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**Specialty section:**

This article was submitted to  
Microbe and Virus Interactions With  
Plants,  
a section of the journal  
Frontiers in Microbiology

**Received:** 16 January 2022

**Accepted:** 03 March 2022

**Published:** 24 March 2022

**Citation:**

Xu J, Li Y, Jia J, Xiong W, Zhong C,  
Huang G, Gou X, Meng Y and  
Shan W (2022) Mutations in PpAGO3  
Lead to Enhanced Virulence of  
*Phytophthora parasitica* by Activation  
of 25–26nt sRNA-Associated Effector  
Genes.  
Front. Microbiol. 13:856106.  
doi: 10.3389/fmicb.2022.856106

# Mutations in PpAGO3 Lead to Enhanced Virulence of *Phytophthora parasitica* by Activation of 25–26 nt sRNA-Associated Effector Genes

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Oomycetes represent a unique group of plant pathogens that are destructive to a wide range of crops and natural ecosystems. *Phytophthora* species possess active small RNA (sRNA) silencing pathways, but little is known about the biological roles of sRNAs and associated factors in pathogenicity. Here we show that an AGO gene, *PpAGO3*, plays a major role in the regulation of effector genes hence the pathogenicity of *Phytophthora parasitica*. *PpAGO3* was unique among five predicted AGO genes in *P. parasitica*, showing strong mycelium stage-specific expression. Using the CRISPR-Cas9 technology, we generated *PpAGO3*<sup>ΔRGG1-3</sup> mutants that carried a deletion of 1, 2, or 3 copies of the N-terminal RGG motif (QRGGYD) but failed to obtain complete knockout mutants, which suggests its vital role in *P. parasitica*. These mutants showed increased pathogenicity on both *Nicotiana benthamiana* and *Arabidopsis thaliana* plants. Transcriptome and sRNA sequencing of *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> showed that these mutants were differentially accumulated with 25–26 nt sRNAs associated with 70 predicted cytoplasmic effector genes compared to the wild-type, of which 13 exhibited inverse correlation between gene expression and 25–26 nt sRNA accumulation. Transient overexpression of the upregulated RXLR effector genes, *PPTG\_01869* and *PPTG\_15425* identified in the mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>, strongly enhanced *N. benthamiana* susceptibility to *P. parasitica*. Our results suggest that PpAGO3 functions together with 25–26 nt sRNAs to confer dynamic expression regulation of effector genes in *P. parasitica*, thereby contributing to infection and pathogenicity of the pathogen.

**Keywords:** *Phytophthora parasitica*, AGO3, small RNA, RXLR effector, RGG domain

## INTRODUCTION

Oomycetes represent a unique group of diploid microorganisms, which resemble but are evolutionarily distant from filamentous fungi (Judelson, 1997). Species in the genus of *Phytophthora* include many economically important plant pathogens, notably *Phytophthora infestans*, *P. sojae*, *P. parasitica*, and *P. ramorum* (Tyler, 2007; Fry, 2008; Grünwald et al., 2008).

During infection, *Phytophthora* secretes a large number of effectors into host plants, including RXLR (Arg–any amino acid–Leu–Arg) effectors and CRN (Crinkling and Necrosis) effectors, to manipulate host physiology and support colonization (Zheng et al., 2014; Fan et al., 2018; Wang and Wang, 2018; Chen et al., 2019). In response, the host plants produce R proteins to recognize the secreted effectors, which are known as avirulence proteins, triggering plant immunity. Nearly all host genotype-specific avirulence factors identified in *Phytophthora* are RXLR effectors (Armstrong et al., 2005; van Poppel et al., 2008; Bozkurt et al., 2011). At the same time, pathogens have evolved ways to evade the perception of effectors by the host R proteins through sequence variation and deletion of avirulence effectors. Apart from that, polymorphisms in the expression levels of effector genes are common in *Phytophthora* and contribute to pathogen plasticity in overcoming host genotype-specific resistance (Shan et al., 2004; Dong et al., 2009; Qutob et al., 2009; Wang et al., 2011a, 2020; Cui et al., 2012; Gijzen et al., 2014; Pais et al., 2018). Increasing evidence suggests that 25–26 nt small RNA (sRNA), the dominant size class of sRNAs in *Phytophthora*, are involved in the regulation of effector gene expression (Vetukuri et al., 2012; Qutob et al., 2013; Jia et al., 2017; Wang et al., 2019). For example, in *P. sojae*, 25 nt sRNAs are associated with *PsAvr3a* gene silencing and the silencing status could be “inherited” to the next generation (Qutob et al., 2013). In addition, up to 125 RXLR effector genes are associated with homologous sRNAs, which correlate with the silencing of the corresponding RXLR effector genes at the mycelium stage (Wang et al., 2019). Similarly, 25–26 nt sRNAs are implicated in the silencing of 40% RXLR and 50% CRN effector genes in *P. parasitica* during vegetative mycelial growth (Jia et al., 2017). However, very little is known about the mechanism and function of sRNA-associated silencing of RXLR effector genes.

The sRNA pathways are conserved in eukaryotes and play important roles in various cellular processes. Depending on the biogenesis pathway and functions, sRNAs are classified into microRNA (miRNA), small interfering RNA (siRNA), and PIWI-interacting RNAs (piRNAs; Girard et al., 2006; Carthew and Sontheimer, 2009; Ye et al., 2016). miRNAs are processed by Dicer or Dicer-like (DCL) proteins from self-folding RNA transcripts and have been widely found in plants and animals (Millar and Waterhouse, 2005), but very few miRNA-like genes exist in fungi and oomycetes (Lee et al., 2010; Fahlgren et al., 2013). piRNAs are only found in animals (Vagin et al., 2006; Brennecke et al., 2007; Czech et al., 2018), whereas siRNAs are present in most eukaryotes, and processed by Dicer or DCL from long double-stranded RNAs (dsRNAs) or hairpin structure (Katiyar-Agarwal et al., 2006; Huang et al., 2019b). To direct gene silencing, sRNAs are loaded to Argonaute (AGO) protein to form an RNA-induced silencing complex (RISC), which then uses the sRNA as guide to direct mRNA cleavage or DNA methylation/histone modification (Kobayashi and Tomari, 2016).

AGO proteins are present in both prokaryotes and eukaryotes (Bohmert et al., 1998; Makarova et al., 2009; Xue et al., 2012; Cervantes et al., 2013; Swarts et al., 2014; Nguyen et al., 2018; Yin et al., 2020), but the number and type of AGO proteins are diverse in different organisms (Tolia and Joshua-Tor, 2007; Hutvagner and Simard, 2008). There are primarily four AGO homologs, including AGO-like, PIWI, Worm-specific AGO

(WAGO), and *Trypanosoma* AGO families (Durand-Dubief et al., 2003; Yigit et al., 2006; Hutvagner and Simard, 2008; Garcia-Silva et al., 2010; Bollmann et al., 2018). Members of the AGO-like family contain primarily the PAZ (PIWI–ARGONAUTE–ZWILLE) and PiWi domain, which contribute to the binding of sRNA 3' end and the cleavage of complementary mRNA, respectively (Lingel et al., 2003; Song et al., 2004). The AGO-like family is primarily involved in miRNA and siRNA-directed silencing and plays a vital role in transcriptional and translational regulation, heterochromatin assembly, and alternative splicing (Baulcombe, 2004; Buker et al., 2007; Azzam et al., 2012; Wei et al., 2012; Marasovic et al., 2013; Huang and Li, 2014). The TbAGO1 in *Trypanosoma brucei* is required for RNAi and plays a role in mitosis and chromosome segregation (Durand-Dubief and Bastin, 2003; Garcia-Silva et al., 2010, 2014). Interestingly, besides the well-known PAZ and Piwi domains, TbAGO1 also contains an N-terminal RGG (arginine–glycine–glycine) repeat motif, which is required for RNA silencing (Shi et al., 2004). Loss of the N-terminal RGG domain can strongly block the association of TbAGO1 with polyribosomes and affect mRNA cleavage (Shi et al., 2004, 2009). The RGG domain of TgAGO in *Toxoplasma gondii* is also functional in the RNA silencing pathway (Musiyenko et al., 2012).

