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Role of Small RNAs in Host-Microbe Interactions

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Abstract

Plant defense responses against pathogens are mediated by activation and repression of a large array of genes. Host endogenous small RNAs are essential in this gene expression reprogramming process. Here, we discuss recent findings on pathogen-regulated host microRNAs (miRNAs) and small interfering RNAs (siRNAs) and their roles in plant-microbe interaction. We further introduce small RNA pathway components, including Dicer-like proteins (DCLs), double-stranded RNA (dsRNA) binding protein, RNA-dependent RNA polymerases (RDRs), small RNA methyltransferase HEN1, and Argonaute (AGO) proteins, that contribute to plant immune responses. The strategies that pathogens have evolved to suppress host small RNA pathways are also discussed. Collectively, host small RNAs and RNA silencing machinery constitute a critical layer of defense in regulating the interaction of pathogens with plants.

Keywords

miRNAs; siRNAs; dicer; argonaute; RNA silencing suppressors

INTRODUCTION

Small RNAs are 20 to 40 nucleotide (nt)-long noncoding RNA molecules present in most eukaryotic organisms that regulate gene expression in a sequence-specific manner either transcriptionally or posttranscriptionally (5, 11, 98, 99). On the basis of their biogenesis and precursor structure, small RNAs are placed in two distinct groups: microRNAs (miRNAs) and small interfering RNAs (siRNAs). Small RNAs regulate a multitude of biological processes in plants, including development, metabolism, maintenance of genome integrity, immunity against pathogens, and abiotic stress responses. Increasing evidence suggests that small RNAs play a critical role in regulating the interaction of pathogens with plants.

SMALL RNA BIOGENESIS PATHWAYS IN PLANTS

Small RNA pathways in plants have been best characterized in the model plant *Arabidopsis*, and seminal pieces of work involving both forward and reverse genetic screens have delineated the cellular proteins that are involved in biogenesis and function of miRNAs and

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siRNAs. In this section, we present a brief summary of different kinds of small RNA pathways known in *Arabidopsis* (Figure 1). For detailed information, please refer to other reviews (11, 44, 47, 53, 66, 92, 98, 99, 107, 115).

miRNA Pathway

miRNAs are derived from the transcripts of miRNA genes generated by RNA polymerase II. The primary miRNA (pri-miRNA) transcript forms a fold-back structure, which is processed into a stem-loop precursor known as precursor miRNA (pre-miRNA). A protein named DAWDLE (DDL) has been proposed to play an important role in miRNA biogenesis by recruiting predominantly DICER-like protein 1 (DCL1) to pri-miRNA for downstream processing (110). The pre-miRNA is acted upon by DCL1 together with HYL1 (HYPOPLASTIC LEAVES 1) and SE (SERRATE) to form the small RNA duplex. The small RNA duplex is then methylated at the 3' ends by HEN1 (HUA ENHANCER 1) and is exported to the cytoplasm by an exportin homolog, HST (HASTY). Mature miRNA is preferentially incorporated into AGO1 (or AGO10) and guides the complex to the target mRNA for cleavage or translational inhibition on the basis of sequence complementarity.

siRNA Pathways

In contrast to miRNAs that are derived from imperfectly base-paired hairpin loop structures, siRNAs are derived from perfectly paired double-stranded RNA (dsRNA) precursors. These dsRNA precursors are derived either from antisense transcription or by the action of a cellular RNA-dependent RNA polymerase (RDR). Four different types of siRNAs are known in plants: trans-acting siRNAs (ta-siRNAs), natural antisense transcripts (NATs)-derived siRNAs (nat-siRNAs), heterochromatic siRNAs (hc-siRNAs) or repeat-associated siRNAs (ra-siRNAs), and long siRNAs (lsiRNAs). RNA Pol II transcribes noncoding TAS genes, and the long primary transcript products are initially cleaved by miRNAs loaded with RNA-induced silencing complexes (RISCs), resulting in a 5' fragment or a 3' fragment. These fragments then act as a template for synthesis of a complementary strand by the concerted action of RDR6 and SGS3 (46, 99). The resulting dsRNA molecule is acted upon by DCL4 and DRB4 to trigger the subsequent production of ta-siRNAs (37, 46). nat-siRNAs are produced from the overlap regions of sense and antisense transcripts of *cis*-NATs. A significant proportion of most eukaryotic genomes encode overlapping *cis*-NAT genes, which have the potential to generate siRNAs when base pairing between sense and antisense transcripts occurs. Though nat-siRNAs have been shown to play an important function in both abiotic and biotic stresses (8, 55), their roles in other plant processes remains to be investigated. The cellular components involved in production of nat-siRNAs are DCL1 and/or DCL2, HYL1, and HEN1 (8, 55). The nat-siRNAs studied also partially depend on RDR6, SGS3, and Pol IV (8, 55). hc-siRNAs or ra-siRNAs are usually 24 nt in length and are primarily derived from transposons, repeat elements, and heterochromatin regions. Their biogenesis is dependent on the DCL3-RDR2-Pol IV pathway (65, 66, 78). hc-siRNAs or ra-siRNAs function in the RNA-dependent DNA methylation (RdDM) pathway by mediating DNA methylation and/or histone modification at the target sites. In addition to 21 to 24 nt siRNAs, a class of lsiRNAs in the size range of 30 to 40 nt was discovered (54). The biogenesis of lsiRNAs is dependent on DCL1, HYL1, HEN1, AGO7, and HST and partially dependent on RDR6 and Pol IV (54). AtlsiRNA-1 is induced by bacterial pathogen *Pseudomonas syringae* and triggers silencing of the target by destabilizing the target mRNA through decapping and 5'–3' degradation (54).

HOST ENDOGENOUS SMALL RNAs IN PLANT-MICROBE INTERACTIONS

Plants have evolved multiple layers of defense in response to pathogen attacks, and bacterial pathogens provide useful examples of how pathogens are encountered at various levels. The

preliminary interaction between the pathogen and its host is responsible for pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in plants (15, 52). Bacteria counteract PTI by secreting and injecting effector proteins into plant cells, which leads to suppression of PTI. Host plants, in turn, have evolved resistance components such as resistance (R) proteins that can recognize effectors and elicit effector-triggered immunity (ETI) (15, 52).

An important role of small RNAs was first demonstrated in plant growth and development (63, 74). There is increasing evidence, however, that small RNAs are involved in regulating plant responses to adverse conditions, including biotic stresses (13, 51). Antiviral defense involving virus-derived small RNAs is an important example of an interaction between plant and pathogen that is mediated by small RNAs. However, these small RNAs are derived from viruses and are therefore exogenous in origin. Unlike viruses that replicate inside the host cell, bacteria, fungi, and other microbes interact with plants without undergoing DNA or RNA replication and transcription inside the plant cell. In these interactions, host endogenous small RNAs play an important role in counteracting these pathogens. Recent reports have shown that plant endogenous small RNAs, including miRNAs and siRNAs, are integral regulatory components of plant defense machinery against bacteria and fungi.

miRNAs

A number of miRNAs have been linked to biotic stress responses in plants. In this section we discuss the role of these miRNAs in plants when infected by different types of pathogens such as bacteria, viruses, and fungi. Moreover, it is now known that these small RNAs are also important in regulating plant-microbe interaction during nitrogen fixation by *Rhizobium* and tumor formation by *Agrobacterium*.

