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CURRENT REVIEW

Small RNAs Add Zing to the Zig-Zag-Zig Model of Plant Defenses

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Plant small RNAs play important roles in transcriptional and posttranscriptional regulation, with ongoing work demonstrating their functions in diverse pathways. Their roles in defense responses are a topic of active investigation, particularly the rich set of micro (mi)RNAs that target disease resistance genes such as nucleotide binding/leucine-rich repeat (*NB-LRR*) genes. The miRNA–*NB-LRR* interactions result in the production of phased, secondary small interfering (phasi)RNAs, and phasiRNAs function in both *cis* and *trans* to propagate negative regulatory effects across additional members of the target gene family. Yet, while phasiRNAs have the capacity to trigger targeted decay of specific targets, both in *cis* and *trans*, their functional relevance in *NB-LRR* regulation remains largely a matter of speculation.

The plant immune system is composed of two layers of defense responses that provide protection against pathogens, including pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). PTI acts as the first layer of defense when membrane-localized pattern recognition receptors (PRR), principally receptor kinases (RK) or receptor-like proteins (RLPs), perceive the presence of PAMPs on the cell surface (Zipfel 2014). PAMPs such as flg22 and Ef-Tu are well-characterized pathogen-derived molecules that trigger PTI responses in the host cell. The PRR-mediated PTI pathway activates a variety of plant immune responses, such as stomatal closure, a burst of reactive oxygen species, increased deposition of callose, signal transduction mediated by mitogen-activated protein kinases, and more (Bigeard et al. 2015; Nicaise et al. 2009). Compared with PTI, ETI is stronger in its amplitude of defense and it is usually mediated by a family of nucleotide-binding/leucine-rich repeat (NB-LRR or NLR) proteins encoded by *NLR* genes. ETI functions in the recognition of effectors secreted by pathogens, activating the hypersensitive response, which is manifested by programmed cell death of the host plant (Cui et al. 2015). In recent years, connections have made between *NB-LRR* genes and small RNA pathways, and this is the focus of our review.

Small RNAs are a type of noncoding RNA that have a variety of biological functions. Plant small RNAs can be divided into several categories according to their distinct biogenesis pathways (Axtell 2013), in every case functioning in gene silencing at the level of either transcriptional gene silencing or posttranscriptional gene silencing (Brodersen and Voinnet 2006). Host small RNAs, such as micro (mi)RNAs and small interfering (si)RNAs, have been described to play crucial roles in plant disease resistance (Katiyar-Agarwal and Jin 2010; Padmanabhan et al. 2009). Plant miRNAs are processed from transcripts forming a stem-loop secondary structure, transcribed from *MIR* genes (Voinnet 2009). A subset of plant miRNAs has been shown to target *NLR* genes regulating plant immunity (Li et al. 2012; Shivaprasad et al. 2012; Zhai et al. 2011). Interestingly, some of the *NLR*-targeting miRNAs are capable of triggering the production of phased secondary (pha)siRNAs from their cleaved target mRNAs, a capability variously attributed either to the 22-nt length of the miRNA triggers or to an asymmetric bulge in the region of the mRNA precursor processed into the miRNA-miRNA* duplex (Chen et al. 2010; Cuperus et al. 2010; Manavella et al. 2012). The more typical 21-nt length of plant miRNAs is less often implicated in the triggering of phasiRNAs, but in such cases, it is typically associated with a pair of target sites in target transcripts, known as 'two-hit' activity (Axtell et al. 2006).

Several years ago, we reviewed phasiRNAs, including aspects of their discovery, their biogenesis pathways, and the classes of genes from which phasiRNAs are generated (Fei et al. 2013). In that review, we focused on the *NLR* gene family, which, in many plants, includes numerous members that yield an abundance of phasiRNAs; we discussed what might be the selective advantage of the production of such a large number of *NLR*-derived secondary siRNAs and how those siRNAs might be utilized. Over the past two years, research on phasiRNAs has advanced, as has the thinking about their roles and functions. Here, we review this recent progress, describing the possible integration of miRNA/phasiRNA-involved gene regulation into the classic 'zig-zag-zig' model of the plant immune system. In addition, we discuss an evolutionary perspective on the roles of miRNA and phasiRNAs in plant defenses.

Roles of miRNAs and phasiRNAs in PTI.