Several AGO proteins have been identified in *Phytophthora* and are implicated in sRNA-mediated regulation of effector genes. For example, in *P. infestans*, silencing of *PiAgo4* or *PiAgo5* caused reduced accumulation of 32 nt sRNAs homologous to *PiAvrblb1*, while silencing of *PiAgo1* resulted in increased accumulation of 32 nt sRNAs to *PiAvrblb1*. Interestingly, the *PiAvrblb1*-derived 32 nt sRNAs were not affected in *PiDCL1* silencing strain (Vetukuri et al., 2011, 2012). Furthermore, co-immunoprecipitation assays showed that CRN effector genes and pseudo-CRN-derived 18–30 nt sRNA were significantly enriched to PiAGO1 and PiAGO5 proteins (Åsman et al., 2016). However, little is known on the specific roles of AGOs in *Phytophthora* biology and pathology.

In this study, we investigated the function of PpAGO3 in the model oomycete organism *P. parasitica* (Meng et al., 2014), where the efficient, complete silencing of many effector genes is associated with accumulation of their homologous 25–26 nt sRNAs during the mycelium stage (Jia et al., 2017). We created mutations in the N-terminal RGG domain repeat region of PpAGO3 by using the CRISPR-Cas9 technology. PpAGO3 mutants (PpAGO3<sup>ΔRGG1-3</sup>), with the deletion of 1–3 copies of RGG domain (QRGGYD), showed enhanced pathogenicity and examined for sRNA accumulation and gene expression. In addition, two PpAGO3/sRNA-regulated RXLR effector genes were further analyzed for their virulence function. Our results provide compelling evidence that PpAGO3 and 25–26 nt sRNAs function together to regulate effector gene expression.

## MATERIALS AND METHODS

### Plant Growing and *Phytophthora parasitica* Cultivation

*Arabidopsis thaliana* and *Nicotiana benthamiana* seeds were sown in a matrix containing soil and vermiculite, and cultured

in a phytotron (23°C) with a photoperiod of 14 h light per day for about 4–5 weeks. *Phytophthora parasitica* strain PpBS042 was isolated from diseased tobacco plant collected from Chongqing, China (Zhang et al., 2020). It was routinely cultured on 5% CA (carrot juice agar) medium with 0.01% CaCO<sub>3</sub> and 0.002% β-sitosterol, in darkness for 3–4 days (23°C). To induce the sporangia production, the culture medium with the fresh mycelia were transformed into 5% CA broth with Petri solution for another 5 days as described before (Huang et al., 2019a). For the zoospore release, it was done as described previously (Wang et al., 2011b).

## Bioinformatics Analysis

The five AGO protein sequences of *P. infestans* were BLASTP aligned with the *P. parasitica* INRA-310 (taxid:761204) database on NCBI, and the five predicted AGO proteins sequences of *P. parasitica* were BLASTP aligned to *P. infestans* T30-4 (taxid:403677) database to ensure the consistency of the sequence alignment. The sequence ID of five AGO proteins in *P. infestans* were PiAGO1 (XP\_002906080.1), PiAGO2 (XP\_002906081.1), PiAGO3 (XP\_002908068.1), PiAGO4 (XP\_002908108.1), PiAGO5 (XP\_002908109.1). The domain architecture was predicted by using Pfam database and displayed by software IBS (Liu et al., 2015). For gene expression pattern analysis, it was conducted by using the RNA-seq data obtained by Jia (2017), and the RNA-seq data analysis would be described later.

## Genome Editing in *Phytophthora parasitica* by CRISPR-Cas9 System

The PpAGO3 mutants were obtained through three main steps: CRISPR-Cas9 vector construction for gene PpAGO3; the plasmid transformation in *P. parasitica*; transformants selection and identification. Firstly, the CRISPR-Cas9 plasmid was constructed and modified using “all-in-one” vector pYF515 developed for *P. sojae* (Fang et al., 2017). The sgRNA for PpAGO3 was designed by using EuPaGDT<sup>1</sup>; then, off-target was analyzed by using FungiDB<sup>2</sup>; finally, RNA secondary structure was predicted online.<sup>3</sup> For PpAGO3, one sgRNA (total score > 0.5) without off-target site and weak RNA secondary structure was chosen for primer design. Through annealing and extension, the synthesized primers were made to dsDNA fragment and were ligated to NheI/BsaI (NEB) digested vector pYF515 by using T4 DNA ligase (Promega). The recombination vector was then transformed into *Escherichia coli* DH5α cells. The sequence-verified plasmid was extracted and concentrated for transformation.

Secondly, CRISPR-Cas9 plasmid for PpAGO3 was transformed into *P. parasitica* strain PpBS042. The transformation was conducted by using PEG-CaCl<sub>2</sub> mediated method as described (Bottin et al., 1999; Meng et al., 2015). The transformation protoplasts were recovered overnight and then were cultured on the 5% CA medium with 13.6 μg/ml G418, 200 μg/ml ampicillin, 20 μg/ml

nystatin, and 20 μg/ml rifampicin. Finally, it was the selection and identification for PpAGO3 mutants. Through 3–7 days selective cultivation (23°C), regenerated mycelium colonies were isolated and transformed to a fresh plate with the same selective medium. Three days later, the G418-resistant transformants were cultivated in 5% CA broth for mycelium collection. Genomic DNA was extracted following the protocol as described previously (Zhang et al., 2020) from each candidate transformants and was examined for target sites by sequence amplification and sequencing.

## Pathogenicity Assay

*Phytophthora parasitica* was cultivated on 5% CA plates as described (Huang et al., 2019a; Zhang et al., 2020). Detached leaves of the 5-week-old *N. benthamiana* and 4-week-old *A. thaliana* Col-0 leaves were used for pathogenicity assays as described previously (Huang et al., 2019a; Zhang et al., 2020). On *N. benthamiana* leaves, the developed lesions were measured 60 h post-inoculation with *P. parasitica* mycelial discs, and the expansion of *P. parasitica* hyphae was visualized by trypan blue staining. For each assay, more than eight leaves were used. On *A. thaliana*, the disease severity index (DSI) was recorded 48 h after inoculation with *P. parasitica* mycelial discs, as described previously (Huang et al., 2019a). For each assay, more than 15 leaves were used. Statistical analysis was performed based on Student's *t*-test between samples and based on a one-way ANOVA.