Bacterial infection—In *Arabidopsis*, the first miRNA discovered to play a role in defense against pathogens was miR393 (70). miR393 is induced by a bacterial flagellin-derived PAMP, Flg22. miR393 negatively regulates auxin signaling by targeting auxin receptors TIR1 (transport inhibitor response 1), AFB2 (auxin signaling F-box protein 2), and AFB3. However, a TIR1 paralog, AFB1, was found to be partially resistant to miR393-mediated cleavage because of extra mismatches in the miRNA target site. Transgenic lines expressing Myc-AFB1 in *tir1-1* background were more susceptible to virulent *Pseudomonas syringae* pv. tomato strain DC3000 (*Pst* DC3000) and displayed enhanced disease symptoms. To determine whether miR393 is involved in race-specific resistance, these transgenic plants were inoculated with avirulent *Pst* DC3000 carrying an effector gene, *avrRpt2*. Bacterial growth in transgenic lines expressing Myc-AFB1 did not differ from non-transformed plants even at 4 days postinoculation (dpi). These data suggest that miR393 has a role in imparting basal resistance but not race-specific resistance. Induction of miR393 was further confirmed by Fahlgren et al. (31) when they carried out deep sequencing of *Arabidopsis* leaves at 1 h and 3 h postinoculation (hpi) with a nonpathogenic strain, *Pst* DC3000 *hrcC*⁻, which has a mutation in the type III secretion system (TTSS). Relative to uninfected leaves, miR393 was induced tenfold in the infected leaves at 3 hpi. Overexpression of miR393a from a strong constitutive promoter resulted in lower levels of TIR1 mRNA in transgenic lines, and these lines exhibited restricted bacterial growth. Besides miR393, two other miRNA families, miR160 and miR167, were also upregulated at 3 hpi. These miRNAs target members of the auxin-response factor (ARF) family of transcription factors that are also involved in auxin signaling (85). Thus, in response to bacterial infection, plants suppress multiple components of the auxin signaling pathways. Another miRNA, miR825, which is predicted to target remorin, zinc finger homeobox family protein, and frataxin-related protein, was also elevated during *Pst hrcC*⁻ infection (31).

The interaction between the plant and another bacterium, *Agrobacterium tumefaciens*, is of general interest because of the widespread use of *A. tumefaciens* for transferring genes into plant genomes. Pruss et al. (79) showed that an oncogenic strain of *A. tumefaciens* induced miR393 at the infiltrated zones, whereas the disarmed strain (i.e., a strain lacking tumor-inducing properties) did not induce miR393. Interestingly, the levels of miR393 and miR167, which repress the auxin signaling pathway, were greatly reduced in tumors induced by *A. tumefaciens* infection. Derepression of the auxin signaling pathway promotes tumor growth. Moreover, roots and stems of miRNA-deficient mutants, *dc11* and *hen1*, were immune to *A. tumefaciens* infection (28). All of these results indicate a possible role of miR393 and miR167 in regulating auxin responses in tumor tissue. However, the levels of all other miRNAs studied were moderately altered in tumor tissue. All these results demonstrate the importance of miR393 and miR167 in coordinating the interaction between plants and *A. tumefaciens*.

Recognition of pathogens by plants can initiate an oxidative burst resulting in enhanced abundance of reactive oxygen species (ROS) (25, 58, 72, 94, 104). Whereas miR398 is down-regulated in response to oxidative stress imposed by high Cu^{2+} or high Fe^{3+} in *Arabidopsis*, the levels of the target mRNAs, Cu/Zn superoxide dismutases 1 and 2 (*CSD1* and *CSD2*), increase significantly (91). To investigate effects of microbe infection on the levels of miR398 and its targets, plants were infected with both virulent *Pst* DC3000 and avirulent *Pst* DC3000 carrying effector *avrRpt2* or *avrRpm1* (48). miR398 was down-regulated by infection with *Pst (avrRpm1)* and *Pst (avrRpt2)* but was unaffected by *Pst* DC3000 infiltration. miR398 cleaves its target mRNA, thereby decreasing the levels of *CSD1* and *CSD2*. During *Pst (avrRpm1)* infection, *CSD1* but not *CSD2* exhibited strong upregulation in transcript levels. The differential response of *CSD2* mRNA to diverse stress conditions suggests the existence of multiple mechanisms that are either dependent or independent of miR398-guided regulation in plants.

Fungal infection—RNA silencing is a robust strategy developed by plants to defend against pathogens, including fungi. Posttranscriptional gene silencing (PTGS) was shown to affect fungal resistance in *Arabidopsis* in studies employing the RNA silencing mutants *sgs2*, *sgs3*, *ago7*, *dcl4*, *nrdp1a*, and *rdr2*, which exhibited enhanced susceptibility to *Verticillium* strains (30).

Lu et al. (61) tested whether small RNAs are involved in the infection of loblolly pine by the endemic rust fungus, *Cronartium quercuum* f. sp. *fusiforme*. Infection with this fungus causes fusiform rust disease, which is characterized by stem and/or branch galls. Small RNAs were cloned from the developing xylem of pine, and 26 miRNAs belonging to four conserved and seven loblolly pine-specific miRNA families were identified. Using small RNA expression profiling, miRNAs involved in disease development were delineated and compared in uninfected pine trees and trees infected with the fusiform rust fungus. The transcript levels for these 11 families of miRNAs were unchanged in roots and in stems above the galls, but transcript levels for 10 of these miRNA families were significantly suppressed in galled stem. These reduced levels of miRNAs in galled stems relative to healthy stems were correlated with increased levels of their target transcripts relative to healthy stems. Interestingly, although the expression of these miRNAs was unchanged in the stem above the gall, their target transcripts were significantly upregulated in stem above the gall as compared with healthy stems. This result suggests that fungal infection at the gall probably immunizes the uninfected stem and may provide protection ahead of the spreading infection. Taken together, these data highlight the complexity of plant-microbe interactions mediated by small RNAs in the galled tissue and the tissue surrounding the gall. The signals responsible for the upregulation of defense responsive genes in the uninfected tissue around the gall remain to be identified.

Viral infection—As discussed earlier, host miRNAs respond to attack by pathogenic fungi and bacteria. This prompts the question of whether host miRNAs respond to viral infection. Two miRNAs, bra-miR158 and bra-miR1885, were greatly upregulated when *Brassica rapa* was infected by Turnip mosaic virus (TuMV) (42). The induction of bra-miR158 and bra-miR1885 is highly specific to TuMV infection because infection of *B. rapa* and *B. napus* with Cucumber mosaic virus, Tobacco mosaic virus (TMV), or the fungal pathogen *Sclerotinia sclerotiorum* had no such change. The putative target for bra-miR1885 is predicted to be a member of the TIR-NBS-LRR class of disease-resistant proteins. It is suggested that bra-miR1885 is a novel miRNA generated from gene-duplication events from the TIR-NBS-LRR class of proteins. Understanding the mechanism of plant defense responses against viruses will require the identification of additional miRNAs that are regulated by viral infection.

Symbiotic nitrogen fixation—Nitrogen fixation in soybean and other legumes is the result of a symbiotic association between the leguminous plant and rhizobial bacteria. This mutually beneficial association involves the exchange of chemical signals leading to the formation of specialized nitrogen-fixing structures known as root nodules (17). Understanding and elucidating this symbiotic association will require the identification of the molecular determinants and their regulators at different stages of the interaction leading to nodule development. Two strategies have been employed for identifying small RNAs that could participate in this interaction: One involves the identification of conserved miRNAs in soybean based on the homology of known miRNAs in other plant species, and the other involves the use of high-throughput sequencing and cloning of small RNAs differentially expressed in soybean roots inoculated with the bacterium *Bradyrhizobium japonicum* compared to mock-inoculated roots (90). Approximately 55 families of miRNAs were identified, of which 35 were found to be novel. Further expression analysis of *B. japonicum*-responsive miRNAs revealed that miR168 and miR172 were transiently upregulated up to 3 hpi but were downregulated to basal levels by 12 hpi. Although miR159 and miR393 exhibited significant upregulation at 3 hpi, miR160 and miR169 showed downregulation. Rhizobial infection changes the levels of miR160, miR393, miR164, and miR168, which target ARFs (ARF10, ARF16, and ARF17), TIR1, NAC1, and AGO1, respectively. These results strongly support the role of auxin homeostasis/signaling in nodulation (Figure 2).

