1982
CMV Malaysian isolate	Disorganized thylakoid system, crystallization of phytoferritin macro molecules and, large starch grains	*Catharanthus roseus*	–	Mazidah et al., 2012
CMV pepo strain with CP_{129} substitutions	Few thylakoid membranes, no granum stacks, abnormal-shaped and hyper-accumulated starch grains	*Nicotiana tabacum*	–	Mochizuki and Ohki, 2011
CMV pepo strain VSR deficient mutant with CP_{129} substitutions	Fewer thylakoid membranes and granum stacks	*Nicotiana tabacum*	–	Mochizuki et al., 2014b
Polerovirus/Luteoviridae				
Beet western yellows virus (BWYV)	Disappearance of grana stacks, stroma lamellae, large starch grains, osmiophilic granules	*Lactuca sativa, Claytonia perfoliata*	–	Tomlinson and Webb, 1978
Sugarcane Yellow Leaf Virus (ScYLV)	Swollen chloroplast, rectangular grana stacks, more plastoglobules	*Saccharum spec.*	–	Yan et al., 2008
Potyvirus/Potyviridae				
Bean yellow mosaic virus (BYMV)	Increased stromal area, swollen chloroplast, loss of envelopes, dilated thylakoids, decreased chloroplast number	*Vicia faba*	–	Radwan et al., 2008
Maize dwarf mosaic virus strain A (MDMV-A)	Small vesicles, deformation of membranes, reduction in grana stack height, disappearance of osmiophilic globules, degeneration of structures	*Sorghum bicolor*	–	Choi, 1996
MDMV Shandong isolate (MDMV-SD)	Thylakoid swelling, envelope broking	*Zea mays*	–	Guo et al., 2004
Plum pox virus (PPV)	Dilated thylakoid, increase in the number and size of plastoglobuli, decreased amount of starch in chloroplasts from palisade parenchyma	*Prunus persica* L.	–	Hernández et al., 2006
	Dilated thylakoids, increased number of plastoglobuli, peculiar membrane configurations	*Pisum sativum*	–	Díaz-Vivancos et al., 2008
	Lower amount of starch granules, disorganized grana structure	*Prunus persica* L.	–	Clemente-Moreno et al., 2013
Potato virus Y (PVY)	Reduced chloroplast number, smaller chloroplasts with exvaginations	*Solarium tuberosum*	–	Pompe-Novak et al., 2001
	Decrease of volume density of starch, increase of volume density of plastoglobuli	*Nicotiana tabacum*	–	Schnablová et al., 2005

(Continued)

TABLE 2 | Continued

Plant Virus*	Chloroplast Abnormality	Plant Host	Virus Factor	References
Sugarcane mosaic virus (SCMV)	Swollen chloroplast, increased number of plastoglobuli	*Sorghum bicolor*	–	El Fattah et al., 2005
Turnip mosaic Virus (TuMV)	Chloroplast aggregation, irregular shaped chloroplast, large osmiophilic granules, poorly developed lamellar system, few or no starch grains,	*Chenopodium quinoa*	Virus particle	Kitajima and Costa, 1973
Zucchini yellow mosaic virus (ZYMV)	Decrease of chloroplasts amount, decreased thylakoids, increased plasto-globule and starch grain in chloroplast	*Cucurbita pepo*	–	Zechmann et al., 2003
Fijivirus/Reoviridae				
Maize rough dwarf virus (MRDV)	Membrane disappearance, swollen grana discs, periphery vesicles	*Zea mays*	Virus particle	Gerola and Bassi, 1966
	Distorted grana and paired membranes.	*Chenopodium quinoa*	Virus particle	Martelli and Russo, 1973
Fabavirus/Secoviridae				
Broad bean wilt virus 2 (BBWV-2) isolate B935	Inhibited lamellar development, membrane vesiculation	*Vicia faba*	–	Li et al., 2006
BBWV-2 isolate PV131	Chloroplast with swollen or disintegrated membrane	*Vicia faba*	–	
Tombusvirus/Tombusviridae				
Artichoke mottled crinkle virus (AMCV)	Distorted grana and paired membranes.	*Chenopodium quinoa*	Virus particle	Martelli and Russo, 1973
Tomato bushy stunt virus (TBSV)	Large plastidial vacuole, disorganized lamellar system, multivesicular bodies originate from chloroplasts, chloroplasts clustered around a group of multivesicular bodies	*Gomphrena globosa*	Virus particle	Appiano et al., 1978
	Large inter-membranous sac, rearrangement of the thylakoids	*Datura stramonium*	–	Bassi et al., 1985
Unassigned/*Tombusviridae*				
Maize necrotic streak virus (MNeSV)	Chloroplast swollen, out membrane invagination	*Zea mays*	–	De Stradis et al., 2005
Tymovirus/Tymoviridae				
Melon rugose mosaic virus (MRMV)	Peripheral vesicles, tendency to aggregate	*Cucumis melo*	–	Mahgoub et al., 1997
Turnip yellow mosaic virus (TYMV)	Peripheral vesicles, reduction of grana number, chlorophyll content; increases in amounts of phytoferritin and numbers of osmiophilic globules	*Brassica rapa*	Viron, Viral RNA	Ushiyama and Matthews, 1970; Hatta and Matthews, 1974
Belladonna mottle virus physalis mottle strain (BeMV-PMV)	Vesicles develop in chloroplasts, vesiculations of the outer membranes	*Datura stramonium*	Viron	Moline, 1973
Wild cucumber mosaic virus (WCMV)	Double membrane vesicles in chloroplasts, single membrane vesicles surrounding chloroplasts	*Marah oreganus*	Virus particle	Allen, 1972
Hordeivirus/Virgaviridae				
Barley stripe mosaic virus (BSMV)	Surrounded chloroplasts, cytoplasmic invaginations into chloroplasts, aggregated chloroplasts, rearrangement of the thylakoids, electron transparent vacuoles in stroma	*Hordeum vulgare*	Viron	Carroll, 1970; Zarzyńska-Nowak et al., 2015
	Peripheral vesicles; Type1: elongated grana or anastomosed lamellae, composed of pellucid stroma, twisted or convoluted membranes forming tubular networks; Type2: swollen and contained disarranged internal membranes; Type3: electron dense stroma, cytoplasmic invaginations.	*Datura stramonium*	Genomic ssRNA	McMullen et al., 1978

(Continued)

TABLE 2 | Continued

Plant Virus*	Chloroplast Abnormality	Plant Host	Virus Factor	References
	Rounded and clustered chloroplasts, cytoplasmic invaginations and inclusions at the periphery	*Nicotiana benthamiana*	TGB2, CP, γb, virus-like particle	Torrance et al., 2006
Pomovirus/Virgaviridae				
Potato mop-top virus (PMTV)	Large starch grains, large cytoplasmic inclusion, terminal extension,	*Nicotianabenthamiana*	Genomic RNA, CP, TGB2	Cowan et al., 2012
Tobamovirus/Virgaviridae				
Ribgrass mosaic virus (RMV)	Disappearance of stroma, decrease in grana lamella, Large starch grains, osmiophilic granules	*Nicotiana tabacum*	–	Xu and Feng, 1998
Tobacco mosaic virus (TMV)	Aggregates and vecuoles in chloroplast	*Lycopersicon esculentum*		Shalla, 1964
	Enlarged plastids, supergranal thylakoids, large accumulations of osmiophilic bodies	*Lycopersicon esculentum*	–	Arnott et al., 1969
	Disappearance of stroma, decrease in grana lamella, large starch grains, osmiophilic granules	*Nicotiana tabacum*	CP	Xu and Feng, 1998
	Swelling, more osmophilic plastoglobuli, loosened thylakoid structure	*Capsicuum anuum*	–	Mel'nichuk et al., 2002
TMV U5 strain	Peripheral vesicles	*Nicotiana tabacum*	Virus particle	Betto et al., 1972
TMV yellow strain	Filled with osmiophilic globules, rearranged, swollen or eliminated lamellar system, extensive chloroplast degradation	*Solanum tuberosum*	–	Liu and Boyle, 1972
TMV *flavum* strain (TMV-Flavum)	Swollen or globular chloroplast, distorted thylakoid membranes, grana depletion, unidentified granular matter	*Nicotiana tabacum*	MP, CP	Lehto et al., 2003
Tomato mosaic Virus (ToMV)	Slightly swollen and distorted cholroplast, large starch grains	*Nicotiana tabacum*	Virus particle	Ohnishi et al., 2009
ToMV L_{11}Y strain (ToMV-L_{11}Y)	Flaccid chloroplast, reduced thylakoid stacks and enlarged spaces between the stacks, cytoplasm penetrates into chloroplast, tubular complexes	*Nicotiana tabacum*	–	Ohnishi et al., 2009
ssRNA NEGATIVE STRAND VIRUSES				
Tospovirus/Bunyaviridae				
Tomato spotted wilt virus (TSWV)	Peripheral vesicles	*Nicotiana tabacum*	–	Mohamed, 1973
***Tenuivirus*/Unassigned**				
Rice stripe virus (RSV)	Reduced sheets of grana stacks, increased amount and size of starch granules	*Oryza Sativa*	Virus particle	Zhao et al., 2016
	Membrane proliferations	*Nicotiana benthamiana*	NSvc4	
ssDNA VIRUSES				
Begomovirus/Geminiviridae				
Abutilon Mosaic Virus (AbMV)	Disorganization of thylakoid system, grana-stroma elimination	*Abutilon spec*	–	Schuchalter-Eicke and Jeske, 1983
	Degenerated thylakoids, more plastoglobuli, less starch, and accumulation of amorphous electron-dense material	*Abutilon selovianum*	Genomic DNA	Gröning et al., 1987
	Generation of stromules	*Nicotiana benthamiana*	MP	Krenz et al., 2012

**Virus taxonomy is in format of Genus/Family. –Not addressed. ssRNA, single-stranded RNA; ssDNA, single-stranded DNA.*

chloroplasts by interfering with the synthesis and transport of CPRPs (Lindbeck et al., 1991, 1992; Ohnishi et al., 2009).

Besides CPs, other viral components are also able to cause chloroplast malformation and contribute to symptom. For example, transgenic expression of CaMV transactivator/viroplasmin (Tav) protein in tobacco plants results in a virus-like chlorosis symptom associated with the abnormal thylakoid stacks (**Figure 1**, **Table 2**) and reduces expression of CPRGs (Waliullah et al., 2014). The potexvirus AltMV TGB3, different from its counterpart

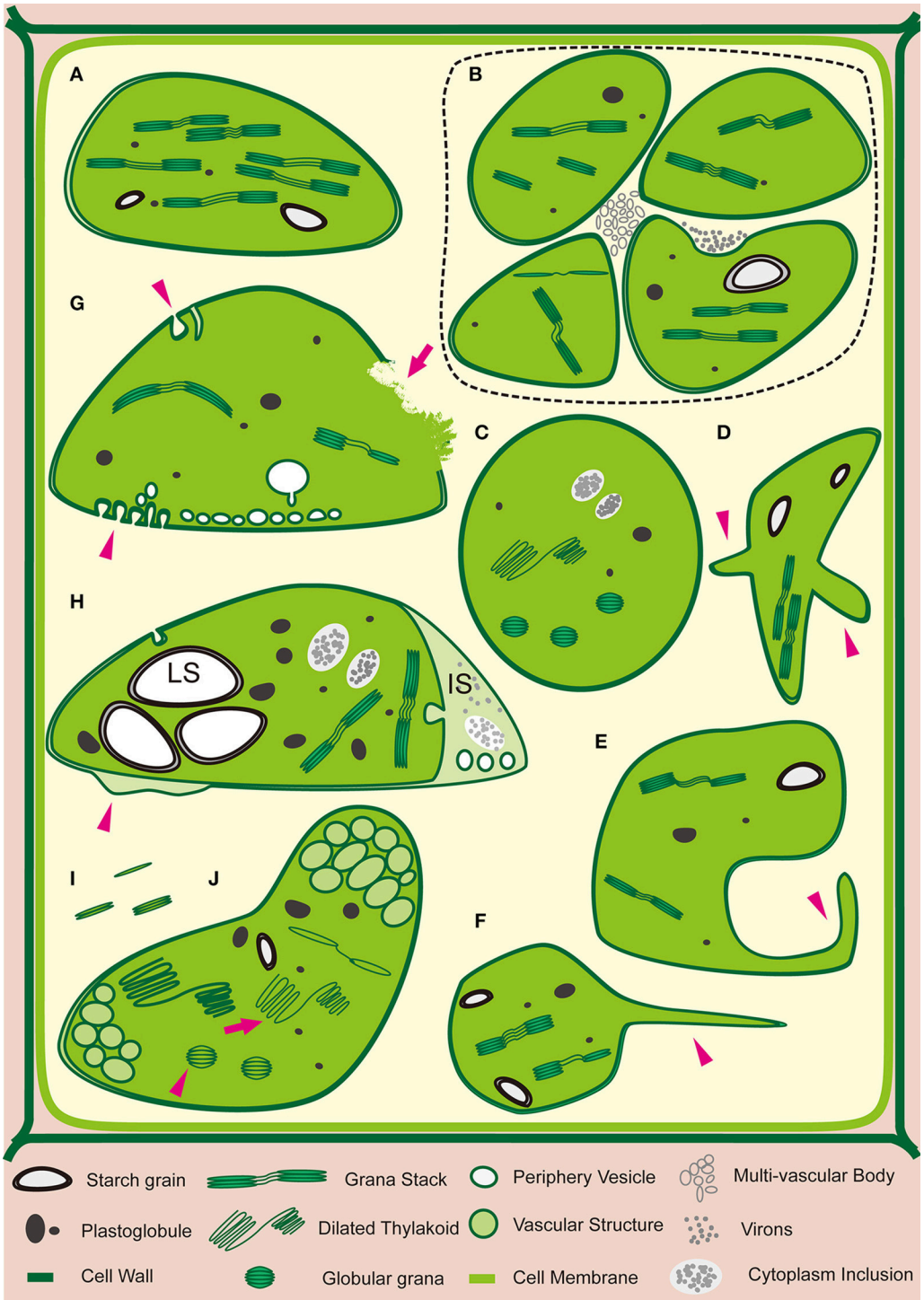

FIGURE 1 | Changes in the Ultrastructure of Chloroplasts Induced by Virus Infection. (A) Normal chloroplast. **(B)** Aggregated chloroplasts (surrounded with dotted line). **(C)** Swollen chloroplast. **(D)** Chloroplast with membrane-bound extrusions. Arrow heads indicate membrane extrusions. **(E)** Amoeboid-shaped chloroplast, arrow head indicates chloroplast membrane extrusions. **(F)** Chloroplast with stromule, arrow head indicates the stromule. **(G)** Chloroplast with irregular out-membrane structures such as peripheral vesicle, cytoplasmic invagination, membrane proliferations and broken envelope. Arrow heads indicates cytoplasmic invaginations, arrow indicates broken envelope of chloroplast. **(H)** Chloroplast with abnormal content changes such as small vesicles, membrane proliferations (arrow head) and inter-membranous sac (IS), large starch grain (LS) and exaggeration of plastoglobules. **(I)** Disorganized grana scattering into the cytoplasm. **(J)** Chloroplast with unusual photosynthetic structures such as dilated thylakoid (arrow) and globular grana (arrow head) and vascular structures.

PVX TGB3, has a chloroplast-targeting signal and preferentially accumulates around the chloroplast membrane (Lim et al., 2010). Overexpression of AltMV TGB3 causes vesiculation at the chloroplast membrane (**Figure 1**, **Table 2**) and veinal necrosis symptom (Lim et al., 2010; Jang et al., 2013). AltMV TGB3 strongly interacts with PS II oxygen-evolving complex protein PsbO and this interaction is believed to have a crucial role in viral symptom development and chloroplast disruption (Jang et al., 2013). In PVY-infected cells, viral multifunctional protein HC-Pro may contribute to the change in the number and size of chloroplast by interfering with the normal activity of the chloroplast division-related factor MinD through direct protein interaction (Jin et al., 2007, **Table 1**). The tenuivirus RSV NSvc4 protein functions as an intercellular movement protein and is localized to PD as well as chloroplast in infected cells. Over-expression of NSvc4 exacerbated malformations of chloroplast (**Figure 1**, **Table 2**) and disease symptoms. Interestingly, the chloroplast localization of NSvc4 is dispensable for the symptom determination while the NSvc4 transmembrane domain probably affects the chloroplast from outside (Xu and Zhou, 2012).

Effect of Virus Infection on Expression of Chloroplast-Targeted Proteins

Studies on the effect of virus infection on expression of chloroplast proteins at the transcriptomic and proteomic levels provide insights into the molecular events during symptom expression. In the susceptible plant response to virus infection, the majority of significantly changed proteins are identified to be located in chloroplasts or associated with chloroplast membranes. Most of them are down-regulated and correlate with the severity of chlorosis (Dardick, 2007; Shimizu et al., 2007; Lu et al., 2012; Rodríguez et al., 2012; Kundu et al., 2013; Wu et al., 2013; Mochizuki et al., 2014a). During virus infection, CPRPs represent the most common viral targets. Among them, the light harvesting antenna complex (Naidu et al., 1984a,b, 1986; Liu et al., 2014) and the oxygen evolving complex (OEC) (Takahashi et al., 1991; Takahashi and Ehara, 1992; Pérez-Bueno et al., 2004; Sui et al., 2006; Wang et al., 2015) of PS II are in thylakoid, while RbCS and RubisCO activase (RCA, an AAA-ATPase family protein) are in chloroplast stroma (Díaz-Vivancos et al., 2008; Pineda et al., 2010; Moshe et al., 2012; Kundu et al., 2013).

As the biosynthesis of CPRPs is a complicated process with a series of steps (Seidler, 1996), plant virus can affect CPRPs at varied levels including transcription, post-transcription, translation, transportation into the chloroplast, assembly and degradation in chloroplast, to contribute to symptom development (Lehto et al., 2003; Pérez-Bueno et al., 2004).

Several plant viruses perturb CPRPs expression at transcription level either in chloroplast or via retrograde signaling into nucleus. Infection of TMV *flavum* strain leads to a total depletion of PS II core complex and OEC, including chloroplast-encoded CPRP PsbA and nuclear-encoded CPRPs LhcB1, LhcB2 (light-harvesting chlorophyll a/b-binding protein B1, B2) and PsbO. However, the *PsbA* mRNA accumulated to a higher level in the infected leaves (Lehto et al., 2003). Thus, TMV *flavum* may block PsbA translation via reducing the level of chloroplast ribosomal RNA (Fraser, 1969) and inhibit the transcription of nuclear-encoded CPRGs through feed-back signaling (Lehto et al., 2003). Similarly, in the case of CMV pepo strain and its CP_{129} mutant isolates, the down-regulation patterns of transcription levels of different CPRGs correlated with the amino acid substitution in the CP protein of the relative isolates, where CMV CP probably repress the transcription of CPRGs via the retrograde signaling from chloroplast into nucleus (Mochizuki et al., 2014a).

It is interesting that plant virus can also exploit host RNA silencing machinery to manipulate CPRGs at post-transcription level. The enlightening evidence is illustrated by CMV-Y satellite (CMV-Y-sat) RNA which can disturb chloroplast function and induce disease symptoms (Shimura et al., 2011; Smith et al., 2011). A 22-nt siRNA derived from CMV-Y-sat RNA targets the *magnesium protoporphyrin chelatase subunit I* (*ChlI*) gene transcripts and down-regulates its expression by RNA silencing (**Table 1**), which leads to a more sever symptom characterized as bright yellow mosaic (Takanami, 1981; Shimura et al., 2011; Smith et al., 2011). In addition, infection by viroids (small non-protein-coding RNAs) results in the production of viroid-derived small RNAs (vd-sRNAs) (Papaefthimiou et al., 2001; Martínez de Alba et al., 2002). Peach latent mosaic viroid (PLMVd) belongs to family *Avsunviroidae* whose members replicate in chloroplast, and may elicit an albino-variegated phenotype (peach calico, PC) with blocked chloroplast development and depletion of chloroplast-encoded proteins (Rodio et al., 2007). The PLMVd variants associated with PC contain an insertion of 12–14 nt that folds into a hairpin with a U-rich tetraloop, the sequence of which is critical for inciting the albino phenotype.. Actually, vd-sRNAs from the hairpin insertion induce cleavage of the mRNA encoding the CPRP chloroplastic heat-shock protein 90 (cHSP90) as predicted by RNA silencing, eventually resulting in PC symptoms (Navarro et al., 2012).

In addition to the virus-derived small RNAs, plant viruses may also modify host microRNA (miRNA) pathway for targeting CPRGs transcripts. The tenuivirus RSV, causing a devastating disease in East Asia countries, hijacks CPRP during infection and perturbs photosynthesis (Satoh et al., 2010; Shi et al., 2016). The perturbation of photosynthesis by RSV is probably caused by up-regulating a special miRNA that targets key genes in chloroplast zeaxanthin cycle, which impairs chloroplast structure and function (Yang et al., 2016).

Viral factors may reduce the level of CPRPs by direct association with target proteins. Tobamoviruses CPs particularly associate with the PS II complex and reduce the levels of PsbP and PsbQ (Hodgson et al., 1989; Pérez-Bueno et al., 2004; Sui et al., 2006). PVY HC-Pro can reduce the amount of ATP synthase complex by interaction with the NtCF1β-subunit in both the PVY-infected (**Table 1**) and the HC-Pro transgenic tobacco plants, leading to a decreased photosynthetic rate (Tu et al., 2015). Potyviruses TuMV, SMV, SYSV, and OYDV may hijack RbCS and/or RbCL via the interaction with P3 or P3N-PIPO during infection to perturb photosynthetic activity (Lin et al., 2011). Potyvirus SCMV infection significantly down-regulates mRNA level of photosynthetic Fd V rather than that of the other

isoproteins (Fd I and Fd II) in maize, while SCMV HC-Pro specifically interacts with the chloroplast precursor of Fd V via TP in cytoplasm outside the chloroplasts (**Table 1**), suggesting that SCMV HC-Pro perturbs the importing of Fd V into chloroplasts and leads to structure and function disturbance of chloroplast (Cheng et al., 2008). Potyvirus SMV P1 (a serine protease) strongly interacts with host plant-derived, but only weakly with non-host *Arabidopsis*-derived, Rieske Fe/S protein of cytochrome b6/f complex, an indispensable component of the photosynthetic electron transport chain in chloroplasts (**Table 1**), suggesting that SMV P1-Rieske Fe/S protein interaction is involved in symptom development (Shi et al., 2007). RSV disease specific protein (SP) is a symptom determinant protein and its overexpression enhances RSV symptom (Kong et al., 2014). During RSV infection, accumulation of SP is associated with alteration in structure and function of chloroplast. SP interacts with 23-kD OEC PsbP, and relocates PsbP from chloroplast into cytoplasm (**Table 1**), while silencing of *PsbP* enhances disease symptom severity and virus accumulation (Kong et al., 2014).

CHLOROPLAST IS INVOLVED IN THE PROCESS OF THE PLANT VIRUS LIFE CYCLE

Increasing studies have unraveled that chloroplast constituents participate in different stages during virus infection. For example, chloroplast is reported to be associated with viral uncoating, an important step of replication (Xiang et al., 2006). Tombusvirus CNV CP harbors an arm region of 38 amino acids that functions as a chloroplast TP to direct CP import to the chloroplast stroma, which is critical for viral disassembly. CNV CP mutant deficient in exposure of the arm region is inefficient to establish infection, highlighting the crucial role of chloroplast targeting in CNV uncoating (Xiang et al., 2006).

Chloroplast and Its Factors Participate in Virus Replication

Chloroplast affords compartment and membrane contents for the replication of plant viruses and probably helps them to evade the RNA-mediated defense response (Ahlquist et al., 2003; Dreher, 2004; Torrance et al., 2006). Plant viruses propagate via RNA-protein complex named viral replication complexes (VRCs), which are the factory for producing progeny viruses (Más and Beachy, 1998, 2000; Asurmendi et al., 2004). During replication of RNA viruses, double-strand RNA (dsRNA) is generated as an intermediate product. As a response against virus infection, the dsRNA replication intermediates can be detected by the host RNA silencing machinery (Angell and Baulcombe, 1997; Baulcombe, 1999). Correspondingly, plant viruses have evolved some mechanisms by encoding viral suppressor of RNA silencing or by associating replication with host membranes (Ahlquist, 2002; Ahlquist et al., 2003). For a large group of viruses, VRCs are associated with the chloroplast envelope, particularly the peripheral vesicles and cytoplasmic invaginations in chloroplast (**Figure 1**, **Table 2**), including alfamovirus AMV (de Graaff et al., 1993), hordeivirus BSMV (Carroll, 1970; Torrance et al., 2006), potyviruses MDMV (Mayhew and Ford, 1974), PPV (Martin et al., 1995), TEV (Gadh and Hari, 1986), TuMV (Kitajima and Costa, 1973), and tymovirus TYMV (Lafleche et al., 1972; Bové and Bové, 1985; Garnier et al., 1986; Lesemann, 1991; Dreher, 2004). The chloroplast membrane associated organization probably helps to shield viral RNAs from recognition by host RNA silencing machinery (Dreher, 2004).

Viral factors, either viral genomic RNAs or proteins, can mediate the chloroplast targeting of VRCs for replication and subsequent virion assembly (Prod'homme et al., 2003; Jakubiec et al., 2004; Torrance et al., 2006). BSMV replicative dsRNA intermediates exist in the chloroplast peripheral vesicles during infection (McMullen et al., 1978; Lin and Langenberg, 1984, 1985; Torrance et al., 2006); in the presence of the viral genome RNA, both TGB2 and γb can be recruited to chloroplasts for virus replication (Torrance et al., 2006). The low pH condition of chloroplast vesicles where TYMV RNA is synthesized is required for the interaction between viral RNA and CP to process virion assembly (Rohozinski and Hancock, 1996). The TYMV VRC-associated membrane vesicles localize at the chloroplast envelope (Prod'homme et al., 2001). TYMV N-terminal replication protein (140 K) is a key organizer of TYMV VRCs assembly and a major determinant for chloroplast localization of TYMV for replication. The 140 K protein can localize to the chloroplast envelope autonomously and interacts with the C-terminal replication protein (66 K) to mediate the targeting of 66 K to the chloroplast envelope (Prod'homme et al., 2003; Jakubiec et al., 2004). TuMV 6 K protein (6 K or 6 K2) can autonomously allocate to chloroplast membrane and promote the adhesion of the adjacent chloroplasts via actomyosin motility system in infected host cells. During the infection, TuMV 6 K induces the formation of 6 K-containing membranous vesicles at endoplasmic reticulum exit sites and sequentially traffic to chloroplast, while the chloroplast-bounded 6 K-vesicles are recruited to VRCs containing viral dsRNA (Wei et al., 2010), supporting the idea that the chloroplast-bound 6 K vesicles are the cellular compartment for TuMV replication. Blocking the fusion of virus-induced vesicles with chloroplasts by the inhibition of SNARE protein Syp71 significantly reduced the viral infection (Wei et al., 2013).

Special chloroplast components are involved in the targeting of VRCs to chloroplast. The lipid in chloroplast membrane can associate with pomovirus PMTV TGB2 (**Table 1**) and facilitate the viral RNA to localize to chloroplast membranes for replication (Cowan et al., 2012). Furthermore, chloroplast factors also participate in the formation of VRCs. Proteomic analysis suggests that sobemovirus RYMV recruits CPRPs such as Ferredoxin-NADP reductase (FNR), RbCS, RCA, and chaperonin 60 to its VRCs during all the infectious stages including replication, long-distance trafficking and symptoms development (Brizard et al., 2006). The 43 kD CPRP chloroplast phosphoglycerate kinase (cPGK) specifically interacts with 3′-UTR of the potexvirus BaMV genomic RNA (Lin et al., 2007, **Table 1**). Silencing of *Nb-cPGK* or mislocalization of cPGK protein reduced BaMV accumulation, suggesting that cPGK may mediate BaMV RNA targeting to chloroplast for replication (Cheng et al., 2013). Interestingly, in *Arabidopsis* genotype Cvi-0

the natural recessive resistance gene *rwm1* against potyvirus WMV encodes a mutated version of cPGK (Ouibrahim et al., 2014), illuminating that the conserved CPRP cPGK may be required for successful replication and infection of a range of plant viruses (Lin et al., 2007; Ouibrahim et al., 2014).

Chloroplast Factors Participate in Viral Movement

The intercellular trafficking and systemic spreading of plant virus need movement proteins (MPs) to fulfill the transport via symplastic routes within plant hosts (Wolf et al., 1989; Ding et al., 1992; Imlau et al., 1999; Lazarowitz and Beachy, 1999). To facilitate virus movement, varied MPs possess common features such as nucleic acid binding activity (Citovsky et al., 1990), specific plasmodesmata (PD) localization (Ding et al., 1992; Fujiwara et al., 1993) and the ability to increase the size exclusion limit of PD (Wolf et al., 1989).

Chloroplast and its factors also participate in virus movement. AltMV TGB3 has a chloroplast-targeted signal and accumulates preferentially in mesophyll cells, which is essential for virus movement. Mutation of the chloroplast-targeted signal in AltMV TGB3 impairs virus movement from epidermal into the mesophyll cells as well as viral long-distance traffic (Lim et al., 2010). Geminivirus AbMV MP interacts with chloroplast-targeted 70-kD heat shock protein (cpHSC70-1) and co-localized to chloroplasts (**Table 1**). Silencing of *cpHSC70*-1 affects chloroplast stability and causes a substantial reduction of AbMV movement but has no effect on viral DNA accumulation (Krenz et al., 2010, 2012). AbMV can replicate in chloroplast (Gröning et al., 1987, 1990) and induce the biogenesis of stromule network (**Figure 1**, **Table 2**). AbMV may use cpHSC70-1 for trafficking along chloroplast stromules into a neighboring cell or from plastids into the nucleus (Krenz et al., 2012).

Viral factors can interact with and hijack chloroplast factors from their normal function and to help viral movement. The CaMV multifunctional P6 protein is the most abundant present in VRCs (Hohn et al., 1997) and associates with PD (Rodriguez et al., 2014). Interestingly, CaMV P6 also interacts with the chloroplast unusual positioning protein1 (CHUP1) (**Table 1**) that is a thylakoid membrane-associated protein for mediating the routine movement of chloroplast on microfilaments in response to light intensity (Oikawa et al., 2003, 2008). Silencing of *CHUP1* slows the formation rate of CaMV local lesion (Angel et al., 2013). Thus, the CaMV P6 protein may mediate the intracellular movement of VRCs to the PD by binding to CHUP1 (Angel et al., 2013). Tobamoviruses ToMV and TMV MPs bind RbCS (**Table 1**) and the interaction occurs at PD (Zhao et al., 2013). Silencing of *RbCS* reduced intercellular movement and systemic trafficking of TMV and ToMV (Zhao et al., 2013). Thus, it may be a common strategy for tobamoviruses to hijack RbCS for efficient movement. In addition to MPs, tobamoviruses need their CPs for efficient long distance movement (Wisniewski et al., 1990; Reimann-Philipp and Beachy, 1993; Ryabov et al., 1999). ToMV CP-interacting protein-L (IP-L) is a chloroplast protein (**Table 1**) and is positively induced by ToMV infection (Zhang et al., 2008). Depletion of *IP-L* delayed ToMV systemic movement and symptoms (Li et al., 2005). Dianthovirus RCNMV MP interacts with chloroplast protein glyceraldehyde 3-phosphate dehydrogenase subunit A (GAPDH-A) (**Table 1**), while silencing of *GAPDH-A* inhibits viral MP localization to the cortical VRCs and reduces RCNMV multiplication in the inoculated leaves (Kaido et al., 2014). Therefore, GAPDH-A is relocated from chloroplast to cortical VRCs to facilitate viral cell-to-cell movement during RCNMV infection.

Based on the current studies, it is clear that plant viruses have evolved to utilize abundant chloroplast proteins to regulate their movement.

CHLOROPLASTS AFFECT PLANT DEFENSE AGAINST VIRUSES

Several hormones regulate plant defense to viruses (Alazem and Lin, 2015). Two of them are salicylic acid (SA) and jasmonic acid (JA). Chloroplast is the crucial site for the biosynthesis of SA (Boatwright and Pajerowska-Mukhtar, 2013; Seyfferth and Tsuda, 2014) and JA (Wasternack, 2007; Schaller and Stintzi, 2009; Wasternack and Hause, 2013). Moreover, chloroplast factors are also involved in the regulation of antagonistic interactions of SA-JA synthesis and signaling (Kunkel and Brooks, 2002; Xiao et al., 2012; Zheng et al., 2012; Lemos et al., 2016). The chloroplast-related regulation of SA and JA biosynthesis is schemed in **Figure 2**.

SA is a small phenolic compound that plays central roles in plant defense against biotrophic pathogens and is essential for the establishment of local and systemic acquired resistance. The majority of pathogen-induced SA is synthesized via the isochorismate pathway in chloroplasts (Boatwright and Pajerowska-Mukhtar, 2013; Seyfferth and Tsuda, 2014). As a key activator of plant defense response, SA biosynthesis and signaling are activated during incompatible plant-virus interaction (Wildermuth et al., 2001; Garcion et al., 2008). Disruption of SA pathway compromises plant resistance against viruses (Alazem and Lin, 2015). In contrast, the application of SA or its analogs often delays the onset of viral infection and disease establishment by improving plant basal immunity (Radwan et al., 2006, 2007, 2008; Falcioni et al., 2014). A chloroplast-localized protein, named calcium-sensing receptor, is found to act upstream of SA accumulation to link chloroplasts to cytoplasmic-nuclear immune responses (Nomura et al., 2012).

JA is an oxylipin, or oxygenated fatty acid and is synthesized from linolenic acid by the octadecanoid pathway, whose biosynthesis starts with the conversion of linolenic acid to 12-oxo-phytodienoic acid (OPDA) in the chloroplast membranes (Turner et al., 2002). JA is thought to play a positive defense role in compatible plant-virus interactions (Alazem and Lin, 2015). For example, silencing of *Coronatine insensitive 1* (*COI1*), a gene involved in the JA signaling pathway, accelerates the development of symptoms caused by co-infection of PVX and PVY, and accumulation of viral titers at early stages of infection (García-Marcos et al., 2013).

The chloroplasts are major sites of the production of reactive oxygen species (ROS), and the photosynthetic electron

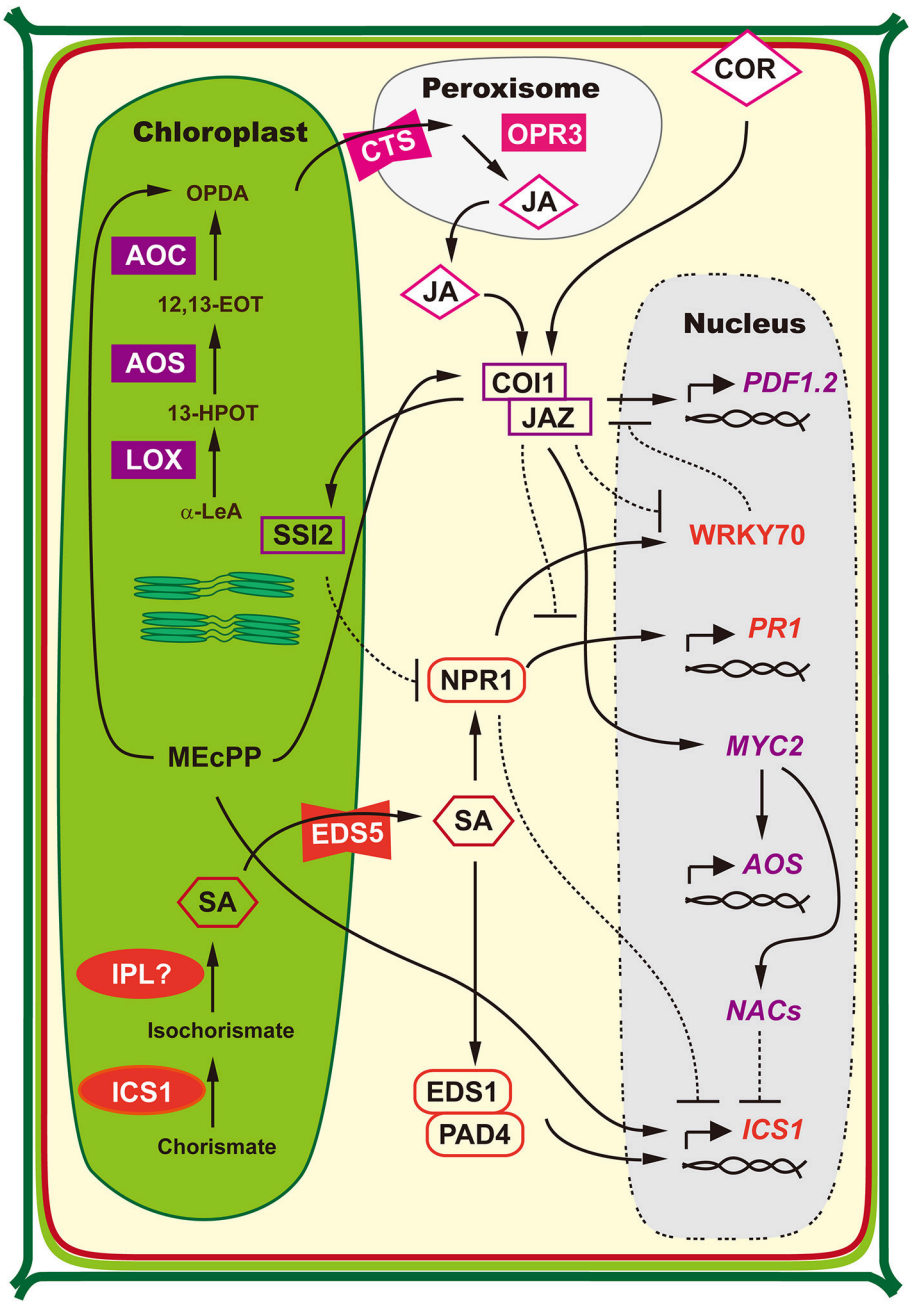

FIGURE 2 | Regulation of SA and JA Biosynthesis is Associated with Chloroplast. SA biosynthesis is predominantly accomplished by nucleus-encoded chloroplast-located isochorismate synthase (ICS1). In chloroplasts, ICS catalyzes the conversion of chorismate into isochorismate, which is further converted to SA by undetermined isochorismate pyruvate lyase (IPL). The MATE-transporter ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) is responsible for SA transportation from chloroplast into cytosol. Defense-elicited ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) complex works in a positive feedback loop to control SA synthesis, which is regulated by SA. While in a negative feedback loop, accumulation of ICS1-produced SA results in the deoligomerization of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1), which is then translocated into nucleus where it suppresses the *ICS1* expression (modified from Boatwright and Pajerowska-Mukhtar, 2013; Seyfferth and Tsuda, 2014). JA biosynthesis originates from polyunsaturated fatty acids released from chloroplast membranes. Firstly, α-linolenic acid (18:3) (α-LeA) is catalyzed by lipoxygenase (LOX) to yield the 13-hydroperoxy derivative 13(S)-hydroperoxy-octadecatrienoic acid (13-HPOT). The dehydration of 13-HPOT by allene oxide synthase (AOS) results in the formation of unstable 12, 13(S)-epoxy-octadecatrienoic acid (12,13-EOT), which is the committed step of JA biosynthesis. Then the 12,13-EOT is converted to 12-oxophytodienoic acid (OPDA) by allene oxide cyclase (AOC) through cyclization and concludes the chloroplast-localized part of JA biosynthesis. Subsequently, OPDA is released from chloroplasts and taken up into peroxisomes by transporter COMATOSE (CTS3). The remaining steps are located in peroxisomes and JA is generated through reduction of the cyclopentenone by OPDA reductase 3 (OPR3) and subsequent three cycles of β-oxidation for side-chain shortening. The JA co-receptor complex of

(Continued)

FIGURE 2 | Continued
CORONATINE INSENSITIVE1 (COI1) and the negative regulator JAZMONATE ZIM DOMAIN (JAZ) proteins regulates the positive feedback loop of JA biosynthesis. Formation of JA subjects JAZ to proteasomal degradation, which allows MYC2 to activate the JA biosynthesis genes such as AOS, AOC, and LOX (modified from Wasternack, 2007; Schaller and Stintzi, 2009; Wasternack and Hause, 2013). NPR1 is the central transcriptional regulator of SA-mediated defense responses and directly regulates *PATHOGENESIS-RELATED 1* (*PR1*) expression (Wang et al., 2006). By wounding or JA treatment, COI1–JAZ co-receptor promotes the degradation of JAZ and release the positively acting transcription factors that binds to JA-responsive promoters to initiate the transcription of JA-responsive genes, such as *PLANT DEFENSIN1.2* (*PDF1.2*) (Chini et al., 2007; Thines et al., 2007; Yan et al., 2009). During the antagonistic interplay between SA and JA, NPR1 suppresses COI1-JAZ mediated induction of JA-responsive genes via WRKY transcription factors, while JA also represses WRKY in COI1-dependent pathway (Li et al., 2004; Gao et al., 2011). On the other hand, the JA signaling proteins, such as chloroplast factor SUPPRESSOR OF SA INSENSITIVITY 2 (SSI2), negatively regulate SA-mediated NPR1-dependent defense responses (Kunkel and Brooks, 2002). Further, the phytotoxin coronatine (COR), a molecular mimic of JA, activates NAC transcription factors via COI1-JAZ and MYC2, which eventually inhibits SA accumulation through repressing *ICS1* expression (Zheng et al., 2012). In addition, the stress-induced methylerythritol cyclodiphosphate (MEcPP) acts as a plastid-to-nucleus retrograde signal to increase the transcription level of *ICS1* (Xiao et al., 2012). Meanwhile, MEcPP increase the level of JA precursor OPDA and induce JA-responsive genes via a COI1-dependent manner in the presence of high SA (Lemos et al., 2016). Solid lines with arrow head represent activation or promotion, dotted lines with bar head to represent deactivation or inhibition.

transport chain is responsible for ROS generation (Asada, 2006; Muhlenbock et al., 2008). Superoxide anion (O_2^-) is the primary reduced product of O_2 photoreduction and its disproportionation produces H_2O_2 in chloroplast thylakoids (Asada, 2006; Muhlenbock et al., 2008). The burst of intracellular ROS can be detected during virus infection in both incompatible and compatible interactions (Allan et al., 2001; Hakmaoui et al., 2012). Chloroplast-sourced ROS are essential for hypersensitive response (HR) induced by incompatible defensive response (Torres et al., 2006; Zurbriggen et al., 2010).

The stromules could function to facilitate the magnification and transport of defensive signals into the nucleus. Interestingly, the stromules can be induced during *N*-mediated TMV resistance response. Further, a number of stromules surround nuclei during plant defense response, which is correlated with the accumulation of chloroplast-localized defense protein NRIP1 and H_2O_2 in the nucleus. In the absence of virus infection, suppression of chloroplast *CHUP1* induces stromules and enhances programmed cell death constitutively (Caplan et al., 2015; Gu and Dong, 2015). In addition, the ultrastructural changes in chloroplast can also be a part of resistant response. For examples, during the hypersensitive reaction of *N*-mediated TMV resistance, the chloroplasts swelled and the membrane burst before tonoplast ruptured (da Graça and Martin, 1975). During the course of lesion development caused by the nepovirus TRSV, the changes in chloroplast ultrastructure (rounding of chloroplasts) enlighten that chloroplast disturbance could reflect plant-virus incompatible responses (White and Sehgal, 1993). The ultrastructure aberrations of chloroplast represent the intensity of apoptotic processes in PVY^{NTN} infection (Pompe-Novak et al., 2001). Thus, the malformation of chloroplast may also indicate a defense response in compatible host-virus interaction.

Removal of the lower epidermis from cowpea and tobacco leaves inoculated with TMV or TNV resulted in reduction of local lesion numbers, indicating that the chloroplast-free epidermal cells possess an active role in virus infection (Wieringabrants, 1981). Further, chloroplast may also have a role in host defense against virus during the compatible plant-virus interaction. Previous studies found that light could influence host susceptibility to virus infection. Despite there is a report that a short burst of light after dark treatment enhances plant susceptibility to TMV infection (Helms and McIntyre, 1967), in most cases, low light and dark treatment is beneficial for viruses to establish infection and increase host's susceptibility compared to light treatment (Bawden and Roberts, 1947; Matthews, 1953; Wiltshire, 1956; Helms, 1965; Helms and McIntyre, 1967; Cheo, 1971; Manfre et al., 2011). The negative correlation between light and infectivity suggest that the robust photosynthesis and chloroplast function play a positive role in defense response during plant-virus interactions.

In compatible plant-virus interactions, some chloroplast factors are sequestrated by virus to block antiviral defense and fuel virus infection. For examples, AMV CP is essential for virus replication and encapsidation, and interacts with the chloroplast protein PsbP in the cytosol (**Table 1**), while mutations that prevent the dimerization of CP abolish this interaction (Balasubramaniam et al., 2014). Interestingly, overexpression of *PsbP* markedly reduced AMV replication in infected leaves, suggesting that there is a potential PsbP-mediated antiviral mechanism which was sequestered by CP-PsbP interaction (Balasubramaniam et al., 2014).

TMV 126-kD replicase associates with several CPRPs (**Table 1**) such as PsbO (Abbink et al., 2002), RCA and ATP-synthase γ-subunit (AtpC) (Bhat et al., 2013). Silencing of *PsbO* results in leaf chlorosis and elevated replication of several viruses including TMV, AMV, and PVX (Abbink et al., 2002). Similarly, suppression of *AtpC* and *RCA* enhances the accumulation of TMV and TVCV (Bhat et al., 2013). In addition, TMV infection specifically decreased the expression levels of *AtpC*, *RCA,* and *PsbO* (Abbink et al., 2002; Bhat et al., 2013). Further, silencing of *RbCS* enhances host susceptibility to ToMV and TMV, which is be accompanied by the reduced expression of pathogen related gene *PR-1a* (Zhao et al., 2013). These findings suggest that these CPRPs (RbCS, AtpC, RCA, and PsbO) play roles in plant defense against TMV, and TMV has evolved a strategy to suppress the defense of host plants for optimizing their own propagation.

The cylindrical inclusion (CI) protein of potyviruses is required for virus replication and cell-to-cell movement. CI protein from PPV and TVMV interacts with photosystem I PSI-K protein (**Table 1**), the product of the gene *psaK* in yeast (Jimenez et al., 2006). Overexpression of PPV CI reduces protein level of PSI-K while silencing or knockout of *psaK* enhances PPV accumulation in *N. benthamiana* and *Arabidopsis*, suggesting that

chloroplast-localized PSI-K protein could have an antiviral role (Jimenez et al., 2006).

AltMV TGB1 can bind several chloroplast factors (**Table 1**), such as light harvesting chlorophyll-protein complex I subunit A4 (LhcA4), chlorophyll a/b binding protein 1 (LHB1B2), chloroplast-localized IscA-like protein (CPISCA) and chloroplast β-ATPase (CF1β) (Seo et al., 2014). Among those chloroplast proteins, CF1β selectively binds the wild type $TGB1_{L88}$ with high RNAi suppressor activity (**Table 1**) but not the natural variant $TGB1_{P88}$ with reduced silencing suppressor activity (Seo et al., 2014). During infection with wild type AltMV, silencing of *CF1β* specifically causes severe necrosis without a significant change of viral RNAs, suggesting a direct role of *CF1β* responding to $TGB1_{L88}$ to induce defense responses (Seo et al., 2014). Taken together, the above reports indicate that the chloroplast plays an important defense role during virus invasion.

During incompatible plant-virus interactions, some chloroplast factors also participate in plant defense against viruses. For examples, in TMV resistance gene *N* containing tobacco, N receptor interacting protein 1 (NRIP1), a rhodanese sulfurtransferase which is destined to chloroplast under normal conditions, associates with both the tobacco N receptor and 126 K replicase during TMV infection; its relocation from chloroplast to cytoplasm and nucleus is required for *N*-mediated resistance to TMV (Caplan et al., 2008). Moreover, depletion of *RbCS* compromises *Tm-2*^{2} mediated extreme resistance against ToMV and TMV (Zhao et al., 2013). In addition, chloroplast-localized calcium-sensing receptor is found to be involved in stromal Ca^{2+} transients and responsible for both basal resistance and *R* gene-mediated defense (Nomura et al., 2012). These observations are consistent with the idea that chloroplasts have a critical role in plant immunity as a major site for the production for ROS, SA, and JA, important mediators of plant immunity.

Taken together, chloroplast factors participate in both basal defense and *R* gene mediated immunity against viruses.

CONCLUSIONS AND FUTURE PERSPECTIVES

The disturbance of chloroplast structure or components is often involved in symptom development and some chloroplast proteins help viruses to fulfill their infection cycle in plants. On the other hand, chloroplast factors seem to play active roles in plant defense against viruses. This is consistent with the idea that ROS, SA, and JA are produced in chloroplast (Heiber et al., 2014).

So far, some chloroplast factors involved in virus symptomology, infection cycle or antiviral defense have been identified, and their roles in virus infection have been characterized. Some findings can explain phenomena observed in early reports. However, our understanding about chloroplast-virus interaction is still quite poor. In the future, we need to identify more chloroplast factors that take part in virus infection and plant defense against viruses, to unravel their precise role and functional mechanism during plant-virus interactions, to investigate how viruses modulate expression of *CPRGs* and chloroplast-derived signaling to affect plant response to viruses, and how viral factors or defense signals traffic between chloroplast and other cellular compartments. Further progress in understanding of chloroplast-virus interactions will open new possibilities in controlling virus infection by regulating host factor's expression level.

AUTHOR CONTRIBUTIONS

JZ wrote most part of this manuscript. XZ helped to write this manuscript. YL, YH supervised, revised and complemented the writing.

FUNDING

This work was supported by the National Natural Science Foundation of China (31530059, 31470254, 31300134, 31270182, and 31370180), the National Basic Research Program of China (2014CB138400), the Special Fund for Agro-scientific Research in the Public Interest of China (201303028), and the China Postdoctoral Science Foundation (2014M550049), the Initial Funding of Zhejiang Academy of Agricultural Sciences, and the Cultural Funding for Youth Talent of Zhejiang Academy of Agricultural Sciences (2015R21R08E03).

ACKNOWLEDGMENTS

We thank Mr. Oliver Terrett at Cambridge University for help in correcting the English of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 23 January 2017
doi: 10.3389/fmicb.2017.00043

Small RNA Based Genetic Engineering for Plant Viral Resistance: Application in Crop Protection

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Small RNAs regulate a large set of gene expression in all plants and constitute a natural immunity against viruses. Small RNA based genetic engineering (SRGE) technology had been explored for crop protection against viruses for nearly 30 years. Viral resistance has been developed in diverse crops with SRGE technology and a few viral resistant crops have been approved for commercial release. In this review we summarized the efforts generating viral resistance with SRGE in different crops, analyzed the evolution of the technology, its efficacy in different crops for different viruses and its application status in different crops. The challenge and potential solution for application of SRGE in crop protection are also discussed.

Keywords: siRNA, miRNA, crop protection, viruses, vegetable, fruit, staple food, genetic engineering

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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *01 December 2016*
Accepted: *06 January 2017*
Published: *23 January 2017*

Citation:
Khalid A, Zhang Q, Yasir M and Li F (2017) Small RNA Based Genetic Engineering for Plant Viral Resistance: Application in Crop Protection. Front. Microbiol. 8:43. doi: 10.3389/fmicb.2017.00043

IMPACT OF VIRAL DISEASE ON CROP PRODUCTION

Modern plant virology commenced at the end of 19th century with the research on tobacco mosaic disease done by Russian scientist Dmittrii Iwanowski and Dutch microbiologist Martinus Beijerinck who discovered the causal agent was much smaller in size compared to other microbes because it can pass bacteria-proof filter candle (Roger, 2014). Later on, this causal agent was termed tobacco mosaic virus (TMV) and became the first virus to be defined. Since then, numerous viruses infecting bacteria, fungi, plants and animals were discovered. Currently more than 6,000 viruses were identified according to the Ninth Report of International Committee on Taxonomy of Viruses, of which about 1,300 are plant viruses (King et al., 2012; Roger, 2014).

Plant viruses impose serious threats to wide range of crops in modern agriculture and it is estimated that economic loss caused by viral pathogen ranks the second compared to those caused by other pathogens (Simon-Mateo and Garcia, 2011). Depending on its nature, some viruses can have very broad host range. For example, tomato spotted wilt virus (TSWV) is reported to infect more than 1000 plant species in 85 families, including many vegetables, peanut, and tobacco (Sherwood et al., 2003) and cucumber mosaic virus (CMV) can infect more than 1200 plant species in 100 families, including many vegetables and ornamentals (Zitter and Murphy, 2009).

Plant viral disease significantly reduces crop quality and yield. It has been estimated that potato leaf role virus (PLRV) resulted in 20 million ton losses in potato production worldwide annually (Kojima and Lapierre, 1988). In most subtropical and tropical areas, tomato leaf curl virus (ToLCV) can cause complete economic loss in a tomato field (Czosnek and Laterrot, 1997). Since late 1980s in central and east Africa, cassava crops in almost 12 different countries were damaged due to

cassava mosaic disease caused by cassava mosaic virus (CsMV) (Legg et al., 2011). In Southeast Asia, Rice tungro virus has been estimated to cause an annual loss of 5–10% of the rice yield (Dai and Beachy, 2009). Broad range of plants including tobacco, tomato, and peanuts has been infected by TSWV (Sherwood et al., 2003) and as a result, the annual economic losses due to this virus are projected to be one billion dollars worldwide (Roger, 2014).

VIRUS INFECTION AND RNA SILENCING IN PLANTS

Due to their devastating threat to crop production, plant viruses has been studied extensively since the first virus, TMV, was discovered. The outcome of a virus infection on a plant is determined both by the genotype of the virus and that of the plant. The plant genetic architecture conferring resistance/tolerance to viruses usually includes so called recessive resistance and active defense. Recessive resistance is usually conferred by lacking positive host factors for virus propagation and accounted by many excellent reviews (Diaz-Pendon et al., 2004; Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012; Nicaise, 2014). In contrast to the passive defense model, plants can also actively attack viruses upon recognition of infection with a plethora of chemical and enzymatic arsenals (Vlot et al., 2009; Ding, 2010; Fu and Dong, 2013; Alazem and Lin, 2015). Among the many active defense mechanisms, RNA silencing was discovered more recently but attracted the most attention in the past decade in plant–virus interaction studies (Li and Ding, 2006; Mlotshwa et al., 2008; Ding and Lu, 2011; Baulcombe, 2015; Carbonell and Carrington, 2015).

Viruses are obligate intracellular parasites and complete their life cycle in living host cells. Plant viruses usually enter plant cells through wounds made by insect vectors or mechanic rubbing, replicate in the initial infected cells, move from cell to cell via plasmadosmata, and spread via phloem into newly emerged young tissue and organ, where they cause disease phenotype and became ready to exit and infect other host plants (**Figures 1A,B**). At the molecular and cellular level, once a virus particle, such as TMV, gets into a host cell, it has to be disassembled to release its genomic (g)RNA. The gRNA then serves as mRNA to produce viral replicase protein, which in turn transcribe gRNA into a complementary (c)RNA and further transcribe more gRNA and subgenomic (sg)RNA using cRNA as template. The amplified gRNA can participate in at least four possible pathways: replication, translation, cell-to-cell movement and assembly (**Figure 1B**).

In the middle 1980s, Sanford and Johnston formulated an elegant concept of pathogen derived resistance (PDR) that "Key gene products from the parasite, if present in a dysfunctional form, in excess, or at the wrong developmental stage, should disrupt the function of the parasite while having minimal effect on the host" (Sanford and Johnston, 1985). It is assumed that all viral activities during infection require that viral proteins interact with different host factors in a proper temporal and spatial manner. Thus PDR was applied to engineer viral resistance in plants by transforming plants with various viral genes since late 1980s and led to successful development of viral resistant crops for commercial application (Baulcombe, 1996). The first PDR in plants was demonstrated by transformation of tobacco plants with TMV coat protein gene (Abel et al., 1986). Numerous attempts were then conducted to generate viral resistance in plants through expression of viral proteins from transgene and in several cases it is consistent with the original idea of PDR, while in many other cases they were not explained by protein based PDR rather led to the discovery of small RNA based RNA silencing mechanism (Baulcombe, 1996; Palukaitis, 2011).

RNA silencing refers to small interfering (si)RNAs or micro(mi)RNAs mediated sequence specific gene silencing mechanisms, which play important role in antiviral defense, development, and maintenance of genome integrity (Ding, 2000; Vance and Vaucheret, 2001; Baulcombe, 2004; Chen, 2012). In plants, several key protein families are involved in RNA silencing, including Dicer-like (DCL), Argonautes (AGO), and RNA-dependent RNA Polymerase (RDR). DCL proteins are type III RNases that process dsRNA or hairpin RNA into siRNA or miRNA, respectively, of 20- to 24-nt long with 2-nt 3′ overhang. AGO proteins are endonucleases that form RNA-induced silencing complex (RISC) with siRNAs or miRNAs. RISC can bind to target mRNA or non-coding RNA by sequence complementarity via its containing siRNA/miRNA, and then silence the target gene expression by cleaving target mRNA and rendering its degradation, or recruiting cofactors and inhibiting mRNA translation, or recruiting DNA and histone modifiers and inhibiting the transcription of target gene. RDR proteins transcribe single-stranded RNA into dsRNAs which is further processed into siRNA by DCL protein. While DCL and AGO proteins present in all organisms where RNA silencing operates, RDR only presents in fungi, plants and very few animals, such as worms and amphioxus (Wassenegger and Krczal, 2006).

In plants DCL, AGO, and RDR are gene families containing multiple members and each functions in different parallel pathways. In *Arabidopsis* many studies have shown that DCL2, DCL4, AGO1, AGO2, RDR1, and RDR6 are the major components in antiviral RNA silencing (Carbonell and Carrington, 2015; Zhang et al., 2015). It is suggested that the double stranded replicative intermediates of RNA viruses or structured single stranded viral RNA can be processed by plant DCL4 or DCL2 into primary viral siRNAs (Voinnet, 2005; Bouche et al., 2006; Deleris et al., 2006; Ding and Voinnet, 2007). These primary viral siRNAs form RISC with AGO1 or AGO2, which target viral mRNAs for degradation. The RDR1 and RDR6 may use the cleaved viral RNA as substrate to synthesize dsRNA, which is further processed by DCL2 and DCL4 into secondary viral siRNA. These secondary viral siRNAs enhance antiviral RNA silencing by forming RISC complexes and targeting viral mRNA in the initial infected cells, or alerting

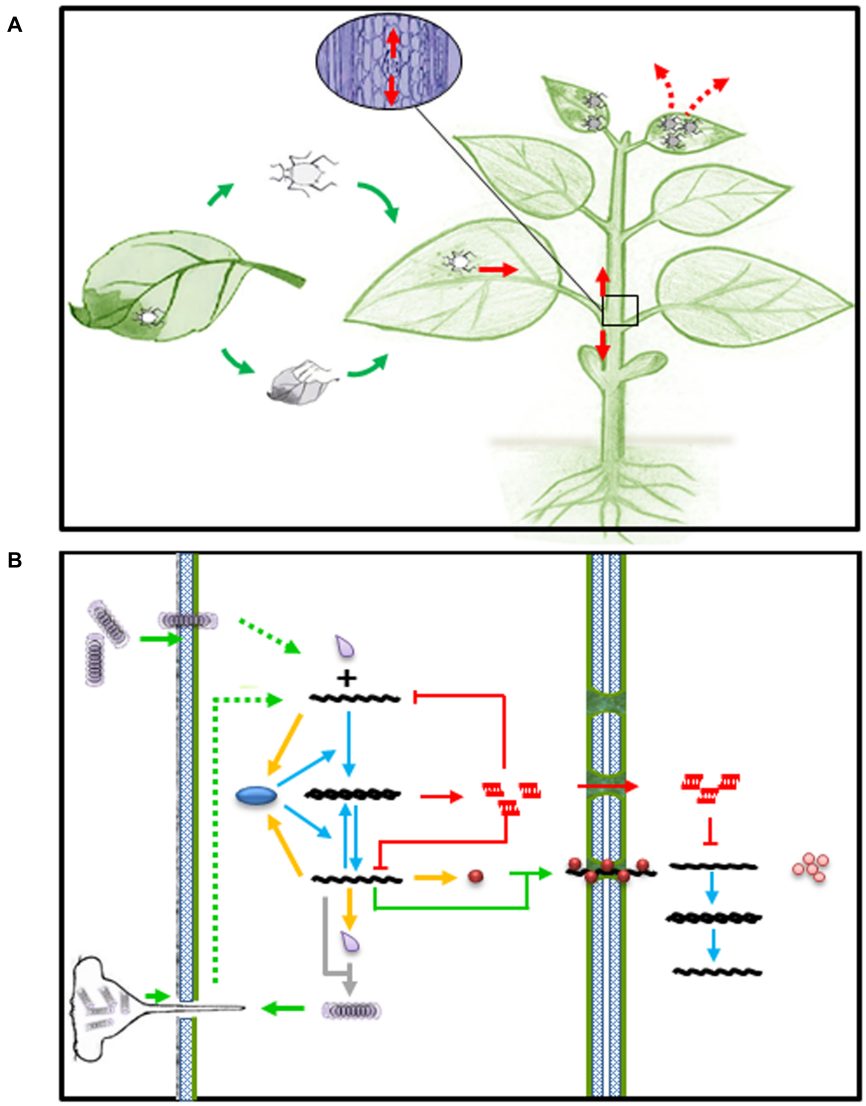

FIGURE 1 | Viral infection and RNA silencing in plants. (A) Virus entry (green arrows), spread (read arrows) and exit (read dashed arrows) in host plant. **(B)** Virus entry and spread (green arrows) in plant cell. Green dashed arrows represent disassembly of virion upon entry into plant cell. Yellow arrows represent expression of viral products, such as replicase (blue oval), movement protein (brown ball), and capsid protein (gray droplet). Blue arrows represent transcription of viral RNAs. Gray arrow depicts virion assembly from newly synthesized capsid and genomic RNA. Red arrows and lines represent activation of small RNA mediated intra and inter-cellular immunity.

the neighboring cell as well as the systemic tissue by the cell-to-cell and systemic movement via plasmadesmata and phloem respectively.

Since generation of dsRNA is a general feature during the replication and gene expression of various types of virus, dsRNA triggered RNA silencing is considered a pathogen molecular pattern (PAMP)- triggered immunity (PTI) in plants (Ding, 2010). In line with the zigzag model of the pathogen–host co-evolution (Jones and Dangl, 2006), virus that can overcome RNA silencing based PTI, usually encode effector that suppresses RNA silencing, which is termed viral suppressor of RNA silencing or VSR (Li and Ding, 2006). Many viruses encode different VSR proteins that suppress RNA silencing using diverse mechanisms (Li and Ding, 2006; Burgyan and Havelda, 2011; Jiang et al., 2012; Csorba et al., 2015).

RNA SILENCING MECHANISMS AND THEIR VIRAL TARGETS IN CROP IMPROVEMENT

RNA silencing has been deployed in crop improvement for viral resistance along the way it has been discovered. Successful resistance was achieved with either full length cDNA encoding functional viral products, or partial, or mutated viral cDNA (**Figures 2A**; Supplementary Table S1). These efforts can be

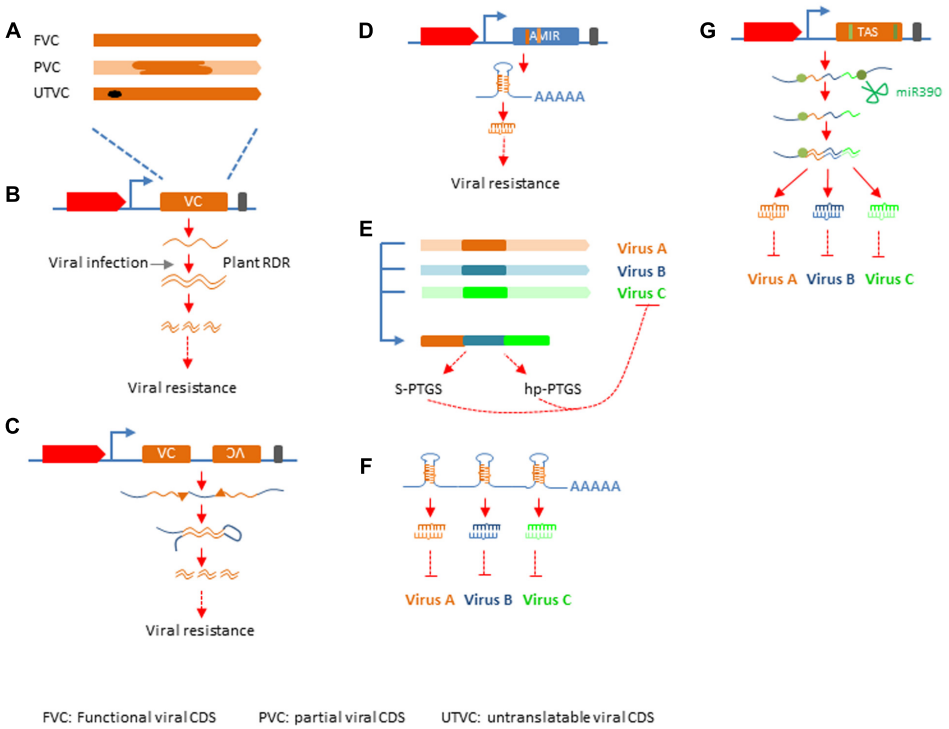

FIGURE 2 | Silencing mechanisms applied in crop protection. (A) Different types of viral sequences used in genetic engineering. FVC, functional viral CDS; PVC, partial viral CDS; UTVC, untranslatable viral CDS. **(B)** S-PTGS, top: structure of silencing construct with red block representing plant promoter, yellow block representing inserted viral sequences, black bar representing transcription terminator. **(C)** hp-PTGS, top: structure of silencing construct as depicted in **(B)**, except there are two viral sequences one in sense and the other in antisense orientation. **(D)** AMIR-PTGS, the structure of AMIR construct is similar to that in **(B,C)**, except that the blue block represent a backbone sequences of a natural miRNA and the dark yellow bar within the blue block depict mature miRNA sequence designed to target viral genome and the light yellow bar represents miRNA star. **(E)** Strategy to generate multiple-viruses resistance in S-PTGS and hp-PTGS. The yellow, blue, and green bars represent different viral sequences. The forth bar with different colors represents the chimeric viral sequences used in S-PTGS and hp-PTGS. **(F)** Cluster of AMIRs for multiple-viruses resistance. **(G)** TAS for multiple-viruses resistance. The TAS gene structure is similar to that described in **(A)**, except the blue block represents natural TAS3 backbone. The green bar in the gene structure and green dots in transcript lines represent miR390 binding sites.

categorized into four groups based on the mechanisms by which antiviral silencing is activated, sense gene induced post-transcriptional gene silencing (S-PTGS), hairpin RNA induced PTGS (hp-PTGS), artificial miRNA induced PTGS (AMIR), and trans-acting siRNA induced PTGS (TAS) (Supplementary Table S1; **Figures 2B–E**).

S-PTGS was practiced very early and very successful in the effort generating viral resistance (Gielen et al., 1991; Fitch et al., 1992; Lindbo et al., 1993). Inspired by the "PDR" hypothesis, researchers tried to generate viral resistance by overexpression of a viral protein in these efforts. However, the mechanism was turn out to be RNA mediated post-transcriptional gene silencing in many cases (Lindbo et al., 1993). The silencing state can be achieved before or after viral infection. In either case, it requires plant RDR protein to transcribe the overexpressed viral sequences into dsRNA, which is processed into siRNA to enhance the antiviral silencing mediated by siRNA derived from viral replication (**Figure 2B**) (Mourrain et al., 2000; Ding and Voinnet, 2007). Protection by S-PTGS type transgene can vary significantly among different lines transformed with the same construct (Supplementary Table S1). Transgenic lines that accumulated high level of viral siRNA and established silencing state in absence of viral infection usually are immune or highly resistant to viral infection (Guo et al., 1998; Masmoudi et al., 2002). On the contrary, transgenic lines express viral transcripts before viral invasion showed variable degree of resistance, ranging from susceptible, delaying in symptom expression, recovery to resistant (Lindbo et al., 1993; Sivamani et al., 2002; Zanek et al., 2008; Reyes et al., 2011). Since early 1990s, expression of antisense RNA was also tested in genetic engineering for viral resistance and various degree of resistance was obtained (Prins et al., 1996, 1997). Silencing mechanism behind these approaches is similar to that of S-PTGS and thus it is categorized as AS-PTGS. Both S-PTGS and AS-PTGS are considered first generation of small RNA based genetic engineering (SRGE) technology for viral resistance which was invented before the RNA silencing mechanism was well-understood and are still widely used till recently (Supplementary Table S1).

Hp-PTGS is the second generation technology developed after dsRNA was recognized as the trigger of RNA silencing. In these practices, researchers constructed silencing vectors with pieces of both sense and antisense viral cDNA under control of plant promoters and terminators. When transformed into plants, these constructs produce transcripts that can fold into dsRNA due

to the complementarity of sense and antisense viral sequences in it. The dsRNA is then processed into siRNAs and confers resistance/immunity to cognate viruses (**Figure 2C**). The first example of hp-PTGS mediated viral immunity was done in tobacco against PVY, which was published at the same year as the seminar paper showing dsRNA is the trigger of RNAi in worms (Fire et al., 1998; Waterhouse et al., 1998). Later on this technology was applied in many crops against diverse viruses and in most cases the degree of resistance to target virus in the transgenic plants was high to immune (Kalantidis et al., 2002) (Supplementary Table S1).

AMIR is considered the third generation technology developed very recently. In the first two generations of small RNA technology, the mature small RNAs that function in viral immunity are not predefined. Since loading of small RNA into the silencing effector AGO proteins requires certain sequence features in those small RNAs (Czech and Hannon, 2011), many small RNAs generated by the first two generation technology may not feed into the effectors. Natural miRNAs are released from well-defined secondary structure in their pri-miRNA transcripts. In the AMIR approach, the mature miRNA sequences in a natural miRNA primary transcript were replaced with specific RNA sequences that are complementary to target viruses and have favorable features for RISC loading, thus to create an artificial miRNA gene. When transformed into plants, the AMIR gene was transcribed and processed into mature miRNA with the designed sequences by the cellular miRNA biogenesis machinery to confer specific virus resistance (**Figure 2D**). The proof-of-concept studies for AMIR mediated viral resistance were reported in *Arabidopsis* and tobacco nearly 10 years ago (Niu et al., 2006; Qu et al., 2007), while its application in crop improvement is very limited and currently only two cases in tomato were reported besides those aforementioned (Zhang et al., 2011a; Vu et al., 2013) (Supplementary Table S1).

Mechanisms for multiple virus resistance were developed since the first generation technology. And there were at least five strategies developed to achieve this goal. The first one was to generate silencing constructs with multiple transcription units each targeting a distinct virus using S-PTGS mechanism (Prins et al., 1995; Shin et al., 2002). The second way was developed by Arif et al. (2009), in which double resistance was obtained by co-transformation with two constructs each target different virus by S-PTGS. In the third way, silencing construct was made with multiple inverted-repeat sequences derived from conserved viral sequences. Each IR structure can produce a dsRNA that can induce hp-PTGS against cognate virus (Zhang et al., 2011b). The forth, also a more widely applied strategy is to piece together partial gene fragments from different viruses first and then generate S-PTGS or hp-PTGS construct with chimeric viral sequences, which will produces siRNAs that target all intended viruses (Supplementary Table S1; **Figure 2E**) (Bucher et al., 2006; Liu et al., 2007; Kung et al., 2009; Lin et al., 2012). Transgenic plants were obtained by this strategy with complete resistance up to six different viruses (Liu et al., 2007). The fifth one is to generate cluster of artificial miRNA precursors and express it from one construct to generate multiple functional miRNAs targeting different viruses (**Figure 2F**). The sixth strategy was using artificial trans-acting siRNA gene to express multiple tasiRNAs targeting different viruses. A stretch of synthetic sequences consists of multiple 21-nt short sequences that complementary to target viruses is inserted between a 3′-cleavable and a 5′-non-cleavable miR390 binding sites to create an artificial TAS transcript. When it is expressed in transgenic plants, the artificial TAS transcript was cleaved by miR390 and the 5′ cleavage product containing the viral sequences is turned into dsRNA by plant RDR6 and diced from its 3′ end successively by DCL4 to release 21-nt tasiRNA that will target cognate viruses (**Figure 2G**). The efficacy of TAS-mediated multiple-viruses resistance has been demonstrated in *Arabidopsis* (Chen et al., 2016).

Viral targets for SRGE include both the virus and the genes or region within a virus that are targeted by small RNAs produced by the transgene. In terms of number of studies, Potyviruses, Tospoviruses, Closteroviruses, and Geminiviruses are among the most studied virus genera (Supplementary Table S1). Viral coat protein or nuclear capsid protein are the most frequently chosen targets for SRGE; viral replicase or replication associated proteins, and VSR protein are also frequently used (Supplementary Table S1). All these targets provide essential function for virus life cycle (**Figure 3**). Besides targeting the coding region, the untranslated region (UTR) in viral genome is also targeted for efficient antiviral silencing (Duan et al., 2008), due to its key role in viral replication and viral mRNA translation.

Choice of promoter in making silencing constructs. In many cases 35S promoter from *Cauliflower mosaic virus* was used to drive the expression of silencing transcripts to produce siRNA or miRNA targeting viruses. The 35S promoter is active in most vegetative tissue and drives gene expression constitutively. Phloem tissue is the highway for viral systemic spread within

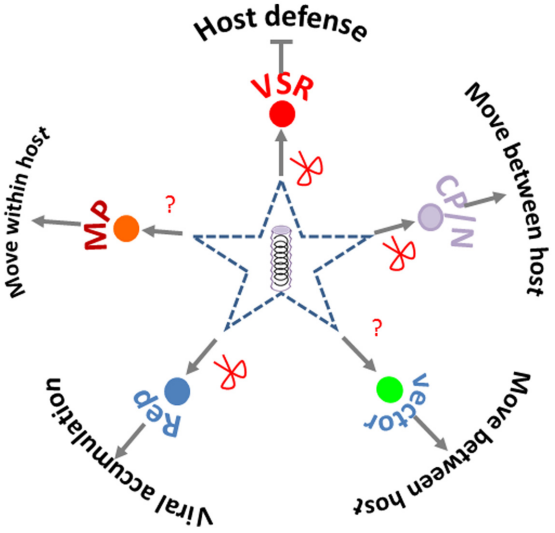

FIGURE 3 | Silencing targets chosen in crop protection. The red scissors point to the viral products (functions) that had been targeted by small RNA based genetic engineering. The question marks point to the viral or vector function yet to be reported as targets for crop protection.

the plants. In a study both phloem-specific promoter and 35S promoter were tested to drive the expression of silencing genes and the 35S promoter driven construct provided better resistance (Ehrenfeld et al., 2004). Expression of virus-targeting small RNA constitutively in all cell types may provide second line of defense in case virus breaks the defense in phloem and evade into newer tissue.

