Filamentous pathogen effectors interfering with small RNA silencing in plant hosts

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Abstract (100 words)

Filamentous eukaryotic pathogens including fungi and oomycetes are major threats of plant health. During the co-evolutionary arms race with the hosts, these pathogens have evolved a large repertoire of secreted virulence proteins, called effectors, to facilitate colonization and infection. Many effectors are believed to directly manipulate targeted processes inside the host cells; and a fundamental function of the effectors is to dampen immunity. Recent evidence suggests that the destructive oomycete pathogens in the genus *Phytophthora* encode RNA silencing suppressors. These effectors play an important virulence role during infection, likely through their inhibitory effect on host small RNA-mediated defense.

Introduction

RNA silencing is a universal gene regulation mechanism in eukaryotes that is also known as RNA interference or RNAi. Central players of RNA silencing are small non-coding RNAs (sRNAs), which guide sequence-specific repression of target genes through transcriptional gene silencing by mediating DNA, histone and chromatin modifications [1], and post-transcriptional gene silencing by mediating transcript cleavage and translational repression [2]. In plants, sRNAs have been extensively studied for their regulatory roles in development [3], abiotic stress response [4], and anti-viral defense [5]. However, there is an accumulating body of evidence suggesting that sRNAs also play an important role during interactions with filamentous eukaryotic pathogens including fungi and oomycetes. In particular, plant mutants in the core RNAi pathway exhibit altered resistance [6-8]; and

specific plant sRNAs influence host-pathogen interactions [9-11]. Recently, effector proteins with RNAi suppression activity were discovered from oomycete pathogens in the genus *Phytophthora* [12]. Together with the well-characterized viral suppressors [13] and the previously reported bacterial suppressors [14], the discovery of *Phytophthora* suppressors of RNA silencing (or PSRs) suggests that manipulating host RNA silencing process is a common virulence strategy employed by pathogens across the kingdoms to facilitate infection [15]. Furthermore, these findings support sRNAs as integral components of plant defense system that have a profound impact on the interactions of plants with *Phytophthora*, and likely other filamentous eukaryotic pathogens including fungi [16].

In this review, we introduce the recent findings on the characterization of PSRs and the role of specific plant sRNA pathways in the interactions with filamentous pathogens. We then discuss the potential mechanisms by which immunity is regulated by these plant sRNAs.

***Phytophthora* effectors with RNA silencing suppression activity**

Compared to viral and bacterial pathogens, plant interactions with filamentous eukaryotic pathogens entail more complex defense-counter defense crosstalk. This is reflected by the hundreds of effectors predicted from oomycete secretomes, especially *Phytophthora*, the destructive plant pathogens [17-19]. Much attention has been focused on the cytoplasmic effectors, which contain a secretion signal peptide and a “host-targeting” motif in the N-terminus. The “RxLR” and the “Crinklers” (CRNs) are two major classes of cytoplasmic effectors that are believed to enter host cells through the specialized infection structure called haustoria [20].

A functional screen of 59 RxLR effectors produced by the soybean pathogen *Phytophthora sojae* revealed two effectors, named PsPSR1 and PsPSR2, that can suppress transgene silencing by inhibiting the accumulation of plant sRNAs [12]. Importantly, ectopic expression of the PSRs in *Nicotiana benthamiana* resulted in hypersusceptibility to *Phytophthora infestans* [12]. Furthermore, expression of a viral suppressor of RNA silencing (VSR) P19 also rendered *N. benthamiana* more susceptible to *P. infestans*, suggesting that these effectors promote infection by targeting host RNA silencing process. This study established a connection between plant sRNAs and the immune response against *Phytophthora* infection.

PsPSR1 broadly influences the accumulation of plant sRNAs

Plants produce two major types of sRNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs), based on their distinctive biogenesis pathways [21]. miRNAs are encoded by the endogenous *MIR* genes; the *MIR* transcripts serve as precursors that are processed by Dicer-like endonucleases (DCLs) to produce miRNAs [22]. On the contrary, siRNAs are derived from invading nucleic acids, such as viruses and transgenes, or from endogenous loci, such as repeats, transposable elements and genes. Precursors of siRNAs are long double-stranded RNAs synthesized by RNA-dependent RNA polymerases (RdRPs); these precursors are then diced into siRNAs. Both miRNAs and siRNAs are loaded into the Argonaute (AGO) proteins for sequence-dependent gene silencing [21,22].