To understand the additional components involved in root nodulation in legumes, researchers have been unraveling the regulatory network at the later phase of the soybean-rhizobial interaction. Cloning and sequencing of miRNAs from functional nodules of soybean identified small RNAs belonging to 11 miRNA families (101). Four of these belonged to conserved miR167, miR172, miR396, and miR399 families, whereas another four families had sequences homologous to gma-miR1507, gma-miR1508, gma-miR1509, and gma-miR1510, which were also reported previously by Subramanian et al. (90). Three novel miRNA families (gma-miR222, gma-miR383, and gma-miR235) that possibly play a role in nitrogen fixation were reported. These miRNAs exhibited differential expression in root nodules. High levels of gma-miR172 and gma-miR222 but low levels of gma-miR1508 and gma-miR1510 were detected in root nodules, prompting the authors to speculate that these miRNAs play critical roles in nodule maturation and nitrogen fixation (Figure 2). Further investigations on functions of these miRNAs and their putative targets would provide insights on legume-rhizobium interactions during symbiosis and ultimately about the mechanism of nitrogen fixation.

The levels of MtHAP2-1, a transcription factor of *Medicago truncatula* strongly upregulated during nodule development, are controlled by miR169 (16). MtHAP2-1 encodes a HAP2-type transcription factor (29) and is abundant in and limited to cells of the nodule

meristematic zone (16), which further suggests its role in nitrogen fixation. MtHAP2-1 RNAi transgenic lines exhibited delayed nodule maturation because of arrested growth and internment of the bacterium *Sinorhizobium meliloti* within the developing nodule. miR169 limits the expression of MtHAP2-1 to the nodule meristematic zone, and the root growth of miR169-resistant MtHAP2-1 transgenic plants was reduced. All of these results strongly suggest that, during rhizobial infection of *M. truncatula*, MtHAP2-1 is critical in differentiation of nodule cells and that its expression levels are temporally and spatially tuned by miR169. The involvement of miR169 in regulating MtHAP2-1 levels further emphasizes the role of small RNAs in symbiotic interaction between bacteria and plants.

Genome-wide small RNA profiling of root apices and nodules of *M. truncatula* identified 100 novel candidate miRNAs in addition to the miRNAs that are homologous to known miRNAs from other species (59). Northern blot analysis and in situ localization studies revealed several miRNAs that were differentially expressed between roots and nodules. miR167 is highly expressed in the nodule peripheral vascular tissues, whereas miR172 and miR398 are specifically localized in the infection zone, which suggests that they have roles in cell differentiation or infection by the symbiotic bacteria. These studies in soybean and *Medicago* indicate that miRNAs indeed contribute to gene regulation of nodulation.

siRNAs

Although plants contain only several hundred miRNAs, they contain huge numbers of endogenous siRNAs. As discussed earlier, these siRNAs have been classified into groups based mainly on their biogenesis: the ta-siRNAs, ra-siRNAs, nat-siRNAs, and lsiRNAs. Their biological roles, however, are not well understood. Our laboratory has identified a nat-siRNA and a lsiRNA specifically induced by the bacterial pathogen *Pst (avrRpt2)*, and these siRNAs contribute to plant antibacterial immunity (54, 55).

nat-siRNAs—The first plant-endogenous nat-siRNA identified as involved in plant immunity is nat-siRNAATGB2, which regulates *R*-gene mediated ETI (55). This small RNA is generated from the overlapping region of a NAT pair, which encodes a Rab2-like GTP-binding protein gene *ATGB2* and a pentatricopeptide protein (PPR)-like gene *PPRL*. The endogenous RNA is specifically and strongly induced by *Pst (avrRpt2)* and cleaves antisense *PPRL* mRNA for silencing. To define the components involved in its biogenesis, we examined the accumulation of ATGB2 nat-siRNAs in *Pst (avrRpt2)*-challenged small RNA-biogenesis mutants. This small RNA is processed by the DCL1-HYL1 complex, stabilized by HEN1-mediated methylation, and amplified by RDR6, SGS3, and RNA Pol IV. We further demonstrated that the induction of nat-siRNAATGB2 by *Pst (avrRpt2)* also requires the cognate host *R* gene *RPS2* and its resistance signaling components, including *NDR1*. Constitutive overexpression of the target gene *PPRL* without the nat-siRNA target site in transgenic *Arabidopsis* plants resulted in a delayed hypersensitive response (HR) and attenuated *RPS2*-mediated disease resistance. These results strongly suggest that *PPRL* acts as a negative regulator of *RPS2*-mediated resistance. *PPRL* is an atypical tandem PPR and is likely to be localized in mitochondria (43, 62), which may contribute to *Pst (avrRpt2)*-triggered oxidative burst, HR, and programmed cell death (PCD). Proteins containing the PPR domain are believed to be involved mainly in posttranscriptional processes in organelles, including RNA editing (87), mRNA silencing by cleavage (103), and translational regulation (86).

lsiRNAs—During the search for pathogen-induced small RNAs by Northern blot analysis, we identified a novel class of endogenous siRNA, the lsiRNAs (54). As the name suggests, lsiRNAs are longer than the normal 21- to 24-nt siRNAs and are in the size range of 30 to 40 nt. We found several lsiRNAs that are mainly induced by bacterial infection or specific

growth conditions. The biogenesis of AtsiRNA-1 involves components of distinct small RNA pathways, including DCL1, HYL1, HEN1, HST, AGO7, RDR6, NRPD1a, and NRPD1b. *Pst (avrRpt2)*-mediated induction of AtsiRNA-1 specifically targets the *AtRAP* gene, which encodes a RNA-binding protein containing a putative RNA-binding RAP domain (RNA binding domain abundant in Api-complexans). AtsiRNA-1 employs a unique mechanism to degrade target mRNA by DCP2-VCS (Decapping 2 and Varicose)-mediated decapping followed by an exoribonuclease XRN4-mediated 5′–3′ decay. AtRAP is a negative regulator of PTI and ETI because the knock-out mutant of this gene resulted in enhanced resistance to both avirulent *Pst (avrRpt2)* and a virulent strain *Pst* DC3000.

To be cost effective, plants have developed a sophisticated regulatory mechanism to suppress the defense response systems under normal conditions, possibly by employing negative regulators of plant defense, such as PPRL and AtRAP. As the defense systems must be switched on rapidly upon pathogen attack, small RNAs such as miRNA393, nat-siRNAATGB2, and AtsiRNA-1, are induced in the early phases of infection and cleave or degrade the mRNA of these negative regulators of plant immunity, thereby mounting rapid counterdefense mechanisms.

siRNAs generated from *R* gene loci (*RPP5* locus and *N* gene MITEs)

Plant resistance (*R*) proteins recognize specific pathogens by directly and indirectly interacting with corresponding avirulence (*avr*) effectors and triggering a cascade of events leading to disease resistance (21, 64). These *R* genes are generally clustered in the genome and encode proteins with common motifs. To keep pace with the continuous and rapid evolution of pathogens, *R* genes undergo coevolution resulting in variable gene clusters. The *Arabidopsis thaliana* ecotype Columbia *RPP4* locus (known as *RPP5* in *Landsberg erecta* for recognition of the oomycetes *Hyaloperonospora parasitica* 5) is composed of seven TIR-NBS-LRR class-*R* genes along with three related and two unrelated genes (73, 109). Two *R* genes in this locus, named *RPP4* and *SNCI* (for suppressor of *npr1-1*, constitutive 1), impart resistance to both *P. syringe* pv. *maculicola* and *H. parasitica* (89, 97, 108, 114). These two *R* genes are coordinately regulated by transcriptional activation and siRNA-mediated RNA silencing. Endogenous siRNAs generated at the *RPP4* locus apparently target *SNCI* because enhanced transcript levels of *SNCI* were observed in small RNA biogenesis-deficient mutants such as *dcl4*, *upf1*, and *ago1* as well as in transgenic plants expressing P1/HC-Pro suppressor. This fine-tuning of *R*-gene expression is important because it substantially reduces the fitness cost for constitutive activation of defense pathways. In other words, the siRNAs generated from the *RPP4* locus may control the resistance responses, thereby enhancing plant health and fitness.

