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Review

Natural Host-Induced Gene Silencing Offers New Opportunities to Engineer Disease Resistance

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RNA silencing is an essential gene-regulation mechanism in eukaryotic organisms. Guided by small RNAs (sRNAs) of 20–25 nt in length, RNA silencing broadly governs a wide range of biological processes. In addition to regulating endogenous gene expression and inhibiting viral infection, accumulating evidence suggests that sRNAs can also function as antimicrobial agents against nonviral pathogens and directly silence gene targets in invading pathogen cells. Here, we summarize current understanding of this host-induced gene silencing (HIGS) process as a defense mechanism during natural infection. Specific focuses will be on recent advancement in the sRNA executors of HIGS and their potential delivery mechanisms from the plant host to filamentous eukaryotic pathogens, including fungi and *Phytophthora* species. Implications of these new findings in the applications of HIGS as a tool for engineering disease resistance is discussed.

Highlights

Small RNAs (sRNAs) produced by plants have emerged as mobile antimicrobial agents during natural infection of nonviral pathogens.

Executors of this natural host-induced gene silencing (HIGS) include specific microRNAs (miRNAs) and a pool of small interfering RNAs (siRNAs).

Secondary siRNAs confer resistance by silencing pathogen genes using a shotgun mechanism, which facilitates a coevolutionary arms race with pathogens.

Pathogen-targeting sRNAs may be secreted through extracellular vesicles and other extracellular vesicle-independent pathways.

Advancement in the understanding of the natural HIGS process through secondary siRNAs offers opportunities to enhance the engineering of disease resistance.

Small RNAs as Regulators of Plant Immune Systems

The primary activity of sRNAs is to silence target gene expression based on sequence complementarity. According to their distinct precursors and biogenetic pathways, plant sRNAs are categorized into two major classes: microRNAs (miRNAs) and small interfering RNAs (siRNAs) [1]. Precursors of miRNAs are primary *MIR* transcripts that form internal stem–loop structures. By contrast, siRNAs are derived from long double-stranded RNA (dsRNA) precursors produced through the activities of RNA-dependent RNA polymerases (RDRs) using exogenous RNA molecules (such as viral RNAs and transgenes) or endogenous transcripts as the templates. Both miRNAs and siRNAs can regulate plant immunity [2].

miRNAs play a prominent role in regulating development, but some can also modulate immune responses. The plant immune system has two branches – pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is activated upon perception of non-self signals known as microbe-associated molecular patterns (MAMPs). Specific miRNAs have been shown to regulate PTI, often by fine-tuning the growth-defense trade-off [3]. For instance, miR393 promotes defense in *Arabidopsis thaliana* against the bacterial pathogen *Pseudomonas syringae* [4] and in soybean against the oomycete pathogen *Phytophthora sojae* [5]. Induction of miR393 results in silencing of auxin receptors TIR1 and AFBs, potentially shifting the balance of energy allocation from growth to defense [6,7]. miRNAs can also directly regulate NB-LRR immune receptors, which play a central role in ETI [8]. In *Arabidopsis*, transcriptional repression of a subset of *MIR* loci led to resistance to the bacterial pathogen *P. syringae*, consistent with a global derepression of *NB-LRR* gene expression [9]. Furthermore, specific miRNA-*NB-LRR* regulations have been reported in tobacco [10], tomato [11], and barley [12].

Compared with miRNAs, siRNAs are well documented regulators of plant defense. A rich literature is available to demonstrate a role of virus-derived siRNAs (vsiRNAs) that guide the cleavage of viral RNAs and silencing transcripts encoding viral proteins [13]. Induction of vsiRNAs is also accompanied by the production of a large population of siRNAs derived from plant transcripts, often coding sequences [14]. Although the function of these so-called virus-activated siRNAs (vasiRNAs) is not understood, one hypothesis is that they could mediate a global transcription reprogramming in infected plants that may confer broad-spectrum resistance. Unlike antiviral defense, the role of siRNAs during the infection of nonviral pathogens has been revealed only recently.