APPLICATION STATUS OF SRGE IN CROP PROTECTION

Small RNA based genetic engineering has been applied in engineering viral resistance for many crops, including major crops of staple food, vegetables, fruits ornamentals, and some cash crop (Supplementary Table S1). *Nicotiana benthamiana* has been widely used as a model species to study the efficacy of constructs for silencing the intended virus (Supplementary Table S1). Stable transgenic plants for a variety of crops were generated expressing small RNAs in different ways and their reactions to targeted viruses were tested in both laboratory and field condition (Supplementary Table S1). In some studies, the durability of resistance was tested for many generations (Wang et al., 2001, 2016; Liu et al., 2007; Cruz et al., 2014; Faria et al., 2014). According to the International Service for the Acquisition of Agri-Biotech Applications (ISAAA) website, dozens of transgenic crops resistance to virus generated with SRGE were approved for commercial release (Supplementary Table S2). Potato and the United States ranks the top among different crops and countries, respectively, in terms of number of lines approved (**Figure 4**). All these commercially released crops were developed based on the first generation SRGE technology.

Papaya provided the first successful example for tackling down the virus threats in agriculture with the SRGE. Papaya is an important tropical fruit with high nutritional value and economic significance. But the papaya industry was nearly destroyed in some regions by Papaya ringspot virus, a potyvirus with positive sense single strand RNA genome, in early 1990s (Supplementary Table S1) (Ferreira et al., 2002). Lack of natural resistance resources and effective disease management strategy made it necessary to the development of transgenic PRSV-resistant papaya and the effort was started late 1980s by Maureen Fitch, Dennis Gonsalves and colleague with the "PDR" approach (Gonsalves, 2006). PRSV-resistant papaya was soon obtained by expressing viral CP through transgene (Fitch et al., 1992) and commercially released in 1998 in Hawaii (Gonsalves, 2006). Due to the specificity of small RNA silencing mediated immunity, the transgene developed in Hawaii did not confer resistance to PRSV strain in Asia and new transgenic papaya lines were developed later with CP genes from local viral strain (Bau et al., 2003). Overcoming of resistance by more virulent PRSV strain was observed and new resistant transgenic papaya was obtained by targeting the viral HcPro protein that suppresses small RNA mediated immunity (Kung et al., 2015). Currently, there are four commercial transgenic papaya lines approved with three in USA and one in China (Supplementary Table S2; **Figure 4A**).

Banana, Citrus and Plum, banana is the largest tropical fruit and BBTV is the most serious viral pathogen for banana cultivation worldwide. BBTV-resistant transgenic banana was developed with hp-PTGS mechanism targeting Rep gene (Shekhawat et al., 2012; Elayabalan et al., 2013). Citrus is a high value fruit crop in international trade for both fresh fruits and juice market. CTV is the most economically important and damaging virus of citrus tree. CTV-resistant citrus was obtained with hp-PTGS targeting multiple VSR genes in the virus genome (Soler et al., 2012) while targeting single VSR is not effective (Batuman et al., 2006). Early efforts with S-PTGS mechanisms also did not work very well in citrus (Supplementary Table S1). Neither banana nor citrus transgenic lines resistant to viruses were approved for commercial release. Plum is one of the oldest domesticated fruit with versatile uses. Plum pox virus is the major viral pathogen of plum. S-PTGS mediated resistance against Plum pox virus was first demonstrated in *N. benthamiana* (Guo et al., 1998; Wittner et al., 1998) and later in Plum (Scorza et al., 2001). PPV-resistant plum was also obtained with hp-PTGS mechanism targeting CP gene (Hily et al., 2007; Ravelonandro

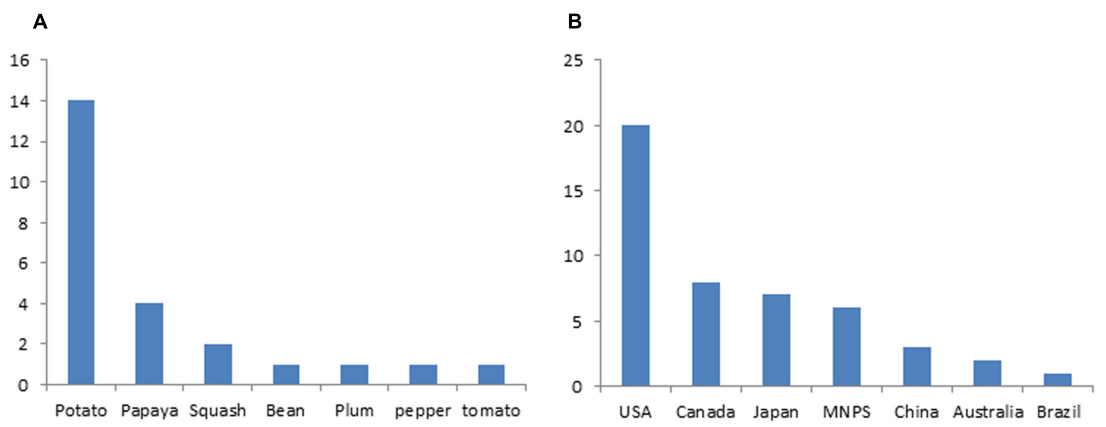

FIGURE 4 | Application status of small RNA based genetic engineering in crop protection. (A) Number of small RNA based transgenic crop varieties that are approved for commercial release. **(B)** Number of small RNA based transgenic crop varieties in different countries that are approved for commercial release.

et al., 2014). The S-PTGS based PPV-resistant plum was approved for commercial release in US (Supplementary Table S2).

Squash, cucumber, and watermelon are common vegetables and fruits belonging to the *Cucubitaceae* family, which suffer from a variety of viral pathogen (Romay et al., 2014). SqMV-resistant squash and CFMMV-immune cucumber were generated by S-PTGS targeting viral CP and Rep gene respectively (Pang et al., 2000; Gal-On et al., 2005). Multiple-viruses resistant Oriental melon and Watermelon were recently reported using S-PTGS with chimeric viral CP sequences (Wu et al., 2010; Lin et al., 2012). PRSV-resistant Cantaloupe was obtained by hp-PTGS mechanism (Krubphachaya et al., 2007). None of these transgenic cucurbita crops were approved for commercial release. Instead, two Squash transgenic lines resistant to CMV and ZYMV were approved for release in Canada and US (Supplementary Table S2).

Potato, tomato, and pepper are important vegetables belonging to *Solanaceae* family and potato is also a very important staple food crop. These crops suffer from a variety of plant viruses and a number of efforts to generate viral resistance with SRGE were reported (Supplementary Table S1). Doreste et al. (2002), Nunome et al. (2002), and Shin et al. (2002) PVX-resistant potato, CMV-resistant tomato and pepper with dual resistance to ToMV and CMV were obtained by means of S-PTGS. Since then PLRV-, PVX-, and PVY-immune potato was developed with hp-PTGS mechanism targeting PLRV-CP, PVX-CP, and PVY-HcPro simultaneously (Arif et al., 2012). TYLCV-immune tomato was also generated with both S-PTGS and hp-PTGS mechanism targeting viral Rep gene (Antignus et al., 2004; Fuentes et al., 2006). Currently, there are 14 transgenic potato lines approved for commercial release in US and other countries and all are developed by the Monsanto Company. One pepper and one tomato line were developed by Peking University and approved for commercial release in China (Supplementary Table S2).

Maize, Wheat, Rice, and Cassava are the major staple food crop and supported calorie consumption for most of the human population. Maize streak virus (MSV) and maize dwarf mosaic virus (MDMV) impose the most frequent viral threat to Maize production. Transgenic maize resistant to MDMV was generated with hp-PTGS mechanism targeting P1 and CP (Zhang et al., 2010, 2013; Zhang Z.Y. et al., 2011) whereas MSV-resistant transgenic maize was created with S-PTGS mechanism targeting viral Rep gene (Shepherd et al., 2007). Transgenic wheat resistant to Wheat streak mosaic virus was created with all three generations of SRGE and newer ones appeared to provide better protection (Sivamani et al., 2000; Fahim et al., 2010, 2012). The most important viral threat for rice production came from *Phytoreoviruses*, *Tenuiviruses*, *Tungroviruses,* and *Waikavirus*, such as RBSDV, RSV, RTBV, and RTSV (Supplementary Table S1). These viral pathogens caused significant losses in rice production in Asia and many resistant transgenic rice lines were generated using hp-PTGS mechanism (Ma et al., 2004; Tyagi et al., 2008; Roy et al., 2012; Sasaya et al., 2014). Some of the resistance traits had been introgressed into cultivated rice varieties (Roy et al., 2012; Valarmathi et al., 2016). Cassava is an important food crop in Africa and *Begmoviruses*, such as ACMV and SLCMV, caused severe problem in Cassava cultivation (Taylor et al., 2004). Initially, ACMV-resistant cassava was created with S-PTGS targeting AC1 gene (Chellappan et al., 2004). Since, ACMV is a DNA virus and its gene expression takes place on viral mini-chromosome structure, viral resistant transgenic cassava was also obtained using hairpin RNA construct targeting the viral promoter for transcriptional gene silencing (hp-TGS) (Vanderschuren et al., 2007). Though, the effectiveness of the transgenic viral resistance has been tested in field trial for many generations (Shimizu et al., 2011; Cao et al., 2013; Wang et al., 2016), currently no SRGE based staple food crop was reported for commercial release.

Peanut, Soybean, and common bean are rich in fatty acid, protein and other nutrients, important for everyday diet, and are all from *Fabaceae* family. PStV- and TSV-resistant peanuts were generated successfully with S-PTGS mechanism targeting (Higgins et al., 2004; Mehta et al., 2013), however, this strategy did not work very well for making transgenic peanut against *Tospoviruses*, such as PBNV and TSWV (Supplementary Table S1). Soybean mosaic virus is the most important viral pathogen to soybean cultivation and several transgenic lines resistant to this virus were generated by hp-PTGS and S-PTGS, targeting HcPro and CP, respectively (Wang et al., 2001; Gao et al., 2015). Multiple-viruses resistant soybean was also generated by expressing multiple short hairpin targeting Rep of AMV, BPMV, and SMV (Zhang et al., 2011b). BGMV-partial-resistant common bean was initially generated with S-PTGS mechanism targeting CP and completely resistant transgenic line was recently obtained using hp-PTGS targeting AC1 gene (Faria et al., 2006, 2014; Aragao et al., 2013). BGMV-resistant common bean was approved for commercial release in Brazil (Supplementary Table S2) while no commercial release of SRGE based viral resistant peanut and soybean were reported.

Tobacco including *Nicotiana tabacum* and *N. benthamiana* were widely used as model plants to study the efficacy of SRGE against various viruses infecting crops (Supplementary Table S1) due to their easiness in transformation. However, result obtained from tobacco is not always consistent with that in the intended crop (Batuman et al., 2006). It is possible that certain virus may be more virulent in its native host due to better fitness. Since small RNA mediated silencing is usually dose dependent, this problem can be solved by targeting multiple viral genes in one construct and screen multiple transgenic lines for better resistance (Soler et al., 2012). It is also important to choose a proper promoter to drive the silencing construct expression in targeted crop as it is shown that small RNA subcellular localization affect antiviral efficiency (Ehrenfeld et al., 2004). Another issue in testing the resistance considered is the method of viral inoculation. Viral saps and *Agrobacterium*-mediated infiltration is widely used for virus inoculation as a routine technique in the lab. It was reported that transgenic tomato showed better resistance when infected via insect than by Agro-infiltration (Antignus et al., 2004), which may due to lower viral dosage in vector mediated infection than in Agro-infiltration. Thus choosing proper viral dosage is important in characterization of transgenic lines.

CHALLENGES AND FUTURE ASPECTS

Early application of the first generation SRGE involves expression of functional viral products, which raises concerns to the human health and the environment. These concerns were well-addressed in the application of PRSV CP transgenic papaya (Fuchs and Gonsalves, 2007). In the newer generation of SRGE technology, only short stretches of viral sequences were expressed and no viral protein product will be expressed in any part of the transgenic crop, thus completely dismiss the concerns, such as heterologous encapsidation, recombination and synergism. However, there still exist real challenges for application of even the second and third generation SRGE.

Crop plants are often subjected to mixed viral infection. VSR from untargeted virus can suppresses the small RNA mediated silencing thus breaks the immunity to SRGE targeted virus (Savenkov and Valkonen, 2001; Simon-Mateo et al., 2003). For the targeted viruses, some isolate has stronger VSR that can break immunity conferred by SRGE (Kung et al., 2015). To solve these problems, multiple virus resistance can be explored with the second and third SRGE technology. It is also necessary to target multiple-genes within one virus to achieve stronger resistance.

Oomycete pathogen was shown to deliver effector into plant cells to suppress small RNA mediated silencing (Qiao et al., 2013), thus possibility exists that SRGE conferred viral immunity may be broken in mixed infection with Oomycete pathogen. Interestingly it was recently reported that miRNA can be exported to fungal cells and inhibit pathogen gene expression thus confer resistance (Zhang et al., 2016). Since Oomycete and fungi are both eukaryotes where silencing operates, thus a possible solution to breaking down SRGE by Oomycete (and possibly fungi as well) is to target it together with viruses by SRGE.

Small RNA mediated silencing is also affected by abiotic stress, such as low and high temperature, drought and salt stress, which are often encountered in crop cultivation. Investigation of molecular mechanism by which those abiotic stresses manipulate silencing pathway, will provide solution to proper compensation strategy for SRGE application in those stress conditions.

It was reported early that small RNA mediated silencing in non-cell autonomous and silencing signal is capable of both cell-to-cell and phloem dependent long distance movement (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). In modern horticulture, grafted seedlings were widely used in vegetable and fruit tree cultivation in which crop scions are grafted onto rootstock of related species. Grafted crops usually perform better compared to their self-rooted counterpart in terms of nutrient efficiency, abiotic stress tolerance and resistance to soil born disease. It is worthwhile to explore the possibility to generate viral resistant rootstock with SRGE to provide protection for different crop scions. This way can save the effort to introduce resistance trait to every commercial varieties or develop transformation system for them, which are time consuming and sometimes not possible for certain species. Though, AMIR mediated resistance failed to cross graft union (Zhang et al., 2011a), many other types of small RNAs remain to be tested for this potential and grafting methods can be further optimized.

Finally, plant genomes encode multiple DCL genes capable of generating miRNA and siRNAs in many ways. Fully dissection of the small RNA biogenesis mechanisms mediated by those different DCL proteins, can help design silencing constructs expressing as many as possible small RNAs, which holds the key for success of SRGE application in crop protection.

AUTHOR CONTRIBUTIONS

AK and FL conducted literature research and analyzed the data. FL and AK wrote the manuscript. QZ contributed **Figure 1**. MY contributed in literature research.

FUNDING

This work is supported by "Yong 1000 talent program" and HZAU start fund (2013RC001 to FL). AK is supported by "CSC scholarship."

ACKNOWLEDGMENT

We apologize to the authors of those references reported relevant research but not cited in this manuscript due to limited space.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00043/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 15 September 2016
doi: 10.3389/fmicb.2016.01458

Interplays between Soil-Borne Plant Viruses and RNA Silencing-Mediated Antiviral Defense in Roots

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Although the majority of plant viruses are transmitted by arthropod vectors and invade the host plants through the aerial parts, there is a considerable number of plant viruses that infect roots via soil-inhabiting vectors such as plasmodiophorids, chytrids, and nematodes. These soil-borne viruses belong to diverse families, and many of them cause serious diseases in major crop plants. Thus, roots are important organs for the life cycle of many viruses. Compared to shoots, roots have a distinct metabolism and particular physiological characteristics due to the differences in development, cell composition, gene expression patterns, and surrounding environmental conditions. RNA silencing is an important innate defense mechanism to combat virus infection in plants, but the specific information on the activities and molecular mechanism of RNA silencing-mediated viral defense in root tissue is still limited. In this review, we summarize and discuss the current knowledge regarding RNA silencing aspects of the interactions between soil-borne viruses and host plants. Overall, research evidence suggests that soil-borne viruses have evolved to adapt to the distinct mechanism of antiviral RNA silencing in roots.

Keywords: soil-borne virus, RNA silencing, antiviral defense, roots, silencing suppressor, *Polymyxa*, *Olpidium*, nematode

OPEN ACCESS

Edited by:
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Reviewed by:
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Ying Wang,
Ohio State University, USA

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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *27 July 2016*
Accepted: *31 August 2016*
Published: *15 September 2016*

Citation:
Andika IB, Kondo H and Sun L (2016) Interplays between Soil-Borne Plant Viruses and RNA Silencing-Mediated Antiviral Defense in Roots. Front. Microbiol. 7:1458. doi: 10.3389/fmicb.2016.01458

INTRODUCTION

Most plant virus transmissions in nature are facilitated by biological vectors, and the site of virus entry into the host plant differs according to these transmission vectors (Hull, 2013). The majority of plant viruses are transmitted into the aerial plant parts by a variety of arthropods, mainly sap-sucking insects such as aphids and whiteflies, while some soil-inhabiting zoosporic organisms and root-feeding nematodes transmit a number of plant viruses into roots (Hull, 2013). Thus, compatibility of the virus with the tissue or cell where it initially enters the host plant is critical for establishing the infection. Each plant organ or tissue has a distinct metabolism and pronounced physiological characteristics. In particular, the features of plant shoots and roots largely diverged from one another; they differ in their anatomical structures, cell compositions, gene expression patterns, and are exposed to contrasting environmental conditions between above and below ground environments. Consequently, antiviral defense in roots may operate differently than that in shoots, and viruses may have evolved to adapt to these mechanistic differences.

Soil-borne viral diseases are generally difficult to control with conventional chemical or agronomical methods because viruliferous vectors could be widespread underground. In particular,

viruliferous resting spores of the zoosporic vectors could be stable and persistent in the infested soil for decades (Rochon et al., 2004; Bragard et al., 2013; Tamada and Kondo, 2013). Consequently, the disease control-measures are mainly dependent on natural plant resistance resources (Kanyuka et al., 2003; Kühne, 2009; McGrann et al., 2009; Ordon et al., 2009), but in agricultural systems, the emergence of resistance-breaking viruses poses a serious threat to crop production (Kühne, 2009; Tamada and Kondo, 2013; Tamada et al., 2016). Nevertheless, studies about the mechanisms by which the plant antiviral defense system combats viruses entering the roots are scarce. This is partly due to the fact that only a limited number of plant-virus–soil-inhabiting vector inoculation systems has been so far successfully established under laboratory conditions.

RNA silencing is a general term for down-regulation of gene expression, mediated by small RNAs in eukaryotes (Baulcombe, 2005). In the cell, RNA silencing is involved in diverse biological processes and operates by targeting DNA/RNA of endogenous or exogenous origin in a nucleic acid sequence-specific manner via inhibition of RNA transcription (involving RNA-directed DNA methylation, RdDM), cleavage of RNA, or translational inhibition of mRNA (Ghildiyal and Zamore, 2009; Voinnet, 2009; Castel and Martienssen, 2013). The important role of RNA silencing in antiviral defense has been well established in plants, insects, fungi, and mammals (Ding, 2010; Li et al., 2013). To counteract antiviral RNA silencing, most of the plant viruses encode silencing suppressor proteins (Li and Ding, 2006; Pumplin and Voinnet, 2013; Csorba et al., 2015).

In this review, we summarize the current information on the molecular aspects of antiviral RNA silencing in roots, with emphasis on the interactions between host antiviral defense and soil-borne viruses. Although the studies and information regarding this topic are still limited and mostly based on analyses using model plant-virus pathosystems, presently available information provides an insight into the divergent action of antiviral RNA silencing defense in roots relative to that already established for shoots. In addition, the effectivity of RNA silencing-based engineered resistance against soil-borne virus infection in plants is also briefly discussed.

DIVERSITIES OF SOIL-BORNE VIRUSES AND THEIR VECTORS

Currently, a number of plant single-stranded RNA (ssRNA) viruses belonging to at least 17 genera in eight virus families, but no DNA or dsRNA virus, are known to be transmitted by soil-inhabiting organisms (**Figure 1**). Considering the possible occurrence of non-vectored soil transmission of plant viruses (Campbell, 1996) and that the natural vectors of many plant viruses are still unknown, it is likely that the members of soil-borne viruses extend beyond these 17 genera. The vectors of soil-borne viruses could be largely categorized into three groups, namely, plasmodiophorids (a class within the kingdom Protista), *Olpidium* spp. (a genus of the order Chytridiales within the kingdom Fungi), and nematodes (a phylum within the kingdom Animalia) (**Figure 1**). *Olpidium* (*Olpidium virulentus*, *O. brassicae,* and *O. brassicae*) vectors transmit viruses from the families *Ophioviridae* (genus *Ophiovirus*), *Rhabdoviridae* [a previously free-floating genus *Varicosavirus,* but has recently been classified into this family (Afonso et al., 2016)], *Alphaflexiviridae* (genus *Potexvirus*), and *Tombusviridae* (genera *Tombus-*, *Aureus-*, *Gamma carmo-*, *Diantho-*, *Alphanecro-*, and *Betanecrovirus*), having flexuous, rod-shaped or icosahedral particles. Plasmodiophorids (*Polymyxa betae*, *P. graminis,* and *Spongospora subterranea*) are vectors of viruses from the families *Benyviridae* (genus *Benyvirus*), *Virgaviridae* (genera *Furo-*, *Peclu-*, and *Pomovirus*) and *Potyviridae* (genus *Bymovirus*), with rod-shaped or filamentous virions (except for two unclassified watercress viruses), while nematodes (*Longidorus* spp., *Paralongidorus maximus*, *Xiphinema* spp., *Trichodorus* spp., and *Paratrichodorus* spp.) are vectors of viruses from the families *Virgaviridae* (genus *Tobravirus*), *Secoviridae* (genus *Nepo-* and *Cheravirus*), and *Tombusviridae* (genus *Dianthovirus*), with rod-shaped or icosahedral particles. Thus, there is no specific association of each vector group with a particular structure of the viruses they transmit and likewise, the same vector species (f. e. *O. virulentus*) can transmit viruses with different particle structures. All known vector-transmitted soil-borne viruses have positive-sense ssRNA genomes except for the members of two genera, *Ophiovirus* and *Varicosavirus,* that have negative sense ssRNA genomes (Verchot-Lubicz, 2003; Kormelink et al., 2011) (**Figure 1**). It appears that the members with multipartite ssRNA genomes dominate the soil-borne viruses as they are more evident in the viruses that belong to the families *Rhabdoviridae* and *Potyviridae*, wherein the members having monopartite genomes and arthropod vectors (such as aphids, whiteflies, leaf- and planthoppers) are the majority in these virus families (Bragard et al., 2013). For soil-borne viruses with icosahedral virion, viral coat protein (CP) is apparently sufficient to mediate the transmission process, which is due to the direct attachment of the virus particles to the surface of vector zoospores or the retention of virions within the nematode feeding apparatus, while those with rod-shaped or filamentous virions involve additional specific proteins or protein domains located in CP read through proteins that facilitate the vector transmission, possibly either through forming a bridge between virus particles and vector or through other unknown mechanisms (Adams et al., 2001; Macfarlane, 2003; Bragard et al., 2013) (**Figure 1**).

Olpidium and nematode vectors transmit viruses to a wide range of hosts, particularly vegetable, ornamental and fruit plants, while viruses transmitted by plasmodiophorid vectors have a more limited range of hosts, but are important food crops such as cereals (furo- and bymoviruses), sugar beet and rice (benyviruses), peanut (pecluviruses), and potato (pomoviruses). For more details and comprehensive reviews regarding the vectors and genomes of soil-borne viruses, readers are referred to Brown et al. (1995), Rush (2003), Rochon et al. (2004), Kühne (2009), Bragard et al. (2013), Tamada and Kondo (2013), and Syller (2014) and references therein.

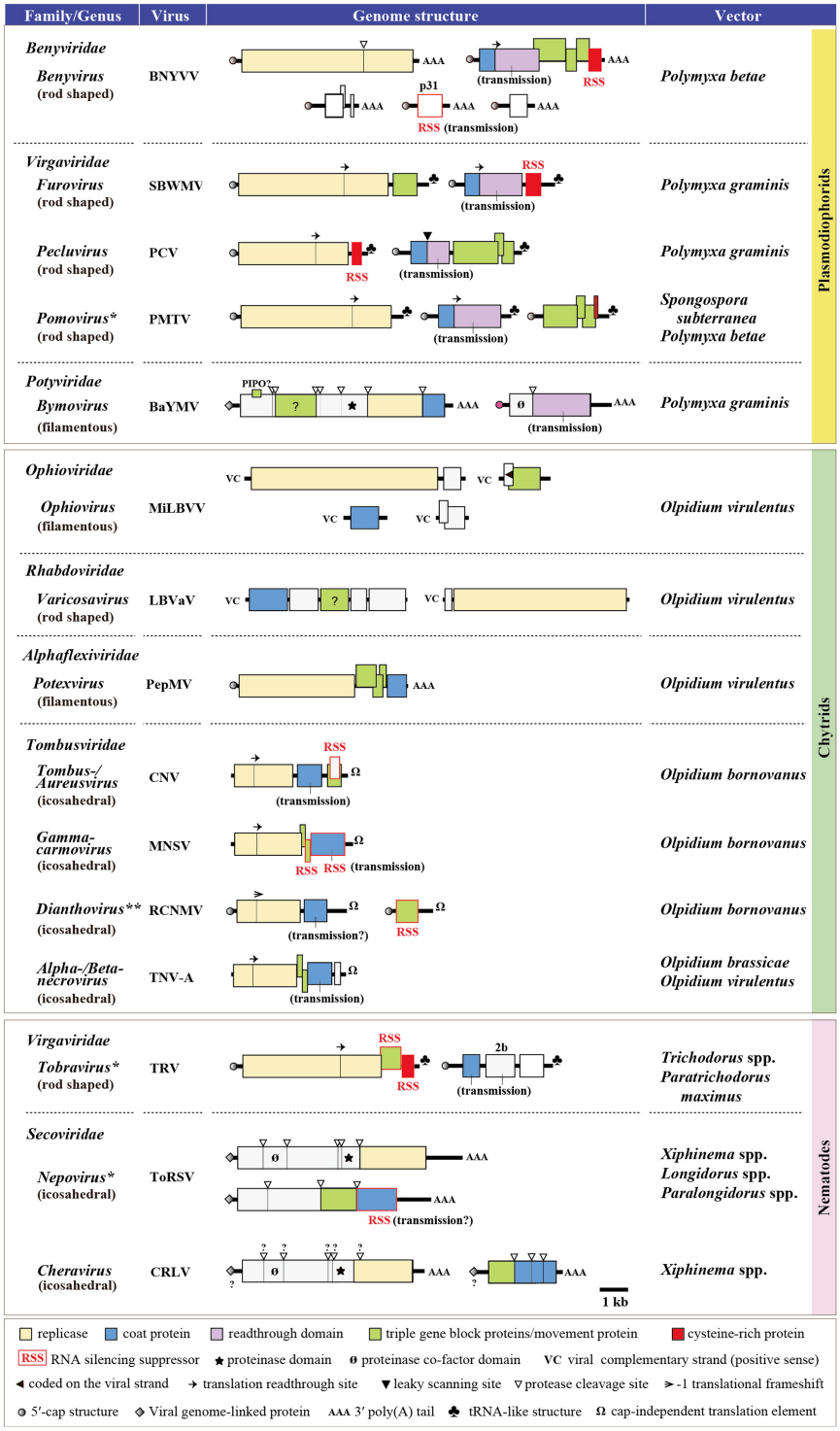

FIGURE 1 | Genome structure of the representative soil-borne plant viruses. The type species member from each virus genus is presented except for the MiLBVV, PePMV, cucumber necrosis virus (CNV), melon necrotic spot virus (MNSV), and RCNMV, which are selected because they are transmitted by soil-borne vectors, while the vector of other members within the same genus is unknown and/or insects. *Some members of these genera are also known as seed transmissible. **A member of this genus (carnation ringspot virus) is transmitted by both *Longidorus* and *Xiphinema* spp. BNYVV, beet necrotic yellow vein virus; SBWMV, soil-borne wheat mosaic virus; PCV, peanut clump virus; PMTV, potato mop-top virus; BaYMV, barley yellow mosaic virus; MiLBVV, mirafiori lettuce big-vein virus; LBVaV, lettuce big-vein associated virus; PepMV, pepino mosaic virus; CNV, cucumber necrosis virus; MNSV, melon necrotic spot virus; RCNMV, red clover necrotic mosaic virus; TNV-A, tobacco necrosis virus-A; TRV, tobacco rattle virus; ToRSV, tomato ringspot virus; CRLV, cherry rasp leaf virus.

DISEASES CAUSED BY SOIL-BORNE VIRUSES IN CROPS

Although soil-borne viruses enter the host plants via the roots, none of the members of this virus group is known to exhibit root tropism within the host plants. After initial infection in the roots, the soil-borne viruses usually travel long distances upward through vasculature and may subsequently induce various viral symptoms in the aerial plant part or may not generate any obvious symptoms, depending on the combination of virus and host plant. Only a few soil-borne viruses cause a particular disease symptom in roots or underground plant organs. Beet necrotic yellow vein virus (BNYVV; genus *Benyvirus*) infection in sugar beet causes the economically significant rhizomania disease which spreads worldwide (Tamada, 2016). It is typically characterized as a massive proliferation of lateral roots and rootlets ("bearded"-like appearance) and severely stunted taproots (Tamada, 1999). Potato mop-top virus (PMTV; genus *Pomovirus*) causes brown arcs or rings in potato tuber flesh (spraing symptoms; Harrison and Reavy, 2002). Viruses belonging to the genera *Furovirus* (type species *Soil-borne wheat mosaic virus*) and *Bymovirus* (type species *Barley yellow mosaic virus*) infect winter cereal crops and cause yellow mosaic symptoms on leaves as well as plant stunting (Kühne, 2009). Peanut clump virus (PCV; genus *Pecluvirus*) infection induces mottling and chlorotic ring symptoms on leaves as well as stunting of the plant (Thouvenel and Fauquet, 1981; Dieryck et al., 2009). The co-infection of lettuce big-vein associated virus (LBVaV; genus *Varicosavirus*) and Mirafiori lettuce big-vein virus (MiLBVV; genus *Ophiovirus*) is associated with lettuce big-vein disease in the field, which is characterized as mottling and chlorophyll clearing along the veins (appearing as big vein), but only MiLBVV is believed to be a sole disease agent (Maccarone, 2013). Viruses of the genera *Tombusvirus* (cucumber necrosis virus; CNV) and *Carmovirus* (i.e., melon necrotic spot virus, MNSV) cause necrosis or necrotic lesions on leaves and stems of Cucurbitaceae plants such as cucumber, melon, and squash (Dias and McKeen, 1972; Hibi and Furuki, 1985). Nepoviruses cause various diseases in a broad range of crops including fruit trees, vegetables, and ornamentals (Sanfaçon, 2008). Grapevine fanleaf virus (GFLV, genus *Nepovirus*) is the main causal agent of fanleaf and yellow mosaic diseases of grapevine worldwide (Andret-Link et al., 2004). Tobacco rattle virus (TRV, genus *Tobravirus*) can infect variety of crops and causes the major diseases of potato (spraing) and ornamental bulbs (Macfarlane, 2008).

GENETIC COMPONENTS OF ANTIVIRAL RNA SILENCING IN PLANTS

In plant, RNA silencing is initiated when imperfect or true double-stranded RNAs (dsRNAs) derived from cellular sequences or viral genomes, are processed by a ribonuclease III-like protein in the Dicer family called "Dicer-like (DCL) proteins" to generate 21–22-nucleotide (nt) microRNAs (miRNAs) or 21–26-nt short interfering RNA (siRNA) duplexes. Each strand of small RNA is then incorporated into the effector complexes termed "RNA-induced silencing complexes (RISCs)," which contain ARGONAUTE (AGO) proteins, to guide the sequence specificity in the downregulation processes (Axtell, 2013; Martínez de Alba et al., 2013; Bologna and Voinnet, 2014). Plant-encoded RNA-dependent RNA polymerases (RDRs) could contribute to the generation of dsRNA substrates for DCL processing, leading to either initiation of RNA silencing or production of secondary small RNAs that further intensify the potency of RNA silencing (Dalmay et al., 2000b; Wang et al., 2010). Plants encode multiple DCL, AGO, and RDR proteins to cope with diverse endogenous RNA-silencing pathways (Zhang et al., 2015). For example, the experimental model plant *Arabidopsis thaliana*, which is widely used for genetic studies on the RNA silencing mechanism, contains 4 DCL, 10 AGO, and 6 RDR proteins (Bologna and Voinnet, 2014). In *A. thaliana*, DCL4 and DCL2, which generate 21 and 22-nt siRNAs, respectively, act hierarchically in antiviral defense against RNA viruses. DCL4 is the primary DCL component for antiviral response, while DCL2 could functionally substitute DCL4 when it is overcome or absent (Deleris et al., 2006; Diaz-Pendon et al., 2007), but in some cases, DCL2 appears to have a specific role in the blocking of the systemic spread of viruses (Garcia-Ruiz et al., 2010; Andika et al., 2015a,b). Among 10 *A. thaliana* AGOs, AGO1 and AGO2 broadly function in antiviral defense against a wide range of RNA viruses, although other AGOs, such as AGO4, AGO5, AGO7, and AGO10, could also show antiviral activities in a more specific virus-host combination (Mallory and Vaucheret, 2010; Pumplin and Voinnet, 2013; Ma et al., 2014; Brosseau and Moffett, 2015; Carbonell and Carrington, 2015; Garcia-Ruiz et al., 2015). *A. thaliana* RDR6 and, to a lesser extent, RDR1, are required for antiviral defense against an RNA virus via amplification of viral siRNAs mechanism (Wang et al., 2010, 2011). In addition to DCL, AGO, and RDR core enzymes, other protein components in the RNA silencing pathway contribute to antiviral defense in *A. thaliana*, such as dsRNA-binding protein 4 (DRB4), a DCL4-interacting protein (Qu et al., 2008; Jakubiec et al., 2012), SUPPRESSOR OF GENE SILENCING 3 (SGS3), a coiled-coil protein (Mourrain et al., 2000; Rajamäki et al., 2014), and HUA ENHANCER 1 (HEN1) which methylates the 2′ hydroxy groups at the 3′-end termini of small RNAs to protect them from degradation (Boutet et al., 2003; Zhang et al., 2012). In *Nicotiana benthamiana* (wild tobacco), which is the most widely used experimental model host of plant RNA viruses, the antiviral activities of RNA silencing components, including the homologs of DCL4, AGO1, AGO2, and RDR6 were also demonstrated (Qu et al., 2005; Schwach et al., 2005; Scholthof et al., 2011; Andika et al., 2015b; Gursinsky et al., 2015; Fátyol et al., 2016).

DISTINCT CHARACTERISTICS OF TRANSGENE AND ENDOGENOUS RNA SILENCING IN ROOTS

The occurrence and mechanism of RNA silencing in the root organ initially received relativity less attention from

plant researchers. However, a growing number of studies have analyzed gene regulation, involving RNA silencing in roots, and revealed some unique characteristics of RNA silencing in roots relative to those observed in leaves or other aerial plant parts. First, lower RNA silencing activities were observed in roots than in leaves when post-transcriptional gene silencing in transgenic plants was induced by the sense transgene. In silenced transgenic *A. thaliana* lines carrying transgene encoding a Fab antibody fragment, suppression of the transgene expression was significantly lower in roots than in leaves (de Wilde et al., 2001). Co-suppression of tobacco endoplasmic reticulum ω-3 fatty acid desaturase (*NtFAD3*) gene by the sense transgene is effective in leaves but not in roots, although transgene-derived siRNAs accumulate in both tissues (Tomita et al., 2004). Likewise, lower levels of transgene silencing in roots than in leaves of silenced transgenic *N. benthamiana* lines carrying the CP read through gene of BNYVV or green fluorescent protein (GFP) gene were observed, as indicated by incomplete degradation of transgene mRNAs and lower levels of transgene siRNAs accumulation (Andika et al., 2005). Moreover, transgene DNA cytosine methylation levels at non-symmetrical CpNpN (N is A, T, or C) but not symmetrical CpG or CpNpG context were lower in roots than in leaves (Andika et al., 2006). Nevertheless, suppression of the target gene appears to be equally effective in mature leaves and roots if inverted repeat (IR) transgenes that are designed to express dsRNAs are used to induce the silencing (Fusaro et al., 2006; Marjanac et al., 2009). The sense- and IR-mediated silencing differ in the initiation step, where sense- but not IR-mediated silencing, requires conversion of ssRNAs into dsRNAs by the activities of RDR6 together with SGS3 and SDE3 (RNA helicase; Dalmay et al., 2000b, 2001; Mourrain et al., 2000; Béclin et al., 2002). It is therefore possible that in roots, either biosynthesis of dsRNA by RDR6 is less efficient or DCL protein(s) do not efficiently process RDR6-dependent dsRNA substrates for siRNA production. Transcriptomic analysis in *A. thaliana*, *N. benthamiana,* and rice showed that the mRNA expressions of RNA silencing core genes in leaves and roots are similar (Kapoor et al., 2008; Nakasugi et al., 2013). Thus, the reason for differential activities of sense transgene silencing between leaves and roots remains unclear.

Recent studies revealed that down-regulation of endogenous gene expressions in root could involve mobile (non-cell autonomous) small RNAs. During the development of *A. thaliana* roots, miR165a and miR166b produced in endodermis cells move to neighboring stele to mediate the suppression of *PHABULOSA* gene transcripts in a dose-dependent manner (Carlsbecker et al., 2010; Miyashima et al., 2011). Grafting experiments using *A. thaliana* plants demonstrated that siRNAs could be transported from shoots to roots and then induce RdDM of transgene promoter (Molnar et al., 2010; Melnyk et al., 2011). Moreover, a portion of endogenous small RNAs in roots are derived from shoots and associated with RdDM of a large number of genome loci, including transposable elements and endogenous genes (Molnar et al., 2010; Lewsey et al., 2016).

ACTIVITIES OF ANTIVIRAL RNA SILENCING IN ROOTS

Some studies have detected the accumulation of siRNAs derived from various ssRNA viruses in the roots of infected plants including *N. benthamiana*, tomato, cucumber, and melon (Andika et al., 2005, 2013, 2015b; Herranz et al., 2015), demonstrating that viruses indeed induce antiviral RNA silencing responses in roots. BNYVV siRNA accumulation is lower in roots than in leaves of *N. benthamiana* and inversely related with RNA genome accumulation (Andika et al., 2005), suggesting that BNYVV may more effectively suppress RNA silencing in roots than in leaves (further discussed in the next section). Potato virus X (PVX, genus *Potexvirus,* natural vector unknown) siRNA accumulation is much lower in roots than in leaves, but this is likely due to the low level of PVX genome replication in roots (Andika et al., 2015b). Analyses using next generation sequencing indicated that siRNAs derived from PVX, Chinese wheat mosaic virus (CWMV, genus *Furovirus*), melon necrotic ringspot virus (MNSV, genus *Carmovirus*), and prunus necrotic ringspot virus (PNRSV, genus *Ilarvirus,* pollen and thrips transmission) are predominantly 21 nt in both leaves and roots (Andika et al., 2013, 2015b; Herranz et al., 2015), indicating that DCL4 is also the major DCL component for biosynthesis of viral siRNAs in roots. Notably, the proportions of MNSV and PNRSV sense siRNAs were higher than those of antisense siRNAs in roots, while the proportions of both strands were equal in leaves (Herranz et al., 2015). This suggests that in roots, DCL proteins preferentially target the sense strand genome of these viruses through cleaving of the secondary structures within viral RNA to generate sense siRNAs (Herranz et al., 2015), although it is generally thought that DCL mainly processes dsRNA replication intermediates formed during RNA virus replication (Ding, 2010). However, we cannot rule out other possibilities, including long-distance movement of sense siRNAs to roots and/or specific processing of viral subgenomic RNAs in roots.

Chinese wheat mosaic virus as well as other members of the genus *Furovirus* requires cool temperatures (below 20°C) to establish infection in the host plants (Ohsato et al., 2003). RDR6 is involved in temperature-dependent antiviral defense against RNA viruses in *N. benthamiana* leaves (Szittya et al., 2003; Qu et al., 2005). Knock-down of RDR6 homolog in *N. benthamiana* enables CWMV accumulation in roots but not in leaves, after a temperature shift to 24°C, and CWMV accumulation is associated with reduced accumulation of viral siRNAs in roots (Andika et al., 2013). This observation suggests that RDR6-dependent RNA silencing activity (probably through production of secondary siRNAs) is mainly responsible for inhibiting CWMV infection in roots at higher temperatures (**Figure 2**), whereas additional mechanism(s) are involved in the suppression of CWMV infection in leaves.

RNA silencing strongly inhibits PVX replication in roots of susceptible plants (Andika et al., 2012, 2015b). *A. thaliana* is not a susceptible host of PVX, but inactivation of DCL4 enables high accumulation of PVX in inoculated leaves, while inactivation of both DCL4 and DCL2 is required for systemic infection of

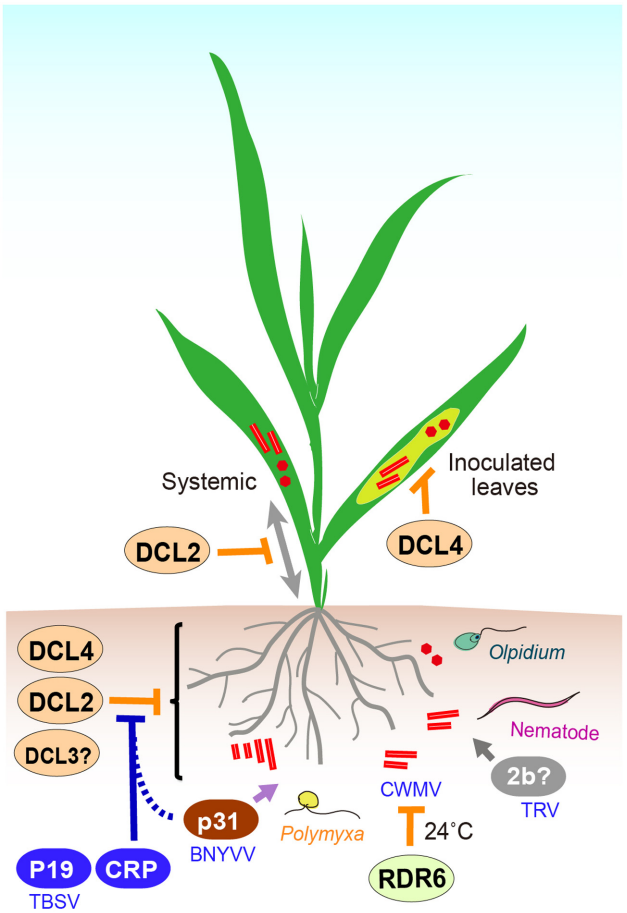

FIGURE 2 | A cartoon presentation illustrating the interplay between viruses and antiviral RNA silencing in roots. In *Arabidopsis thaliana*, DCL4 is essential for the inhibition of PVX accumulation in inoculated leaves, while DCL2 particularly functions in blocking of PVX systemic infection. DCL4 is the primary DCL protein component involved in intracellular antiviral silencing in roots, but it can be functionally compensated for by DCL2 or possibly partially, DCL3. At higher temperatures (after a temperature shift to 24°C, see main text), RDR6 is involved in inhibition of CWMV multiplication in *Nicotiana benthamiana*, whereas at the same temperature other mechanism(s) is mainly responsible for CWMV inhibition in shoots. Cysteine rich proteins (CRPs) encoded by TRV and BNYVV more effectively suppress RNA silencing in roots than in leaves. BNYVV p31 exhibits root-specific RNA silencing suppression activity and contributes to efficient virus transmission by *Polymyxa betae* vector into roots. TBSV P19 expression is essential for TBSV infection via root mechanical inoculation in *N. benthamiana*.

PVX in upper leaves and roots. Another set of experiments was performed using a transgenic *A. thaliana* line that carries a replication-competent PVX cDNA transgene (AMP243 line; Dalmay et al., 2000a). Inactivation of DCL4 in AMP243 plants, where PVX replication is strongly suppressed in the cell due to intracellular antiviral silencing, is sufficient to enable high levels of PVX replication throughout the aerial organs, but not in roots (Andika et al., 2015b). These observations demonstrate that while DCL4 is critical for intracellular antiviral silencing against PVX replication in shoots, there are strong functional redundancies among DCL proteins, in which other DCLs (most probably DCL2) functionally complement DCL4 in roots (Andika et al., 2015a) (**Figure 2**). These strong redundancies may result in potent inhibition of PVX replication in roots, likely by providing multiple layers of antiviral defense. Thus, these observations suggest that to some degree, antiviral RNA silencing in roots may operate differently from that in shoots.

SUPPRESSION OF RNA SILENCING BY SOIL-BORNE VIRUSES

Numerous RNA silencing suppressors (RSSs) encoded by soil-borne viruses have been identified (listed in **Table 1**). Notably, the small cysteine-rich proteins (CRPs) located in a 3′proximal open reading frame (ORF) of the genome segment of viruses belonging to the genera *Beny-*, *Furo-*, *Peclu-*, and *Tobravirus* [as well as genera *Hordeivirus* (Yelina et al., 2002) and *Goravirus* (Atsumi et al., 2015) in the family *Virgaviridae*, some members are transmitted by seed transmission and no known biological vectors] (**Figure 1**), similarly function as an RSS, and some of them have been subjected to detailed studies. The CRP is also encoded by viruses belonging to the genus *Pomovirus,* but CRP encoded by PMTV does not exhibit RSS activity (Lukhovitskaya et al., 2005). The CRPs are characterized by the presence of multiple cysteine residues in their N-terminal or central portions, but they do not show a notable amino acid sequence similarity among different genera (Koonin et al., 1991). CRPs encoded by furo-, peclu- tobra-, and hordeiviruses contain a highly conserved CGxxH (Cys–Gly–x–x–His, x is any amino acid residue) motif (Te et al., 2005). Mutational analyses on CWMV 19K CRP and TRV 16K indicate that CGxxH motif as well as other conserved cysteine residues are critical for protein stability and/or RSS activity (Sun et al., 2013a; Fernández-Calvino et al., 2016). Similarly, cysteine residues located in a putative C4 (Cys4) zinc-finger domain of BNYVV p14, which are also conserved among other benyviruses, are essential for protein stability and RSS function (Chiba et al., 2013). Each of these CRPs shows distinct subcellular localization, for example BNYVV p14 localizes to nucleous (Chiba et al., 2013); CWMV 19K is associated with endoplasmic reticulum through amphipathic α-helix domain, and PCV P15 localizes to peroxisomes via C-terminal SKL (Ser-Lys-Leu) motif (Dunoyer et al., 2002; Sun et al., 2013a), although none of those organelle targeting is required for RSS activities. CWMV 19K and PCV P15 self-interact (dimerization) through coiled-coil domain (Dunoyer et al., 2002; Sun et al., 2013a), while the self interaction of BNYVV p14 is mediated by the C4 zinc-finger domain (Chiba et al., 2013) and importantly, the ability of those CRPs to form dimers is essential for RSS activities. CWMV 19K binds to the large form of CP (CUG-initiated extension to the N-terminal of CP), but the biological role of this interaction is unknown (Sun et al., 2013b). TRV 16K is not needed for the systemic spread of the virus, but is necessary for transient meristem invasion (Martín-Hernández and Baulcombe, 2008). In addition, TRV 16K inhibits the *de novo* formation of RISC and binds AGO4

TABLE 1 | Properties of RNA silencing suppressors (RSSs) encoded by soil-borne viruses.

Genus Virus[1]	RSS	Protein category[2]	Local/cell-to-cell[3]	Motif, domain/target[4]	Subcellular localization	Di-mer	Reference
Benyvirus							
BNYVV	p14	CRP	Weak/–	NoLS, zinc-finger/–	Cytoplasm, nucleous	Yes	Andika et al., 2012; Chiba et al., 2013
	p31	–[7]	No/–		–	–	Rahim et al., 2007
BSBMV	p14	CRP	Weak/–	Zinc-finger/–	–	–	Chiba et al., 2013
BdMoV	p13	CRP	Weak/–	NLS, zinc-finger/–		–	Guilley et al., 2009; Andika et al., 2012
Furovirus							
SBWMV	19K	CRP	Weak/–	CGxxH, coiled-coil/–	–	–	Te et al., 2005
CWMV	19K	CRP	Weak/strong	CGxxH, coiled-coil, amphipathic α-helix/–	Endoplasmic reticulum	Yes	Sun et al., 2013a
Pecluvirus							
PCV	P15	CRP	Strong/–	CGxxH, coiled-coil, SKL/–	Peroxisomes	Yes	Dunoyer et al., 2002
Tobravirus							
TRV	16K	CRP	Weak/–	CGxxH/AGO4	Cytoplasm, nucleus	Yes	Ghazala et al., 2008; Andika et al., 2012; Fernández-Calvino et al., 2016
	29K[5]	MP	No/–				Deng et al., 2013
PepRSV	12K	CRP	Strong/–	–/–	–	–	Jaubert et al., 2011
Tombusvirus							
CNV	p20	(RSS)	Weak/–	–/–	–	–	Hao et al., 2011
Gammacarmovirus							
MNSV	p42	CP	Weak/strong	–/–	–	Yes	Genoves et al., 2006
	p7B	MP	Weak/–	–/–	–	–	Genoves et al., 2006
Dianthovirus							
RCNMV	RNA[6]		Strong/–	–/–	–	–	Takeda et al., 2005
	MP	MP	No/strong	–/–	Endoplasmic reticulum, cell wall	–	Tremblay et al., 2005; Powers et al., 2008; Kaido et al., 2009
Nepovirus							
ToRSV	CP	CP	Weak/–	WG/AGO1	–	Yes	Karran and Sanfaçon, 2014

[1]*BNYVV, beet necrotic yellow vein virus; BSBMV, beet soil-borne mosaic virus, BdMoV, burdock mottle virus; SBWMV, soil-borne wheat mosaic virus; CWMV, Chinese wheat mosaic virus; PCV, peanut clump virus; TRV, tobacco rattle virus; PepRSV, pepper ringspot virus; CNV, cucumber necrosis virus; TBSV, tomato bushy stunt virus; MNSV, melon necrotic spot virus; RCNMV, red clover necrotic mosaic virus, ToRSV, tomato ringspot virus.*

[2]*CRP, cysteine-rich protein; CP, coat protein; MP, movement protein.*

[3]*Suppression activities on local silencing in Agrobacterium co-infiltration assay relative to well-known strong suppressors such as p19 of tomato bushy stunt virus and HC-Pro of potato virus Y/ability to promote cell-to-cell movement of a suppressor-defective virus in the trans-complementation assay.*

[4]*NoLS, nucleolar-localization signal; NLS, nuclear-localization signal; CGxxH, cysteine-glycine-two any amino acid residues-histidine motif; SKL, serine-lysine-leucine motif; WG, tryptophan-glycine motif.*

[5]*Silencing suppression by 29K occurred in the context of RNA1 replication.*

[6]*RNA silencing suppression is mediated by the replication of RCNMV RNA1.*

[7]*"–", not determined.*

(Fernández-Calvino et al., 2016), but does not cause a global deregulation of the microRNA-regulatory pathway (Martínez-Priego et al., 2008). Likewise, tomato ringspot virus (ToRSV, genus *Nepovirus*) CP binds and destabilizes AGO1 through the recognition involving WG/GW (Try-Gly/Gly-Try) motif (Karran and Sanfaçon, 2014). Nevertheless, the mechanism of action of other RSSs encoded by soil-borne viruses remains unclear.

It is important to point out that in *Agrobacterium* co-infiltration assay using a GFP reporter gene in *N. benthamiana* (Voinnet et al., 2000), a method that is most commonly used for identification of viral RSS, the majority of RSSs encoded by soil-borne viruses exhibit weak suppression activities against local silencing relative to suppression activities of well-known potent suppressors such as HC-Pro of potato virus Y (PVY, a potyvirus, aphid transmission) and p19 of tomato bushy stunt virus (TBSV, a tombusvirus, natural vector unknown; **Table 1**) (Verchot-Lubicz, 2003). However, some of those RSSs show strong activities to promote cell-to-cell movement of a suppressor-defective virus in *trans*-complementation assays (Genoves et al., 2006; Powers et al., 2008; Sun et al., 2013a), suggesting that those RSSs are more effective in inhibition of cell-to-cell spread of silencing signals that move ahead of the virus (intracellular silencing) rather than inhibition

of local (intercellular) silencing in leaves. Interestingly, in a silencing reversal assay using transgenic *N. benthamiana* line 16c systemically silenced for the GFP gene, BNYVV or TRV infection restored GFP expression in roots but not in leaves, while infection of tobacco mosaic virus (TMV, genus *Tobamovirus*) and two aphid-borne (non-soil-borne) viruses, PVY and cucumber mosaic virus (genus *Cucumovirus*), restored GFP expression in both tissues. Moreover, BNYVV and TRV elevated PVX RNA accumulation in a co-infection experiment and this stimulating effect was due to the activity of p14 or 16K RSS encoded by those viruses (Andika et al., 2012). In another co-infection experiment, TRV also showed an activity to suppress antiviral silencing-like responses that inhibit the replication of TMV in lateral root primordia (Valentine et al., 2002). Collectively, these observations suggest that some RSS encoded by soil-borne viruses might be more effective in roots than in leaves. Further supporting evidence for this notion comes from the analyses of accumulations of some soil-borne viruses in plants. CWMV and MNSV accumulate to higher levels in roots than in leaves (Gosalvez-Bernal et al., 2008; Andika et al., 2013). Nepo- or tobraviruses have unusual ability to infect meristems and often show a recovery phenotype, which is manifested as a drastic reduction in virus symptoms and titer in newly developed leaves (Ratcliff et al., 1997, 1999). The recovery phenotype is thought to be mediated by RNA silencing-related mechanisms and mutations in the viral RSS can result in viruses that exhibit a recovery-like phenotype in the host plants (Ratcliff et al., 1997; Szittya et al., 2002). Similarly, BNYVV infection in *N. benthamiana* exhibited reduced viral accumulation similar as the "recovery" phenomenon in leaves but not in roots (Andika et al., 2005). Therefore, it is suggested that the weak RSS encoded by these viruses could not effectively inhibit the induction of antiviral systemic silencing, leading to recovery in upper leaves (Martín-Hernández and Baulcombe, 2008; Ghoshal and Sanfaçon, 2015).

Only a few studies have examined the relevance of silencing suppression in the context of virus infection through roots. The p31 encoded by RNA 4 of BNYVV is not essential for virus multiplication, but is required for efficient virus transmission by *P. betae* vector into roots (Tamada et al., 1989). Interestingly, in a silencing reversal assay, p31 showed an activity to suppress GFP transgene silencing in roots but not in leaves, proving that p31 has a root-specific RSS function (Rahim et al., 2007) (**Figure 2**). TRV 2b is a nematode transmission helper protein (Macfarlane, 2003) and is also required for extensive root (and also shoot) meristem invasion (Valentine et al., 2004). In a more recent study on TBSV, which is also considered a soil-borne virus because soil solarization and fumigation could reduce disease incidence (Gerik et al., 1990; Campbell, 1996), TBSV p19 suppressor is required for TBSV to infect *N. benthamiana* via root mechanical inoculation but not via leaves mechanical inoculation (Manabayeva et al., 2013) (**Figure 2**). Together, these observations suggest that suppression of RNA silencing or other antiviral defense mechanism is one of the factors that determine the efficiency of virus transmission to the roots.

EFFECTIVITY OF RNA-BASED TRANSGENIC RESISTANCE AGAINST SOIL-BORNE VIRUSES

Using the transgenic approach, RNA silencing has been successfully applied to generate plant resistant against infection with diverse viruses (Simon-Mateo and Garcia, 2011; Cillo and Palukaitis, 2014; Saurabh et al., 2014). Several researches have introduced a portion of genome sequence derived from soil-borne viruses into either experimental models or crops plants and evaluated the responses of the transgenic plants to virus infection. Although the silencing of viral sequences in the transgenic plants could in general provide a high degree of protection against the soil-borne viruses (e.g., for crops, Dong et al., 2002; Pavli et al., 2010; Zare et al., 2015; Kawazu et al., 2016), some other studies similarly observed that upon roots inoculation, virus resistance was less effective in roots than in shoots. Inoculation of roots of transgenic *N. benthamiana* carrying CP gene of PMTV using viruliferous *S. subterranea* resulted in virus accumulation in roots but no systemic movement of PMTV to shoots (Germundsson et al., 2002). *N. benthamiana* plants transformed with CP read through domain of BNYVV were immune to viral infection following leaf mechanical infection, but BNYVV accumulated at a low level in the roots of the same plants upon challenged by viruliferous *P. betae* vector (Andika et al., 2005). Transgenic *N. tabacum* carrying 57-kDa read through domain of the replicase gene of TRV was highly resistant to manual leaf inoculation, but the virus could be detected in roots following root manual inoculation or nematode vector inoculation (Vassilakos et al., 2008). Likewise, MiLBVV was detected in roots, but not in leaves of transgenic lettuce carrying IR transgene of MiLBVV CP following roots inoculation by *Olpidium* vectors (Kawazu et al., 2009). However, transgenic sugar beet plants carrying 0.4 kb IR sequence of BNYVV replicase gene showed high resistance to BNYYV infection through vector inoculation (Lennefors et al., 2008). This suggests that the potency of transgenic resistance against root inoculation could be affected by various factors including construct design, viral gene sequence, and plant species. A recent report showed that a high and durable transgenic wheat resistance against wheat yellow mosaic virus (WYMV, genus *Bymovirus*) infection in the field is obtained by transformation with antisense nuclear inclusion b (NIb) replicase of WYMV (Chen et al., 2014). Transgene siRNAs are not detected in transgenic plants, indicating that the resistance is not mediated by transgene silencing, although it is possible that the resistance resulted from cleavage of dsRNAs that are formed through annealing of antisense transcripts with viral genome RNA by DCLs or other cellular RNases (Chen et al., 2014). It is necessary to further explore the antiviral efficacy of antisense transgenes in different soil-borne virus-host plant pathosystems. In addition, the effectivity of artificial miRNAs in conferring virus resistances (Niu et al., 2006; Qu et al., 2012; Ramesh et al., 2014) has not been tested against soil-borne viruses.

CONCLUDING REMARKS

Overall, the observations from the studies described in this review provide evidence for divergent operations of RNA silencing in roots, although the primary factors responsible for the distinct regulation of RNA silencing activities in roots remain an open question. Moreover, the antiviral roles of RNA silencing components in the context of virus infection through roots are yet to be examined. Interestingly, those studies also demonstrated that some soil-borne viruses appear to have adapted to the mechanistic differences of antiviral RNA silencing in roots by evolving their RSS with more active function in facilitating viral transmission and accumulation in roots than in leaves. Further studies are needed to investigate the possibility that RSS encoded by soil-borne viruses specifically target certain molecular components of antiviral silencing in roots. It is worth mentioning that many plant viruses vectored by sap-sucking insects that usually penetrate their stylets into the phloem tissue, exhibit phloem-limited accumulation within their host plants (Omura et al., 1980; Latham et al., 1997; de Zoeten and Skaf, 2001; Shen et al., 2016). This also goes along with the opinion that the vectors influence virus evolution and adaptation within the host plants.

An agronomic practice for the effective control of soil-borne diseases is not available, while the use of methyl bromide (bromomethane), which is the most popular pre-plant soil fumigant against soil-borne fungi and nematodes, has been withdrawn worldwide under the Montreal protocol (Bell, 2000). Thus, harnessing the plant natural antiviral defense could potentially become a feasible alternative method for protecting the crop plants against these diseases. In fact, the results of several studies have opened the possibility of RNA silencing enhancement in plants, for example by chemical (ascorbic acid derivatives) treatments (Fujiwara et al., 2013), environmental (light intensity and temperature) modifications (Kotakis et al., 2010; Patil and Fauquet, 2015), overexpression of endogenous plant RNA silencing enhancers (Dorokhov et al., 2006; Meyer et al., 2015) and deactivation of plant endogenous suppressor of RNA silencing (Sarmiento et al., 2006; Gy et al., 2007; Shamandi et al., 2015; Liu and Chen, 2016). With the notion that RNA silencing plays an important role in defense against virus invasion via roots, it is anticipated that more detailed studies on antiviral RNA silencing mechanisms in roots could provide a solid basis for the future development of effective control measures of soil-borne virus diseases. Lastly, the advent of novel molecular tools for functional genomics and expanding understanding of plant innate immunity will allow greater options for the development of virus resistant crops.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

The authors are grateful for financial supports to Yomogi Inc., the Project for Extramural Scientists of State Key Laboratory of Agrobiotechnology (2015SKLAB6-23), National Key Research and Development Foundation, Ministry of Science and Technology of China (2016YFD0300705), and a Grant-in-Aid for Scientific Research (grant nos. 15K07312 and 25252011), Japan Society for the Promotion of Science (JSPS). We also thank Drs. Tetsuo Tamada, Nobuhiro Suzuki, Chengui Han, and Jianping Chen for fruitful discussions and the two reviewers for their valuable suggestions and comments.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 21 December 2016
doi: 10.3389/fmicb.2016.02068

Cross-Talk in Viral Defense Signaling in Plants

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Viruses are obligate intracellular parasites that have small genomes with limited coding capacity; therefore, they extensively use host intracellular machinery for their replication and infection in host cells. In recent years, it was elucidated that plants have evolved intricate defense mechanisms to prevent or limit damage from such pathogens. Plants employ two major strategies to counteract virus infections: resistance (*R*) gene-mediated and RNA silencing-based defenses. In this review, plant defenses and viral counter defenses are described, as are recent studies examining the cross-talk between different plant defense mechanisms.

Keywords: defense responses, *R* gene, RNAi, viral suppressor of RNAi, virus

OPEN ACCESS

Edited by:
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Reviewed by:
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Elizabeth P. B. Fontes,
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *30 September 2016*
Accepted: *07 December 2016*
Published: *21 December 2016*

Citation:
Moon JY and Park JM (2016) Cross-Talk in Viral Defense Signaling in Plants. Front. Microbiol. 7:2068. doi: 10.3389/fmicb.2016.02068

INTRODUCTION

Plant viruses comprise an important group of pathogens causing a range of plant diseases that are often responsible for significant losses in crop production. Among the wide range of known plant viruses, most viruses have a very limited host range and only a few viruses cause severe disease symptoms (Dawson and Hilf, 1992). Even though viruses contain relatively simple genomes, the molecular basis of the mechanisms by which plant viruses infect their hosts and the signaling components involved in host resistance are not well defined.

The immune response against bacterial or fungal pathogens often relies on recognition of the conserved molecules associated with a group of pathogens, designated pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors (PRRs) (Boller and He, 2009). Upon PAMP recognition, activated PRRs induce PAMP-triggered immunity (PTI) (Monaghan and Zipfel, 2012). PTI against viral pathogens has been primarily described in mammalian cells, but not in plant cells (Calil and Fontes, 2016). However, several recent studies provided evidence that PTI and related components are also involved in antiviral defense responses in plants (Korner et al., 2013; Nicaise, 2014; Iriti and Varoni, 2015; Calil and Fontes, 2016; Nicaise and Candresse, 2016; Niehl et al., 2016). In general, plants defense responses triggered against viral pathogens are based on RNA- or protein-mediated resistance. The RNA-mediated resistance response is a basal defense response to viral invasion that mainly involves the RNA silencing pathway of the host, which mediates the cleavage of viral RNA. Compared to this basal defense response, the host resistance (R) protein-mediated defense response against viral pathogens is far more robust, in most cases limiting viral replication and spread to inoculated leaves (Zhou and Chai, 2008; Verlaan et al., 2013; Nakahara and Masuta, 2014). In this review, we summarize molecular mechanisms underlying two major defense pathways employed during plant resistance to viral pathogens and highlight a few studies addressing the cross-talk between these defense pathways.

RNA SILENCING IN VIRAL DEFENSE

RNA gene silencing, also termed RNA interference (RNAi), which acts as a basal defense mechanism against viruses, is one of the main plant immune responses against viral pathogens (Vaucheret, 2006; Ding and Voinnet, 2007). Most viruses that cause disease in plants have RNA genomes containing imperfect regulatory stem-loops, which are copied into complementary double-stranded RNA (dsRNA) replication intermediates by virus-encoded RNA-dependent RNA polymerases (RDRs) (Ruiz-Ferrer and Voinnet, 2009). The dsRNAs are then recognized by a host ribonuclease III-like protein, namely, Dicer-like (DCL), and then processed into 21–24-nucleotide short interfering RNAs (siRNAs). The siRNAs are recruited to a functional RNA-induced silencing complex (RISC) and then act as guides to direct RISC to their target viral RNA molecules, which have complementary sequences (Ruiz-Ferrer and Voinnet, 2009). Consequently, viral RNAs are degraded by the core components of RISC, which are members of the Argonaut (AGO) protein family (Vaucheret, 2008). The antiviral RNAi response is effective in various species (Katiyar-Agarwal and Jin, 2010), even though it is slow and thus viral infections are often not completely cleared.

The concept of PTI against viral pathogens is currently confined to animals because receptors that sense RNA or DNA viruses as ligands have only been identified in animals (Takeuchi and Akira, 2009). In plants, dsRNAs produced during virus infection are also regarded as viral PAMPs (Ding, 2010; Jensen and Thomsen, 2012). The RNA silencing pathway was assumed to play a role in the immune responses that recognize such dsRNAs in plants, unlike in animals (Ding and Voinnet, 2007). However, a few recent publications indicate that the known PTI components are involved in dsRNA recognition and that the reaction is an immune response distinct from the RNA silencing pathway. Therefore, these studies claim that PTI against viral pathogens is preserved in plants and animals (Korner et al., 2013; Nicaise, 2014; Nicaise and Candresse, 2016; Niehl et al., 2016). However, there is no direct evidence to explain how dsRNAs are recognized in plants; therefore, further studies are needed to determine whether an animal-like mechanism underlies dsRNA-mediated PTI in plants.

To overcome RNAi-mediated host defense, plant viruses frequently encode viral suppressors of RNAi (VSRs) that perturb the plant RNA silencing pathway (Ding and Voinnet, 2007). VSRs have been isolated from nearly all plant virus families. In addition to suppressing RNAi silencing during viral pathogenesis, most VSRs identified to date play important roles in replication, assembly, or movement of viruses. Although the primary sequences and structures of these VSR proteins vary considerably, most VSR-mediated suppression is thought to occur via two general mechanisms (**Figure 1**). Some VSRs, such as potyviral HcPro, *Beet Yellow Virus* P21 protein, *Peanut Clump Virus* P15 protein, and TCV coat protein (CP or P38), sequester small RNA duplexes by binding to short or long viral dsRNAs, which then leads to impaired assembly of AGOs into RISCs (Lakatos et al., 2006; Carbonell and Carrington, 2015). Alternatively, some VSRs impede the activity of AGO proteins that have a central role in the anti-viral RNA silencing pathway (Carbonell and Carrington, 2015). For example, *Cucumber Mosaic Virus* 2b protein suppresses RISC activity through a physical interaction with the PAZ domain of AGO1 (Duan et al., 2012). Similarly, two other viral VSR proteins, *Sweet Potato Mild Mottle Virus* (SMMV) P1 and TCV CP, also directly interact with AGO proteins through glycine/tryptophan (GW/WG) repeat motifs, which resemble the AGO1-binding peptides on RISC (Azevedo et al., 2010; Giner et al., 2010). These findings demonstrate that VSR suppression of RNAi silencing might involve independently evolved VSR proteins that show functional overlap (Carbonell and Carrington, 2015). Studies on VSRs will not only improve our understanding of plant–virus interactions, but they will also help elucidate the signaling mechanism underlying host RNA silencing pathways.

RESISTANCE GENE-MEDIATED DEFENSE RESPONSES AGAINST VIRAL PATHOGENS

To circumvent PTI, pathogens produce effectors that suppress immune responses triggered by active PRRs (Deslandes and Rivas, 2012). The bacterial pathogens usually encode ∼20–30 highly regulated effectors that are secreted directly into the host cytoplasm. Although individual effectors from closely related bacterial strains exhibit functional diversity, they possess highly redundant activities and extensive interchangeability (Cunnac et al., 2009; Deslandes and Rivas, 2012). This also applies to viral proteins such as movement proteins (MPs), and replicase proteins, which act as avirulent (Avr) factors (Kachroo et al., 2006).

Resistance (*R*) genes have evolved in plants as a countermeasure to the effect of pathogen effectors on PTI (Jones and Dangl, 2006). *R* genes mediate effector-triggered immunity (ETI), which is a highly amplified version of PTI (Jones and Dangl, 2006). Many *R* genes have been identified, which confer resistance to diverse pathogens including bacteria, fungi, oomycetes, insects, and viruses (Jones and Dangl, 2006; Kachroo et al., 2006). Notable examples of *R* genes conferring resistance against viral pathogens include tobacco "N" against TMV, "Rx1/2" in potato against Potato Virus X (PVX), and "HRT" and "RCY1" against TCV and CMV in *Arabidopsis*, respectively (Whitham et al., 1994; Bendahmane et al., 1999, 2000; Cooley et al., 2000; Takahashi et al., 2001). The *R* genes are largely dominant, whereas some genes exhibit recessive, tolerance, or partial resistance characteristics. Moreover, dominant R genes *HRT* and *RCY1* require recessive factors to confer resistance (Cooley et al., 2000; Takahashi et al., 2001). Since viruses require host factors for their infection (termed susceptibility factors), loss of these can also confer resistance to viral pathogens. Such resistance is often recessive (Truniger and Aranda, 2009). Notably, most such recessive *R* genes have been analyzed in potyviruses and encode translation initiation factors of the 4E or 4G family (eIF4E/eIF4G) (Kang et al., 2005; Truniger and Aranda, 2009). Interestingly, EF1A is required for Soybean Mosaic virus (SMV)-induced endoplasmic reticulum (ER) stress and, therefore, replication of SMV (Luan et al., 2016).

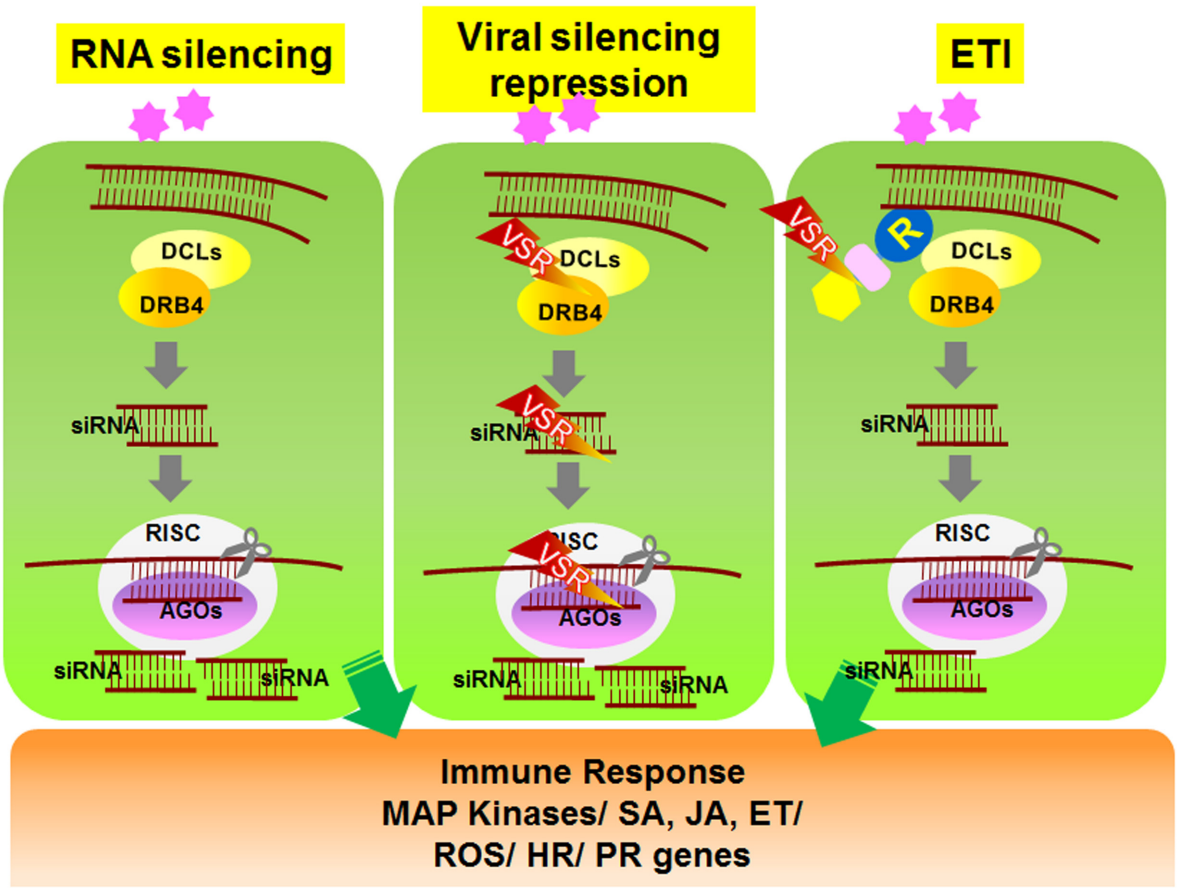

FIGURE 1 | Schematic model of RNA silencing- and *R*-mediated responses in plant cells. Upon amplification of viruses in plant cells, viral double-stranded RNAs (dsRNAs) activate RNA silencing mechanisms. Viral dsRNAs are processed into small RNA fragments (siRNAs) by DCL1 and its cofactor DRB4. The siRNAs are recruited to RISC, which is associated with AGO protein. RISC/AGO/siRNA then targets and degrades complementary viral transcripts (left panel). Viruses express genes encoding VSR proteins that inhibit the regulation and activation of gene silencing mechanisms (center panel). In response, several R proteins recognize the VSRs and induce downstream ETI responses (right panel). DCL, Dicer-like; DRB, dsRNA-binding protein; siRNA, small interfering RNA; AGO, Argonaute; RISC, RNA-induced silencing complex; VSR, viral suppressors of RNA silencing.

Consequently, silencing of *EF1A* inhibits SMV replication and confers resistance against SMV.