MITEs (miniature inverted repeat transposable elements) are truncated DNA transposons that are generally fewer than 600 base pairs (bp) long, lack open reading frames, and depend on the activity of transposons for their mobility (49, 50). MITEs are present in high copy numbers in several plant genomes and are predicted to be regulatory elements in plant gene expression (33, 50). They are also thought to serve as a major evolutionary element in transposon-mediated gene regulation in plants by generating small RNAs. The complexity of the TMV *R* gene *N* is maintained by MITE-mediated creation of new gene structures (56). Virus infection may lead to temporary inhibition of PTGS for *N* expression and siRNA-guided cleavage. This may induce premature translation termination, provide a polyadenylation signal, or introduce deletions/insertions leading to gene diversity. Kuang et al. (56) suggested that biogenesis of most MITE-derived small RNAs (MiS) depends on the NR-PD1a/RDR2/DCL3/AGO4 pathway. DCL4 is also implicated in the accumulation of MiS small RNAs (56). It will help our understanding of *R* gene evolution to elucidate the mechanisms of generating small RNAs from MiS and their functionality in gene regulation in plants.

COMPONENTS OF THE SMALL RNA BIOGENESIS PATHWAY PLAY AN IMPORTANT ROLE IN PLANT DEFENSE

Many plant genomes encode multiple DCLs, RDRs, and AGOs in the RNAi silencing machinery. The components within the same family have distinct or sometimes partially overlapping functions in different small RNA pathways. *Arabidopsis* has four DCLs, six RDRs, and ten AGOs, many of which are involved in plant-defense signaling pathways.

Dicer-Like Proteins and Their Associated Proteins

Arabidopsis contains four DCLs that process dsRNA or fold-back RNA precursors to generate siRNAs and miRNAs, respectively. Genetic experiments using single, double, or triple mutants of DCLs have dissected the roles of individual DCLs and their compensatory functions in the production of virus-derived small RNAs (viRNAs). Recent studies demonstrate that loss-of-function mutations in both DCL4 and DCL2 are necessary and sufficient to make plants highly susceptible to several (+)ssRNA viruses (9, 22, 24, 35).

Deleris et al. (22) employed virus-induced gene silencing (VIGS) to knock out endogenous phytoene desaturase (PDS) by inoculating *Arabidopsis* plants with modified tobacco rattle virus (TRV). The virus was modified such that the plant PDS gene replaced the RNA2-encoded 2b and 2c sequences in the viral genome. After infection, the modified virus did not cause disease because of the siRNA-mediated antiviral response. That is, the siRNAs targeted TRV-PDS and generated PDS-targeting siRNAs. These PDS siRNAs initiated degradation of endogenous *PDS* mRNA, which results in extensive photo-bleaching of the plants. The inoculation of the modified TRV-PDS in wild-type and *dcl* mutants showed that DCL4 is the primary sensor and produces 21-nt siRNAs that program a RISC effector complex against viruses. In the absence of DCL4, DCL2 acts as a subordinate antiviral defense protein by producing 22-nt siRNAs. Moreover, double mutants of *dcl2* and *dcl4* exhibited hypersusceptibility to the viral infection, which suggests the combined action of these two specific proteins in antiviral defense. Unlike DCL2 and DCL4, DCL1 and DCL3 were not found to be involved in antiviral immunity in this study. A similar study by Qu et al. (82), which aimed to determine the contribution of key RNA-silencing pathway components in antiviral silencing, found that all four DCL proteins are involved in mounting an antiviral defense in plants. This study confirmed that DCL2 and DCL4 are primary proteins, whereas DCL3 has a minor role in this process. Interestingly, DCL1 was also implicated in antiviral silencing but apparently plays a negative role, as it down-regulates the expression of DCL4 and DCL3. These studies corroborate that there is a functional hierarchy of different plant DCL proteins (DCL4>DCL2>DCL3>DCL1) in processing viral RNAs into viRNAs (22). DCL3 also has clear antiviral roles in natural DNA virus infections (7, 67). *Arabidopsis* plants infected with CaMV had an increased viral load in *dcl2-dcl3-dcl4* mutants. However, viral load determined by immunoblot analysis of coat protein was not significantly different between wild-type plants and single mutants (*dcl2*, *dcl3*, *dcl4*) or double mutants (*dcl2-dcl3*, *dcl2-dcl4*, *dcl3-dcl4*). DCL1 plays a positive role in antiviral defense by facilitating synthesis of viRNAs derived from the intramolecular hairpins formed in the 35S-leader sequence of CaMV. These hairpins resemble miRNA precursors, and DCL1 probably identifies this structure, excises it, and then presents it to other Dicers for further processing.

Small dsRNA-binding proteins (DRBs) are essential cofactors of DCL proteins (45, 69). These DRBs, however, do not exhibit hierarchical redundancy as do DCLs (19). DCL1 and DCL4 interact with DRB1/HYL1 and DRB4, respectively (45, 69). DRB4 contributes to antiviral defense, possibly by interacting with DCL4 (82). In contrast, DCL2 and DCL3 do not require any DRB for production of viRNAs (19). Another protein that contains a dsRNA

binding domain is HEN1, which plays an important role in small RNA metabolism (75). The *Arabidopsis hen1* mutant exhibits hyper-susceptibility to CMV by a fivefold increase in the accumulation of the viral RNA when compared with that in wild type, which suggests that HEN1 contributes to resistance against the virus (10).

DCL proteins are also involved in the generation of small RNAs that contribute to antibacterial immunity in plants. The *dcl1* mutant showed enhanced susceptibility to *Pst* DC3000 *hrcC*⁻, a nonpathogenic strain that can elicit PTI (71). DCL1 is required for generating miR393. The accumulation of the target transcripts of miR393 (TIR1, AFB2, and AFB3) is increased in the *dcl1-9* mutant (70) when treated with flg22 peptide. DCL1 is also involved in the generation of nat-siRNAATGB2 (55) and AtlsiRNA-1 (54).

HYL1, the dsRNA-binding protein associated with DCL1, is also involved in resistance against bacterial infection, as the *hyl1* mutant was susceptible to *Pst (avrRpt2)* (55). Moreover, the *hyl1* mutant exhibited compromised accumulation of nat-siRNAATGB2 and AtlsiRNA-1 (54, 55).

RNA-Dependent RNA Polymerases

In plants, RDRs are important for siRNA formation because they synthesize dsRNAs for downstream processing by DCL. Initial studies showed that virus infection enhances RDR activity, which led to the hypothesis that RDRs are one of the many host factors that assist virus replication (27). Subsequently, extensive studies have implicated RDRs in antiviral defense in plants (4, 106, 112). Xie et al. (106) found that RDR1 activity is induced not only by virus infection but also by defense signaling compounds such as salicylic acid (SA). Reducing the expression levels of RDR1 in transgenic antisense *Arabidopsis* plants resulted in enhanced accumulation of viral RNAs and increased susceptibility to TMV and potato virus X (PVX) infection. AtRDR1, an ortholog of NtRDR1, is also known to impart defense against tobamovirus and tobnavirus because *Arabidopsis rdr1* mutant plants had enhanced levels of viral RNAs (112). NtRDR1 is also involved in combating potato virus Y (PVY) infection; knocking down expression of RDR1 in transgenic tobacco plants resulted in reduced expression of other defense-related genes such as *AOX1* and *ERF5* (84). These studies raise the question of whether different RDR proteins (for instance, the six members in *Arabidopsis thaliana*) have distinct or overlapping functions.