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An Emerging Role for Secondary siRNAs in Plant Immunity

siRNA-mediated silencing often involves a signal amplification process in which primary sRNAs guide long double-stranded RNA synthesis from target transcripts and the subsequent production of 21- and 22-nt secondary siRNAs, which can regulate target genes in *trans* [15,16]. In land plants, a select group of miRNAs (often 22-nt in length) trigger secondary siRNA production from both coding and noncoding RNAs through a highly conserved pathway [17]. This pool of secondary siRNAs, consisting of diverse sequences and accounting for a significant portion of the endogenous siRNA population, has recently been shown to contribute to plant defense. Mutants of the secondary siRNA pathway do not have severe developmental defects; rather, they exhibit enhanced susceptibility to eukaryotic pathogens. In *Arabidopsis*, mutants diminished in secondary siRNA production were hypersusceptible to the fungal pathogens *Botrytis cinerea, Verticillium dahliae*, and the oomycete pathogen *Phytophthora capsici* [18–20]. A similar hypersusceptibility phenotype was reported in rice when infected with the fungal pathogen *Magnaporthe oryzae* [21]. This genetic evidence supports a widespread role of the secondary siRNA pathway in plant immunity.

Known primary targets of miRNAs that trigger secondary siRNA production include noncoding *TAS* (*trans*-acting siRNAs) loci and protein-coding genes, especially those that encode pentatricopeptide repeat (PPR) proteins and NB-LRR immune receptors [8,22]. Both *PPR* and *NB-LRR* are large gene families. Through the production of secondary siRNAs from a small number of *PPR* and *NB-LRR* transcripts, the silencing signal is spread into flaking sequences of the primary targets; as such, an extended number of genes in the same family can be regulated. For example, attenuation of miR482/2118 in tomato led to compromised production of secondary siRNAs from specific *NB-LRR* transcripts and enhanced disease resistance, probably through activation of multiple *NB-LRR* genes [11]. Similar miRNA-*NB-LRR*-siRNA circuits have also been identified from *Medicago truncatula*, soybean, potato, and barley [8,12]. These results suggest that specific secondary siRNAs can modulate endogenous genes that contribute to defense response, especially the *NB-LRR* genes.

While siRNA-mediated regulation of *NB-LRR* genes forms a direct link between secondary siRNAs and plant immunity, it does not provide an easy explanation for the disease susceptibility phenotype observed in the plant mutants that are defective in secondary siRNA production. Recent studies in *Arabidopsis* shed light on this dilemma where secondary siRNAs were found to enhance disease resistance by silencing target genes in invading pathogens.

Secondary siRNAs as Executors of Host-Induced Gene Silencing

The term 'host-induced gene silencing' (HIGS) was coined to refer to the observation that plants producing artificial sRNAs designed to target specific gene(s) in a nonviral pathogen could silence the target gene(s) and confer resistance to the pathogen. First applied to root-knot nematodes and insects, HIGS has been used to engineer several agronomically important crops in order to control various filamentous eukaryotic pathogens and parasitic plants [2]. However, it was only recently that the molecular details of HIGS during natural infections as an integral component of plant immunity were discovered.

The first example of HIGS as a natural defense mechanism was reported in cotton, where two miRNAs, miR159 and miR166, were found to silence virulence-related genes in the fungal pathogen *Verticillium dahliae* [23]. In this study, the authors provided strong evidence that these plant-derived miRNAs were present in the fungal mycelia and available for gene silencing in the pathogen. Following this initial discovery, studies in *Arabidopsis* suggest that secondary siRNAs could also silence pathogen genes during infection. In particular, two tasiRNAs, derived from noncoding *TAS* transcripts, were shown to silence target genes in the fungal pathogen *Botrytis cinerea* [18]; and a pool of siRNAs derived from a subset of *PPR* transcripts target genes in the *Phytophthora* pathogen *P. capsici* [24]. These results are consistent with the hypersusceptibility phenotype of the *Arabidopsis rdr6* mutant, which is abrogated in the secondary siRNA production, to *B. cinerea* and *P. capsici* [18,20,24]. It is noteworthy that the *rdr6* mutant, as well as other mutants in the same secondary siRNA pathway,

was also hypersusceptible to *V. dahliae* [19], suggesting that, in addition to the miRNAs, secondary siRNAs also contribute to plant defense against this fungal pathogen.

Consistent with a role of secondary siRNAs in plant immunity, pathogens have evolved effector proteins that target the secondary siRNA pathway in order to promote disease. Effectors are essential virulence factors that directly manipulate host targets for the benefit of disease development. Both *Phytophthora* pathogens and the rust-causing fungal pathogen *Puccinia graminis* encode effectors that suppress the RNA silencing pathway inside their host plants [25–29]. Intriguingly, the *Phytophthora* effector PSR2 specifically affects the secondary siRNA biogenesis in *Arabidopsis* [24,25], and the *P. graminis* effector PgtSR1 also has a significant impact on secondary siRNA levels [27]. Effectors are considered molecular probes and have facilitated the identification of host immune components and mechanisms. The virulence activities of PSR2 and PgtSR1 indicates an important role for secondary siRNAs in plant defense during fungal and *Phytophthora* infection.