The majority of dominant *R* proteins contain nucleotide-binding site (NBS) and leucine rich repeat (LRR) domains (Collier and Moffett, 2009), which is also the case for *R* genes that confer resistance against viral pathogens (de Ronde et al., 2014). The NBS-LRR R proteins can be further subcategorized as putative coiled-coil- or toll-interleukin-1 receptor-like (TIR)-type proteins based on the presence of these domains at their N-termini (Collier and Moffett, 2009). TIR, NBS, and LRR domains are also found in *Drosophila* and human receptor proteins involved in innate immunity (Nürnberger et al., 2004), suggesting that the animal and plant proteins evolutionarily diverged from a common ancestor and that and that similar modules were selected to regulate innate immune responses.

While only selected R proteins show direct interactions with Avr factors (Dodds et al., 2006; Ueda et al., 2006; Cesari et al., 2013), most R proteins are thought to act indirectly via other intermediary host proteins. This is further explained by the "guard/decoy" model, which describes how R proteins guard host accessory proteins (guardees), and pathogen effector-mediated alteration of the guardees results in the activation of R protein (Jones and Dangl, 2006; Collier and Moffett, 2009; Dodds and Rathjen, 2010). For example, N protein from tobacco indirectly recognizes a p50 helicase fragment of the TMV replicase protein via a chloroplast-localized N receptor-interacting protein 1 (NRIP1) (Caplan et al., 2008). Upon TMV infection, NRIP1 residing in the chloroplast translocates to the cytoplasm and nucleus. Cytosolic NRIP1 associates with TMV replicase and then recruits N protein through a direct interaction between NRIP1 and the TIR domain of N (Ueda et al., 2006).

Unlike viral pathogens, both fungal and bacterial genomes encode multiple Avr factors, which are thought to play a role in the suppression of PTI. However, viruses appear to compensate for the presence of a single Avr effector by undergoing frequent alterations in the critical amino acid sequences without drastically changing the protein structure. Host R protein-mediated recognition of the modified Avr factor then depends

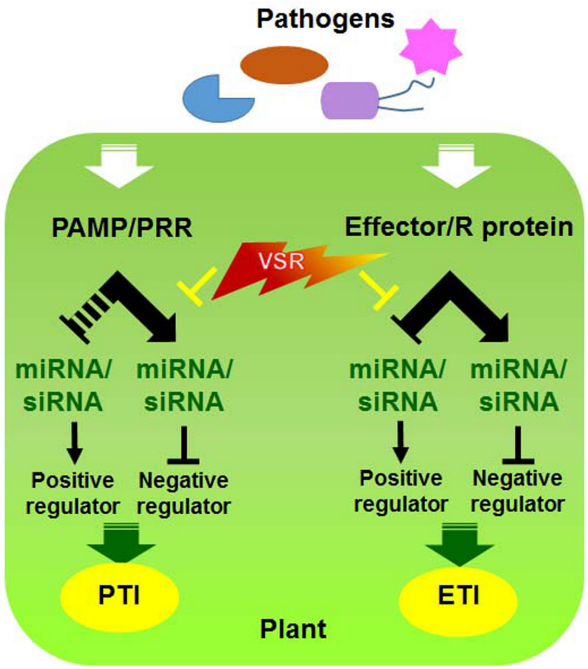

FIGURE 2 | Interactions between viral silencing suppression and host factors involved in PTI and ETI. Model of molecular virus–host interactions in RNA silencing and PRR/R-mediated resistance [modified from (Katiyar-Agarwal and Jin, 2010)].

on the relative affinity between R protein and the modified Avr factor. For instance, several hypervirulent strains of TCV isolated from *in planta*-propagated TCV are able to escape HRT-mediated recognition and cause disease in resistant plants (Wobbe et al., 1998; Zhu et al., 2013).

CROSS-TALK BETWEEN RNAi- AND *R* GENE-MEDIATED ANTI-VIRAL DEFENSE RESPONSES

Since both RNAi and *R* gene-mediated pathways participate in antiviral defense, it is plausible that these pathways undergo cross-talk to maximize the efficiency of defense responses against viral infections (Nakahara and Masuta, 2014). Indeed, viral pathogens often encode a single protein that functions as a suppressor of RNAi as well as an Avr effector (**Figure 2**) (Palanichelvam et al., 2000; Eggenberger et al., 2008; Katiyar-Agarwal and Jin, 2010; Wen et al., 2012; Zhu et al., 2013). For example, TMV replicase and TCV CP function as VSRs and are recognized by N and HRT, respectively, to induce the HR (Wang et al., 2012; Zhu et al., 2013). However, it is currently unclear how they communicate with each other and whether they assist each other to increase disease resistance or have sequential defense functions and thereby act individually.

Recently, a few studies provided molecular evidence that these two defense mechanisms are associated with each other (Li et al., 2012; Shivaprasad et al., 2012; Verlaan et al., 2013; Zhu et al., 2013). Several components involved in host RNA silencing mechanisms have recently been shown to be required for *R* gene-mediated defense. For example, double-stranded RNA binding protein (DRB) four interacts with HRT and is required for HRT stability (Zhu et al., 2013). In addition, *R* genes against Tomato yellow leaf curl virus were recently shown to encode DFDGD-class RDR (Verlaan et al., 2013). Interestingly, activation of HRT-mediated resistance is not dependent on the RNA silencing suppressor activity of CP and is not associated with the accumulation of TCV-specific small RNA. This finding suggests that the HRT-mediated signaling pathway recruits components of the RNA silencing pathway, but this resistance response is not associated with the cleavage of viral RNA.

It is likely that alteration of small RNAs derived from viral infections plays a role in regulating *R* gene expression levels, thereby regulating resistance signaling (Li et al., 2012; Shivaprasad et al., 2012), rather than direct regulation by VSR activity. For instance, Li et al. (2012) observed that *miR6019* and *miR6020* in tobacco cause specific cleavage of transcripts of *N* and its homologs by binding to the complementary sequence of a conserved region encoding the TIR domain of the N protein. Furthermore, phasiRNA synthesis from the *N* coding sequence via overexpression of *miR6019* was accompanied by reductions in *N* transcript levels and *N*-mediated resistance against TMV (Li et al., 2012). In addition, a group of 22 nt miRNAs from the *miR482/2118* superfamily targets numerous NLRs within *Solanaceae* species. These miRNAs target highly conserved sequences in the genes encoding predicted NLR proteins (Zhai et al., 2011; Shivaprasad et al., 2012). Activation of VSR induces quantitative changes of whole small RNA species in host cells. Interestingly, VSRs upregulate the transcript levels of the targeted NLRs by attenuating the production or activity of *miR482/2118* family members. The *miR482/2118* family members are thought to ordinarily down-regulate their target *NLR* genes but upregulate these genes only when they are required for plant resistance via the VSRs of viral pathogens (Padmanabhan and Dinesh-Kumar, 2014). Altogether, these studies suggest that the RNA silencing response is integrated with *R* gene-mediated anti-viral resistance responses; however, it is not yet clear whether degradation of the viral genome via host RNA silencing-mediated defense is necessary for *R* gene-mediated defense.

CONCLUDING REMARKS

Since the zig-zag model was first proposed by Jones and Dangl (2006), many interactions between plant and bacterial pathogens have been reported, in which a pathogen suppresses or alters PTI by effectors, and plants have developed induced ETI, a stronger type of defense against effectors, during evolution (Boller and He, 2009). Long-term plant disease resistance studies of viral pathogens have revealed RNA silencing and *R* gene-mediated defense responses. In recent years, studies of the relationship between these two resistance responses have enhanced understanding of the interaction between plants and viruses. As genome analysis techniques are developed,

understanding of plant–virus interactions increases. Kontra et al. (2016) recently reported that the tombusviral P19 suppressor preferentially affects virus-derived small interfering RNAs rather than endogenous host miRNAs in virus-infected plants. The authors suggested that the relationship between VSRs and host RNA silencing, as well as their contribution to the virulence of viruses, should be reconsidered. In parallel, Li et al. (2013) revealed a role for miRNAs in translational inhibition as well as silencing in plants and demonstrated that this process occurs in the ER. It would be interesting to integrate our knowledge of the roles of the ER in viral pathogenesis and in *R* gene-mediated defense responses (Jheng et al., 2014; Verchot, 2014; Moon et al., 2016). Uncovering the subcellular localization of small RNAs, VSR, and R protein will be critical for understanding how the two antiviral pathways interact. Although the concept of PTI and ETI is less clear in viral pathogenesis than in bacterial pathogenesis at present, future in-depth studies of the two anti-viral defenses and cross-talk between them will enhance understanding of plant immune responses, as well as to bacteria and fungi.

AUTHOR CONTRIBUTIONS

JM wrote the paper. JP wrote and edited the paper.

ACKNOWLEDGMENTS

This work was supported by the KRIBB Research Initiative Program, Ministry of Science, ICT, and Future Planning, Republic of Korea.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 04 October 2016
doi: 10.3389/fmicb.2016.01552

Diverse Functions of Small RNAs in Different Plant–Pathogen Communications

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RNA silencing is a conserved mechanism that utilizes small RNAs (sRNAs) to direct the regulation of gene expression at the transcriptional or post-transcriptional level. Plants utilizing RNA silencing machinery to defend pathogen infection was first identified in plant–virus interaction and later was observed in distinct plant–pathogen interactions. RNA silencing is not only responsible for suppressing RNA accumulation and movement of virus and viroid, but also facilitates plant immune responses against bacterial, oomycete, and fungal infection. Interestingly, even the same plant sRNA can perform different roles when encounters with different pathogens. On the other side, pathogens counteract by generating sRNAs that directly regulate pathogen gene expression to increase virulence or target host genes to facilitate pathogen infection. Here, we summarize the current knowledge of the characterization and biogenesis of host- and pathogen-derived sRNAs, as well as the different RNA silencing machineries that plants utilize to defend against different pathogens. The functions of these sRNAs in defense and counter-defense and their mechanisms for regulation during different plant–pathogen interactions are also discussed.

Keywords: small RNA, RNA silencing, plant immunity, pathogen virulence, plant–pathogen interaction

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *25 July 2016*
Accepted: *16 September 2016*
Published: *04 October 2016*

Citation:
Huang J, Yang M, Lu L and Zhang X (2016) Diverse Functions of Small RNAs in Different Plant–Pathogen Communications. Front. Microbiol. 7:1552. doi: 10.3389/fmicb.2016.01552

INTRODUCTION

Small RNAs (sRNAs) are 20–30 nucleotide (nt)-long non-coding RNA molecules, which are widely present in eukaryotic organisms. It is well established that sRNAs are involved in the regulation of gene expression through a process generally termed RNA silencing. RNA silencing contributes to almost all eukaryotic cellular processes, including preventing the invasion of viruses or transgenes, inhibiting the movement of transposable elements, and regulating developmental and physiological processes (Itaya et al., 2008; Wang et al., 2011a; Castel and Martienssen, 2013; Bond and Baulcombe, 2014; Holoch and Moazed, 2015).

Plant sRNAs are divided into two major classes: microRNAs (miRNAs) and small interfering RNAs (siRNAs). Most miRNAs are 21–24 nt in length and derived from RNAs with imperfectly base-paired hairpin structures (Chen, 2009), while siRNAs are generated from perfectly complementary long dsRNAs (Xie et al., 2004). Plant siRNAs are grouped into four subclasses: *trans*-acting siRNAs (ta-siRNAs), heterochromatic siRNAs (hc-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNAs), and long siRNAs (lsiRNAs). Proteins, such as

Dicer-like proteins (DCLs), HYPONASTIC LEAVES 1 (HYL1), HUA ENHANCER 1 (HEN1), and Serrate (SE) are involved in sRNA biogenesis pathways (Katiyar-Agarwal and Jin, 2010; Rogers and Chen, 2013; Holoch and Moazed, 2015). Some siRNAs require RNA-dependent RNA polymerases (RDRs) and suppressor of gene silencing 3 (SGS3) for amplification (Sijen et al., 2007). After processing and amplification, sRNA duplexes are sorted and loaded into Argonaute (AGO) proteins, and the passenger strand is discarded. In animals, the passenger strand is removed by slicing or unwinding in an ATP-dependent reaction (Liu and Paroo, 2010). In plant, however, the removing mechanism of the passenger strand is still unclear. Matured RNA-induced silencing complexes (RISCs) with the guide strands anneal to its complementary sequence and regulate gene expression at transcriptional and post-transcriptional levels through DNA methylation, chromatin modification, mRNA slicing, mRNA degradation, or translational inhibition (Ghildiyal and Zamore, 2009; Zhang X. et al., 2011).

One of the important functions of RNA silencing is to suppress the infection of pathogens. The RNA silencing machinery of host plants can directly target the genomic RNA and transcripts of viruses, viroids, and virus satellites to suppress their RNA accumulation. However, plants are also susceptible to other pathogens, such as bacteria, fungi, oomycetes, and nemotodes, which unlike viruses, do not replicate or expose their genome in host cells during any part of the infection process. To defeat these pathogens, plants have evolved complicated defense systems, including PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). When successful pathogens evolve new effectors to suppress the host ETI response, plants respond by evolving novel resistance (R) proteins to recognize the effectors and trigger ETI responses in this endless arms race.

Both miRNAs and siRNAs contribute to PTI and ETI by fine-tuning plant hormones and/or silencing the genes involved in pathogen virulence (Navarro et al., 2006; Zhang W. et al., 2011). While host sRNAs play important roles in pathogen resistance, pathogens also encode sRNAs to manipulate host defense responses, as well as mediate pathogen virulence. sRNAs in fungi, oomycetes, and bacteria have been shown to function in promoting pathogen virulence. In fungi and oomycetes, sRNAs are mostly generated from transposable element (TE) regions (Nunes et al., 2011; Vetukuri et al., 2012; Weiberg et al., 2013). Key proteins in the RNA silencing machineries, such as DCLs, AGOs, and RDRs, are also present in these eukaryotic plant pathogens and are involved in the biogenesis and function of some sRNAs (Murata et al., 2007; Vetukuri et al., 2011). However, the biogenesis of sRNAs in fungi is more diverse than in plants. Both DCL-dependent and DCL-independent siRNA biogenesis mechanisms were identified in fungi *Neurospora crassa* (Lee et al., 2010). Furthermore, at least four different mechanisms that use distinct combinations of proteins, including Dicers, QDE-2, the exonuclease QIP, and an RNAse III domain-containing protein MRPL3, were proposed to be involved in the biogenesis of miRNA-like small RNAs (milRNAs) in *N. crassa* (Lee et al., 2010). Bacterial non-coding sRNAs are different from sRNAs in eukaryotes (Weiberg et al., 2014). They functionally associate with distinct RNA-binding protein complexes, including the clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) system (CRISPR-Cas) (Fahlgren et al., 2007; Li et al., 2010; Zhang X. et al., 2011; Wiedenheft et al., 2012), the RNA chaperone Hfq (Schu et al., 2015), and CsrA/RsmA (Schu et al., 2015), and regulate the expression of target mRNA through short and impacted base-pair (10–25 nt). Meanwhile, viroids, the smallest known pathogen, which does not code for proteins, have been proposed to encode specific sRNAs that target host genes and result in disease symptoms (Wang et al., 2004). Furthermore, some virus-derived siRNAs (vsiRNAs), which are generated to target viral RNAs, may target host genes, and subsequently mediate the viral disease symptom. Whether viral fitness would be increased by vsiRNAs remains unknown (Qi et al., 2009; Xia et al., 2014). Viruses, oomycetes, and bacteria have RNA silencing suppressors and other effectors that directly inhibit host sRNAs, while some fungi that localize in the intercellular space of plants deliver fungal sRNAs as effectors into plant cells to inhibit the plant PTI response. In this review we will discuss our current understanding of sRNAs in plants and plant pathogens, focusing on their functional differences in plant–pathogen interactions.

PLANTS ENCODE sRNAs THAT FINE-TUNE PLANT HORMONES AND ANTIMICROBIAL ACTIVITY TO DEFEND AGAINST PATHOGEN ATTACK

Although a potent immune system is necessary for plants to survive pathogen infections, it also deprives the limited resources available for plant growth and development. Although more studies need to be done, a constitutively active immune system in plants may result in reduced growth and seed yield (Tian et al., 2003; Walters and Heil, 2007). Thus, plant immune responses must be tightly regulated, and one strategy is to generate endogenous sRNAs that silence specific genes involved in plant hormone production or antimicrobial activity. Upon infection, the biogenesis and/or the accumulation of these sRNAs are regulated, which subsequently fine-tune plant hormone levels and the expression of genes involved in plant resistance (**Figures 1A** and **2A**).

As far as we know, bacteria, fungi, and oomycetes infect plants without direct genome and RNA interaction with the host RNA silencing machineries. To these pathogens, fine-tuning of the plant immune system is critical for host resistance. Various plant miRNAs and siRNAs play critical roles in anti-bacterial resistance (**Figure 1A**). miR393 is the first miRNA shown to function in anti-bacterial defense. The accumulation of miR393 is up-regulated upon the treatment of the conserved N-terminal part of flagellin, flg22, or the infection of bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. miR393 enhances host resistance to *Pst* DC3000 by negatively regulating the expression of F-box auxin receptors, including Transport Inhibitor Response 1 (TIR1), Auxin signaling F-Box proteins 2 (AFB2), and 3 (AFB3) (Navarro et al., 2006). Further

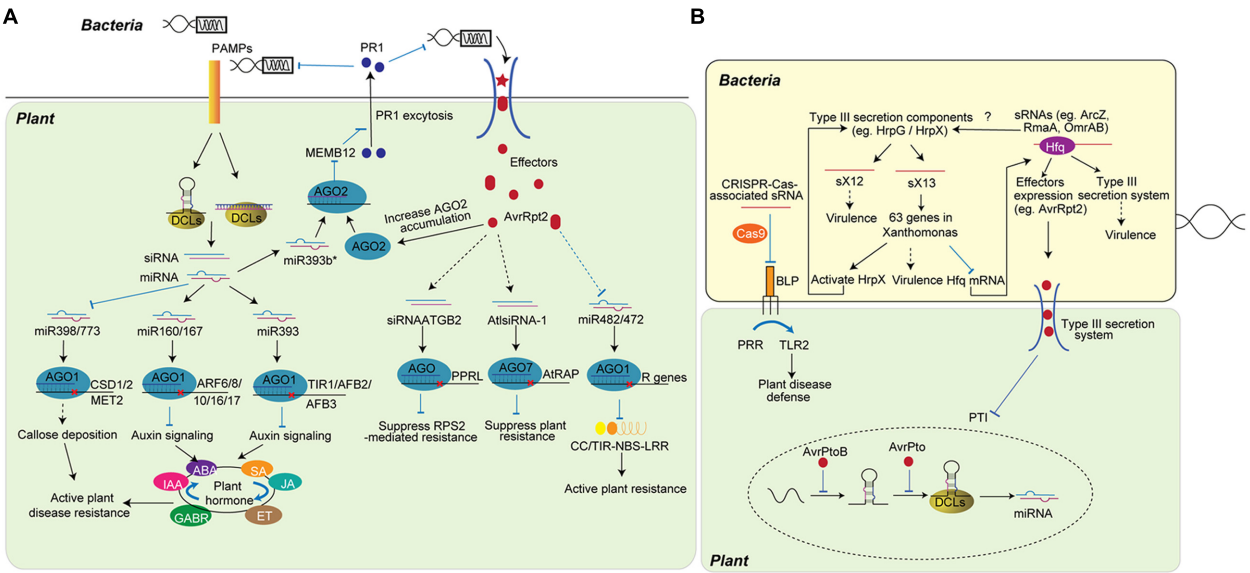

FIGURE 1 | Role of sRNAs in plant-bacteria interaction. (A) Plant sRNAs defend bacteria attack by fine-tuning plant hormone and disease resistance activity. Upon the infection, plants detect PAMPs and modulate the accumulation of miRNA and siRNA. miRNAs, such as miR393, miR160, and miR167, regulate disease resistance by fine-tuning plant hormone networks, while other miRNAs regulate the activation of R protein (miR482/miR472) or the slicing of genes inhibiting plant immunity (miR398/miR773). miR393b*, the pairing strand of miR393, increases plant immunity by promoting the exocytosis of antimicrobial protein. siRNAs, including siRNAATGB2 and AtlsiRNA-1, are induced by bacteria effectors and enhance ETI by silencing genes that negatively regulate plant disease resistance. **(B)** Bacteria non-coding sRNAs (ncRNAs) regulate bacteria gene expression to improve virulence. Through imperfect base-pairing of short regions (10- to 25-nt), bacteria ncRNAs bind to target mRNAs and guide the suppression of genes or proteins that are involved in virulence. ncRNAs can regulate bacteria virulence by inhibiting proteins that trigger host defense (BLP) or affecting the expression of effectors (AvrRpt2). Bacteria effectors translocate into host plant cell and inhibit the regulation of plant sRNA (bottom). The AvrPtoB effector specifically represses the accumulation of miR393 at the transcriptional level, while AvrPto reduces the processing of miR393.

studies in rice determined that miR393 is a *bona fide* stress-related miRNA that is widely involved in plant resistance to other pathogens and abiotic stresses, such as salt and drought (Bian et al., 2012; Xia et al., 2012; Campo et al., 2013). In addition to miR393, miR160 and miR167 also target Auxin response factor (ARF) family transcription factors and are induced by infection with *Pst* DC3000 *hrcC*$^{-}$, a strain with a mutated type III secretion system, to improve plant antibacterial defense (Fahlgren et al., 2007). Further studies uncovered that miR160a and 15 other miRNAs are induced upon flg22 treatment. On the other hand, miR398b, miR773, and 9 other miRNAs are down-regulated upon flg22 treatment (Li et al., 2010). Over-expression of miR398b and miR773 attenuates PTI by repressing flg22 or bacteria-induced callose deposition, indicating miRNAs play important roles in disease resistance. However, the over-expression of miR160, which increases PAMP-induced callose deposition, did not significantly change the basal defense of plant to *Pst* DC3000 bacteria, suggesting a complicate miRNA regulatory network in plant disease responses (Li et al., 2010). Furthermore, miR393b*, the complementary strand of miR393, is loaded into AGO2 and regulates plant resistance by suppressing the expression of MEMB12. MEMB12 is a Golgi-localized SNARE protein, and its down-regulation leads to increased exocytosis of PR1, which subsequently enhances plant resistance (Zhang X. et al., 2011). Thus, miR393 and miR393b*, two sRNAs generated from a same sRNA duplex, bind AGO1 and AGO2 respectively to regulate distinct hormone pathways and coordinately increase plant immunity (Navarro et al., 2006; Zhang X. et al., 2011). Another interesting finding about miRNA in bacterial defending is that one miRNA can target both negative and positive regulators of immunity depending on the timing and the amplitude of defense responses. miR863-3p improves plant defense by silencing a typical receptor-like pseudokinase1 (ARLPK1) and ARLPK2 during early infection, and negatively regulates defense by silencing *SE* gene during later infection (Niu et al., 2016).

In response to sRNA-mediated PTI, successful pathogens deliver effectors into host cells to interfere with PTI. For detailed information about the role of pathogen effectors, several reviews are available (Dou and Zhou, 2012; Feng and Zhou, 2012). To counteract pathogen effectors, plants induce ETI. As ETI is more robust and usually triggers a hypersensitive response (HR), the ETI reaction is strictly regulated by siRNAs and miRNAs. siRNA nat-siRNAATGB2, which is specifically induced by *Pst* DC3000 effector protein AvrRpt2, enhances ETI by suppressing the expression of pentatricopeptide repeats (PPR) protein-like gene (PPRL) and preventing the negative effect of PPRL on the resistance pathway mediated by *RPS2*, a resistance gene that specifically recognizes effector AvrRpt2 (Katiyar-Agarwal et al., 2006). AtlsiRNA-1, which is also induced by AvrRpt2, improves disease resistance by silencing the expression of AtRAP, a negative regulator of plant disease resistance (Katiyar-Agarwal et al., 2007). In addition to these sRNAs, genome-wide sRNA

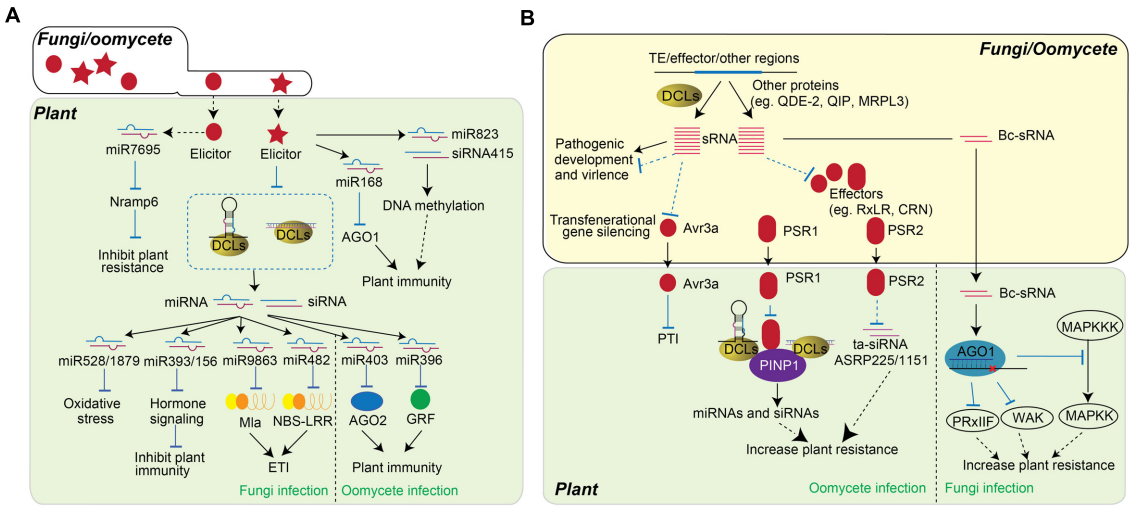

FIGURE 2 | Role of sRNAs in plant-fungi/oomycete interaction. (A) Plant sRNAs regulate PTI and ETI in response to fungi or oomycete infection. The infection of fungi (left) and oomycete (right) alters the accumulation of miRNAs, by which changes the expression of genes contribute to plant resistance. Fungi elicitorsor fungi infections triggers the accumulation of some sRNAs, such as miR7695, miR168, miR823 and siRNA415, while miR528, miR1879, miR9863, and miR482 are down-regulated to improve plant resistance. The accumulations of miR403 and miR396 are down-regulated upon oomycete infection. **(B)** Schematic representation of the function of fungi/oomycete sRNAs in pathogen virulence. sRNAs encoded by fungi and oomycetes are usually generated from TE region, effector coding region, and other regions. These sRNA can be either DCL-dependent or DCL-independent and are involved in the regulation of pathogen development and virulence. In particular, sRNA regulate the expression of effectors, which further influence the accumulation of host miRNA and siRNA. sRNAs generated from Avr3a region of oomycete can transgenerationally change the pathogen virulence. The PSR1 and PSR2 effectors of oomycete are secreted into plant cells and alter host RNA silencing machineries as RNA silencing suppressor to decrease host immunity. On the other hand, fungi sRNAs, Bc-sRNAs, translocate into host cell and utilize plant RNA silencing component to reduce the expression of host immune genes and facilitate fungi infection.

deep sequencing indicates that the accumulation of more than 20 miRNAs and various nat-siRNAs are significantly altered upon ETI (Zhang M. et al., 2011; Zhang et al., 2012). Some targets of these miRNAs are key genes contributing to the hormone biosynthesis and signaling pathways involved in plant resistance. A TE-siRNA, TE-siR815, generated from the intron of WRKY45-1, represses ST1 and subsequently attenuates WRKY45-mediated resistance to *Xanthomonas oryzae pv. Oryzae*, which results in the opposite functions of *WRKY45-1* and *WRKY45-2* (Zhang et al., 2016).

Host sRNAs contribute to ETI not only by regulating the expression of genes involved in plant resistance but also by directly regulating the activation of R proteins. For instance, *RPP4* and *SNC1*, two *R* genes located in the *RPP5* locus, are involved in disease resistance against bacterial and fungal pathogens (Baldrich et al., 2014, 2015). A study demonstrated that these *R* genes are negatively regulated by RNA silencing. The *SNC1* gene was up-regulated in *dcl4* and *ago1* mutants (Yi and Richards, 2007). When a pathogen interferes with host RNA silencing, it may subsequently disturb the sRNA-mediated inhibition of *R* genes and activate the function of these R proteins. However, sRNAs complementary to the *SNC1* region are not increased in *dcl4* and *ago1* mutants, suggesting that other sRNAs may contribute to the up-regulation of *SNC1* in these mutants (Yi and Richards, 2007). The accumulation of miR482 is decreased in plants infected with *Pst* DC3000 but not *Pst* DC3000 *hrcC*$^{-}$ (Shivaprasad et al., 2012). Further study predicted that miR482 can target mRNAs of 58 coiled-coil, nucleotide-binding site, leucine rich repeat proteins (CC-NBS-LRR). Meanwhile, the production of secondary siRNA, caused by the targeting in a RDR6 dependent manner, may target other mRNAs of a defense-related protein. Thus, upon the infection of virus or bacteria, the accumulation of miR482 is decreased to suppress the miR482-mediated silencing cascade, and subsequently increase the expression of defense-related mRNAs (Shivaprasad et al., 2012). miR482 in cotton was also reported to target more than 10% of NBS-LRR genes and triggers the production of secondary siRNAs. Infection with fungal pathogen *Verticillium dahliae* down-regulates miR482 accumulation and increases NBS-LRR gene expression in cotton (Zhu et al., 2013). Interestingly, *Arabidopsis* miR472, which targets RPS5 CC-NBS-LRR genes, modulates both PTI and ETI pathways. Mutation in miR472 results in increased resistance to both *Pst* DC3000 and *Pst* DC3000 *avrPphB* (Boccara et al., 2014).

Host sRNAs also regulate PTI and ETI upon various fungal and oomycete attack (**Figure 2A**). *Magnaportbe oryzae* is a rice blast fungus that causes rice blast disease. The accumulation of rice miRNA528/miR1879 is down-regulated by treatment with a *M. oryzae* elicitor, resulting in up-regulation of their target genes that control oxidative stress (Baldrich et al., 2015). Meanwhile, the accumulation of miR393b/miR156 are also negatively altered upon the elicitor treatment of *M. oryzae* on rice (Campo et al., 2013). On the other hand, a novel DCL4-processed miRNA, osa-miR7695, was identified in rice to target an alternatively spliced transcript of *Nramp6* (Natural resistance associated macrophage protein 6) gene and its overexpression results in

enhanced resistance to *M. oryzae* infection (Campo et al., 2013). Further study identified a group of small RNAs, including miR156, miR165/166, miR170, and miR172 in *Arabidopsis* that were regulated by elicitors of the fungus *Fusarium oxysporum*. Particularly, miR168, which is known to regulate plant abiotic responses via control of AGO1, was transcriptionally activated, and its upregulation negatively correlated with AGO1 transcripts (Baldrich et al., 2014). In addition, miR823 and siRNA415, both of which are involved in RNA-directed DNA methylation (RdDM), were also found to be induced by fungal elicitors (Baldrich et al., 2014). The regulation of these sRNAs by fungal elicitors suggests their functions in PTI. *Puccinia graminis* f.sp. *tritici* (*Bgt*) is a powdery mildew fungus that causes devastating disease in wheat, barley, and other plants. Eight different miRNAs, miR159, miR164, miR167, miR171, miR444, miR408, miR1129, and miR1138, that regulate three different defense response processes are significantly induced at the early, but not the late, stage of *Bgt* infection. Thus, these miRNAs may play a key role in HR at the onset of disease (Gupta et al., 2012). The roles of sRNA in plant immune response were further demonstrated in powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) (Liu et al., 2014). *Mildew resistance locus a* (*Mla*), encoding a group of CC-NBS-LRR proteins that respond to *Bgh*, are targeted by the miRNA family miR9863. miR9863 was shown to guide the cleavage of *Mla1* transcripts in barley, and down-regulate the accumulation of MLA1 protein in the *Nicotiana benthamiana* expression system. In addition, miR9863 can trigger the biogenesis of 21-nt phased siRNAs (phasiRNAs) and further repress the expression of *Mla1*. Over-expression of miR9863 specifically attenuates Mla1-mediated cell death and disease resistance (Liu et al., 2014). miR482 in potato can also target NBS-LRR genes. *V. dahliae* infection down-regulates the accumulation of miR482, which in turn increases NBS-LRR gene expression (Yang et al., 2015). The silencing of NBS-LRR genes by these specific 22-nt miRNAs, and their activation after miRNA down-regulation upon bacteria, fungal, or viral treatments, have been widely studied in different plants (He et al., 2008; Xin et al., 2010; Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012; Zhu et al., 2013; Boccara et al., 2014; Liu et al., 2014; Fei et al., 2015). *Phytophthora sojae* is a notorious oomycete that infects soybean root and stem. *P. sojae* infection down-regulates the expression of miR403, a miRNA that targets AGO2, a positive regulator of plant immunity (Guo et al., 2011). Similarly, the accumulation of sRNAs and their targets are also differently regulated in susceptible and resistance soybean cultivars. The expression of miR396 in *Solanaceae* is down-regulated upon infection with another oomycete, *Phytophthora infestans*. Over-expression of miR396 resulted in the down-regulation of GRF targets and increased susceptibility to *P. infestans* (Chen et al., 2015). It is clear that plant sRNAs play a critical role in regulating the expression of genes involved in plant defense and immunity. However, each sRNA has distinct function in plant immune response, and the accumulation and the function of sRNAs are pathogen-dependent. Therefore, in order to obtain the systematic role of RNA silencing in plant resistance, the function of more sRNAs needs to be further investigated.

PLANTS UTILIZE sRNAs TO DEFEND AGAINST PATHOGEN BY DIRECTLY TARGETING ON VIRAL AND VIROID GENOMES AND TRANSCRIPTS

Viruses and viroids infect plants by replicating their genomes inside the host cells. Post-transcriptional gene silencing (PTGS) was first identified in both transgenes processing and *Potato virus X* (PVX) infection. sRNAs complementary to the sense transcript of the transgene and the positive strand of PVX were discovered, indicating that sRNAs participate in PTGS transgene silencing and viral defense (Hamilton and Baulcombe, 1999). Further studies revealed that the replication of viruses and viroids, and the folding of their RNA genomes and transcripts, produce dsRNAs that recruit RNA silencing machinery (Ding, 2009).

Viruses contain either single-stranded RNA (ssRNA), double-stranded RNA (dsRNA), ssDNA, or dsDNA genomes (Ding and Voinnet, 2007). During the replication of an ssRNA viral genome, a complementary strand of RNA is synthesized, which forms a long dsRNA with the original viral genome. The dsRNA replicative intermediate forms of ssRNA viruses and the dsRNA genomes of dsRNA viruses can be targeted by host RNA silencing machineries (**Figure 3A**). Nearly equal amounts of positive and negative strand vsiRNAs without positional bias were derived from *Cucumber yellows closterovirus* (CuYV), *Turnip mosaic potyvirus* (TuMV), CMV, *Watermelon mosaic virus* (WMV), PVX, and *Tomato yellow leaf curl virus* (TYLCV), all positive ssRNA viruses from different families (Yoo et al., 2004; Ho et al., 2006; Donaire et al., 2009; Wang et al., 2010). It was also shown that vsiRNAs were nearly equally derived from the positive and negative genome of *Rice stripe virus* (RSV), an ambisense virus with four genomic ssRNAs (Yan et al., 2010). However, more than 80% of vsiRNAs derived from *Cymbidium ringspot virus* (CymRSV) are generated from the positive strand (Molnar et al., 2005). Similar phenomena are also observed in plants infected with other ssRNA viruses such as TCV, *Tobacco mosaic virus* (TMV), *Tobacco rattle virus* (TRV), and *Pepper mild mottle virus* (PMMoV), in which some positive strand vsiRNAs can account for 97% of total vsiRNAs (Ho et al., 2006; Donaire et al., 2009; Qi et al., 2009). There are no dsRNA intermediate replicative forms for ssDNA and dsDNA viruses. Some vsiRNAs generated from DNA viruses display strong strand bias, indicating that these vsiRNAs may be processed from the structured region of the viral RNA transcripts. 62% of the vsiRNAs match the transcript polarity of *Cauliflower mosaic virus* (CaMV), a virus from which the commonly used constitutive 35S promoter is derived. Although up to 82% of vsiRNAs are generated from the leader region, these exhibit no strand bias (Blevins et al., 2011). *Tomato yellow leaf curl China virus* (TYLCCNV) is a *Geminiviridae* that has an ssDNA genome. Although the vsiRNAs derived from TYLCCNV display site bias, they map nearly equally to the positive and negative genomes (Yang et al., 2011a). Thus, both the dsRNA replicative form and the secondary structure of viral genomes can processed by host RNA silencing machineries. However, the implication of these findings on viral pathogenicity or evolution is still unknown.

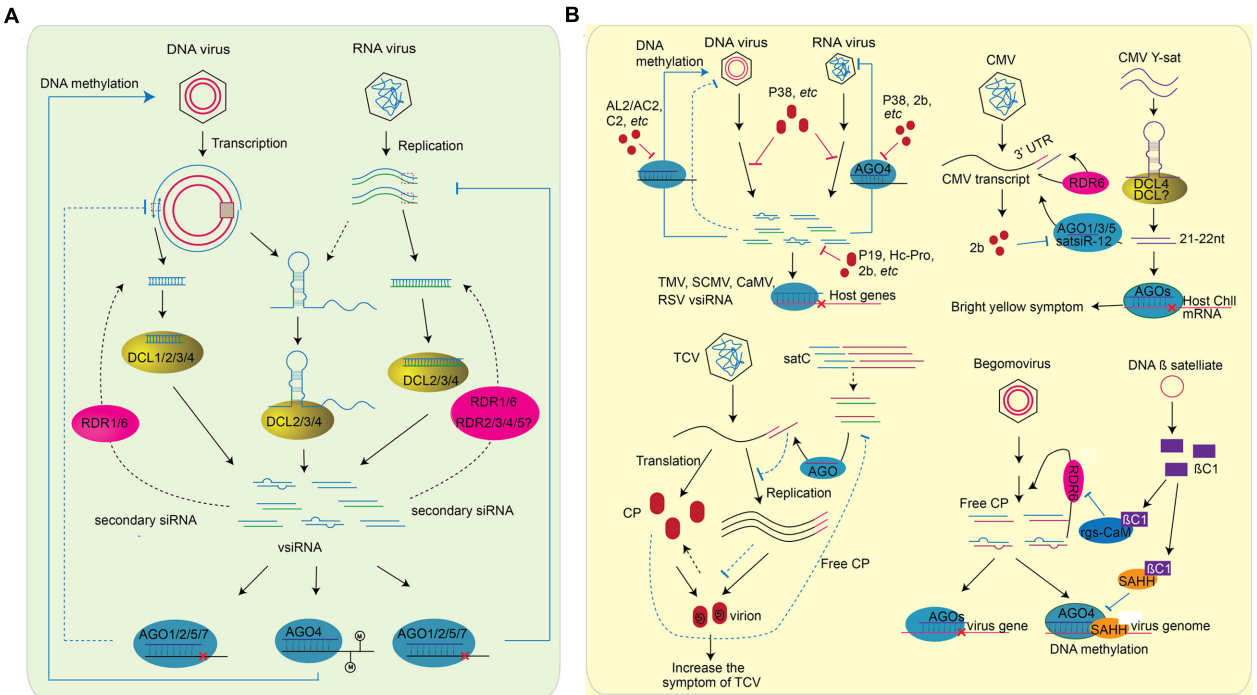

FIGURE 3 | Role of sRNAs in plant-virus interaction. RNA silencing inside plant cells can be divided into two parts: **(A)** Plant generate vsiRNAs, targeting on virus genome directly to defend viral infection. The generation of vsiRNA are slightly different for RNA virus or DNA virus. For RNA virus, the structure region of virus genome, dsRNA replicative intermediate forms of ssRNA viruses, and the dsRNA genomes of dsRNA viruses can be processed by DCL proteins (right). The vsiRNAs of DNA virus, on the other hand, can be processed from the structured region of the transcript and the overlapping region of the bi-direction transcription (left). In both cases, RDR1 and RDR6 are involved in the generation of secondary vsiRNA (shown in blank dash line). After generation, vsiRNAs are loaded into different AGOs and perform the silencing of virus genome. vsiRNAs target on RNA virus to slice the genomic RNA, while perform DNA methylation on the genome of DNA virus. Whether vsiRNA targets on the transcription of DNA virus remains unknown (blue dash line). **(B)** The counter-defense of virus to plant RNA silencing machinery. As plant generates vsiRNA to silence virus genome, viruses encode suppressors, such as 2b, Hc-Pro, P19, AL2/AC2, P38, and etc., as a counter-defense (left above). The effect of suppressor on RNA silencing include the interfere of DCL slicing, the blocking of methylation, the binding of vsiRNsA, the preventing of RISC assembly, and etc. vsiRNAs encoded by TMV, CMV, CaMV, and RSV can also target the host genes to decrease plant defense. In addition, plant viruses are often accompanied with a variety of subviral RNA/DNAs. These satellite RNA/DNAs affect virus pathogenicity by generating satRNA-derived siRNAs (satsiRNAs). CMV Y satellite (Y-sat) produces a 22-nt satsiRNA that targets Chll, a key gene involved in chlorophyll synthesis, resulting in bright yellow symptom. sat-siR-12, another satsiRNA can loaded into AGO1/3/5 and regulate CMV transcripts accumulation with the function of RDR6. As counter defense, CMV encodes VSR 2b to inhibit the function of AGOs (right above). TCV is often accompanied with a single strand satellite RNA (satC) that is composed of the 3′ end of TCV helper virus (left bottom). Because of the sequence similarity of satsiRNA and the 3′ end of TCV helper virus, the presence of satC-siRNA represses the accumulation of TCV genomic RNA. At the same time, TCV genomic RNA and the CP protein assemble to a virion. CP is a VSR encode by TCV. The down-regulation of TCV transcripts by satC-siRNAs result in the increase of free CP protein, which subsequently suppresses the accumulation of satC-siRNAs (shown in dash line). DNA ß satellites are circular ssDNA that associate with many monopartit begomoviruses. The ßC1 protein encoded by DNAß satellite is a VSR that suppresses TGS by the interaction with SAHH, and PTGS through the interaction with rgs-CaM (right bottom).

Viroids, the smallest pathogen that can replicate in the nucleus or chloroplast, consists of naked, single-stranded, closed circular RNAs with sizes ranging from 250- to 400-nt (Ding, 2009). More than two decades ago, people noticed that *Potato spindle tuber viroid* (PSTVd) infection results in full methylation of the PSTVd cDNA sequence that is inserted into the tobacco genome (Wassenegger et al., 1994). This methylation occurs by viroid-induced RNA silencing and RdDM. Later studies detected siRNAs in PSTVd-infected tomato and tobacco plants and proved that viroids are the activator and target of RNA silencing (**Figure 4A**) (Itaya et al., 2001; Papaefthimiou et al., 2001). Viroid-associated siRNAs (vdsiRNAs) of PSTVd are generated from both polarities in the left and right domains. By profiling PSTVd vdsiRNAs through deep sequencing, Itaya et al. (2007) uncovered that PSTVd vdsiRNAs predominately map to the positive strand of the left and right terminal regions, indicating that these sRNAs are generated from the secondary structure of plus-strand RNAs. Some vdsiRNAs are also generated from the negative strand of the central part, indicating they may be processed from the secondary structure of the negative-strand viroid genomic RNA (Itaya et al., 2007). *Citrus exocortis viroid* (CEVd) replicates in the nucleus and mainly generates 5′-phosphorylated and 3′-methylated vdsiRNAs with positive polarity. Most CEVd vdsiRNAs are located within the right-end domain, suggesting that structured RNA is the main substrate of DCL enzymes (Martin et al., 2007). *Avocado sunblotch viroid* (ASBVd), *Peach latent mosaic viroid* (PLMVd), and *Chrysanthemum chlorotic mottle viroid* (CChMVd) are three viroids that replicate in the chloroplast. CChMVd and PLMVd

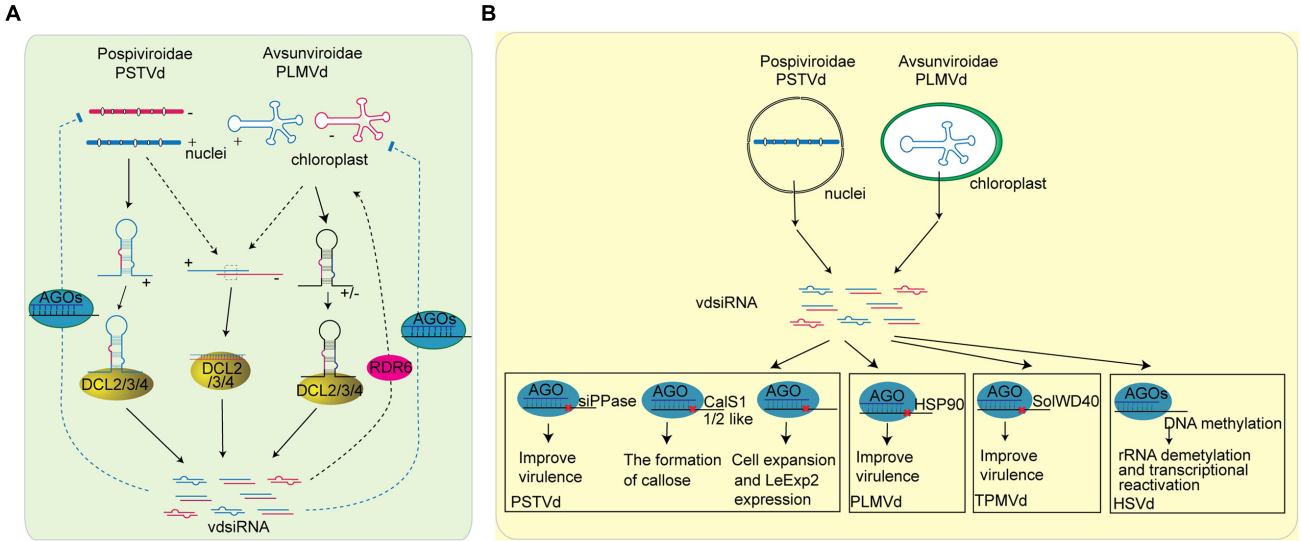

FIGURE 4 | Role of sRNAs in plant-viroid interaction. (A) The biogenesis of vdsiRNAs in plant and the possible function of vdsiRNA in plant defense to viroid. PSTVd is mainly found in nucleolus, and its vdsiRNAs predominately map to the positive strand of the left and right terminal regions. It is most likely that PSTVd-vdsiRNAs are generated from the hairpin or stem-loop structure of plus-strand of PSTV transcripts. The secondary structure of PSTVd transcripts are targeted by DCL protein and sliced into vdsiRNA. Another possible source of vdsiRNA are the accidental association of (+) and (–) strand replication, which are further target by DCL protein. On the other hand, PLMVd, viroid that replicate in the chloroplast, generate vdsiRNAs from both polarities. The stem-loop structures of PLMVd are processed by DCL protein to generate vdsiRNAs. Furthermore, some research indicate that vdsiRNAs can be amplified through the activity of RDRs. After generation, vdsiRNA may be loaded into plant AGO proteins and target viroid RNAs. **(B)** The function of vdsiRNAs in producing viroid symptom. Some of the viroid symptom maybe caused by vdsiRNAs that target host genes. vdsiRNA generated by PSTVd can target on various plant genes including soluble inorganic pyrophosphatase (siPPase) gene, callose synthase genes CalS11-like and CalS12-like, and LeExp2 gene, while PLMVd vdsiRNA has been reported to target HSP90 and trigger signal transduction that eventually leads to viroid disease symptoms. TPMVd vdsiRNA has also been shown to slice the SolWD40 gene. In addition, HSVd vdsiRNAs are involved in TGS by inducing DNA methylation of the promoter region of rRNA genes.

generate vdsiRNAs from both polarities (de Alba et al., 2002; St-Pierre et al., 2009). ASBVd also generates vdsiRNAs in leaves displaying bleached symptoms (Markarian et al., 2004). Thus, both *Pospiviroidae* and *Avsunviroidae* viroid families can produce vdsiRNAs in plants (Ding and Itaya, 2007; Ding, 2009; Hammann and Steger, 2012). The fact that vdsiRNAs can be generated from both the positive and the negative strand of the viroid genome with strand and position bias indicates that vdsiRNAs are predominately processed from the secondary structure of the viroid genomic RNAs. However, it is important to point out that the discoveries of vdsiRNAs may be biased due to the current methods for sRNA cloning.

dsRNA inducers are processed by plant DCL proteins to generate sRNAs. *Arabidopsis* encodes four DCL proteins that generate different sRNAs: DCL1 processes hairpin pri-miRNAs and pre-miRNAs into 21-nt miRNAs; DCL3, DCL4, and DCL2 process long dsRNAs into 24-nt hc-siRNAs, 21-nt siRNAs, and 22-nt siRNAs, respectively. For RNA viruses and viroids, the perfectly paired dsRNA intermediate replication form and the hairpin structure of the single genomic RNA are predominant dsRNA inducers. Indeed, DCL4, DCL2, and DCL3 process ssRNA viruses (e.g., CMV, TuMV, and TCV) into 21-nt, 22-nt and 24-nt vsiRNAs, respectively (Bouche et al., 2006; Deleris et al., 2006; Fusaro et al., 2006; Diaz-Pendon et al., 2007; Garcia-Ruiz et al., 2010). The newly emerging systemic leaves of PSTVd-infected plants only accumulate shorter (21–22-nt) vdsiRNAs, while the older leaves contain both shorter and longer (24-nt) vdsiRNAs (Machida et al., 2007; Schwind et al., 2009). Similar vdsiRNA accumulation patterns are also present in *Hop stunt viroid* (HSVd)- and *Hop latent viroid* (HLVd)-infected plants. However, very little is known about the biogenesis of vdsiRNAs. 21-nt, 22-nt, and 24-nt vsiRNAs also accumulate in plants infected with *Cabbage Leaf Curl Virus* (CalCuV), *Beet curly top virus* (BCTV), and *Pepper golden mosaic virus* (PepGMV), which are all ssDNA viruses, and CaMV, a dsDNA virus (Blevins et al., 2006; Rodriguez-Negrete et al., 2009; Raja et al., 2014). 24-nt vsiRNAs are the predominant vsiRNAs produced from DNA viruses. DCL3, DCL4, and DCL2 are required for the accumulation of 24-nt, 21-nt, and 22-nt CalCuV vsiRNAs, respectively. DCL3 and DCL4 are also responsible for accumulation of 24- and 21-nt vsiRNAs derived from BCTV, respectively (Raja et al., 2014). Although the hairpin structure of viral or viroid genomes is one of the main sources of vsiRNA and vdsiRNAs, DCL1-dominant hairpin processing is not involved in vsiRNA and vdsiRNAs accumulation or anti-RNA-viral resistance. However, DCL1, but not DCL4, is required for the accumulation of 21-nt vsiRNAs from CaMV (Blevins et al., 2006).

After the initial processing of dsRNA inducers, the antiviral and antiviroid signals are amplified by host RDRs. *Arabidopsis* encodes six RDRs, among which the function of RDR1, RDR2, and RDR6 have been well studied. RDR1 is induced by

salicyclic acid (SA) treatment and TMV infection in tobacco and *Arabidopsis*, and a mutation in RDR1 permits efficient multiplication of ssRNA viruses (Xie et al., 2001; Yu et al., 2003; Yang et al., 2004). Furthermore, the *Arabidopsis rdr6* mutant is more susceptible to infection with ssRNA and ssDNA viruses (Dalmay et al., 2000; Mourrain et al., 2000; Dalmay et al., 2001; Muangsan et al., 2004). Infection with RSV (a negative ssRNA virus) and RDV (a dsRNA virus) decreases the expression of rice RDR6. Down-regulation of rice RDR6 by antisense transformation results in increased susceptibility to RDV (Jiang L. et al., 2012; Hong et al., 2015). Both RDR1 and RDR6 are required for secondary CMV vsiRNA production in *Arabidopsis*: RDR1 is required for the production of vsiRNAs from 5′-terminal ends of the genome, while RDR6 is required for the production of vsiRNAs from the 3′-terminal ends (Wang et al., 2010). However, expression of *Nicotiana tabacum* RDR1 in *N. benthamiana* plants (which do not encode RDR1) showed that RDR1 suppresses RNA silencing mediated by RDR6 and enhances viral infection in transgenic plants (Ying et al., 2010). In addition, accumulation of HSVd and PSTVd genomic RNAs was higher in RDR6-silenced plants, indicating that RDRs also contribute to anti-viroid resistance (Gomez et al., 2008; Di Serio et al., 2010). Systematic analysis via profiling of vsiRNAs and vdsiRNAs in pathogen infected plants have revealed that sRNAs processed from pathogen genomic RNAs indeed decrease in *rdr* knock-out mutants or RDR-silenced plants (Gomez et al., 2008; Di Serio et al., 2010; Garcia-Ruiz et al., 2010; Wang et al., 2010; Hong et al., 2015). The decreased accumulation of vsiRNAs and vdsiRNAs and increased susceptibility of *rdr* mutant plants demonstrate the anti-viral/viroid role of RDRs. Although RDR2 is responsible for 24-nt hc-siRNA accumulation, mutation in *RDR2* has little or no effect on the accumulation of vsiRNAs of DNA viruses CalCuV and CaMV (Blevins et al., 2006). Tomato *Ty-1* and *Ty-3* are alleles of the same gene that encodes RDRs with sequence similarity to *Arabidopsis* RDR3, RDR4, and RDR5. They are TYLCV resistance genes, and susceptible tomato lines without these loci produce lower levels of TYLCV vsiRNAs and accumulate higher viral titers (Verlaan et al., 2013; Butterbach et al., 2014). However, in *Arabidopsis,* the antiviral functions of RDR3, RDR4, and RDR5 have not yet been uncovered. Therefore, the function of RDR2 and other RDRs in host-virus/viroid interactions needs to be further explored.

After processing and amplification, vsiRNAs and vdsiRNAs are loaded into AGO proteins to inhibit the replication and movement of viruses and viroids. AGO1 (Morel et al., 2002; Zhang et al., 2006), AGO2 (Takeda et al., 2008; Harvey et al., 2011; Jaubert et al., 2011; Wang X.B. et al., 2011), AGO3 (Schuck et al., 2013), AGO5 (Takeda et al., 2008), AGO7 (Qu et al., 2008), and AGO10 (Garcia-Ruiz et al., 2015) have been shown to bind vsiRNAs or be involved in anti-viral RNA silencing pathways. Recovery from infection with a DNA virus requires the function of host AGO4 (Raja et al., 2008). Thus, the 24-nt vsiRNAs of DNA viruses may associate with AGO4 to methylate the viral genome. A mutant defective in DRB3, a double-stranded RNA binding protein that interacts with DCL3 and AGO4, displays lower methylation of the viral DNA genome and increased hyper susceptibility to germinivirus, further demonstrating the function of the DCL3-AGO4 RdDM pathway in resistance against DNA viruses (Raja et al., 2014). Furthermore, AGO18, a novel AGO that is conserved in monocot plants, is induced by RSV and required for rice antiviral resistance (Wu et al., 2015). In regards to the vdsiRNAs, the 21-nt and 22-nt vdsiRNAs are predominately loaded into AGO1, AGO2, and AGO3 (Minoia et al., 2014); the 24-nt vdsiRNAs are loaded into AGO4, AGO5, and AGO9; while AGO6, AGO7, and AGO10 do not bind vdsiRNAs (Minoia et al., 2014). However, the anti-viroid function of these AGOs needs to be further determined.

Although vdsiRNAs processed by DCLs are loaded into plant AGOs, their regulation of viroid genomes is not well known. PSTVd, CEVd, and CChMVd in plants can be silenced by transgenic dsRNAs or co-inoculated dsRNAs. This silencing is sequence-specific, temperature-dependent and, in some cases, dose-dependent (Vogt et al., 2004; Carbonell et al., 2008; Schwind et al., 2009). However, further studies indicate that viroids may have evolved a mechanism to avoid the silencing of sRNA. Dr. Biao Ding's group found that PSTVd replicates easily in infected plants even with the present of high accumulation of vdsiRNAs (Itaya et al., 2001). Studies on PSTVd and HSVd show that the circular genome of the viroid is resistant to RNA silencing (Wang et al., 2004; Gomez and Pallas, 2007). A possible explanation is the structured viroid RNA can be processed into active vdsiRNAs, but the viroid RNA is resistant to RISC-mediated degradation due to its secondary structure (Itaya et al., 2007).

PATHOGEN sRNAs REGULATE PATHOGEN GENE EXPRESSION TO INCREASE VIRULENCE

During plant–microbial pathogen interaction, host miRNAs and siRNAs play a role in modulating host immunity while some sRNAs derived from pathogens can decrease host defense or increase pathogen virulence. Fungi, oomycetes, bacteria, viruses, viroids, and satellite RNAs all produce sRNAs that are either similar to or distinct from plant sRNAs (**Figures 1B**, **2B**, **3B**, and **4B**). During the counter-defense response, these pathogen sRNAs facilitate infection by adjusting pathogen gene expression to increase virulence.

Fungi and oomycetes encode siRNAs that are mainly derived from transposons, inverted, tandem, or other repeat regions, and effector coding regions. These sRNAs display diverse biogenesis pathways, and some require typical RNA silencing components, such as DCLs, AGOs, and RDRs for accumulation (Murata et al., 2007; Lee et al., 2010; Jiang N. et al., 2012; Fahlgren et al., 2013; Qutob et al., 2013; Raman et al., 2013; Weiberg et al., 2013). sRNAs in fungal pathogens have been shown to mediate pathogenic virulence by traveling into host cells and silencing host genes (**Figure 2B**) (Weiberg et al., 2013). Although there is indirect evidence that links sRNAs to pathogen virulence, the function of sRNAs in pathogen cells has not been well studied. The differential accumulation of *M. oryzae* sRNAs in vegetative and specialized-infection tissues suggests that sRNAs in *M. oryzae* may be involved in growth, development, and virulence (Nunes et al., 2011).

Moreover, sRNA profiling of *M. oryzae* identified a set of genes that are transcriptionally regulated by sRNAs. One of these is *ACE1*, a known avirulence gene that has increased expression in the *dcl1* mutant (Raman et al., 2013). The sRNAs in three *Phytophthora* species, *P. infestans*, *P. sojae* and *Phytophthora ramorum*, were analyzed, and they were predominantly 21-nt and 25-nt long (Fahlgren et al., 2013). The 21-nt sRNAs were found to be derived from gene families including Crinkler (CRN) effectors and type III fibronectins. Some of these 21-nt sRNAs are predicted to target amino acid/auxin permeases, but their exact functions are still unknown (Fahlgren et al., 2013). sRNAs generated from RxLR and CRN effectors loci were also identified. The expression levels of these effectors and the sRNAs, vary in *P. infestans* strains that differ in virulence, suggesting that these sRNAs may affect the accumulation of effectors, thus alter the virulence (Vetukuri et al., 2012). Some sRNAs map to the tRNA loci of fungi and oomycetes (Nunes et al., 2011; Asman et al., 2014). The biogenesis of these sRNAs requires pathogen DCLs and AGOs. The accumulations of these sRNAs are significantly changed during the infection progress, which suggests that these sRNAs may function in pathogen-host interaction. Moreover, recent study have identified sRNAs associated with *P. infestans* AGO proteins (Asman et al., 2016). PiAGO1-associated 20–22 nt sRNAs, were generated from genes encoding host cell death-inducing CRN effectors, while 24–26 nt sRNAs, which bound to PiAGO4, were derived mainly from Helitron, Crypton, PiggyBac and Copia transposons. The essential role of PiAGO1 in gene regulation, together with its associated sRNAs, which derived from CRN gene family, implicating 20–22 nt sRNAs may bind to AGO1 to regulate the expression of genes in CRN family and subsequently mediate the pathogen virulence (Asman et al., 2016). In addition, sRNAs that are derived from the effector regions can transgenerationally alter the virulence of the pathogen. Avirulence (Avr) gene *Avr3a* of *P. sojae* encodes an effector protein that can be detected by the host *R* gene. The expression of *Avr3a* gene in *P. sojae* attenuated the virulence of plants carrying the *R* gene *Rps*3a. Qutob et al. (2013) observed non-Mendelian inheritance of transgenerational gene silencing of *Avr3a* and gain of virulence in soybean plants. Meanwhile, increased accumulation of 25-nt sRNAs was seen in gene-silenced strains but not in strains with *Avr3a* mRNA, indicating there is sRNA-associated transgenerational gene silencing (Qutob et al., 2013).

Until now bacteria have not been found to encode typical sRNAs as plants, but they produce 50- to 300-nt non-coding sRNAs (ncRNAs) that regulate the expression of target mRNAs through imperfect base-pairing of short regions (10- to 25-nt) (**Figure 1B**) (Altuvia, 2007; Weiberg et al., 2014). There is an emerging body of evidence suggesting that ncRNAs are involved in bacterial virulence. Bacterial lipoprotein (BLP) triggers cell activation and host defense through toll-like receptors (TLRs). CRISPR-Cas-associated sRNAs from *Francisella novicida* guide the Cas9 protein to suppress BLP, which subsequently facilitates evasion of TLR2 (Sampson et al., 2013). The Cas9 system acting with a small, CRISPR/Cas-associated RNA (scaRNA) also controls virulence of *Francisella tularensis* (Sampson et al., 2013). However, a direct link between the Cas system and plant bacterial pathogenesis has not yet been found. Genome-wide transcriptome analysis has identified 16 intergenic sRNAs and seven *cis*-encoded antisense sRNAs in the plant pathogen *Xanthomonas campestris* pv. *vesicatoria* (Xcv) (Schmidtke et al., 2012). The expression of half of these intergenic sRNAs is controlled by components of the type III secretion system, and some are involved in virulence. The deletion of sX12 delays the development of disease symptoms and HR in pepper plants (Schmidtke et al., 2012). The 115-nt sRNA sX13 regulates 63 genes, which are involved in signal transduction, motility, transcriptional and posttranscriptional regulation, and virulence. Deletion of *sX13* strongly delayed development of disease symptoms in susceptible and resistant pepper plants (Schmidtke et al., 2013). However, the function of sX13 is not dependent on Hfq, a hexameric RNA-binding protein that globally interacts with sRNAs to post-transcriptionally regulate gene expression and virulence traits in many animal and plant pathogenic bacteria. Hfq can bind up to 100 sRNAs in *Salmonella* (Chao and Vogel, 2010). Hfp-dependent sRNAs in *Erwinia amylovora* were also identified, and 40 of them were found to associate with Hfq. sRNAs ArcZ, RmaA, and OmrAB all contribute to virulence by positively modulating type III secretion system attachment, amylovoran production, and motility (Zeng et al., 2013; Zeng and Sundin, 2014).

PATHOGENS ENCODE sRNAs TARGETING HOST GENES TO IMPROVE VIRULENCE

Another strategy of pathogens to counteract host defenses is the production of sRNAs that target host genes to decrease host immune responses. Viroids do not code any protein or peptide and yet are able to replicate, travel cell-to-cell and long distance through phloem, resist plant defense responses, and cause disease in certain hosts (Ding and Itaya, 2007; Ding, 2009). For a long time, the question of how viroids produce disease symptoms without any open reading frames has intrigued scientists. Early studies focus on explaining the molecular mechanism of viroid pathogenesis by determining the interaction of genomic RNA of viroid with host factors, including host proteins or nucleic acids (Navarro et al., 2012a,b). While a few proteins or RNAs were determined to interact with viroid RNA, their roles in viroid pathogenesis is largely inclusive. In recent years, the new hypothesis that viroids cause disease symptoms by producing sRNAs to target host genes was raised and there are many studies supporting this hypothesis (**Figure 4B**). Over-expression of PSTVd hairpin RNA, which produces sRNAs, results in similar phenotypes as PSTVd infection, suggesting that PSTVd may cause disease symptoms by sRNA-mediated silencing (Wang et al., 2004). Large-scale sequencing uncovered that two genes involved in gibberellin or jasmonic acid biosynthesis contain binding sites for PSTVd vdsiRNAs (Wang et al., 2011b). Moreover, DCL4, which should reduce PSTVd levels by slice or dice its genome RNA to produce vdsiRNA, seems to benefit

the accumulation of PSTVd (Dadami et al., 2013). Expression of an artificial miRNA containing the sequence of the PSTVd virulence modulating region down-regulates the expression of a *Nicotiana* soluble inorganic pyrophosphatase (siPPase) gene and leads to a PSTVd infection phenotype (Eamens et al., 2014). In addition, a recent study showed that single vdsiRNA is able to silence multiple host mRNAs. vdsiRNAs derived from PSTVd can target two callose synthase genes, CalS11-like and CalS12-like, which are essential for the formation of callose. The efficiency of suppression depends on the viroid variants and the target gene (Adkar-Purushothama et al., 2015). PLMVd is a chloroplast-replicating viroid and an insertion of a 12- to 13-nt fragment inhibits chloroplast development (Rodio et al., 2007). Further study uncovered that in *Prunus persica*, two vdsiRNAs containing the insertion sequence target the chloroplast heat shock protein 90 (HSP90) and triggers signal transduction that eventually leads to viroid disease symptoms (Navarro et al., 2012a). A single U257A change in the PSTVd central conserved region also strongly increases PSTVd virulence by restricting host cell expansion. The lethal phenotype of PSTVd is correlated with the down-regulation of *LeExp2* gene expression (Qi and Ding, 2003). It is not clear whether the U257A mutation also produces a novel sRNA that targets some essential host genes that is critical for cell expansion and *LeExp2* expression. Furthermore, upon viroid infection, vdsiRNAs generated by *Tomato planta macho viroid* (TPMVd) targets and slices the SolWD40 gene, the function of which is unknown (Avina-Padilla et al., 2015). Although HSVd genomic RNA is higher in RDR6-silenced plants, the viroid-induced symptoms are absent. Meanwhile, HSVd vdsiRNA accumulation is decreased in RDR6-silenced plants, suggesting that the symptoms of HSVd is dependent on vdsiRNAs (Gomez et al., 2008). The symptom severity of CEVd is also correlated with the level of vdsiRNAs but not the viroid genome level, further supporting that vdsiRNAs are not simply by-pass products of anti-viroid RNA silencing reactions, but they have a purpose in producing disease symptoms karian (Markarian et al., 2004). In addition, there are some evidence that link viroid infection to transcriptional gene silencing (TGS). Wassenegger et al. (1994) discovered that PSTVd cognate DNA sequences were methylated in PTSVD-expressing transgenic tobacco plant, while the T-DNA and the genomic plant DNA remained unaltered. Further studies also demonstrate the correlations between viroid infection and host genes transcriptional alteration. For instance, cucumbers infected with HSVd accumulate high levels of sRNAs derived from ribosomal transcripts, as well as ribosomal RNA (rRNA) precursors. This was caused by altered DNA methylation in the promoter region of rRNA genes, resulting in demethylation and transcriptional reactivation of normally inactive rRNA genes (Martinez et al., 2014). *N. benthamiana* carrying an HSVd dimeric sequence develops similar phenotype to HSVd-infected plants (Gomez et al., 2008). This plant also accumulates high levels of sRNAs derived from ribosomal transcripts along with a decrease in rDNA methylation, suggesting that this may be a general phenomenon (Castellano et al., 2015). However, the correlation between sRNA accumulation and DNA methylation needs to be further determined.

It is noteworthy that although some studies suggest that symptoms produced by viroids in plants are associated with vdsiRNAs and the RNA silencing machinery, there is no uniform correlation between the levels of vdsiRNA and symptoms (Ding and Itaya, 2007; Ding, 2009; Kovalskaya and Hammond, 2014). Moreover, in contrast to early observation that symptoms similar to those of PSTVd infection were developed in some transgenic tomato lines expressing non-infectious PSTVd hairpin RNA (Wang et al., 2004), no disease symptoms were found in other tomato lines, despite the accumulation of PSTVd hairpin-derived siRNA (Schwind et al., 2009). Whether vdsiRNA indeed results in viroid disease symptoms requires further investigation.

Plant viruses are often accompanied with a variety of subviral RNA/DNAs, which have no or little sequence similarity to plant viruses. Most satellite RNAs do not encode proteins but can significantly alter viral disease symptoms (**Figure 3B**) (Collmer and Howell, 1992; Simon et al., 2004). More and more studies indicate that the pathogenicity of satellite RNA/DNA may due to host gene silencing induced by satRNA-derived siRNAs (satsiRNAs). CMV Y satellite (Y-sat) causes a bright yellow mosaic phenotype. Replication of Y-Sat is resistant to RNA silencing, but expression of viral suppressors of RNA silencing (VSR) reduces the disease symptoms (Wang et al., 2004). The hairpin structures of CMV satellite RNA are processed by DCL4 and other DCL proteins to form 21-nt and 22-nt satsiRNAs (Du et al., 2007). Y-sat produces a 22-nt satsiRNA that targets Chll, a key gene involved in chlorophyll synthesis, and cleaves Chll mRNA post-transcriptionally, causing the bright yellow mosaic phenotype. Transformation of *N. tabacum* with a silencing-resistant version of Chll greatly reduces the Y-Sat symptoms (Shimura et al., 2011; Smith et al., 2011). satsiR-12, another satsiRNA generated from SD-CMV satellite RNA, targets the upstream region of the CMV 3′ UTR for slicing. satsiR-12 is loaded into AGO1/2/5 for RDR6-mediated regulation, which can be suppressed by 2b encoded by CMV (Zhu et al., 2011). However, the accumulation of 2b coding subgenomic RNA, RNA4A and 2b proteins is also reduced by SD-CMV satellite RNA, which attenuates the D-CMV yellow symptom in *N. benthamiana* (Hou et al., 2011). TCV is often accompanied with a single strand satellite RNA, satC, that is composed of the 3′ end of TCV helper virus. The presence of satC represses the accumulation of TCV genomic RNA and virion, which leads to increased levels of free CP proteins. CP is a VSR encoded by TCV that targets the DCL2/4 silencing pathway and suppresses satC accumulation. The satC-mediated enhancement of free CP proteins then increases the symptoms of TCV (Zhang and Simon, 2003; Manfre and Simon, 2008). Thus, sRNAs generated from satellite RNAs produce species-specific disease symptoms by targeting host genes or viral genomes. On the other hand, in the presence of SD-CMV satellite RNA, the infection of CMV-Δ2b lead to high accumulation of satsiRNA, while the accumulation of CMV siRNA was reduced. Thus, the dice and slice of host RNA silencing machinery on SD-CMV satellite RNA may decrease its efficiency on CMV RNAs (Hou et al., 2011). DNA β satellites are circular ssDNA that associate with many monopartit begomoviruses and are essential for viral disease symptoms (Briddon et al., 2001; Jose and Usha,

2003; Cui et al., 2004). The ßC1 protein encoded by DNA β satellite is a VSR that suppresses methylation-mediated TGS and RDR6-mediated PTGS through the interaction of *S*-adenosyl homocysteine hydrolase (SAHH) and rgs-CaM, which will be discussed later (Cui et al., 2005; Yang et al., 2011b; Li et al., 2014). Thus, viral satellite RNA/DNA can alter the symptoms caused by the helper virus with different sRNA related mechanisms.

Upon infection with a virus, host plants process viral genomic or transcript RNAs into vsiRNAs and load them into RISC complexes to inhibit the amplification and movement of the virus. However, depending on the similarity of vsiRNA-target gene and host genes, some vsiRNAs can target host genes, which subsequently increase viral pathogenicity (**Figure 3B**). Deep sequencing and bioinformatics studies indicate that 16 TMV vsiRNAs potentially target *Arabidopsis* genes. Two of these vsiRNAs target and slice transcripts of a polyadenylation specificity factor and an unknown protein similar to translocon-associated protein alpha. The slicing of these two genes only happens upon TMV infection, revealing that they are real vsiRNA targets (Qi et al., 2009). Dozens of *Zea mays* genes are predicted targets of vsiRNAs encoded by *Sugarcane mosaic virus* (SCMV). Some vsiRNA targets that contribute to biotic/abiotic stress responses and ribosome biogenesis are down-regulated upon SCMV infection (Xia et al., 2014). In addition, vsiRNAs originating from the leader region of CaMV 35S RNA were found to increase the accumulation of CaMV. Like other vsiRNAs, these leader-derived vsiRNAs are DCL-dependent and subsequently loaded into AGO1 (Blevins et al., 2011). These vsiRNAs may also facilitate CaMV accumulation by suppressing *Arabidopsis* gene expression. RSV infection causes plant stunting, chlorosis, and other symptoms. A recent study showed that vsiRNAs can be generated from RSV RNA4, and further targeting host gene *eIF4A*. The infection of RSV down-regulated eIF4A expression. Interestingly, eIF4A suppression by artificial miRNAs leads to rice leaf-twisting and stunting (Shi et al., 2016). Thus, vsiRNAs can directly cause virus pathogenicity, as with vdsiRNAs. Nine chloroplast-related genes (ChRGs) are also down-regulated upon RSV infection and silencing them with artificial miRNAs causes plant chlorosis symptoms, similar to viral infection. However, whether the down-regulation of ChRGs upon RSV infection is also mediated by RSV vsiRNAs need to be further studied (Xia et al., 2014). In contrast to siRNAs, there are relatively few studies done on virus-encoded miRNAs in plants. Studies on *Sugarcane streak mosaic virus* (SCSMV) and *Hibiscus chlorotic ringspot virus* (HCRSV) suggest the existence of virus-encoded miRNAs that may target plant genes, but their detailed functions remain unknown (Gao et al., 2012; Viswanathan et al., 2014).

Fungi, omycetes, and bacteria that localize in the intercellular region in the early infection stages can deliver pathogen sRNAs into plant cells to target host genes as counter-defense. Infecting *Arabidopsis* and *Solanum lycopersicum* with a destructive fungal plant pathogen, *Botrytis cinerea*, results in the presence of a set of *B. cinerea* sRNAs (Bc-sRNAs) in both plants. Among these sRNAs, 73 Bc-sRNAs are able to target host genes in both *Arabidopsis* and *S. lycopersicum*. These Bc-sRNAs are processed by fungi DCLs and loaded into a host AGO1 protein to slice host targets. A mutation in *Arabidopsis AGO1* reduces the susceptibility of the plant to *B. cinerea,* and a mutation in *B. cinerea DCLs* decreases fungi pathogenicity. Multiple Bc-sRNA target genes were identified, including *Arabidopsis* mitogen-activated protein kinase genes *MPK1* and *MPK2*, a cell wall-associated kinase (*WAK*), a peroxiredoxin (*PRXIIF*), and the tomato MPK-kinase kinase 4 (*MAPKKK4*). Suppression of these genes increases the disease susceptibility of the plant (Weiberg et al., 2013). This is the first study showing that sRNAs from a eukaryotic pathogen mediate pathogen virulence using host RNA silencing machinery; however, it is still unclear how these fungal siRNAs are delivered into plant cells. Pathogen sRNAs have been shown to be delivered into animal cells though RNA transporters. Two membrane-associated RNA transporters, systemic RNAi defective-1 (SID1) and SID2, were identified in *C. elegans* (Shih and Hunter, 2011; McEwan et al., 2012). However, no membrane-associated RNA transporters have yet been identified in plants.