Arabidopsis RDR6 (SDE1/SGS2) was initially considered to be important for transgene-induced PTGS and apparently had no role in plant antiviral response because a RDR6 mutant allele *sde1* did not exhibit enhanced viral susceptibility (20, 68). Extensive studies by Qu et al. (81), however, demonstrated that NbRDR6 (a functional homolog of AtRDR6) plays an important role in antiviral defense. The transgenic tobacco plants wherein NbRDR6 was downregulated showed hypersusceptibility to many different viruses, and this response was more pronounced at elevated temperatures. This was consistent with earlier reports, which demonstrated that there is increased siRNA generation at higher temperatures (93). This study provided strong evidence that NbRDR6 contributes to general antiviral defense by RNA silencing and that environmental conditions influence the plant-virus interactions. In a recent elegant study, Wang et al. (102) examined single, double, and triple mutants of RDR1, RDR2, and RDR6 using a mutated CMV with no viral suppressor (VSR) 2b, and demonstrated the role of both RDR1 and RDR6 in secondary viRNA formation. Small RNA profiling revealed that RDR1 preferentially amplifies viRNAs that mapped at the 5'-terminal viral RNAs, whereas RDR6-dependent viRNAs mapped to the 3'-terminal half of viral RNAs. RDR6 interacts with a coiled-coil protein, SGS3, to produce secondary viRNAs (57, 99). An *sgs3* mutant shows enhanced susceptibility to cucumovirus (68), which indicates that SGS3 also contributes to antiviral resistance in plants. The generation of nat-siRNAATGB2 (55) and AtlsiRNA-1 (54) requires RDR6. *Pst* DC3000 (*avrRpt2*) displayed

enhanced growth in the *rdr6* mutant (55), which provided direct evidence for the function of RDR6 in plant immunity.

Argonautes

AGOs are associated with small RNAs and form RISC complexes to induce silencing of target genes (41). *Arabidopsis* contains 10 AGOs, and their role in plant immunity is yet to be determined. There is emerging evidence that the methylation status of plant genomes is altered in response to attack by pathogens, including viruses, bacteria, and fungi (34, 39, 76, 88). hc-siRNAs trigger transcriptional gene silencing (TGS) by guiding RNA-directed DNA methylation (RdDM) and histone modification in plants (47, 66, 96). AGO4 is a major nuclear RNAi effector associated with hc-siRNAs or ra-siRNAs that direct DNA methylation (60, 80). Involvement of AGO4 in the disease-resistance response links DNA methylation and plant defense. When attacked by viruses, plants employ DNA methylation to repress viral transcription and/or replication. Upon infection by either of two geminiviruses, cabbage leaf curl virus (CaLCuV) or beet curly top virus (BCTV), *Arabidopsis* plants silence viral chromatin by both cytosine and histone methyltransferases (83). This is evident from the hypersusceptibility of the methylation-deficient mutants to geminiviruses, including mutants of cytosine methyltransferases (*drm1*, *drm2*, *cmt3*, and *met1*), histone H3K9 methyltransferase (*kyp2*), RdDM pathway components (*ago4*, *ddm1*, and *nrdp2A*), or methyl cycle enzymes (*adk1* and *adk2*) (83). Viral suppressors AL2 and L2 inhibit the activity of adenosine kinase (ADK), a cellular enzyme involved in the generation of S-adenosylmethionine (a methyltransferase cofactor). Therefore, plants infected with virus lacking L2 had hypermethylation of viral DNA. Additionally, recovery of the virus-infected plants from the disease symptoms required AGO4 (83). Chromatin methylation may be a generalized process adopted by plants to evade infection by DNA. VIGS of two *Nicotiana benthamiana* homologs of *Arabidopsis* AGO4 blocked the R gene-mediated antiviral responses through translational suppression of viral RNAs (6). This result suggests that AGO4 may have additional functions besides its role in the RdDM pathway. It is also possible that the NbAGO4 may not function the same way as *Arabidopsis* AGO4, or there might be another unidentified *N. benthamiana* AGO that is more closely related to *Arabidopsis* AGO4.

AGO4 is also involved in antibacterial defenses. Mutant *ago4-2* was identified from a genetic screening using a H₂O₂-responsive Ep5C promoter-driven GUS reporter (2). Assessment of disease susceptibility revealed that *ago4-2* exhibits reduced resistance to virulent *Pst* DC3000 as well as to avirulent *Pst* (*avrRpm1*). However, mutants of DCL3 and RDR2, the up-stream components of AGO4, and mutants of chromomethylase 3 (CMT3) and defective in RNA-directed DNA methylation (DRD1) and domains rearranged methyltransferase 1 and 2 (DRM1 and DRM2), the downstream components of AGO4 in the RdDM pathway, showed no change in *Pst* growth. These results suggest that either these components are functionally redundant with their close homologous genes or AGO4 simply has an unidentified RdDM-unrelated function in plant defense. These possibilities should be investigated by testing the disease resistance responses of double and triple mutants of these components in the RNAi and RdDM pathways.

In addition to AGO4, Qu et al. (82) conclusively showed that AGO1 and AGO7 have an important role in slicing viral RNAs. AGO1 is the primary slicer because it targets viral RNAs with more compact structures, but AGO7 is a surrogate slicer whose targets are less structured (82). The biogenesis of AtlsiRNA-1 is known to involve AGO7, as *ago7* mutant does not accumulate AtlsiRNA-1 (54). However, other *ago* mutant plants, including *ago3*, *ago4*, and *ago9*, showed no significant change in the level of AtlsiRNA-1 as compared with wild type. AGO7 is also associated with TAS3 ta-siRNA (1, 32, 36). The accumulation of

bacteria-induced AtlsiRNA-1 is dependent on AGO7, suggesting a role of AGO7 in antibacterial defense.

RNA SILENCING SUPPRESSORS OF PATHOGENS

Viral Suppressors of RNA Silencing

Many viruses encode specific proteins that suppress the host antiviral silencing response and thereby benefit viral infection. These viral suppressors of RNA silencing (VSRs) can act at three levels, i.e., they can (a) inhibit generation of viRNAs, (b) inhibit loading of viRNAs in RISC by binding to the viRNA, and (c) inhibit components of RISC. Ectopically expressed VSRs, in conjugation with a sensor, have been used to decipher functions of many VSRs. The use of viruses with disabled or modified VSRs, however, has recently proven to be a very effective approach for determining VSR function. Two recent reviews provide comprehensive coverage on this topic (23, 26). Table 1 summarizes what is known about the mode of action of VSRs in plants.

Bacteria-Encoded Suppressors of RNA Silencing

As noted in the previous paragraph, viruses encode VSRs that suppress host antiviral silencing machinery and thereby promote their pathogenesis. Because small RNAs and RNA-silencing machinery are also important for antibacterial defense, the question arises as to whether bacterial pathogens also developed similar silencing suppressors to counter antibacterial defense responses in plants. Recently, Navarro et al. (71) identified several *Pst* type III secretion effectors that suppress host RNA silencing machinery and therefore increase disease susceptibility (Figure 3). AvrPtoB represses transcription of miRNA genes and results in a low level of pri-miR393. Some pri-miRNAs were unaffected, however, and it is therefore unlikely that AvrPtoB is a general transcriptional suppressor of the miRNA pathway. AvrPtoB might suppress a specific component involved in plant defense that is required for transcription of miR393 genes. Another effector, AvrPto, interferes with processing of some miRNA precursors and downregulates the level of mature miR393. It remains to be determined whether AvrPto directly suppresses miRNA-processing components, such as DCL1 and HYL1, or the components required for miRNA stability, such as HEN1. In addition, HopT1 inhibits the action of the AGO1 protein in the RISC complex. Thus, as with viruses, bacteria have evolved an array of effectors that target different steps of the miRNA pathway. We speculate that other pathogens, such as fungi and oomycetes, have also developed RNAi suppressors to counteract host antipathogen RNA-silencing mechanisms.