A Comparison between siRNAs and miRNAs as Gene Silencing Agents during the Host–Pathogen Arms Race

Both demonstrated to be involved in HIGS, miRNAs and siRNAs have their own advantages in silencing target genes in pathogens. In general, miRNAs have a higher abundance, which facilitates silencing efficiency. Intriguingly, miR166 and miR159, that contribute to target gene silencing in *V. dahliae*, are among the most abundant miRNAs in cotton and *Arabidopsis*, and their corresponding *MIR* genes are further induced during infection [23]. Considering that sRNA-mediated gene regulation is dosage-dependent, the highly abundant miRNAs could be advantageous for use in HIGS.

Compared with miRNAs, secondary siRNAs have their own specific features that are suitable for functioning as antimicrobial agents. Secondary siRNAs represent a pool of diverse sequences that could target multiple sites in a transcript, multiple transcripts in a pathogen, and also multiple pathogens. A good example is *PPR*-derived siRNAs in *Arabidopsis* [24]. A significant portion of secondary siRNAs in *Arabidopsis* are derived from ~10 *PPR* transcripts, and their biosynthesis requires a small number of sRNA triggers [16,30]. Although *PPR*-siRNAs are constitutively produced, one of these sRNA triggers, miR161, is induced upon pathogen perception, leading to enhanced production of a pool of ~4000 *PPR*-derived siRNAs with diverse sequences. Target prediction identified 249 genes in the pathogen *P. capsici* that could potentially be silenced by 437 *PPR*-derived siRNAs [24]. The simultaneous silencing of multiple targets presumably increases the efficiency of pathogen inhibition. Potential targets of *PPR*-derived siRNAs could also be predicted from the fungal pathogen *V. dahliae*, and likely other pathogens [24], indicating that this pool of siRNAs could confer broad-spectrum resistance.

sRNAs guide gene silencing through sequence complementarity. During the constant arms race with plants, individuals in a pathogen population that evolve mutated bases in the host sRNA target site are expected to be selected to avoid HIGS-based defense. In order to maintain the antimicrobial activities, sequences of the host sRNAs also need to constantly change. Distinct from this evolutionary dynamic driven by an arms race, miRNAs are often conserved, consistent with their role in regulating endogenous targets (Figure 1A). In addition to targeting pathogen genes, both miR159 and miR166 are conserved miRNAs that regulate conserved gene targets in plants. miR159 regulates vegetative-phase changes by targeting MYB transcription factors [31], and miR166 is involved in drought response by regulating abscisic acid homeostasis [32]. Since they are not 'designated' for HIGS, coevolution of these miRNAs with pathogens is restricted. Sequence diversification of miRNAs is also constrained because they are encoded by *MIR* genes and the primary transcripts must form a characteristic stem–loop structure. On the contrary, sequence changes in secondary siRNAs could be easily accomplished as long as the miRNA-target site(s) remains conserved in the primary transcripts to initiate the production of long dsRNA precursors. Noncoding genes have less constraint on sequence diversification. Intriguingly, some of the coding transcripts that spawn siRNAs also exhibit evolutionary dynamics that may reflect an arms race. For example, the RFL (restorer-of-fertility-like) subfamily of *PPR* genes that produce secondary siRNAs undergoes diversifying selection, which is distinct from the purifying selection observed in other *PPR* genes whose functions

depend on their protein products [33,34]. Furthermore, the 'shotgun' approach used by secondary siRNAs is less likely to be quickly defeated by pathogens through target site mutation (Figure 1B). Taken together, secondary siRNAs help to maintain a diverse pool of antimicrobial sRNAs that would be beneficial to HIGS-mediated plant defense in the context of the host–pathogen arms race.