RNA silencing inhibits the infection, replication, and movement of same viruses at different steps. Thus, pathogens also encode RNA silencing suppressors to decrease the accumulation of sRNAs or inhibit the function of sRNAs (Qi et al., 2004). Many VSRs are viral pathogenicity determinants, indicating that the suppression function is important for pathogenicity. Some VSRs bind viral dsRNA or vsiRNAs and decrease the number of functional sRNAs targeting viral genomes. Other VSRs directly or indirectly target RNA silencing pathway components such as DCLs, RDRs, and AGOs to inhibit the accumulation and function of endogenous miRNAs and siRNAs, thus increasing the severity of infection symptoms (Csorba et al., 2015). It is believed that plant viruses encode multiple VSRs or a multi-functioning VSRs and express them in host cells to counteract host defenses. For examples, the AL2 VSRs encoded by DNA virus CalCuV silences both transcription-dependent PTGS (transcription activation with the interaction with WEL1 and rgs-CaM) and transcription-independent PTGS (ADK inactivation with the interaction with ADK) (Wang et al., 2003; Trinks et al., 2005; Yong Chung et al., 2014). A recent study uncovered that AL2 also reverses TGS by a transcription-activation- and ADK inactivation-independent mechanism (Jackel et al., 2015). Pns10 encoded by RDV can not only bind siRNAs but also down-regulate RDR6 expression to suppress RNA silencing for viral replication and movement (Cao et al., 2005; Ren et al., 2010). While the replication of RNA viruses is suppressed by PTGS, the replication of DNA viruses is inhibited by both PTGS and TGS (Raja et al., 2008). The function of DNA VSR in the accumulation of 24-nt TGS siRNAs and RdDM pathway components has also been determined recently: C2 (also known as AL2 or AC2) inhibits the ADK function and attenuates the degradation of SAMDC1; C4 down-regulates the accumulation of MET1 but not CMT3; Rep represses the expression of MET1 and CMT3; V2 of TYLCV and AC5 of *Mungbean yellow mosaic India virus* (MYMIV) decreases the methylation of transgenic and endogenous loci by an unknown function; betasatellite βC1 inhibits the activity of SAHH (Wang et al., 2003, 2014; Yang et al., 2011b; Zhang Z. et al., 2011; Rodriguez-Negrete et al., 2013; Li et al., 2015).

Some bacteria and oomycetes also deliver effector proteins into host cells to suppress RNA silencing. Although miR393 is induced upon *Pst* DC3000 infection, the AvrPtoB effector specifically represses the induction of miR393 at the transcriptional level. AvrPto also reduces miR393 accumulation. However, the accumulation of pri-miR393 is not changed in transgenic plants, which indicates that AvrPto may post-transcriptionally down-regulate the processing of miR393 (Navarro et al., 2008). Oomycete *P. sojae* encodes two RNA silencing suppressors: PSR1 down-regulates the accumulation of both host miRNAs and siRNAs, while PSR2 specifically decreases the accumulation of host siRNAs (Qiao et al., 2013). Both of them are effector proteins and their over-expression enhances the infection of *Phytophthora* and viruses. PSR1 interacts with PINP1, a RNA helicase that regulates the accumulation of both miRNAs and siRNAs. The over-expression of *PSR1* or the down-regulation of *PINP1* impairs the localization of the DCL1 protein complex (Qiao et al., 2015). Another PSR2 protein encoded by *P. infestans* can also suppress RNA silencing and enhance the plant susceptibility to *Phytophthora* (Xiong et al., 2014). Thus, the RNA silencing suppressors encoded by oomycetes might be a general counter-defense mechanism. It will be interesting to see whether fungi also deliver effector proteins to inhibit host resistance.

CONCLUSION

There is an increasing amount of evidence that shows communication occurs between plants and different pathogens via sRNAs. The importance of sRNAs in regulating plant immunity and pathogen virulence allows scientists to utilize and manipulate RNA silencing machinery to improve plant immunity, impair pathogen virulence, and thus increase crop production. RNAi technology has been employed to manipulate plant metabolites, develop plants with improved resistance to environment stresses, and engineer plants to defend against pathogen infections (Koch and Kogel, 2014). In plants, expression of pathogen dsRNAs is widely used for plant resistance to viruses that replicate in plant cells. The different roles of sRNAs have also been demonstrated in anti-fungal, anti-insect, anti-nematode resistance, pointing to the existence of cross-kingdom RNA silencing (Baum et al., 2007; Mao et al., 2007; Nowara et al., 2010; Ibrahim et al., 2011; Koch et al., 2013; Panwar et al., 2013). However, RNA silencing is a complicated system, and there are two sides to the coin. For instance, while plants utilize vsiRNAs to silence viral RNA as a defense strategy, vsiRNAs can also target plant mRNAs to promote viral virulence. The never-ending arms race drives the co-evolution of pathogen and hosts, resulting in the variety of sRNAs and RNAi components. To utilize RNA silencing machinery, further investigation is required to explore this complicated and fascinating sRNA world.

AUTHOR CONTRIBUTIONS

JH wrote the introduction, the summary of sRNA in plant and virus. MY wrote the summary of sRNA in bacteria and fungi. Both authors contributed to the figures and figure legend. XZ and LL supervised and complemented the writing.

ACKNOWLEDGMENTS

We apologize to our colleagues whose studies were unable to be discussed for space restrictions. We would like to thank Dr. Yifan Lii for helpful comments. We would also like to thank Juan Du for figure correction. This work was supported by the Ministry of Agriculture transgenic major projects (2016ZX08010001), the Strategic Priority Research program of the CAS (No. XDB11050700), National Natural Science Foundation of China (No. 31471782, No. 91540116, No. 31622046), and Open research Fund Program of State Key Laboratory of Integrated Pest Management (Chinese IPM1503).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 05 January 2017
doi: 10.3389/fmicb.2016.02139

Immune Receptors and Co-receptors in Antiviral Innate Immunity in Plants

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Plants respond to pathogens using an innate immune system that is broadly divided into PTI (pathogen-associated molecular pattern- or PAMP-triggered immunity) and ETI (effector-triggered immunity). PTI is activated upon perception of PAMPs, conserved motifs derived from pathogens, by surface membrane-anchored pattern recognition receptors (PRRs). To overcome this first line of defense, pathogens release into plant cells effectors that inhibit PTI and activate effector-triggered susceptibility (ETS). Counteracting this virulence strategy, plant cells synthesize intracellular resistance (R) proteins, which specifically recognize pathogen effectors or avirulence (Avr) factors and activate ETI. These coevolving pathogen virulence strategies and plant resistance mechanisms illustrate evolutionary arms race between pathogen and host, which is integrated into the zigzag model of plant innate immunity. Although antiviral immune concepts have been initially excluded from the zigzag model, recent studies have provided several lines of evidence substantiating the notion that plants deploy the innate immune system to fight viruses in a manner similar to that used for non-viral pathogens. First, most R proteins against viruses so far characterized share structural similarity with antibacterial and antifungal R gene products and elicit typical ETI-based immune responses. Second, virus-derived PAMPs may activate PTI-like responses through immune co-receptors of plant PTI. Finally, and even more compelling, a viral Avr factor that triggers ETI in resistant genotypes has recently been shown to act as a suppressor of PTI, integrating plant viruses into the co-evolutionary model of host-pathogen interactions, the zigzag model. In this review, we summarize these important progresses, focusing on the potential significance of antiviral immune receptors and co-receptors in plant antiviral innate immunity. In light of the innate immune system, we also discuss a newly uncovered layer of antiviral defense that is specific to plant DNA viruses and relies on transmembrane receptor-mediated translational suppression for defense.

Keywords: resistance genes, receptor NIK1, PAMP-triggered immunity, effector-triggered immunity, antiviral immunity, ETI, PTI, NSP-Interacting kinase 1

OPEN ACCESS

Edited by:
Yule Liu,
Tsinghua University, China

Reviewed by:
Xiaorong Tao,
Nanjing Agricultural University, China
Magdalena Krzymowska,
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *27 September 2016*
Accepted: *19 December 2016*
Published: *05 January 2017*

Citation:
Gouveia BC, Calil IP, Machado JPB, Santos AA and Fontes EPB (2017) Immune Receptors and Co-receptors in Antiviral Innate Immunity in Plants. Front. Microbiol. 7:2139. doi: 10.3389/fmicb.2016.02139

INTRODUCTION

Plants recognize potential pathogens mainly through two classes of distinct immune receptors (Schwessinger and Ronald, 2012; Spoel and Dong, 2012; Zvereva and Pooggin, 2012; Dangl et al., 2013). The first class consists of cell-surface associated pattern recognition receptors (PRRs), which are often represented by receptor-like kinases (RLKs) and receptor-like proteins (RLPs; **Figure 1**).

PRRs recognize conserved structural motifs present in microbes, which are known as microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs), or endogenous danger signals released by the plant during wounding or pathogenic attack, which are termed damage-associated molecular patterns (DAMPs; Macho and Zipfel, 2014). Perception of PAMPs by PRRs activates PAMP-triggered immunity (PTI), a transduction signal cascade that culminates with transcriptional reprograming and biosynthesis of specific defense molecules (Hogenhout et al., 2009; Bigeard et al., 2015). Activation of this immune response enables plants to respond rapidly and efficiently to a large range of pathogens (Roux et al., 2014). The second class of immune receptors includes intracellular immune receptors called R proteins (Jones and Dangl, 2006; Tsuda and Katagiri, 2010; **Figure 1**). These intracellular receptors directly or indirectly recognize effectors secreted by pathogens into the

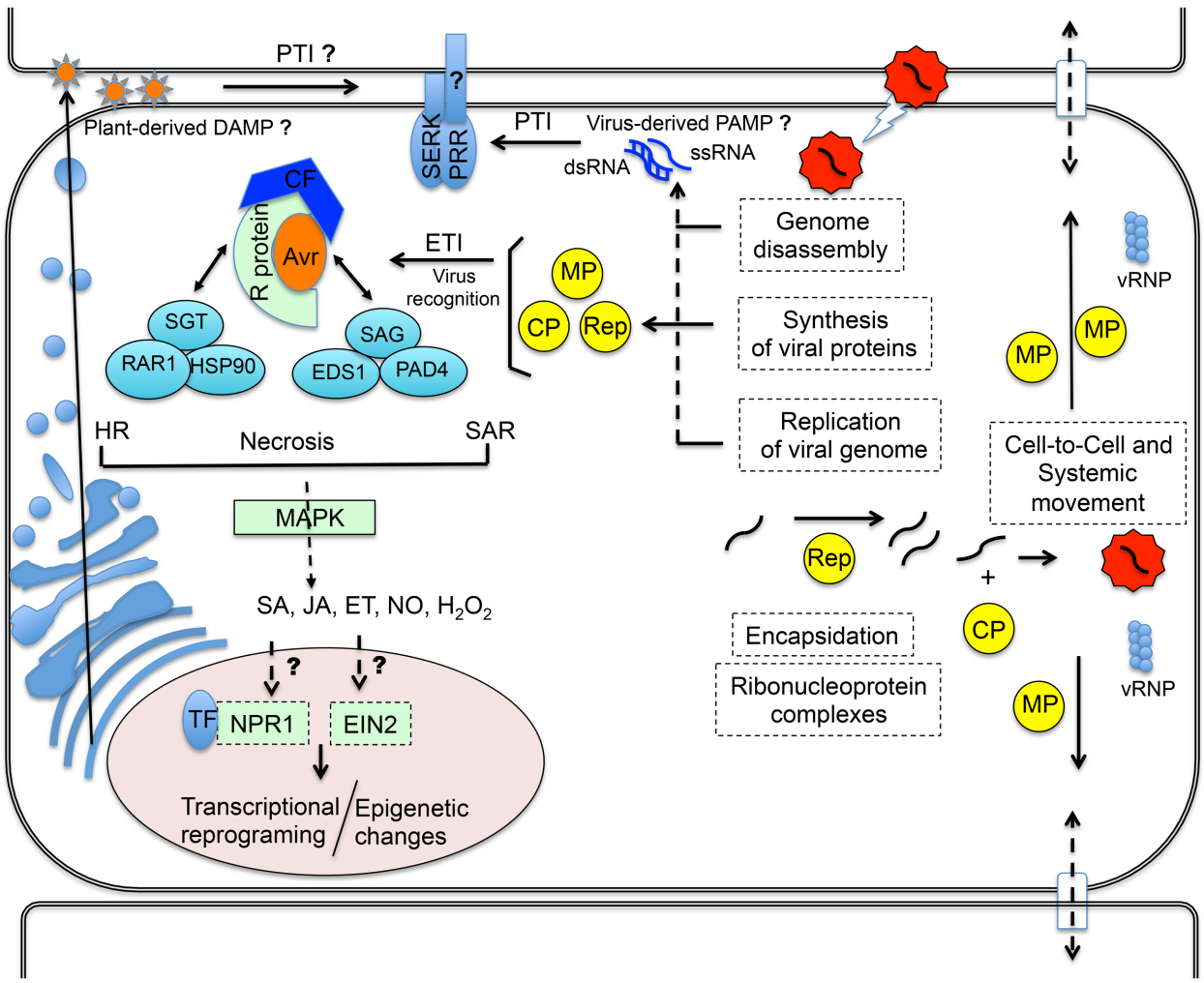

FIGURE 1 | Antiviral innate immunity with conserved features with antibacterial and antifungal immune responses. Plant viruses are obligate, biographic parasites and as such their life cycles start with the penetration of the virions in the host cells via wound sites (lightening arrow). Within the host cells, the virion is disassembled and then host cells mediate the expression of the viral genome by providing a translation apparatus for all viruses and transcription machinery for DNA viruses **(Figure 2)**. The viral mRNAs are translated into the cytoplasm, producing at least three viral proteins absolutely required for completion of the viral life cycle, replication protein (Rep), movement protein (MP) and coat protein (CP). The viral replication proteins combine with cellular proteins to produce multiple copies of the virus genome. These newly made genomes interact with CPs to form new virions or viral ribonucleoprotein complexes (vRNP). The next step is movement of the virus into neighboring cells, which requires the MP. The intracellular translated viral proteins (Avr) may also provide recognition sites for cytosolic NB-LRR receptors (e.g., R proteins), triggering ETI, which results in HR, necrosis or SAR similarly to non-viral ETI. R proteins, R co-factors (CF) and Avr factors form an interacting complex with the SGT1/RAR1/HSP90, and EDS1/PAD4/SAG101 modules to mediate downstream changes in SA, JA, ET, NO and H_2O_2 levels or signaling via MAP Kinases cascades, culminating in the induction of defense genes. NPR1 complexes TF to induce defense genes via SA signaling, whereas EIN2 is a regulator of ET signaling. Virus infection may also trigger epigenetic changes. At the first line of defense, replication of viral RNA genomes may provide non-self RNA motifs (ssRNA or dsRNA) as virus-derived PAMPs to activate PTI. Alternatively, plant cells may sense viral infection and secrete plant-derived DAMPs, recognized by PRRs extracellularly. Members of the SERK family also function as co-receptors in viral PTI. Arrows denote unknown or putative paradigms in viral innate immunity. Adapted from Mandadi and Scholthof (2013).

host intracellular environment and activate effector-triggered immunity (ETI; Howden and Huitema, 2012), which is often manifested in the hypersensitive response (HR) associated with rapid cell death, production of reactive oxygen species (ROS) and salicylic acid (SA) as well as expression of defense-related genes (Win et al., 2012). This is considered to be a more robust defense compared to PTI (Coll et al., 2011; Reimer-Michalski and Conrath, 2016). The effectors that are specifically detected by matching R proteins to activate ETI are termed avirulence (Avr) proteins. Pathogens containing Avr genes are avirulent to plants carrying the cognate R genes and virulent to plants without the R genes. Due to the limitation of the coding capacity of viral genomes, virtually all virus proteins, such as replicase, movement proteins (MPs), coat proteins (CPs), can act as Avr determinants. Therefore, virus Avr proteins are usually necessary for successful infection and are almost invariably virulence factors in a susceptible host.

Studies in plant–virus interactions have pioneered the description of paradigms in plant immune response, including the HR and systemic acquired resistance (SAR; Holmes, 1929, 1938; Ross, 1961). Nevertheless, current semantics and concepts regarding plant immunity models were built to fill the findings on bacterial and fungal infections and hence antiviral immune concepts were initially excluded from these models (Jones and Dangl, 2006; Bent and Mackey, 2007; Boller and Felix, 2009; Dodds and Rathjen, 2010; Schwessinger and Ronald, 2012; Spoel and Dong, 2012). Recently, Mandadi and Scholthof (2013) proposed reconciling the differences and perpetuating the analogy between antiviral and anti-non-viral immune concepts into definitions of viral effectors, viral ETI and viral PTI. These definitions, as described below, integrate antiviral immune concepts into current plant immunity models.

Typical bacterial and fungal effectors are delivered into host cells via microbial secretion systems, whereas viral effectors encoded by the viral genome are directly translated into the host cytoplasm. These factors share similar functions because bacterial and fungal effectors interfere with PTI or other immune regulators and viral effectors promote virulence by interfering with host defense pathways. Although not covered in this review, viral suppressors of RNA silencing are also included in this category. Similar to non-viral pathogen effectors, in resistant genotypes, the intracellularly translated viral effectors are recognized by R proteins, triggering immune responses that often are associated with hallmarks of ETI, such as HR, SA accumulation, ROS production and SAR. Therefore, virus Avr factors, which interfere with defenses, are also referred to as viral effectors, and the immune response they trigger is also referred to as ETI. However, viral ETI is independent with regard to the nature of the immune response, which may or may not be associated with hallmarks of bacterial or fungal ETI. The notion that viruses encode PAMPs recognized by PRRs, such as virus-derived nucleic acids, is well documented in animal systems, and recent evidence has extended the concept of viral PTI to plant–virus interaction systems.

An additional recently uncovered virus-specific defense mechanism relies on suppression of host translation mediated by the transmembrane immune receptor NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE 1 (NIK1), which was first identified as a virulence target of begomovirus NSP (**Figure 2**). Activation of NIK1-mediated antiviral signaling leads to translocation of the ribosomal protein L10 (RPL10) to the nucleus, where it interacts with L10-INTERACTING MYB DOMAIN-CONTAINING PROTEIN (LIMYB) to fully repress expression of translational machinery-related genes and global host translation. Begomovirus mRNAs are unable to escape this translational regulatory mechanism of plant cells and hence are not efficiently translated, which compromises infection upon activation of NIK1-mediated defense. Although the NIK1-mediated defense response is remarkably dissimilar from the PTI response, structural components and activation of the NIK1 immune receptor as well as its interaction with virus infection exhibit features reminiscent of the plant innate immunity mechanism.

This review focuses on the concepts of viral ETI and viral PTI, describing antiviral immune receptors and co-receptors involved in antiviral innate immunity in plants. Furthermore, we describe NIK1-mediated antiviral signaling, a newly discovered layer of antiviral defense, which is specific to plant DNA viruses and relies on transmembrane receptor-mediated translational suppression for defense. This latter level of antiviral defense is discussed within the context of the innate immune system.

EFFECTOR-TRIGGERED IMMUNITY IN ANTIVIRAL DEFENSE: R GENE-MEDIATED RESPONSES TO VIRUS INFECTION

Activation of ETI, involving strain-specific recognition of a virus-encoded effector through direct or indirect interaction with a corresponding resistance gene (R gene) product, can lead to the hypersensitive reaction (HR). HR is considered a resistance response against several different pathogens that, to the some extent, occurs through similar mechanisms. Similar to non-viral infections, the HR response during viral infection is initiated by direct or indirect Avr-R interactions and is frequently associated with accumulation of SA in both infected and non-infected tissues (Culver and Padmanabhan, 2007; Carr et al., 2010; Pallas and García, 2011; Mandadi and Scholthof, 2012). HR is also associated with perturbation in Ca^{++} homeostasis, membrane integrity and activation of caspase-like proteases, such as the vacuolar processing enzyme that is considered an executioner of cell death during HR (Mur et al., 2008). Although cell death is often associated with HR-mediated resistance, HR may be uncoupled from resistance, an interpretation that arises from compelling biochemical and genetic studies of *Potato virus X* (PVX), *Tomato bushy stunt virus* (TBSV), *Cauliflower mosaic virus* (CaMV) and *Tomato mosaic virus* (ToMV; Bendahmane et al., 1999; Chu et al., 2000; Cole et al., 2001; Ishibashi et al., 2007, 2009). For instance, the tomato resistance protein Tm-1 relays resistance against ToMV by inactivating the ToMV replicase protein

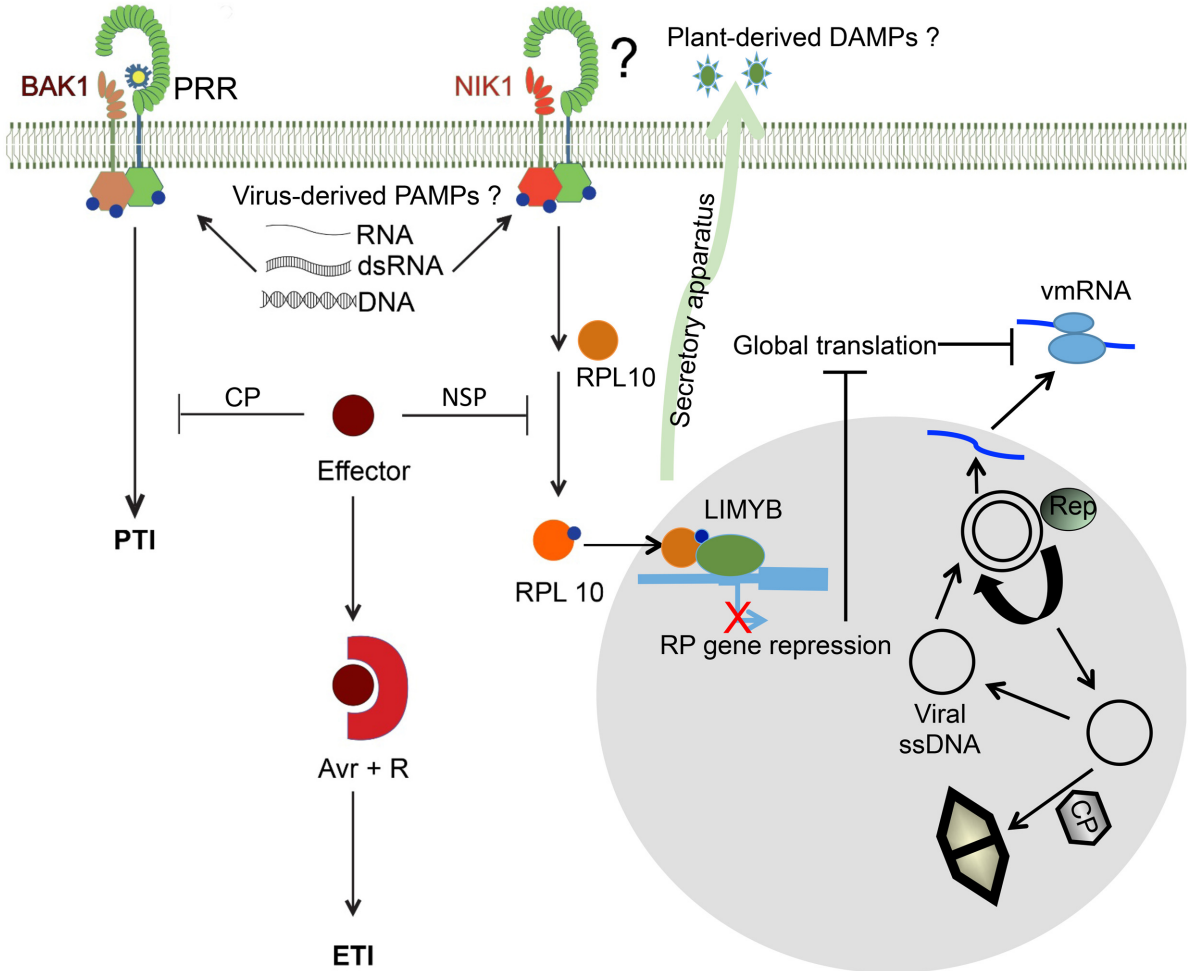

FIGURE 2 | Similarities between viral PTI and NIK1-mediated antiviral signaling. Replication and expression of viral genomes lead to the accumulation of non-self DNA or RNA motifs (virus-derived PAMPs), which may be recognized by PRRs that in turn heteromultimerize with co-receptors (BAK1 or SERK1) to trigger viral PTI. Alternatively, PTI may be activated by endogenous DAMPs, which are induced by virus infection and delivered to the apoplast via the secretory apparatus. In addition to PTI, in the case of DNA viruses (begomoviruses), plant cells may also elicit the translational control branch of the NIK1-mediated antiviral signaling as an innate defense. The mechanism of NIK1 transmembrane receptor activation is unknown. Structural organization and biochemical properties of NIK1 may suggest an activation mechanism dependent on recognition of viral PAMPs or endogenous DAMPs by PRR partners, similarly to a typical viral PTI. In this case, one may consider virus derived-dsDNA as possible PAMPs. The viral single-stranded DNA form begomoviruses replicates via double-stranded DNA intermediates that are transcribed in the nucleus of plant-infected cells. NSP binds to the nascent viral DNA and facilitates its movement to the cytoplasm and acts in concert with the classical MP to transport the viral DNA to the adjacent, uninfected cells. Activation of NIK1 in incompatible interactions promotes phosphorylation and subsequent translocation of RPL10 to the nucleus, where it interacts with LIMYB to fully repress the expression of RP genes, leading to global translation suppression, which also impairs viral mRNA (vmRNA) translation. In begomovirus-host compatible interactions, NSP binds to NIK1 and suppresses its activity. In any case, RNA or DNA viruses, a successful infection implicates in accumulation of virus effectors (for example, CP from PPV and NSP from begomoviruses) to suppress PTI, leading to disease. In resistant genotypes, however, the resistance genes specifically recognize, directly or indirectly, the viral effectors, called avirulence (Avr) factors, activating ETI and conferring resistance. Adapted from Machado et al. (2015).

without eliciting HR-associated cell death (Ishibashi et al., 2007, 2009).

As for non-viral pathogens, most plant antiviral R genes encode NB-LRR [nucleotide-binding-leucine-rich repeat (LRR)] proteins that mediate resistance via specific (direct or indirect) recognition of a virus Avr factor (Win et al., 2012) (**Table 1**). Based on their variable N-terminal domain, these plant NB-LRR proteins are further classified into coiled-coil (CC)-NB-LRR or Toll/interleukin 1 receptor-like (TIR)-NB-LRR protein families (Bonardi et al., 2012). Most of the known antiviral R proteins are CC-NB-LRR-like, whereas only a small number belong to the TIR-NB-LRR class (Zvereva and Pooggin, 2012; de Ronde et al., 2014). Recognition of effectors by R proteins may occur through direct ligand-receptor interactions (gene-for-gene model; Flor, 1971) or through indirect interactions (Guard Model; Jones and Dangl, 2006; Oßwald et al., 2014). In the Guard Model, the resistance protein guards a target host protein, the guardee, and perceives alterations in this target protein upon interaction

TABLE 1 | Plant antiviral NB-LRR resistance genes and the cognate avirulence determinants.

Gene	Plant	R protein signature	Virus	Avr factor	Reference
N	*Nicotiana glutinosa*	TIR-NB-LRR	Tobacco mosaic virus (TMV)	Replicase	Whitham et al., 1994; Padgett et al., 1997
Rx1	*Solanum tuberosum*	CC-NB-LRR	Potato virus *X* (PVX)	Coat Protein	Bendahmane et al., 1999
Rx2	*S. tuberosum*	CC-NB-LRR	PVX	Coat Protein	Bendahmane et al., 2000
HRT	*Arabidopsis thaliana* ecotype Dijon-17	CC-NB-LRR	Turnip crinkle virus (TCV)	Coat Protein	Cooley et al., 2000; Ren et al., 2000
RCY1	*A. thaliana* ecotype C24	CC-NB-LRR	Cucumber mosaic virus strain y	Coat Protein	Takahashi et al., 2001, 2002
Sw-5	Solanum peruvianum	SD-CC-NB-LRR	Tomato spotted wilt virus	Movement protein (NS)	Brommonschenkel et al., 2000; Spassova et al., 2001; Hallwass et al., 2014; Peiro et al., 2014
Y-1	*S. tuberosum*	TIR-NB-LRR	Potato virus *Y*	?	Vidal et al., 2002
Tm-22	*Solanum lycopersicum*	CC-NB-LRR	Tomato mosaic virus (ToMV)	Movement protein	Lanfermeijer et al., 2003
BcTuR3	*Brassica campestris*	TIR-NB-LRR	Turnip mosaic virus	?	Ma et al., 2010
Rsv1	*Glycine max*	CC-NB-LRR	Soybean mosaic virus	P3 + HC-Pro	Hayes et al., 2004; Wen et al., 2013
Pv1	*Cucumis melo*	TIR-NB-LRR	Papaya ringspot virus	?	Anagnostou et al., 2000
Pv2	*Cucumis melo*	TIR-NB-LRR	Papaya ringspot virus	?	Brotman et al., 2013
Cv (locus)	*Poncirus trifoliata*	CC-NB-LRR	Citrus tristeza virus	?	Yang et al., 2003
CYR1	*Vigna mungo*	CC-NB-LRR	Mungbean yellow mosaic virus	Coat Protein	Maiti et al., 2012
Pvr4	*Capsicum annuum*	CC-NB-LRR	Potato virus *Y* Pepper mottle virus	RNA-dependent RNA polymerase (NIb)	Kim et al., 2015, 2016
Tsw	*Capsicum chinense*	CC-NB-LRR	Tomato spotted wilt virus	NSs RNA silencing suppressor	Margaria et al., 2007; Ronde et al., 2013, 2014; Kim et al., 2016

Avr, avirulence; CC, coiled- coil; NB, nucleotide binding; LRR, leucine-rich repeat; SD, solanaceous-specific domain; TIR, Toll/interleukin 1 receptor-like.

with the pathogen effectors. Therefore, the modification of the guardee by the effector causes activation of the R protein to initiate a resistance response. Implicit in the Guard Model is the notion that the guarded effector target is indispensable for the virulence function of the effector protein in the absence of the cognate R protein (Dangl and Jones, 2001; Jones and Dangl, 2006). Alternatively, in the Decoy Model, a decoy (effector target mimic) evolved to act as a molecular sensor to only detect a pathogen without having any other role in the basic cellular machinery of the host (Van der Hoorn and Kamoun, 2008). Therefore, effector alteration of the decoy triggers innate immunity in plants that carry the cognate R protein but does not result in enhanced pathogen fitness in plants that lack the R protein.

The R signaling cascade in plant–virus interactions consists of rapid activation of MAP kinases and involvement of molecular chaperone complexes controlling R protein stabilization and destabilization (Kadota and Shirasu, 2012; Hoser et al., 2013). Convergence between viral and non-viral ETI is observed at the chaperone protein complex containing HEAT SHOCK PROTEIN 90 (HSP90), SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1) and REQUIRED FOR MLA12 RESISTANCE1 (RAR1). The HSP90/RAR1/SGT1 chaperone complex contributes to the stability and proper folding of R proteins during activation, mediating downstream MAP kinase activation, changes in defense gene expression and hormone levels (Liu et al., 2004; Dodds and Rathjen, 2010). Examples of R proteins against viruses that use the HSP90/RAR1/SGT1signaling module to mediate antiviral resistance are the N protein and Rx protein, which confer resistance against Tobacco mosaic virus (TMV) and PVX, respectively (**Table 1**) (Liu et al., 2004; Botër et al., 2007). Another functional module comprising ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1; Aarts et al., 1998; Falk et al., 1999), PHYTOALEXIN DEFICIENT4 (PAD4; Feys et al., 2001, 2005) and SENESCENCE-ASSOCIATED GENE101 (SAG101) mediates HR against viral and non-viral pathogens in a similar manner. In Arabidopsis, the EDS1/PAD4/SAG101 complex regulates HRT-mediated resistance against *Turnip crinkle virus* (TCV; **Table 1**) (Zhu et al., 2011). The HRT-mediated resistance also requires a functional SA-mediated signaling pathway (Chandra-Shekara et al., 2004). Disruption of SA signaling compromises HRT-mediated resistance without affecting HRT-mediated HR, providing further evidence that HR and resistance, albeit closely related, are unlinked processes. Therefore, virus-triggered ETI responses also involve functional SGT1/RAR1/HSP90 (Liu et al., 2004) and EDS1/PAD4/SAG101 (Zhu et al., 2011) protein complexes.

The tobacco *N* gene (for necrotic-type response), which confers resistance against TMV and encodes a TIR-NB-LRR

protein, was the first identified R gene (Holmes, 1938; Whitham et al., 1994). TMV is a positive-sense single-stranded RNA virus of 6.3–6.5 kb that encodes at least four proteins (Goelet et al., 1982; Osman and Buck, 1996). They include a 126-kDa replicase (with methyltransferase and RNA helicase domains), which is encoded by the 5′ORF of TMV and is directly translated from genomic RNA; the stop codon of which is read through to give a 183-KDa RNA-dependent RNA polymerase (RDR). The other two viral proteins, a MP and a capsid protein (CP), are expressed from separate subgenomic RNAs. The N resistance protein directly interacts with the helicase domain (the p50 effector) of TMV replicase to trigger resistance (Ueda et al., 2006). In fact, ectopic expression of the C-terminal 50 kDa portion (p50) of the 126 kDa replicase is sufficient to induce HR in tobacco carrying the *N* gene (Erickson et al., 1999). Full resistance to TMV, however, depends on N receptor-interacting protein 1 (NRIP1), which is recruited from chloroplasts to the cytoplasm and nucleus by the p50 effector and interacts directly with the N resistance protein (Caplan et al., 2008). The nuclear localization of the N resistance protein, which has been demonstrated to be critical for N-mediated resistance, is controlled by upstream events of receptor activation (Burch-Smith et al., 2007; Hoser et al., 2013). As a plant NB-LRR, the N protein requires the conserved chaperone complex consisting of HSP90, RAR1 and SGT for proper folding, accumulation and regulation (Liu et al., 2004). The assembly of this chaperone complex with the N protein occurs in the cytoplasm and SGT controls the nucleocytoplasmic partitioning of the immune receptor (Hoser et al., 2013). Upon TMV infection, p50 binds first to the TIR domain and then to the NB and LRR domains of the N protein leading to conformational changes and oligomerization of the immune receptor (Mestre and Baulcombe, 2006). Phosphorylation of SGT1 by an activated SIPK, a tobacco MAPK6 homolog, shifts the balance toward its nuclear distribution and consequently the N receptor complex is distributed to the nucleus (Burch-Smith et al., 2007; Hoser et al., 2013). Within the nucleus, N protein interacts with transcriptional factors (TFs) to modulate the expression of defense-related genes. The SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-domain transcription factor SPL6 is an example of TF that interacts with the N immune receptor and positively regulates a subset of defense genes (Padmanabhan et al., 2013). This association is detected only when the TMV effector, p50, is present in the cell and is required for N-mediated resistance. SPL6 from Arabidopsis also functions in resistance against the bacterial pathogen *Pseudomonas syringae* expressing the AvrRps4 effector, as SPL6 is required for the R protein RPS4-mediated resistance (Padmanabhan et al., 2013). Therefore, the SPL6-mediated modulation of defense gene expression represents another convergent point in R-mediated resistance against both viruses and bacteria.

The *Rx* gene in potato encodes a well-characterized representative of the CC-NB-LRR class of R proteins, which mediates extreme resistance against PVX elicited by the viral CP. PVX is also a monopartite positive-sense single-stranded RNA virus (Huisman et al., 1988). Unlike other disease resistance responses, this extreme resistance is not associated with HR at the site of infection but rather is associated with an early arrest of viral accumulation in single cells (Bendahmane et al., 1999). The Rx protein also associates with the molecular chaperone HSP90 and its signaling proteins SGT1 and RAR1 to modulate the innate immune response in plants (Botër et al., 2007). The cochaperone SGT1 also interferes with the nucleocytoplasmic distribution of Rx protein (Slootweg et al., 2010; Hoser et al., 2014). Accordingly, silencing the cochaperone SGT1 impaired the accumulation of Rx1 protein in the nucleus and Rx distribution exactly mirrored that of ectopic AtSGT1b variants with forced cytoplasmic or nuclear localization. The Rx nucleocytoplasmic partitioning is also controlled by the Rx interacting partner RanGAP2 (Tameling et al., 2010). The Rx N-terminal CC domain interacts intramolecularly with the Rx NB-LRR region and intermolecularly with the Rx cofactor RanGAP2 (Ran GTPase-activating protein 2; Rairdan et al., 2008; Tameling et al., 2010). In fact, the crystal structure of the CC domain of Rx in complex with the Trp-Pro-Pro (WPP) domain of RanGAP2 reveals that the Rx CC domain forms a heterodimer with RanGAP2, which may prevent Rx self-association (Hao et al., 2013). The C-terminus of the LRR domain is thought to be involved in specific recognition of the viral effector, CP, although direct interaction between CP and Rx has not been demonstrated (Bendahmane et al., 1995; Dangl and Jones, 2001; Farnham and Baulcombe, 2006; Candresse et al., 2010). The Rx-interacting protein RanGAP2 controls Rx nucleocytoplasmic distribution and can act as a cytoplasmic retention factor for Rx. CP of PVX is recognized in the cytosol, and signaling is also activated in this compartment. Concentrating Rx in the cytosol via RanGAP2 overexpression enhances resistance signaling, whereas sequestering Rx in the nucleus through interaction with a nuclear-localized version of RanGAP2 inhibits resistance signaling (Slootweg et al., 2010; Tameling et al., 2010). However, nuclear export signal-mediated expulsion of Rx from the nucleus moderately reduced resistance, indicating that the nuclear pool of Rx also functions in immunity. These results demonstrate that both nuclear and cytoplasmic pools of NB-LRR Rx1 are necessary for full immune responses to PVX. Therefore, in both Rx-mediated resistance and N-mediated resistance, the R protein is activated in the cytoplasm, yet full functionality of the Rx and N R proteins depends on their nucleocytoplasmic distribution.

A few dominant resistance genes encoding the non-NB-LRR class of proteins have been characterized; these proteins have been found to function as sensors of virus infection but do not induce typical ETI-like defense responses, such as HR (**Table 2**). One such example is the tomato *Tm-1* gene, which confers dominant resistance to ToMV and contains two conserved domains: an uncharacterized N-terminal region (residues M1–K431) and a TIM-barrel-like C-terminal domain (residues T484–E754; Ishibashi et al., 2012, 2014; Yang et al., 2016). Tm-1 binds to ToMV replication proteins and inhibits ToMV multiplication without inducing a defense response: binding of Tm-1 to ToMV replication proteins inhibits the RNA-dependent RNA replication of ToMV and replication complex assembly on membranes that

TABLE 2 | Plant antiviral non-NB-LRR resistance genes and the cognate avirulence determinants.

Gene	Plant	R protein signature	Virus	Avr determinant?	Reference
JAX1	*Arabidopsis thaliana*	Jacalin-like [lectin gene]	Broad resistance against potexvirus	?	Yamaji et al., 2012
RTM1	*Arabidopsis thaliana*	Jacalin-like	Tobacco etch virus	Coat Protein	Chisholm et al., 2000
RTM2	*Arabidopsis thaliana*	Jacalin-like	Plum pox virus	Coat Protein	Whitham et al., 2000; Decroocq et al., 2009
Ty-1, Ty-3	*Solanum chilense*	RDR	Tomato yellow leaf curl virus	?	Verlaan et al., 2013; Butterbach et al., 2014
Tm-1	*Solanum hirsutum*	TIM-barrel-like domain protein	ToMV	Replicase, Helicase domain	Ishibashi et al., 2007; Kato et al., 2013

Avr, avirulence; RDR, RNA-dependent RNA polymerase.

precedes negative-strand RNA synthesis (Ishibashi and Ishikawa, 2013, 2014). Another recently characterized example of non-NB-LRR R proteins is the sensor proteins Ty-1 and Ty-3, which confer resistance to *Tomato yellow Leaf Curl Virus* (TYLCV). The *Ty-1* and *Ty-3* genes are allelic and code for an RDR of the RDRc type, which has an atypical DFDGD motif in the catalytic domain (Verlaan et al., 2013). The mechanism of resistance is completely uncoupled from ETI and appears to be linked to the RNA silencing strategy of antiviral defense (Butterbach et al., 2014). Accordingly, *Ty-1/Ty-3* plants display enhanced siRNA levels that coincide with hypermethylation of the TYLCV V1 (CP) promoter, indicating that *Ty-1*-based resistance against TYLCV involves enhanced transcriptional gene silencing.

In summary, most antiviral dominant resistance genes so far characterized encode typical NB-LRR R proteins (**Table 1**), which specifically recognize viral effectors or Avr factors and utilize signaling modules such as SGT1/RAR1/HSP90 and EDS1/PAD4/SAG101 complexes to mediate resistance responses, similar to non-viral pathogens. Therefore, plants appear to have evolved strategies and signaling modules to defend themselves against a large spectrum of pathogen types, such as bacteria, viruses and fungi. This interpretation allows us to integrate some aspects of the antiviral immune concepts into the typical bacterial and fungal immunity models to classify viral effectors and viral ETI.

PAMP-TRIGGERED IMMUNITY IN ANTIVIRAL DEFENSES: CO-RECEPTORS PAVE THE WAY

Plant innate defense responses are also activated upon perception of conserved PAMPs, which are pathogen-derived conserved motifs. In addition, endogenous molecules released by the host during pathogenic attack or wounding, which are known as DAMPs, can also elicit plant defense (Zipfel, 2014). Detection of different PAMPs/DAMPs by the corresponding PRRs at the plasma membrane activates signaling cascades, leading to transcriptional and physiological changes in host cells that prevent pathogen infection and establish PTI (Jones and Dangl, 2006; Macho and Zipfel, 2014; Bartels and Boller, 2015). In plants, PRRs are represented by RLKs and RLPs located at the cell surface, both of which require a co-receptor to form an active complex and initiate signaling (Machado et al., 2015). The best characterized co-receptors for PRR are members of LRR subfamily II of the RLK superfamily (LRRII-RLK subfamily). This family is represented by 13 members in the Arabidopsis genome, which can be divided into three closely related clusters: one representing five SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERK1-5), a cluster of LRR-RLKs of unknown function and a cluster of NUCLEAR-SHUTTLE PROTEIN-INTERACTING KINASES (NIK1-3; Zhang et al., 2006; Sakamoto et al., 2012). Among SERKs, SERK3, which is also termed BRASSINOSTEROID INSENSITIVE 1 (BRI1)-ASSOCIATED KINASE 1 (BAK1), is the most well-characterized subfamily member. SERK3 functions as a co-receptor of several PRRs, such as FLAGELLIN SENSING 2 (FLS2), ELONGATION FACTOR-thermo unstable (EF-Tu) receptor (EFR) or PEP1 receptor 1 (PEPR1), which perceive specific PAMPs/DAMPs and trigger or amplify bacterial/fungal PTI (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011; Wang et al., 2014). Upon PAMP perception, FLS2 and EFR form a ligand-induced complex with BAK1, which leads to rapid phosphorylation of both proteins (Chinchilla et al., 2007; Heese et al., 2007; Roux et al., 2011; Sun et al., 2013) and activation of immune responses, including production of ROS by the NADPH oxidase RBOHD, activation of the mitogen-activated protein kinase (MAPK) cascade, transcriptional reprogramming of defense genes and immunity to pathogens (Kadota et al., 2014; Li et al., 2014; Macho and Zipfel, 2014).

The mechanism of PTI in virus–host interactions is well characterized in animals. One of the best studied PRRs in mammals, Toll-like receptors (TLR), have important roles in antiviral defense via recognition of a different range of MAMPs, such viral RNA and DNA (Song and Lee, 2012). In contrast, the PTI pathway in plants remains unclear with regard to resistance against viruses, although studies describing an association of PTI in antiviral immunity have been recently reported (Yang et al., 2010; Kørner et al., 2013; Nicaise, 2014; Machado et al., 2015; Nicaise and Candresse, 2016; Niehl et al., 2016). Indeed, a complex set of typical PTI responses is induced in plants upon virus infection, including SA accumulation, ROS production, ion fluxes, defense gene (e.g., PR-1) activation, and callose deposition (for a review, see Nicaise, 2014). The PRR co-receptors BAK1

or BAK1-LIKE1 (BKK1) are required for antiviral immunity in Arabidopsis, and loss-of-function mutations in *BAK1* and *BKK1* result in enhanced susceptibility to TCV infection (Yang et al., 2010). Consistently, Arabidopsis *bak-1* mutants show increased susceptibility to three different RNA viruses, and crude extracts of virus-infected leaf tissues induce typical PTI responses in a BAK1-dependent manner (Kørner et al., 2013). The Arabidopsis double mutant *bak1-5 bkk1* displays increased viral accumulation when inoculated with *Plum pox virus* (PPV; Nicaise and Candresse, 2016). Furthermore, MAPK4, a negative regulator of plant PTI signaling, suppresses soybean defense against *Bean pod mottle virus* (BPMV; Liu et al., 2011), and chitosan, a deacetylated chitin derivative elicitor, is able to stimulate the plant immune response against viruses (Iriti and Varoti, 2014).

The current data suggest that viral components can act as PAMPs but do not eliminate the possibility that DAMPs produced in response to virus can potentially elicit PTI-based antiviral responses in plants. Recently, double-stranded RNA (dsRNA) and virus-derived dsRNA have been shown to function as viral PAMPs in Arabidopsis and to induce the PTI pathway (Niehl et al., 2016). Indeed, application of dsRNA to Arabidopsis leaf disks resulted in the induction of typical PTI responses, including MAPK activation, ethylene synthesis and defense gene expression. Furthermore, dsRNA treatment confers protection against viruses because plants inoculated with the synthetic dsRNA analog polyinosinic–polycytidylic acid, poly(I:C) together with *Oilseed rape mosaic virus* (ORMV) showed significantly reduced viral accumulation in treated leaves. Interestingly, dsRNA-mediated PTI appears to be independent of the RNA silencing pathway but does involve the co-receptor kinase SERK1. These findings relate membrane-associated signaling events with dsRNA-mediated PTI in plants (Niehl et al., 2016). Although plasma membrane-localized co-receptors of PRRs, such as BAK1, BKK1 and SERK1, have been shown to be involved in viral PTI, it remains to be determined how intracellular pathogens, which deliver PAMPs intracellularly, are perceived extracellularly.

The PTI pathway also contributes to antiviral immunity against PPV in Arabidopsis (Nicaise and Candresse, 2016). As a counteraction strategy, the CP from PPV appears to act as a PTI suppressor, impairing early immune responses such as the oxidative burst and enhanced expression of PTI-associated marker genes *in planta* during infection (Nicaise and Candresse, 2016). Therefore, PPV CP displays a virulence function that acts at the PTI level and antagonizes the Avr functions of many viral recognized by antiviral R proteins during elicitation of ETI (**Table 1**). These observations suggest that plant viruses also fit into the zigzag model of co-evolving pathogenic virulence strategies and plant defense responses that shape the two-branched innate immune system (Jones and Dangl, 2006).

Collectively, these data suggest the existence of PTI signaling mechanism targeting plant viruses and may represent a conserved process between plants and animals. Identification of PRR-mediated pathways as well as characterization of nucleic acid-sensing PRRs will shed light on the mechanism by which PTI is elicited in plants and its role in antiviral resistance.

TRANSMEMBRANE RECEPTOR-MEDIATED TRANSLATIONAL SUPPRESSION IN ANTIVIRAL IMMUNITY: UNIQUE AND SHARED PTI-LIKE FEATURES OF THE NIK1-MEDIATED ANTIVIRAL RESPONSE

The transmembrane receptor NIK was first identified as a virulence target of Nuclear Shuttle Protein (NSP) from *Begomovirus,* the largest genus of the *Geminiviridae* family (Fontes et al., 2004; Mariano et al., 2004). Similar to the PTI co-receptors BAK1 and SERK1, NIK receptors (NIK1, NIK2 and NIK3) belong to the LRRII-RLK subfamily and are involved in plant defenses against viruses (Fontes et al., 2004). Nevertheless, the mechanism by which NIK1 transduces the antiviral signal is completely different from the typical PTI signaling mediated by BAK1 or SERK1 and PRRs. Nonetheless, some similarities between these transduction pathways with regard to receptor activation, suppression and association with ETI have been observed (Machado et al., 2015, **Figure 2**).

NUCLEAR SHUTTLE PROTEIN-INTERACTING KINASE 1-mediated antiviral signaling is activated upon perception of begomovirus infection, which leads to phosphorylation of the NIK1 kinase at key threonine residues at positions 468 and 474 (Santos et al., 2009; Zorzatto et al., 2015). Thr-468 and Thr-474 are located within the conserved activation loop and align to the same positions as conserved BAK1 residues Thr-449 and Thr-455 and SERK1 residues Thr-462 and Thr-468, which are intramolecular targets for BAK1 and SERK1 kinase activation (Shah et al., 2001; Wang et al., 2005, 2008; Yun et al., 2009). Phosphorylation of the functional analogs NIK1 Thr-474, SERK1 Thr-468 and BAK1 Thr-455 is essential for receptor/co-receptor signaling, which may underscore a similar mechanism for activation (Shah et al., 2001; Wang et al., 2008; Santos et al., 2009; Yun et al., 2009; Brustolini et al., 2015). Nevertheless, unlike BAK1 or SERK1, phosphorylation at NIK1 Thr-474 leads to phosphorylation at Thr-469, which has an inhibitory effect, thereby providing a mechanism by which NIK1 modulates the extent of auto- and substrate phosphorylation. Although NIK1 is activated upon perception of virus infection, the molecular bases of such elicitation are unknown. Indeed, there is a complete lack of information with respect to the nature and identity of possible ligands or mechanisms that trigger or stabilize NIK1 dimerization or multimerization with receptors. Because viruses are intracellular pathogens and may not have access to the apoplast, it remains to be determined how the NIK1 extracellular domain, which is expected to drive ligand-dependent oligomerization of receptors and co-receptors, senses viruses intracellularly. Possible ligands that could perform this function are DAMPs, which would be secreted by plant cells upon virus perception. Alternatively, viral nucleic acid-derived PAMPs could intracellularly activate NIK1 kinase, a mechanism that would resemble virus-derived dsRNA-mediated activation of mammalian intracellular protein kinase R (PKR; Balachandran et al., 2000). Virus-derived nucleic acid PAMPs

could also activate NIK1-associated nucleic acid-sensing PRRs in endosomes derived from receptor internalization via endocytic pathways. In plant cells, the PRRs FLS2, ERR and PEPR have been shown to be internalized in a clathrin-dependent manner. Endocytosis requires the co-receptor BAK1 and depends on receptor activation (Mbengue et al., 2016). In a similar manner, the Avr factor Avr4 induces association of Cf-4 RLP with BAK1 to initiate receptor endocytosis and plant immunity (Postma et al., 2016).

In general, ligand-dependent phosphorylation and activation of RLKs require homo or heterodimerization of the receptors. In the case of BAK1 and SERK1, compelling evidence has revealed that they function primarily as co-receptors for receptor signaling not only in defense but also in development (Ma et al., 2016). As a member of the LRRII-RLK subfamily sharing conserved structural organization and biochemical activation properties with SERKs, NIK1 may also function as a co-receptor in immune active complexes. However, NIK1-containing antiviral signaling complexes have not been isolated, and a receptor partner for NIK1 has yet to be identified.

Begomovirus NSP binds *in vitro* and *in vivo* with the kinase domain of NIK1 to suppress NIK1 activity (Fontes et al., 2004; Brustolini et al., 2015). The NSP binding site corresponds to an 80-amino acid stretch (positions 422–502) of NIK1 that encompasses the putative Ser/Thr kinase active site (subdomain VIb–HrDvKssNxLLD) and the activation loop (subdomain VII–DFGAk/rx, plus subdomain VIII–GtxGyiaPEY; Fontes et al., 2004). Binding of NSP to the kinase domain promotes steric constraints that impair intermolecular phosphorylation at Thr-474 within the A-loop of NIK1, thereby suppressing its kinase activity. The NSP-mediated suppression of NIK1 kinase prevents activation of the NIK-mediated pathway and hence enhances the pathogenicity of begomoviruses in their hosts (Santos et al., 2009, 2010). In addition to acting as a virulence factor suppressing NIK1-mediated antiviral signaling, NSP from the begomovirus *Bean dwarf mosaic virus* (BDMV) has been demonstrated to function as an Avr gene and elicit HR in *Phaseolus vulgaris* (Garrido-Ramirez and Gilbertson, 1998). According to the zigzag evolutionary model of plant innate immunity (Jones and Dangl, 2006), the involvement and activation of ETI in plant-virus interactions (NSP in resistant bean genotypes) is conceptually associated with successful PTI inhibition (NIK1 signaling) by a viral effector (NSP). This interpretation further substantiates the notion that NIK1-mediated antiviral signaling shows features of PTI-like mechanisms.

Despite similarities in the activation and suppression mechanisms of PTI and NIK1-mediated antiviral signaling, the downstream events of NIK1 activation are quite distinct from the typical PTI response. In fact, activation of NIK1 signaling by constitutive or inducible expression of the gain-of-function T474D NIK1 mutant, which is not inhibited by viral NSP, results in a massive down-regulation of translation machinery-related genes, suppression of host global translation and enhanced broad-spectrum tolerance to begomoviruses in Arabidopsis and tomato (Brustolini et al., 2015; Zorzatto et al., 2015). T474D-mediated suppression of global translation is associated with a decrease in host and viral mRNA in actively translating polysomes. Therefore, begomovirus is not capable of sustaining high levels of viral mRNA translation in T474D-expressing lines, indicating that suppression of global protein synthesis may effectively protect plant cells against DNA viruses.

Progress toward deciphering the mechanism of the translational control branch of NIK1 signaling includes identification of the downstream effectors, RPL10 and LIMYB (Rocha et al., 2008; Zorzatto et al., 2015). RPL10 was isolated based on its capacity to interact with NIK1 and was genetically and biochemically linked to the NIK1-mediated signaling pathway (Carvalho et al., 2008; Rocha et al., 2008). Consistent with a role for RPL10 in antiviral defense, loss of *RPL10* function recapitulates the *nik1* enhanced susceptibility phenotype to begomovirus infection, as *rpl10* knockout lines develop severe symptoms similar to those of *nik1* and display a similar infection rate (Carvalho et al., 2008; Rocha et al., 2008). NIK1 activation mediates RPL10 phosphorylation and consequent translocation of the RP from the cytoplasm to the nucleus. The regulated nucleocytoplasmic shuttling of RPL10 depends on NIK1 kinase activity and on the phosphorylation status of the RP (Carvalho et al., 2008). Mutations that impact NIK1 activity similarly affect the capacity of NIK1 to mediate translocation of RPL10 to the nucleus and to transduce an antiviral signal. In the nucleus, RPL10 interacts with LIMYB to form a transcription-repressing complex that specifically down-regulates expression of translational machinery-related genes, such as RP genes. This down-regulation of RP genes results in global suppression of host translation and enhanced tolerance to begomoviruses. Expression of the gain-of-function T474D mutant also results in repression of the same set of LIMYB-regulated RP genes, but T474D requires the function of LIMYB for RP repression. In addition, loss of LIMYB function releases the repression of translation-related genes and increases susceptibility to *Cabbage leaf curl virus* (CaLCuV) infection (Zorzatto et al., 2015). Collectively, these results provide both genetic and biochemical evidence that LIMYB functions as a downstream component of the NIK1-mediated signaling pathway linking NIK1 activation to global translation suppression and tolerance to begomoviruses.

Although NIK1 is structurally similar to SERKs, the mechanism of NIK1-mediated antiviral defense is distinct from that of BAK1-mediated PTI. The current model for NIK1-mediated antiviral signaling states that, in response to virus infection, NIK1 undergoes homo- or heterodimerization to promote phosphorylation of the activation loop (**Figure 2**). Activated NIK1 mediates phosphorylation and consequent translocation of RPL10 to the nucleus, where it interacts with LIMYB to fully repress RP gene expression. Prolonged down-regulation of RP gene expression leads to suppression of global host translation. DNA viruses, such as begomoviruses, cannot escape this translational regulatory mechanism of plant cells, and viral mRNAs are not translated efficiently, thereby compromising infection. NSP acts as a virulence factor and suppresses the kinase activity of NIK1 to overcome the NIK1-mediated immune response. NSP from the begomovirus BDMV has also been shown to function as an Avr factor that activates typical ETI responses in resistant bean genotypes. Therefore, NSP may link the suppression of NIK1 signaling with activation of ETI

responses in accordance with the zigzag evolutionary model of plant innate immunity, although the NIK1-mediated antiviral signaling may represent a new evolved branch of plant antiviral immunity, which relies on suppression of translation for defense.

CONCLUSION

Innate immunity against plant viruses and its underlying mechanisms have attracted the attention of breeders and scientists. Accordingly, there is a growing list of R genes against viruses, and our knowledge regarding the mechanisms of R gene-mediated defenses has advanced considerably over the last decade. However, in comparison with R genes against non-viral pathogens, the number of well-studied examples of antiviral R genes is still limited with respect to an understanding of the level of specialization of dominant resistance against viruses and the boundaries of features shared with non-viral ETI. Even more limited is our understanding of viral PTI in plants. Recent studies have provided insights into plant viral PTI. For example, it is now known that several components of bacterial and fungal PTI participate also in viral PTI. These include the co-receptor SERKs, BAK1 and SERK1, and the MAPK4 negative regulator, in addition to common effects of non-viral PTI that are also elicited during virus infection. Nevertheless, our knowledge about the dynamics between the virulence strategy of viruses and the plant immune system is still rudimentary, and several steps in the mechanism of antiviral innate immunity are still unknown. Indeed, although non-self RNA motifs appear to function as PAMPs from RNA viruses, we do not know the identities of virus-derived PAMPs or plant-derived DAMPs that would induce antiviral PTI. The repertoire of viral suppressors of PTI is limited to the CP from PPV and perhaps to NSP from begomoviruses. Furthermore, antiviral PRRs have not been identified, and mechanisms by which intracellular pathogens that have no access to the apoplast are sensed extracellularly are unknown. A better understanding of the repertoire of virus effectors (Avr factor) and NB-LRR host targets (R proteins) and their mode of action in activating ETI and/or suppressing PTI will help to define the evolutionary pressure acting upon the host and viruses and to determine how to deploy the immune system for more efficient control of virus infection. We also need to define NIK1-mediated suppression of translation as a general or virus-specific antiviral strategy in plants. To date, a sustained NIK1 pathway has been shown to be effective against begomoviruses, one of the largest groups of plant DNA viruses, which cannot circumvent the regulatory mechanism of host translation. In this regard, the intrinsic capacity of agronomically relevant crops to withstand the deleterious effects of suppression of global translation must be considered as a relevant agronomic trait if we are to use the translational control branch of NIK1-mediated antiviral signaling for crop protection against begomoviruses.

AUTHOR CONTRIBUTIONS

BG and IC wrote the first draft of the manuscript; JM wrote the ETI section; AS edited the manuscript and EF edited the manuscript.

ACKNOWLEDGMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico [573600/2008-2 and 447578/2014-6 to E.P.B.F.) and the Fundação de Amparo à Pesquisa do Estado de Minas Gerais [CBB-APQ-03175-13 and CBB-APQ-01491-14 to EF]. BG and IC were supported by CNPq graduate fellowships, and JM is a recipient of a CAPES postdoctoral fellowship.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 26 October 2016
doi: 10.3389/fmicb.2016.01695

Recessive Resistance to Plant Viruses: Potential Resistance Genes Beyond Translation Initiation Factors

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The ability of plant viruses to propagate their genomes in host cells depends on many host factors. In the absence of an agrochemical that specifically targets plant viral infection cycles, one of the most effective methods for controlling viral diseases in plants is taking advantage of the host plant's resistance machinery. Recessive resistance is conferred by a recessive gene mutation that encodes a host factor critical for viral infection. It is a branch of the resistance machinery and, as an inherited characteristic, is very durable. Moreover, recessive resistance may be acquired by a deficiency in a negative regulator of plant defense responses, possibly due to the autoactivation of defense signaling. Eukaryotic translation initiation factor (eIF) 4E and eIF4G and their isoforms are the most widely exploited recessive resistance genes in several crop species, and they are effective against a subset of viral species. However, the establishment of efficient, recessive resistance-type antiviral control strategies against a wider range of plant viral diseases requires genetic resources other than eIF4Es. In this review, we focus on recent advances related to antiviral recessive resistance genes evaluated in model plants and several crop species. We also address the roles of next-generation sequencing and genome editing technologies in improving plant genetic resources for recessive resistance-based antiviral breeding in various crop species.

Keywords: plant virus disease control, host resistance, recessive resistance, translation initiation factors, genetic resources, antiviral breeding

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Edited by:
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *26 August 2016*
Accepted: *11 October 2016*
Published: *26 October 2016*

Citation:
Hashimoto M, Neriya Y, Yamaji Y and Namba S (2016) Recessive Resistance to Plant Viruses: Potential Resistance Genes Beyond Translation Initiation Factors. Front. Microbiol. 7:1695. doi: 10.3389/fmicb.2016.01695

INTRODUCTION

Plant viruses are obligate parasitic microbes that can be characterized by their distinct life cycles depending on host plant machinery. Their genomes are the simplest among plant-associated microbes: a single, or multiple, DNA or RNA molecule(s) encoding several proteins, some of which encapsidate the DNA or RNA to form viral particles. Plant viruses do not deploy specific structures to enter into plant cells and, in general, passively enter through wounds or are transmitted by other organisms including insects, mites, and fungi. Frequent mutations due to error-prone genome replications enable viruses to circumvent plant defense systems and cause severe crop production losses (Kobayashi et al., 2014). Thus far, agrochemicals that directly target viral life cycles have not been developed, and, consequently, it remains difficult to control plant viral diseases. Furthermore, due to worldwide climate change and international trade, there is an increasing risk of plant virus outbreaks.

Great efforts have been made to control plant viral diseases to enhance crop production (Nicaise, 2014; Tsuda and Sano, 2014). Measures used to control these diseases can be categorized into those that depend on plant defense machinery and those that do not. Resistant cultivars, whose traits have been introduced by crossing, are commonly used as crop species to control plant viruses. Plant host resistance is achieved in two ways: one method involves dominant *Resistance* (*R*) genes and the other depends on recessive alleles of genes that are critical for plant viral infection. Most of the dominant *R* genes encode proteins with nucleotide-binding sites and leucine-rich repeats (NB-LRR), and other proteins from the same family confer resistance to bacterial and fungal pathogens (Moffett, 2009; Padmanabhan and Dinesh-Kumar, 2014). In addition, several genes that are distinct from the conventional NB-LRR–type *R* genes have been described (Chisholm et al., 2000; Ishibashi et al., 2007; Yamaji et al., 2012). The second mechanism of plant resistance to viruses, referred to as recessive resistance, is also widely exploited in many crops (Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012). In fact, about half of the alleles responsible for virus-resistance in crops are recessive (Kang et al., 2005). Recessive resistance traits can be introduced into crop species by crossing, or random mutagenesis and selection (Piron et al., 2010). Recessive resistance breeding has the practical advantages of not requiring the introduction of transgenes and not being restricted by the selection of naturally occurring traits only. However, most of the recessive resistance genes isolated to date are eukaryotic translation initiation factors (eIF) 4E and eIF4G, and their isoforms (hereafter eIF4Es).

Mutations in eIF4Es confer loss-of-susceptibility to potyviruses and several other viruses. To enable recessive resistance-based crop breeding against a wide range of plant viruses, it is important to improve the genetic resources available for recessive resistance other than eIF4Es. In the absent of naturally occurring recessive resistant cultivars, and if eIF4Es-mediated resistance is not effective in a plant–virus interaction, a mutation in a potential recessive resistance gene can be introduced. This review focuses on our current understanding of the genetic resources for recessive resistance, and how to enhance them using technologies such as next-generation sequencing (NGS) and genome editing for recessive resistance-based antiviral breeding in various crop species.

eIF4Es-MEDIATED RECESSIVE RESISTANCE

Recessive resistance is based on the molecular interactions between viruses and host plants. Plant viruses propagate their genomes in plant cells by hijacking large numbers of host cell proteins, and then spread to adjacent healthy cells and tissues (Hyodo and Okuno, 2014; Wang, 2015). Mutations in the plant genes encoding factors necessary for viral infection can interfere with viral propagation in plants. Another possible mechanism of recessive resistance against plant viruses is based on the autoactivation of plant defense responses when there is a deficiency in a negative regulator of defense signaling (Truniger and Aranda, 2009). However, no experimental evidence has been obtained to directly support the latter hypothesis in naturally occurring cultivars (Orjuela et al., 2013).

Recessive resistance mediated by eIF4Es was first found in mutants of *Arabidopsis thaliana* exhibiting loss-of-susceptibility to tobacco etch virus (TEV; *Potyvirus*), which is due to deficiency in the *eIFiso4E* gene, an isoform of *eIF4E* (Lellis et al., 2002). Subsequent studies revealed that eIF4Es-mediated resistance against potyviruses is found in several resistant crop cultivars including pepper (*Capsicum annuum*), lettuce (*Lactuca sativa*), and wild tomato (*Solanum habrochaites*) (Ruffel et al., 2002, 2005; Nicaise et al., 2003). In addition to potyviruses, eIF4Es-mediated resistance to other viruses has been observed. These include cucumber mosaic virus (CMV; *Cucumovirus*) in *Arabidopsis* (Yoshii et al., 2004); two carmoviruses, turnip crinkle virus (TCV) in *Arabidopsis* (Yoshii et al., 1998) and melon necrotic spot virus (MNSV) in melon (*Cucumis melo*) (Nieto et al., 2006); two bymoviruses, barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BYMV) in barley (*Hordeum vulgare*) (Kanyuka et al., 2005; Stein et al., 2005); and rice yellow mottle virus (RYMV; *Sobemovirus*) in rice (*Oryza sativa*) (Albar et al., 2006) (this information is also summarized in Truniger and Aranda, 2009 and Sanfaçon, 2015). Unsurprisingly, eIF4Es-mediated resistance is only effective against viruses that interact specifically with at least one of the eIF4Es. Remarkably, in *Arabidopsis*, selective involvement of eIF4Es is found even in closely related viruses in the same genera, including *Potyvirus* and *Polerovirus* (Sato et al., 2005; Nicaise et al., 2007; Reinbold et al., 2013), suggesting that the specific interactions between these viruses and eIF4Es developed after these species diverged from one another. Conservation of translation initiation factors in plants indicates that a wide range of plant viruses may take advantage of host eIF4Es; however, due to partial functional redundancy among eIF4E isoforms, deficiency of an individual in eIF4Es does not always confer resistance to all plant viruses (Mayberry et al., 2011; Martínez-Silva et al., 2012). Moreover, because of the essential roles of eIF4Es in plant viability, knockout mutations of either eIF4E or eIF4G and its corresponding isoform result in an embryo-lethal phenotype (Nicaise et al., 2007; Patrick et al., 2014). Because the utility of eIF4Es as recessive resistance genes is limited, it is important to identify and characterize additional genetic targets that may mediate recessive resistance against a wider range of viral species.

POSITIVE REGULATORS OF VIRAL INFECTION: GENETIC RESOURCES FOR RECESSIVE RESISTANCE

Over the past few decades, a large number of host factors have been isolated and functionally characterized to generate a better understanding of virus life cycles (Nagy and Pogany, 2011; Hyodo and Okuno, 2014; Wang, 2015). To identify host factors, forward and reverse genetic approaches using *Arabidopsis* and other model plants have been used (Ishikawa et al., 1991; Yoshii et al., 2009; Castelló et al., 2010). In addition, other host factors have been identified by screening for interactors with viral proteins

and components of protein complexes containing viral factors (Mine et al., 2010; Nishikiori et al., 2011; Xiong and Wang, 2013). Genome-wide screening using the heterologous yeast system with brome mosaic virus (BMV; *Bromovirus*) and also with tomato bushy stunt virus (TBSV; *Tombusvirus*), has revealed that viral infections are affected by more than 100 host genes in each case; these genes encode a distinct set of host factors for each of the two viruses (Kushner et al., 2003; Gancarz et al., 2011; Nagy, 2016). Among the identified host proteins, several give rise to loss-of-susceptibility phenotypes when the corresponding genes are mutated. In addition, other host proteins identified from naturally occurring resistant cultivars are important genetic resources for recessive resistance. They are discussed separately in the next section.

With several exceptions (Fujisaki and Ishikawa, 2008; Cheng et al., 2009; Huh et al., 2013), many of the host factors characterized so far in plants positively control viral infection; herein, we refer to them as "positive regulators." These positive regulators have been characterized predominantly through transient knockdown experiments. Knockdown of a gene encoding a positive regulator of viral infection results in a decrease of viral accumulation. This phenotype is equivalent to recessive resistance, and leads us to expect that the corresponding host factor could be a recessive resistance gene in crop species, especially if deficiency of the host factor has no adverse effect on plant growth. However, there could be a qualitative difference between the transient knockdown of a host factor by RNA silencing and the null mutation. When a host factor is indispensable for plant viability or is encoded by functionally redundant genes, the transient knockdown of the factor and the null mutation may produce different phenotypes (Wei et al., 2013; Xiong and Wang, 2013). Alternatively, even if a host factor plays an essential role in plant viability, a conserved amino acid substitution could confer viral resistance without an adverse effect on plant growth (Ouibrahim et al., 2014). This scenario would suggest that the substituted amino acid is critical for molecular plant–virus interaction, but not for plant viability. Further molecular analyses will be necessary to reveal the availability of positive regulators as recessive resistance genes.

Some of the positive regulators identified so far are common among distantly related viruses (Nagy et al., 2014). Although confirmatory molecular studies will be required, deficiency of these host factors could generate recessive resistance against a wide range of viruses. For example, HSP90 is required for viral replication of red clover necrotic mosaic virus (RCNMV; *Dianthovirus*) (Mine et al., 2012) and bamboo mosaic virus (BaMV; *Potexvirus*) (Huang et al., 2012). Infection by rice stripe virus (RSV; *Tenuivirus*) (Jiang et al., 2014), turnip mosaic virus (TuMV; *Potyvirus*) (Jungkunz et al., 2011) and RCNMV (Mine et al., 2012) is not supported efficiently after silencing of HSP70. eEF1A seems to be commonly involved in viral replication via interaction with a viral replicase in tobacco mosaic virus (TMV; *Tobamovirus*) (Yamaji et al., 2006, 2010), TuMV (Thivierge et al., 2008), and TBSV (Li et al., 2009) as well as with viral RNA in turnip yellow mosaic virus (TYMV; *Tymovirus*) (Dreher et al., 1999), TMV (Zeenko et al., 2002), and TBSV (Li et al., 2009). Noted that these host factors are also involved in plant growth, gene expression, and plant hormone signaling (Ransom-Hodgkins, 2009; Clément et al., 2011; Jungkunz et al., 2011; Zhang X.C. et al., 2015). In plants, cytosolic HSP70 and HSP90 are important for disease resistance against pathogens other than viruses (Kanzaki et al., 2003; Takahashi et al., 2003). Therefore, some mutations of these genes not only confer recessive resistance to a plant virus but may also have unexpected adverse effects on plants.

PROMISING GENETIC RESOURCES FOR RECESSIVE RESISTANCE

If a host factor for viral infection can be mutated in one plant species without any adverse effects on plant growth at least under controlled greenhouse conditions, one would expect that this might be possible in other plant species, too, and that such host factors would be promising genetic targets for recessive resistance. In this section, we discuss host factors that have been identified as potential targets for recessive resistance either from loss-of-susceptibility mutants or from naturally occurring resistant cultivars (**Table 1**). It is noteworthy that some translation factors, including polyA-binding protein (PABP) and DEAD-box RNA helicase (referred to as DDXs or RHs), are promising genetic targets for recessive resistance (Dufresne et al., 2008; Li et al., 2016), but because they have been discussed in detail elsewhere (Sanfaçon, 2015), they are not covered in this section.

Tobamovirus multiplication 1 (TOM1) has been identified using *Arabidopsis* mutants with loss-of-susceptibility to youcai mosaic virus [YoMV; *Tobamovirus* (previously referred to as TMV-Cg)] (Yamanaka et al., 2000). The *tom1-1* mutation does not completely suppress YoMV accumulation unless the *TOM3* gene is also mutated (Yamanaka et al., 2002). However, CMV and TCV accumulation are unaffected in the *tom1tom3* double mutant (Yamanaka et al., 2002). TOM1 and TOM3 are closely related, seven-pass membrane proteins, and TOM1 interacts with the helicase domain of YoMV replicase (the current model of tobamovirus replication is well documented in another review; Ishibashi and Ishikawa, 2016). Although *TOM1* and *TOM3* homologs are encoded in *Nicotiana* spp., tomato (*S. lycopersicum*), pepper and rice (Kumar et al., 2012), functional validation of these proteins in tobamovirus accumulation has only been performed in *Nicotiana* spp. (Asano et al., 2005; Chen et al., 2007). Asano et al. (2005) demonstrated that knockdown of both *TOM1* and *TOM3* genes in *N. tabacum* completely suppresses three distinct tobamoviruses other than YoMV. The genes identified from the *tom2-1 Arabidopsis* mutant are *TOM2A* and *TOM2B* (Tsujimoto et al., 2003). TOM2A is a four-pass membrane protein and TOM2B is a basic protein. Although the molecular function of TOM2B is unknown, TOM2A is thought to be involved in tobamovirus accumulation via its interaction with TOM1 (Tsujimoto et al., 2003; Ishibashi and Ishikawa, 2016).

ARL8, a small GTP-binding ARF-family protein, has been co-purified with a replicase from tomato mosaic virus (ToMV; *Tobamovirus*) (Nishikiori et al., 2011). ARL8, together with TOM1, is involved in ToMV replication through regulating

TABLE 1 | The genetic resources for recessive resistance found in loss-of-susceptibility mutants and naturally occurring resistant cultivars.