## RNA Extraction, Library Construction, and Sequencing

For library construction, *P. parasitica* strain PpBS042 was firstly cultured on 5% CA solid medium with 0.01% CaCO<sub>3</sub> and 0.002% β-sitosterol, in darkness for 3–4 days (23°C). Then, the culture medium with the fresh mycelia were transformed into 5% CA broth with Petri solution for another 3 days. The total RNA was extracted from fresh mycelia of both the wild-type and PpAGO3<sup>ARGG</sup> mutants by using the RNA extract kit (Aidlab, RN40). The RNA concentration and quality were examined by using NanoDrop 2000 and gel electrophoresis. Construction and sequencing of small RNA and RNA libraries were performed by Biomarker Technologies (Beijing, China) with Illumina novaseq 6000 platform. For construction of sRNA library, only 18–45 nt size small RNAs were used for sequencing. The wild-type and the two PpAGO3<sup>ARGG</sup> mutants contain three biological replicates, respectively.

## High-Throughput Sequencing Data Analysis

According to sRNA raw data, adaptor sequence (AGATCGGA AGAGCACACGTCTG) was first filtered. Then, clean reads were mapped to the *P. parasitica* genome (INRA-310 version 3.0, Assembly Dev initiative, Broad Institute) with Bowtie with no mismatches (-v 0 -a; Langmead et al., 2009; Jia et al., 2017). Reads mapped to rRNA, tRNA, mitochondrial DNA were also filtered by using bowtie (-v 0). The sRNAs that mapped to the gene locus (gene body plus 500 bp upstream and downstream region) were counted and regarded as the sRNA accumulation. Depending on the strand (sense/antisense)

<sup>1</sup><http://grna.ctegd.uga.edu/>

<sup>2</sup><https://fungidb.org/fungidb/>

<sup>3</sup><http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html>

the sRNAs derived and the sRNA size, the detailed analysis was further divided. The sRNA counts were calculated by using self-write perl scripts. For sRNAs differentially expressed between the wild-type and *PpAGO3*<sup>ΔRGG</sup> mutants, the mapping counts were analyzed by using R package, DESeq2 (Love et al., 2014). For each gene, the corresponding sRNA expression level was calculated by using RPKM, reads Per Kilobase per Million.

According to the RNA-seq raw data, the adapter sequence and low-quality reads were filtered by using software Trimmomatic (-phred33 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36; Bolger et al., 2014). The clean reads were mapped to *P. parasitica* genome (INRA-310-version 3.0) by using software Hisat2 (-min-intronlen 20 -max-intronlen 3,000; Kim et al., 2015). Software featureCounts was used for mapping counts calculation (Liao et al., 2014). The differential expression analysis was conducted by using DESeq2 (Love et al., 2014). Gene annotation of differentially expressed genes (DEGs) was done by Blast2go (Conesa et al., 2005), and GO enrichment analysis was realized by using software Ttools (Chen et al., 2020). FeatureCounts output data were used to calculate gene expression level, FPKM. The heatmap for gene/sRNA expression level was performed by using Ttools (Chen et al., 2020). The expression pattern of *PpAGO* family genes was obtained by analyzing the RNA-seq data obtained previously (Jia, 2017). The RNA sequencing was conducted by collecting the 6-week-old *N. benthamiana* leaves infected with *P. parasitica* strain Pp016 at 3, 6, 12, 24, and 48 h post-inoculation (Jia, 2017). The FPKM values were calculated and extracted by using Cuffquant and Cuffnorm (Trapnell et al., 2012).

## Vector Construction and *Agrobacterium tumefaciens*-Mediated Transient Expression Assay

RXLR effector genes PPTG\_01869 and PPTG\_15425 were fused with *GFP*. The effector gene fragment and *GFP* were both amplified by using *P. parasitica* cDNA and DNA polymerase FastPfu (TransGen Biotech). The generated fragments were ligated to the restriction sites (KpnI and XbaI) in the vector pKannibal (Wesley et al., 2001) by using T4 DNA ligase (Promega). The fusion construct was further transferred to the *NotI* sites in vector pART27 (Gleave, 1992). The resulted constructs *PPTG\_01869-GFP*, *PPTG\_15425-GFP*, and *GFP* were transferred into *Agrobacterium tumefaciens* GV3101 cells, respectively, and cultured in Luria Bertani (LB) liquid broth, harvested and suspended in infiltration buffer as described (Huang et al., 2019a). The agroinfiltration was performed at concentration (OD<sub>600</sub> 0.04) on *N. benthamiana* leaves by using needleless syringes (Meng et al., 2015). The infiltrated *N. benthamiana* leaves were detached and gene expression state was examined by using the fluorescence microscope (Olympus-BX-51TRF) 24–36 h post-infiltration, and pathogenicity assays were performed using procedures as described (Huang et al., 2019a). Eight leaves were used for each experiment and the experiments were repeated three times.

## RESULTS

### Mutations in PpAGO3 Led to Enhanced Pathogenicity of *Phytophthora parasitica*

*Phytophthora* genomes encode several AGO homologs that phylogenetically clustered with the AGO-like family (Åsman et al., 2016; Bollmann et al., 2016, 2018). In *P. parasitica*, five AGO proteins, designated as PpAGO1–5 (Supplementary Figure 1), were shown to have high sequence homologies to PiAGOs of *P. infestans*, a sister species of *P. parasitica*. Searching the Pfam database (Finn et al., 2010) revealed that they all contain the typical domains of AGO, including the N-terminal PAZ, Mid (middle), and Piwi domains with one or two linkers (L1 and L2; Supplementary Figure 1). In addition, they all contain the arginine–glycine–glycine (RGG) repeat motif at the N terminus. However, the number of RGG motif is highly variable among five PpAGO proteins, with one for PpAGO1, three for PpAGO2, 24 for PpAGO3, two for PpAGO4, and 13 for PpAGO5 (Supplementary Figure 1).