Secretion of Pathogen-Targeting sRNAs from Plant Cells

A key process in HIGS is the translocation of plant sRNAs from host cells to the invading pathogen, where the RNA silencing machinery is hijacked to silence pathogen target(s) contributing to infection. Although sRNAs are known to function as highly mobile signaling molecules, the mechanisms underlying sRNA movement remain unclear [35]. It is noteworthy that RNA mobility may not directly correlate with abundance. For example, the highly abundant miR168 in cotton was not detected in the cells of the invading fungal pathogen *V. dahliae* [23]. Similar observation was also made in mRNA exchange between the parasitic plant *Cuscuta pentagona* and its host *Arabidopsis*. Although most of the mobile transcripts were highly abundant, many abundant transcripts showed low mobility [36]. These findings indicate a sorting mechanism that may determine sRNA and mRNA trafficking between plant hosts to invading pathogens/parasites.

The first step in HIGS sRNA trafficking is the export of sRNAs from plant cells. Knowledge of plant secretory pathways is mainly acquired from research on protein secretion. The conventional secretion pathway involves the classic endoplasmic reticulum (ER)–Golgi route in which proteins are translated in membrane-bound polysomes (MBPs) on rough ER and transferred to Golgi apparatus through budding vesicles. These vesicles are eventually fused to the plasma membrane after going through the *trans*-Golgi network (TGN) to release the content, which can be taken up by another cell through endocytosis [37]. Another route of secretion is through the unconventional pathway, independent of the Golgi apparatus, that involves extracellular vesicles (EVs). EVs are membrane-bound small organelles that are released by cells into their surrounding environment. A major class of EVs is the

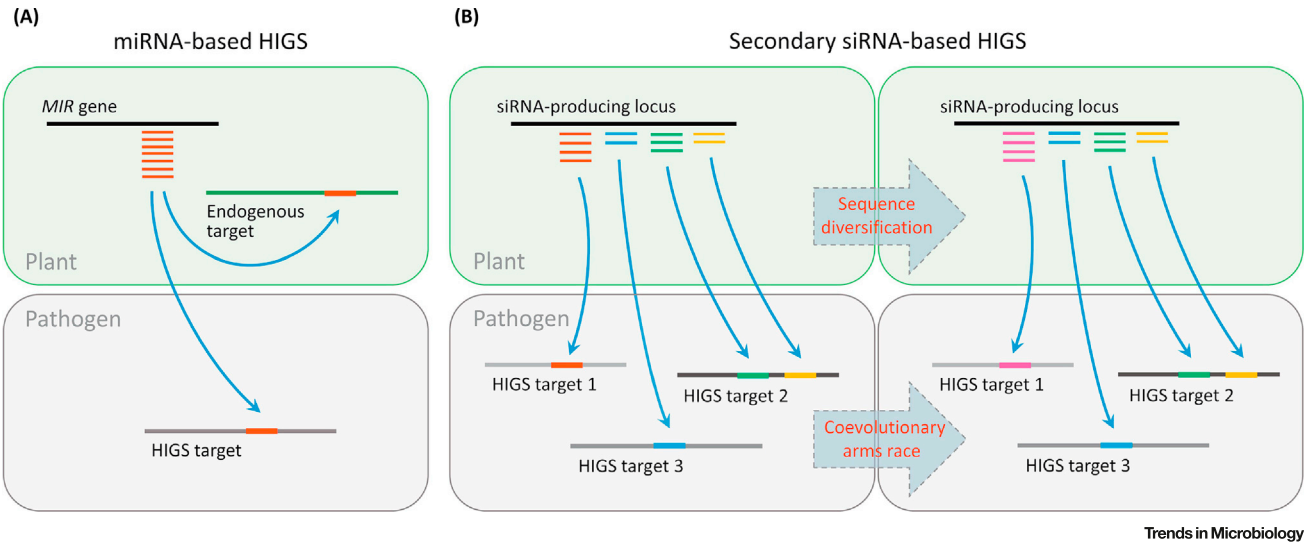

Figure 1. A Comparison of miRNA- and Secondary-siRNA-Mediated Host-induced Gene Silencing (HIGS).
(A) miRNAs are derived mainly from one conserved sequence in the precursor transcripts and thereby have a limited number of target(s) in both the plant and the invading pathogen(s). Coevolution of miRNAs with pathogens is constrained because sequence complementarity must be maintained to regulate endogenous target(s). (B) A population of secondary siRNAs with diverse sequences are produced from primary coding or noncoding transcripts and regulate multiple gene targets in an invading pathogen. This 'shotgun' mechanism increases the sustainability of secondary siRNA-mediated defense because it is more resistant to pathogen evolution through sequence changes (e.g., target-site changes illustrated in HIGS target 1). Secondary siRNAs could also engage in the arms race with pathogens through sequence diversification of the primary transcripts. Lines with different colors represent different siRNA sequences or their complementary sequences in target transcripts.

exosomes, which originate as intraluminal vesicles (ILVs) formed in the cytosol and are enclosed into endosomal compartments called multivesicular bodies (MVBs). These vesicles are released from MVBs when they are fused with plasma membrane to the apoplast [38]. The contents of EVs are subsequently delivered into a recipient cell through endocytosis [39].