In *Arabidopsis*, treatment with the PAMP known as flg22 induces the accumulation of miR393, which in turn targets F-box auxin receptors, including TIR1, AFB2, and AFB3 (Navarro et al. 2006). This repression of auxin signaling correlates with enhanced disease resistance in plants, reflecting an enhancement of PTI (Navarro et al. 2006). miR393 is conserved across plant species, as are its targets and target sites (Bian et al. 2012). It is possible that its function in suppressing auxin signaling and enhancing PTI is also conserved. There is some support for this hypothesis of a conserved function; for example, in soybean, miR393 is upregulated upon infection by the oomycete pathogen *Phytophthora sojae* and a miR393-knockdown in the transient 'hairy root' system displayed enhanced susceptibility to *Phytophthora sojae* (Wong et al. 2014). Interestingly, as a 22-nt miRNA, miR393 triggers phasiRNA biogenesis from the target transcripts of *AFB2* and *AFB3* in *Arabidopsis*, and

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these secondary siRNAs together with miR393 repress transcript levels of *TIR1*, *AFB2*, and *AFB3* (Si-Ammour et al. 2011 Consequently, via hormone crosstalk, both plant development and basal defense responses are regulated by miR393 and miR393-triggered phasiRNAs, a situation that fits in a modified version of the now-classic 'zig-zag-zig' model of plant-pathogen signaling and responses (Fig. 1) (Jones and Dangl 2006). Therefore, miR393-involved regulation of the auxin signaling pathway has crosstalk with immune responses at the level of PTI. Although other plant hormones, such as gibberellic acid, brassinosteroid, and abscisic acid, have crosstalk with PTI, and salicylic acid– and jasmonic acid–mediated defense pathways (Denancé et al. 2013; Huot et al. 2014; Kazan and Manners 2009; Spoel and Dong 2008), these hormone-mediated pathways apparently include few roles for miRNAs in defenses, making miR393 the only miRNA that is a verified participant in PTI via hormone crosstalk.

miRNAs other than miR393 that suppress auxin signaling also play a role in plant immune responses. For example, overexpression of miR160a, a miRNA targeting auxin response factors, increases callose deposition in *Arabidopsis* treated with either flg22 or the *hrcC* mutant defective in the type III secretion system of *Pseudomonas syringae* (Li et al. 2010) and, in rice, enhances plant immunity against *Magnaporthe oryzae* (Li et al. 2014). In addition, miR398b, a miRNA targeting mRNAs of copper and zinc superoxide dismutases, has different roles in plant disease resistance. In *Arabidopsis*, miR398b accumulation was reduced upon flg22 treatment, overexpression of miR398b showed decreased callose deposition, and thereby, increased susceptibility to *P. syringae* DC3000 (Li et al. 2010). In contrast, in rice, overexpression of miR398b yielded increased hydrogen peroxide production and inhibited fungal growth (Li et al. 2014). The effects of this miRNA on immune responses are quite variable; however, it is not clear whether this difference is because different immune responses are triggered by diverse types of pathogens or because of a fundamental distinction in the immune systems in dicots and monocots.

microRNA and phasiRNA involvement in ETI.

Apart from miRNA roles in PTI via hormonal regulation, there are numerous miRNAs that directly target transcripts from *NLR* genes, a class of genes predominantly involved in ETI (Fig. 1). Moreover, many or most of these miRNAs are 22 nt and trigger the production of phasiRNAs from their *NLR* targets (Fei et al. 2013). In the legume *Medicago truncatula*, several hundred *NLR* genes are targeted by just five miRNAs (miR1507, miR2109, and miR2118a, b, and c) at sequences encoding conserved motifs of resistance (R) proteins, triggering widespread phasiRNAs (Fei et al. 2015; Zhai et al. 2011). Due to a close evolutionary relationship with *Medicago* spp., the regulatory network for *NLR* genes in soybean utilizes a similar but somewhat expanded repertoire of miRNAs (Arikit et al. 2014). In Solanaceous species, including tomato, potato, and tobacco, *NLR*-targeting miRNAs have also been well-characterized as triggering abundant phasiRNAs, including the miRNAs miR482, miR5300, miR6019, and miR6027 (Li et al. 2012; Shivaprasad et al. 2012). Perennial woody plants have also been reported to employ the miR482/2118 superfamily and other miRNA families to repress *NLR* genes. For example, in peach, about 94 *NLR* genes were identified as *PHAS* loci, predominantly triggered by the miR482 family (Zhu et al. 2012). A recent study identified a novel miRNA that targets an *R* gene in apple (Ma et al. 2014); this *R* gene is expressed at a higher level in the resistant than the susceptible cultivar, and interestingly, *Agrobacterium*-mediated infiltration of the *R* gene in the leaf of a susceptible apple cultivar enhanced plant immunity against the fungal pathogen *Alternaria alternata* f. sp. *mali* (Ma et al. 2014). In spruce, poplar, and grape, a large proportion of *NLR* genes produce 21-nt phasiRNAs (Källman et al. 2013). In a recently published study in spruce, we showed that spruce *NLR* genes are targeted by both the conserved miR482/2118 superfamily and a large number of other miRNAs (Xia et al. 2015), indicating that *NLR* genes are targeted by a variety of miRNA families in different plants. Indeed, there is substantial variation