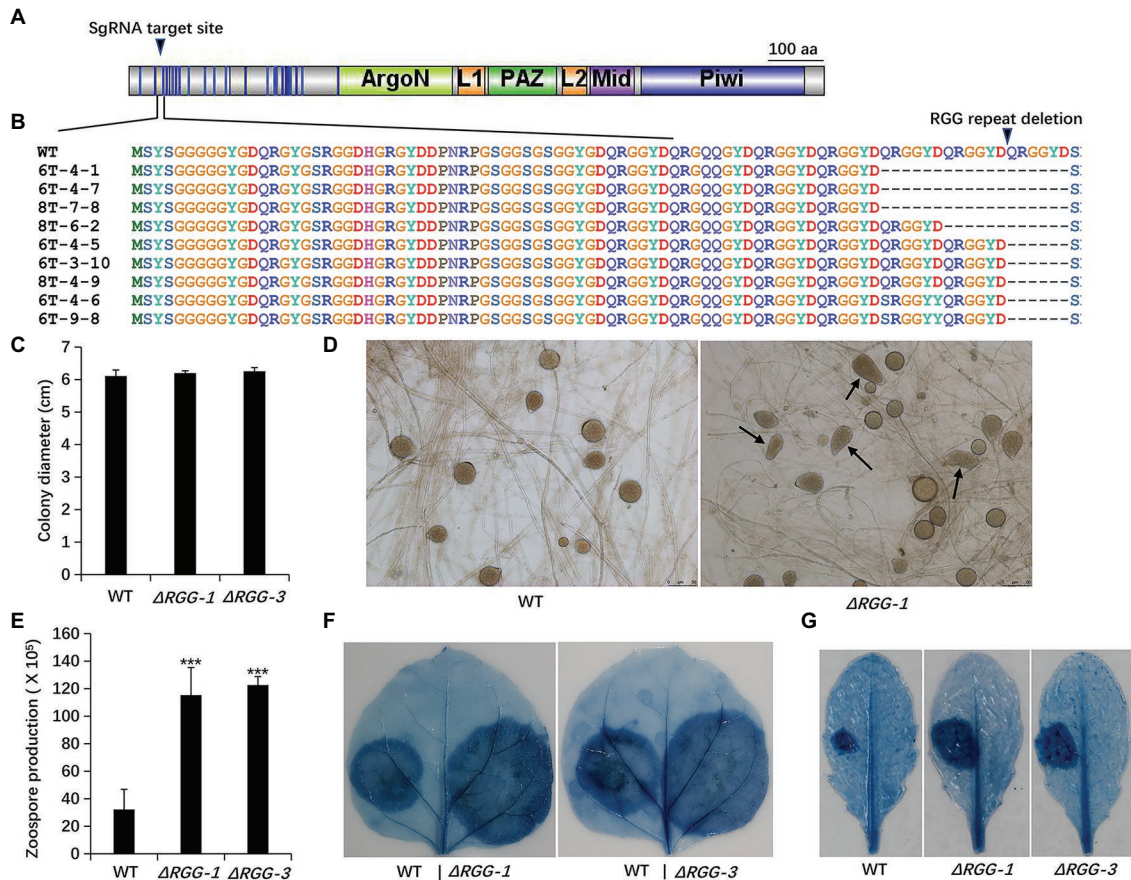
We examined the expression pattern of five *PpAGO* genes using RNA-seq data of *P. parasitica* Pp016 before and after infection of *N. benthamiana* (Jia, 2017). The results showed that *PpAGO3* is highly expressed in mycelia (Supplementary Figure 1) during which the class of the 25–26 nt sRNA is abundant (Jia et al., 2017).

To investigate the potential biological function of *PpAGO*, we employed CRISPR-Cas9 gene editing system (Fang et al., 2017; Zhang et al., 2020) to mutate *PpAGO3*. The N-terminal sequence of PpAGO3 contains 24 copies of the RGG motif. Through two independent transformation experiments, we obtained nine *PpAGO3* mutants all with three types of mutations that carry a deletion of 1–3 copies of the RGG domain in the N terminus without frame shifts or complete knockout (Figures 1A,B), suggesting PpAGO3 is vital to *P. parasitica*.

Phenotypic analysis showed that the *PpAGO3*<sup>ΔRGG</sup> mutants displayed stronger growth vigor than the wild-type strain, showing more dense and thicker mycelia on 5% CA agar plates, though the colony diameters remained unchanged (Figure 1C). In addition, *PpAGO3*<sup>ΔRGG</sup> mutants always produce some abnormal sporangia (Figure 1D) and released many more zoospores than wild-type strain (Figure 1E). More interestingly, *PpAGO3*<sup>ΔRGG</sup> mutants were more invasive than the wild-type *P. parasitica* on *N. benthamiana* leaves (Figure 1F). Similarly, *A. thaliana* leaves infected with *PpAGO3*<sup>ΔRGG</sup> mutants also showed stronger disease phenotypes (Figure 1G). These results suggest that *PpAGO3* plays vital role in the pathogenicity and development of *P. parasitica*.

### The Expression of 25–26 nt sRNAs Derived From Effector Genes Is Significantly Changed in the Mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>

Our previous study showed that the 25–26 nt sRNAs are enriched at the vegetative mycelium stage and is associated with high level of silencing of numerous effector genes in *P. parasitica*, including 40% RXLR (226) and 50% CRN (147) effector genes



**FIGURE 1 |** Generation and phenotypic analysis of *Phytophthora parasitica* mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>. **(A)** The designed sgRNA targeting sites on *PpAGO3* protein. **(B)** Mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> were generated by CRISPR-Cas9 technology. Three types of mutation were obtained: an 18 aa (amino acids) deletion (three QRGYD repeats) in mutants 6T-4-1, 6T-4-7, and 8T-7-8; a 12 aa deletion (two repeats) for mutant 8T-6-2; and a six aa deletion (one repeat) for mutants 6T-4-5, 6T-3-10, 8T-4-9, 6T-4-6, and 6T-9-8. Besides that, a single amino acid substitution within RGG motif was notable for mutants 6T-4-6 and 6T-9-8. Mutants *PpAGO3*<sup>ΔRGG1</sup> (*ΔRGG-1*) and *PpAGO3*<sup>ΔRGG3</sup> (*ΔRGG-3*) showed similar growth rate to the wild-type, as shown by *t*-test **(C)**, produced abnormal sporangium **(D)** and more zoospores than the wild-type (Student test: \*\*\**p* < 0.001) **(E)**, and caused larger lesions than the wild-type on *Nicotiana benthamiana* **(F)** and on *Arabidopsis thaliana* **(G)**. The detached 5-week-old *N. benthamiana* and 4-week-old *A. thaliana* leaves were inoculated with mycelial discs and the water-soaked lesions were examined by trypan blue staining at 48 hpi. Similar results were obtained from three independent experiments.

(Jia et al., 2017). The high level of *PpAGO3* expression in vegetative hyphae, together with the enhanced virulence of the *PpAGO3*<sup>ΔRGG</sup> mutants, led us to assume that *PpAGO3* may interact with 25–26 nt sRNAs to regulate expression of effector genes. Small RNA sequencing was therefore conducted by using RNA isolated from fresh hyphae tissues of the wild-type and mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>.

Clean reads of 18–45 nt sRNAs that mapped to *P. parasitica* genome (*P. parasitica* INRA-310 version 3.0, Assembly Dev initiative, Broad Institute)<sup>4</sup> excluding the rDNA, mtDNA, and tRNA sequences, were obtained for further analysis. The overall sRNA counts and 25–26 nt sRNA percent did not seem to be affected in mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> (Supplementary Table 1). Importantly, this analysis detected differential accumulation of the 25–26 nt

sRNAs specific to some RXLR and CRN effector genes (Supplementary Table 2) compared to the wild-type strain. Of the 226 RXLR effector genes, 36 (15.9%) contained differentially accumulated 25–26 nt sRNAs in the gene body and 500 nt upstream and downstream flanking sequences in mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>, of which 24 genes showed increased and 12 decreased sRNA accumulation (Figure 2A). For the 147 predicted CRN effector genes, 34 (23.13%) displayed differential 25–26 nt sRNA accumulation in the mutants compared to the wild-type strain (Supplementary Table 2), with 17 showing increased and 17 reduced sRNA accumulation (Figure 2A).