In plants, EVs are enriched with antimicrobial proteins and metabolites [40,41], indicating that they contribute to plant defense. Because cargos of EVs in animal cells include various RNA molecules, and transportation of miRNAs through EVs has been shown to play a key role in intercellular communications [42], EVs of plants have also been investigated for sRNA transportation. Recent analyses confirm the presence of sRNAs in EVs [18,43]; however, the composition of sRNA cargos remains to be defined. A recent sRNA profiling analysis suggests that the overall abundance of miRNAs and siRNAs is low in EVs of *Arabidopsis* [43]; rather, 10–17 nt 'tiny RNAs (tyRNAs)' are highly enriched. These tyRNAs seem to be RNA degradation products and have unknown functions in gene regulation [43]. Although this result dampened the idea that EV is the major secretion pathway for sRNA translocation during HIGS in plants, specific miRNAs and secondary siRNAs are present in EVs. For example, *PPR*-derived siRNAs and tasiRNAs that were shown to silence pathogen genes were present, although not enriched, in EVs of *Arabidopsis* [43]. Interestingly, the abundance of a *PPR*-derived siRNA that targets a *P. capsici* gene increased in EVs isolated from *P. capsici*-infected *Arabidopsis* leaves [24], raising the possibility that EV cargos may change during infection as a defense response.

The observation that some miRNAs and many secondary siRNAs are enriched in apoplastic spaces but not in EVs indicates that sRNAs could be secreted through EV-independent pathway(s) [43]. Cytoplasmic partitioning of different classes of sRNAs has been observed in plants. Intriguingly, a group of miRNAs and siRNAs are associated with MBPs, and this association is required for the initiation of secondary siRNA production, which may also occur on rough ER [44]. It is therefore tempting to speculate that the biosynthesis of secondary siRNAs might be linked to their secretion, putatively related to the ER–Golgi route. Indeed, essential enzymes for secondary siRNA production, including RDR6, have been found to be present in the so-called 'siRNA bodies' that are often adjacent to Golgi [45,46]. The potential linkage of biosynthesis and secretion of secondary siRNAs would benefit their function as HIGS agents. It is noteworthy that this large, diverse siRNA population is constantly produced, although it can be further induced upon pathogen perception, possibly as a surveillance mechanism. Their secretion would be important to avoid unintended silencing of endogenous genes.

Translocation of sRNAs at the Interface of Plant–Pathogen Interactions

After secretion, plant sRNAs need to enter pathogen cells to silence target gene(s). An important battleground with active material exchange is at the specialized infection structures – called haustoria – formed by biotrophic/hemibiotrophic filamentous eukaryotic pathogens; these structures facilitate nutrient uptake and effector delivery [47–49]. Haustoria are also portals targeted by antimicrobial agents produced and exported from the host plants [48]. Enveloped by a modified plant plasma membrane called the extrahaustorial membrane (EHM), haustoria are separated from the host cell by the extrahaustorial matrix (EHMx) where the plant cell wall is absent (Figure 2). In plant cells, Golgi stacks, ER, secretory vesicles, and MVBs accumulate in the vicinity of EHM, indicating that sRNAs could be actively transported through EHM and then taken up by pathogen cells through endocytosis [50,51]. sRNAs produced by parasitic plants for silencing host targets were also found to be enriched in haustoria [52]. These observations indicate that haustoria might be a major gateway for sRNA translocation. It is perceivable that sRNAs secreted from plant cells through EV-dependent and/or -independent pathways could be concentrated at the EHMx. This is important because the efficiency of gene silencing is determined by the quantity of sRNAs; and the amount of pathogen-targeting sRNAs must be sufficient to make a significant impact on target gene expression.

sRNA transportation between plants and necrotrophic generalist pathogens that do not produce haustoria or other specialized infection structures might have different interaction dynamics. The model necrotrophic generalist fungal pathogen *B. cinerea* has been shown to take up externally applied sRNAs, long double-stranded RNAs, as well as purified EVs from the environment [18,53].