Gene	Plant species encoding homologs	Cause of resistance	Affected virus[1]	Non-affected virus[1]	Reference
TOM1; *TOM3*	*Nicotiana* spp. *Solanum lycopersicum* *Capsicum annuum* *Oryza sativa*	Loss-of-susceptibility by ethyl methanesulfonate (EMS) mutagenesis	YoMV ToMV TMV TMGMV PMMoV	CMV TCV TYMV	Ishikawa et al., 1991 Ishikawa et al., 1993 Yamanaka et al., 2000 Yamanaka et al., 2002 Kumar et al., 2012
TOM2A; *TOM2B*	*Arabidopsis thaliana*	Loss-of-susceptibility by fast neutron mutagenesis	YoMV ToMV	CMV TCV TYMV	Ohshima et al., 1998 Tsujimoto et al., 2003
ARL8	*Arabidopsis thaliana* *Nicotiana tabacum*	Loss-of-susceptibility by simultaneous null mutation of *ARL8a* and *ARL8b* by T-DNA insertions	ToMV YoMV	CMV	Nishikiori et al., 2011
RIM1	*Oryza sativa* *Arabidopsis thaliana*	Loss-of-susceptibility by Tos17-based insertional mutagenesis	RDV	RTYV RSV	Yoshii et al., 2009
DBP1	*Arabidopsis thaliana* *Nicotiana tabacum* *Zea mays* *Oryza sativa* *Mesembryanthemum crystallinum*	Loss-of-susceptibility in a T-DNA mutant	TuMV PPV	CMV	Carrasco et al., 2005 Castelló et al., 2010
cPGK	*Nicotiana* spp. *Solanum lycopersicum* *Solanum tuberosum* *Populus trichocarpa* *Sorghum bicolor* *Oryza sativa* *Triticum aestivum* *Zea mays*	Natural resistance gene, *rwm1*, found in *Arabidopsis* Cvi-0 ecotype	WMV PPV BaMV	PVX CMV	Lin et al., 2007 Ouibrahim et al., 2014 Poque et al., 2015
EXA1	*Arabidopsis thaliana* *Oryza sativa* *Solanum lycopersicum*	Loss-of-susceptibility by EMS mutagenesis	PlAMV PVX AltMV	CMV TCV YoMV	Hashimoto et al., 2016
PVIP1; PVIP2	*Arabidopsis thaliana* *Pisum sativum* *Nicotiana benthamiana*	Loss-of-susceptibility in a knockdown mutant of each *PVIP*	TuMV	–	Dunoyer et al., 2004
PDLP1; PDLP2; PDLP3	*Arabidopsis thaliana*	Loss-of-susceptibility by triple mutation of *PDLP1*, *PDLP2* and *PDLP3* by T-DNA insertions	GFPV CaMV	ORMV	Amari et al., 2010
PCaP1	*Arabidopsis thaliana*	Loss-of-susceptibility in a T-DNA mutant	TuMV	ORMV	Vijayapalani et al., 2012
SYTA	*Arabidopsis thaliana*	Loss-of-susceptibility in a T-DNA mutant	CaLCuV TVCV TuMV	CaMV	Lewis and Lazarowitz, 2010 Uchiyama et al., 2014
Sec24a	*Arabidopsis thaliana*	Loss-of-susceptibility in an EMS-induced mutant	TuMV	–	Jiang et al., 2015
RHD3	*Arabidopsis thaliana*	Loss-of-susceptibility in a T-DNA mutant	TSWV	–	Feng et al., 2016
PDIL5-1	All plant species	Natural resistance gene, *rym11*, found in barley	BaYMV BaMMV	–	Yang et al., 2014
IRE1	All plant species	Loss-of-susceptibility by double mutation of *IRE1a* and *IRE1b* by T-DNA insertions	TuMV	–	Zhang L. et al., 2015
bZIP60	All plant species	Loss-of-susceptibility in a T-DNA mutant	TuMV PVX	–	Ye et al., 2011 Zhang L. et al., 2015
HAT1; HAT2; HAT3	*Arabidopsis thaliana*	Loss-of-susceptibility by triple mutation of *HAT* genes by T-DNA insertions	CMV	–	Zou et al., 2016
CPR5	*Oryza glaberrima* *Arabidopsis thaliana*	Natural resistance gene, *rymv2*, found in African rice	RYMV	–	Orjuela et al., 2013

[1]*Virus abbreviations not provided in the text: TMGMV (tobacco mild green mosaic virus; Tobamovirus), PMMoV (pepper mottle mosaic virus, Tobamovirus), AltMV (alternanthera mosaic virus; Potexvirus).*

the enzymatic activity of a ToMV replicase in RNA synthesis and capping (Nishikiori et al., 2011). While a deletion in any one of three *ARL8* genes does not alter ToMV accumulation in *Arabidopsis*, mutation of both the *ARL8a* and *ARL8b* genes completely suppressed viral accumulation without any adverse effect on plant growth (Nishikiori et al., 2011). *ARL8* demonstrates that host factor genes and their functionally redundant homologs may be good targets for joint mutations that together produce recessive resistance. Alternatively, as demonstrated by the eIF4Es (Sato et al., 2005; Nicaise et al., 2007; Reinbold et al., 2013), when a distinct protein among a functionally related group has established a specific interaction with a virus, the corresponding gene alone could be targeted for mutation to generate recessive resistance.

Rice dwarf virus multiplication 1 (rim1) mutant is produced by a retrotransposon Tos17 insertion in an NAC-domain transcription factor and shows loss-of-susceptibility to rice dwarf virus (RDV; *Phytoreovirus*) (Yoshii et al., 2009). However, *rim1* mutants are susceptible to two other rice viruses, rice transitory yellowing virus (RTYV; *Rhabdovirus*) and RSV (Yoshii et al., 2009). The RIM1 protein is closely related to an *Arabidopsis* NAC domain protein, ANAC028. Yoshii et al. (2009) also demonstrated that the *rim1* mutation has a small negative effect on the survival of green rice leafhopper (*Nephotettix cincticeps*), an insect vector of RDV. This may be related to observations of jasmonic acid (JA)-induced phenotypes in some *rim1* mutants (Yoshii et al., 2010). Although the molecular function of RIM1 in RDV infection is unclear, the protein could be critical for RDV infection without being a general defense repressor if RIM1-mediated resistance is specific for RDV (Yoshii et al., 2009).

Knockout mutation in *DNA-binding protein phosphatase 1* (*DBP1*) gene does not influence plant growth in *Arabidopsis*, but does result in resistance to two potyviruses, TuMV and plum pox virus (PPV) (Castelló et al., 2010). The domain structure of DBP1 suggests that it functions in signal transduction as well as in transcriptional regulation (Carrasco et al., 2006). *DBP1*-related genes are present in dicotyledons and monocotyledons, including *N. tabacum*, maize (*Zea mays*), and rice (Carrasco et al., 2005). As DBP1 forms a stabilizing interaction with eIFiso4E, the loss of susceptibility of *dbp1* mutants may be related to the low-level accumulation of eIFiso4E (Castelló et al., 2010). DBP1 also interacts with 14-3-3 family protein GRF6, regulating its phosphorylation status, and the *grf6* mutant is resistant to PPV (Carrasco et al., 2006, 2014). The DBP1 interaction with GRF6 may regulate the phosphorylation status of eIFiso4E, thereby altering its cap-binding activity (Khan and Goss, 2004). Further studies are needed to confirm the mechanism of DBP1-mediated resistance.

A recessive allele conferring resistance to watermelon mosaic virus (WMV; *Potyvirus*) has been identified in the *Arabidopsis* ecotype Cvi-0 and designated *resistance to watermelon mosaic virus 1* (*rwm1*) (Ouibrahim et al., 2014). Map-based cloning identified an amino acid substitution in a nuclear-encoded chloroplast phosphoglycerate kinase, cPGK2 (Ouibrahim et al., 2014). *cPGK2* gene homologs are found in dicotyledons and monocotyledons including: *Nicotiana* spp., tomato, potato (*S. tuberosum*), poplar (*Populus trichocarpa*), sorghum (*Sorghum bicolor*), rice, wheat (*Triticum aestivum*), and maize. Downregulation of *cPGK* genes in *N. benthamiana* compromises WMV (Ouibrahim et al., 2014) and PPV accumulation (Poque et al., 2015). Remarkably, cPGK is associated with the 3′-untranslated region of BaMV genomic RNA and is required for the efficient accumulation of BaMV in *N. benthamiana* (Lin et al., 2007). Recently, Cheng et al. (2013) demonstrated that cPGK recruits BaMV genomic RNA to chloroplasts to support BaMV replication in *N. benthamiana*. Consistent with this, some potyviruses are thought to replicate their genomic RNA in chloroplasts (Wei et al., 2013). Further studies are needed to reveal the role of cPGK in potyvirus and potexvirus infection.

More recently, Hashimoto et al. (2016) demonstrated that deficiencies in *essential for potexvirus accumulation 1* (*EXA1*) gene were present in a loss-of-susceptibility *Arabidopsis* mutant that did not support plantago asiatica mosaic virus (PlAMV; *Potexvirus*) accumulation. *EXA1* is an unannotated gene in plants, but contains a putative eIF4E-binding motif and a GYF domain, which binds to proline-rich peptides (Kofler and Freund, 2006). Based on sequence comparisons with other related genes, *EXA1* homologs are encoded in rice and tomato and are structurally related to human GIGYF2 protein (Hashimoto et al., 2016). T-DNA insertion of *EXA1* gene, forming *exa1-1* mutant, does not affect accumulation of CMV, TCV, or YoMV, but does suppress the accumulation of two distinct potexviruses other than PlAMV (Hashimoto et al., 2016). Because human GIGYF2 regulates mRNA translation (Morita et al., 2012), it is conceivable that EXA1 might also regulate the translation of a viral protein during early infection. Further studies are needed to reveal the role of EXA1 in virus infection and whether EXA1-mediated resistance is effective in other plant species and against viruses other than potexviruses.

Functional studies on the host factors that play a critical role in viral transport to healthy plant cells have shed light on several potential recessive resistance genes conferring resistance to plant viruses. Once the viral genomes are replicated in the initially infected cells, the viruses must transport their genomes through plasmodesmata (PD), which are plant-specific intercellular nanopores that connect neighboring cells. To transport infectious entities to PD, viral movement proteins (MPs) recruit host factors and host machineries, such as cellular trafficking pathways. Viruses that are able to reach the phloem by continuous transport to neighboring cells systemically spread through the sieve tube, depending on host factors. Potyvirus VPg-interacting protein from pea (PVIPp) was isolated through yeast two-hybrid screening of a cDNA library from pea (*Pisum sativum*). PVIPp interacts with VPg protein of pea seed-borne mosaic virus (PSbMV; *Potyvirus*) (Dunoyer et al., 2004). In *Arabidopsis*, PVIP1 and PVIP2 are closely related homologs, and their knockdown in plants confers loss-of-susceptibility to TuMV (Dunoyer et al., 2004). A TuMV mutant with a point mutation in VPg that affects the interactions with PVIP1 compromises cell-to-cell transport (Dunoyer et al., 2004). Since PVIP1 and PVIP2 interact with VPg proteins of other potyviruses, PVIPs-mediated resistance may also be effective against other potyviruses. *Arabidopsis* PCaP1 and COPII coatomer Sec24a interact with P3N-PIPO and $6K_2$ of TuMV, respectively (Vijayapalani et al., 2012; Jiang et al.,

2015). Both host factors are involved in distinct steps in TuMV cell-to-cell transport. A mutation in *PCaP1* or *Sec24a* gene in *Arabidopsis* impairs TuMV infection (Vijayapalani et al., 2012; Jiang et al., 2015). Knockout of *root hair defective 3* (*RHD3*), whose gene product is involved in the formation of the tubular ER network structure, significantly inhibits the systemic infection of tomato spotted wilt virus (TSWV; *Tospovirus*) (Feng et al., 2016). PD-located protein 1 (PDLP1) was originally identified as a cell wall-associated membrane protein in *Arabidopsis* and was isolated from a highly purified cell wall fraction (Bayer et al., 2006; Thomas et al., 2008). A *PDLP1*, *PDLP2* and *PDLP3* triple mutant inhibits systemic infection of grapevine fanleaf virus (GFLV; *Nepovirus*) and cauliflower mosaic virus (CaMV; *Caulimovirus*) but not oilseed rape mosaic virus (ORMV; *Tobamovirus*) (Amari et al., 2010). Although GFLV and CaMV are distantly related viruses, MPs of both viruses form a specific structure, called a tubule used in cell-to-cell transport. These results imply that the loss-of-susceptibility of *pdlp1/2/3* triple mutant is also applicable to other viruses that employ the tubule-based transport strategy. *Arabidopsis* synaptotagmin (SYTA), a plant homolog of calcium sensors widely studied in animals, has been shown to interact with MP of cabbage leaf curl virus (CaLCuV; *Begomovirus*) (Lewis and Lazarowitz, 2010). Remarkably, a *syta* mutant significantly inhibits systemic infection of a diverse spectrum of plant viruses, including CaLCuV, turnip vein clearing virus (TVCV; *Tobamovirus*) and TuMV, but not of CaMV (Lewis and Lazarowitz, 2010; Uchiyama et al., 2014), suggesting that SYTA and the involved cellular machinery are promising candidates for recessive resistance against a wide range of viruses. In spite of the above-mentioned results, no study has reported a naturally occurring recessive resistant cultivar that targets viral transport. Thus, the targeting of viral transport for recessive resistance may well be technically challenging.

The unfolded protein response (UPR) is a highly conserved cellular machinery that allows both animals and plants to cope with an overload of unfolded proteins in the endoplasmic reticulum (ER) (Howell, 2013). Recently, several studies have suggested the relevance of the UPR in plant–virus interactions (Ye et al., 2011; Yang et al., 2014; Zhang L. et al., 2015; Arias Gaguancela et al., 2016). In barley, the recessive resistance genes *rym4/rym5*, which are alleles of *eIF4E*, have been overcome by resistance-breaking isolates of BaMMV and BaYMV (Hariri et al., 2003; Kühne et al., 2003), whereas *rym11* resistance cultivars are highly durable against both virus isolates. Positional cloning has revealed that a mutation in *protein disulfide isomerase like 5-1* (*PDIL5-1*) is responsible for the recessive resistance gene *rym11* (Yang et al., 2014). The natural variation among *HvPDIL5-1* genes suggests that most of the *rym11* cultivars collected from eastern Asia are the result of frequent interactions with highly divergent forms of BaMMV and BaYMV (Yang et al., 2014). PDIL5-1 is a conserved protein in plants and animals, which functions as an endoplasmic reticulum-localized chaperone in the UPR (Howell, 2013). *Arabidopsis bzip60-2* mutant and *ire1a/ire1b* double mutant, which are mutants of other UPR components, show loss-of-susceptibility to TuMV (Zhang L. et al., 2015). Silencing of *bZIP60* gene significantly suppresses the accumulation of potato virus X (PVX; *Potexvirus*) in *N. benthamiana* (Ye et al., 2011). Although the mechanism of the resistance mediated by the UPR components remains unclear, the striking conservation of UPR components and the consistency of their roles in viral infection imply that they are promising genetic targets for recessive resistance to a wide range of viruses.

Several lines of evidence suggest that a mutation in a gene encoding a component of plant defense responses could confer resistance to viruses. *Arabidopsis ssi2* mutant, which accumulates high levels of plant defense hormone salicylic acid (SA), confers resistance to CMV (Sekine et al., 2004). Based on the experimental evidences, Sekine et al. (2004) demonstrated that the resistance to CMV in *ssi2* mutant is unrelated to SA production and the dwarf phenotype. Some *Arabidopsis* mutants related to the defense hormone ethylene, such as *acs6* mutant, also shows resistance to YoMV (Chen et al., 2013). Although the loss-of-susceptibility of the mutants related to defense responses may be due to elevated antiviral defense signaling(s), mutants such as *ssi2* mutant frequently show an abnormal growth phenotype (Sekine et al., 2004). Remarkably, a triple mutant of *homeodomain-leucine zipper protein 1* (*HAT1*) and its related genes *HAT2* and *HAT3* confers loss-of-susceptibility to CMV without any growth defect despite the high level of SA and JA accumulation (Zou et al., 2016). However, as discussed earlier, if a deficiency in a specific defense signaling molecule confers recessive resistance to a plant virus, there could be unexpected adverse effects on the plants because of the complex nature of the plant defense signaling network (Mine et al., 2014). The *RYMV2* gene, identified using the resistant Tog7291 accession of African rice (*O. glaberrima*), encodes a recessive resistance gene that is responsible for durable resistance to rice yellow mottle virus (RYMV; *Sobemovirus*). The *rymv2* mutant is deficient in a rice homolog of the *Arabidopsis CPR5* gene (Orjuela et al., 2013), which has a repressive role in plant defense responses (Yoshida et al., 2002). Alleles of the *rymv2* mutant have also been found in seven additional African rice accessions that were resistant to RYMV (Orjuela et al., 2013). Due to the role of *Arabidopsis* CPR5 in defense responses, the activation of defense responses by *rymv2* alleles presumably contributes to RYMV resistance.

STRATEGIES FOR IMPROVING THE GENETIC RESOURCES FOR RECESSIVE RESISTANCE

Despite their importance, few host factors have successfully been identified by forward genetic screening or as naturally occurring recessive resistant alleles (**Table 1**). In part, this is because genetic screening and traditional gene mapping approaches are labor intensive and costly; it is also difficult to identify particular types of gene (for example, those that are functionally redundant or those that are essential for plant viability) using a genetic approach. Moreover, even after genes of interest have been identified, there may be substantial delays before these can be used to generate recessive resistance in crop species. Establishing resistant cultivars targeting a specific gene using random mutagenesis and screening, and introducing

traits through crossing, are both technically challenging and time-consuming procedures. However, the emergence of NGS, genome editing, and other technologies have provided new opportunities for improving and utilizing genetic resources for recessive resistance breeding.

As discussed above, loss-of-susceptibility to viral infection produced by random mutagenesis is genetically equivalent to recessive resistance found in natural variants. Performing random mutagenesis in crops and model plants circumvents the limitations imposed by relying on genetic variation found only in naturally occurring cultivars. In addition, the recessive resistance discussed earlier including that mediated by eIF4Es, is effective in several plant species. Therefore, random mutagenesis and selection for loss-of-susceptibility mutants in model plants, including *Arabidopsis*, is still an attractive option for improving genetic resources to apply recessive resistance in crops. Model plants facilitate the isolation of loss-of-susceptibility mutants and the subsequent identification of corresponding genes due to the availability of whole-genome sequence information and their characteristically simple genetics (Yamanaka et al., 2000; Yoshii et al., 2009). By contrast, random mutagenesis performed in polyploid plants (e.g., wheat and soybean) presents difficulties that include obtaining mutants with discernible phenotypes, often due to functional complementation by redundant genes. However, to ultimately apply mutant screening in *Arabidopsis* to recessive virus resistance-based crop breeding, it is important to select a virus species that can infect *Arabidopsis*, and comes from the same viral genus as the target virus (Ishikawa et al., 1991; Fujisaki et al., 2004; Yamaji et al., 2012). Additionally, to reliably and rapidly detect viral infection, the introduction of green fluorescent protein into an infectious viral clone is desirable (Baulcombe et al., 1995; Minato et al., 2014). The rationale for this is based on the expectation that viruses from the same genus have similar life cycles. In fact, it is known that some host factors, including eIF4Es, play a similar role in infection by different viruses from the same genus (Asano et al., 2005; Ouibrahim et al., 2014; Yang et al., 2014; Poque et al., 2015; Hashimoto et al., 2016). However, there are many exceptions that challenge this rationale (for example, see the section on eIF4Es-MEDIATED RECESSIVE RESISTANCE). Thus, validation of the results obtained from *Arabidopsis* mutant screening in other host–virus interactions is essential.

Next-generation sequencing technologies have made it easy for many plant scientists to access whole-genome plant sequencing (Morrell et al., 2012). Simultaneously, genomics-based crop breeding using NGS technologies is expected to overcome the challenge of feeding an increasing world population. As suggested by Varshney et al. (2014), NGS technologies, which have rarely been applied to antiviral breeding using natural variants (Zuriaga et al., 2013; Mariette et al., 2016), would be quite useful for identifying loci in naturally resistant variants and also for breeding to introduce resistant loci into specific cultivars. Several studies have identified loci of interest from *Arabidopsis* mutants using whole-genome sequencing of pooled mutant F2 populations (Schneeberger et al., 2009; Austin et al., 2011; Uchida et al., 2011). The *EXA1* gene was identified successfully from a loss-of-susceptibility mutant by combining conventional map-based cloning and whole-genome sequencing of mutant plants (Hashimoto et al., 2016). Methods based on a similar concept have also been established in rice (Abe et al., 2012; Takagi et al., 2013). These studies suggest that a resistance locus could be identified rapidly from a loss-of-susceptibility mutant based on whole-genome sequencing in *Arabidopsis* and rice.

Genome editing based on sequence-specific nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effectors (TALENs), and CRISPR-associated protein 9 (Cas9) in clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas systems has recently been developed to enable targeted mutagenesis and gene insertion in eukaryotic genomes (Gaj et al., 2013). The applications of these genome editing technologies in plants are well summarized elsewhere (Araki and Ishii, 2015; Luo et al., 2016; Ma et al., 2016). Importantly, genome editing technologies have been employed not only in model plants but also in several crop species, and they are now being applied even more widely. One of the outstanding points of genome editing in terms of its application to crop breeding is that the original transgenes for genome editing can be removed via segregation after editing. Recently, the CRISPR/Cas9 system was used to establish eIFiso4E-deficient *Arabidopsis* mutants that were free from transgenes and exhibited recessive resistance to TuMV (Pyott et al., 2016). More importantly, the CRISPR/Cas9 system was also applied to cucumber (*Cucumis sativus*) to disrupt the *eIF4E* gene, and the non-transgenic, eIF4E-deficient plant lines were resistant to the cucumber vein yellowing virus (CVYV; *Ipomovirus*) and two potyviruses (Chandrasekaran et al., 2016). Even in polyploid soybean, duplicated genes have been mutagenized using ZFNs (Curtin et al., 2011). In allohexaploid wheat, simultaneous mutation of three *MILDEW RESISTANCE LOCUS* genes by TALENs resulted in resistance to a powdery mildew fungal pathogen (Wang et al., 2014). Editing of multiple genes using the CRISPR-Cas9 system is applicable to *Arabidopsis* and rice (Ma et al., 2015). These studies suggest that genetic targets for recessive resistance may be mutagenized in various crop species (including polyploid crops) using genome editing technologies. Previously, the only methods for introducing a recessive resistant locus from a natural variant into a specific cultivar were crossing and random mutagenesis. Because targeted mutagenesis by genome editing involves a small deletion or insertion in a specific genomic site through non-homologous end-joining (NHEJ), genome editing technologies are compatible with developing and applying genetic resources for recessive resistance in crop species.

CONCLUSION AND FUTURE PERSPECTIVES

In this article, we focused on emphasizing the importance of recessive resistance in future anti-viral breeding. Significant fundamental research efforts have been invested in identifying host factors involved in plant virus infection. The corresponding genes are potential targets for recessive resistance, in addition to the eIF4Es. The application of this information to crop research should result in the development of new recessive resistance

traits. However, to avoid the unforeseeable effects of mutations and to expand the possible application range of each host factor, further studies are essential and should focus on the molecular function of each factor in viral infection and also in that of the relevant viruses. In addition, it is necessary to identify plants that are susceptible to the viruses through each particular host factor. Currently, extensive characterization studies have been limited to only a few plant virus species, and so it is critical to expand this research to include other viruses with agricultural impact (Rybicki, 2015). Genome editing technologies are promising methods for introducing recessive resistance into various crop species. Moreover, some genome-edited crops have already been made available without restriction by the US Department of Agriculture, one of the agencies responsible for the regulation of genetically modified organisms (GMOs) in the USA (Waltz, 2016a,b). However, it remains unclear whether resources created by genome editing are subject to regulations associated with GMOs in other countries (Hartung and Schiemann, 2014; Araki and Ishii, 2015). Based on the possible regulatory guidelines that take into account mutation patterns and modification mechanisms, as suggested by Araki and Ishii (2015), mutation mechanisms capable of producing recessive resistance should be prioritized into categories that may be most easily accepted. Further research to support and enhance the safety of genome editing technologies for recessive resistance-based crop breeding is extremely important.

AUTHOR CONTRIBUTIONS

MH, YY, and SN designed the research. MH, YN, and YY surveyed and discussed on the previous researches. MH, YY, and SN wrote the paper with the support by YN.

ACKNOWLEDGMENTS

This research was supported by JSPS KAKENHI Grant Number 25221201 (category 'S') to SN and Grant-in-Aid for JSPS Fellows (Grant Number 15J04093) to MH.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 30 November 2016
doi: 10.3389/fmicb.2016.01906

The Current Status of the Soybean-*Soybean Mosaic Virus* (SMV) Pathosystem

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Soybean mosaic virus (SMV) is one of the most devastating pathogens that cost huge economic losses in soybean production worldwide. Due to the duplicated genome, clustered and highly homologous nature of *R* genes, as well as recalcitrant to transformation, soybean disease resistance studies is largely lagging compared with other diploid crops. In this review, we focus on the major advances that have been made in identifying both the virulence/avirulence factors of SMV and mapping of SMV resistant genes in soybean. In addition, we review the progress in dissecting the SMV resistant signaling pathways in soybean, with a special focus on the studies using virus-induced gene silencing. The soybean genome has been fully sequenced, and the increasingly saturated SNP markers have been identified. With these resources available together with the newly developed genome editing tools, and more efficient soybean transformation system, cloning SMV resistant genes, and ultimately generating cultivars with a broader spectrum resistance to SMV are becoming more realistic than ever.

Keywords: soybean, *soybean mosaic virus*, disease resistance, virus-induced gene silencing, SNP, mapping

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *30 September 2016*
Accepted: *15 November 2016*
Published: *30 November 2016*

Citation:
Liu J-Z, Fang Y and Pang H (2016) The Current Status of the Soybean-Soybean Mosaic Virus (SMV) Pathosystem. Front. Microbiol. 7:1906. doi: 10.3389/fmicb.2016.01906

OVERVIEW

Soybean [*Glycine max* L. (Merrill)] is one of the most important sources of edible oil and proteins. Pathogen infections cause annual yield loss of $4 billion dollars in the United States alone[1]. Among these pathogens, *Soybean mosaic virus* (SMV) is the most prevalent and destructive viral pathogen in soybean production worldwide (Hill and Whitham, 2014). SMV is a member of the genus *Potyvirus* in the *Potyviridae* family and its genome is a single-stranded positive-sense RNA, encoding at least 11 proteins (**Figure 1**): potyvirus 1 (P1), helper-component proteinase (HC-Pro), potyvirus 3 (P3), PIPO, 6 kinase 1(6K1), cylindrical inclusion (CI), 6 kinase 2 (6K2), nuclear inclusion a-viral protein genome-linked (NIa-VPg), nuclear inclusion a-protease (NIa-Pro), nuclear inclusion b (Nib), and coat protein (CP) (Eggenberger et al., 1989; Jayaram et al., 1992; Wen and Hajimorad, 2010). Numerous SMV isolates have been classified into seven distinct strains (G1 to G7) in the United States based on their differential responses on susceptible and resistant soybean cultivars (Cho and Goodman, 1979, **Table 1**), while in China, 21 strains (SC1–SC21) have been classified (Wang et al., 2003; Guo et al., 2005; Li et al., 2010). The relationship between G strains in the United States and SC strains in China has not been fully established yet. SMV resistance is conditioned by complex gene families. Multiple independent resistance loci with different SMV strain specificities have been identified, and most of them are non-Toll interleukin receptor- nucleotide binding

[1]http://aes.missouri.edu/delta/research/soyloss.stm

site-leucine rich repeat (TIR-NBS-LRR) type R genes (Hill and Whitham, 2014). So far, three independent loci, *Rsv1*, *Rsv3*, and *Rsv4* in the United States and many *Rsc* loci in China, have been reported for SMV resistance. However, none of these genes has been cloned and their identities remain to be revealed.

MAPPING OF SMV RESISTANT LOCI

Complex Nature of *Rsv1* Loci in Soybean

Rsv1 was originally identified in the soybean line PI 96983 (Kiihl and Hartwig, 1979), and it confers extreme resistance (ER) to SMV-G1 through G6 but not to SMV-G7 (Chen et al., 1991; Hajimorad and Hill, 2001; **Table 1**). Multiple *Rsv1* alleles including *Rsv1-y*, *Rsv1-m*, *Rsv1-t*, *Rsv1-k,* and *Rsv1-r* have been identified from different soybean cultivars with differential reactions to SMV G1–G7 strains (Chen et al., 2001). *Rsv1* was initially mapped to soybean linkage group F on chromosome 13 (Yu et al., 1994) and two classes of NBS-LRR sequences (classes b and j) were identified in this resistance gene cluster (Yu et al., 1996). A large family of homologous sequences of the class j Nucleotide biinding site-leucine rich repeat (NBS-LRRs) clustered at or near the *Rsv1* locus (Jeong et al., 2001; Gore et al., 2002; Peñuela et al., 2002). Six candidate genes (1eG30, 5gG3, 3gG2, 1eG15, 6gG9, and 1gG4) in PI96983 were mapped to a tightly clustered region near *Rsv1*, three of them (3gG2, 5gG3, and 6gG9) were completely cloned and sequenced (GenBank accession no. AY518517–AY518519). Among the three genes, 3gG2 was found to be a strong candidate for *Rsv1* (Hayes et al., 2004). When 3gG2, 5gG3, and 6gG93 were simultaneously silenced using *Bean pod mottle virus* -induced gene silencing (BPMV-VIGS), the *Rsv1*-mediated resistance was compromised, confirming that one or more of these three genes is indeed the *Rsv1* (Zhang et al., 2012). Because, the sequence identities of these three *R* genes are extremely high along the entire cDNAs, it is impossible to differentiate which one(s) is *Rsv1*.

Several studies indicate that two or more related non-TIR-NBS-LRR gene products are likely involved in the allelic response of several *Rsv1*-containing lines to SMV (Hayes et al., 2004; Wen et al., 2013; Yang et al., 2013). Wen et al. (2013) generated two soybean lines, L800 and L943, derived from crosses between PI96983 (*Rsv1*) and Lee68 (*rsv1*) with distinct recombination events within the *Rsv1* locus. The L800 line contains a single PI96983-derived member 3gG2, confers ER against SMV-N (an avirulent isolate of G2 strain). In contrast, the line L943 lacks 3gG2, but contains a suite of five other NBS-LRR genes allows limited replication of SMV-N at the inoculation site. Domain swapping experiments between SMV-N and SMV-G7/SMV-G7d demonstrate that at least two distinct resistance genes at the *Rsv1* locus, probably belonging to the NBS-LRR class, mediate recognition of HC-Pro and P3, respectively (Khatabi et al., 2013; Yang et al., 2013).

Rsv3 Is Most Likely a NBS-LRR Type Resistant Gene

Rsv3 was originated from "L29," a 'Williams' isoline derived from Hardee (Bernard et al., 1991; Gunduz et al., 2000). The diverse soybean cultivars carrying *Rsv3* alleles condition resistance to SMV G5 through G7, but not G1 through G4 (Jeong et al., 2002; **Table 1**). *Rsv3* locus was firstly mapped between markers A519F/R and M3Satt on MLG B2 (chromosome 14) by Jeong et al. (2002), and was subsequently mapped on MLG-B2 with a distance of 1.5 cM from Sat_424 and 2.0 cM from Satt726 (Shi et al., 2008). The 154 kbp interval encompassing *Rsv3* contains a family of closely related coiled-coil NBS-LRR (CC-NBS-LRR) genes, implying that the *Rsv3* gene most likely encodes a member of this gene family (Suh et al., 2011).

Rsv4 Likely Belongs to a Novel Class of Resistance Genes

Rsv4 confer resistance to all 7 SMV strains (Chen et al., 1993; Ma et al., 1995). It was identified in soybean cultivars V94-5152 and mapped to a 0.4 cM interval between the proximal marker Rat2 and the distal marker S6ac, in a ~94 kb haplotype block on soybean chromosome 2 (MLG D1b++W) (Hayes et al., 2000; Saghai Maroof et al., 2010; Ilut et al., 2016). A haplotype phylogenetic analysis of this region suggests that the *Rsv4* locus in *G. max* is recently introgressed from *G. soja* (Ilut et al., 2016). Interestingly, this interval did not contain any NB-LRR type *R* genes. Instead, several genes encoding predicted transcription factors and unknown proteins are present within the region, suggesting that *Rsv4* most likely belongs to a novel class of resistance gene (Hwang et al., 2006; Ilut et al., 2016).

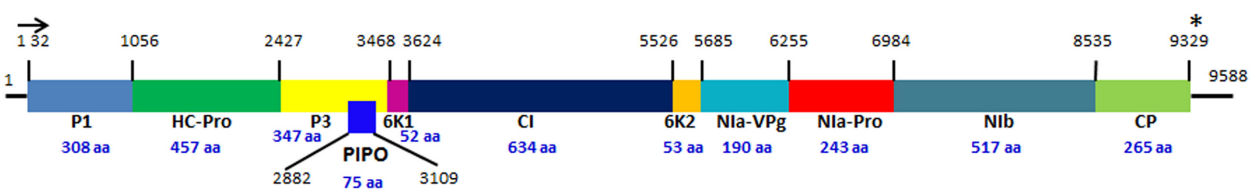

FIGURE 1 | The genome organization of *Soybean mosaic virus*. The diagram was drawn based on the nucleotide sequence of SMV N strain (Eggenberger et al., 1989). The colored boxes represent 11 proteins encoded by SMV genome. The black lines at the 5′ and 3′ ends represent 5′ and 3′ untranslated region (UTR). The horizontal arrow and the star indicate the start and stop codons of the SMV polypeptide, respectively. The numbers above the vertical lines indicate the start positions of the SMV proteins. The sizes of the SMV proteins (the numbers of amino acids) are indicated by the blue numbers below the protein names. The PIPO embedded in the P3 is shown by the overlapping dark blue box with the start and stop positions labeled, respectively. The diagram is not drawn in scale.

TABLE 1 | Summary of soybean-SMV studies.

Resistant locus	Chromosome location	Type of resistance gene	Strain specificity	Avirulent factor(s)
Rsvl	13 (Yu et al., 1994; Gore et al., 2002)	NBS-LRR type (Yu et al., 1994, 1996; Khatabi et al., 2013; Yang et al., 2013)	Resistant to: GI–G4 susceptable to: G5–G7 (Chen et al., 1991)	He-Pro and P3 (Eggenberger et al., 2008; Hajimorad et al., 2008) CI (Chowda-Reddy et al., 2011b; Wen et al., 2011) P3 (Chowda-Reddy et al., 2011b)
Rsv3	14 (Jeong et al., 2002; Shi et al., 2008)	CC- NBS-LRR type (Suh et al., 2011)	Resistant to: G5–G7 susceptable to: G1–G4 (Jeong et al., 2002)<	CI (Seo et al., 2009; Zhang et al., 2009a; Chowda-Reddy et al., 2011b) P3 (Chowda-Reddy et al., 2011a,b)
Rsv4	2 (Hayes et al., 2000; Saghai Maroof et al., 2010)	Novel class (Ilut et al., 2016)	Resistantto: GI–G7 (Chen et al., 1993; Ma et al., 1995)	P3 (Chowda-Reddy et al., 2011a,b; Khatabi et al., 2012; Wang et al., 2015)

THE OTHER SMV RESISTANT GENES

Many *Rsc* loci have been identified. The resistance genes *Rsc-8* and *Rsc-9*, which confer resistance to strains *SC-8* and *SC-9* respectively, have been mapped to the soybean chromosomes 2 (MLG D1b+W) (Wang et al., 2004). The interval of *Rsc-8* was estimated to be 200 kb and contains 17 putative genes and five of them, Glyma02g13310, 13320, 13400, 13460, and 13470 could be the candidates of *Rsc-8* based on their predicted functions and expression patterns (Wang et al., 2011). The *Rsc-15* resistant gene was mapped between Sat_213 and Sat_286 with distances of 8.0 and 6.6 cM to the respective flanking markers on chromosome 6 (Yang and Gai, 2011). The resistance gene *Rsc-7* in the soybean cultivar Kefeng No.1 was mapped to a 2.65 mega-base (Mb) region on soybean chromosome 2 (Fu et al., 2006) and was subsequently narrowed down to a 158 kilo-base (Kb) region (Yan et al., 2015). Within 15 candidate genes in the region, one NBS-LRR type gene (Glyma02g13600), one HSP40 gene (Glyma02g13520) and one serine carboxypeptidase-type gene (Glyma02g13620) could be the candidates for *Rsc-7*. The allelic relationship between the *Rsv* loci and the *Rsc* loci has yet to be determined.

Despite numerous efforts, none of the SMV resistant genes has been cloned and their identities remain to be identified. This reflects the complex nature of the resistant genes in palaeopolyploid soybean, in which 75% of the genes are present in multiple copies (Schmutz et al., 2010). This statement is reinforced by a recent finding that the soybean cyst nematode (SCN) resistance mediated by the *Rhg1* is conditioned by copy number variation of a 31-kilobase segment, in which three different novel genes are present (Cook et al., 2012). There are 1–3 copies of the 31-kilobase segment per haploid genome in susceptible varieties, but 10 tandem copies in resistant varieties (Cook et al., 2012). The presence of more copies of the 31-kb segment in resistant varieties increases the expressions of this set of the 3 genes and thus conferes the resistance (Cook et al., 2012, 2014).

IDENTIFICATION OF AVIRULENT FACTORS IN DIFFERENT SMV STRAINS THAT ARE SPECIFICALLY RECOGNIZED BY DIFFERENT *Rsv* GENE PRODUCTS

Avirulent Factors for *Rsv1*

SMV isolates are classified into seven strains (G1–G7) based on phenotypic reactions on a set of differential soybean cultivars (Cho and Goodman, 1982). The modification of avirulence factors of plant viruses by one or more amino acid substitutions can convert avirulence to virulence on hosts containing resistance genes and therefore, can be used as an approach to determine the avirulence factor(s) of a specific resistant gene.

Rsv1, a single dominant resistance gene in soybean PI 96983 (*Rsv1*), confers ER against SMV-G1 through G6 but not to SMV-G7 (Chen et al., 1991; Hajimorad and Hill, 2001; **Table 1**). SMV-N (an avirulent isolate of strain G2) elicits ER whereas strain SMV-G7 provokes a lethal systemic hypersensitive response (LSHR) (Hajimorad et al., 2003; Hayes et al., 2004). SMV-G7d, an evolved variant of SMV-G7 from lab, induces systemic mosaic (Hajimorad et al., 2003). Serial passages of a large population of the progeny in PI 96983 resulted in emergence of a mutant population (vSMV-G7d), which can evade *Rsv1*-mediated recognition and the putative amino acid changes that potentially responsible for the mutant phenotype is initially tentatively narrowed down to HC-Pro, coat protein, PI proteinase or P3 (Hajimorad et al., 2003; Seo et al., 2011) and was later mapped to P3 through domain swapping between the pSMV-G7 and pSMV-G7d (Hajimorad et al., 2005). The amino acids 823, 953, and 1112 of the SMV-G7d are critical in evading of *Rsv1*-mediated recognition (Hajimorad et al., 2005, 2006). By generating a series of chimeras between SMV-G7 and SMV-N in combination with site-directed mutagenesis, Eggenberger et al. (2008) and Hajimorad et al. (2008) independently showed that gain of virulence on *Rsv1*-genotype soybean by an avirulent SMV strains requires concurrent mutations in both P3 and HC-Pro and HC-Pro complementation of P3 is essential for

SMV virulence on *Rsv1*-genotype soybean (**Table 1**). A key virulence determinant of SMV on *Rsv1*-genotype soybeans that resides at polyprotein codon 947 overlaps both P3 and a PIPO-encoded codon. This raises the question of whether PIPO or P3 is the virulence factor. Wen et al. (2011) confirmed that amino acid changes in P3, and not the overlapping PIPO-encoded protein, which is embedded in the P3 cistron, determine virulence of SMV on *Rsv1*-genotype soybean. Chowda-Reddy et al. (2011b) constructed a chimeric infectious clone of G7, in which the N-ternimal part of CI was swapped with the corresponding part of G2. Compared with wildtype G7, this chimeric strain lost virulence on *Rsv1*-genotype plant but gained infectivity on *Rsv3*-genotype plant, indicating an essential role of CI for breaking down both *Rsv1*- and *Rsv3*-mediated resistance (Chowda-Reddy et al., 2011b). Together, it appears that P3, HC-Pro and possibly CI are virulent determinants for *Rsv1*-mediated resistance (**Table 1**).

Avirulent Factors for *Rsv3*

It has been proven that cytoplasmic inclusion cistron (CI) of SMV serves as a virulence and symptom determinant on *Rsv3*-genotype soybean and a single amino acid substitution in CI was found to be responsible for gain or loss of elicitor function of CI (Seo et al., 2009; Zhang et al., 2009a). Analyses of the chimeras by exchanging fragments between avirulent SMV-G7 and the virulent SMV-N showed that both the N- and C-terminal regions of the CI cistron are required for *Rsv3*-mediated resistance and the N-terminal region of CI is also involved in severe symptom induction in soybean (Zhang et al., 2009a). In addition to CI, P3 has also been reported to play an essential role in virulence determination on *Rsv3*-mediated resistance (Chowda-Reddy et al., 2011a,b; **Table 1**).

Avirulent Factor for *Rsv4*

Gain of virulence analysis on soybean genotypes containing *Rsv4* genes showed that virulence on *Rsv4* carrying cultivars was consistently associated with Q1033K and G1054R substitutions within P3 cistron, indicating that P3 is the SMV virulence determinant on *Rsv4* and one single nucleotide mutation in the P3 protein is sufficient to compromise its elicitor function (Chowda-Reddy et al., 2011b; Khatabi et al., 2012; Wang et al., 2015). However, the sites involved in the virulence of SMV on *Rsv4*-genotype soybean vary among strains (Wang et al., 2015).

It is clear now that P3 plays essential roles in virulence determination on *Rsv1, Rsv3,* and *Rsv4* resistant loci, while CI is required for virolence on *Rsv1* and *Rsv3* genotype soybean plants (Chowda-Reddy et al., 2011a,b). These results imply that avirulent proteins from SMV might interact with the soybean *R* gene products at a converged point. This evolved interactions sometimes could give SMV advantage in breaking resistance conferred by different SMV resistant genes simply by mutations within a single viral protein. On the other hand, since multiple proteins are involved in virulence on different resistant loci, concurrent mutantions in multiple proteins of SMV are required to evade the resistance conferred by different SMV resistant genes. The likelihood of such naturally occuurred concurrent mutations in different viral proteins is low. Therefore, integration of all three SMV resistant genes in a single elite soybean cultivar may provide long-lasting resistance to SMV in soybean breeding practice (Chowda-Reddy et al., 2011b).

GAIN OF VIRULENCE BY SMV ON A RESISTANT SOYBEAN GENOTYPE RESULTS IN FITNESS LOSS IN A PREVIOUSLY SUSCEPTIBLE SOYBEAN GENOTYPE

It seems that it is a common phenomenon that gain of virulence mutation(s) by an avirulent SMV strain on a resistant genotype soybean is associated with a relative fitness loss (reduced pathogenicity or virulence) in a susceptible host (Khatabi et al., 2013; Wang and Hajimorad, 2016). The majority of experimentally evolved mutations that disrupt the avirulence functions of SMV-N on *Rsv1*-genotype soybean also results in mild symptoms and reduced virus accumulation, relative to parental SMV-N, in Williams82 (*rsv1*), demonstrating that gain of virulence by SMV on *Rsv1*-genotype soybean results in fitness loss in a previously susceptible soybean genotype, which is resulted from mutations in HC-Pro, and not in P3 (Khatabi et al., 2013; Wang and Hajimorad, 2016). It has been also demonstrated that gain of virulence mutation(s) by all avirulent viruses on *Rsv4*-genotype soybean is associated with a relative fitness penalty for gaining virulence by an avirulence strain (Wang and Hajimorad, 2016). Thus, it seems that there is a cost for gaining virulence by an avirulence strain.

THE SOYBEAN LINES CARRYING MULTIPLE *Rsv* GENES DISPLAY BROADER SPECTRUM OF RESISTANCE AGAINST SMV

Soybean line PI486355 displays broad spectrum resistance to various strains of SMV. Through genetic studies, Ma et al. (1995) identified two independently inherited SMV resistant genes in PI486355. One of the genes allelic to the *Rsv1* locus (designated as *Rsv1-s*) has dosage effect: the homozygotes conferring resistance and the heterozygotes showing systemic necrosis to SMV-G7. The other gene, which is epistatic to the *Rsv1*, confers resistance to strains SMV-G1 through G7 and exhibits complete dominance over *Rsv1*. The presence of this gene in PI486355 inhibits the expression of the systemic necrosis conditioned by the *Rsv1* alleles.

Soybean cultivar Columbia is resistant to all known SMV strains G1-G7, except G4. Results from allelism tests demonstrate that two genes independent of the *Rsv1* locus are present in Columbia, with one allelic to *Rsv3* and the other allelic to none of the known *Rsv* genes (Ma et al., 2002). Plants carrying both genes were completely resistant to both G1 and G7, indicating that the two genes interact in a complementary fashion (Ma et al.,

2002). The resistance conditioned by these two genes is allele dosage-dependent, plants heterozygous for either gene exhibiting systemic necrosis or late susceptibility.

Tousan 140 and Hourei, two soybean accessions from Japan, and J05, a accession from China, carry both *Rsv1* and *Rsv3* alleles and are resistant to SMV-G1 through G7 (Gunduz et al., 2002; Zheng et al., 2006; Shi et al., 2011).

These results indicate that integration of more than one *Rsv* genes into one cultivar can confers a broader spectrum of resistance against SMV. Therefore, pyramiding multiple *Rsv* genes in elite soybean cultivars could be one of the best approaches to generate durable SMV resistance with broader spectrum.

THE HOST FACTORS THAT ARE INVOLVED IN SMV RESISTANCE

The Host Components in *R* Gene-Mediated Defense Responses Are Conserved in *Rsv1*-Mediated ER Against SMV

The key components in *R* gene mediated disease resistant signaling pathway have been identified in model plant *Arabidopsis*, among which, RAR1 (*Required for Mla 12 Resistance*), SGT1(*Suppressor of G2 Allele of Skp1*) and HSP90 (*Heat Shock Protein 90*) are the most important ones (Belkhadir et al., 2004). Using BPMV-VIGS, it has been shown that *Rsv1*-mediated ER against SMV in soybean requires RAR1 and SGT1 but not GmHSP90, suggesting although soybean defense signaling pathways recruit structurally conserved components, they have distinct requirements for specific proteins (Fu et al., 2009). However, Zhang et al. (2012) showed that silencing *GmHSP90* using BPMV-VIGS compromised *Rsv1*-mediated resistance. In addition, silencing *GmEDR1* (*Enhanced Disease Resistance 1*), *GmEDS1* (*Enhanced Disease Susceptibility 1*), *GmHSP90*, *GmJAR1* (*Jasmonic Responsive 1*), *GmPAD4* (*Phytoalexin Deficient* 4), and two genes encoding *WRKY* transcription factors (WRKY6 and WRKY 30), all of which are involved in defense pathways in model plant *Arabidopsis*, *Rsv1*-mediated ER was also compromised (**Table 2**). These results suggest that the host components required for *R* gene-mediated resistant signaling pathways are conserved across plant species.

Conserved but Divergent Roles of MAPK Signaling Pathway in SMV Resistance

Mitogen-activated protein kinase (MAPK) cascades play important roles in disease resistance (Meng and Zhang, 2013). The function of MAPK signaling pathways in disease resistance was investigated in soybean using BPMV-VIGS (Liu et al., 2011, 2014, 2015). Among the plants silenced for multiple genes in MAPK pathway, the plants silenced for the *GmMAPK4 and GmMAPK6* homologs displayed strong phenotypes of activated defense responses (Liu et al., 2011, 2014). Consistent with the activated defense response phenotypes, these plants were more resistant to SMV compared with vector control plants (Liu et al., 2011, 2014), indicating that both genes play critical negative roles in basal resistance or PAMP-triggered immunity (PTI) in soybean. The constitutively activated defense responses has been reported for *mpk4* mutant in *Arabidopsis* (Petersen et al., 2000) and the positive role of MPK6 in defense responses is well-documented (Meng and Zhang, 2013). However, the negative role of MAKP6 homologs has not been reported previously (Liu et al., 2014), indicating that both conserved and distinct functions of MAPK signaling pathways in immunity are observed between *Arabidopsis* and soybean.

Identifications of the Other Host Factors that Play Critical Roles in SMV Resistance

Numerous host factors participate in defense responses in plants. Identification of these factors may facilitate rationale design of novel resistant strategies. Recently, it has been shown that silencing *GmHSP40.1*, a soybean nuclear-localized type-III DnaJ domain-containing HSP40, results in increased infectivity of SMV, indicating a positive role of GmHSP40.1 in basal resistance (Liu and Whitham, 2013). A subset of type 2C protein phophatase (PP2C) gene family, which participate ABA signaling pathway, is specifically up-regulated during *Rsv3*-mediated resistance (Seo et al., 2014). Synchronized overexpression of GmPP2C3a using SMV-G7H vector inhibits virus cell-to-cell movement mediated by callose deposition in an ABA signaling-dependent manner, indicating that GmPP2C3a functions as a key regulator of *Rsv3*-mediated resistance (Seo et al., 2014). An ortholog of *Arabidopsis* K^+ weak channel encoding gene AKT2, was significantly induced by SMV inoculation in the SMV highly resistant genotype, but not in the susceptible genotype (Zhou et al., 2014). Overexpression of *GmAKT2* not only significantly increased K^+ concentrations in young leaves but also significantly enhanced the resistance against SMV, indicating alteration of K^+ transporter expression could be a novel molecular approach for enhancing SMV resistance in soybean (Zhou et al., 2014). Molybdenum cofactor (Moco) is required for the activities of Moco-dependant enzymes. Cofactor for nitrate reductase and xanthine dehydrogenase (Cnx1) is known to be involved in the biosynthesis of Moco in plants. Soybean plants transformed with *Cnx1* enhanced the enzyme activities of nitrate reductase (NR) and aldehydeoxidase (AO) and resulted in an enhanced resistance against various strains of SMV (Zhou et al., 2015). The differentially expressed genes in *Rsv1* genotype in response to G7 infection have been identified (Chen et al., 2016a). Knocking down one of the identified genes, the eukaryotic translation initiation factor 5A (*eIF5A*), diminished the LSHR and enhanced viral accumulation, suggesting an essential role of eIF5A in the *Rsv1*-mediated LSHR signaling pathway. Eukaryotic elongation factor 1A (eEF1A) is a well-known host factor in viral pathogenesis. Recently, Luan et al. (2016) showed that silencing *GmeEF1A* inhibits accumulation of

TABLE 2 | Host factors participate in SMV resistance.

Host factors	Biological functions	Type of resistance	Positive or negative Roles	Reference
GmHSP90, GmRARI	Defense signaling	Rsvl-mediated	Positive	Fu et al., 2009;
GmSGTI, GmEDSI, GmEDRI, GmJARI, GmPAD4, GmWRKY6, GmWRKY30				Zhang et al., 2012
GmMPK4	Defense signaling	Basal	Negative	Liu et al., 2011
GmMPK6	Defense signaling	Basal	Positive/negative	Liu et al., 2014
GmHSP40.1	Co-chaperone	Basal	Positive	Liu and Whitham, 2013
GmPP2C	ABA signaling	Rsv3-mediated	Positive	Seo et al., 2014
GmAKT2	K^+ channel	Basal	Positive	Zhou et al., 2014
GmCNXI	Moco biosynthesis	Basal	Positive	Zhou et al., 2015
GmeIF5A	Translation initiation	flsi/3-mediated	Positive	Chen et al., 2016a
GmeEFIa	Translation elongation	Basal	Negative	Luan et al., 2016
GmAGOI	Gene silencing	Silencing-mediated	Positive	Chen et al., 2015
GmSGS3	Gene silencing	Silencing-mediated	Positive	Chen et al., 2015

SMV and P3 protein of SMV interacts with GmeEF1A to facilitate its nuclear localization and therefore, promotes SMV pathogenicity.

SMALL RNA PATHWAYS IN SMV RESISTANCE

miRNAs Participate in SMV Resistance

Small RNAs play a fundamental role in anti-viral defense. Three miRNAs, miR160, miR393 and miR1510, which have been previously shown to be involved in disease resistance in other plant species, have been identified as SMV-inducible miRNAs through small RNA sequencing approach (Yin et al., 2013), implying that these three miRNAs might play roles in SMV resistance. Chen et al. (2015) recently showed that the expression of *miRNA168* gene is specifically highly induced only in G7-infected PI96983 (incompatible interaction) but not in G2- and G7-infected Williams 82 (compatible interactions). Overexpression of *miR168* results in cleavage of miR168-mediated *AGO1* mRNA and severely repression of AGO1 protein accumulation (Chen et al., 2015). Silencing *SGS3*, an essential component in RNA silencing, suppressed AGO1 siRNA, partially recovers the repressed AGO1 protein, and alleviates LSHR severity in G7-infected *Rsv1* soybean (Chen et al., 2015). These results strongly suggest that miRNA pathway is involved in G7 infection of *Rsv1* soybean, and LSHR is associated with repression of AGO1.

Chen et al. (2016b) recently performed small RNA (sRNA)-seq, degradome-seq and as well as a genome-wide transcriptome analysis to profile the global gene and miRNA expression in soybean in response to three different SMV isolates. The SMV responsive miRNAs and their potential cleavage targets were identified and subsequently validated by degradome-seq analysis, leading to the establishment of complex miRNA-mRNA regulatory networks. The information generated in this study provides insights into molecular interactions between SMV and soybean and offer candidate miRNAs and their targets for further elucidation of the SMV infection process.

Improving SMV Resistance through Generating RNAi Transgenic Lines Targeted for SMV Genome

The multiple soybean cultivars transformed with an RNA interference (RNAi) construct targeted for SMV HC-Pro displayed a significantly enhanced resistance against SMV (Gao et al., 2015). Soybean plants transformed with a single RNAi construct expressing separate short hairpins or inverted repeat (IR) (150 bp) derived from three different viruses (SMV, Alfalfa mosaic virus, and Bean pod mottle virus) confer robust systemic resistance to these viruses (Zhang et al., 2011). This strategy makes it easy to incorporate additional short IRs in the transgene, thus expanding the spectrum of virus resistance. As the cases in the other plant species, these studies demonstrate that RNA silencing is obviously the most effective approach for SMV resistance.

VIGS Is a Powerful Tool to Overcome Gene Redundancy in Soybean

Bean pod mottle virus -induced gene silencing system has been proven successful in gene function studies in soybean (Zhang et al., 2009b, 2010; Liu et al., 2015). There are four *GmMAPK4* homologs that can be divided into two paralogous groups (Liu et al., 2011). The sequence identities of ORFs within the groups are greater than 96%, whereas the identities between the groups are 88.7% (Liu et al., 2015). The BPMV-VIGS construct used for silencing *GmMAPK4* by Liu et al. (2011) actually can silence all four of the isoforms simultaneously. When only one parologous group was silenced by using construct targeted for the 3′ UTR (the sequence identity of the 3′ UTRs between the two parologous groups is less than 50%), the activated defense response was not observed, indicating that silencing the four *GmMAPK4* isoforms simultaneously

is necessary for activating defense responses in soybean. Using the same approach, it has been differentiated that *GmSGT1-2* but not *GmSGT1-1* is required for the *Rsv1*-mediated ER against SMV (Fu et al., 2009). Thus, VIGS is currently the most powerful tool in overcoming the gene redundancy in soybean.

CONCLUDING REMARKS

As none of the SMV resistant gene has been cloned so far, it is not possible to generating resistant soybean plants simply by transforming the resistant genes. In addition, due to the rapid evolution in avirulence/effector genes, the resistance conditioned by *R* genes will be overcome quickly (Choi et al., 2005; Gagarinova et al., 2008). Therefore, there is urgent need for a better solution in generating long-lasting SMV resistance with wide spectrums. As the first step, the identities of different *Rsv* genes need to be revealed and the key components in SMV resistant signaling pathway need to be identified. Cutting edge functional genomics tools and technologies have been proven successful in cloning of SCN resistant genes *Rhg4* (Liu et al., 2011). TILLING coupled with VIGS and RNA interference confirmed that a mutation in the *Rhg4*, a serine hydroxymethyltransferase (*SHMT*) gene, is responsible for *Rhg4* mediated resistance to SCN (Liu et al., 2011). VIGS has been proven useful in interrogating gene functions and can overcome gene redundancy in soybean (Liu et al., 2015). It has been shown recently that knocking out all three *TaMLO* homoeologs simultaneously in hexaploid bread wheat using TALEN and CRISPR-CAS9 resulted in heritable broad-spectrum resistance to powdery mildew (Wang et al., 2014). We believe that the same strategy can be applied to soybean in the near future. These new functional genomics approaches and genome editing tools will greatly facilitate the cloning of SMV resistant genes and elucidating the SMV resistant signaling pathways. Marker assisted selection (MAS) has become very useful in the effort of tagging genes for SMV resistance. Single nucleotide polymorphism (SNP) is a powerful tool in genome mapping, association studies, and cloning of important genes (Clevenger et al., 2015) and the increasingly saturated SNPs are being established in soybean (Wu et al., 2010; Lee et al., 2015). With all these tools and resources available, pyramiding multiple SMV resistance genes in elite soybean cultivars to generate durable resistance with broad spectrum is more realistic than ever.

AUTHOR CONTRIBUTIONS

J-ZL wrote most part of this manuscript and prepared the figure and tables. YF and HP helped to write part of this manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (31571423 and 31371401 to J-ZL), Qianjiang Talent Program of Zhejiang Province (2013R10074 to J-ZL) and Xin Miao Program of Zhejiang Province.

ACKNOWLEDGMENT

We regret that we were unable to cite all the references in the field due to space limitations. We thank Ray Liu for proofreading and editing this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 23 January 2017
doi: 10.3389/fmicb.2017.00047

Antiviral Defenses in Plants through Genome Editing

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Plant–virus interactions based-studies have contributed to increase our understanding on plant resistance mechanisms, providing new tools for crop improvement. In the last two decades, RNA interference, a post-transcriptional gene silencing approach, has been used to induce antiviral defenses in plants with the help of genetic engineering technologies. More recently, the new genome editing systems (GES) are revolutionizing the scope of tools available to confer virus resistance in plants. The most explored GES are zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats/Cas9 endonuclease. GES are engineered to target and introduce mutations, which can be deleterious, via double-strand breaks at specific DNA sequences by the error-prone non-homologous recombination end-joining pathway. Although GES have been engineered to target DNA, recent discoveries of GES targeting ssRNA molecules, including virus genomes, pave the way for further studies programming plant defense against RNA viruses. Most of plant virus species have an RNA genome and at least 784 species have positive ssRNA. Here, we provide a summary of the latest progress in plant antiviral defenses mediated by GES. In addition, we also discuss briefly the GES perspectives in light of the rebooted debate on genetic modified organisms (GMOs) and the current regulatory frame for agricultural products involving the use of such engineering technologies.

Keywords: antiviral defense, CRISPR/CAS9, genome editing technologies, plant viruses, TALEN, ZFN

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *30 September 2016*
Accepted: *06 January 2017*
Published: *23 January 2017*

Citation:
Romay G and Bragard C (2017) Antiviral Defenses in Plants through Genome Editing. Front. Microbiol. 8:47. doi: 10.3389/fmicb.2017.00047

INTRODUCTION

Viruses are well-known to be one of the major concerns for agricultural production and food security throughout the world. It is estimated that viral agents are responsible for the half of emerging diseases reported in plants (Anderson et al., 2004). The control of plant viruses is often dependent on the use of pesticides; however, such strategy has many adverse environmental effects (Bragard et al., 2013). In many plant virus-related outbreaks, the disease management is difficult to accomplish due to the variability of factors affecting the development of the disease, such as local climate conditions, plant aging, crop varieties, vector transmission efficiency and severity of viral strains. Unlike other pathogens (i.e., fungi and bacteria), plant viruses cannot be controlled chemically and a combination of cultural practices, biosecurity measures, organism-vector management and plant genetic resistance is needed to deal with the disease (Nicaise, 2014).

The use of viral resistance factors from plants is considered one of the most important alternatives to face virus infections (Hull, 2014; Ziebell, 2016). The pioneer works on resistance to *Tobacco mosaic virus* in *Nicotiana glutinosa* led to the initial understandings on plant viral immune responses and the introgression of resistance genes from wild to cultivated plants (Mandadi and Scholthof, 2013). Over the past decades, virus resistance genes have been used to improve the most of cultivated plants and many cultivars are commercially available (Gómez et al., 2009). The main drawbacks of approaches using resistance genes are the considerable time and cost to develop a durable resistant crop variety (Kang et al., 2005).

Plants, like other eukaryotes, are able to deploy an alternative strategy to face viruses: RNA interference (RNAi). The RNAi is a biological mechanism whereby small RNA molecules, such as small interfering RNA (siRNA) or microRNA (miRNA), can regulate gene functions via post-transcriptional gene silencing. A critical breakthrough was the demonstration that double-strand RNA (dsRNA) molecules trigger the RNAi pathway to regulate gene expression in *Caenorhabditis elegans* (Fire et al., 1998). This model was subsequently tested and confirmed in many other organisms. Nowadays, it is known that these dsRNAs are targeted and cleaved by the endoribonuclease Dicer producing 21 to 25-nucleotide small RNAs (siRNA or miRNA) which are bound to an Argonaute protein into the RNA-induced silencing complex (RISC) (Wilson and Doudna, 2013). The RISC is guided by the Argonaute-bound strand to a single strand RNA, perfectly complementary to the dsRNA, which is degraded or translationally inhibited (Bartel, 2009; Wilson and Doudna, 2013). In plants, dsRNA molecules from either RNA or DNA viruses may be produced and afterward processed by the host RNAi machinery to induce an antiviral response (Szittya and Burgyán, 2013). Although the RNAi triggering molecule (i.e., dsRNA) had not been discovered in the 1980s, it was known that the inhibition of gene expression could be generated by expression of antisense RNA in plant cells (Ecker and Davis, 1986). The application of this strategy to induce pathogen resistance, involving the pathogen genome itself, was called parasite-derived resistance (Sanford and Johnston, 1985), and currently referred as pathogen derive resistance (PDR). Since then, this approach has been used to derive viral resistance through transgenic expression of virus genes in plants and, in some cases, with commercial applications (Baulcombe, 1996; Simón-Mateo and García, 2011; Younis et al., 2014). However, most viruses have developed silencing suppressor mechanisms to counteract the RNAi-mediated defense of plants. Hence, an RNAi-mediated resistance in transgenic plants could be overcome by the targeted virus after inoculation with a non-target virus possessing a silencing suppressing gene (Simón-Mateo and García, 2011). Besides, RNAi technology is based on knockdown gene function(s), which can be incomplete, varies between different experiments and have unpredictable off-target effects (Gaj et al., 2013). Therefore, other approaches involving stable gene modification have been gaining attention over the last decade due to their versatility to manipulate any gene from any organism (Gaj et al., 2013; Boettcher and McManus, 2015). These approaches are referred as genome editing systems (GES). In this review, we provide insights about the latest progress on the different technologies based on GES used to control plant viruses and its perspective for a broad application in crop improvement.

MECHANISMS OF GENOME EDITNG SYSTEMS

Prior to GES development, the genetic engineering of virus resistant plants has been mainly undertaken using viral sequences, which are introduced in the genome of the susceptible plant through genetic transformation methods (Saharan et al., 2016). At least 25 viruses have been used to develop virus resistant plants by inserting the viral sequence itself (Wani and Sanghera, 2010; Saharan et al., 2016). Although several techniques for genetic transformation of plants are available, the most popular techniques are *Agrobacterium* infection and ballistic bombardment (Ye, 2015). *Agrobacterium*-mediated transformation is based on the transfer and insertion of a given DNA sequence into a plant genome by plasmids Ti (tumor-induced) or Ri (rhizogenic) from the bacterium (Gelvin, 2003). Genetic transformation trough ballistic bombardment is based on delivery of DNA-coated metal particles accelerated with a biolistic device to introduce the DNA into the target cells or tissues (Kikkert et al., 2005). Both methods have been used to develop antiviral strategies focused on PDR approach. When the complete sequence of a virus gene is inserted into the host plant genome, to interfere with the life cycle of the target virus, the PDR approach is referred as viral protein mediated resistance (Saharan et al., 2016). Within such approach the major viral proteins used are the coat protein, replicase protein, movement protein, and replication-associated protein (Prins et al., 2008). As mentioned above, the PDR approach have been also applied using small sequences from the viral genome to activate the RNAi mechanism of the host plant, via post-transcriptional gene silencing (Simón-Mateo and García, 2011).

Genome editing systems approaches against plant viruses have been mainly developed using *Agrobacterium* infection to introduce such systems into the plant cells for stable or unstable transformation. The applications of GES are based on the use of sequence-specific nucleases, which lead to DNA modifications by double-strand breaks (DSBs) in a targeted gene (Gaj et al., 2013; Voytas, 2013). After the DSBs DNA is repaired by two different mechanisms: (i) the non-homologous end-joining (NHEJ), in which the ends of the broken DNA are re-joined without use of a repair template, and (ii) the homologous recombination (HR) whereby two homologous DNA molecules exchange nucleotide sequences (Wyman and Kanaar, 2006). Thus, DNA modifications mediated by sequence-specific nucleases are possible in a particular genomic location (Voytas, 2013). Four major platforms for GES have been developed using sequence-specific nucleases (**Figure 1**), meganucleases, zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs) and more recently the clustered regularly interspaced short palindromic repeats (CRISPR) along with the CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9).

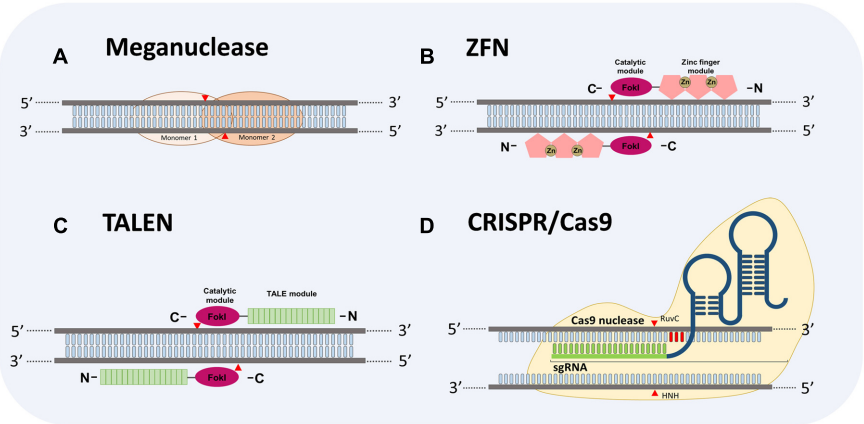

FIGURE 1 | Schematic diagram depicting four Genome Editing Systems (GES) to target DNA. (A) Homodimers structure of a meganuclease system. **(B)** Zinc finger nuclease (ZFN) showing two monomers bound to DNA. The ZFN contains a catalytic FokI domain (ellipse in pink) and a zinc finger DNA-binding domain (DBD) (pentagons in rose). **(C)** Transcription activator-like effector nuclease (TALEN) showing two monomers bound to DNA. Like ZFN, TALEN comprises a catalytic FokI domain (ellipse in pink). Light green rectangles represent the DNA bind domain containing the repeat variable di-residue (RVD) arrays of amino acids to recognize DNA specific sequences. **(D)** Clustered regularly interspaced short palindromic repeats (CRISPR) CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9). Typically CRISPR/Cas9 system comprises a Cas9 protein (depicted in light gold) with two nuclease domains, referred as HuvC and HNH, and a chimeric single guide RNA (sgRNA). The sgRNA consists of a CRISPR RNA (crRNA, 21 nucleotides in light green) to direct the Cas9 protein to the complementary sequences of the DNA target and a *trans*-activating crRNA (RNA sequence represented in dark blue) involved in the processing of pre-crRNA into a mature crRNA. Arrowheads in red indicate cleave sites to each GES.

Meganucleases or homing endonucleases are encoded by introns and inteins that recognize DNA sequences between 12 and 42-base-pair (bp) in length, unlike restriction enzymes (Jurica and Stoddard, 1999). The meganucleases are characterized by a high specificity, even though they could tolerate single mutations in the targeted sequence (Silva et al., 2011). Due to the high specificity of meganucleases, the repertoire of targetable sequences is very limited (Pâques and Duchateau, 2007). In specific cases their utility relies on the previous insertion of the recognition site in the targeted genome to undertake a high-efficiency recombination (Carroll, 2011). To overcome this drawback, residues-specific mutations in engineering meganucleases are introduced to alter their DNA recognition sites allowing to increase the use of these proteins in gene targeting experiments, however, the production of customized meganucleases still remains too complex (Silva et al., 2011).

The second GES are the ZFNs, which are chimeric proteins created by fusing the DNA-binding domain (DBD) of a zinc-finger protein with the DNA cleavage domain of the *Fok*I restriction enzyme (Urnov et al., 2010). *Fok*I works as a dimer and its catalytic domain cleaves the DNA sequence outside of the recognition site (Bitinaite et al., 1998). A ZFN is engineered with two monomers separated by a spacer sequence of 5–7 bp wherein the catalytic domains of the chimeric proteins cleave each DNA brand to produce the DSB (Christian et al., 2010). An effective ZFN should contain more than three zinc-finger domains in each DNA-binding module to increase specific DNA recognition (Gaj et al., 2013).

Transcription activator-like effector nucleases is the third GES. This system is a fusion of a transcription activator-like effector (TALE) and the non-specific cleavage domain of the enzyme *Fok*I (Cermak et al., 2011; Mahfouz et al., 2011; Pesce et al., 2015). TALEs are proteins encoded by phytopathogenic bacteria *Xanthomonas* spp. and delivered into the plant host cells to promote pathogen growth through manipulation of plant processes (Boch and Bonas, 2010; Schornack et al., 2013). Once TALEs are injected into the cells, they translocate to the nucleus, bind to their DNA targets and mimic host transcription factors to reprogram host gene expression (Mahfouz et al., 2011). TALE proteins are composed by an N-terminal secretion and translocation domain, a central DBD and a C-terminal transcription activation domain carrying nuclear localization signal (Schornack et al., 2013). The DNA-binding specificities of these proteins were solved by Boch et al. (2009) who showed that the DBD is an array of tandem repeat units consisting of 34 amino acids with two hypervariable amino acids at the position 12 and 13 that constitute a repeat variable diresidue (RVD). A specific amino acid arrangement in the RVD region determine a specific nucleotide recognition in the DNA target (Boch et al., 2009). Thus, this characteristic of TALEs, along with the previous knowledge on biotechnology applications of *Fok*I enzyme, have allowed the TALEN system's design.

The fourth and most recent GES is CRISPR/Cas9, which is an RNA-guided nuclease technology. This genome editing approach is based on the CRISPR/Cas system found in most archaea and many bacteria that confers immunity against foreign DNA elements such as viruses and plasmids (Barrangou and Marraffini, 2014; Makarova et al., 2015). Barrangou et al. (2007) demonstrated that after viral challenge on several strains of *Streptococcus thermophilus* the bacteria are able to generate virus-resistant mutants through integration of viral genome sequences

into the CRISPR loci in association with *cas* genes expression. Among CRISPR/Cas systems, the CRISPR/Cas9 from *S. pyogenes* is the most studied model (Dominguez et al., 2016). Two elements are essential to engineer a CRISPR/Cas9 system: (i) the *cas*9 protein containing two nuclease domains (RuvC and HNH) that cleave both strands of the DNA target leading to DSBs and site mutations, and (ii) a guide RNA (gRNA) whose role is direct *cas*9 protein to the DNA target. A gRNA is composed by two different RNA molecules: a CRISPR RNA (crRNA), which contains complementary sequences to the DNA target, and a *trans*-activating crRNA (tracrRNA) involved in processing of precursor crRNA molecules to a mature crRNA (Brouns et al., 2008).

A milestone in the development of CRISPR/Cas as a biotechnological tool was the engineering of a chimeric RNA containing the crRNA and tracrRNA in a single guide RNA (sgRNA), which was also able to direct Cas9 to the DNA target (Jinek et al., 2012). After such a finding, CRISPR-Cas9 technology became the most popular GES. The specificity of the CRISPR/Cas9-mediated DNA cleavage also relies on recognition of a trinucleotide sequence of DNA target referred as protospacer adjacent motif (PAM) (Sternberg et al., 2014). As showed in **Figure 2**, CRISPR-Cas technology is rapidly advancing and expanding its potential application not only targeting DNA molecules, like the precedent genome editing technologies, but also RNA molecules including RNA viruses.

As mentioned above, GES such as ZFN, TALEN, or CRISPR-Cas9 are provided of DNA-binding and catalytic domains. Meanwhile, TALE and artificial zinc finger protein (AZP) have DBDs and lack of catalytic domains. TALE and AZP can be engineered to prevent viral multiplication by blocking specific DNA sites in the viral genomes, which are essential for DNA-binding proteins of the virus and subsequent interactions with the replication machinery of host cell.

GENOME TARGETING TECHNOLOGIES AND GES AGAINST PLANT VIRUSES

In **Table 1** are summarized the several genome targeting technologies that have been explored to provide potential control against plant viruses. It is important to note that the most evaluated species belong to the families *Geminiviridae* and *Potyviridae*. According to the latest report of the International Committee on Taxonomy of Viruses (ICTV), *Geminiviridae* and *Potyviridae* families represent the two largest groups of plant viruses containing 326 and 195 species, respectively (ICTV, 2015). Hence the use of these technologies to enhance plant resistance against a large number of viruses might increase significantly in the near future.