Our previous studies showed that most of sRNAs in *P. parasitica* are derived from both sense and antisense strands of the genes typical of double-stranded RNA (dsRNA) processing (Jia et al., 2017). For 49 of the 70 effector genes (36 RXLR and 34 CRN effector genes), 25–26 nt sRNAs of both sense

<sup>4</sup><https://www.broadinstitute.org/>

and antisense polarities were differentially expressed in the *PpAGO3*<sup>ΔRGG</sup> mutants compared to the wild-type strain. For example, the effector genes *PPTG\_18147*, *PPTG\_18920*, and *PPTG\_22243*, showed a large number of 25–26nt sense and antisense sRNA in the wild-type strain, but these 25–26nt sRNAs could not be detected in mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> (Figures 2B–D). On the contrary, both the sense and antisense 25–26nt sRNAs matching the effector genes *PPTG\_07435*, *PPTG\_12078*, and *PPTG\_11304*, showed increased accumulation in the mutants (Figures 2E–G). These results indicated that PpAGO3 is involved in the accumulation of 25–26nt sRNAs in *P. parasitica* that have both sense and antisense polarities typical of dsRNA-derived sRNAs.

### Expression Changes of 25–26nt sRNAs Associated With Non-effector Genes in the Mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>

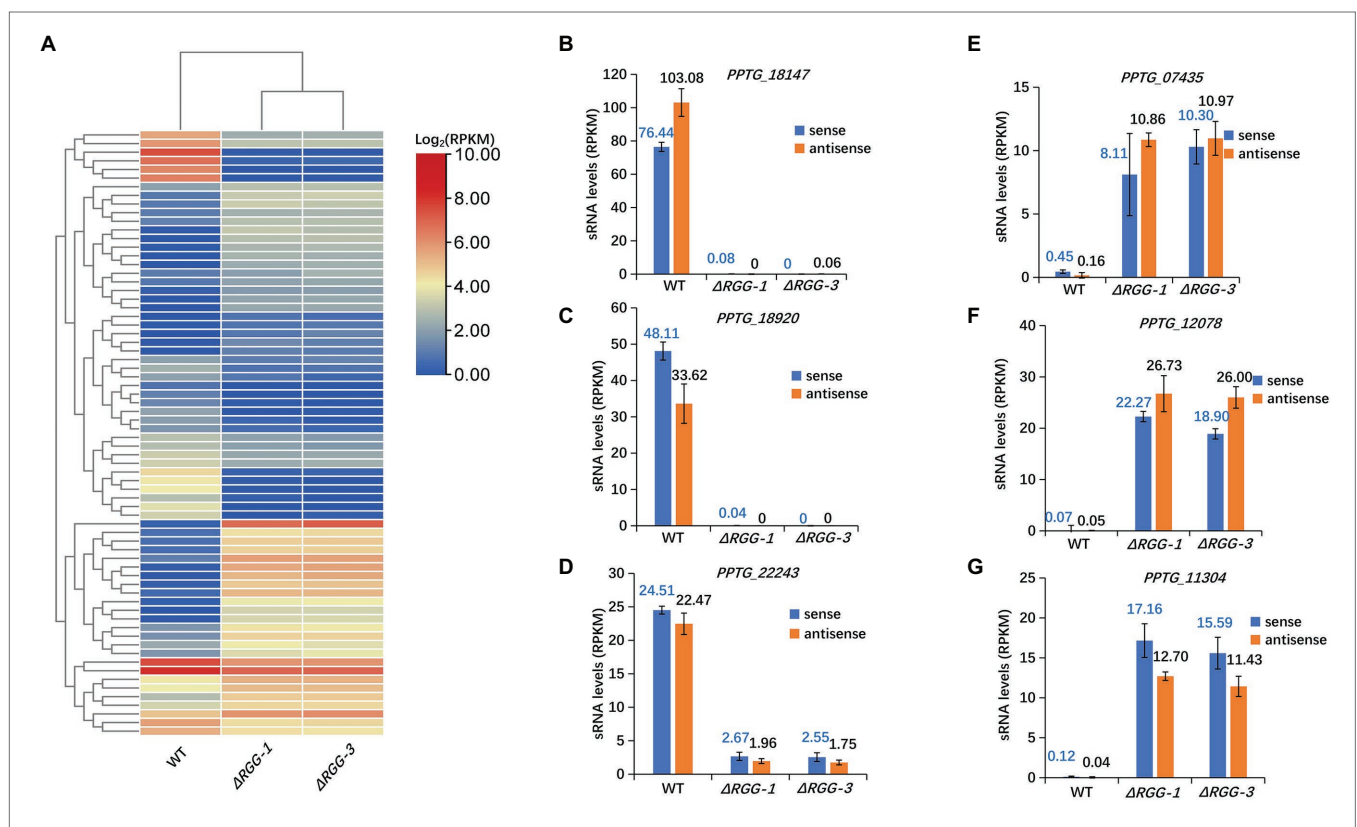
In addition to effector genes, differential accumulation of 25–26nt sRNA was also observed for 2,508 non-effector genes. Of these genes, 1,607 showed increased sRNA accumulation and 901 showed reduced accumulation (Supplementary Table 2).

The differentially accumulated sRNAs from 1808 genes contained both sense and antisense populations (Figure 3A), which provided more evidence that these differentially expressed sRNAs are generated from dsRNA and associated with PpAGO3 protein.

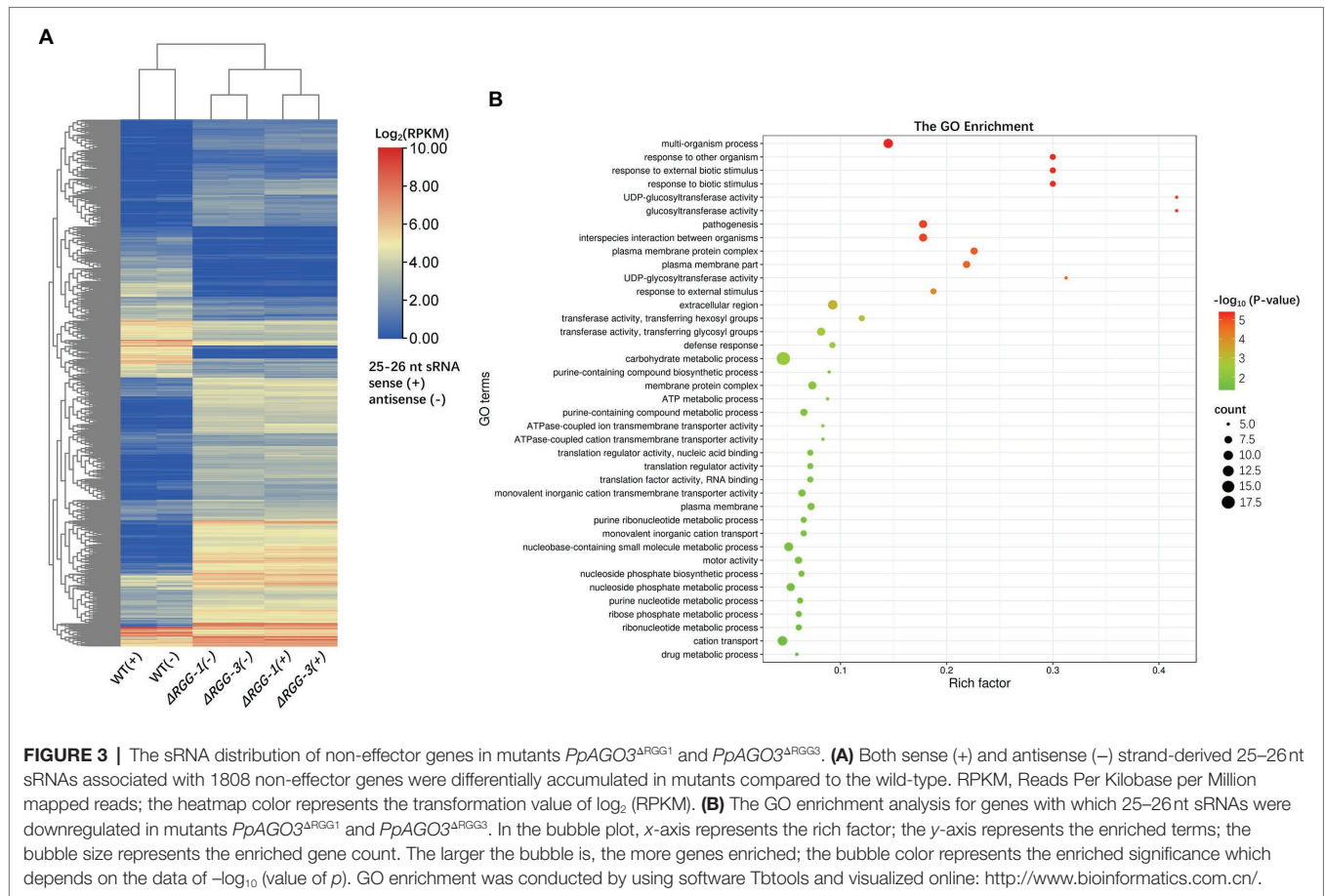
GO enrichment analysis of these 2,508 non-effector genes showed that the downregulated sRNAs were mainly associated with genes in the Biological Process level category, including multi-organism process, response to external biotic stimulus, response to biotic stimulus and to other organism, pathogenesis, and interspecies interaction between organism terms (Figure 3B). The association of differentially accumulated 25–26nt sRNAs with biotic response and pathogenesis genes, in addition to effector genes, further suggest the importance of these sRNAs in the regulation of *Phytophthora* pathogenesis.