Since *B. cinerea* produces a variety of cell wall degrading enzymes and phytotoxic metabolites to damage host cells [54], timing of the sRNA production and secretion in relation to the cell damage/death events is critical for efficient defense through HIGS.

Future Perspectives and Application of HIGS to Engineer Disease Resistance

Research on plant immune mechanisms has provided exciting opportunities to engineer disease resistance in crops. Although there have been continuous efforts to use HIGS to enhance resistance, the results are variable. Recent discoveries in cross-organismal gene silencing during natural infection offer ways for improvement.

The most common strategy of HIGS has been to generate transgenic plants carrying a construct that harbors DNA fragments based on the sense and antisense sequences of a selected target gene [2,55]. After transcription, long double-stranded RNA or long hairpin molecules are produced, which are expected to serve as precursors for the production of artificial sRNAs. If these sRNAs are translocated into pathogen cells during infection, they may silence the target genes. Another strategy is to generate transgenic plants producing artificial miRNAs using the backbone of a known *MIR* gene [2]. Successful examples have been mainly reported from the long hairpin constructs; however, large variations in gene silencing efficiency have also been observed [56,57]. Often, these variations seem to be in a case-by-case manner and the underlying mechanism is unknown.

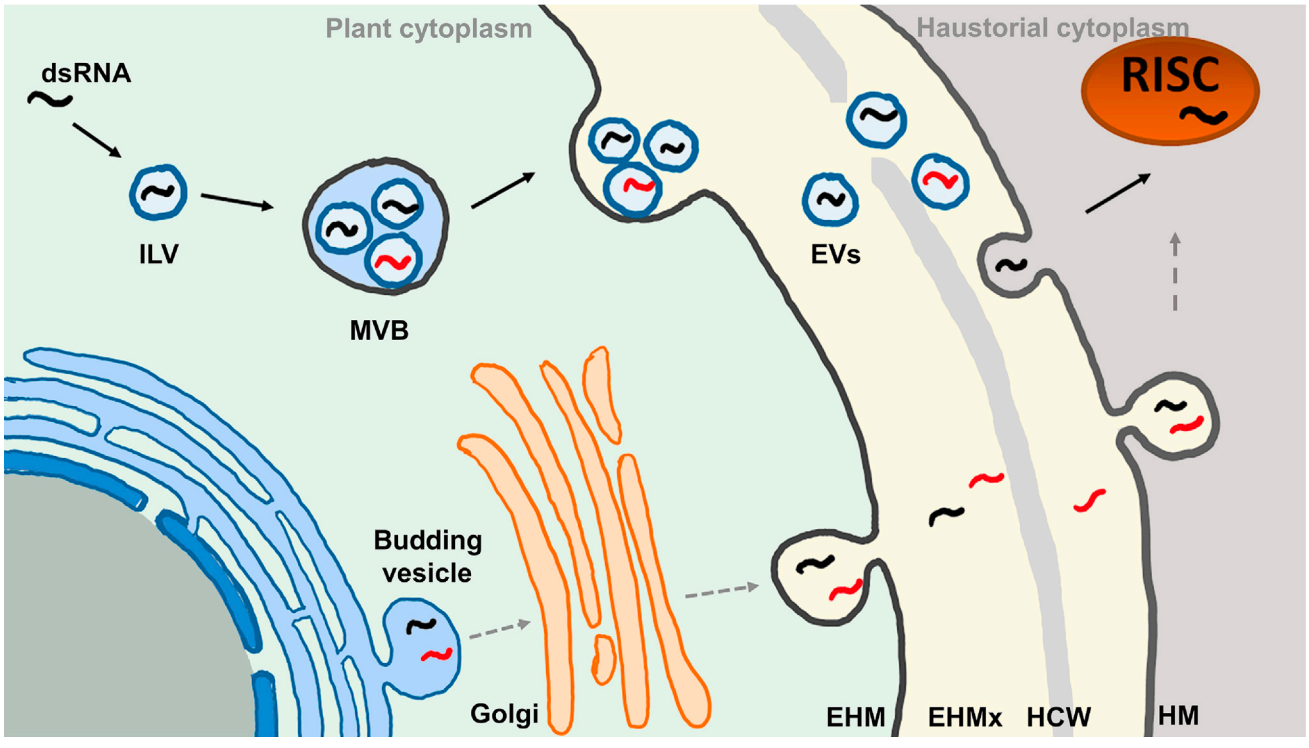

Figure 2. A Speculative Model of Small (s)RNA Trafficking between Plant and Pathogen Cells through Haustoria.
In plant cytoplasm, sRNAs as duplexes (dsRNA) may be incorporated into intraluminal vesicles (ILVs), which are subsequently internalized by multivesicular bodies (MVBs). MVBs may fuse with the extrahaustorial membrane (EHM) and release extracellular vesicles (EVs) into the extrahaustorial matrix (EHMx). The EVs may fuse with the haustorial membrane through endocytosis and unload their sRNA cargos in the invading pathogen cells. Once inside the pathogen cytosol, plant sRNAs may silence target gene(s) by hijacking the RNA-induced gene silencing (RISC) complexes. Another potential secretion route of sRNAs is based on the conventional secretion pathway for proteins where secondary siRNAs synthesized on the rough ER could be internalized into budding vesicles and then trafficked through the ER–Golgi. These sRNAs are released into EHMx as free sRNAs and taken up by haustoria through endocytosis. Abbreviations: HCW, haustorial cell wall of the pathogen; HM, haustorial membrane.