Using transgenic *Arabidopsis* plants expressing *PsPSR1*, the abundance of representative endogenous sRNAs was quantified in order to determine the host targets of this effector. A cross-the-board reduction in sRNA accumulation was observed in the transgenic plants, indicating that PsPSR1 has a general effect on the biogenesis of both miRNAs and siRNAs [12]. Indeed, characterization of the PsPSR1 virulence targets in *Arabidopsis* and soybean revealed a conserved nuclear protein that is required for the accumulation of all major classes of endogenous sRNAs [8]. This protein, designated PSR1-Interacting Protein 1 (PINP1), contains the aspartate-glutamate-alanine-histidine-box RNA helicase domain and appears to be involved in the assembly of sRNA processing complexes. Importantly, *PINP1*-silenced plants (null mutants cause embryonic lethality) were also hypersusceptible to *Phytophthora* infection, likely as a consequence of the reduced sRNA levels [8]. At present, how PINP1 regulates the sRNA processing complexes and whether the RNA helicase activity is required for this function remains unknown. Nonetheless, these findings started to form a link between the levels of sRNAs and *Phytophthora* resistance in *Arabidopsis*.

PsPSR2 specifically affects secondary siRNAs

PsPSR1 and PsPSR2 do not share sequence similarity. In contrast to the general inhibitory activity of PsPSR1, PsPSR2 only affects the accumulation of secondary siRNAs in *Arabidopsis* [12]. Generated from miRNA-targeted transcripts, secondary siRNAs can amplify the original silencing effect on the target genes due to increased sRNA abundance and expanded target sites [23]. This mechanism is particularly effective on regulating multiple members in large

gene families. Importantly, secondary siRNAs have been shown to play a prominent role in regulating nucleotide-binding leucine-rich repeat (*NB-LRR*) genes, which encode the canonical disease resistance proteins [24-30]. Another gene family that is regulated by secondary siRNAs encodes pentatricopeptide repeat (PPR) proteins [31-32]. Similar to *NB-LRR*, *PPR* genes also form a large family (~450 in *Arabidopsis* and >400 in most plant species). These nucleus-encoded regulator proteins are targeted to mitochondria and chloroplasts and have a profound impact on the function of these organelles [33]. Mitochondria and chloroplasts are tightly linked to immunity by producing reactive oxygen species (ROS) and the defense hormone salicylic acid (SA) [34,35]. For example, *Arabidopsis* mutants of a *PPR* gene, *PPR2*, showed enhanced ROS production shortly after bacterial infection [36]. Interestingly, *PPR2* is regulated by a siRNA, miR400, which is also able to trigger secondary siRNA production [31,36]. As such, secondary siRNAs could determine plant resistance levels by acting as the central regulators of *NB-LRR* and *PPR* genes.

The contribution of *PsPSR2* to virulence was indicated by the observation that *PsPSR2*-silenced *P. sojae* mutants exhibited reduced virulence on soybean [12]. Consistently, soybean roots expressing *PsPSR2* were hypersusceptible to *P. sojae* infection. In addition, *Arabidopsis* transgenic plants expressing *PsPSR2* also showed hypersusceptibility to *Phytophthora capsici*, although the plants did not have observable developmental deficiency [37]. Interestingly, *PsPSR2*-expressing *Arabidopsis* showed wild-type-like phenotype concerning response to the infection of the bacterial pathogen *Pseudomonas syringae* [37]. It therefore seems that the secondary siRNA pathway is particularly important for plant defense against *Phytophthora*.

PsPSR2 belongs to a large RxLR effector family in *Phytophthora*

Sequence clustering of all predicted RxLR proteins from four *Phytophthora* species (*P. sojae*, *P. infestans*, *P. capsici*, and *Phytophthora ramorum*) suggests that *PsPSR2* belongs to a single subgroup of 66 RxLR effectors from the four *Phytophthora* species. *PiPSR2*, a close homolog of *PsPSR2* in *P. infestans*, can also suppress RNA silencing and promote *Phytophthora* infection in *N. benthamiana* [37], indicating that they may manipulate similar targets in plant hosts.

A common observation of pathogen effectors is that they are rapidly evolving during the

co-evolutionary arms race with host immune system [38]. The RxLR effectors are highly variable among *Phytophthora* species and sequence-conserved effectors are rare [19]. However, PsPSR2 exhibits a relatively high conservation level when compared to homologs from other *Phytophthora* species (Fig. 1A). In particular, the PsPSR2-like effectors share a similar protein structure, which contains three conserved motifs, namely W, Y, and L, that are arranged in a specific, regular manner (Fig. 1B). PsPSR2 contains five repeated L-W-Y modules, one W-Y module at the N-terminus, and one L-W module at the C-terminus. This arrangement is preserved in PiPSR2 with the only difference being that PiPSR2 has six repeated L-W-Y modules in the central region. Similar motif modules could also be observed in nearly all the identified PsPSR2-like effectors in *P. sojae* and other *Phytophthora* species (Fig. 1B). Remarkably, the WY-(LWY)_n-LW arrangement is the dominant pattern that is present in 53% of the effectors in this subgroup. This raises the question that whether some of these effectors also possess RNA silencing suppression activity. A survey on the functions of effectors belonging to this subgroup and further characterization on their intra- and inter-species variation will provide important information on this conserved effector family and shed light into the conservation of RNA silencing suppression as a virulence strategy of *Phytophthora*.