### Upregulated Effector Genes in the Mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>

The expression variation of effector gene-associated 25–26nt sRNAs in *PpAGO3*<sup>ΔRGG</sup> mutants and increased virulence of the mutants prompted us to examine if expression of effector genes were influenced. RNA sequencing (RNA-seq) was



**FIGURE 2 |** Significant changes in accumulation of 25–26nt sRNAs homologous to 70 cytoplasmic effector genes in mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> compared to the wild-type *P. parasitica*. (A) Heatmap for 25–26nt sRNA accumulation in wild-type, and mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>. The accumulation of 25–26nt sRNAs was increased for those associated with 24 RXLR and 17 CRN effector genes, and was reduced for those associated with 12 RXLR and 17 CRN effector genes in mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>. The color represents the transformation value of log<sub>2</sub> (RPKM). (B–G) The expression levels of sense and antisense 25–26nt sRNAs associated with six representative effector genes in wild-type, and mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>. Both sense and antisense 25–26nt sRNAs associated *PPTG\_18147* (B), *PPTG\_18920* (C), and *PPTG\_22243* (D) were highly accumulated in the wild-type but reduced dramatically in mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>. On the contrary, the sense and antisense 25–26nt sRNAs associated with *PPTG\_07435* (E), *PPTG\_12078* (F), and *PPTG\_11304* (G) were significantly increased in *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> mutants compared to the wild-type.



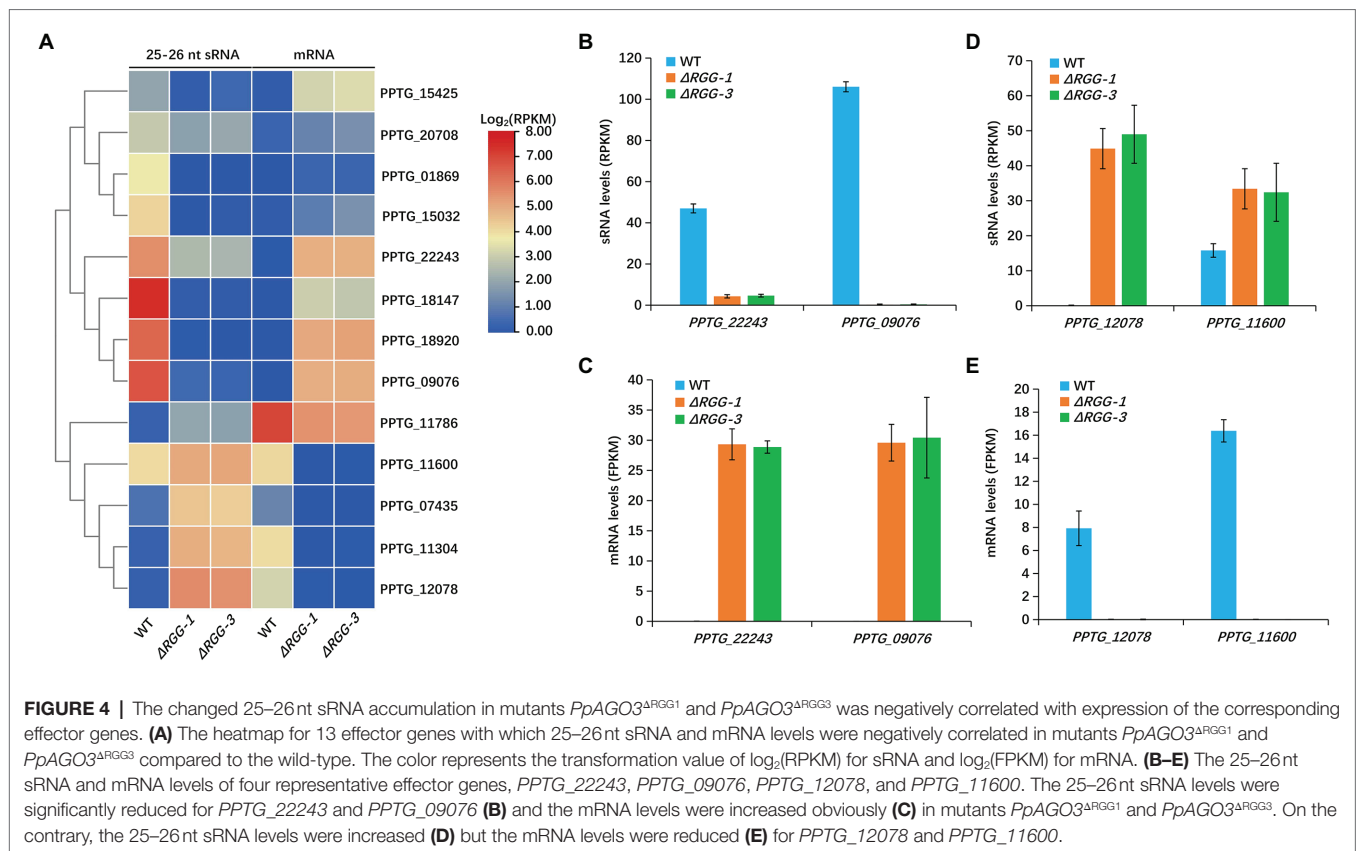
performed on wild-type and mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> using RNA samples extracted from fresh mycelia. Analysis of the RNA-seq data revealed that 24 RXLR effector genes, 10.6% of the 226 predicted RXLR effector genes in *P. parasitica*, were differentially expressed ( $|\log_2$  Fold change| > 1 and Padj < 0.05; **Supplementary Table 3**). Of these 24 genes, 12 showed increased mRNA level while the other 12 reduced mRNA level (**Supplementary Table 3**). For the CRN effector genes, 18.4% (27/147) genes exhibited differential expression with 17 being upregulated and 10 downregulated (**Supplementary Table 3**). In total, 51 effector genes were differentially expressed in the *PpAGO3*<sup>ΔRGG</sup> mutants compared to the wild-type strain.