CONCLUDING REMARKS

More and more studies have shown that many host miRNAs and siRNAs are induced or suppressed by various pathogen challenges and that modulation of miRNA and siRNA levels plays an important role in gene expression reprogramming and fine-tuning plant responses against a wide range of pathogens (Figure 4). These pathogen-responsive small RNAs induce posttranscriptional gene silencing by guiding mRNA cleavage/degradation or translational repression, or may guide transcriptional gene silencing by direct DNA methylation or chromatin modification. This idea is supported by observations that many components in the small RNA pathways are required for plant defense responses and immunity. As a countermeasure, viruses and bacteria have developed VSRs and BSRs to suppress host RNAi machinery and compromise disease resistance in plants. To combat continuously evolving pathogens, plants have also evolved components, such as R proteins, that can recognize VSRs and BSRs and trigger robust and rapid resistant responses, which are referred to as ETI.

The study of small RNA-mediated regulatory mechanisms in plant immunity is an emerging field, and we expect that many more pathogen-responsive small RNAs will be identified using new technologies, such as high-throughput deep sequencing. Characterization of these small RNAs and their target genes will reveal new components in plant resistance signaling pathways and help us understand the molecular mechanisms of plant immunity. We also expect that more silencing suppressors will be identified from viruses, bacteria, fungi, and oomycetes, and that such identification will increase our understanding of the interaction and coevolution between pathogens and plant hosts. These studies will elucidate the molecular mechanisms of plant defense responses and will ultimately lead to the development of effective tools for controlling disease in the field.

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Glossary

RISC	RNA-induced silencing complex
PAMP	pathogen-associated molecular pattern
PTI	PAMP-triggered immunity
ETI	effector-triggered immunity
TTSS	type III secretion system
R proteins	resistance proteins

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SUMMARY POINTS

1. Endogenous small RNAs play pivotal roles in reprogramming of host gene expression in response to infection by a wide range of microbes, including viruses, bacteria, and fungi.
2. Small RNAs contribute to PTI or basal defense, as well as ETI or race-specific resistance.
3. Small RNAs are generated from pathogen-derived nucleic acids as seen in viruses and *Agrobacterium*. These pathogen-derived small RNAs may regulate the interaction of pathogens with plants.
4. Endogenous small RNAs are induced or suppressed during legume-rhizobia symbioses. These small RNAs are thought to facilitate the plant-bacterium interaction by regulating different steps in the development of nitrogen-fixing nodules.
5. Different components of small RNA pathways directly play important roles in mediating host immune responses against pathogens.
6. RNA silencing is activated in plants to counteract pathogen infection. To counter-counteract effects of silencing and to establish infection, rapidly evolving pathogens secrete suppressors of RNA silencing (VSRs and BSRs) that inhibit different steps of small RNA pathways.
7. To combat continuously evolving pathogens, plants have evolved components such as R proteins that can recognize pathogen-derived suppressors to mount robust and rapid resistance responses.

FUTURE ISSUES

1. Deep sequencing technologies will allow small RNA profiling of plants infected with various pathogens and identification of small RNAs involved in plant-microbe interactions.
2. It is a challenge to identify and characterize the target genes of newly discovered small RNAs that exhibit alteration in their expression level upon pathogen infection. Overcoming this challenge will allow rapid deciphering of new components in plant-pathogen interactions and lead to elucidation of the complex regulatory network of host immune systems.
3. Identification of pathogen-derived small RNAs and their potential host targets will help us understand how pathogens cause disease. Whether there is any functional interaction between pathogen-derived small RNAs and host mRNAs is an interesting area of research.
4. Identification of different components of small RNA machinery during disease susceptibility and resistance responses remains an active area of research. It is still not known whether components of the disease resistance pathway also regulate generation of small RNAs.
5. Studying the changes in the DNA methylation profiles of host plants after infection with various pathogens may help us understand the evolution of new components in plant defense for combating rapidly evolving pathogens.
6. Microbes such as viruses and bacteria encode suppressors that counteract the plant defense system. The identification of additional silencing suppressors from different pathogens and the elucidation of their functions and how they act as suppressors will shed light on the generality of suppression of disease resistance. It is interesting and useful to identify the targets of these suppressors and see whether these suppressors affect other pathways in plants besides small RNA pathways. Another tempting projection is that the pathogen-encoded suppressors could affect transposon activation and affect plant gene expression. Understanding the action of these suppressors will unravel the diversity and evolution of pathogens as well as RNA silencing pathways.
7. Information generated from characterization of new small RNAs and the regulatory network of host immune systems needs to be scrutinized for development of tools to enhance plant resistance against pathogens.

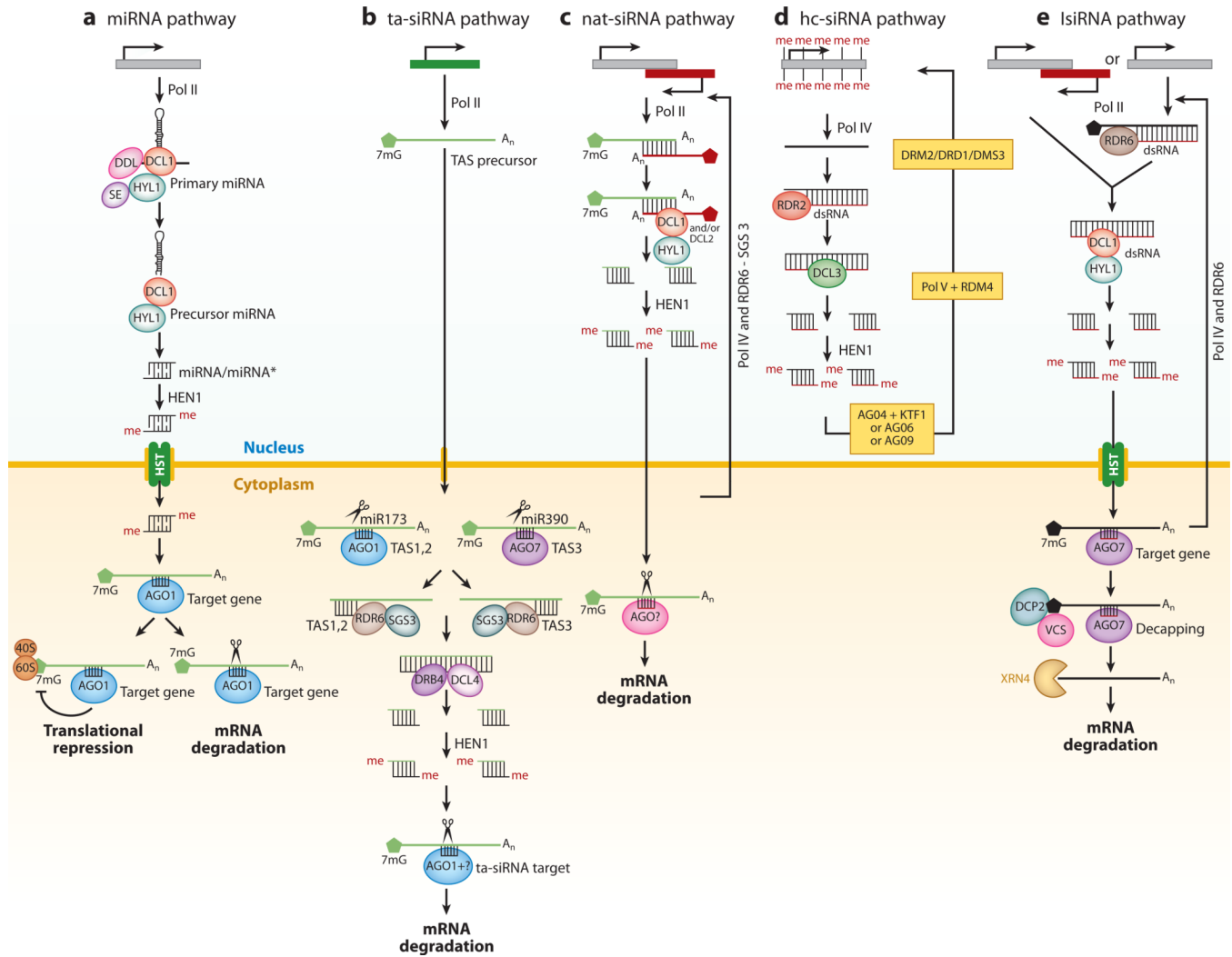


Figure 1.