Antiviral Resistance in Plants Based on Zinc Finger Technology

The first efforts to introduce a viral inhibition factor in plants, through zinc finger technology, were carried out in the mid-2000s. Sera (2005) developed an AZP *in planta* targeting a 19-bp fragment in the intergenic region (IR) of *Beet severe curly top virus* (BSCTV, genus *Curtovirus*). BSCTV, like all members of family *Geminiviridae*, has an IR containing a binding site recognized by the replication initiator protein (Rep) to initiate viral replication (Rizvi et al., 2015). BSCTV replication was reduced in transgenic *Arabidopsis thaliana* plants carrying the AZP that was efficient to block the binding site of the BSCTV Rep (Sera, 2005). *In vitro* assays, using AZP technology, have also been performed to predict inhibition of *Tomato yellow leaf curl virus* (TYLCV, genus *Begomovirus*) by blocking the Rep binding site of TYLCV (Takenaka et al., 2007; Koshino-Kimura et al., 2009; Mori et al., 2013); however, its efficient application *in planta* remains to be confirmed. Zinc finger technology was also applied to reduce replication of *Rice tungro bacilliform virus* (RTBV, genus *Tungrovirus*) in transgenic *A. thaliana* plants carrying an AZP which was able to recognize and block promoter sequences of RTBV (Ordiz et al., 2010). AZP for antiviral applications have been designed to bind and block specific DNA sites that are crucial for DNA-binding proteins of viruses (Sera, 2005; Koshino-Kimura et al., 2009). Unlike AZP, the ZFN technology, for antiviral applications, involves not only the DBD but also the DNA catalytic domains of *Fok*I restriction enzyme to introduce deleterious mutations in the viral genome, as previously explained. More recently, ZFN strategy against *Tomato yellow leaf curl China virus* (TYLCCNV, genus *Begomovirus*) was developed to target the AC1 gene of the virus that codes the Rep protein (Chen et al., 2014). In that study, agroinfiltrations of *N. benthamiana* plants with TYLCCNV infectious clone and antiviral ZFN showed a significant reduction of viral replication as compared with agroinfiltrations of the viral clone alone. Furthermore, the same ZFN developed for TYLCCNV was tested against *Tobacco curly shoot virus* (TbCSV), another begomovirus, showing an inhibition of the replication of the virus and, thus, suggesting a possible resistance strategy to be broadly used against begomoviruses.

Antiviral Resistance in Plants Based on TALE Technology

Recently Cheng et al. (2015) developed a TALE platform to evaluate a broad-spectrum resistance against begomoviruses. In such study, two conserved 12-nucleotide motifs among begomoviruses (into the IR and the AC1 gene, respectively) were used to engineer TALEs and, afterward, were challenged with three begomoviruses: TbCSV, *Tomato leaf curl Yunnan virus* (TLCYnV) and *Tomato yellow leaf curl China virus* (TYLCCNV). Transgenic plants of *N. benthamiana* carrying the TALEs displayed resistance to TbCSV and TYLCCNV, while the resistance to TLCYnV was partial. Although a broad-spectrum approach for resistance to a virus group is desirable, it seems difficult to predict a unique TALE system to control a large group of virus like geminiviruses. TALE technologies including nuclease domains (TALEN) have not been still reported against plant viruses. For human viruses, the potential antiviral applications of TALEN have been explored for several viruses such as Human immunodeficiency virus (HIV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), however, the generic challenges for gene therapy (e.g., adequate specificity, viral escape, efficient delivery to virus infected tissues, or limited

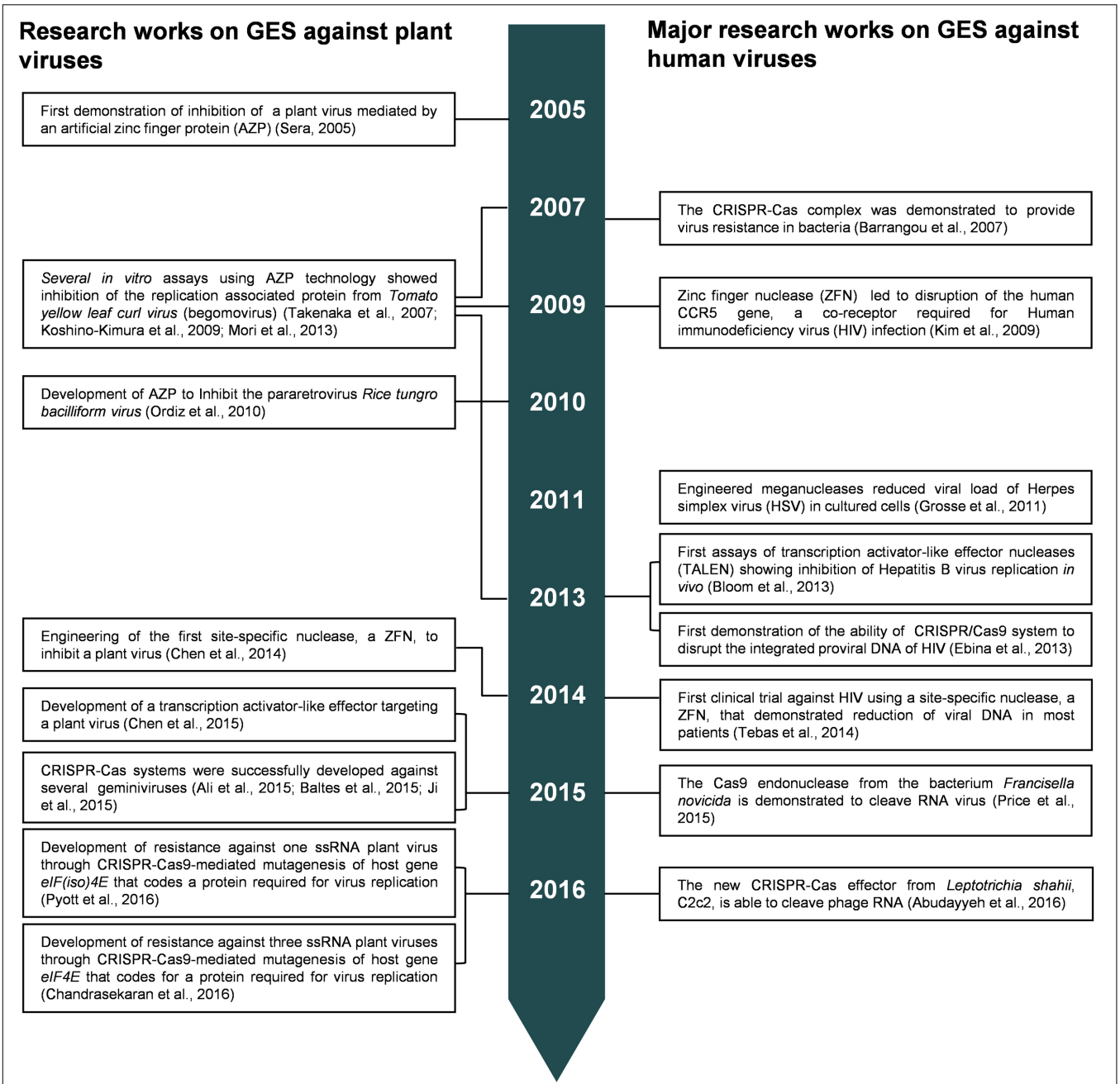

FIGURE 2 | Highlights on developments of genome editing technologies against viruses.

immune host responses) remain to be solved (Bloom et al., 2015).

Antiviral Resistance in Plants Based on CRISPR-Cas Technology

Since the advent of CRISPR-Cas system as a biotechnology tool for genome editing, many labs working with eukaryotic viruses have directed their interest on this technology due to its affordability, simplicity and efficiency as compared with precedent GES like ZFN or TALEN. Thus, many efforts are being undertaken to shed light on the potential application of CRISPR-Cas9 to control human viruses such as HIV, HBV, Human papillomavirus, Epstein-Barr and plant viruses (Price et al., 2016).

As showed in **Table 1**, four and six viruses in the families *Potyviridae* and *Geminiviridae*, respectively, have been used for developing antiviral defenses in plants, using CRISPR-Cas systems (Ali et al., 2015, 2016; Baltes et al., 2015; Chandrasekaran et al., 2016; Pyott et al., 2016). Interestingly, independent studies evaluating geminivirus resistance showed that the most promising sgRNAs were those targeting the IR

TABLE 1 | Genome targeting technologies developed to confer viral resistance in plants.

Genome targeting platform	Virus	Genus	Family	DNA targeted	Reference
CRISPR/Cas9	*Merremia mosaic virus*, *Cotton leaf curl Kokhran virus*, *Tomato yellow leaf curl virus* (TYLCV)	*Begomovirus*	*Geminiviridae*	Viral	Ali et al., 2015, 2016
	Beet curly top virus (BCTV); *Beet severe curly top virus* (BSCTV)	*Curtovirus*		Viral	Ali et al., 2015; Ji et al., 2015
	Bean yellow dwarf virus	*Mastrevirus*		Viral	Baltes et al., 2015
	Cucumber vein yellowing virus	*Ipomovirus*	*Potyviridae*	Host	Chandrasekaran et al., 2016
	Zucchini yellow mosaic virus, *Papaya ringspot virus*, *Turnip mosaic virus*	*Potyvirus*		Host	Chandrasekaran et al., 2016; Pyott et al., 2016
TALE	*Tomato yellow leaf curl China virus* (TYLCCNV), *Tobacco curly shoot virus* (TbCSV), *Tomato leaf curl Yunnan virus*	*Begomovirus*	*Geminiviridae*	Viral	Cheng et al., 2015
ZFN	TYLCCNV, TbCSV	*Begomovirus*	*Geminiviridae*	Viral	Chen et al., 2014
AZP	BSCTV, TYLCV	*Begomovirus*	*Geminiviridae*	Viral	Sera, 2005; Takenaka et al., 2007; Koshino-Kimura et al., 2009; Mori et al., 2013
	Rice tungro bacilliform virus	*Tungrovirus*	*Caulimoviridae*	Viral	Ordiz et al., 2010

of these viruses (Ali et al., 2015, 2016; Baltes et al., 2015). The IR of geminiviruses contain a stem-loop structure in which an invariant nonanucleotide motif is involved in the viral replication (Hanley-Bowdoin et al., 2000). Baltes et al. (2015) observed that sgRNAs designed near to the stem-loop structure of *Bean yellow dwarf virus* were less efficient to generate insertions/deletions (indels), suggesting a possible interference of the secondary structure on the sgRNA-Cas9 cleavage. Despite that, viral load was reduced probably by blocking of the Rep binding site (Baltes et al., 2015). Furthermore, Ali et al. (2015) engineered a sgRNA-Cas9 that was efficient to target the IR of TYLCV and also that of other geminiviruses like *Beet curly top virus* and *Merremia mosaic virus*. More recently, sgRNA-Cas9 targeting the IRs and the coat protein genes of the TYLCV and *Cotton leaf curl Kokhran virus* showed that CRISPR-Cas9 directed to coding sequences can generate viral variants which are able to replicate and spread in the plants, while CRISPR-Cas9 directed to non-coding intergenic sequences produced viral interference and a low recovery of mutated viral variants (Ali et al., 2016). One of the most interesting feature of CRISPR-Cas9 system is its flexibility to assemble multiple gRNA modules for targeting several genes simultaneously (Xing et al., 2014; Xie et al., 2015). Baltes et al. (2015) showed that a CRISPR-Cas9 system containing two gRNA modules targeting the same viral genome was more effective to reduce the infection than their relative gRNA delivered in separated constructs.

Given that GES target DNA sequences, these technologies seem to be mainly suitable for plant DNA viruses. However, an interesting CRISPR-Cas9 approach has been successfully used to develop resistant plants against RNA viruses, demonstrating that CRISPR-Cas9 system is a promising and powerful tool to be considered in the near future for crop improvement programs. Two recent studies showed the CRISPR-Cas9-mediated disruption of plant genes encoding eukaryotic translation initiation factors in cucumber and *A. thaliana* (Chandrasekaran et al., 2016; Pyott et al., 2016, respectively). Interactions between eukaryotic translation initiation factors 4E (eIF4E) or its isoform eIF(iso)4E and the viral genome-linked protein (VPg) of potyviruses are required for the virus infection (Robaglia and Caranta, 2006). Natural resistance to potyviruses are generally associated with mutations of host eIF4E or eIF(iso)4E that hamper their interaction with the VPg protein (Estevan et al., 2014; Sanfaçon, 2015). Chandrasekaran et al. (2016) used CRISPR-Cas9 systems to mutate the *eIF4E* gene in cucumber plants conferring resistance to the ipomovirus *Cucumber vein yellowing virus*, and to the potyviruses *Zucchini yellow mosaic virus* and *Papaya ring spot virus*. Similarly, Pyott et al. (2016) developed another CRISPR-Cas9 construct to introduce site specific-mutations in the *eIF(iso)4E* gene of *A. thaliana,* which were efficient to confer resistance to the potyvirus *Turnip mosaic virus* (TuMV) In the light of these studies, CRISPR-Cas9 systems could be developed to target host genes coding for other members in the family of plant translation factors such as eIF4G, eIF(iso)GE, eIF4A-like helicases, eIF3, eEF1A, and eEF1B that are also identified to interplay with protein and viral RNAs (Nicaise, 2014; Sanfaçon, 2015).

THE PLANT VIRUS RANGE THAT GES CAN TARGET

Genome editing systems are known to bind to double-stranded DNA (dsDNA) and subsequently introduce a DSB in a sequence-specific manner. Hence, the first studies using genome targeting or genome editing platforms for plant viruses aimed to control DNA viruses (**Table 1**). Although the members of the plant virus families *Geminiviridae* and *Nanoviridae* are composed of single-stranded DNA (ssDNA), they also contain replicative intermediate forms of dsDNA which can be targeted by GES. The other plant virus family possessing DNA genomes is *Caulimoviridae*. Unlike geminiviruses and nanoviruses, the caulimoviruses have a dsDNA genome. Currently, there are 432 virus species that belong to the DNA virus families *Geminiviridae*, *Nanoviridae,* and *Caulimoviridae* (ICTV, 2015). Others viruses in the families *Metaviridae* and *Pseudoviridae* that infect plants could be directly targeted by GES. Metaviruses and pseudoviruses are reverse transcribing RNA viruses. Although their genomes are single-stranded RNA (ssRNA), they possess replicative dsDNA forms (Boeke et al., 2012; Eickbush et al., 2012), being good candidates to be targeted by GES. In fact, the reverse transcribing RNA virus HIV has already been subjected to GES by targeting its replicative DNA forms known as provirus (Price et al., 2016).

The majority of plant viruses have RNA genomes. More specifically, 836 out of 1268 species recognized by the ICTV are RNA viruses (ICTV, 2015). Although all GES are able to bind DNA molecules, it was shown that Cas9 protein is able to bind and cleave ssRNA when using specially designed PAM-presenting oligonucleotides (O'Connell et al., 2014). Therefore, the development of an RNA-targeting CRISPR/Cas9 complex offers a promising platform to control RNA viruses. Interestingly, two recent discoveries showed that CRISPR-Cas system can directly interfere with RNA virus infections. In the first study, Price et al. (2015) developed a CRISPR-Cas9 system to target HCV (ssRNA virus) using a new variant of Cas9 endonuclease, called FnCas9, capable to cleave ssRNA molecules. FnCas9 is a Cas protein from the bacterium *Francisella novicida,* which is able to repress a endogenous mRNA (Sampson et al., 2013). In the second study, a new Cas protein from the bacterium *Leptotrichia shahii* was characterized and named C2c2. This protein contains two HEPN (Higher Eukaryotic and Prokaryotic nucleotide-binding) RNase domains (Shmakov et al., 2015). In *L. shahii* C2c2 provides resistance to an RNA phage and it was recently demonstrated to be guided by a single crRNA and programmable against ssRNA (Abudayyeh et al., 2016). Taking into account that 784 out of 1268 plant virus species possesses ssRNA, these discoveries could play a prominent role, in the near future, to enhance the current tools for plant virus control.

APPLICATIONS OF GES BEYOND PLANT VIRUS CONTROL IN AGRICULTURE

The great success of the RNAi technology was based on the ability to modulate gene expression by post-transcriptional gene silencing mechanisms whereby the role of many genes has been unveiled. GES are not only able to modulate gene expression, but also are useful to introduce nucleotide modifications into the genome of almost every organism. Hence, gene editing technologies seem to have no limits on their applications. Such applications have been extensively reviewed (Gaj et al., 2013; Boettcher and McManus, 2015; Govindan and Ramalingam, 2016; Steinert et al., 2016). In agriculture, the applications of GES have been explored for many purposes in addition to plant virus control. For example, a meganuclease system was developed to confer herbicide tolerance in cotton lines (D'Halluin et al., 2013). Herbicide resistant plants were also generated using zinc finger technologies to target tobacco acetolactate synthase genes that are involved in resistance to imidazolinone and sulphonylurea herbicides (Townsend et al., 2009). TALENs have been successfully used to inhibit the vacuolar invertase gene in potato, which is associated with the accumulation of reducing sugars and high levels of acrylamide, a potential carcinogen, in tubers (Clasen et al., 2016). TALEN and CRISPR/Cas9 systems were demonstrated to target several genes implicated with the phytic acid production in maize seeds (Liang et al., 2014). Phytic acid is a major storage for phosphorus and mineral cations in several crops and it is poorly digested by monogastric animals (Shi et al., 2003). In wheat, TALENs and CRISPR/Cas9 were also used to confer fungi resistant in wheat lines by disrupting of the mildew locus O (*MLO*) gene, which is related with mildew powder susceptibility (Wang et al., 2014). Besides GES targeting plant genomes, CRISPR/Cas9 system was used to inhibit genes encoding polyphenol oxidase that causes browning in common white mushroom (*Agaricus bisporus*) (Waltz, 2016). In general, many successful examples of GES targeting crops have demonstrated the broad spectrum of applications that exhibit these technologies in agriculture beyond plant virus control.

GES-MEDIATED PLANT VIRUS DEFENSES VIS-À-VIS GMO REGULATIONS

The insights on the scope of GES is rapidly advancing throughout the scientific community. For virologists and plant breeders, genome editing technologies is offering an encouraging approach to circumvent labor-intensive and time-consuming methods used in conventional genetic engineering and traditional breeding techniques. Nevertheless, one of the major hurdles of the GES approaches is the public perception in which a product obtained from these technologies is considered as a genetic modified organism (GMO). The GMO definition according to the Cartagena Protocol on Biosafety and the European Union was mainly conceived to distinguish products obtained by conventional plant breeding technologies and those obtained by recombinant DNA technologies (Sprink et al., 2016). Development of transgenic plants implies the transfer of foreign DNA into host cells. For example, several cases of virus-resistant plants have been generated by insertion of partial sequences from viral genomes based on RNAi technology (Simón-Mateo and García, 2011). Unlike, some genome editing approaches allow the insertion of point mutations in the genome of recipient species

and afterward it is possible the removal of the encoding sequences of the programmable nucleases. Indeed, Chandrasekaran et al. (2016) introduced mutations in the factor eIF4E of cucumber plants using the CRISPR/Cas9 system, to induce resistance to potyviruses, and subsequently the gene encoding the GES was removed by breeding, leading to transgene-free generations. Likewise, Pyott et al. (2016) generated TuMV resistant plants of *A. thaliana* lacking the CRISPR/Cas9 system after two generations. Insertion of point mutations are also feasible using mutagenic agents. For example, using the technique TILLING (targeting induced local lesions in genomes) point mutations in tomato eIF4 were induced by ethyl methanesulfonate to confer immunity to two potyviruses (Piron et al., 2010). Besides this, crop improvement derivate by mutagenic agents are not considered as GMO due to the lack of foreign DNA. Thus, some genome editing approaches are considered to generate non-transgenic plants (plant without foreign DNA). However, the controversy whether the products generated by these type of technologies should be considered GMOs or not still remains (Sprink et al., 2016). In another example of transgene-free plants modified by GES, a preassembled CRISPR/Cas9 complex was successfully delivered to induce mutations into protoplasts of *A. thaliana*, tobacco, lettuce and rice producing regenerated plants with the expected mutations and without foreign DNA (Woo et al., 2015). Such approach could be useful to eliminate pararetroviruses that are able to integrate their genomes in the host genome. One of the most important cases is the banana streak viruses (BSVs), because the cultivated banana species are only reproduced by vegetative propagation and BSVs remain inserted into genome indefinitely. Along with BSVs, *Banana bunchy top virus* (BBTV, family *Nanoviridae*) are the most economically important viruses for banana production worldwide (Rybicki, 2015; Mukwa et al., 2016). Interestingly, BBTV is a DNA virus that could be directly targeted using the same transgene-free GES approach.

Currently there is a growing worldwide debate about to how to regulate research and use of plant produced with the novel genome editing technologies (Sprink et al., 2016). In the meantime, contrasting scenarios are offered. For example, on the one hand, in the European Union, many research groups are directly concerned and waiting for the European Commission' answer regarding the legal status of gene-edited plants and whether these plants should be regulated as GMOs (Abbott, 2015). On the other hand, in the USA, several gene-edited products have already been deregulated, including the first CRISPR-edited organism, a gene-edited mushroom (Wolt et al., 2015; Waltz, 2016). Overall, regardless of the swift progresses on genome editing technologies that are expanding our boundaries on plant virus control, other factors such as regulatory frameworks, biosafety and public perception of gene-edited organisms are also important to take into account. "At the dawn of the recombinant DNA era, the most important lesson learned was that public trust in science ultimately begins with and requires ongoing transparency and open discussion" (Baltimore et al., 2015).

CONCLUDING REMARKS

Altogether, genome editing technologies are revolutionizing the current tools for crop improvement programs, including the antiviral arsenal of plants. Nowadays, the proof of concept of zinc finger (including AZP and ZFN), Tale and CRISPR-Cas9 technologies against plant viruses have been done for at least 14 different species, mainly in the families *Geminiviridae* and *Potyviridae*. Among these technologies, CRISPR-Cas9 has been already explored for most of the viruses tested, despite the fact that it is the more recently developed GES. This fact might be explained by the simplicity of CRISPR-Cas9 designing, which can be adopted by standard biotechnological laboratories. However, off-targets effects (possible breaks in non-targeted DNA sites) continue as a major concern for application of CRISPR-Cas9 technology. A next generation sequencing (NGS) approach can be useful to provide a compelling profile of off-target cleavage sites for a given GES (Gaj et al., 2013). Likewise, NGS approaches will be useful to gain insights on pathosystems involving CRISPR-Cas9-edited plants and targeted viruses (Hadidi et al., 2016). Beyond the debate on regulation of GES-edited plant products, the use of GESs to induce antiviral defenses raises the question on durable resistance mediated by these technologies and the generation of challenging viral isolates. Although promising, the genome editing studies aiming to generate plant virus resistance have been carried out just to evaluate resistance against specific viruses. However, further studies addressing a larger spectrum of viral isolates in a same species and longer period of viral exposition are needed to better understand the durable resistance mediated by GES and the possible mechanisms deployed by some viral isolates to overcome the induced antiviral defenses.

AUTHOR CONTRIBUTIONS

GR wrote the draft version of manuscript and CB reviewed the manuscript providing substantial contributions. Both authors read and approved the final version of the work.

FUNDING

GR was supported by Move-In Louvain fellowship of Université catholique de Louvain.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 November 2016
doi: 10.3389/fmicb.2016.01797

Different Virus-Derived siRNAs Profiles between Leaves and Fruits in Cucumber Green Mottle Mosaic Virus-Infected *Lagenaria siceraria* Plants

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OPEN ACCESS

Edited by:
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Zhejiang Normal University, China

Reviewed by:
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China Agricultural University, China
Xuefeng Yuan,
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *03 June 2016*
Accepted: *25 October 2016*
Published: *09 November 2016*

Citation:
Li J, Zheng H, Zhang C, Han K, Wang S, Peng J, Lu Y, Zhao J, Xu P, Wu X, Li G, Chen J and Yan F (2016) Different Virus-Derived siRNAs Profiles between Leaves and Fruits in Cucumber Green Mottle Mosaic Virus-Infected Lagenaria siceraria Plants. Front. Microbiol. 7:1797. doi: 10.3389/fmicb.2016.01797

RNA silencing is an evolutionarily conserved antiviral mechanism, through which virus-derived small interfering RNAs (vsiRNAs) playing roles in host antiviral defense are produced in virus-infected plant. Deep sequencing technology has revolutionized the study on the interaction between virus and plant host through the analysis of vsiRNAs profile. However, comparison of vsiRNA profiles in different tissues from a same host plant has been rarely reported. In this study, the profiles of vsiRNAs from leaves and fruits of *Lagenaria siceraria* plants infected with Cucumber green mottle mosaic virus (CGMMV) were comprehensively characterized and compared. Many more vsiRNAs were present in infected leaves than in fruits. vsiRNAs from both leaves and fruits were mostly 21- and 22-nt in size as previously described in other virus-infected plants. Interestingly, vsiRNAs were predominantly produced from the viral positive strand RNAs in infected leaves, whereas in infected fruits they were derived equally from the positive and negative strands. Many leaf-specific positive vsiRNAs with lengths of 21-nt (2058) or 22-nt (3996) were identified but only six (21-nt) and one (22-nt) positive vsiRNAs were found to be specific to fruits. vsiRNAs hotspots were only present in the 5′-terminal and 3′-terminal of viral positive strand in fruits, while multiple hotspots were identified in leaves. Differences in GC content and 5′-terminal nucleotide of vsiRNAs were also observed in the two organs. To our knowledge, this provides the first high-resolution comparison of vsiRNA profiles between different tissues of the same host plant.

Keywords: virus-derived small RNAs, CGMMV, tissue-specific, NGS data analysis, *Lagenaria siceraria*

INTRODUCTION

RNA silencing is a natural antiviral mechanism in plants and other eukaryotic organisms. Through the process, virus-infected plants produce virus-derived small interfering RNAs (vsiRNAs) which play important roles in host antiviral defense (Zhu et al., 2011; Szittya and Burgyán, 2013; Zhang et al., 2015). The endoribonuclease activity of the dicer-like proteins (DCLs) 2 and 4 is essential for the production of these vsiRNAs (Deleris et al., 2006). DCL4 mainly targets virus RNA to produce 21 nucleotide (nt) vsiRNAs, while DCL2 is responsible for the processing of 22-nt vsiRNAs when DCL4 is absent or its activity is inhibited (Xie et al., 2004; Deleris et al., 2006). Both vsiRNAs can guide the RNA-induced silencing complex (RISC) to slice viral RNA in a sequence-specific manner. In addition, two other plant DCLs, DCL1 and DCL3, are essential for the production of small RNAs (Henderson et al., 2006). DCL1 is mainly responsible for excising the stem-loop structures of primary microRNAs (miRNAs) into mature approximately 21-nt miRNAs that play key roles in post-transcriptional gene silencing (Blevins et al., 2006; Dong et al., 2008). The functions of these DCLs are overlapping and can be complemented. Very low levels of 21-nt vsiRNAs were produced by DCL1 in *dcl2*/*dcl3*/*dcl4* triple mutant plants infected with Cucumber mosaic virus (CMV), while 24-nt vsiRNAs were produced by DCL3 in *dcl2*/*dcl4* double mutant plants, indicating a compensatory role for these DCLs (Bouché et al., 2006; Deleris et al., 2006).

The biogenesis of vsiRNAs has attracted much attention over the past decade, but is still not comprehensively understood. Early studies indicated that vsiRNAs are mostly produced from double stranded viral RNA (dsRNA) replicative intermediates (RIs) in a process that generates almost equal numbers of vsiRNAs from the positive and negative strands (Ahlquist, 2002). In addition, the highly structured regions in a single stranded viral RNA (ssRNA) can also contribute to the biogenesis of vsiRNAs, resulting in many more vsiRNAs derived from positive strand rather than negative strand (Molnár et al., 2005; Szittya et al., 2010; Wang et al., 2010).

Next Generation Sequencing (NGS) technology has recently been used to investigate the vsiRNA profiles of various combinations of viruses and plants. In general, 21-nt vsiRNAs usually predominate in the population, there is a strong A/U bias at the first nucleotide of vsiRNAs, and vsiRNA-producing hotspots can be identified within the viral genome (Miozzi et al., 2013; Visser et al., 2014; Xia et al., 2014; Yang et al., 2014; Kutnjak et al., 2015; Li et al., 2016). Previous studies indicated that vsiRNAs are predominantly responsible for RNA silencing-mediated antiviral immunity and the main function of vsiRNAs is to target and degrade viral mRNA through post-transcriptional gene silencing in plants (Zhu et al., 2011; Zhang et al., 2015). Moreover, recent studies have shown that vsiRNAs may also occasionally regulate host mRNAs with near perfect complementarity. The first report of this phenomenon was the targeting of the chlorophyll biosynthetic gene (CHLI) of *Nicotiana* by siRNAs derived from CMV Y-satellite, resulting in the yellowing of the plant (Shimura et al., 2011; Smith et al., 2011). It has also recently been shown that the eukaryotic translation initiation factor 4A (eIF4A) of *Nicotiana benthamiana* can be targeted by siRNA derived from Rice stripe virus (RSV), resulting in leaf-twisting and stunting (Shi et al., 2016). These results indicate the complicated function of vsiRNAs during virus-host interaction.

Cucumber green mottle mosaic virus (CGMMV) is a member of the genus *Tobamovirus*, family *Virgaviridae*, and causes a serious disease of cucurbit crops with significant economic losses in several countries including Israel, China, Korea and Russia (Antignus et al., 1990; Ugaki et al., 1991; Kim et al., 2003; Slavokhotova et al., 2007; Liu et al., 2009). Recently, it was reported on melon in the United States (Tian et al., 2014). CGMMV can be transmitted mechanically on seeds and pollen, causing typical mosaic and mottling symptoms on leaves, as well as fruit distortion (Mink, 1993). Similar to other tobamoviruses, CGMMV is a single-stranded positive RNA virus with a 3′ tRNA-like structure, encoding four polypeptides including a 124- to 132-kDa protein, a 181- to 189-kDa read-through protein, a 28- to 31-kDa movement protein (MP) and a 17- to 18-kDa coat protein (CP) (King et al., 2011). The profile of CGMMV-derived siRNAs in infected leaves of cucumber was reported recently (Li et al., 2016). The present study reports markedly different vsiRNA profiles (abundance, polarity and hotspot distribution) between infected fruits and leaves of *Lagenaria siceraria*.

MATERIALS AND METHODS

Sample Collection and Total RNA Extraction

Seeds of bottle gourd (*Lagenaria siceraria*, accession "Hangzhou gourd") were sown in soil rich in organic matters in a greenhouse with the ambient temperatures between 20 and 25°C, and watered every 3 days to maintain ample soil moisture. At the two and a half leaf stage plants were mechanically inoculated with CGMMV virions on the two expanding leaves using sap from a previously infected plant. Approximately 100 mg of tissue was homogenized in 20 volumes of inoculation buffer (0.1M phosphate buffer, pH7.5, 0.2% sodium sulfite and 0.01M 2-mercaptoethanol), while the mock plants were only inoculated with inoculation buffer.

Three replicate samples of fruit and leaves from plants with typical CGMMV symptoms and from mock controls were collected for RNA extraction. Total RNAs were extracted from each sample using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The presence of CGMMV infection in the tissues was confirmed with a One Step RT-PCR Kit (TOYOBO, Japan) following the product's protocol and using CGMMV specific primers (CG-F: 5′-GCTTACAAT CCGATCACAC-3′; CG-R: 5′-ATTATCTATCTCAGCCCTAG-3′). The RNA quantity and quality from each sample was evaluated by denaturing agrose gel electrophoresis and a 2100 Bioanalyzer (Agilent, USA).

Small RNA Sequencing and Raw Data Pre-Processing

Approximately 5 μg of total RNA was extracted for the preparation of a small RNA library according to the protocol of TruSeq Small RNA Sample Prep Kits (Illumina, USA). Briefly,

total RNA was resolved using denatured 8% polyacrylamide gel electrophoresis (PAGE) and small RNA fragments were isolated. After ligation of the 5′ and 3′ adaptors, the short RNA fragments were reverse transcribed using SuperScript II Reverse Transcriptase (Life Technologies, USA) and amplified by PCR. Finally, single-end sequencing (36 bp) was performed on an Illumina Hiseq2500 at LC-BIO (Hangzhou, China) following the protocol of the manufacturer.

After parsing small RNA sequences from the 3′ adaptor sequence, low quality and junk sequences, including transfer RNAs (tRNAs), ribosomal RNA (rRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), and repetitive sequences, were removed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The remaining sRNA reads were collapsed to uniread sets and the reads of > 30-nt or < 18-nt were discarded. Clean sRNA reads were used for further bioinformatics analysis.

Bioinformatics Analysis of Sequencing Data

To identify CGMMV-derived siRNAs, processed reads from each of the 12 *L. siceraria* libraries were mapped to the CGMMV reference genome (NCBI Accession No: KP868654) using Bowtie software (http://bowtie-bio.sourceforge.net) with one mismatch. To facilitate comparisons across different libraries, vsiRNA read numbers were normalized to "Reads Per Million" (RPM) based on the total small RNA read numbers of the corresponding library. All of the downstream analyses were performed using custom perl scripts and linux (Cent OS 6.5) bash script. For statistical analysis of the three biological replicates, one-way ANOVA analysis using Originpro 8.5 software was performed and values of $P < 0.01$ were considered significant. To avoid the inaccuracy of low copy sequences, sequences with <10 raw reads in each of the three replicates were removed (for the analysis of leaves or fruits specific vsiRNAs). Specific (Unique) vsiRNAs were extracted from the three replicates of each sample during this analysis. RNA secondary structures were predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) with default parameters.

Northern Blot

Total RNA was isolated from plants with Trizol (Invitrogen, USA) according to the manufacturer's instructions. For northern blot of CGMMV RNAs, a DNA probe targeting CGMMV CP was synthesized with primers (5′-GCTTACAATCCG ATCACAC-3′ and 5′-ATTATCTATCTCAGCCCTAG-3′) and labeled with DIG according to the manufacturer's protocol (DIG Oligonucleotide 3′-end labeling Kit, Roche, USA). For northern blot of positive-stranded CGMMV RNAs in leaves, a sequence (5′-CAACACAGGACCGTTGAGGAAAGCGTA AAAACCCGCACCTGGGAATCTAGAATTAATATCTACGAC AGACGAGGGTAACGCA-3′) was synthesized and labeled as DNA probe, and its complementary sequence was used for detecting negative-stranded CGMMV RNAs. Another sequence (5′-CATAGCTCTGAGCTTTAACTACACTAAAGT CAGTTATAGATAAATACTTAAGAATGGAAAAATAGT TAGGGAGCAACTTATC-3′) was used for detecting positive-stranded CGMMV RNAs in fruits, and its complementary sequence was used for detecting negative -stranded CGMMV RNAs in fruits. Pre-hybridization, hybridization and signal detection were done according to the protocol of the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, USA).

Tissue Immunoblot

Tissue immunoblot was carried out as described previously (Andika et al., 2005). Primary anti-CP (1: 5000) polyclonal serum and secondary polyclonal AP-conjugated goat anti-rabbit IgG (1: 10 000) (Sigma, USA) were used for blotting according to the methods described before (Peng et al., 2011).

RESULTS AND DISCUSSION

Overview of Small RNA Deep Sequencing Data

Cucumber green mottle mosaic virus (CGMMV)-infected leaves of bottle gourd showed the typical green mottle mosaic symptom 14 days after inoculation (**Figure 1A**), while the infected fruits had only a slight green mottle on the skin. Leaves and fruits were collected from three replicate virus-infected plants and infection with CGMMV was confirmed in each by RT-PCR (**Figure 1B**). Leaves and fruits from three mock plants were also collected as controls. Small RNAs isolated from extracted total RNAs of these tissues were then used for Illumina high-throughput sequencing.

After the removal of the junk, adapter and repeat reads, total numbers of small RNAs 18–30 nt long obtained from the three virus-infected fruits were 8,558,357 (6,160,591 unique),

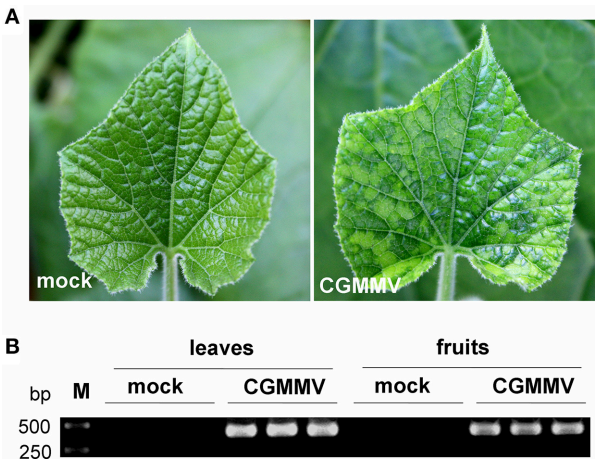

FIGURE 1 | Symptoms of CGMMV on leaves of *L. siceraria* and detection of CGMMV in leaves and fruits of *L. siceraria* through RT-PCR. (A) the typical green mottle mosaic symptom on CGMMV-infected leaves 14 days after inoculation (right panel), but not on mock leaves (left panel). **(B)** RT-PCR detection of CGMMV in CGMMV-infected and mock leaves and fruits of *L. siceraria* (three replicates). Clear bands were observed (confirmed by sequencing) in both leaves and fruits of CGMMV-infected samples, whereas no bands were detected in mock samples.

TABLE 1 | Summary of deep sequencing results of small RNA libraries from virus-infected and healthy *L. siceraria*.

Category	Fruits						Leaves					
	Replicate 1		Replicate 2		Replicate 3		Replicate 1		Replicate 2		Replicate 3	
	Mock (%)	Infected (%)	Mock (%)	Infected (%)	Mock (%)	Infected (%)	Mock (%)	Infected (%)	Mock (%)	Infected (%)	Mock (%)	Infected (%)
Junk reads (unique)	38,257 (0.52)	41,406 (0.58)	40,243 (0.50)	49,056 (0.61)	24,652 (0.34)	29,063 (0.57)	72,490 (1.25)	115,414 (1.49)	42,723 (1.34)	80,746 (1.37)	50,833 (1.18)	118,790 (1.35)
Junk reads (total)	41,065 (0.33)	48,287 (0.40)	43,306 (0.32)	55,305 (0.42)	26,649 (0.21)	33,359 (0.36)	86,896 (0.89)	144,998 (0.88)	51,772 (0.71)	103,055 (0.82)	61,625 (0.73)	155,882 (0.88)
Adapter and Length filter (unique)	777,629 (10.52)	757,192 (10.56)	1,162,594 (14.51)	935,667 (11.71)	766,948 (10.53)	759,138 (14.98)	375,488 (6.48)	842,828 (10.89)	348,862 (10.98)	686,222 (11.68)	378,319 (8.75)	901,498 (10.24)
Adapter and Length filter (total)	2,322,327 (18.66)	2,217,325 (18.59)	3,190,255 (23.73)	2,823,459 (21.57)	2,505,998 (19.82)	2,872,114 (30.77)	798,597 (8.14)	2,542,072 (15.43)	2,975,305 (40.82)	2,266,513 (18.05)	1,635,724 (19.33)	2,610,433 (14.66)
Rfam (unique)	46,796 (0.63)	35,433 (0.49)	48,433 (0.60)	35,086 (0.44)	38,572 (0.53)	32,089 (0.63)	18,787 (0.32)	55,100 (0.71)	21,552 (0.68)	54,019 (0.92)	14,789 (0.34)	47,635 (0.54)
Rfam (total)	424,682 (3.41)	433,138 (3.63)	550,979 (4.10)	413,185 (3.16)	661,347 (5.23)	405,919 (4.35)	73,128 (0.75)	487,950 (2.96)	104,472 (1.43)	418,871 (3.34)	51,803 (0.61)	250,177 (1.41)
Repeats (unique)	3932 (0.05)	3665 (0.05)	3991 (0.05)	3765 (0.05)	3416 (0.05)	2797 (0.06)	1175 (0.02)	5607 (0.07)	1063 (0.03)	4931 (0.08)	1107 (0.03)	5929 (0.07)
Repeats (total)	17,930 (0.14)	8759 (0.07)	12,199 (0.09)	7660 (0.06)	11,231 (0.09)	5984 (0.06)	1971 (0.02)	34,455 (0.21)	1568 (0.02)	25,181 (0.20)	1514 (0.02)	13,177 (0.07)
Clean reads (unique)	6,399,113 (86.55)	6,160,591 (85.93)	6,614,229 (82.58)	6,799,565 (85.12)	6,335,102 (86.98)	4,129,482 (81.48)	5,268,422 (90.98)	6,413,925 (82.85)	2,720,910 (85.64)	4,780,352 (81.37)	3,837,794 (88.76)	7,429,239 (84.36)
Clean reads (total)	9,442,756 (75.87)	8,558,357 (71.74)	9,432,345 (70.17)	9,240,205 (70.60)	9,268,640 (73.31)	5,654,646 (60.59)	8,766,823 (89.32)	10,250,660 (62.22)	4,064,336 (55.76)	7,513,459 (59.85)	6,644,494 (78.51)	12,265,181 (68.90)

9,240,205 (6,799,565 unique) and 5,654,646 (4,129,482 unique). Corresponding numbers from the mock fruits were 9,442,756 (6,399,113 unique), 9,432,345 (6,614,229 unique) and 9,268,640 (6,335,102 unique). From three virus-infected leaves, totals were 10,250,660 (6,413,925 unique), 7,513,459 (4,780,352 unique) and 12,265,181 (7,429,239 unique) and from three mock leaves, there were 8,766,823 (5,268,422 unique), 4,064,336 (2,720,910 unique) and 6,644,494 (3,837,794 unique). An overview of the deep sequencing results is presented in **Table 1**. In addition, different types of non-coding sRNAs including tRNAs, rRNAs, snoRNAs, and snRNAs were identified while mapping to the Rfam database (Version 12.0) (**Figure S1**). Interestingly, the numbers of these non-coding sRNAs reads in CGMMV-infected leaves were much larger than in mock leaves, whereas no such pattern was observed for fruits (**Figure S1**).

The size distribution of these 12 small RNA libraries was similar. Reads with 24-nt length accounted for most (60–70%) of the total sRNAs, followed by 23-nt (**Figure 2**). Notably, the percentage of 21- and 22-nt reads in virus-infected leaf samples were significantly larger than in the mock, whereas 24-nt reads were obviously fewer, similar to previous reports (Xia et al., 2014; Li et al., 2016). However, no significant differences of length distribution were observed between mock and infected fruits (**Figures 2A,B**).

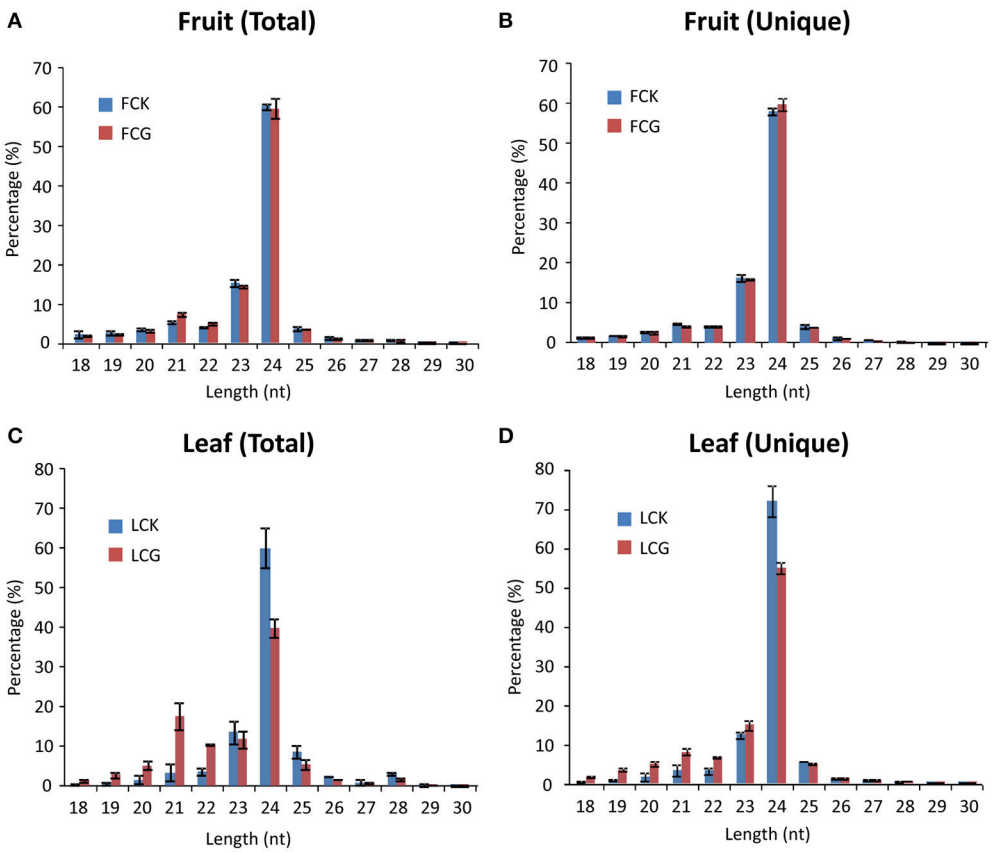

FIGURE 2 | Size distribution of sRNAs from healthy and CGMMV-infected *L. siceraria*. (A) Total sRNAs from fruit. **(B)** Unique sRNAs from fruit. **(C)** Total sRNAs from leaves. **(D)** Unique sRNAs from leaves. FCK, Healthy fruit; FCG, CGMMV Infected fruit; LCK, Healthy leaf; LCG, CGMMV Infected leaf. Error bars indicate ± SD calculated from three biological replicates. The numbers in the horizontal axis indicate length of vsiRNAs, and numbers in the vertical axis indicate percentage of vsiRNAs in healthy or CGMMV-infected samples.

TABLE 2 | Summary of Cucumber green mottle mosaic virus-derived small interfering RNAs (vsiRNAs) from virus-infected *L. siceraria.**

	Fruits			Leaves		
	Replicate 1	**Replicate 2**	**Replicate 3**	**Replicate 1**	**Replicate 2**	**Replicate 3**
vsiRNAs (unique)	67,105 (1.09%)	64,450 (0.95%)	47,522 (1.15%)	190,382 (2.97%)	165,911 (3.47%)	166,372 (2.24%)
vsiRNAs (total)	535,904 (6.26%)	424,062 (4.59%)	253,195 (4.48%)	3,176,423 (30.99%)	2,321,842 (30.90%)	2,526,004 (20.59%)

**Small RNA reads from L. siceraria were mapped to the Cucumber green mottle mosaic virus genome with full match and 1 mismatch.*

More vsiRNAs Are Produced in Leaves than in Fruits

To identify CGMMV-derived siRNAs in infected plants, the clean sRNA libraries were mapped to the virus reference genome. The total vsiRNAs accounted for 4.48–6.26% (5.11 ± 0.99%) of the total sRNAs of virus-infected fruits, while the corresponding figures for unique vsiRNAs were 0.95–1.15% (1.06 ± 0.10%). These values are much lower than those from infected leaves where total vsiRNAs accounted for 20.59–30.99% (27.49 ± 5.98%) of the total sRNAs and the corresponding figures for unique vsiRNAs were 2.24–3.47% (2.89 ± 0.62%) (**Table 2**, **Figure 3**), indicating that many more vsiRNAs were produced in leaves than fruits. An earlier study of *N. benthamiana* plants infected with Beet necrotic yellow vein virus (BNYVV) showed that the antiviral response was more effective in leaves than in roots; vsiRNAs accumulated more in leaves than in roots, whereas BNYVV mRNA levels were lower in leaves than in roots (Andika et al., 2005). We therefore compared the CGMMV RNA abundance in fruits and leaves using northern blot. Interestingly, higher levels of both genomic RNA and particularly subgenomic RNAs (sgRNAs) accumulated in leaves than in fruits (**Figure 3B**), correlating positively with the abundance of vsiRNAs (**Figure 3A**). Our results are consistent with a previous report that increased levels of Rice black-streaked dwarf virus (RBSDV) derived siRNAs in doubly-infected insects (RBSDV and RSV) compared to those infected only with RBSDV was positively correlated with the elevated levels of RBSDV RNA (Li et al., 2013). However, the reasons for these positive or negative relationships between

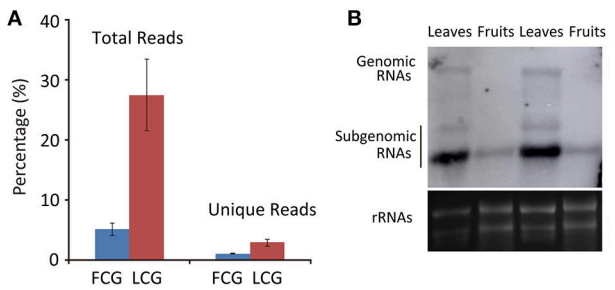

FIGURE 3 | Abundance of CGMMV derived siRNAs from infected *L. siceraria*. (A) Percentage of vsiRNAs in fruit and leaves of *L. siceraria* infected with CGMMV. Error bars indicate ± SD calculated from three biological replicates. FCG, CGMMV Infected fruit; LCG, CGMMV Infected leaf; **(B)** Northern blot detection of CGMMV RNA accumulation in fruit and leaf. Accumulation of CGMMV is much more in leaves compare to fruits for both genomic and subgenomic RNAs. rRNAs were used as control.

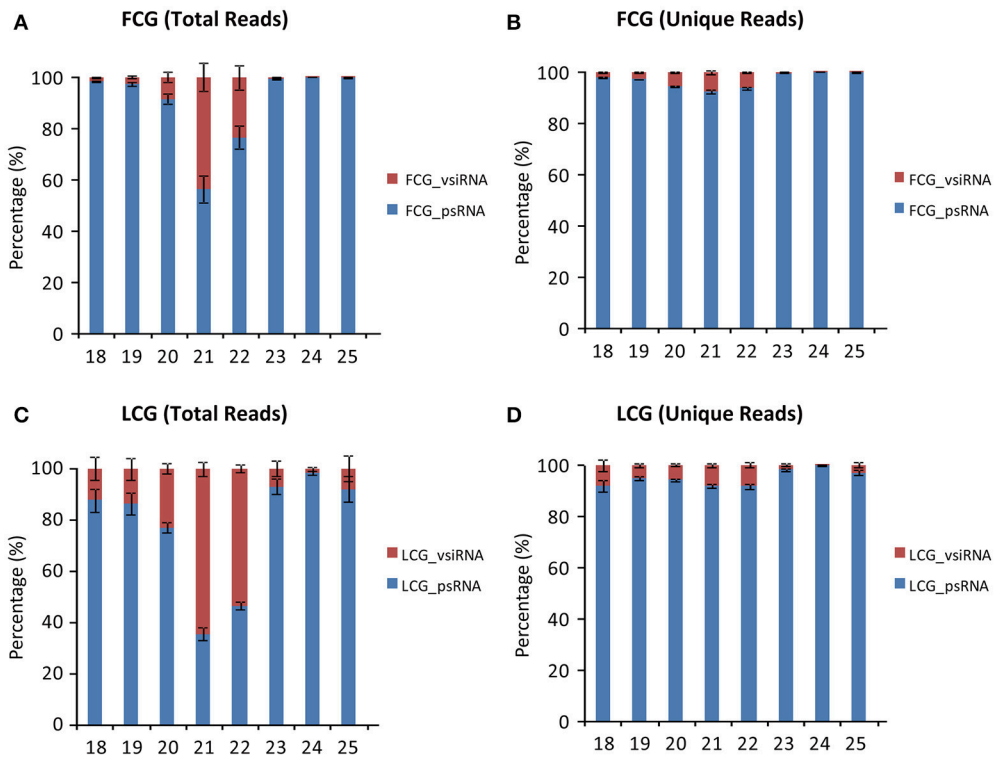

FIGURE 4 | Percentage of virus derived siRNAs and plant sRNA with different lengths (18–30 nt) from *L. siceraria* infected with CGMMV. (A) Total reads from fruit. **(B)** Unique Reads from fruit. **(C)** Total reads from leaves. **(D)** Unique reads from leaves. FCG_vsiRNA: CGMMV-derived siRNA from infected fruit; FCG_psRNA: Plant sRNA from infected fruit. LCG_vsiRNA: CGMMV-derived siRNA from infected leaf; LCG_psRNA: Plant sRNA from infected leaf. Error bars indicate ± SD calculated from three biological replicates. The numbers in the horizontal axis indicate length of vsiRNAs, and numbers in the vertical axis indicate percentage of vsiRNAs and psRNA in CGMMV-infected samples.

vsiRNAs and mRNA levels in different samples are still not clear.

Since CGMMV can be transmitted by seeds and pollen, it is interesting to investigate whether viruses only in seeds contribute to vsiRNAs production in fruits or viruses in any parts of fruits had such contribution. We hence detected the virus distributions in fruits through tissue immunoblot with antibody of CGMMV. Results showed that viruses were detectable in any parts of virus-infected fruits, indicating the ubiquitous localization of CGMMV in virus-infected fruits (**Figure S2**). And these results also suggested that, in addition to seeds, viruses in other parts of fruits could also contribute to the production of vsiRNAs in fruits.

Most vsiRNAs Are 21 and 22 nt Long

Although 24 nt sRNAs accounted for the largest percentage of total sRNAs, a remarkably high percentage of the 21 and 22 nt sRNAs in infected plants are vsiRNAs, especially in the leaves (64.44 ± 2.62% for 21 nt and 53.54 ± 1.52% for 22 nt) (**Figures 4A,C**). The increased numbers of 21 and 22 nt sRNAs in infected leaves (as compared to mock-inoculated) may therefore be mainly due to the presence of vsiRNAs (**Figures 2C,D**). Interestingly, the percentages of unique vsiRNAs, are relatively low (<10%) in both infected leaves and fruits (**Figures 4B,D**), suggesting that there are very high copy numbers of vsiRNAs in infected plants. The predominance of 21 and 22 nt vsiRNAs has been reported in various eukaryotic organism (Deleris et al., 2006; Donaire et al., 2008; Yan et al., 2010; Li et al., 2013; Mitter et al., 2013; Xia et al., 2014; Yang et al., 2014). This suggests that homologs of DCL4 (production of 21 nt vsiRNA) and DCL2 (production of 22-nt vsiRNA) in *L. siceraria* are actively involved in antiviral defense and play important roles in response to CGMMV infection (Xie et al., 2004; Deleris et al., 2006).

vsiRNAs Are Predominantly Produced from Viral Positive Strand RNAs in Leaves but Not In Fruits

The numbers of vsiRNAs derived from positive or negative strand viral RNA were also compared. In infected leaves, many more vsiRNAs were produced from the positive strand viral RNA irrespective of vsiRNA length (**Figures 5C,D**), which is similar to the results from Cymbidium ringspot virus (CymRSV) and Tobacco rattle virus where vsiRNAs were predominantly from the viral positive strand RNA (Molnár et al., 2005). Furthermore, it has been demonstrated experimentally that secondary structures within the CymRSV single-stranded RNA strands could serve as substrates for DCL-mediated cleavage (Molnár et al., 2005), which might be also one of the reasons for the asymmetry in strand polarity of vsiRNAs in CGMMV infected leaves. Here, we tried to predict the potential secondary

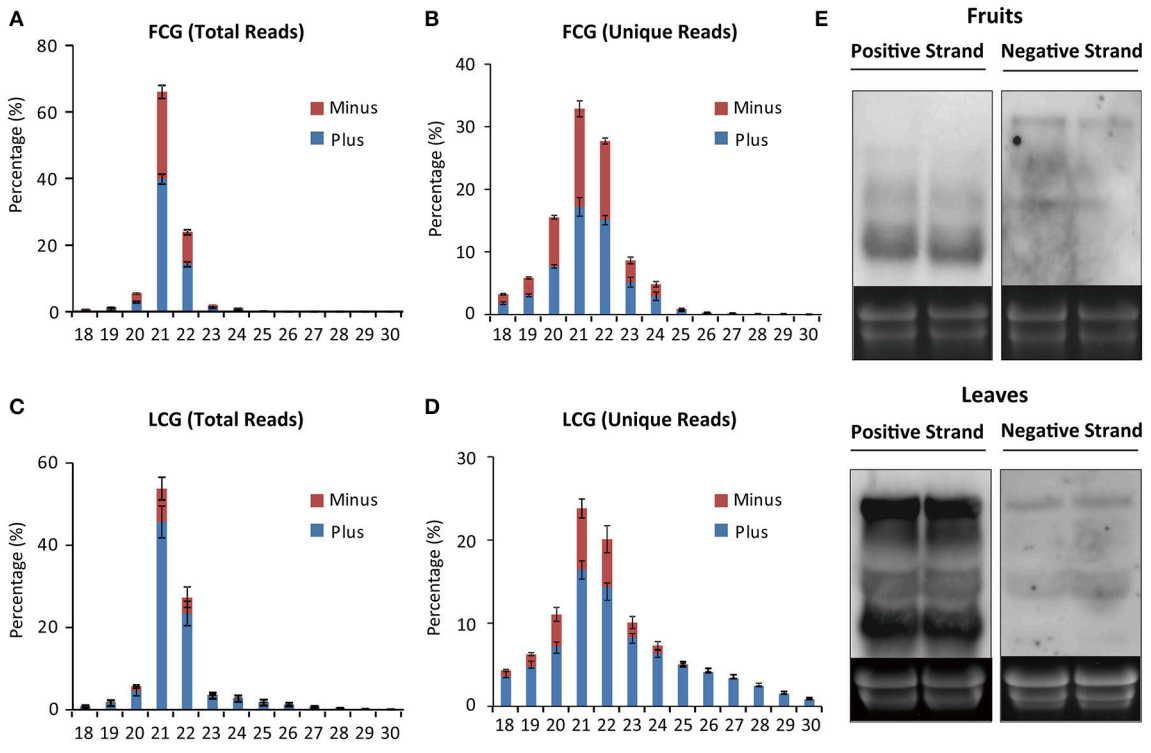

FIGURE 5 | Size distribution of CGMMV-derived siRNAs from infected *L. siceraria*. (A) Total vsiRNAs from fruit. **(B)** Unique vsiRNAs from fruit. **(C)** Total vsiRNAs from leaves. **(D)** Unique vsiRNAs from leaves. FCG, CGMMV Infected fruit; LCG, CGMMV Infected leaf. Error bars indicate ± SD calculated from three biological replicates. The numbers in the horizontal axis indicate length of vsiRNAs, and numbers in the vertical axis indicate vsiRNAs percentage of different lengths in CGMMV-infected samples. **(E)** Northern blot detection of positive and negative-stranded CGMMV RNAs in fruits and leaves infected *L. siceraria*. Two samples were used for each analysis.

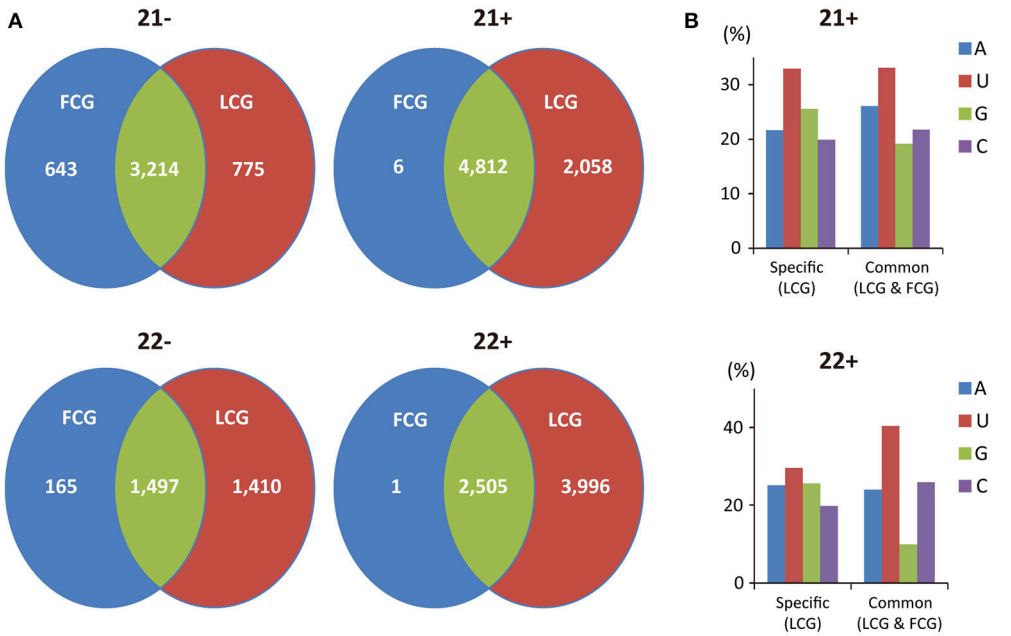

FIGURE 6 | Profile of tissue-specific and common CGMMV-derived siRNAs in infected *L. siceraria*. (A) Abundance of tissue-specific and common vsiRNAs with the lengths of 21 and 22 nt in *L. siceraria*. Much higher numbers of vsiRNAs are specifically produced in leaves compare to fruits, especially for plus strand of vsiRNAs. **(B)** Distribution pattern of the 5′ nt in leaf-specific and common positive vsiRNAs with lengths of 21 and 22 nt. FCG, CGMMV Infected fruit; LCG, CGMMV Infected leaf. 22- or 21- indicate vsiRNAs with length of 22 or 21 nt derived from negative strand of viral genome, and 22+ or 21+ indicate vsiRNAs with length of 22 or 21 nt derived from positive strand of viral genome.

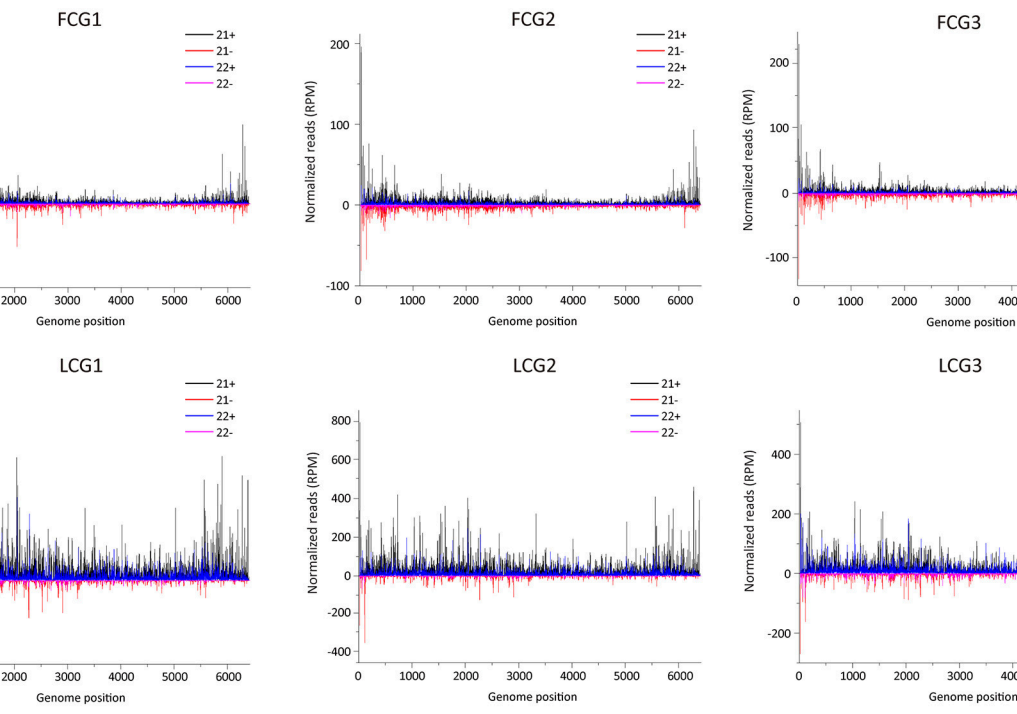

FIGURE 7 | Distribution of CGMMV-derived siRNAs along the viral genome. FCG1, FCG2, FCG3: CGMMV Infected fruit (three replicates); LCG1, LCG2, LCG3: CGMMV Infected leaf (three replicates); Color coding indicates viral sRNAs derived, respectively, from the positive (+) and negative genomic strands (–). All reads in this analysis were redundant and normalized.

TABLE 3 | Nucleotide composition (%) of the unique CGMMV-derived small RNAs (with lengths of 21 and 22 nt) and of the CGMMV genomic sequence *.

	−GC(%)	−A(%)	−U(%)	−G(%)	−C(%)	GC(%)	A(%)	U(%)	G(%)	C(%)
Fruit 21 nt	42.83 ± 0.35	31.13 ± 0.30	26.04 ± 0.13	18.91 ± 0.16	23.92 ± 0.20	43.76 ± 0.25	25.54 ± 0.19	30.70 ± 0.07	23.84 ± 0.09	19.92 ± 0.16
Fruit 22 nt	42.07 ± 0.41	31.39 ± 0.31	26.54 ± 0.27	18.35 ± 0.18	23.73 ± 0.30	43.06 ± 0.44	25.85 ± 0.25	31.09 ± 0.19	23.38 ± 0.20	19.68 ± 0.25
Leaf 21 nt	39.33 ± 0.82	31.76 ± 0.19	28.91 ± 0.63	17.15 ± 0.47	22.18 ± 0.38	43.28 ± 0.20	25.43 ± 0.26	31.29 ± 0.46	23.26 ± 0.30	20.02 ± 0.10
Leaf 22 nt	38.54 ± 1.02	31.94 ± 0.27	29.52 ± 0.82	16.71 ± 0.63	21.83 ± 0.41	42.64 ± 0.17	25.73 ± 0.18	31.64 ± 0.33	22.91 ± 0.23	19.73 ± 0.08
CGMMV 5′-300 bp	–	–	–	–	–	42.3	34.7	23.0	19.7	22.7
CGMMV 3′-300 bp	–	–	–	–	–	48.3	20.7	31.0	26.7	21.7
CGMMV	–	–	–	–	–	42.99	25.69	31.32	23.82	19.17

**Small RNA reads from L. siceraria were mapped to the CGMMV genome with full match and 1 mismatch.*

structure within the CGMMV positive strand RNA, but no clear relationship was observed between the predicted secondary structure and vsiRNAs with relative high abundance (data not shown). Thus, secondary structure might not the main reason for the asymmetry in strand polarity of vsiRNAs in CGMMV infected leaves. We next detected the accumulation of positive and negative-stranded CGMMV RNAs in leaves to investigate whether this vsiRNA asymmetry polarity was related with the different ratio of positive and negative-stranded CGMMV RNAs in leaves. Results showed that positive-stranded CGMMV RNAs were accumulated much more than negative ones (**Figure 5E**), which suggests that the vsiRNA asymmetry polarity in leaves might resulted from the high ratio of positive-stranded RNAs to negative ones.

Meanwhile, interestingly, in fruits, vsiRNAs were almost equally from the positive and negative strands of viral RNA (**Figures 5A,B**). Northern blot showed that the total positive-stranded CGMMV RNAs were accumulated at a similar level to negative ones in fruits of *L. siceraria* according to the size and density of the bands (**Figure 5E**). However, bands with high density for positive-stranded RNAs were clearly lower compare to negative-stranded ones in blotting (**Figure 5E**), which probably indicates the complicated composition of CGMMV RNAs with positive-stranded or negative-stranded forms. For a dsRNA virus, almost equal numbers of positive and negative vsiRNAs were generated, suggesting that the dsRNA genome or dsRNA RIs are the target of host Dicer as reported previously (Wu et al., 2010; Li et al., 2013). For ssRNA viruses, approximately equal proportions of positive and negative vsiRNAs have sometimes also been reported where vsiRNAs were mainly derived from viral dsRNA RIs (Aliyari et al., 2008; Wu et al., 2010). This suggests that dsRNA RIs of CGMMV may serve as the major substrates for vsiRNAs production in fruits. Here, we found that the different ratio of positive and negative-stranded CGMMV RNAs in leaves and fruits might be positively correlated to the proportions of positive and negative vsiRNAs.

The tissue-specific distribution of vsiRNAs was analyzed further for the 21 and 22 nt vsiRNAs which composed the majority of all vsiRNAs. Only six 21 nt and one 22 nt positive vsiRNAs were produced specifically in fruits while 2058 and 3996 positive vsiRNAs were identified to be specifically produced in leaves for those lengths (**Figure 6A**), which might be also due to the different ratio of positive and negative-stranded CGMMV RNAs in fruits and leaves of *L. siceraria*.

vsiRNAs Hotspots in Fruits, but Not in Leaves, Were Only Present in the 5′-Terminal and 3′-Terminal Regions of the Positive Strand

To examine the distribution pattern of vsiRNAs within the CGMMV genome, 21 and 22 nt long vsiRNAs of all infected libraries were aligned to the virus genome. These vsiRNAs (from both leaves and fruits) cover the entire CGMMV genome (**Figure 7**), consistent with the previous report (Li et al., 2016). There were strong vsiRNAs preferences to the 5′ terminal of

TABLE 4 | First Nucleotide (%) of the unique CGMMV-derived small RNAs (21 and 22 nt) *.

	−A(%)	−U(%)	−G(%)	−C(%)	A(%)	U(%)	G(%)	C(%)
Fruit 21 nt	30.32 ± 0.38	26.66 ± 0.70	16.19 ± 0.80	26.83 ± 0.46	25.04 ± 0.28	31.20 ± 0.63	20.84 ± 0.68	22.92 ± 0.56
Fruit 22 nt	30.05 ± 0.51	26.87 ± 1.39	13.09 ± 0.69	30.00 ± 1.25	25.19 ± 0.47	31.27 ± 1.64	17.15 ± 0.73	26.38 ± 1.59
Leaf 21 nt	28.75 ± 1.28	31.82 ± 2.90	11.51 ± 2.04	27.92 ± 0.58	23.04 ± 1.26	35.79 ± 2.71	15.34 ± 2.09	25.82 ± 0.65
Leaf 22 nt	27.28 ± 1.81	32.45 ± 2.70	9.62 ± 1.97	30.65 ± 1.03	22.98 ± 1.40	34.24 ± 1.69	14.41 ± 1.14	28.36 ± 0.85

**Small RNA reads from L. siceraria were mapping to CGMMV genome with full match and 1 mismatch.*

viral negative strand in both fruits and leaves, suggesting that these regions are preferentially cleaved by the host Dicer in fruits (**Figure 7**). For positive strand, vsiRNAs hotspots were only present in the 5′-terminal and 3′-terminal in fruits, while multiple hotspots were identified for leaves (**Figure 7**). Recent reports indicated that the production of vsiRNA hotspots in the 3′ region of a virus genome could be ascribed to the presence of viral sgRNAs (Ruiz-Ruiz et al., 2011; Silva et al., 2011; Visser et al., 2014). The CP of CGMMV is expressed from a 3′ terminal sgRNA which might explain the presence of vsiRNA hotspots in the CP region. In addition, we found that many vsiRNAs produced in the 3′ tRNA-like structure region, and the mechanism for this needs further investigation. Previous studies indicated that hairpin structures in single stranded viral genomes can also contribute to the production of vsiRNAs (Molnár et al., 2005; Du et al., 2007). To identify potential secondary structures that might be related to the generation of the vsiRNA hotspots, approximately 300 bp of the CGMMV 5′ and 3′ regions were selected and analyzed. However, no obvious relationship was found between the predicted secondary structures and vsiRNA hotspots region (data not shown). The correlation between vsiRNAs hotpots and secondary structure of the viral genome is still not clear (Donaire et al., 2009). The identification of hotspots for CGMMV derived siRNAs may help select efficient target regions within the genome that can be targeted with artificial siRNA hairpins in future research.

Different Distribution Patterns of GC Content and 5′-Terminal Nucleotide of vsiRNAs in Leaves and Fruits

Previous studies have shown that vsiRNAs are preferentially produced from GC-rich regions and vsiRNAs tend to have a higher GC content than that of the entire viral genome (Ho et al., 2007; Yan et al., 2010). However, the GC content of vsiRNAs (21 and 22 nt) from the positive strand of fruits and leaves was similar to that of the CGMMV genome (**Table 3**). Interestingly, the GC content of vsiRNAs (21 and 22 nt) from the negative strand was higher in fruits than in leaves (**Table 3**), indicating a tendency for these negative strand vsiRNAs in leaves to be produced from regions with lower GC content. Furthermore, since vsiRNAs hotspots were commonly identified in the CGMMV 5′ and 3′ regions, 300 bp of these region were also examined. Surprisingly, the 5′-end has GC content of 42.3% which is similar to the GC content of the full genome (43.0%), while the 3′-end has higher GC content (48.3%; **Table 3**) which might explain the hotspots for vsiRNAs in fruit.

The 5′ terminal nucleotide of small RNAs is important for the sorting of small RNAs into AGO complexes in plants (Mi et al., 2008; Takeda et al., 2008). Our results indicated that 5′ terminal nucleotide of vsiRNAs (21 and 22 nt) from the negative strand was mostly frequently A in fruits or U in leaves, while for the positive strand, the nucleotide was mostly U in both fruits and leaves (**Table 4**). A 5′ terminal G is underrepresented in both leaves and fruits irrespective of polarity (**Table 4**). A U preference for the 5′ terminal nucleotide has also been demonstrated in other plants (Donaire et al., 2009; Qi et al., 2009). In *Arabidopsis*, AGO2 and AGO4 preferentially recruit small RNA with a 5′ terminal A, while AGO1 harbors miRNAs with a 5′ terminal of U (Mi et al., 2008). Our data suggest that both AGO2 and AGO4 actively recruit vsiRNAs in leaves and fruits, while AGO1 tends to be involved in the recruitment of negative strand vsiRNAs in fruits. The different 5′ terminal nucleotide preference of vsiRNA (negative strand) for A in fruits and U in leaves suggests that multiple AGO complexes might be involved in varying degrees during anti-viral defense in different tissues.

Finally, we compared the distribution patterns of the 5′ nt between leaf-specific and common positive vsiRNAs with lengths of 21 and 22 nt. Leaf-specific positive vsiRNAs 21 nt in length had an increased percentage of G at the 5′ compared with common vsiRNAs, while the percentage of A was decreased (**Figure 6B**). For 22 nt vsiRNAs, the percentage of G was also increased but at the expense of C and U (**Figure 6B**). The different distributions of the 5′ nt for leaf-specific and common vsiRNAs may suggest the irreplaceable roles of leaf-specific vsiRNAs in antiviral defense.

CONCLUSION

In this study, NGS sequencing of sRNAs was performed to investigate profiles of CGMMV-derived siRNAs in infected leaves and fruits of *L. siceraria*. Different vsiRNA patterns of abundance, polarity, hotspot distribution, GC content and 5′-terminal nucleotide were observed in infected leaves and fruits. Furthermore, infected leaves have large numbers of leaf-specific vsiRNAs with a distinct 5′ nt. To our knowledge, this provides the first high-resolution comparison of vsiRNA profiles between different tissues of the same host plant.

AUTHOR CONTRIBUTIONS

JC and FY conceived and designed the experiments. JL, HZ, CZ, KH, JP, and YL performed the experiments on viruses. JZ, PX,

XW, and GL performed the experiments on plants. JL, HZ, JC, and FY analyzed data and wrote the manuscript.

FUNDING

This work was financially supported by the Special Fund for Agro-scientific Research in the Public Interest (201303028, 201403032), the International Science & Technology Cooperation Program of China (2015DFA30700) and Fund for Research in the Public Interest of Zhejiang Province (2015C32042).