We then investigated whether the effector genes with differential mRNA level were associated with differentially accumulated 25–26 nt sRNAs. Overlapping the 51 differentially expressed effector genes (**Supplementary Table 3**) with the 70 effector genes that had differentially accumulated 25–26 nt sRNAs (**Figure 2A**) identified 14 effector genes that showed significant changes in both 25–26 nt sRNA accumulation and mRNA level. Interestingly, except for *PPTG\_01844*, the remaining 13 effector genes, including 4 RXLR and 9 CRN effector genes, all showed a negative correlation between sRNA accumulation and mRNA expression level (**Figure 4A**). In particular, 8 genes showed reduced accumulation of 25–26 nt sRNA that correlated

with increased gene expression level in *PpAGO3*<sup>ΔRGG</sup> mutants compared to the wild-type strain, whereas five genes showed increased sRNA accumulation with reduced gene expression (**Figure 4A**). For instance, *PPTG\_22243* and *PPTG\_09076* both showed a dramatic reduction in 25–26 nt sRNA abundance in *PpAGO3*<sup>ΔRGG</sup> mutants (**Figure 4B**), which correlated with strong upregulation of gene expression at the transcript level (**Figure 4C**). On the contrary, *PPTG\_12078* and *PPTG\_11600* showed an increase in 25–26 nt sRNA level but with reduction on mRNA level (**Figures 4D,E**). Taken together, these results suggest that PpAGO3 interacts with 25–26 nt sRNA to regulate the expression of the sRNA-associated effector genes.

### The PpAGO3-Regulated RXLR Effector Genes *PPTG\_01869* and *PPTG\_15425* Both Contribute to *Phytophthora parasitica* Virulence

We next examined if these upregulated effector genes contribute to the enhanced pathogenicity of the mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup>, by analyzing the virulence function of the effector genes using *A. tumefaciens* infiltration-delivered transient expression. We selected two RXLR effector genes, *PPTG\_01869* and *PPTG\_15425*, which showed strong upregulation of mRNA levels in *PpAGO3*<sup>ΔRGG</sup> mutants with dramatic reduction of 25–26 nt



sRNA accumulation (Figures 5A–D). *Nicotiana benthamiana* leaves infiltrated with the *PPTG\_01869*-overexpression construct and inoculated with *P. parasitica* mycelial disks developed significantly larger lesions at 2 days post-inoculation than leaves infiltrated with the control GFP construct (Figure 5E). Similarly, *A. tumefaciens*-mediated overexpression of *PPTG\_15425* strongly enhanced *P. parasitica* infection (Figure 5F). These results indicated that the *PpAGO3*-regulated *PPTG\_01869* and *PPTG\_15425* effector genes play a positive role in *P. parasitica* virulence.

## DISCUSSION

A number of reports have suggested that sRNA pathways participate in the silencing of effector genes in *Phytophthora* species (Vetukuri et al., 2012; Qutob et al., 2013; Jia et al., 2017; Wang et al., 2019). AGO proteins, as the key component of RNAi pathway, were reported to be associated with effector gene-derived sRNA (Vetukuri et al., 2011, 2012; Åsman et al., 2016). These findings suggested the potential function of AGO proteins and sRNAs in the effector genes regulation in *Phytophthora* but the real functional role of the RNA silencing pathways in *Phytophthora* pathogenicity has yet to be confirmed.

In this study, we used the model organism *P. parasitica* to examine the function of AGO in effector gene regulation and pathogenicity. Through sequence analysis, five AGO proteins were identified which all consisted of the conserved domains,

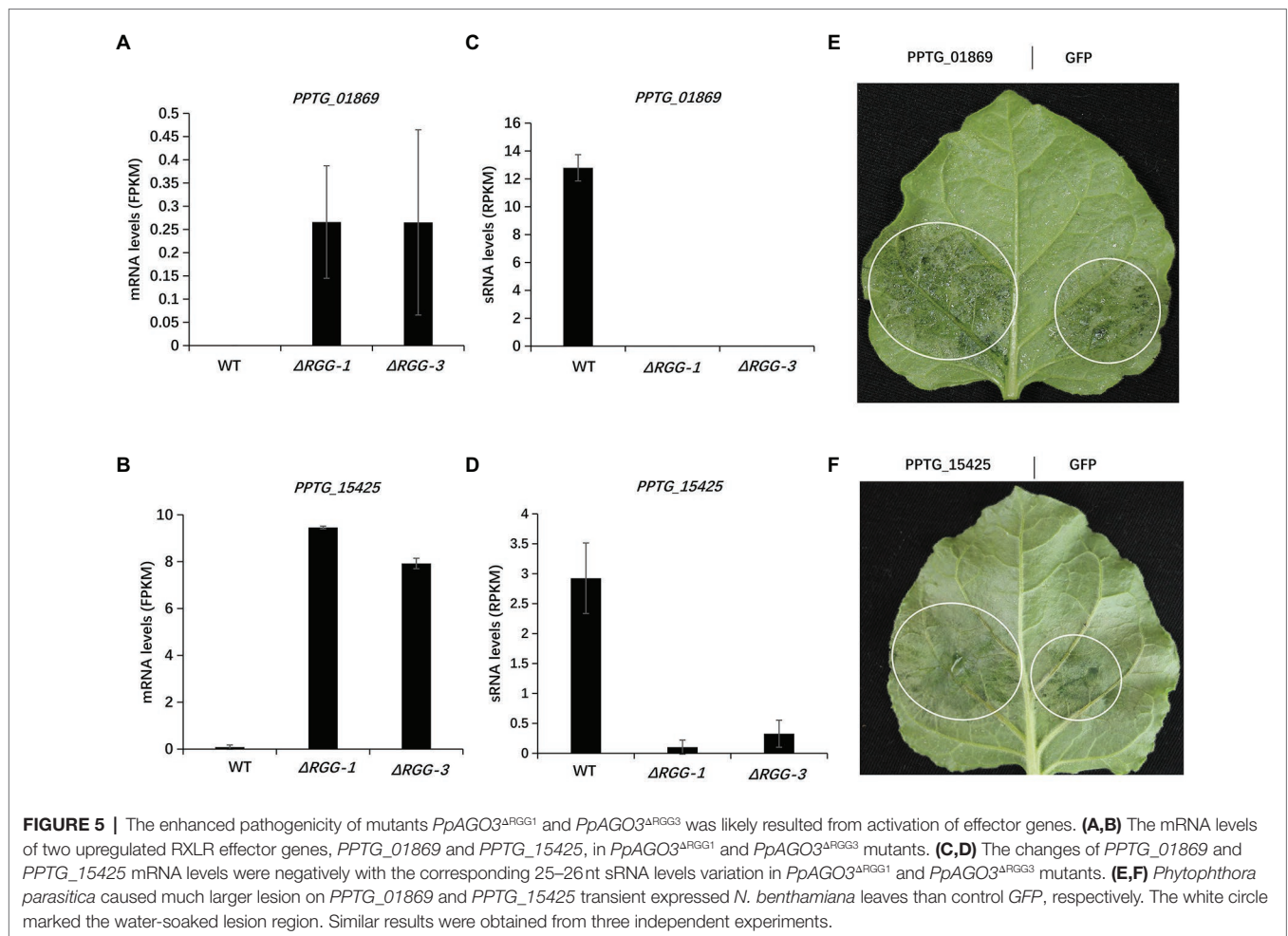
PAZ, Mid and Piwi. Functional studies focused on *PpAGO3*, a unique member with 24 RGG repeats at the beginning of the N terminus and highly expressed in vegetative hyphae.