Endogenous small RNA pathways in *Arabidopsis*. (a) microRNA (miRNA) pathway. miRNAs are generated by transcription of noncoding miRNA genes by RNA Pol II. The primary miRNAs possess stem-loop structures that are acted upon by DCL1-HYL1-SE protein complex. DDL protein is known to be involved in the formation of precursor miRNA (pre-miRNA). The DCL1-HYL1 complex further processes pre-miRNA into 21-nucleotide (nt) miRNAs. The miRNA (miRNA:miRNA*) duplexes are then methylated at their 3' ends by HEN1. These methylated miRNAs are transported into cytoplasm by an exportin homolog, HASTY (HST). The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) containing AGO1 protein. The RISC is recruited to the target gene on the basis of sequence complementarity, and AGO1 represses gene expression by either mRNA degradation or translational repression. (b) *trans*-acting small interfering RNA (ta-siRNA) pathway. The process of TAS precursor is triggered by a miRNA-mediated cleavage. The resulting 5' fragment (in case of TAS1a–c and TAS2) and 3' fragment (in case of TAS3) act as templates for the formation of long double-stranded RNA (dsRNA) by concerted action of RDR6 and SGS3. These long dsRNAs are then recognized by the DCL4-DRB4 complex and cut into phased 21-nt small RNAs that undergo further methylation by HEN1. The ta-siRNAs are incorporated into a RISC containing AGO7 (in the case of TAS3) or AGO1 (in the case of TAS1 and 2), which results in target cleavage. (c)

natural antisense transcript-derived siRNA (nat-siRNA) pathway. Natural antisense transcripts produced by Pol II form dsRNA within their overlapping regions. The dsRNAs are processed by DCL1 and/or DCL2 into siRNAs that target antisense transcripts through an unidentified AGO protein containing RISC complex. RDR6-SGS3, together with Pol IV, forms an amplification loop to generate more nat-siRNAs, which reinforce the cleavage of antisense transcript. (d) heterochromatic siRNA (hc-siRNA) pathway. Transcription of heterochromatic regions, repeat regions or transposons by Pol II and/or Pol IV results in the formation of single-stranded RNA(ssRNA), which is converted into dsRNA by the action of RDR2. This dsRNA is processed into predominantly 24-nt long hc-siRNAs by DCL3. These 24-nt siRNAs associate with AGO4 (or AGO6, or AGO9) through an adaptor protein, KTF1, to form an RNA-directed DNA methylation (RdDM) effector complex that directly or indirectly recruits proteins involved in heterochromatin formation, including DRM2, DRD1, and DMS3, to the hc-siRNA target loci. (e) long siRNA (lsiRNA) pathway. lsiRNAs are generated by DCL1 from coding or noncoding genes, or overlapping regions of antisense transcription, or dsRNAs from the action of Pol IV and RDRs. lsiRNAs are methylated by HEN1 and repress the expression of target genes by guiding mRNA decapping mediated by DCP2 (decapping 2) and VCS (Varicose) and 5'-3' RNA decay mediated by exoribonuclease XRN4.

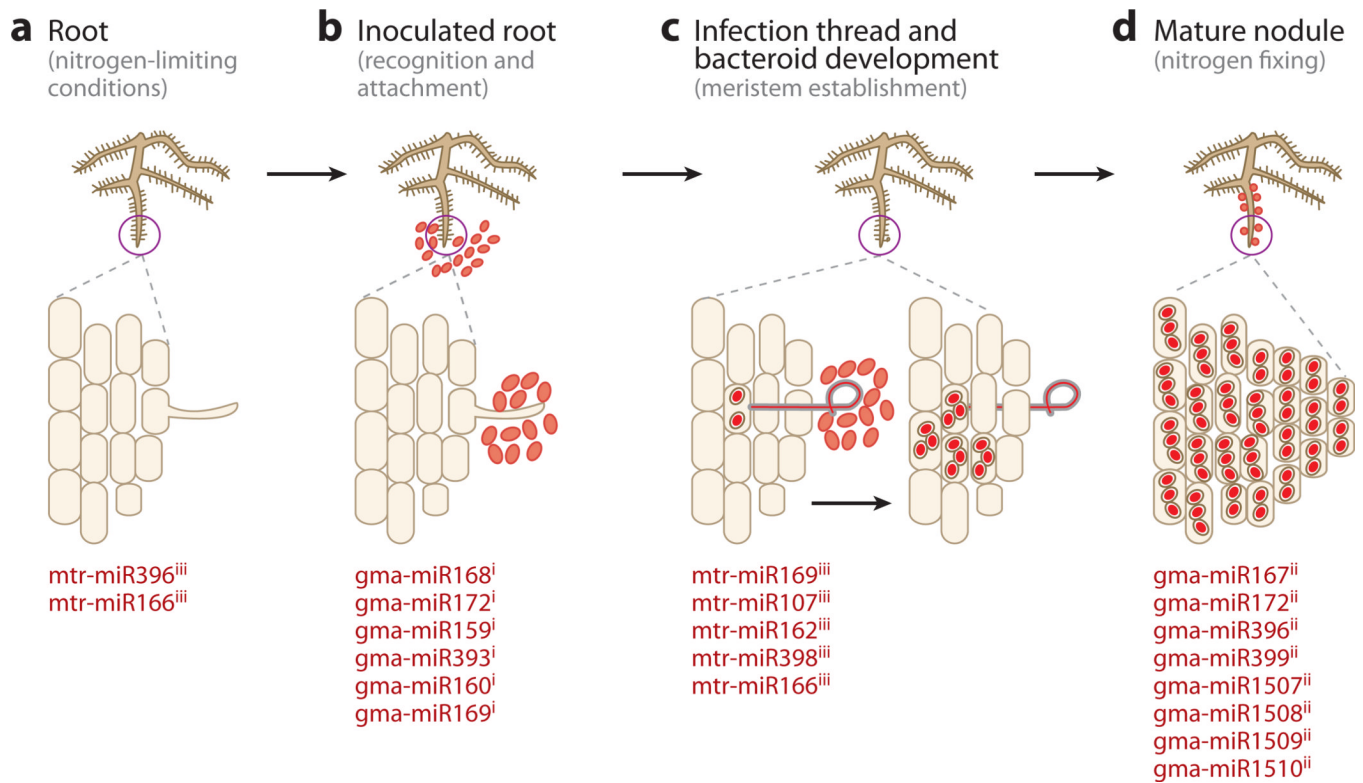


Figure 2. Small RNAs regulate rhizobia-legume symbioses, resulting in nodule development for nitrogen fixation. Different steps in nodule formation are shown along with the miRNAs predicted to be involved at specific steps. (a) The interaction of nitrogen-starved plants with rhizobial bacteria results in the exchange of chemical signals as plants secrete flavonoids and bacteria produce lipochitoooligosaccharides. (b) The recognition of signals results in the attachment of bacterial cells to root hairs. (c) Changes in ionic equilibrium lead to the deformation of root hairs and transcription of nodulation-specific genes. Curling of root hairs to engulf bacteria results in the formation of infection thread that transports bacteria deep into the root tissue followed by bacteroid development. (d) Within 2 to 3 weeks postinoculation, mature nitrogen-fixing nodules are formed. Superscripts *i*, *ii*, and *iii* represent the miRNAs identified by Subramanian et al. (90), Wang et al. (101), and Lelandais-Brière et al. (59), respectively. *mtr*, *Medicago truncatula*; *gma*, *Glycine max*.