ACKNOWLEDGMENTS

We thank Prof. M. J. Adams, Stevenage, Herts, UK, for correcting the English of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01797/full#supplementary-material

Figure S1 | Categories of non-coding sRNA in small RNA libraries of healthy and CGMMV-infected *L. siceraria*. FCK1, FCK2, FCK3: Healthy fruit (three replicates); FCG1, FCG2, FCG3: CGMMV Infected fruit (three replicates); LCK1, LCK2, LCK3: Healthy leaf (three replicates); LCG1, LCG2, LCG3: CGMMV Infected leaf (three replicates).

Figure S2 | Tissue immunoblot analysis for detection of CGMMV in virus-infected fruit. The transaction of fresh virus-free (MOCK) and virus-infected and fruits were shown in upper panels, while the corresponding tissue immunoblot with antibody of CGMMV were shown in bottom panels, which revealed the obvious blue signals in any parts of virus-infected fruits, indicating the ubiquitous localization of CGMMV in virus-infected fruits.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 04 November 2016
doi: 10.3389/fmicb.2016.01771

Improved Pathogenicity of a Beet Black Scorch Virus Variant by Low Temperature and Co-infection with Its Satellite RNA

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Co-infection of none-coding satellite RNAs (sat-RNAs) usually inhibits replication and attenuates disease symptoms of helper viruses. However, we find that the sat-RNA of *Beet black scorch virus* (BBSV) and low temperature (18°C) additively enhance the systemic infection of BBSV in *Nicotiana benthamiana*. Northern blotting hybridization revealed a relatively reduced accumulation of BBSV-derived small interfering RNAs (siRNAs) in presence of sat-RNA as compared to that of BBSV alone. Cloning and sequencing of total small RNAs showed that more than 50% of the total small RNAs sequenced from BBSV-infected plants were BBSV-siRNAs, whereas the abundance of sat-siRNAs were higher than BBSV-siRNAs in the sat-RNA co-infected plants, indicating that the sat-RNA occupies most of the silencing components and possibly relieves the RNA silencing-mediated defense against BBSV. Interestingly, the 5′ termini of siRNAs derived from BBSV and sat-RNA were dominated by Uridines (U) and Adenines (A), respectively. Besides, the infection of BBSV alone and with sat-RNA induce down-regulation of miR168 and miR403, respectively, which leads to high accumulation of their targets, *Argonaute 1* (*AGO1*) and *AGO2*. Our work reveals the profiles of siRNAs of BBSV and sat-RNA and provides an additional clue to investigate the complicated interaction between the helper virus and sat-RNA.

Keywords: beet black scorch virus, satellite RNA, RNA silencing, temperature, siRNAs

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Edited by:
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Reviewed by:
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Fei Yan,
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *16 August 2016*
Accepted: *21 October 2016*
Published: *04 November 2016*

Citation:
Xu J, Liu D, Zhang Y, Wang Y, Han C, Li D, Yu J-L and Wang X-B (2016) Improved Pathogenicity of a Beet Black Scorch Virus Variant by Low Temperature and Co-infection with Its Satellite RNA. Front. Microbiol. 7:1771. doi: 10.3389/fmicb.2016.01771

INTRODUCTION

The satellite RNAs (sat-RNAs) of plant viruses rely on the helper virus for replication and encapsidation but share little or no sequence similarity with the helper virus genome (Murant and Mayo, 1982; Hu et al., 2009). Based on genome size, the sat-RNAs are divided into two classes: large sat-RNAs encoding a nonstructural protein and small sat-RNAs without coding capacity (Simon et al., 2004). Sat-RNAs usually inhibit the replication of helper viruses and may alter the disease symptoms of the helper viruses depending on the interaction among host factors, helper viruses, and sat-RNAs (Collmer and Howell, 1992; Roossinck et al., 1992; Hull, 2002). Despite the fact that there are sat-RNAs that reduce the disease symptoms without influencing the accumulation of helper viruses, most sat-RNAs reduce both disease symptoms and titer of the helper virus, probably

by competing with the helper virus for common replication factors (Roossinck et al., 1992; Hull, 2002). However, there are exceptions in which sat-RNAs enhance the disease symptoms during co-infections. For example, a few isolates of Cucumber mosaic virus (CMV) sat-RNAs can exacerbate the disease symptoms in tobacco and tomato hosts because of a specific sequence carried by the sat-RNAs (review in Roossinck et al., 1992; Simon et al., 2004). Similarly, sat-RNA of *Groundnut rosette virus* (GRV) is largely responsible for the groundnut rosette symptom and the transmission between natural hosts of GRV (Murant et al., 1988; Robinson et al., 1999). Interestingly, unlike the sat-RNAs of CMV and GRV, the sat-RNA C of *Turnip crinkle virus* (TCV) often enhances the visible symptoms of TCV in a host-dependent manner (Li, 1990). Since sat-RNA C is capable of interfering the encapsidation of TCV genomic RNAs thereby to boost the accumulation of free coat proteins, which are RNA silencing suppressor (Wang and Simon, 1999; Thomas et al., 2003; Zhang and Simon, 2003), these observations suggest that some sat-RNAs enhance helper virus disease symptoms by directly or indirectly suppressing RNA silencing. So far, to our knowledge, there is no report to document an enhancement of helper virus titers during co-infection with sat-RNAs (Hull, 2002; Simon et al., 2004).

In the past few years, the progressive understanding of RNA silencing results in more information about the interaction between plants and the pathogens. The RNA silencing in plants has been found to have numerous functions in the course of plant growth (Baulcombe, 2004; Meister and Tuschl, 2004; Chapman and Carrington, 2007), among which one of the important function is to provide an adaptive immune system counteracting pathogens, including viruses, bacteria, and so on (Ding et al., 2004; Voinnet, 2005; Ding and Voinnet, 2007). In *Arabidopsis*, the cascade of DCL2/3/4, AGO1/2/3/5/7/10, and RDR1/6 have been shown to be involved in antiviral RNA silencing pathways (review in Huang et al., 2016). Accordingly, the viruses have evolved diverse viral suppressors of RNA silencing (VSRs) to inhibit distinct steps in the silencing pathway (Li and Ding, 2006; Díaz-Pendón and Ding, 2008). At present, about 35 individual VSR families have been reported in plant viruses (Ding and Voinnet, 2007). The co-evolution processes of silencing and suppressing reveal complex interaction between virus and host plant in the long history of co-existence (Ding and Voinnet, 2007). Some subviral pathogens, such as viroids and satellites, are also influenced by the pressure of RNA silencing and evolved effective secondary structures to avoid or minimize the small RNA-mediated silencing (Wang et al., 2004). The secondary structure of *Potato spindle-tube viroid* (PSTVd) was also found to induce silencing but could be resistant to RISC-mediated cleavage (Itaya et al., 2007). Recent studies demonstrate that sat-RNAs-derived siRNAs can directly silence host genes, which is responsible for sat-RNA-induced disease symptom (Shimura et al., 2011; Smith et al., 2011).

Beet black scorch virus (BBSV) was firstly reported in northern China in the late 1980s and lately identified as a new species of genus *Betanecrovirus* (Cao et al., 2002; Yuan et al., 2006; King et al., 2011). BBSV induced the symptom of black scorched leaves and necrotic fibrous roots in the sugar beet plants in late spring, causing severe yield loss in the plantation areas. Two isolates from the provinces of Ningxia and Xinjiang have been reported in China, designated as BBSV-N and BBSV-X respectively (Cao et al., 2002; Xi et al., 2006), which exhibit 99.45% similarity in nucleotide sequence. In addition, BBSV-Co was reported in Colorado of Unite States (Weiland et al., 2006, 2007), which shared 93% similarity with BBSV-N, and different BBSV isolates were identified in Iran and Spain (Koenig and Valizadeh, 2008; González-Vázquez et al., 2009). During the serial propagation of BBSV, a gain-of-function mutant harboring a single nucleotide substitution at nucleotide (nt) 3477 in the 3′UTR induce higher infectivity than wild-type BBSV in *N. benthamiana* (Xu et al., 2012). In addition to the viral genome RNA, another 615 nt single stranded RNA has been identified as a satellite RNA in the isolate of BBSV-X (Guo et al., 2005). During the replication of sat-RNA of BBSV, various forms such as monomers, dimers, and tetramers are accumulated, and the dimer form plays an intermediate role in replication (Guo et al., 2005).

In this study, we first showed that the satellite RNA enhance the pathogenesis and accumulation of BBSV in *N. benthamiana* plants under at or below room temperature. Further analyses including cloning and sequencing of siRNAs derived from BBSV and its sat-RNA, suggest that sat-RNA may alleviate RNAi mediated antiviral silencing to enhance the systemic infection of BBSV by acting as surrogacy of the helper virus.

MATERIALS AND METHODS

Plant Materials and Virus Inoculation

N. benthamiana plants were grown in a growth chamber with a 16-h-light/8-h-dark cycle at 25°C. Three leaves of *N. benthamiana*, typically at the four-leaf stage in 1-month-old, were used for inoculation (Xu et al., 2012). A BBSV variant, BBSV-m294 (abbreviated as Bm, GenBank accession no. JN635330.1) that caused severe symptom in *N. benthamiana* and obtained after a passage of propagation (Xu et al., 2012). The sat-RNA (GenBank accession no. NC_006460.1) of BBSV were used for inoculation by the method reported previously (Xu et al., 2012). After inoculation, plants were grown at 18 and 25°C conditions. Three systemic leaves were harvested at 12 dpi for northern blot analysis and sequencing of small RNA.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted using TRIzol® Reagent (Invitrogen, USA). For detection of viral genomic RNA and mRNA of BBSV and its sat-RNA, 2 μg total RNA extracted from mock or virus-infected plants was used for hybridization using indicated gene-specific ^{32}P-radiolabled cDNA probes corresponding to the 3′UTR fragment of BBSV or the full-length sat-RNA, respectively as described (Xu et al., 2012). For small RNA gel blots, 10 μg total RNA were separated on 17% denaturing polyacrylamide gel (PAGE) and transferred to nylon membranes (GE Healthcare, UK). DNA oligonucleotides corresponding to the sequences of BBSV (nt 155–176, nt 769–788, nt 823–842, nt 1259–1278, nt 1762–1781, nt 2020–2039, nt 2266–2287, and nt 3115–3134) or sat-RNA (nt 132–152, nt 395–416 and nt 506–527) were synthesized respectively. The mixtures of

antisense oligonucleotides corresponding to BBSV and sat-RNA were labeled with [γ-^{32}P] ATP as probes used for hybridization at 40°C for 16–20 h in PerfectHyb plus buffer (Sigma-Aldrich). The membranes were washed in 2 × SSC (0.3 M NaCl and 0.03 M sodium citrate) containing 0.2% SDS for 30 min and then twice with 1 × SSC containing 0.1% SDS for 20 min both at 50°C.

Small RNA Library Sequencing and Analysis

The small RNA libraries were generated following the manufacturer's protocol (Illumina, California, USA). Briefly, separated by electrophoresis, RNA fractions with sizes between 18–30 nt corresponding to the small RNA population were purified, and cloned using NEBNext® Multiplex Small RNA Library Prep Set for Illumina kit. The final products were quantified on the Agilent DNA 1000 chip and sequencing was performed by use of an Illumina Hiseq 2500-SE50 (Illumina, California, USA). The Illumina sequencing reads were first trimmed to remove the adaptor sequence to get clean reads. The trimmed sequencing reads were then blasted to the Bm (JN635330.1) and sat-RNA (NC_006460.1) and the sequences with full matched were considered as Bm or sat-RNA small RNAs. The clean reads were also blasted in miRBase for miRNAs. The data of small RNA libraries was deposited in GenBank with accession number GSE80694.

Quantitative RT-PCR Analyses

To measure expression levels of miR168 and miR403, stem-loop quantitative RT-PCR technique was used as previously described (Varkonyi-Gasic and Hellens, 2011). The quantitative expression of DCLs, AGOs and RDR6 mRNAs were checked by real-time RT-PCR as described previously (Kotakis et al., 2010). Primers used for quantitative analysis above are listed (Table S1).

RESULTS

Low Temperature or sat-RNA Co-infection Enhances the Infection by BBSV Variant m294

In our previous studies, a series of BBSV spontaneous variants were isolated from serial propagation of wild-type BBSV in *Chenopodium amaranticolor* and *N. benthamiana*. The typical variant BBSV-m294 (abbreviated as Bm, GenBank accession no. JN635330.1) causes more severe symptoms than wild-type BBSV at low temperature (18°C) (Xu et al., 2012). To determine the impact of environment temperature on Bm infection, *N. benthamiana* plants were mechanically inoculated with Bm and maintained at 18 or 25°C, respectively. At 12 dpi, the infected plants were photographed as shown in **Figure 1**. The Bm-infected plants induced typical yellow curling symptoms on systemic leaves at 18°C, whereas the Bm induced very few yellow chlorotic mottle spots in upper leaves at 25°C (**Figure 1**, middle panel).

BBSV infection is naturally associated with satellite RNAs (sat-RNAs), which depend on BBSV for replication and movement but share no sequence homology with the helper viral genome (Guo et al., 2005). To determine if the sat-RNA affects the pathogenicity of BBSV in different temperatures, we further inoculated *N. benthamiana* plants with Bm alone or with its sat-RNA at 18 or 25°C. In contrast with very few infection lesions by Bm alone, clearly visible disease symptom was observed in the systemically infected leaves infected by Bm and its sat-RNA at 25°C (**Figure 1**, bottom panel). Moreover, the viral symptom induced by Bm and its sat-RNA was further enhanced at 18°C than at 25°C (**Figure 1**, upper panel).

Collectively, both low temperature and co-infection with sat-RNA additively enhance the pathogenicity of BBSV.

Sat-RNA Co-infection Enhances Bm Accumulation but Reduces the Production of Bm-Derived siRNAs

In order to examine the accumulation of BBSV along with or without its sat-RNA in different temperature, we further carried out northern blot hybridizations to detect the genomic and subgenomic RNA of Bm, as well as sat-RNA. In consistence with symptom observations, the genomic and subgenomic RNA of Bm accumulated to significantly higher levels at 18°C than that of Bm at 25°C (**Figure 2A**, lane 2 and 5). In addition, the accumulation level of Bm genomic RNA was higher in sat-RNA co-infection samples than Bm alone at either low or room temperature (**Figure 2A**, upper panel). Thus, these results indicate that both low temperature and co-infection with its sat-RNA additively enhance Bm accumulation in *N. benthamiana* plants.

To investigate the RNA silencing-mediated antiviral defense, the accumulation of the small interfering RNAs derived from the sat-RNA and its helper virus were analyzed through northern blot hybridizations. The Bm- and sat-RNA-derived siRNAs were readily detected in systemic leaves and all the viral siRNAs were mostly 22-nt in length followed by 21-nt (**Figure 2B**), which revealed that the replications of Bm and its sat-RNA strongly triggered the host RNA silencing. The accumulation level of BBSV-derived siRNAs was similar in the plants infected by Bm alone and co-infection with sat-RNA at 18°C (**Figure 2B**, compare lane 2 and 3), despite of the fact that BBSV genomic RNA accumulated to higher levels in the presence of sat-RNA than that of Bm alone (**Figure 2A**, compare lane 2 and 3). This finding indicated that the presence of sat-RNA relatively decreased the production of Bm-derived siRNAs in the co-infected plants at 18°C. Significantly, sat-siRNAs accumulated to very high levels in the co-infected leaves, regardless of temperature conditions (**Figure 2B**, sat-siRNAs lane 3 and 6). These results demonstrated that the high-level accumulation of the sat-RNA and its derived siRNAs may saturate the potency of antiviral silencing targeting Bm, which relieves the silencing targeting to Bm.

Sat-RNA Reduces the Production of Bm-Derived siRNAs by Saturating DCL2 and DCL4 Function during Co-infection

To characterize the population of the siRNAs derived from Bm and its sat-RNA, total small RNAs were cloned from the systemically infected leaves of *N. benthamiana* plants maintained at different temperatures. After trimming the linker

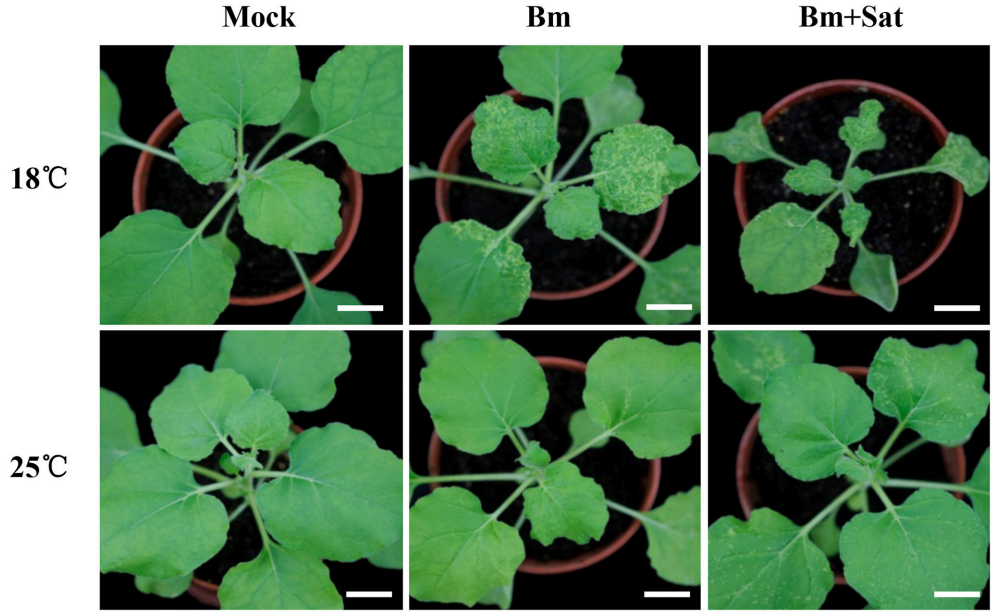

FIGURE 1 | Co-infection of sat-RNA and low temperature additively improved the systemic infectivity of BBSV. Symptom development in *N. benthamiana* inoculated by buffer (Mock), Bm alone and co-infected with its sat-RNA at 18 and 25°C. The infected seedlings were photographed at 12 days after inoculation with purified Bm or sat-RNA-associated Bm at a concentration of 100 μg/mL. Bars = 2 cm.

sequences, the small RNA reads of 18- to 30-nt in length were further analyzed. More than 16 million small RNA reads, including endogenous small RNAs and virus-derived siRNAs, were obtained from each sample (**Table 1**). Notably, in the Bm-infected leaves, approximately 69.2% of total sequenced small RNAs were perfectly match or complementary to the genome of Bm (**Table 1**), indicating that Bm genomic and subgenomic RNAs served as the major substrates of the host Dicer enzyme(s) in *N. benthamiana* plants infected with Bm alone. In the BBSV/sat-RNA co-infected plants at 18°C, however, 29.6 and 43.2% of total small RNAs were mapped to Bm and its sat-RNA, respectively (**Table 1**). The similar results were obtained in the systemic leaves infected by Bm alone or with sat-RNA at 25°C (**Table 1**). These results are consistent with the above northern blot analysis and strongly indicate that sat-RNA is the predominant substrate of the host Dicer enzyme(s), leading to reduced production ratio of the helper viral siRNAs in the BBSV/sat-RNA co-infected plants.

We further analyzed the polarity of virus-derived siRNAs and found different profiles of viral siRNAs derived from Bm and sat-RNA. Nearly equal amount of positive and negative stranded Bm-siRNAs accumulated in all small RNA samples (**Figure 3A**). However, in sat-RNA co-infected leaves, a clear prevalence for sense strand of sat-siRNAs was observed under both 18 and 25°C temperature conditions, representing 97.6 and 96.2% of the total sat-siRNAs, respectively (**Figure 3B**). The distinct polarity of Bm- and sat-siRNAs might due to their different replication processes, in which, positive-stranded RNA viruses usually use dsRNA as an intermediate template for genomic RNA synthesis (Kovalev et al., 2014), and most circular satellite RNAs utilize rolling cycle mechanism for its replication that produce abundant plus strands and few minus templates (Branch and Robertson, 1984; Bruening et al., 1991).

With regard to size distribution, both Bm-siRNAs and sat-siRNAs are dominated by 22-nt reads (65.3–73.6%), followed by 21-nt reads (16.3–28.9%) and other length reads (**Figures 3C–E**), which suggests that the *N. benthamiana* homologs of DCL2 and DCL4 are the predominant Dicers involved in the biogenesis of viral siRNA from both Bm and sat-RNA. The dominance of 22-nt siRNAs of BBSV and sat-RNA is consistent with *Cymbidium ring spot virus* (CymRSV)-derived vsRNAs in *N. benthamiana* plants (Donaire et al., 2009).

The previous studies have reported that the selective loading of small RNAs into specific AGOs is determined by the 5′-terminal nucleotides of siRNAs (Mi et al., 2008; Montgomery et al., 2008). To determine potential interactions between viral siRNAs and distinct AGO complexes, we analyzed the 5′ terminial of viral siRNAs derived from Bm- and sat-siRNAs (**Figures 3F–H**). Bm-siRNAs are dominated by uridines (5′U) with the ratio of 31.0–33.2%, and followed in order by adenines (A), cytidines (C), and guanines (G), which is consistent in the samples infected by Bm alone or with sat-RNA under different temperature (**Figures 3F,G**). In contrast, sat-RNA-derived siRNAs exhibited a clear predominance of A at 5′ end (46.2–48.0%) (**Figure 3H**). These trends were not affected by two temperature conditions. In contrast, there is no obvious preference of nucleotides in the composition of BBSV and sat-RNA genome (BBSV: A 24.8%, C 23.7%, G 25.8%, U 25.7%; sat-RNA: A 26.0%, C 22.0%, G 24.4%, U 27.6%). Considering different AGOs preferred different 5′-terminal first nucleotide

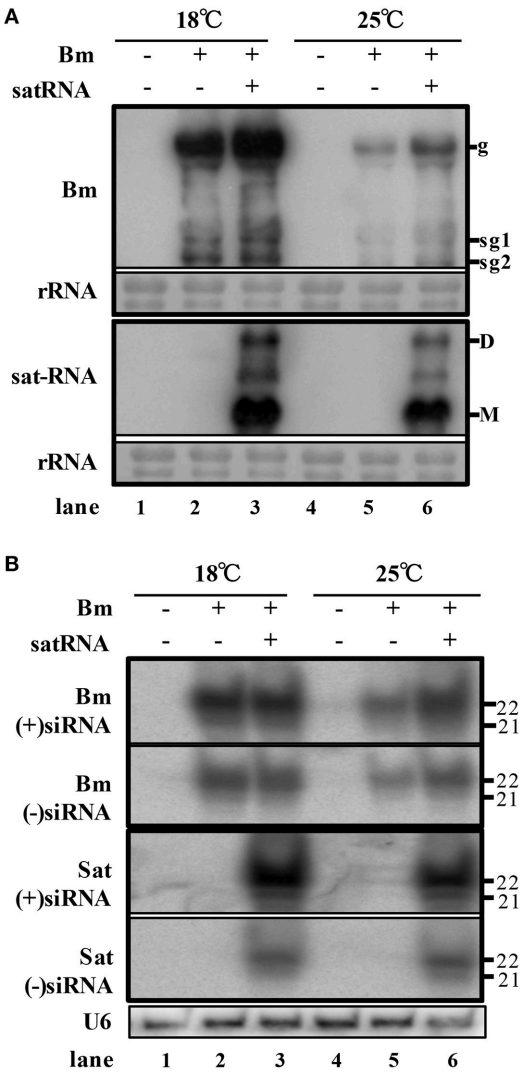

FIGURE 2 | Low temperature and co-infection with its sat-RNA relatively relieve the silencing potency targeting to BBSV. (A) Accumulation of viral RNA from Bm (top panel) and sat-RNA (bottom panel) in systemically infected leaves. Genomic (g) and sub-genomic RNA1 and RNA2 (sg1 and sg2) were indicated on the right at the top panel. Sat-RNA monomer (M) and dimer (D) were indicated on the right at the bottom panel. Methylene blue-stained rRNA was used as a loading control. **(B)** northern blot analysis of Bm- and sat-RNA -derived siRNAs by probes hybridizing to sense and anti-sense genome regions. U6 were indicated as loading controls. The positions of 21 and 22 nt RNAs are indicated on the right.

(Mi et al., 2008; Montgomery et al., 2008), these results indicate an involvement of different AGOs, mainly AGO1, and AGO2, in the antiviral silencing targeting Bm and sat-RNA, respectively.

Sat-RNA-Derived siRNAs are Predominantly Processed from the Highly Structured Region of the sat RNA Genome

To further explore the frequencies of Bm-siRNAs and sat-siRNAs in the Bm and sat-RNA genomes, we mapped the positive- and negative-stranded viral siRNAs to the top and bottom of genomes of Bm and sat-RNA, respectively. Note that two different scales were used to accommodate the high abundance of siRNAs. The Bm-siRNAs were almost continuously but heterogeneously distributed along Bm genome and exhibited similar patterns with or without sat-RNA (**Figure 4A**). However, the sat-siRNAs exhibited several peak distribution features in all sat-RNA co-inoculation samples (**Figure 4B**). The most abundant sat-siRNAs were peaked in the positive strand of nt 396–417 (**Figure 4B**), where a highly structured region was predicted to be formed by using Mfold software (Figure S1). Notably, the distribution patterns of Bm- and sat-RNA-derived siRNAs remained unchanged in all the virus-inoculated samples at both 18 and 25°C, indicating that the profiles of Bm- and sat-siRNAs were not a result of sequencing biases (**Figure 4**, Figure S2).

Bm Infection or Co-infection with Bm Sat-RNA Perturbs the Expression of Antiviral Silencing Genes

We also analyzed miRNA expression from total small RNA reads. Virus infection induced down-regulation of miR164, miR166, miR167, miR168, and miR403, and up-regulation of miR172 and miR397, after inoculation of Bm alone or with sat-RNA at two temperatures (**Figures 5A,D**; Figure S3), indicating that a series of host miRNAs were affected by virus infection. Among these miRNAs, miR168, and miR403 target the mRNAs of *AGO1* and *AGO2*, respectively, which are the main antiviral silencing components. Therefore, the expression levels of miR168 and miR403 were further confirmed by quantitative RT-PCR (**Figures 5B,E**). Interestingly, the down-regulation of miR168 level was more obvious in Bm samples than that in sat-RNA co-inoculation samples, which is consistent in 18 and 25°C (**Figure 5B**). Accordingly, *AGO1*, the target of miR168, was up-regulated and the mRNA level was higher in Bm samples than that with sat-RNA (**Figure 5C**), suggesting a main role of antiviral AGO1 in Bm inoculation leaves. However, miR403, which negatively regulates AGO2 mRNAs, exhibited down-regulation level to a larger extent in sat-RNA co-inoculation samples than that in Bm samples (**Figure 5E**), and its target *AGO2* were up-regulated higher in sat-RNA co-inoculation leaves (**Figure 5F**), indicating AGO2 as a major antiviral component of RNA silencing in sat-RNA involving leaves. These results were consistent with the results of 5′ nucleotide bias analysis of viral siRNAs, in which predominant U in Bm-siRNAs were mediated by AGO1, whereas prominent A preference in sat-siRNAs were mainly AGO2 involved (**Figures 2A,B**).

We also detected the accumulation of other RNA silencing components *DCL2*, *DCL4*, and *RDR6* mRNA levels in *N. benthamiana* by quantitative real-time RT-PCR (**Figure 6**). Compared with mock, Bm alone or with sat-RNA consistently induced relatively high levels of *DCL2* and *DCL4* mRNA accumulation (**Figures 6A,B**), which is consistent with the sequencing data of dominant 22- and 21-nt length siRNAs. It is interesting that no significant changes in the accumulation of *RDR6* mRNAs in sat-RNA co-inoculation samples compared with Bm alone (**Figure 6B**), perhaps due to its primary function of stably producing ta-siRNAs that is most important for plant growth. All these data suggest that the host plants exert different expression patterns to Bm and sat-RNA for antiviral silencing.

TABLE 1 | The amount of small RNAs isolated from systemic leaves of *N. benthamiana* inoculated with buffer (Mock), Bm alone or co-inoculated with the sat-RNA at low (18°C) and room (25°C) temperature.

	18°C Mock	18°C Bm	18°C Bm + Sat	25°C Mock	25°C Bm	25°C Bm+Sat
Total	16,913,661	17,910,527	19,097,617	16,593,227	17,191,750	17,781,409
BBSV		12,401,345 (69.2%)	5,651,525 (29.6%)		9,683,060 (56.3%)	5,438,352 (30.6%)
Sat-RNA			10,575,344 (43.2%)			8,688,464 (30.8%)

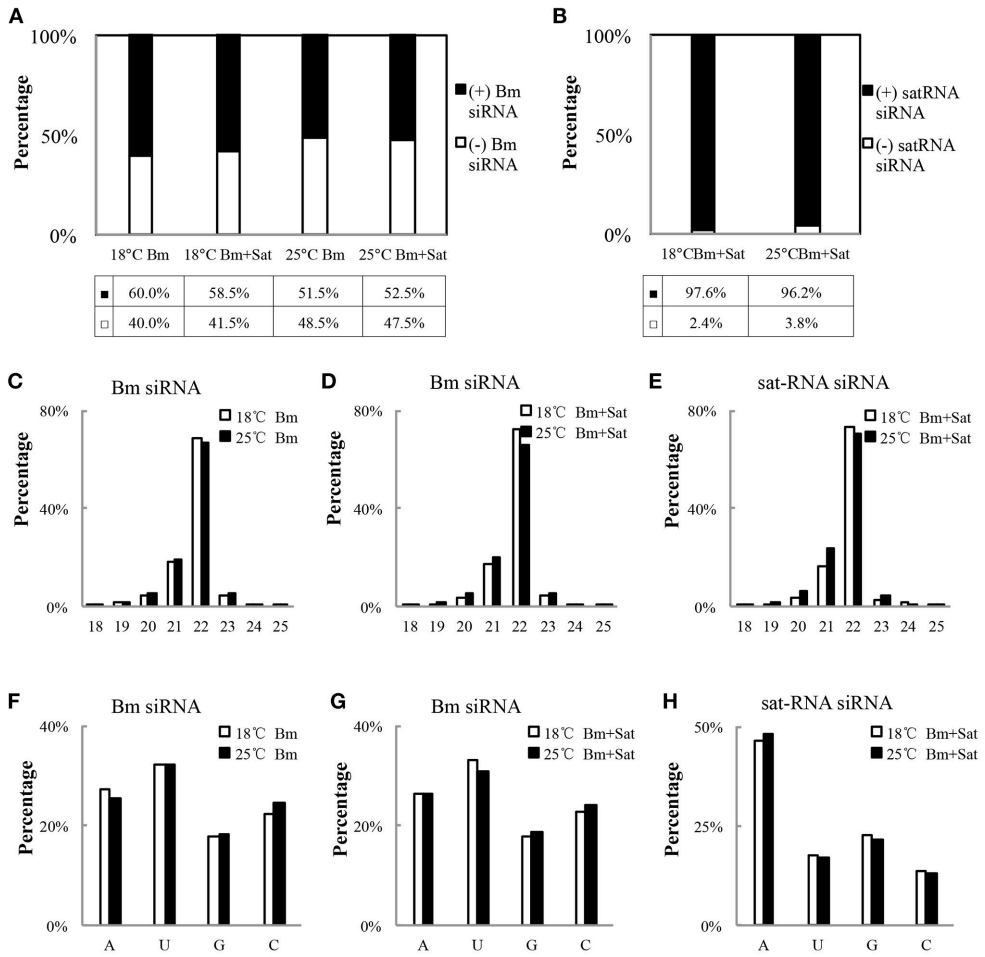

FIGURE 3 | Profiles of the Bm- and sat-RNA -derived siRNAs. The total siRNAs were isolated from Bm-inoculated and sat-RNA co-inoculated systemic leaves grown at 18 and 25°C conditions at 12 dpi. The 18- to 30-nt siRNA from Bm- and sat-RNA were analyzed. Relative abundance of siRNA from the positive strand (black column) and the negative strand (white column) of BBSV genomic RNA **(A)** or sat-RNA **(B)**. The relative percentages of (+) siRNA and (−) siRNA to total siRNAs are shown in the bottom. **(C–E)** showed the size distributions of Bm- and sat-RNA- derived siRNAs in the different treatment as indicated. **(F–H)** showed relative frequency of 5′-terminal nucleotide of Bm- and sat-RNA -derived siRNAs with respect to the amount of individual nucleotides in the Bm genome **(F,G)** or sat-RNA genome **(H)**.

DISCUSSION

Sat-RNAs are viral parasites and depend on their helper viruses for replication, encapsidation and movement in the host plants. Sat-RNAs are usually involved in the interaction between their helper viruses and plant hosts by modulating the accumulation level of helper viruses and symptom induction. In this study, we found that the sat-RNA of BBSV facilitated the systemic infection of the helper virus in *N. benthamiana*. Our results also show that BBSV is temperature sensitive and the systemic infection is enhanced at lower temperature. Analysis of siRNAs derived from the sat-RNA and the helper virus by

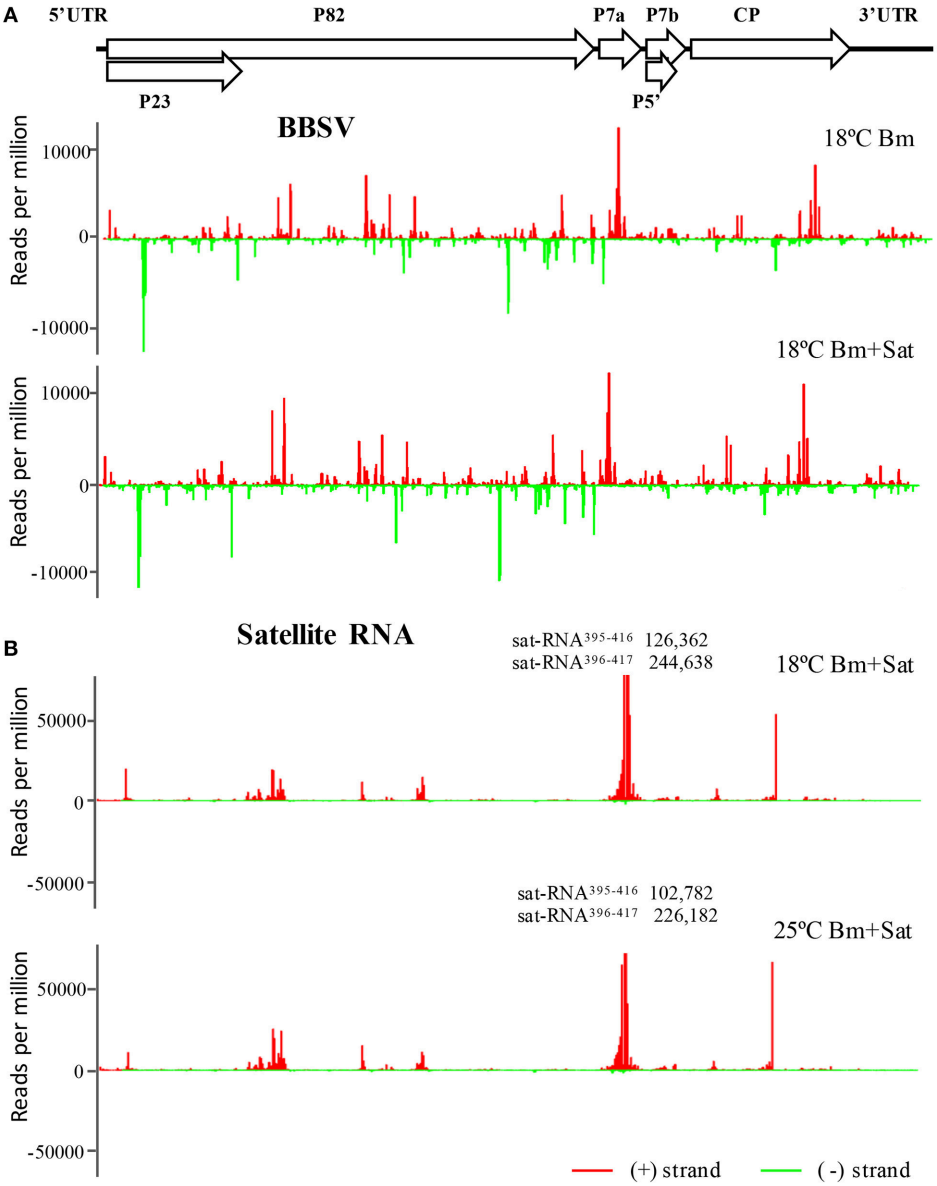

FIGURE 4 | The abundant distribution of siRNAs on Bm and sat-RNA genomes. siRNAs derived from the viral genome of Bm **(A)** and sat-RNA **(B)** are shown in red above (positive strand) or green below (negative strand) the horizontal line. X axis represents the length of the genome, and Y axis the counts of the siRNAs per million of total viral-derived siRNAs. The Bm genome organization is shown schematically above the graphs with coding open reading frames indicated as open arrows. Note that the read counts of two (+) sat-siRNAs (sat-RNA$^{395-416}$ and sat-RNA$^{396-417}$) are out of scale and indicated **(B)**.

both northern blotting and cloning/sequencing revealed that virus infection triggers high levels of RNA silencing and that sat-RNA co-replication produces highly abundant sat-siRNAs to relieve the silencing pressure for helper virus. Meanwhile, virus infection induced high levels of expression of DCLs and AGOs of RNA silencing as main antiviral elements. Our findings reveal that the helper virus (Bm) is probably mainly targeted by AGO1-associated complex, and the sat-RNA is silenced by AGO2-associated complex. These results illustrate the sat-RNA could benefit the helper virus, especially when the helper virus is confronted with strong defense of host plants.

It is known that plant RNA-directed RNA polymerases (RDRs) were involved in antiviral silencing and that high temperature enhances antiviral silencing in *N. benthamiana* because RDR6 may be inactive at the low temperature (Xie et al., 2001; Szittya et al., 2003; Qu et al., 2005). In *Arabidopsis* and *N. benthamiana*, RDR6 has been shown playing an important role in the host antiviral defense (Dalmay et al., 2000; Mourrain et al., 2000; Schwach et al., 2005). Thus, our observation

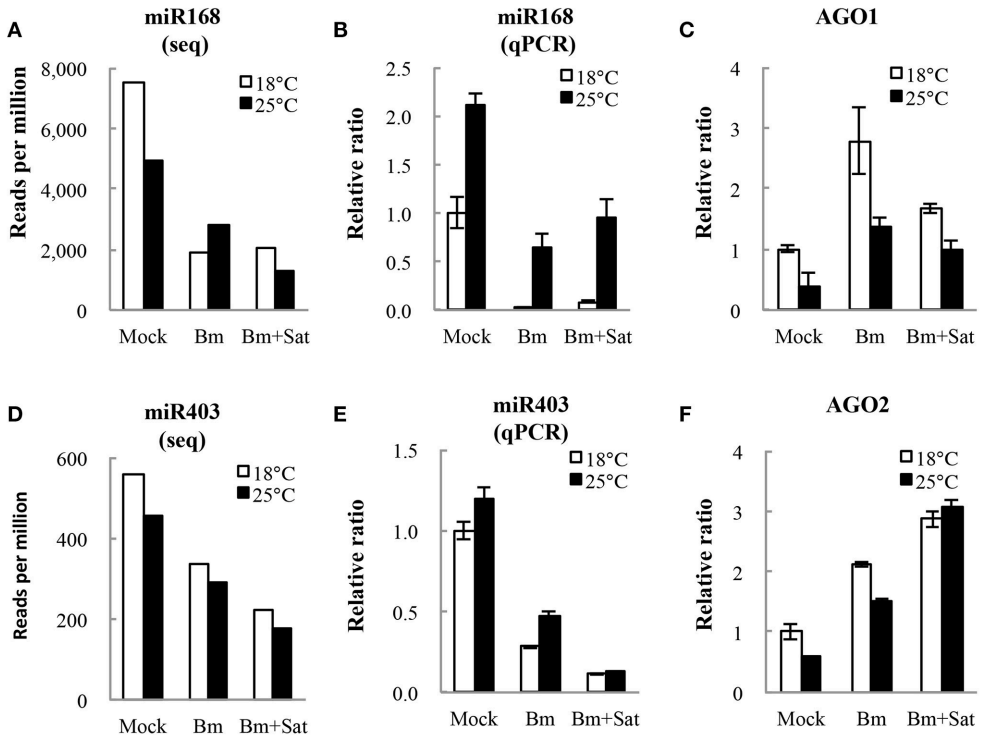

FIGURE 5 | miR168 and miR403 expression levels and their targets AGO1 and AGO2 analysis. (A,D) showed the amount of miR168 and miR403 per million total reads in small RNA library. **(B,E)** showed quantitative RT-PCR validation of the relative expression levels of miR168 and miR403. **(C,F)** showed relative expression of AGO1 and AGO2 transcripts targeted by miR168 and miR403, respectively. Error bars represent mean standard error calculated from three biological replicates.

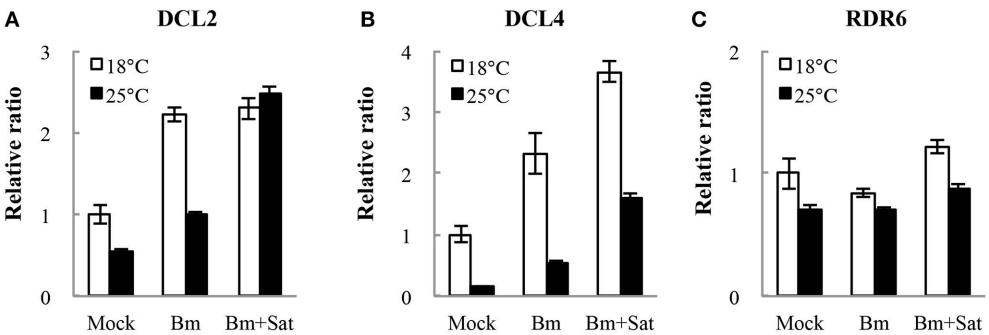

FIGURE 6 | Quantitative RT-PCR analysis of the expression of RNA silencing-related *N. benthamiana* DCL2 (A), DCL4 (B), and RDR6 (C) mRNAs. Error bars represent mean standard error calculated from three biological replicates. The information and primer sequences used for amplification of DCLs and RDR6 were listed in Table S1.

that milder symptom by BBSV in *N. benthamiana* at the higher temperature might be due to the improvement of RDR6 functions, but not of its mRNA levels shown in **Figure 6C**. The incomplete or aborted viral RNAs produced in the process of BBSV replication would be recognized as aberrant RNAs to be converted into dsRNAs *de novo* by RDR6 or other RDRs, strengthening the silencing to degrade the viral RNAs.

Although the replication of defective interfering RNA (DI RNAs), sat-RNAs and viroids produces abundant siRNAs, these sub-viral RNAs are resistant to RNA silencing, because highly structured RNAs may be poor targets for RNA cleavages by RNA-induced silencing complex (Wang et al., 2004; Du et al., 2007; Gomez and Pallas, 2007; Itaya et al., 2007). Unlike DI RNAs, however, sat-RNAs have no sequence homology with the helper virus so that the abundant siRNAs derived from the sat-RNA are not able to enhance BBSV silencing. Therefore, we propose that the efficient sat-RNA replication yields abundant substrates for host Dicers, which compete for the silencing factors and thereby facilitate infection and spread of the helper virus. It should be pointed out that sat-RNA modulation of helper virus silencing

reported in this study is independent of the sequence homology between sat-RNA and the helper virus reported previously for TCV sat-RNA C, which is 3′ co-terminal with the helper viral RNAs (Zhang and Simon, 2003).

It is known that sat-RNAs compete for the replication machinery with the helper virus, which could enhance the survival of the host to the benefit of the helper virus (Hull, 2002). Our work suggests a new function for sat-RNAs in the antiviral silencing of the helper virus that is also beneficiary to the infection and spread of the helper virus. We propose that these properties of sat-RNAs play a key role in the emergence and evolution of sat-RNAs.

AUTHOR CONTRIBUTIONS

XW and JY conceived and designed the experiments; JX and DeL performed the experiments; JX and XW analyzed the data and drafted the manuscript. YZ, YW, CH and DaL participated in experimental coordination and revision of the manuscript; XW and JY proofread and finalized the manuscript.

FUNDING

This work was supported by grants from National Science Foundation of China (31370176, 31500123, and 31322004).

ACKNOWLEDGMENTS

We thank Songyu Liu for analyzing the small RNA libraries of BBSV and its sat-RNA.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01771/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 24 August 2016
doi: 10.3389/fmicb.2016.01329

CMV2b-AGO Interaction Is Required for the Suppression of RDR-Dependent Antiviral Silencing in *Arabidopsis*

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: 20 July 2016
Accepted: 11 August 2016
Published: 24 August 2016

Citation:
Fang Y-Y, Zhao J-H, Liu S-W, Wang S, Duan C-G and Guo H-S (2016) CMV2b-AGO Interaction Is Required for the Suppression of RDR-Dependent Antiviral Silencing in Arabidopsis. Front. Microbiol. 7:1329. doi: 10.3389/fmicb.2016.01329

Using a transient plant system, it was previously found that the suppression of *Cucumber mosaic virus* (CMV) 2b protein relies on its double-strand (ds) RNA binding capacity, but it is independent of its interaction with ARGONAUTE (AGO) proteins. Thus, the biological meaning of the 2b-AGO interaction in the context of virus infection remains elusive. In this study, we created infectious clones of CMV mutants that expressed the 2b functional domains of dsRNA or AGO binding and tested the effect of these CMV mutants on viral pathogenicity. We found that the mutant CMV2b$_{(1–76)}$ expressing the 2b dsRNA-binding domain exhibited the same virulence as wild-type CMV in infection with either wild-type Arabidopsis or *rdr1/6* plants with RDR1- and RDR6-deficient mutations. However, remarkably reduced viral RNA levels and increased virus (v)siRNAs were detected in CMV2b$_{(1–76)}$-infected *Arabidopsis* in comparison to CMV infection, which demonstrated that the 2b$_{(1–76)}$ deleted AGO-binding domain failed to suppress the RDR1/RDR6-dependent degradation of viral RNAs. The mutant CMV2b$_{(8–111)}$ expressing mutant 2b, in which the N-terminal 7 amino acid (aa) was deleted, exhibited slightly reduced virulence, but not viral RNA levels, in both wild-type and *rdr1/6* plants, which indicated that 2b retained the AGO-binding activity acquired the counter-RDRs degradation of viral RNAs. The deletion of the N-terminal 7 aa of 2b affected virulence due to the reduced affinity for long dsRNA. The mutant CMV2b$_{(18–111)}$ expressing mutant 2b lacked the N-terminal 17 aa but retained its AGO-binding activity greatly reduced virulence and viral RNA level. Together with the instability of both 2b$_{(18–111)}$-EGFP and RFP-AGO4 proteins when co-expressed in *Nicotiana benthamiana* leaves, our data demonstrates that the effect of 2b-AGO interaction on counter-RDRs antiviral defense required the presence of 2b dsRNA-binding activity. Taken together, our findings demonstrate that the dsRNA-binding activity of the 2b was essential for virulence, whereas the 2b-AGO interaction was necessary for interference with RDR1/6-dependent antiviral silencing in *Arabidopsis*.

Keywords: CMV, viral suppressor, 2b, ARGONAUTE, RDR, RNA silencing

INTRODUCTION

RNA silencing (RNA interference, RNAi) is an evolutionarily conserved regulatory mechanism of gene expression in eukaryotes mediated by 20–25-nucleotides (nt) small interference RNAs (siRNAs; Meister and Tuschl, 2004; Baulcombe, 2005). These siRNAs are processed from double-stranded (ds) or hairpin (hp) RNA by Dicer or Dicer-like (DCL) protein. To induce silencing, one strand of a siRNA is loaded into an Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC) and guides the RISC to bind to complementary single-stranded RNA and cleave the RNA. siRNAs-guided AGO-cleaved target RNA may be recognized by RNA-dependent RNA polymerase (RDR), which amplifies the dsRNA substrate for DCLs to produce secondary siRNAs and reinforce the RNA silencing process (Peragine et al., 2004; Axtell et al., 2006; Baulcombe, 2007).

In plants, viral infection also triggers the siRNA-mediated RNA silencing as a natural antiviral defense mechanism. RDR-dependent amplification is a crucial step toward achieving an efficient antiviral defense response in plants. Two of the six *Arabidopsis thaliana* RDRs, RDR1, and RDR6, have been implicated in defense against many viruses, including *Cucumber mosaic virus* (CMV; Dalmay et al., 2000; Qu et al., 2005; Schwach et al., 2005; Vaistij and Jones, 2009; Garcia-Ruiz et al., 2010; Qu, 2010; Ying et al., 2010; Li et al., 2014).

CMV is a tripartite positive-strand RNA virus, which contains three genomic RNAs and two subgenomic RNAs that encode five proteins (Palukaitis and Garcia-Arenal, 2003): two RNA-dependent RNA polymerases, 1a and 2a proteins, and movement protein (MP) encoded by genomic RNA1, RNA2, and RNA3. The 2b protein and the coat protein (CP) are expressed from subgenomic RNA4A and RNA4, which are transcribed from genomic RNA2 and RNA3, respectively (Schwinghamer and Symons, 1975; Ding et al., 1994). The 2b protein expressed from subgenomic RNA4A plays an important role in diverse processes, including symptom induction as a viral virulence determinant, host-specific virus accumulation, the inhibition of RNA silencing and the systemic spread of silencing (Ding et al., 1995; Lucy et al., 2000; Guo and Ding, 2002; Shi et al., 2002). As a viral suppressor of RNA silencing (VSR), the 2b protein has been identified to directly interact with both the long/short dsRNA and AGO proteins (Zhang et al., 2006; Goto et al., 2007; González et al., 2010, 2012; Duan et al., 2012; Hamera et al., 2012), attributed to its complex biochemical and subcellular targeting activity (Duan et al., 2012). In our previous study of the 2b protein encoded by the severe SD isolate from CMV subgroup I, we uncoupled the domain requirements for dsRNA binding and nucleolar targeting from the physical interactions with AGO proteins. We found that dsRNA sequestration is the predominant mechanism by which 2b suppresses silencing and that the 2b-AGO interaction is not essential for its suppressor function. We also found that the direct in *vivo* interactions of the 2b protein with AGO proteins require the functional nucleolar localization signal (NoLS) and redistribute the 2b protein in the nucleus (Duan et al., 2012).

The roles of RNAi-mediated viral immunity against CMV were mostly illustrated using the mutant of CMV that does not express the 2b protein or mutate by amino acid substitution in the N-terminal dsRNA binding domain of the 2b (Diaz-Pendon et al., 2007; Wang et al., 2010; Xu et al., 2013; Dong et al., 2016). These mutants of CMV reduce virulence and virus accumulation in wild-type Arabidopsis plants, but are efficiently rescued in mutant plants defective in RNAi components, such as RDR1, RDR6, or DCL4, which shows that the 2b protein plays critical roles in anti-RNAi defense and that its N-terminal dsRNA binding domain is required for the induction of virulence and virus accumulation in the CMV-infected plants (Diaz-Pendon et al., 2007; Wang et al., 2010; Xu et al., 2013; Dong et al., 2016). We previously found that the 2b-AGO interaction was not essential for the 2b in suppression of silencing, however, we wondered what is the biological significance of the 2b-AGO interaction in the context of virus infection. To this end, we created mutants of CMV from the SD strain that expressed the 2b functional domains of dsRNA- or AGO- binding activity (Duan et al., 2012) and tested the effect of these CMV mutants on viral pathogenicity. We found that the dsRNA-binding activity of the 2b was essential for virulence, whereas the 2b-AGO interaction was necessary for interference with RDR1/6-dependent antiviral silencing in *Arabidopsis*. The possible benefit of the 2b-AGO interaction in CMV infectivity is discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

N. benthamiana plants were grown in a greenhouse at 25°C with 16-h light/8-h dark cycles. *rdr1/6* and wild-type *Arabidopsis* plants were grown in a greenhouse at 22°C with 16-h light/8-h dark cycles.

Plasmid Constructs

35S-R1, 35S-R2, 35S-R3, and 35S-RΔ2b(R2aΔ2b) were described in a previous study (Hou et al., 2011). For R2a2b$_{(1–76)}$, R2a2b$_{(8–111)}$, and R2a2b$_{(18–111)}$ point mutant constructs, mutagenesis was introduced using QuikChange® Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, 210518) according to the manufacturer's instructions. The templates were 35S-R2, 35S-R2, and R2a2b$_{(8–111)}$, and the primer pairs were R2-2b77TGAF/R, R2-2b8-111F/R, and R2-2b18-111F/R, respectively (Table S1).

For the constructs used in dsRNA binding activities, pGEX-4T-2-SD2b was described in a previous study (Duan et al., 2012). The constructs pGEX-4T-2-SD2b$_{(8–111)}$ and pGEX-4T-2-SD2b$_{(18–111)}$ were generated with the pGEX-4T-2-SD2b template using QuikChange® Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, 210518) according to the manufacturer's instructions. The primer pairs were GST2b8-111F/R and GST2b18-111F/R for the constructs pGEX-4T-2-SD2b$_{(8–111)}$ and pGEX-4T-2-SD2b$_{(18–111)}$, respectively (Table S1).

For the constructs used in suppression activity, pBI121-35S-SD2b was described in a previous study (Duan et al., 2012). To generate pBI121-35S-SD2b$_{(8–111)}$, pBI121-35S-SD2b$_{(18–111)}$, and pBI121-35S-SD2b$_{(1–76)}$, the 2b8-111F/2bR, 2b18-111F/2bR, and 2b1-76F/R primers (Table S1) were used to amplify the 2b$_{(8–111)}$/2b$_{(18–111)}$/2b$_{(1–76)}$ mutant fragments with the template

of pBI121-35S-SD2b; the resulting fragments were cut by XbaI-SacI and inserted into the XbaI-SacI digested pBI121-35S-SD2b vector to yield pBI121-35S-SD2b$_{(8-111)}$, pBI121-35S-SD2b$_{(18-111)}$, and pBI121-35S-SD2b$_{(1-76)}$.

For constructs used in the subcellular localization and SD2b-AGO colocalization, pBI121-35S-SD2b-EGFP and RFP-AGO4 were described in a previous study (Duan et al., 2012). The constructs pBI121-35S-SD2b$_{(8-111)}$ and pBI121-35S-SD2b$_{(18-111)}$-EGFP were generated with the pBI121-35S-SD2b-EGFP template using QuikChange® Lightning Site-Directed Mutagenesis Kits (Agilent Technologies, 210518) according to the manufacturer's instructions. The primer pairs were 2B8-111-EGFPF/R and 2B18-111-EGFPF/R for the pBI121-35S-SD2b-EGFP and RFP-AGO4 constructs, respectively (Table S1).

Agrobacterium tumefaciens-Mediated Transient Expression and Virus Inoculation

35S-R1, 35S-R3 and different R2 mutant constructs were co-infiltrated into the leaves of 5-week-old *N. benthamiana* plants, as described in a previous study (Hou et al., 2011). Systemically infected leaves were harvested from pools of 15 to 20 plants for sap extraction for viral infection. Similar levels of viral RNAs in each sap sample estimated by RNA gel blotting were inoculated to *N. benthamiana* and *A. thaliana* seedlings.

RNA Extraction and RNA Gel Blot Analysis

Plant total RNA used for RNA gel blotting was extracted by the hot-phenol method as previously described (Fernández et al., 1997). For the detection of viral RNAs, three 1-kb fragments at the 3′-terminus of each cDNA clone (35S-R1, 35S-R2, and 35S-R3) were amplified, which were then labeled with [a-^{32}P] dCTP using the Rediprime II system (GE Healthcare, RPN1633) and were mixed as probes. For the detection of siRNAs, 30 mg of total RNA was separated on 17% polyacrylamide-8 M urea gels. The probes were labeled with [r-^{32}P]ATP using T4 PNK (NEB, M0201V). VsiRNAs were detected using mixtures of labeled DNA oligonucleotides specific to RNA3. Signal intensity was quantified using ImageQuant TL software (GE Healthcare).

Expression and Purification of Recombinant Proteins

For the expression of fusion proteins in *Escherichia coli*, recombinant plasmids were transformed into BL21 cells and induced at 0.3 mM isopropyl b-D-1thiogalactopyranoside (Sigma-Aldrich) in Luria-Bertani medium at 28°C for 3 to 6 h. GST-tagged fusion proteins were purified using Glutathione Sepharose 4B (GE Healthcare, 17-0756-01) according to the manufacturer's instructions.

EMSAs

21/24-bp ds-siRNA and 55-bp ds-RNA, which were described in a previous study (Duan et al., 2012), were radiolabeled in 50-pmol quantities with 0.3 mM [r-^{32}P]ATP and 20 units of T4 PNK (NEB, M0201V). Binding reactions were performed with 1 ng of radiolabeled ds-siRNA and 1 nmol of protein in binding buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 300 mM NaCl, 0.1% Nonidet P-40, and protease inhibitor cocktail. After 40 min at room temperature, 1 mL of 50% glycerol and dye was added, and protein RNA complexes were resolved on 6% native polyacrylamide gel. The gels were then exposed to a storage phosphor screen (GE Healthcare).

Subcellular Localization Assays

The subcellular localization of 2b mutants and their colocalization with AGO proteins were determined by infiltrating binary plasmids of pBI121-35S-SD2b-EGFP mutants and RFP-AGO4 into 5-week-old *N. benthamiana* leaves, which were maintained for 3 days at 25°C (16-h light/8-h dark). The nuclei were stained with 100 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) for 10 min before confocal microscopy. Confocal fluorescence of GFP, RFP, and DAPI were captured with a Leica SP8 confocal microscope.

RESULTS

Construction of the Infectious Clones of SD-CMV with Different Mutations in the 2b Coding Sequence

We previously constructed infectious clones of SD-CMV with the viral genomic RNA1, RNA2, and RNA3 under the 35S promoter (**Figure 1A**) and a chimeric RNA2 (Δ2b) infectious clone, in which 2b protein expression was abolished by nucleotide substitution in the start codon ATG, as well as four other ATG codons in the 2b coding sequence, but 2a protein expression was unaffected, designated as CMVΔ2b (**Figure 1A**; Hou et al., 2011). To investigate the biological functions of the different biochemical properties of the 2b protein in the context of CMV infection in plants, in this study, we further created mutations of 2b of the genomic RNA2 according to the two main biochemical properties of double-stranded RNA (dsRNA) and AGO binding activities (Duan et al., 2012). As shown in **Figure 1A**, in addition to the above Δ2b, three 2b mutants were created by deleting the C-terminal 35 amino acids (aa) and creating a stop codon in the 2b coding sequence to yield 2b$_{(1-76)}$ without affecting the overlapping portion of the 2a polymerase. Another two mutants were created by deleting the N-terminal 7 or 17 aa to yield 2b$_{(8-111)}$ and 2b$_{(18-111)}$ by nucleotide substitution in the 1st or both the 1st and 8th "ATG" codons of the 2b coding sequence without affecting the 2a protein. To obtain viral sources of chimeric CMV with different 2b mutant, each of these constructs was transformed into *Agrobacterium* for the infiltration of *N. benthamiana* in the presence of 35S-RNA1 and 35S-RNA3 to examine the infectious properties of the chimeric CMV with 35S-2bx (x represents different 2b mutations shown in **Figure 1A**), and the related viruses were referred to as wild-type CMV, CMVΔ2b, CMV2b$_{(1-76)}$, CMV2b$_{(8-111)}$, and CMV2b$_{(18-111)}$ (**Figure 1B** and Supplementary Figure S1).

Typical symptoms were observed in CMV-, CMV2b$_{(1-76)}$-, and CMV2b$_{(8-111)}$-inoculated plants, whereas CMVΔ2b- and CMV2b$_{(18-111)}$-inoculated plants did not develop visible symptoms (Supplementary Figure S1). However, as shown in **Figure 1B**, RNA gel blot analysis confirmed that the three CMV

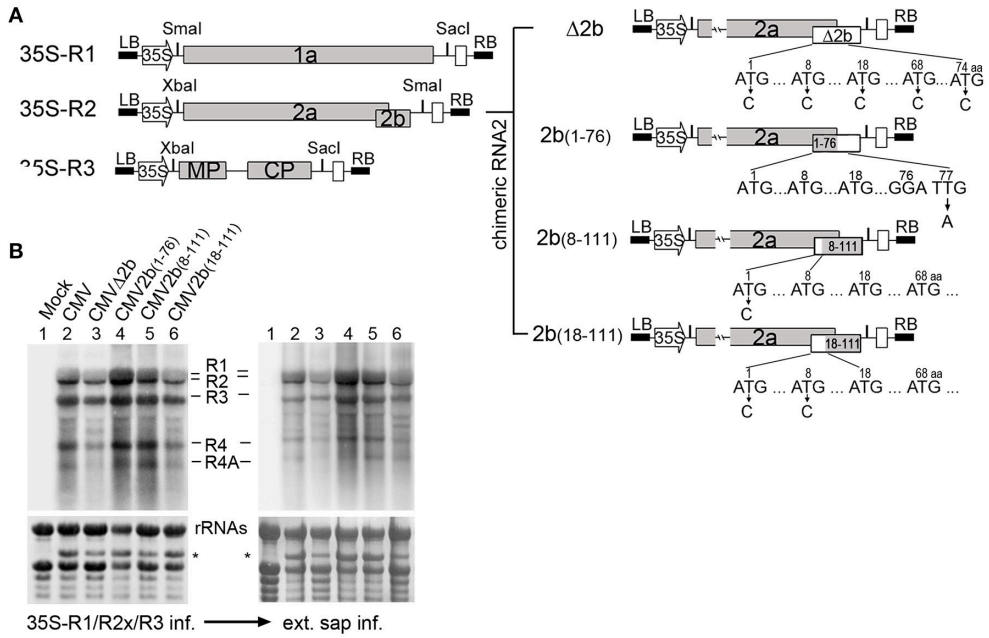

FIGURE 1 | Construction and biological activities of 2b mutants in the context of CMV infection. (A) Diagram of SD-CMV infectious clone construction. 35S-R1, 35S-R2, and 35S-R3 were the three full-length clones of SD-CMV genomic RNA1, RNA2, and RNA3 as well as four chimeric RNA2 mutants. R2aΔ2b, 2b protein expression was abolished; R2a2b$_{(1–76)}$, with deletion of the C-terminal 35 amino acids; R2a2b$_{(8–111)}$, with deletion of the N-terminal seven amino acids; and R2a2b$_{(18–111)}$, with deletion of the N-terminal 17 amino acids. Substituting "C" for each "T" in the start codon ATG, as well as other ATG codons, or "A" for "T" in creating stop codon present in the 2b coding sequence are indicated with the numbers of the amino acid positions. **(B)** RNA gel blot detection of CMV accumulation in *Agrobacterium*-inoculated leaves (left panel) and the plants inoculated with sap extracted from each CMV2b$_{(x)}$-infected *Nb* leaf (right panel). SD-CMV genomic RNA 3′ UTR was used as a probe. Methylene blue-stained ribosomal rRNA was used as loading control. *A stained viral RNA used as an indicator of SD-CMV infection.

genomic RNA1, RNA2, and RNA3 transcripts, as well as both subgenomic RNA4 and 4A transcripts, were accumulated in non-inoculated systemic leaves for all chimeric viruses detected using the 3′-untranslated region (3′-UTR) as a probe (**Figure 1B**). This indicated that the infiltration of the mixture could sustain the replication of both subgenomic RNA4 and 4A. RT-PCR and sequencing analysis of viral RNAs isolated from infected plants confirmed that all chimeric 2bx mutations were genetically stable in *N. benthamiana* (Nb) plants. Similar symptom development and the accumulation of viral RNAs were obtained in the Nb plants inoculated with sap extracted from these each chimeric CMV-infected Nb leaves (**Figure 1B**).

Detection of the dsRNA and AGO Binding Activities of the Deletion Mutants of the 2b Protein

We previously uncoupled the 2b domain requirements for dsRNA binding and nucleolar targeting from the physical interaction with AGO proteins (Duan et al., 2012). The 61 aa N-terminal end [2b$_{(1–61)}$], which contains the complete α1-linker-α2 structure involved in dsRNA binding, retained the wild-type 2b ability to bind 21- and 24-nt siRNA duplexes, whereas 2b$_{(13–111)}$ exhibited weak affinity for 21-nt siRNA but showed no detectable affinity for 24-nt siRNA (Duan et al., 2012). To characterize the dsRNA binding activities of 2b$_{(8–111)}$ and 2b$_{(18–111)}$, which corresponded to the deletion mutants constructed in the context of the CMV RNA2 genome (**Figure 1A**), we performed electrophoretic mobility shift assays (EMSAs) with the full-length of 2b, 2b$_{(8–111)}$, and 2b$_{(18–111)}$ expressed and purified as GST fusion proteins (**Figures 2A,B**). Similar to previous results (Duan et al., 2012), 2b exhibited high affinity for either small 21- and 24-nt siRNA duplexes or long dsRNA (**Figure 2B**). 2b$_{(8–111)}$ was almost as active as 2b in binding to the 21- and 24-nt siRNA duplex but exhibited reduced affinity for long dsRNA (**Figure 2B**). Deletion of 17 aa from the N terminus abolished both of the siRNA dsRNA binding activities (**Figure 2B**), which indicated that further deletion of 4 aa (from 13 to 17 aa) in the N-terminal α1 helix completely abolished the weak affinity for 21-nt siRNA of 2b$_{(13–111)}$. Consistently, 2b$_{(13–111)}$ retained the partial silencing suppression activity detected using an *Agrobacterium* coinfiltration assay (Duan et al., 2012); however, 2b$_{(18–111)}$ failed to suppress GFP silencing as indicated by the lack of green fluorescence in the co-infiltrated leaves, in which 2b$_{(8–111)}$ was almost as active as 2b in the suppression of GFP silencing (**Figure 2C**).

We next investigated the subcellular localization of 2b$_{(8–111)}$ and 2b$_{(18–111)}$ and their possible interaction with the AGO protein in *N. benthamiana* leaf epidermal cells. 2b$_{(8–111)}$ and 2b$_{(18–111)}$ fused at their C-termini with enhanced green fluorescent protein (EGFP) were transiently expressed in *N. benthamiana* via *Agrobacterium*-mediated infiltration. The 2b-EGFP (Duan et al., 2012) was used as a control. Similar to 2b-EGFP, both 2b$_{(8–111)}$-EGFP, and 2b$_{(18–111)}$-EGFP were mainly detected in the nucleus with dense fluorescence in the

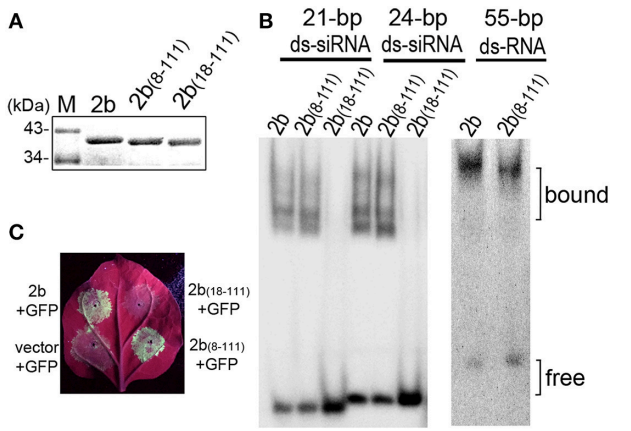

FIGURE 2 | Detection of the suppression of the transgene-induced silencing and dsRNA-binding activity of 2b and its mutations. (A) Purified GST-tagged 2b, $2b_{(8–111)}$, and $2b_{(18–111)}$. **(B)** Gel mobility shift assays for the detection of the dsRNA binding affinity. GST-tagged 2b, $2b_{(8–111)}$, and $2b_{(18–111)}$ were incubated with siRNA duplexes and long dsRNA. $2b_{(8–111)}$ showed a high affinity for 21/24-nt siRNA duplexes, similar to 2b, but showed a reduced affinity for long dsRNA; $2b_{(18–111)}$ showed no binding to 21/24-nt siRNA duplexes. **(C)** GFP fluorescence in the leaves of *Nb* plants coinfiltrated with GFP and 2b or mutants. Coinfiltration of GFP with vector was used as control. Photographs were taken under UV light at 4 dpi.

nucleoli (**Figure 3A**), consistent with both deletion mutants containing NoLS from the 13 to 37 region, including both NLSs (**Figure 3C**). This allowed for the accumulation of the fusion protein in the nucleoli and nucleus (Duan et al., 2012).

Coexpression of 2b-EGFP and RFP-AGO4 resulted in their colocalization in the nucleus with dense fluorescence in nucleus-associated bodies (**Figure 3B**), which was consistent with our previous finding that the redistribution of 2b-EGFP in the nucleus when coexpressed with AGO proteins (Duan et al., 2012). Similar nuclear colocalization and redistribution was observed for $2b_{(8–111)}$-EGFP, but not $2b_{(18–111)}$, when they were coexpressed with RFP-AGO4 (**Figure 3B**). Intriguingly, the densities of fluorescence were greatly reduced for both $2b_{(18–111)}$-EGFP and RFP-AGO4 when they were coexpressed compared to that of each when expressed alone (cf. **Figures 3A,B**). One of the possible explanations might be that the interaction of $2b_{(18–111)}$-EGFP and RFP-AGO4 caused the instability of both proteins due to $2b_{(18–111)}$'s lack of binding to ds-siRNA and suppression of silencing (**Figures 2B,C**). Nevertheless, these data demonstrate that the deletion of 17 aa from the N terminus of the 2b protein completely abolished both the long dsRNA and ds-siRNA binding activity, whereas the deletion of 7 aa from the N terminus retained the wild-type 2b abilities in binding both ds-siRNA and AGO protein but reduced the affinity for binding long dsRNA compared to wild-type 2b.

Correlation of Virulence and Viral RNA Levels with Different 2b Mutations in Wild-Type *Arabidopsis* Plants

To investigate the biological functions of the different biochemical properties of the 2b protein in the context of CMV infection in plants, wild-type *Arabidopsis* plants were inoculated with sap extracted from the above *N. benthamiana* leaves infected with CMV, CMVΔ2b, $CMV2b_{(1–76)}$, $CMV2b_{(8–111)}$, or $CMV2b_{(18–111)}$. The development of disease symptoms in *Arabidopsis* plants was monitored. At 9 days post-inoculation (dpi), plants infected with wild-type CMV displayed severe developmental defects, including reduced leaf size and a shortened petiole, and all new leaves were aggregatecd in the center of the plants as observed at 21 dpi (**Figure 4A**). Plants inoculated with CMVΔ2b displayed no symptoms and normal growth compared to mock infection plants. $CMV2b_{(1–76)}$-infected plants displayed severe developmental defects similar to CMV-infected plants, and the development of both CMV- and $CMV2b_{(1–76)}$-infected plants was arrested with short and defective inflorescence at 21 dpi (**Figure 4A**). $CMV2b_{(8–111)}$-infected plants also showed typical defects in development and inflorescence, albeit less stunting. However, $CMV2b_{(18–111)}$-infected plants exhibited very mild symptoms, and plant growth was not arrested (**Figure 4A**). RT-PCR and sequencing analysis of viral RNAs isolated from infected plants confirmed that all chimeric 2bx mutations are genetically stable in *Arabidopsis*.

RNA gel blot analysis confirmed that three CMV genomic (RNA1, RNA2, and RNA3) and both subgenomic (RNA4 and 4A) transcripts were accumulated in the systeimic leaves of these infected plants (**Figure 4B**). Consistent with the degree of disease, minimal and small quantities of viral RNAs in CMVΔ2b- and $CMV2b_{(18–111)}$-infected plants were detected (**Figure 4B**). Intriguingly, similar severities of disease symptoms were observed for CMV-, $CMV2b_{(1–76)}$-, and $CMV2b_{(8–111)}$-infected plants; however, the level of viral RNAs in $CMV2b_{(1–76)}$-infected plants was obviously lower than that in CMV- and $CMV2b_{(8–111)}$-infected plants (**Figure 4B**). $2b_{(1–76)}$ lacked the AGO binding domain but retained the dsRNA-binding domain and the silencing suppression activity (**Figure 4C**). Therefore, we examined the production of viral siRNAs (vsiRNAs) in these infected plants. High levels of vsiRNAs were detected in $CMV2b_{(1–76)}$-infected plants compared to wild-type and those infected with other chimeric viruses (**Figure 4B**), which was consistent with the low level of viral RNAs in these plants. No major differences in the accumulation of miR173 were detected following infection with either wild-type or each mutant CMV, which supported an earlier observation that CMV infection does not alter miRNA accumulation (Diaz-Pendon et al., 2007). These results demonstrate that N terminal dsRNA binding activity is responsible for the induction of the virulence of CMV, which does not necessarily correlate with the accumulation of viral RNAs, and 2b-AGO binding is likely required for CMV to suppress the silencing of viral RNAs in *Arabidopsis* plants.