W, Y, and L motifs are known to be enriched in oomycete RxLR effectors [19,39-40]. So far, such motifs have not been found in fungi or other organisms. Previous structural analysis on effectors containing the W-Y motif module revealed a three or four α -helices bundle that is important for effector recognition by corresponding NB-LRR proteins in *Arabidopsis* [41-43]. Recent functional analysis of an RxLR effector with five W-Y repeats demonstrated that its virulence function was dependent on the C-terminal domain, outside the repeat region [44]. It would be interesting to determine the impact of the WY-(LWY)_n-LW module on the RNA silencing suppression activity of PSR2 and the virulence activity of other effectors in this family. Analysis of protein structure and the plant targets of PSR2-like effectors will provide molecular basis of the function of this specific repeat arrangement.

The role of secondary siRNAs in plant response to filamentous pathogen infection

Several lines of evidence support a particularly important role of secondary siRNAs in plant response to *Phytophthora* infection: 1) secondary siRNAs are induced during the early

infection stages in soybean inoculated with *P. sojae* [9]; 2) *PsPSR2*-expressing *Arabidopsis* shows a specific reduction on the accumulation of secondary siRNAs [12], and these plants are highly susceptible to *Phytophthora* infection [37]; 3) silencing of *PsPSR2* leads to largely reduced virulence of *P. sojae* [12]; 4) *PsPSR2*-like effectors are widely produced by *Phytophthora*.

In addition to *Phytophthora*, there are indications that secondary siRNAs also influence plant defense against fungal pathogens. This is mainly based on genetic characterizations of mutants defective in the biogenesis of secondary siRNAs. For example, the RNA-dependent RNA polymerase RDR6 is required for the synthesis of double-stranded RNA precursors together with a RNA-binding proteins SGS3; the precursor RNAs are subsequently processed by Dicer-like 4 (DCL4) to produce secondary siRNAs. In *Arabidopsis*, secondary siRNAs are absent from *rdr6*, *sgs3*, and *dcl4* mutant plants [45-48]. Interestingly, these mutants are also hypersusceptible to the fungal pathogen *Verticillium dahliae* [6]. A recent paper on the characterization of an *rdr6* mutant of rice also reported that larger lesions were formed after infection by the fungal pathogen *Magnaporthe oryzae* [49]. On the contrary, *dcl4* mutant *Arabidopsis* plants showed unchanged resistance to the bacterial pathogen *Pseudomonas syringae* [14]. This is in line with the observation that *PsPSR2*-expressing *Arabidopsis* also exhibited wild-type during *P. syringae* infection [37]. These findings suggest a specifically important contribution of the secondary siRNA pathway to plant defense against filamentous pathogens.

RNAi manipulating effectors produced by fungal pathogens?

Similar to oomycetes, fungal pathogens also produce a large repertoire of effectors [50]. Given the evident contribution of plant sRNAs in anti-fungal defense, it would not be surprising if some fungal effectors possess RNA silencing suppression activities. So far, the virulence function and the underlying molecular basis of fungal effectors are largely unknown; nonetheless, two lines of evidence indicate that they may manipulate host RNAi pathway. Firstly, miR9863 in barley represses the expression of the R gene *Mla* through secondary siRNA production [10]. During the infection by the powdery mildew fungal pathogen *Blumeria graminis* f. sp. *Hordei*, the transcript levels of *MIR9863* and the abundances of miR9863-derived secondary siRNAs were increased at the early infection

stage; however, the expression level of *Mla* was also increased [10]. This leads to an interesting possibility that the induction of *Mla* was due to a potential RNA silencing suppressor encoded by the fungal pathogen. In a second study, two miRNAs targeting *NB-LRR* genes were found to be specifically repressed in a resistant tomato cultivar infected by *Fusarium oxysporum*, leading to activation of these resistance genes [11]. Intriguingly, the reduced accumulation of these two miRNAs was not observed from a susceptible cultivar [11], suggesting that it could represent a defense strategy of the tomato plants against a potential activity of fungal RNA silencing suppressors [16]. A similar explanation could also apply to the barley-*B. graminis* f. sp. *Hordei* interaction where miR9863 and its secondary siRNAs only repress the expression of a specific group of *Mla* alleles, reflecting a potential host-pathogen arms race [10]. These studies suggest that suppression of sRNA accumulation and/or function by fungal pathogens could benefit infection in certain plant genotypes.