Evidence for the involvement of *PpAGO3* in the pathogenicity of *P. parasitica* was first suggested by the increased growth vigor of the mutants *PpAGO3*<sup>ΔRGG1-3</sup> deleted with 1–3 copies of the RGG domain, which were generated using the CRISPR-Cas9 system. These mutants displayed increased growth on medium and produced abnormal sporangia and greatly increased number of zoospores. More significantly, the *PpAGO3*<sup>ΔRGG</sup> mutants were much more invasive in the tested host plants *N. benthamiana* and *A. thaliana* than the wild-type strain.

AGO proteins regulate gene expression through interactions with sRNAs. Consistent with this, our sRNA sequencing analysis of the mutants *PpAGO3*<sup>ΔRGG1</sup> and *PpAGO3*<sup>ΔRGG3</sup> both detected altered accumulation of 25–26 nt sRNAs homologous to 70 effector genes, and 13 of these effector genes showed an inverse correlation between their expression levels and the abundance of the associated 25–26 nt sRNAs. The role of *PpAGO3* in regulating effector gene expression is further supported by the opposite developmental stage expression profiles of the *PpAGO3* and some of the effector genes. Three such effector genes showed low expression levels at the mycelium stage where the level of *PpAGO3* expression was the highest. In contrast, these genes were induced during the infection where the expression of *PpAGO3* is reduced.

The functional significance of *PpAGO3* regulation of effector genes was shown by the transient overexpression assay of two





*PpAGO3*-regulated effector genes, *PPTG\_01869* and *PPTG\_15425*. These two genes were strongly upregulated in the *PpAGO3*<sup>ΔRGG</sup> mutants, which correlated inversely with reduced abundance of 25–26 nt sRNAs. Overexpression of both of these two effector genes, using agrobacterium infiltration in *N. benthamiana* leaves, enhanced the infection of *P. parasitica*. This result suggests that the enhanced virulence of the *PpAGO3*<sup>ΔRGG</sup> mutants is related to the activated expression of the sRNA-regulated effector genes. Taken together, our results provided compelling evidence that *PpAGO3*, in conjunction with 25–26 nt sRNAs, plays a major role in the regulation of effector gene expression in *P. parasitica*, and this regulation contributes to the virulence of the pathogen in host plants. However, how *PpAGO3* interact with 25–26 nt sRNAs and how the effector genes are regulated, remain to be further studied.

One question is why these effector genes need to be regulated by the sRNA silencing pathways. Here we propose three possible scenarios. First, it was considered that some effectors could trigger plant immunity as avirulence factors, and suppression of these effectors would help to evade the perception by host plants. Consistently, some avirulence effector genes are suppressed in *P. infestans* and *P. sojae* (Shan et al., 2004; Wang et al., 2019), and the silencing of *PiAGO1*, *PiAGO4*, or *PiAGO5* in

*P. infestans* is associated with the accumulation of *PiAvrblb1* homologous 32 nt sRNA (Vetukuri et al., 2011, 2012). Second, some effectors might be super-virulent to host plants, and silencing of these effector genes is required to establish a balance between successful infection and continuous evolution in the host. The last possible scenario is that, in order to conserve energy, some effectors are suppressed at the mycelium stage but only derepressed when needed during the infection process, like the effector genes *PPTG\_01869* and *PPTG\_15425* shown in this study. Further studies are needed to investigate these hypotheses.

Besides the predicted effector genes, *PpAGO3* appears to regulate many other genes in *P. parasitica*. The RNA-seq analysis identified a total of 3,047 genes with differential expression in *PpAGO3*<sup>ΔRGG</sup> mutants (Supplementary Table 3), and sRNA-seq data detected 2,578 genes with differential accumulation of 25–26 nt sRNA (Supplementary Table 2). In total, across the *P. parasitica* genome, 509 non-cytoplasmic effector genes were identified to show negative correlation between 25 and 26 nt sRNA abundance and mRNA level (Supplementary Figure 2A). These genes include three INF-like genes (Supplementary Figures 2B,C), one of which could induce cell death shown by our unpublished work. The function of

these non-cytoplasmic effector genes in *Phytophthora*–host plant interactions is worth further investigation.

Some of the genes downregulated in *PpAGO3*<sup>ARGG</sup> mutants were associated with increased abundance of 25–26 nt sRNA accumulation, suggesting a role of these sRNAs in the high-level silencing of these genes. It is possible that other AGO members also interact with the 25–26 nt sRNAs, and in the *PpAGO3*<sup>ARGG</sup> mutants, these AGOs function to repress the expression of these genes. Future studies should examine the interactions of 25–26 nt sRNAs with the different AGO members in *P. parasitica*.

For AGO proteins, a diverse number of RGG motifs was found localized in the N-terminal region of each PpAGO proteins (**Supplementary Figure 1**). The N-terminal RGG domain was reported to play an important role in AGO-mediated RNA silencing in *T. brucei* and *T. gondii* (Shi et al., 2004, 2009; Musiyenko et al., 2012), and is also important for the function of other RGG-containing proteins in pre-mRNA splicing, transcription and mRNA translation processes (Rajyaguru and Parker, 2012; Thandapani et al., 2013; Chong et al., 2018). In this study, our results suggested that the RGG domain is also functionally important in *P. parasitica*.

In conclusion, the RGG domain-changed *PpAGO3*<sup>ARGG</sup> mutants have allowed us to generate compelling evidence that PpAGO3 plays an important role in regulating effector gene expression in *P. parasitica* through interaction with the 25–26 nt class of sRNAs. Future studies should investigate the interactions between PpAGO3 and the 25–26 nt sRNAs and how the 25–26 nt sRNAs are generated and how they direct silencing of the effector genes. Moreover, a detailed functional analysis of the RGG and other domains as well as the full-length protein of PpAGO3 were also necessary, especially for the role in *Phytophthora*–host plant interactions.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories

and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

WS, YM, and JX designed the experiment. JX, YL, JJ, WX, CZ, GH, XG, and YM performed the experiment. JX, WS, JJ, and CZ analyzed the data. JX and WS wrote the paper with suggestions from all authors. All authors contributed to the article and approved the submitted version.

## FUNDING

This study received funding from National Natural Science Foundation of China (31561143007), China Agriculture Research System (CARS-09), and the State Administration of Foreign Experts Affairs (#B18042). The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

## ACKNOWLEDGMENTS

We thank Brett Tyler (Oregon State University, United States) for providing the CRISPR-Cas9 vector and helpful suggestions, Ming-Bo Wang (CSIRO Agriculture and Food, Canberra, Australia) for suggestions and manuscript revision, and Patrick Schäfer (Ulm University, Germany) for useful suggestions.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2022.856106/full#supplementary-material>

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