Correlation of Virulence and Viral RNA Levels with Different 2b Mutations in RDR1/RDR6-Deficient Mutants

Previous studies showed that the 2b gene of the CMV Fny strain is required for interference with RDR1- and RDR6-dependent antiviral silencing (Diaz-Pendon et al., 2007; Dong

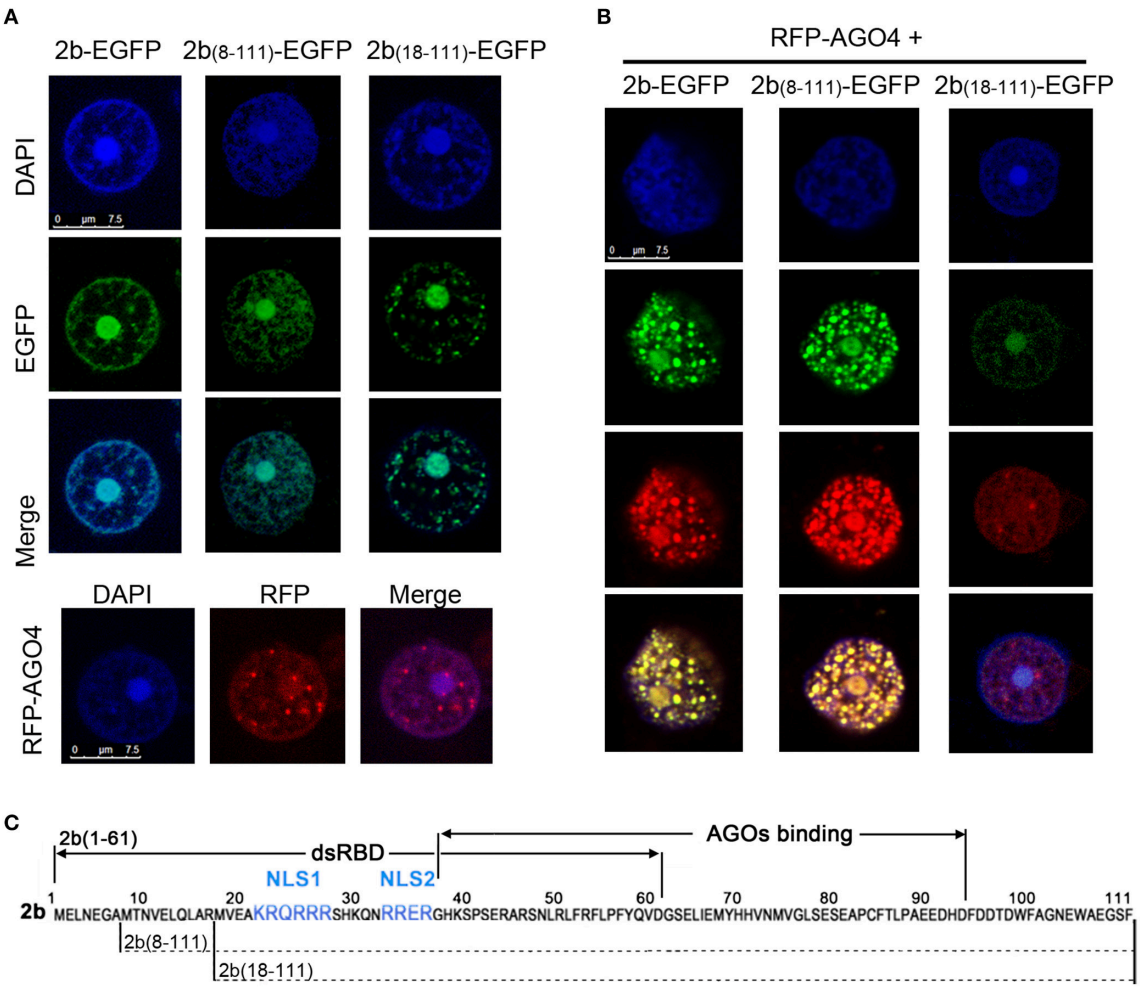

FIGURE 3 | Colocalization pattern of 2b or its derivative mutants with AGO4 in Nb leaf epidermal cells. (A) Subcellular localization of 2b-EGFP, $2b_{(8–111)}$-EGFP, $2b_{(18–111)}$-EGFP, and RFP-AGO4 in *Nb* leaf epidermal cells. DAPI staining was performed to represent the nuclei. Bars = 7.5 μm. **(B)** Subcellular location of the coexpression of 2b-EGFP, $2b_{(8–111)}$-EGFP, or $2b_{(18–111)}$-EGFP with RFP-AGO4. **(C)** Diagram of the 2b function domain as previously reported (Duan et al., 2012). NLS, nuclear localization signal.

et al., 2016). To examine whether the accumulation of CMV-derived vsiRNAs in $CMV2b_{(1–76)}$-infected plants was dependent on RDR proteins, we inoculated *Arabidopsis* with double mutants of RDRs (RDR1 and RDR6) with wild-type CMV, CMVΔ2b, $CMV2b_{(1–76)}$, $CMV2b_{(8–111)}$, or $CMV2b_{(18–111)}$. *Trans-acting* siRNA tasiR255 was absent in the tested *rdr1/6* plants, verifying that the *RDR* mutant alleles (**Figure 5B**). CMVΔ2b remained defective in inducing virulence in *rdr1/6* mutant; CMVΔ2b-infected plants displayed no symptoms and normal growth compared to mock infection plants (**Figure 5A**). Similar to infected wild-type *Arabidopsis* plants, *rdr1/6* plants infected with CMV-, $CMV2b_{(1–76)}$-, and $CMV2b_{(8–111)}$ displayed severe developmental defects, and whole plant development was arrested, although less stunting was observed in $CMV2b_{(8–111)}$-infected *rdr1/6* plants (**Figure 5A**). $CMV2b_{(18–111)}$-infected *rdr1/6* mutant plants exhibited mild but clear disease symptoms (**Figure 5A**). The inflorescences of $CMV2b_{(18–111)}$-infected *rdr1/6* plants were shorter and defective compared to those of $CMV2b_{(18–111)}$-infected wild-type plants (**Figure 4A**), which demonstrated that $2b_{(18–111)}$ retained partial activity in the suppression of RDR6- and/or RDR1-mediated antiviral defense.

We further conducted RNA gel blot hybridizations to compare the accumulation of viral RNAs in these infected *rdr1/6* mutant plants. Consistent with the asymptomatic phenotype, minimal levels of viral RNAs in CMVΔ2b-infected plants were detected (**Figure 5B**). Interestingly, a similar severity of disease symptoms was observed for CMV-, $CMV2b_{(1–76)}$-, and $CMV2b_{(8–111)}$-infected *rdr1/6* plants; however, the accumulation level of viral RNAs in $CMV2b_{(1–76)}$-infected plants was clearly higher than that in CMV- and $CMV2b_{(8–111)}$-infected mutant plants (**Figure 5B**), opposite of that detected in infected wild-type *Arabidopsis* plants, in which a lower level of viral RNAs accumulated in $CMV2b_{(1–76)}$-infected plants than in CMV- and $CMV2b_{(8–111)}$-infected plants (**Figure 4B**).

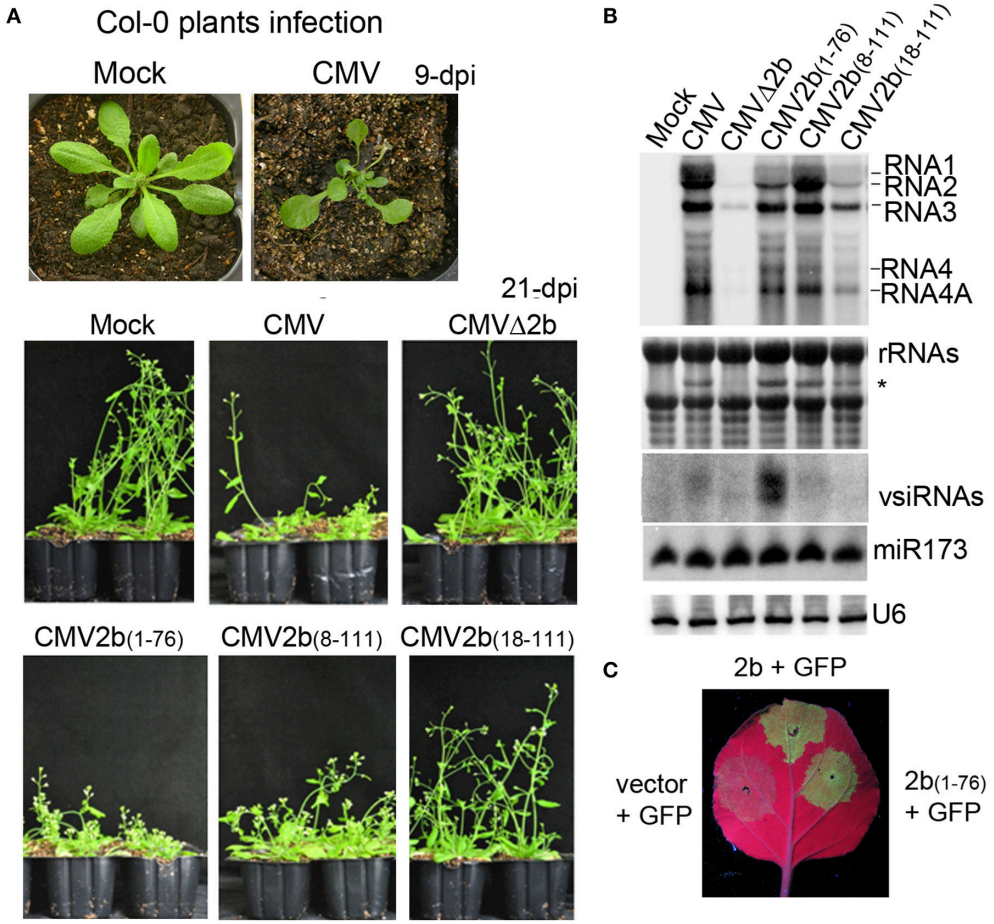

FIGURE 4 | Effects of 2b and its mutants on the virulence and accumulation of viral RNAs in infection with wild-type *Arabidopsis* plants. (A) Disease symptoms in wild-type *Arabidopsis* plants inoculated with CMV and CMV2b$_{(x)}$ at 9 dpi and 21 dpi. **(B)** RNA gel blot detection of viral genomic RNAs and vsiRNAs. Methylene blue-stained ribosome rRNA and U6 were used as the loading control. **(C)** GFP fluorescence in the leaves of *Nb* plants coinfiltrated with GFP and 2b or mutants. Coinfiltration of GFP with vector was used as control.

We then examined the production of vsiRNAs. Remarkably, vsiRNAs in CMV2b$_{(1-76)}$-infected *rdr1/6* plants (**Figure 5B**) were greatly reduced compared to CMV2b$_{(1-76)}$-infected wild-type Col-0 plants (**Figure 4B**). These results indicated that RDR6 and/or RDR1 play role(s) in partially silencing CMV RNAs disrupted by 2b-AGO binding activity. Taking into account of the low level of viral RNAs and the high level of vsiRNAs in CMV2b$_{(1-76)}$ infected wild-type plants, our data demonstrate that the 2b-dsRNA binding activity is insufficient to suppress the host degradation of viral RNA that requires the functions of RDR6 and/or RDR1.

DISCUSSION

In our previous study, we characterized the SD-CMV 2b protein in terms of subcellular localization, RNA binding, AGO interaction, and the suppression of RNA silencing (Duan et al., 2012). We found that dsRNA sequestration is the predominant mechanism by which 2b suppresses silencing and that the 2b-AGO interaction is not essential for its suppressor function. In this study, we further explored the biological significance of different functional activities of the 2b protein in the context of virus infection. By creating mutants of SD-CMV that expressed different 2b functional domains, either retaining the dsRNA-binding activity or the AGO-binding activity, we found that 2b's dsRNA-binding activity was essential for virulence and viral RNA propagation, whereas the 2b-AGO interaction was necessary for interference with RDR-dependent antiviral silencing in *Arabidopsis*.

The 2b$_{(8-111)}$ mutant protein with 7 aa deleted from the N terminus was almost as active as wild-type 2b in binding to 21- and 24-nt ds-siRNA duplex and in the suppression of transgene *GFP* silencing (**Figure 2**). Therefore, CMV2b$_{(8-111)}$ caused wild-type CMV-like to impact both the viral RNA level and the virulence in either wild-type *Arabidopsis* or *rdr1/6* mutant plants. However, we also noted that the stunted phenotype was less severe in CMV2b$_{(8-111)}$-infected plants. This might be related

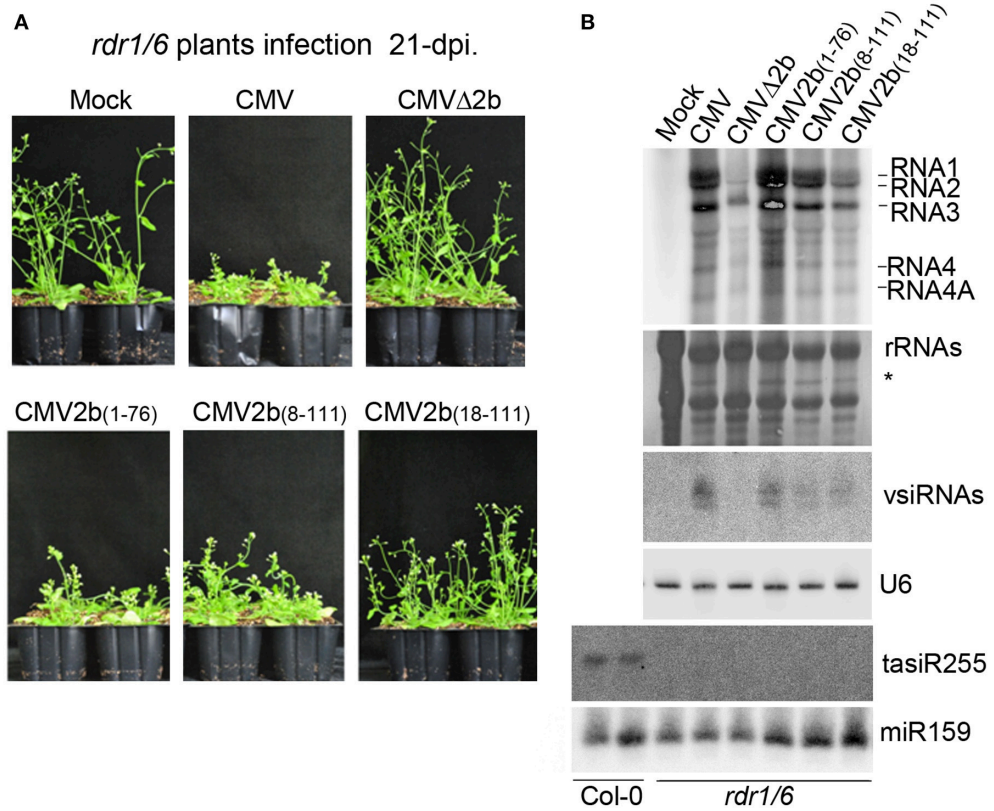

FIGURE 5 | Effects of 2b and its mutants on the virulence and accumulation of viral RNAs in infection of *rdr1/6* plants. (A) Disease symptoms in *rdr1/6* plants inoculated with CMV and CMV2b$_{(x)}$ at 21 dpi. **(B)** RNA gel blot detection of viral genomic RNAs and vsiRNAs. tasiR255 was used to verify the *RDR* mutant alleles. Methylene blue-stained ribosome rRNA and U6 as well as miR159 were used as the loading controls.

to its reduced activity in binding long dsRNA compared to that of wild-type 2b (**Figure 2B**). This observation suggests that the first 7 aa of the 2b protein might affect the N-terminal α1 helix, which is followed by the short linker and the α2 helix structure involved in long dsRNA binding (Chen et al., 2008). Indeed, we previously found that 2b$_{(13-111)}$ with the deletion of the N-terminal 12 aa was defective in binding long dsRNA and 24-nt ds-siRNA but retained very weak 21-nt ds-siRNA binding ability, which revealed that the N-terminal α1 helix is essential for binding to long dsRNA (Duan et al., 2012). Thus, even 2b$_{(8-111)}$ was as active as wild-type 2b in binding both 21- and 24-nt ds-siRNA, it was defective in binding long dsRNA. Thus, 2b$_{(8-111)}$ may be defective in binding, for example, endogenous long non-coding RNAs that have emerged as new regulatory elements with essential roles in plant development and stress signaling pathways (Wu et al., 2013; Zhu et al., 2014; Wang et al., 2015), which may explain the less stunted phenotype in CMV2b$_{(8-111)}$-infected plants.

Deletion of 17 aa from the 2b N terminus abolished both the siRNA-binding and silencing suppressor activities (**Figure 2**). CMV2b$_{(18-111)}$ infection never achieved the wild-type CMV level of viral RNAs in either wild-type *Arabidopsis* or *rdr1/6* mutant plants (**Figures 4**, **5**). However, in comparison with CMVΔ2b infection, the level of viral RNAs was clearly higher in CMV2b$_{(18-111)}$-infected wild-type *Arabidopsis*, but not obvious in *rdr1/6* plants, which implied that the 2b$_{(18-111)}$ had a role in countering the RDR-dependent defense against CMV accumulation. We previously found that *in vivo*, the 2b-AGO interaction depends also on the nucleolar targeting of the 2b protein (Duan et al., 2012). 2b$_{(18-111)}$ retained the AGO-binding domain, and its nucleolar targeting was evident (**Figure 3A**). However, taking into account that the instability of both the 2b$_{(18-111)}$–EGFP and RFP-AGO4 fusion proteins when they were co-expressed (**Figure 3B**), we speculated that it would decrease the effect of 2b$_{(18-111)}$ on countering RDR-dependent resistance in the absence of dsRNA binding activity. A previous study that examined the 2b-AGO4 interaction using the BiFC assay found that the fluorescent signal of the 2b-AGO4 interaction was reduced in *rdr2* mutant plants, which compromised the accumulation of 24-nt siRNAs (Hamera et al., 2012). Taken together, these findings suggest that a lack of siRNAs in the formation of the 2b$_{(18-111)}$-AGO4 complex might result in the degradation of both proteins.

The effect of the 2b-AGO interaction in counteracting RDR-dependent antiviral silencing was substantiated by comparing the levels of viral RNAs and siRNAs in CMV2b$_{(1-76)}$-infected wild-type *Arabidopsis* and *rdr1/6* mutant plants (**Figures 4**, **5**). Retaining the dsRNA-binding activity but lacking the

AGO-binding domain, $2b_{(1–76)}$ failed to suppress the RDR-dependent degradation of viral RNAs, resulting in the production of a large quantity of vsiRNAs and a decrease in the viral RNA level (**Figure 4B**). This finding clearly demonstrated that the 2b-AGO binding activity is required for CMV to counter the host's RDR-dependent degradation of viral RNAs. The physical interaction of 2b with AGOs requires the region encompassing residues 62 to 94 (Duan et al., 2012). Although this region is highly variable in sequence, it is present among all of the cucumoviral 2b proteins (Ding et al., 1994), which reveal its important in *vivo* function for CMV infection.

We found that the 2b dsRNA binding activity is responsible for the induction of virulence, which did not necessarily correlate with the level of CMV RNAs (**Figure 4**). This is consistent with a previous finding that Fny-CMV2bNLS, an Fny strain expressing the 2b mutant with an additional NLS and enhanced nuclear targeting, increased viral virulence but decreased virus accumulation and increased vsiRNAs (Du et al., 2014a). The authors therefore proposed that partitioning the 2b protein between the cytoplasmic and nuclear/nucleolar compartments allows CMV to regulate the balance between virus accumulation and damage to the host (Du et al., 2014a). We previously found that the 2b-AGO interaction redistributed both the 2b and AGO proteins in the nucleus (Duan et al., 2012). The nucleus/nucleolus-localized 2bNLS failed to increase virus accumulation in Fny-CMV2bNLS infection, which might be attributed to the disrupted redistribution of 2b-AGO. This likely resembled the $2b_{(1–76)}$ failure in interactions with AGO proteins and the inhibition of viral RNA degradation in Fny-CMV2bNLS infection. It was also reported that Fny-CMV2blm expressing the 2b mutant defective in ds-siRNA binding activity drastically attenuated the virulence in wild-type *Arabidopsis* plants (Dong et al., 2016). Virulence could be efficiently rescued in CMV2blm-infected plants harboring RDR6-deficient mutations, including *rdr6*, *rdr1/6*, *rdr2/6,* and *rdr1/2/6*, but not *rdr1* and *rdr2* mutant plants. Viral RNAs also accumulated to higher levels in *rdr6* and *rdr1/6* than in wild-type *Arabidopsis* plants (Dong et al., 2016). Unlike the wild-type N terminus of 2b, which was required to form dimers, tetramers and oligomers, 2blm could only form dimers (Dong et al., 2016). Therefore, the rescued virulence and viral RNA level in CMV2blm-infected RDR6-deficient mutant plants was suggested due to the low oligomerization of the 2blm that directly weakened ds-siRNA binding activity (Dong et al., 2016). The 2blm contained the full-length sequence with double alanine substitution (L15A and M18A) mutations at the N-terminus but retained the long dsRNA binding domain (Dong et al., 2016). Thus, unlike $2b_{(18–111)}$, which caused the instability of the interacting $2b_{(18–111)}$-AGO proteins (**Figure 3B**), 2blm might bind to AGO but retain the stability of two proteins due to its long dsRNA binding activity. This may rescue the virulence in *rdr6* mutant plants, in which the RDR1-mediated degradation of viral RNAs might be suppressed by the 2blm-AGO interaction. In our study, the N-terminal 17 aa deletion mutant $2b_{(18–111)}$ contained the AGO-binding domain but was defective in binding long and short dsRNA. Neither severe disease symptoms nor high viral RNA level were obtained with $CMV2b_{(18–111)}$ infection in *rdr1/6* plants (**Figure 5**), which demonstrated the failure of the *in vivo* $2b_{(18–111)}$-AGO-dependent suppression of the host degradation of CMV RNAs in the absence of 2b dsRNA binding activity.

In summary, although the silencing suppression activity of 2b relies on its dsRNA binding capacity and is independent of its interaction with AGO (Duan et al., 2012), we found that in the context of virus infection, the 2b-AGO interaction was indispensable for interference with RDR-dependent antiviral silencing in *Arabidopsis,* and the effect was remarkable in the presence of the 2b dsRNA-binding activity. The 2b dsRNA-binding activity was essential for virulence, probably being related to its effect on the alteration of miR159-regulated transcript levels (Du et al., 2014b). However, in agreement with the dual edge of VSR in the virus-host interactions (Zhao et al., 2016), the 2b protein exerted a precise effect on the regulation of balance between virus accumulation and virulence-induced damage to the host. Binding to AGO proteins might weaken the nucleus/nucleolus localization of 2b and inhibit the RDR-dependent degradation of viral RNAs in the cytoplasm, presumably to maximize the benefit for the virus. The multiple biochemical properties of the 2b protein exerted essential roles in diverse silencing suppressor activities, which cooperatively or independently contributed to the accumulation and virulence of viral RNAs.

AUTHOR CONTRIBUTIONS

HG, CD, and YF conceived the study and designed the research. YF and JZ performed molecular work. YF, JZ, and SL performed the sampling and analyzed the data. SW assisted construction and infection. HG, YF, and JZ wrote the manuscript and discussed the results and all the authors commented on the manuscript.

FUNDING

This study was supported by the Ministry of Science and Technology (2014CB138402) and the Natural Science Foundation of China (No.91519327).

ACKNOWLEDGMENTS

We thank Dr. Shou-Wei Ding for *rdr1/6* Arabidopsis mutant seeds and Dr. Xian-Bing Wang for anti-2b antiserum.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01329

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 09 November 2016
doi: 10.3389/fmicb.2016.01796

The Roles of Alpha-Momorcharin and Jasmonic Acid in Modulating the Response of *Momordica charantia* to *Cucumber Mosaic Virus*

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Alpha-momorcharin (α-MMC) is a type-I ribosome inactivating protein with a molecular weight of 29 kDa that is found in *Momordica charantia*, and has been shown to be effective against a broad range of human viruses as well as having anti-tumor activities. However, the role of endogenous α-MMC under viral infection and the mechanism of the anti-viral activities of α-MMC in plants are still unknown. To study the effect of α-MMC on plant viral defense and how α-MMC increases plant resistance to virus, the *M. charantia–cucumber mosaic virus* (CMV) interaction system was investigated. The results showed that the α-MMC level was positively correlated with the resistance of *M. charantia* to CMV. α-MMC treatment could alleviate photosystem damage and enhance the ratio of glutathione/glutathione disulfide in *M. charantia* under CMV infection. The relationship of α-MMC and defense related phytohormones, and their roles in plant defense were further investigated. α-MMC treatment led to a significant increase of jasmonic acid (JA) and vice versa, while there was no obvious relevance between salicylic acid and α-MMC. In addition, reactive oxygen species (ROS) were induced in α-MMC-pretreated plants, in a similar way to the ROS burst in JA-pretreated plants. The production of ROS in both ibuprofen (JA inhibitor) and (α-MMC+ibuprofen)-pretreated plants was reduced markedly, leading to a greater susceptibility of *M. charantia* to CMV. Our results indicate that the anti-viral activities of α-MMC in *M. charantia* may be accomplished through the JA related signaling pathway.

Keywords: alpha-momorcharin, jasmonic acid, salicylic acid, reactive oxygen species, *Cucumber mosaic virus*, *Momordica charantia*

OPEN ACCESS

Edited by:
Yule Liu,
Tsinghua University, China

Reviewed by:
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *29 August 2016*
Accepted: *25 October 2016*
Published: *09 November 2016*

Citation:
Yang T, Meng Y, Chen L-J, Lin H-H and Xi D-H (2016) The Roles of Alpha-Momorcharin and Jasmonic Acid in Modulating the Response of Momordica charantia to Cucumber Mosaic Virus. Front. Microbiol. 7:1796. doi: 10.3389/fmicb.2016.01796

Abbreviations: α-MMC, alpha-momorcharin; ABT, 1-aminobenzotriazole; ASA, ascorbic acid; CAT, catalase; CMV, *cucumber mosaic virus;* DHA, dehydroascorbic acid; dpi, days post-inoculation; EDTA, ethylenediaminetetraacetic acid; Fv/Fm, the maximal quantum efficiency of PSII; GSH, glutathione; GSSG, glutathione disulfide; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; H_2O_2, hydrogen peroxide; JA, jasmonic acid; MDA, malondialdehyde; NBT, nitro blue tetrazolium; O^{2-}, situ accumulation of superoxide; *PAL,* phenylalanine ammonia-lyase; POD, peroxidase; PVP, polyvinylpyrrolidone; qRT-PCR, quantitative real-time polymerase chain reaction; *RBOH,* respiratory burst oxidase homolog; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase; ΦPSII, quantum efficiency of PSII.

INTRODUCTION

Plants and pathogens have been engaged in an ongoing game of one-upmanship for millions of years. Plant viruses utilize multiple strategies to eliminate plant defenses and then promote their replication in host plants. To survive, plants have evolved a range of defense mechanisms, such as hormone-mediated signaling pathways and gene silencing pathways, to increase their defenses against pathogen attack (Gaffney et al., 1993; Pieterse et al., 2009; Chen et al., 2013). Hormones such as JA, SA, and abscisic acid (ABA) are primarily involved in the plant defense responses in plant–virus interactions (Zhu et al., 2014; Alazem and Lin, 2015). These pathways can be cooperative, or antagonistic through a complex network. Thus, plants can adjust the level of cross-talk to maintain an effective defense under pathogen attack (Koornneef and Pieterse, 2008; Robert-Seilaniantz et al., 2011). Although, plants have evolved a range of defense mechanisms to increase their defenses against pathogen attack, agricultural crops worldwide still suffer from a vast array of diseases, which cause tremendous yield and quality losses (Culbreath et al., 2003; Rodoni, 2009). It is known that some plants possess specific metabolic pathways to synthesize the number of valuable proteins that can be used for the prevention and treatment of diseases (Calixto, 2000). For example, in recent years it has been reported that plant genes encoding ribosomal inactivating proteins (RIPs) could affect the disease tolerance and resistance of plants (Huang et al., 2007; Dowd et al., 2012).

Ribosomal inactivating proteins are toxic *N*-glycosidases that function by irreversibly inhibiting protein synthesis through the removal of one or more adenine residues from 28S ribosomal RNA (Stirpe and Battelli, 2006; Puri et al., 2012). It has been reported that many RIPs have anti-viral, anti-cancer, deoxyribonuclease, and antibacterial activities (Stirpe, 2004; Kaur et al., 2011). RIPs could enhance the resistance of plants against viruses, bacterium, and fungi *in vitro* and *in vivo*. For example, it has been clearly demonstrated that the expression of α-MMC in transgenic rice plants can prevent rice blast (Qian et al., 2014). α-MMC is a member of the type I family of RIPs, with a molecular weight of approximately 29 kDa, and is derived from *Momordica charantia.* It has important biological properties in animals, including DNA hydrolase, rRNA *N*-glycosidases, anti-HIV, antibacterial, and antiviral activities (Fang et al., 2012; Pan et al., 2014). Our previous study showed that foliar spraying of α-MMC on tobacco plants exhibited multiple antiviral activities against phytopathogenic viruses and antifungal activity (Zhu et al., 2013). In addition, several reports describing the relationship between RIPs and abiotic stress responses, such as drought, salinity, and heavy metal contamination, have been published (Jiang et al., 2012; Wang et al., 2016). Moreover, numerous studies have shown the involvement of α-MMC in bacterial defense responses (Lodge et al., 1993; Yuan et al., 2002).

Plant response to various stresses is frequently associated with the generation of ROS (Zhang et al., 2001; Baxter et al., 2014). For a long time, ROS were considered to be harmful and to cause damage to plants. Notably, several lines of evidence proved that high levels of ROS caused cell death (Choi et al., 2007; Yang et al., 2011). For example, brassinosteroids-induced abiotic stress resistance was reduced after H_2O_2 treatment in *cucumber* plants (Wei et al., 2015). However, other studies have suggested that ROS, especially H_2O_2 encoded by *RBOH* genes, have a positive effect on plant responses to biotic and abiotic stresses (Xia et al., 2011; Deng et al., 2016a). Many studies have shown that low levels of ROS act as a defense signal in plants. For example, in *Nicotiana benthamiana*, ROS generation was shown to inhibit virus replication (Deng et al., 2016a). In another study, H_2O_2 was shown to decrease the susceptibility of *zucchini* to CMV (Tao et al., 2015). Hence, the role of ROS remains uncertain in plant–virus interactions.

Cucumber mosaic virus is considered to be one of the world's most important viruses due to its wide range of hosts. It is also one of the few viruses that can infect *M. charantia* both naturally and experimentally. α-MMC is a natural secondary metabolite encoded by the endogenous gene of *M. charantia*. Therefore, the *M. charantia*–CMV interaction system could be an excellent model for investigating α-MMC induced host responses to viral infection. In this study, a chemical treatment demonstrated that α-MMC could inhibit virus replication by inducing a plant defense that was dependent on JA. Moreover, α-MMC played a positive role in plant resistance and the activation of JA. Additionally, JA in turn influenced the accumulation of α-MMC in *M. charantia*–CMV interactions. Notably, our results showed that ROS acted as a second messenger in α-MMC and JA-induced CMV defense responses.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Momordica charantia plants were grown in a temperature-controlled growth room under a 16 h-light/8 h-dark cycle (100 mol m^{-2} s^{-1}) at 20–25°C. Experiments were performed at the stage when the second leaves of *M. charantia* plants were fully expanded.

Chemical Treatments and Virus Inoculation

The α-MMC was extracted from seeds of *M. charantia* according to the method of Bian et al. (2010). JA, SA, ibuprofen, and ABT were purchased from Sigma-Aldrich (St. Louis, MO, USA). The hormone and inhibitor solutions were prepared in water containing 0.02% (vol/vol) Tween 20. The following concentrations of chemicals were used: JA (100 μM), SA (100 μM), α-MMC (0.5 mg/ml), ibuprofen (100 μM), and ABT (1 nM). Distilled water containing 0.02% (vol/vol) Tween 20 was used as a control treatment. For the α-MMC+ ABT+SA treatment, seedlings were pretreated with ABT, then 12 h later were treated with α-MMC for 24 h, and were then exposed to the virus before being treated with SA 3 days later. To investigate the roles of hormones in the resistance, leaves were pretreated with ibuprofen or ABT, and 12 h later these plants were exposed to the virus. For the ibuprofen+JA treatment, plants were pretreated with ibuprofen, then 12 h later were infected with CMV before being treated with JA three days later.

In infection experiments, the chemicals were sprayed 24 h before virus inoculation. CMV was maintained in an aqueous suspension of 0.02 M sodium phosphate buffer (PBS, pH 7.0) at 4°C. The inoculation with virus was implemented as described previously (Deng et al., 2016b). PBS buffer was used as a control. All experiments were repeated three times.

Superoxide, H_2O_2 Staining, and Determinations

Superoxide and H_2O_2 staining were detected with NBT and the H_2O_2 fluorescence probe H_2DCFDA (Sigma-Aldrich). *M. charantia* leaves were vacuum infiltrated with NBT (0.5 mg/mL) solutions for 2 h. Leaves were then decolorized in boiling ethanol (90%) for 15 min. The method used for H_2O_2 fluorescence probe staining was described by Deng et al. (2016b). The Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen, Waltham, MA, USA) was used to determine H_2O_2 accumulation.

Damage Estimation

Electrolyte leakage was measured as described by Yang et al. (2004). After measuring the conductivity of the fresh leaves, they were boiled for 60 min to achieve 100% electrolyte leakage. Lipid peroxidation was estimated by measuring the MDA as previously described (Velikova et al., 2000). The lipid peroxides were expressed as MDA content.

Determination of Antioxidant Enzymes

For the enzyme assays, 0.3 g of leaf material was ground with 3 mL ice-cold 25 mM Hepes buffer (pH 7.8) containing 0.2 Mm EDTA, 2 mM ascorbate and 2% PVP. The homogenates were centrifuged at 4°C for 20 min at 12,000 *g* and the resulting supernatants were used for the determination of enzymatic activity. SOD, CAT, APX, and POD activities were assayed as described by Wang et al. (2011). ASA, DHA, GSH, and GSSG were extracted and determined as described by Xu et al. (2012).

Analysis of Chlorophyll Fluorescence

Chlorophyll fluorescence was determined with an imaging pulse amplitude modulated fluorometer (IMAG-MINI; Heinz Walz, Effeltrich, Germany). For the measurement of Fv/Fm, plants were first dark adapted for 30 min. Minimal fluorescence (Fo) was measured during the weak measuring pulses, and maximal fluorescence (Fm) was measured by a 0.8 s pulse of light at about 4000 l mol m^{-2} s^{-1}. An actinic light source was then applied to obtain a steady-state fluorescence yield (Fs), after which a second saturation pulse was applied for 0.7 s to obtain the light-adapted maximum fluorescence (Fm_0). Fv/Fm and ΦPSII were calculated as Fm – Fo/Fm and (Fm_0 – Fs)/Fm_0, respectively.

JA and SA Determination

Momordica charantia plants were grown in soil and inoculated following the different treatments and systemic leaves were used for hormone determination. SA and JA were quantified by high-performance liquid chromatography–mass spectrometry (HPLC–MS) from crude plant extracts according to the method of Zhu et al. (2013). As internal standards, 2-hydroxybenzoic acid-[$^2H_6$] (d_6-SA) was obtained from Sigma-Aldrich, and dihydrojasmonic acid (H_2JA) was obtained from OlChemim (Olomouc, Czech Republic) (Zhu et al., 2014).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total cellular RNA was extracted using a previously described method (Wang et al., 2010). The RNA content was calculated by measuring the absorbance value taken at 260 nm. All RNA samples were treated with DNase I before PCR. The qRT-PCR analysis was performed with the primers shown in Supplementary Table S1. Relative quantitation of the target gene expression level was performed using the comparative Ct. Three technical replicates were performed for each experiment, including at least three independent plants. 18S RNA was used as an internal control.

Protein Extraction and Western Blot Analysis

The total proteins were extracted with extraction buffer (50 mM Tris-Cl, pH 6.8, 5% mercaptoethanol, 10% glycerol, 4% SDS, and 4 Murea) in an ice bath. The protein concentrations were determined through the Bradford method, using bovine serum albumin as the standard (Xi et al., 2007). Western blot analysis was performed according to the protocol described Xi et al. (2007).

Statistical Analysis

The data were expressed as the mean ± SD and statistically analyzed using a one-way analysis of variance (ANOVA). A difference was considered to be statistically significant when $P < 0.05$.

RESULTS

Spatiotemporal Expression of α-MMC in *M. charantia*

The qRT-PCR and Western blot analysis were used to detect the expression of α-MMC in different parts of the *M. charantia* plants. Seven-day old *M. charantia* plants were divided into four parts: root, stem, leaf, and cotyledon. The qRT-PCR results with α-MMC specific primers indicated that the highest transcription level of α-MMC was in the leaf. The gene expression level of α-MMC in cotyledons was slightly reduced compared with the leaf. The lowest transcription of α-MMC was in the root (five times lower than the leaf and about 35% lower than the stem (**Figure 1A**). As shown in **Figure 1B**, the Western blot analysis results indicated that the highest accumulation of α-MMC protein occurred in the cotyledon, with intermediate levels in the root, which confirmed the results of previous reports (Stirpe and Battelli, 2006).

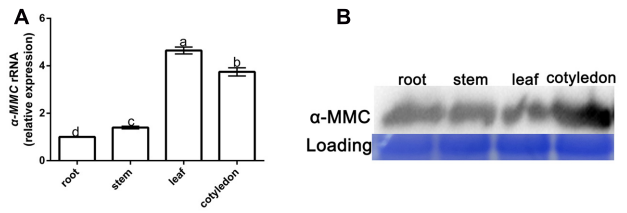

FIGURE 1 | Spatiotemporal expression of α-MMC in *Momordica charantia*. **(A)** A qRT- PCR analysis of α-MMC accumulation levels in the root, stem, leaf, and cotyledon, respectively. 18S RNA was used as an internal control. Bars represent the mean and standard deviation of values obtained from three biological replicates per genotype and time point. Significant differences ($P < 0.05$) are denoted by different lower case letters. **(B)** Western blot analysis of α-MMC in the root, stem, leaf, and cotyledon. Rubisco proteins were used as loading controls and were stained by Coomassie Brilliant Blue.

α-MMC Suppressed CMV Replication and Accumulation in Inoculated and Systemic Leaves

To investigate the role of α-MMC in the *M. charantia* defense against CMV, plants were pretreated with α-MMC and water before CMV infection and the CMV replication and accumulation levels were then detected by qRT-PCR and Western blot analysis in inoculated and newly grown leaves (systemic leaves). As shown in **Figure 2A**, the transcription level of CMV decreased significantly in α-MMC-pretreated plants compared with water-pretreated plants. The qRT-PCR result was also confirmed by Western blot analysis (**Figure 2B**). The trends of the replication and accumulation of CMV on systemic leaves and inoculated leaves were consistent. These results confirmed the role of α-MMC in the *M. charantia* defense against CMV.

The MDA content and electrolyte leakage indicated the degree of damage in plants caused by biotic stresses. To verify the damage to the plasma membrane caused by the plant virus infection, the MDA content and electrolyte leakage were measured in inoculated and systemic leaves. As shown in **Figure 2C**, water pretreated plants had more serious damage and a greater occurrence of cell-death than α-MMC pretreated plants. The change of electrolyte leakage and the MDA content in systemic and inoculated leaves were consistent (**Figure 2D**). At 8 dpi, *M. charantia* infected leaves developed strong disease symptoms characterized by yellow spots and mosaics compared with control plants. Water pretreated plants displayed more serious symptoms than α-MMC-pretreated plants (**Figure 2E**). The results indicated that α-MMC played a positive role in *M. charantia* resistance to CMV infection.

Effects of α-MMC on the Antioxidant Capacity under CMV Inoculation

Activation of antioxidant capacity was important to avoid oxidative damage in plants under virus infection. To investigate whether antioxidant systems participate in an α-MMC induced CMV defense response, we examined the activity of the antioxidant enzymes SOD, CAT, POD, and APX-POD. As shown in **Figure 3**, the enzyme activities of all treated plants were elevated under CMV infection, but α-MMC pretreatment did not boost the activity of antioxidant enzymes. In contrast, the enzyme activities were lower than in plants pretreated with water under virus infection. These results suggested that α-MMC may reduce the oxidative damage in some way that is independent of antioxidant capacity.

Effects of Exogenous α-MMC on Endogenous α-MMC and Hormone Production

Hormones play vital roles in plant–pathogen interactions, with SA, and JA known to be involved in defense responses. We investigated whether α-MMC-induced CMV resistance was dependent on these hormones by quantifying the levels of SA and JA in pretreated plants. As shown in **Figures 4B,C**, there was no significant difference in the accumulation of SA in control and inoculated plants, and CMV infection caused little variation in SA levels. However, α-MMC pretreatment and CMV infection could induce JA accumulation. Furthermore, the JA content increased more in α-MMC-pretreated plants than in water-pretreated plants under virus infection (**Figure 4A**). Based on these results, we speculated that α-MMC induced a CMV defense that was dependent on JA content, but not on SA content.

To further investigate the roles of hormones in limiting CMV infectivity, we used a JA inhibitor (ibuprofen) and an SA inhibitor (ABT) to determine their functions in CMV infection (Zhu et al., 2006; Wang et al., 2012). To test the effect of the inhibitors, we evaluated hormone contents and the transcription of *PAL*, which plays a crucial role in SA synthesis in pretreated plants. As shown in **Figures 4D–F**, these inhibitors could effectively inhibit hormone biosynthesis. CMV replication and accumulation increased in ibuprofen-pretreated plants compared with plants that were only pretreated with α-MMC. However, there was no significant difference in ABT-pretreated plants (**Figures 4G,H**). These results also proved that the α-MMC induced virus defense depends on JA, but not SA.

Because α-MMC exhibits anti-inflammatory and anti-viral effects in animals, we further investigated its expression in plants after chemical treatment and CMV inoculation. To identify the molecular mechanisms involved in the α-MMC response to CMV, we examined the transcription of the α-MMC synthesis gene and its protein accumulation. The qRT-PCR and Western blot results showed that foliar applications of α-MMC induced the up-regulation of endogenous α-MMC (**Figures 4I,J**). Transcription and accumulation of α-MMC were greater in α-MMC-pretreated plants than in water-pretreated plants under CMV infection, while all of them were up-regulated. As shown in **Figure 4G**, CMV accumulation in α-MMC+ ibuprofen-pretreated plants was increased compared with α-MMC-pretreated plants, but was less than in water-pretreated plants. The expression of α-MMC was inversely proportional to CMV replication and accumulation (**Figures 4G,H**). Taken together,

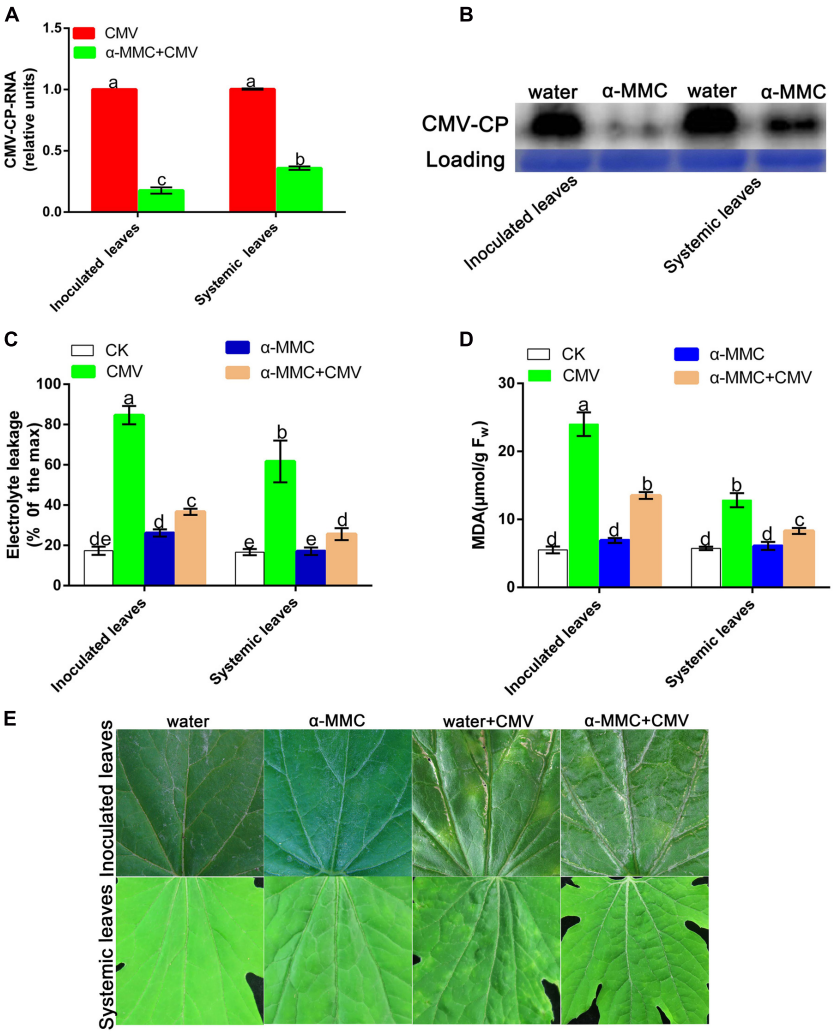

FIGURE 2 | α-MMC suppressed CMV replication and accumulation in inoculated and systemic leaves. (A) A qRT- PCR analysis of CMV mRNA accumulation in inoculated leaves at 5 dpi and systemic leaves at 9 dpi. 18S RNA was used as an internal control. Bars represent the mean and standard deviation of values obtained from three independent biological replicates. **(B)** Western blot analysis of coat protein accumulation of CMV in inoculated leaves at 5 dpi and systemic leaves at 9 dpi. Rubisco proteins were used as loading controls and were stained by Coomassie Brilliant Blue. Changes in electrolyte leakage **(C)** and MDA content **(D)** under CMV infection. Bars represent the mean and standard deviation of values obtained from three independent biological replicates. **(E)** Phenotype of water and α-MMC-pretreated plants with or without CMV infection at 8 dpi. Experiments were repeated three times with similar results. Significant differences ($P < 0.05$) are denoted by different lowercase letters.

these results suggest that α-MMC was a positive regulator in α-MMC-induced viral resistance in *M. charantia*.

Involvement of ROS in α-MMC-Induced CMV Defense

To determine the possible role of ROS in α-MMC-induced virus resistance in *M. charantia*, we attempted to detect the *in situ* accumulation of superoxide (O^{2-}) and H_2O_2 using NBT and H_2DCF-DA staining procedures, respectively. The results showed that both O^{2-} and H_2O_2 increased in α-MMC-pretreated leaves compared with water-pretreated leaves (**Figures 5A,B**). We further detected H_2O_2 levels in these leaves. Similarly, in α-MMC-pretreated plants, the H_2O_2 content was significantly higher than in water-pretreated plants infected with CMV, which was consistent with the *RBOH* gene (**Figures 5C,D**). The results suggested that α-MMC could induce an ROS burst in response to CMV infection. Importantly, α-MMC-induced ROS accumulation was again largely inhibited by ibuprofen, but not by ABT.

JA Plays a Positive Role in Photosystem Protection in α-MMC-Induced CMV Resistance

Pathogens and environmental stress can disturb the photochemistry of photosystem II (PSII) and induce a photoprotection mechanism. F_V/Fm and ΦPSII are indicators

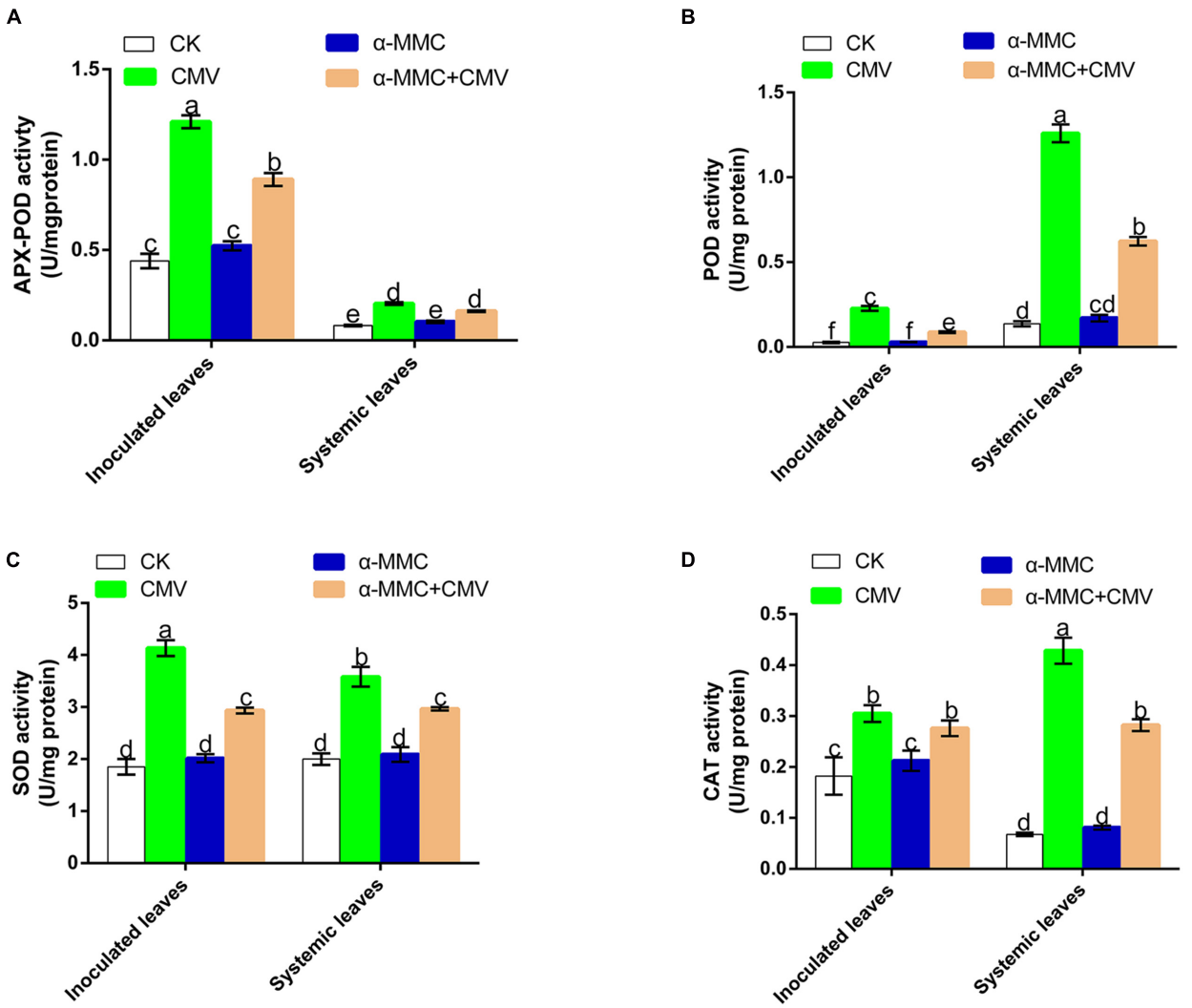

FIGURE 3 | Analysis of antioxidant enzyme activities under CMV infection at 5 dpi in inoculated and at 9 dpi in systemic leaves. APX-POD **(A)**, POD **(B)**, SOD **(C)**, CAT **(D)**. Bars represent the mean and standard deviation of values obtained from three independent biological replicates. Experiments were repeated three times with similar results. Significant differences ($P < 0.05$) are denoted by different lowercase letters.

of PSII photochemical activity. To determine the roles of the hormone in the α-MMC-induced defense response to CMV, we investigated the effects of ibuprofen, JA inhibitor and ABT, SA inhibitor on the α-MMC-induced resistance to CMV challenge. As shown in **Figures 6A,C**, the Fv/Fm of water-pretreated plants was significantly lower than in α-MMC-pretreated plants. In contrast, non-photochemicalexciton quenching (NPQ) was higher in water-pretreated plants, but lower in α-MMC-pretreated plants under CMV infection at 9 dpi (**Figures 6B,D**). The lower Fv/Fm indicated a decline of photosynthesis, while the higher NPQ implied some degree of photo-damage suffered by the plant. Taken together, the results indicated that α-MMC could protect the photo-system of plants under virus infection. However, α-MMC-induced resistance to photo-oxidative stress was largely inhibited if the plants were pretreated with ibuprofen, but were not influenced by ABT (**Figures 6C,D**). The application of JA almost rescued the decrease in stress resistance due to ibuprofen. These results showed that JA played a positive role in photo-system protection in α-MMC-induced CMV resistance.

JA Improves Plant Resistance under CMV Infection

Salicylic acid and JA are important natural hormones that have a function in plant resistance against virus infection. To explore the effects of JA and SA on plant resistance under CMV infection, we used hormones and relevant inhibitors in this experiment (**Supplementary Figure S2**). There was less accumulation of CMV in JA-pretreated plants than in water-pretreated plants (**Figures 7A,B**). However, the level of viral replication was significantly higher in ibuprofen-pretreated plants than in water-pretreated plants, but this level of viral replication could be

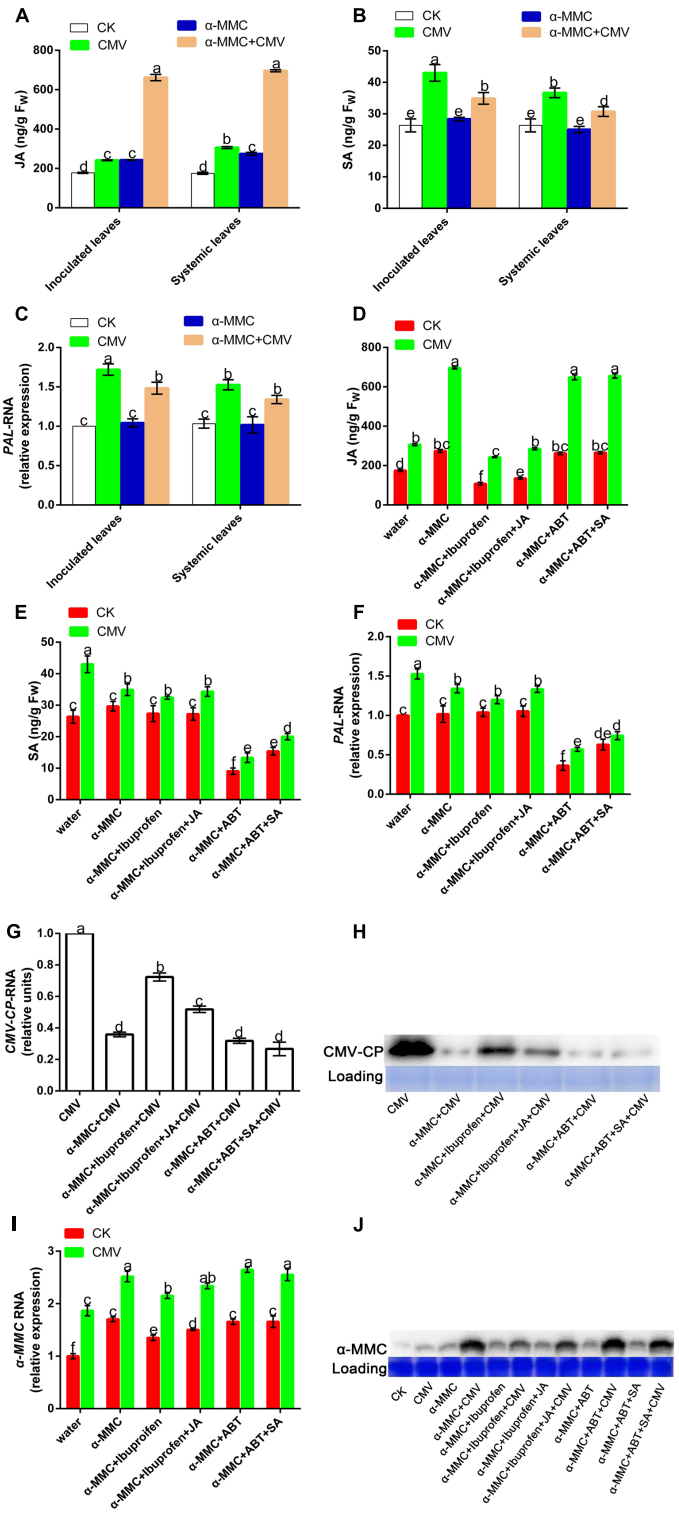

FIGURE 4 | Effects of exogenous α-MMC on endogenous α-MMC and hormone production. JA and SA accumulation **(A,B)** in the water and α-MMC-pretreated plants with or without CMV infection, and the expression of the SA biosynthesis gene **(C)** at 5 dpi in inoculated leaves and 9 dpi in systemic leaves. JA and SA accumulation **(D,E)** in different pretreatments and the expression of the SA biosynthesis gene **(F)** at 9 dpi in systemic leaves. A qRT-PCR analysis of CMV and α-MMC mRNA accumulation levels **(G,I)** and Western blot analysis of the CMV coat protein and α-MMC accumulation **(H,J)** in systemic leaves collected at 9 dpi. 18S RNA was used as the internal control. Error bars represent the mean and standard deviation of values obtained from three independent biological replicates. Experiments were repeated three times with similar results. Significant differences ($P < 0.05$) are denoted by different lowercase letters.

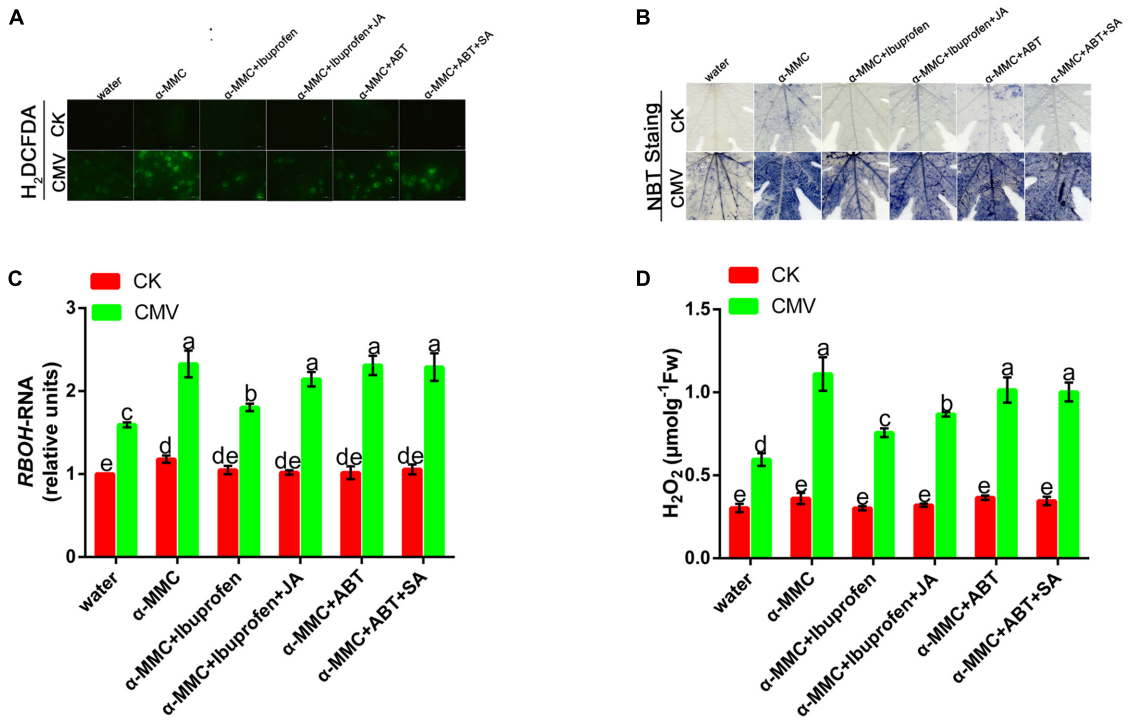

FIGURE 5 | Involvement of ROS in α-MMC-induced CMV defense. H_2DCFDA- **(A)** and NBT-stained **(B)** control or CMV inoculated *M. charantia* leaves pretreated with water, α-MMC, α-MMC+Ibuprofen, α-MMC+Ibuprofen+JA, α-MMC+ABT, or α-MMC +ABT+SA at 5 dpi in systemic leaves. **(C)** A qRT-PCR analysis of the *RBOH* gene at 5 dpi. 18S RNA was used as the internal control. **(D)** H_2O_2 level in control or CMV inoculated leaves determined at 5 dpi. Error bars represent the mean and standard deviation of values obtained from three independent biological replicates. Experiments were repeated three times with similar results. Significant differences ($P < 0.05$) are denoted by different lowercase letters.

reduced by applying JA. As shown in **Figure 7B**, there was no significant difference in the level of virus accumulation among SA, SA inhibitor, and water-pretreated plants. These results showed that plant resistance could be induced by JA but not SA.

From the above results, we concluded that ROS participated in the α-MMC-induced CMV defense. To further investigate whether JA activated plant innate immunity related to ROS, NBT, and H_2DCF-DA staining were used in this experiment. The results indicated that JA pretreatment led to a substantial increase in the production of ROS compared with water pretreatment, but this could be inhibited by ibuprofen (**Figures 7C,D**). All of the results suggested that ROS were involved in JA-induced CMV resistance.

DISCUSSION

The use of α-MMC to treat human diseases such as tumors, HIV, and fungal infections has been well-studied in the past (Bian et al., 2010; Puri et al., 2012). However, studies of the role of α-MMC in plant virus resistance have rarely been reported. Therefore, the role of α-MMC in plant defense and the mechanisms involved are not well-understood. This study provides an insight into the characterization of the role of the α-MMC-mediated defense response in plants using *M. charantia* and the CMV interaction system. We revealed that α-MMC, JA, and ROS played important roles in α-MMC-mediated CMV defense in *M. charantia.*

Spatial Differences in the Expression of α-MMC in *M. charantia*

The α-MMC level was associated with disease resistance in plants, but its gene transcription and protein accumulation were not the same in different organs. Therefore, the spatiotemporal expression of α-MMC was investigated in *M. charantia* by qRT-PCR and protein hybridization. Western blot analysis revealed that a higher accumulation of α-MMC existed in the cotyledon and root, which was consistent with the results of previous research (Kaur et al., 2012). The highest level of transcription of α-MMC appeared in the leaf and the least was in the root. Based on these results, we hypothesized that because the root and stem were lignified and the cotyledon was a vestigial organ, their level of gene transcription was lower than in the leaf.

α-MMC-Induced CMV Defense Was Dependent on JA Levels in *M. charantia*

Plants are endowed with an innate immune system, and phytohormones such as SA and JA have been reported to play an important role in plant immunity. In this study, the JA content increased in α-MMC-pretreated plants under virus infection, along with a higher viral resistance. This result confirmed

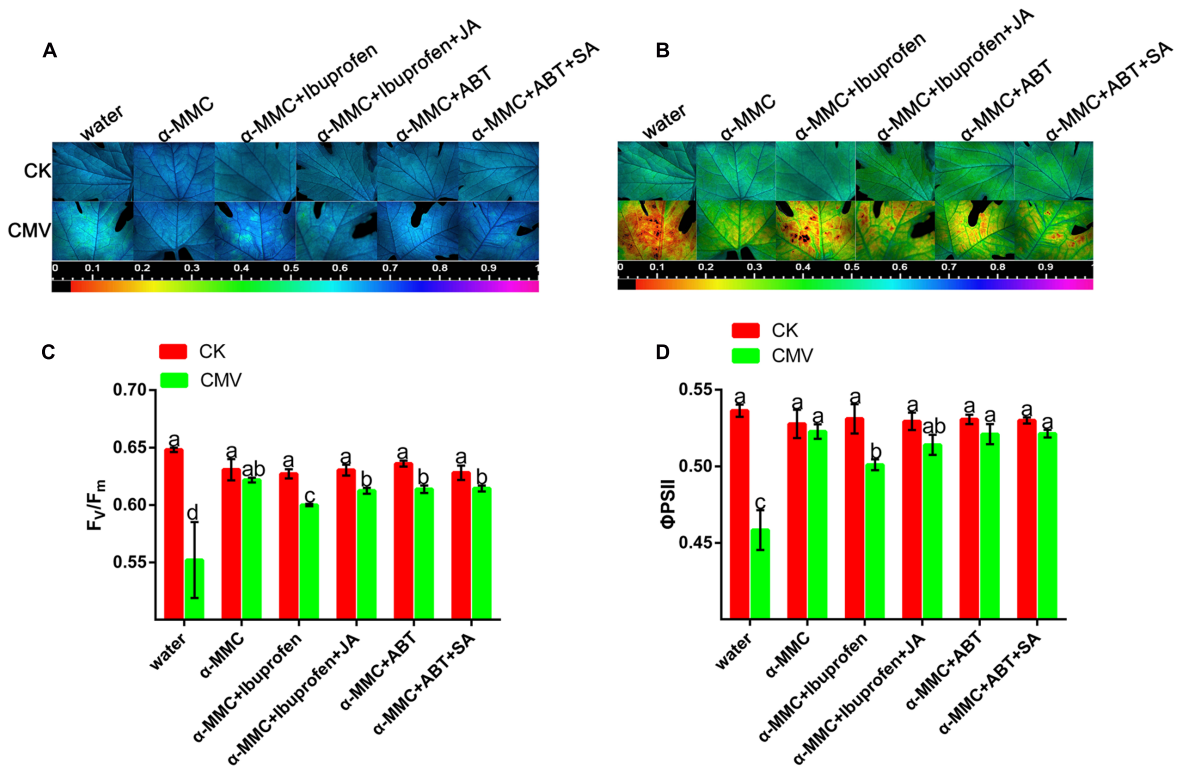

FIGURE 6 | Jasmonic acid plays a positive role in photosystem protection in α-MMC-induced CMV resistance. (A,B) Images of Fv/Fm and ΦPSII at 9 dpi. Four plants were used for each treatment and a picture of one representative plant is shown. **(C,D)** Average values for the respective chlorophyll fluorescence image replicates. Error bars represent the mean and standard deviation of values obtained from three independent biological replicates. Experiments were repeated three times with similar results. Significant differences ($P < 0.05$) are denoted by different lowercase letters.

the previous reports that hormones contribute to plant biotic stresses resistance (Alazem and Lin, 2015; Tao et al., 2015). We further revealed that only JA, and not SA, was involved in the α-MMC-induced defense in *M. charantia*. When JA biosynthesis was inhibited by its inhibitor, virus accumulation increased (**Figures 4G,H**). In contrast, the SA content and the expression of *PAL* showed little variation among the different treatments infected by CMV, while ABT pretreatment did not affect the resistance induced by α-MMC (**Figures 4B,G**). These results suggest that the response of JA accumulation to virus infection was activated in α-MMC-pretreated plants, therefore they displayed an enhanced insensitivity to CMV infection.

JA Content Was Increased by α-MMC Pretreatment and Could also Enhance α-MMC Accumulation in the *M. charantia*–CMV Interaction System

In this study, we tried not only to reveal the potential mechanisms of α-MMC's function in CMV resistance, but also to investigate the relationship between α-MMC-induced resistance and phytohormone mediated defense pathways. Interestingly, under the exogenous application of JA the accumulation of CMV was significantly suppressed, and under the exogenous application of ibuprofen the replication of CMV was increased. However, the expression of the virus was almost unaffected by the SA or ABT treatment compared with the control (**Figures 7A,B**). These results showed that virus infection was inhibited by JA, but not SA. In the α-MMC-induced resistance, α-MMC could enhance the JA content under CMV infection (**Figure 4A**). In hormone-pretreated plants, JA could induce the expression of α-MMC, but SA could not (**Figures 7E,F**). Taken together, our results indicate that α-MMC is involved in JA-induced CMV resistance in *M. charantia*, while SA was unlikely to be involved in JA and α-MMC activation.

As described above, JA was involved in α-MMC-induced CMV resistance in *M. charantia* and played a vital role in the response to CMV. Therefore, we tested the relationship of α-MMC and hormones in the response of *M. charantia* to CMV. To achieve this, JA, ibuprofen, SA, ABT, and α-MMC were used. The results showed that α-MMC expression increased in JA-pretreated plants, but decreased in the ibuprofen pretreated group (**Figures 7E,F**). In α-MMC-pretreated plants, JA accumulation also increased. The accumulation of SA was not affected by α-MMC, and it could not induce the expression of α-MMC (**Figures 4A,B** and **7E,F**). These data indicated that α-MMC could not only affect the JA content, but JA could also regulate the expression and accumulation of α-MMC. However, α-MMC and SA could not be induced by each other.

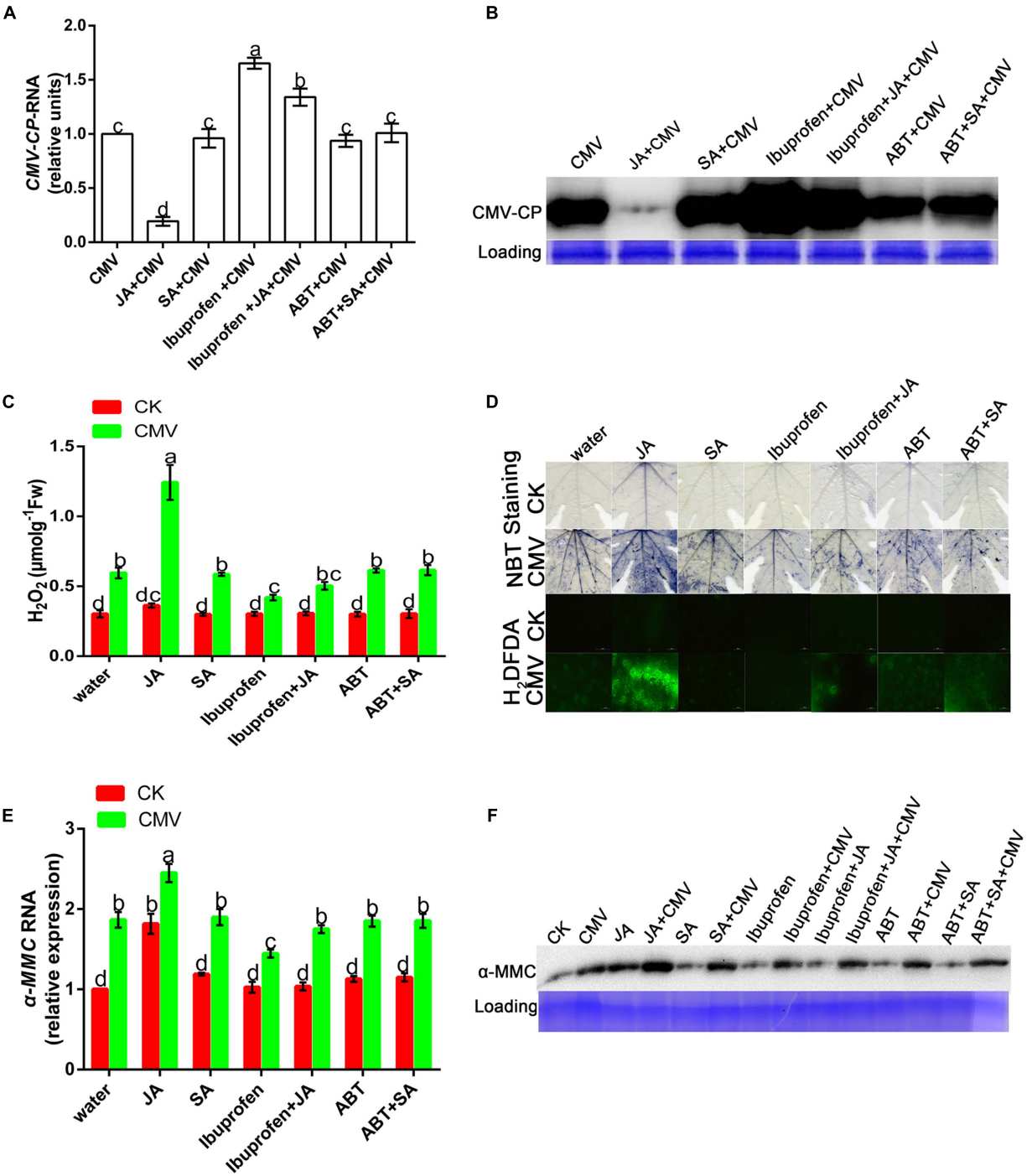

FIGURE 7 | Jasmonic acid improves plant resistance under CMV infection. (A,E) A qRT-PCR analysis of CMV and hormone-induced α-MMC mRNA accumulation in systemic leaves at 9 dpi. 18S RNA was used as an internal control. Bars represent the mean and standard deviation of values obtained from three independent biological replicates. **(B,F)** Western blot analysis of coat protein accumulation of CMV and α-MMC-protein accumulation in systemic leaves at 9 dpi. Rubisco proteins were used as loading controls and were stained by Coomassie Brilliant Blue. **(C)** H_2O_2 content. Error bars represent the mean and standard deviation of values obtained from three independent biological replicates. Significant differences ($P < 0.05$) are denoted by different lowercase letters. **(D)** Superoxide contents were detected by NBT staining and H_2O_2 levels were detected by H_2DCFDA staining at 5 dpi in water-, hormone- or hormone inhibitor pretreated plants with or without CMV infection. Experiments were repeated three times with similar results.

ROS Act as a Second Messenger in α-MMC and JA-Induced CMV Defense

Most forms of biotic or abiotic stress disrupt the metabolic balance of cells, resulting in the enhanced production of ROS. The roles of ROS in incompatible plant–virus interactions have been studied previously (Moeder et al., 2005; Király et al., 2008). However, ROS involvement in compatible plant–virus interactions is still controversial (Fu et al., 2010; Shi et al., 2012). Compared with other pretreated plants, in α-MMC or JA-pretreated plants, the levels of ROS were the highest, but plant damage was the least (**Figures 5**, **6**, and **7B,C**). Virus replication and accumulation were inversely related to ROS in the JA and α-MMC-induced defense (**Figures 4G**, **5**, and **7A,C**). These results suggest that ROS acted as a second messenger to mediate the JA and α-MMC signal during the induction of stress resistance. Although ROS are considered to be an important cellular signal, they can be cytotoxic. However, there was no obvious up-regulation of the activities of several antioxidant enzymes compared with the control. Conversely, there was a significant up-regulation in CMV-infected plants (**Figure 3**). Interestingly, the ratios of GSH/GSSH and ASA/DHA were increased in α-MMC-pretreated plants compared with water-pretreated plants, but they were decreased in plants pretreated with α-MMC+ ibuprofen under CMV infection (**Supplementary Figure S1**). The results showed that α-MMC may depend on reducing substances to avoid oxidative damage, but not antioxidant enzymes.

In summary, the results presented in this study provide evidence that α-MMC-induced plant resistance depends on the JA content under CMV infection, while the SA pathway did not display a relationship with the α-MMC-regulated viral defense response. ROS acted as a second messenger in the plant defense response to CMV infection. Our study presents new evidence that in the *M. charantia*–CMV interaction system, the molecular resistance mechanism induced by α-MMC was similar to that induced by JA. Thus, our results have revealed the novel roles of α-MMC and JA in plants against CMV infection and clarified the relationships between JA, ROS, SA, and α-MMC during CMV infection in *M. charantia*, although an understanding of the detailed mechanism needs further investigation.

AUTHOR CONTRIBUTIONS

D-HX and TY contributed to the experimental design of the study. TY and YM performed the experiments, data analysis, and drafted the manuscript. L-JC and H-HL read and corrected the manuscript.

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (NSFC) (31270290, 31171835).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01796/full#supplementary-material

FIGURE S1 | Changes in the activities of GSH/GSSH (A) and ASA/DHA **(B)** in *M. charantia* under CMV infection at 9 dpi. The JA pathway was inhibited by ibuprofen or the SA pathway was inhibited by ABT pre-treatment in these α-MMC-treated plants. Error bars represent the mean and standard deviation of values obtained from three independent biological replicates. Experiments were repeated three times with similar results. Significant differences ($P < 0.05$) are denoted by different lowercase letters.

FIGURE S2 | Detection of the JA content (A) and SA content **(B)** in hormone-pretreated and hormone inhibitor-pretreated *M. charantia* at 9 dpi. Error bars represent the mean and standard deviation of values obtained from three independent biological replicates. Experiments were repeated three times with similar results. Significant differences ($P < 0.05$) are denoted by different lowercase letters.

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