The recent sRNA profiling in EVs and apoplast indicates selective sRNA secretion, which inevitably affects HIGS efficiency and may contribute to the observed variation in gene silencing efficiency. Possible factors that influence sRNA secretion include sRNA sequences and biosynthetic pathways. In general, secondary siRNAs tend to be enriched in apoplast, possibly through an EV-independent secretion pathway. On the contrary, many miRNAs are relatively enriched in plant cytoplasm, indicating a low tendency to be secreted to extracellular spaces. Although some miRNAs are enriched in EVs or apoplast, what determines this selectivity remains elusive. In animal cells, specific sequence motifs in mature miRNAs have been found to determine selective loading in exosomes by RNA-binding proteins [58,59]. A specific extracellular Argonaute protein (exWAGO) was shown to be an EV cargo in a gastrointestinal nematode and has the potential to selectively load sRNAs that affect host immunity [60]. Identification and characterization of plant sRNA-binding proteins that may facilitate sRNA secretion through EV-dependent and -independent pathways will fill this important knowledge gap of HIGS. However, before the mechanism by which specific sRNAs are sorted for secretion is identified, an endogenous siRNA-based approach should have a better chance for success than artificial sRNAs.

Genetic evidence and pathogen effector target analysis strongly support a role for secondary siRNAs in plant defense. The 'shotgun' approach used by secondary siRNAs to target multiple pathogen genes may increase the sustainability of the resistance. Secondary siRNA production is a deeply conserved function in land plants [61]. It is therefore exciting to hypothesize that broad-spectrum resistance may be achieved by manipulating the endogenous secondary siRNA pathway in a way that the pool of siRNAs is optimized for targeting a particular pathogen(s) (Figure 3). For example, *PPR*-derived siRNAs in *Arabidopsis* have the potential to target multiple pathogens; and the production of secondary siRNAs from miRNA-targeted *PPR* transcripts is widespread in dicots through a conserved miRNA-*PPR*-siRNA circuit [17]. Using a synthetic biology approach, the endogenous secondary siRNA-producing *PPR* genes may be modified by replacing native siRNA sequences with sequences designed to target specific gene(s) in one or more pathogens in order to increase silencing efficiency. This secondary siRNA replacement strategy has proved to be feasible for silencing the