Future perspectives

Pathogens depend on a multitude of effectors to subvert host immunity. Accumulating evidence demonstrated that the host RNA silencing pathway is a common target that is manipulated by viral, bacterial and oomycete effectors. An important future research area is to identify and characterize RNA silencing suppressors from fungi and oomycetes.

Characterization of viral suppressors of RNA silencing (VSRs) showed that VSRs arose independently multiple times and target diverse RNA silencing components through different mechanisms [15]. Similar convergent evolution will likely be observed in effectors produced by filamentous pathogens that interfere with host RNA silencing network.

Therefore, the only effective approach to identify these potential suppressors is by functional screens, such as the transgene-induced RNA silencing suppression system used to identify viral and *Phytophthora* effectors. This research will determine whether modulating host sRNA pathways has a general significance in the virulence of filamentous eukaryotic pathogens.

Effectors have been used as molecular probes to facilitate understanding of basic cellular processes. Target analysis of PsPSR1 led to the identification of PINP1, a novel regulator of sRNA biogenesis. Research on the host targets of additional RNA silencing suppressors will most definitely identify novel components and regulatory mechanisms in sRNA silencing.

1 These studies will also advance our understanding of eukaryotic pathogenesis and set the
2 foundation to enhance resistance against these devastating diseases.

3 A key piece of puzzle that has not been solved is the target genes regulated by plant sRNAs,
4 especially the secondary siRNAs, that eventually exert influence on the outcome of
5 plant-pathogen interactions. Current evidence suggests that these endogenous plant sRNAs
6 can directly regulate defense-related genes and fine-tune their expression at different
7 infection stages; interference with this regulation may benefit the pathogens by inducing
8 aberrant gene expression. Plant sRNAs could also indirectly influence immunity by regulating
9 the growth/defense tradeoff. Perception of pathogens usually leads to compromised growth
10 as the expense of defense activation [51]. Disrupting sRNA-mediated growth repression is an
11 effective virulence strategy that benefits infection. Another exciting possibility is that plant
12 sRNAs may regulate pathogen genes through Host-induced Gene Silencing (discussed in
13 [16]). Although transfer of endogenous plant sRNAs and direct manipulation of pathogen
14 gene expression has not been shown in filamentous pathogens, the successful silencing of
15 targeted genes in fungal and oomycete pathogens using sRNA constructs expressed in plant
16 hosts [52-54] indicates that plants may produce anti-pathogen sRNAs as a defense
17 mechanism. Identification of these sRNAs will provide important insight into the
18 mechanisms by which plant sRNAs, including secondary siRNAs, affect the outcome of
19 host-pathogen interactions and advance our understanding of the virulence function of RNA
20 silencing suppressors.

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Figure 1. Conservation and protein structure of PsPSR2 and PsPSR2-like effectors.

A, Relative conservation levels of 379 *P. sojae* RxLR effectors with other oomycete species. TBLASTN was used with an E-value cutoff $< 1e-5$ to compare each predicted *P. sojae* RxLR effector protein sequence (excluding the Sec secretion signal peptide) with translated genome sequences of the indicated species. Blast Score Ratio (calculated by dividing the query score with the self-comparison score for each protein) of the best hit was calculated for each effector to determine a relative conservation level. High BSR values indicate relatively high conservation levels. The oomycete species included in this analysis are *Phytophthora infestans*, *Phytophthora capsici*, *Phytophthora parasitica*, *Phytophthora ramorum*, *Hyaloperonospora arabidopsidis*, *Peronospora hyoscyami* f. sp. *tabacina*, *Plasmopara halstedii*, *Pythium ultimum*, and *Saprolegnia parasitica*.

B, Protein motif structure of PsPSR2 and its homologs in four *Phytophthora* species. PsPSR2 homologs were identified from the predicted RxLR effector repertoires of *P. sojae*, *P. infestans*, *P. ramorum*, and *P. capsici* using BLASTP (E-value $< 1e-50$). The predicted locations of the W, Y and L motifs in individual proteins are presented. The MEGA5-constructed Neighbor-Joining tree was compressed from 66 PsPSR2-like effectors to 30 by presenting one representative effector if multiple effectors from the same species form a cluster. PsPSR2 and PiPSR2, with RNA silencing suppression activities, are labeled by *.