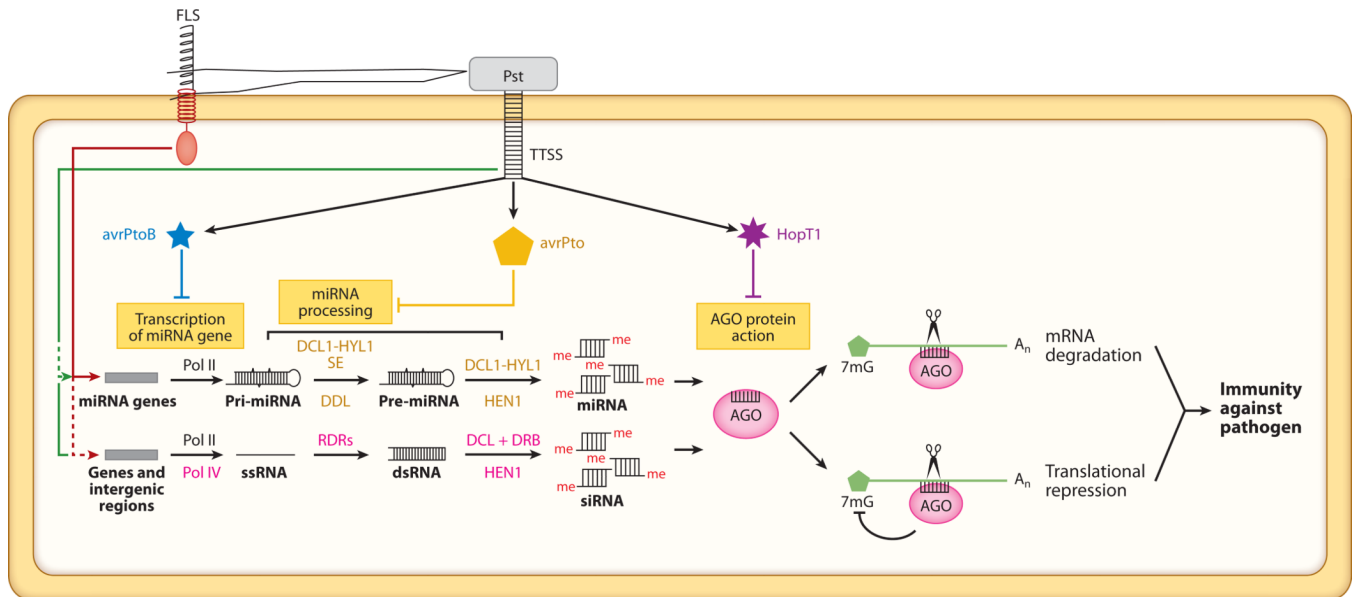


Figure 3. Mechanism of action of bacterial suppressors of RNA silencing (BSRs) in plants. Different steps in small RNA pathways are suppressed by different effectors encoded by *Pseudomonas syringae*. FLS: flagellin receptor; TTSS: type III secretion system of bacterial pathogen; Pst: *Pseudomonas syringae* pv. *tomato* DC3000.

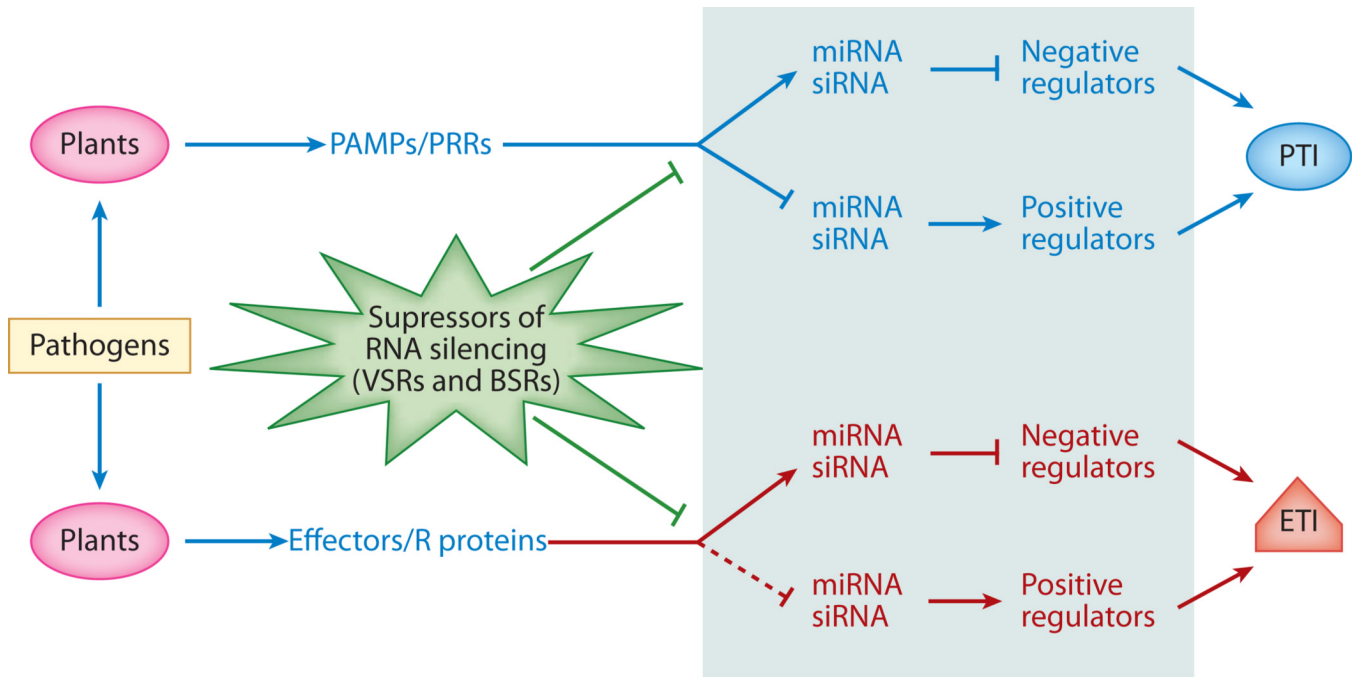


Figure 4. Immunity against pathogens is regulated by small RNAs in plants. In PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) regulated by small RNAs, RNA silencing suppressors (VSRs and BSRs) repress the small RNA silencing pathway. PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; VSRs, viral suppressors of RNA silencing; BSRs, bacteria-encoded suppressors of RNA silencing.

Table 1

Mode of action of viral silencing suppressors in plants

Suppressor	Source	Mode of action	Reference
AC4	Geminivirus	Competes with AGOs by binding to single-stranded siRNA and thereby preventing RISC assembly	12
AC2	Begomovirus	Transcriptional activator. Induces expression of any gene, which might be a silencing suppressor.	95
HcPro	Potyvirus	Mimics <i>hen1</i> mutations. viRNAs are oligo-uridylated and partially degraded due to lack of 2'-O-methylation. Interacts with a calmodulin-related protein, overexpression of which suppresses silencing. Amino acids 180, 205, and 396 of HcPro are critical for suppression of miRNA, ta-siRNA, and VIGS pathway but not for sense PTGS.	111, 3, 105
P6	Cauliflower mosaic virus	Is imported in the nucleus and binds to DRB4 protein. Suppresses RNA silencing pathway, possibly by inactivating DRB4, which is an essential component required for DCL4 action.	40
2b	Cucumber mosaic virus	Interacts physically with siRNA-loaded RISC and inhibits its slicing action. In vitro assays suggest that 2b binds to siRNAs to a lesser extent than to long dsRNAs. 2b inhibits the production of RDR1-dependent viral siRNAs.	113, 38, 24
P0	Polerovirus	Promotes ubiquitin-dependent proteolysis of AGO1.	77
P69	Tymovirus	Inhibits viRNA amplification.	14
AL2	Curtovirus	Interacts with adenosine kinase, whose inhibition possibly prevent methylation of viral DNA.	100
p126	TMV	Encodes methyltransferase and helicase. Binds duplex siRNA and inhibits HEN1-dependent methylation and degradation.	7
RNAse III	Closteroviridae	In vitro assays suggest that RNAse III suppresses siRNA silencing by cleaving 21, 22, and 24 bp siRNAs into 14-bp fragments.	18