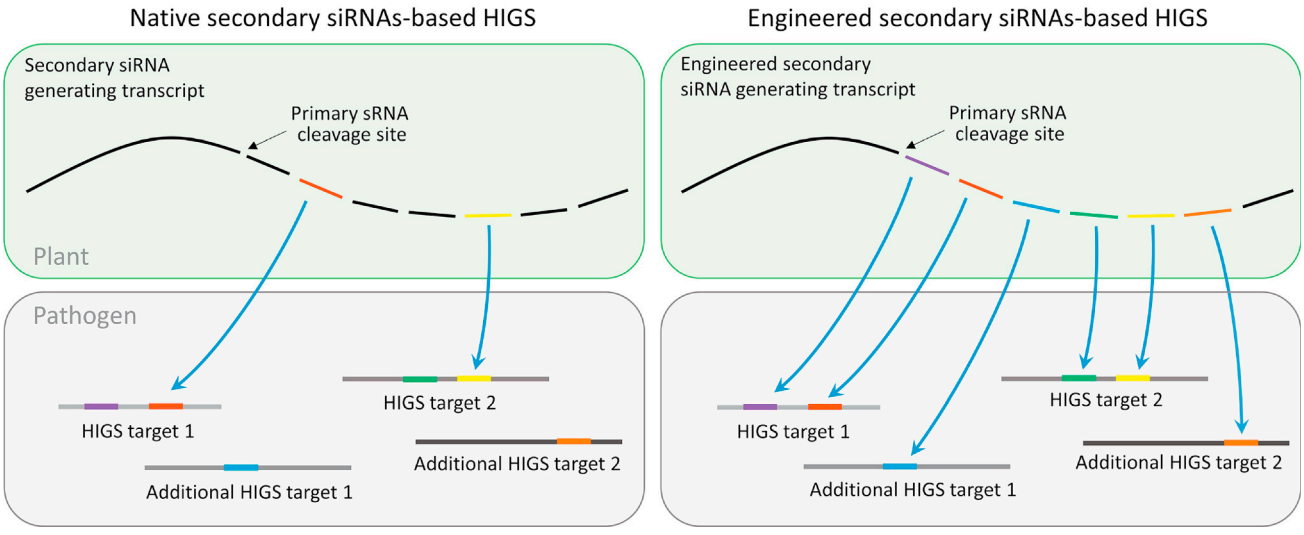

Figure 3. A Synthetic Biology Approach Can Be Used to Increase Host-Induced Gene Silencing (HIGS) Efficiency by Engineering Secondary Small Interfering (si)RNA-Producing Loci.

The siRNA-generating region of an RNA molecule is replaced by sequences that are designed to target specific sequences in pathogen genes. The primary sRNA cleavage site remains unchanged to initiate the synthesis of a dsRNA precursor, which would produce a pool of siRNAs that silence the target gene(s) with higher efficiency. As a result, the plants producing these siRNAs should be resistant to the pathogen. Lines with different colors represent different siRNA sequences or their complementary sequences in target transcripts.

FAD2 gene in *Arabidopsis* [62]. In combination with CRISPR/Cas9-based engineering technology, this approach has the promise to generate gene-edited crops with enhanced resistance.

Concluding Remarks

The research field of host–pathogen interactions has seen major breakthroughs in the arms race centered on trans-species sRNA silencing. In plants, both miRNAs and siRNAs have been shown to function as antimicrobial agents through a HIGS mechanism. However, unique features of secondary siRNAs, especially the potential of sequence diversification during coevolution with pathogen target genes, make them particularly suitable as antimicrobial agents. However, a major unanswered question is the sorting and secretion of sRNAs, which may be determined by EV-dependent or -independent pathways and could also be coupled with siRNA biogenesis. Nonetheless, these recent findings offer exciting new opportunities to develop disease resistance in crops by engineering natural sRNA-based defense. We expect many more exciting discoveries in this area of research to come out in the near future (see Outstanding Questions).

Outstanding Questions

Does HIGS represent a basal surveillance mechanism in plants? If so, how do pathogens overcome this defense during the arms race with the hosts? Is RNA silencing suppression activity a common virulence function required for pathogen infection?

Does the same pool of plant sRNAs function in HIGS against a variety of eukaryotic pathogen infections? What is the evolutionary dynamic of these sRNA-generating loci in plants?

How are plant sRNAs translocated into pathogen cells? If they are shuttled by extracellular vesicles (EVs), what determines the selectivity of sRNA loading? Do sRNAs secreted through EV-dependent or -independent pathways change during the infection of different pathogens? How is directional trafficking of EVs accomplished, especially during the infection of generalist pathogens that do not form specialized infection structures? Are specific RNA-binding proteins and vesicle receptors involved in the sRNA trafficking process?

Can plant sRNAs be loaded in the RNA-induced gene silencing complexes in pathogens? If so, can they effectively compete with pathogen endogenous sRNAs? Are the plant sRNA levels in the pathogen cells high enough to silence target genes to an extent that significantly impacts their functions? Are specific infection structures, such as haustoria, involved in the sRNA targeting process?

What is the evolutionary dynamic of plant sRNA-targeted genes in the pathogens? Some pathogens do not have canonical RNA-induced gene silencing complexes; can they still be affected by plant sRNAs?

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