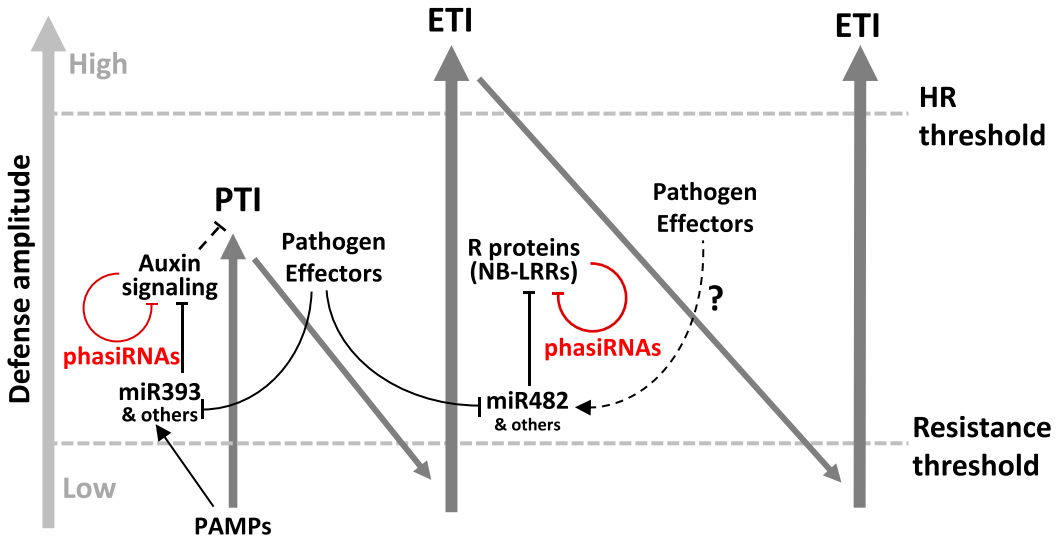

Fig. 1. The integration of micro (mi)RNAs and phased secondary small interfering (phasi)RNAs in the 'zig-zag-zig' model of the plant immune system. The original model by Jones and Dangl (2006) describes a stepped, evolutionary model for defenses that describes the quantitative nature and molecular evolution of disease resistance in plants. In a variation on that model, pathogen-associated molecular patterns (PAMPs) induce the accumulation of miRNAs that participate in PAMP-triggered immunity (PTI) via hormone crosstalk. For example, miR393, which targets genes that are involved in auxin signaling (*TIR1*, *AFB2*, and *AFB3*) is induced upon treatment with flg22. The repression of auxin signaling during infection enhances host PTI by hormone crosstalk. PhasiRNAs are triggered by miR393, which enhances the activity of this miRNA by targeting genes involved in the auxin signaling pathway. Effectors from pathogens can suppress the levels of plant miRNAs, such as miR393, to enhance susceptibility. However, the miR482 family, a negative regulator of plant resistance (*R*) genes, can also be repressed upon detection of effectors to enhance effector-triggered immunity (ETI). Some miRNAs can trigger phasiRNA biogenesis from *R* genes, and these phasiRNAs may function synergistically with miRNAs either in *cis* or *trans* to suppress *R*-gene transcript levels. Some effectors may also promote miRNA stability or production by targeting the cellular machinery involved in small RNA biogenesis or turnover. Some aspects of this model are speculative (indicated by a question mark), for example, whether effectors may specifically activate miR482 expression to attenuate host ETI.

across species in both the presence and absence of these miRNAs and in the breadth of their targets (and of the resulting phasiRNAs). *Arabidopsis* has just two miRNAs, miR472 and miR825*, that target only a few *NLR* genes (Chen et al. 2010; Howell et al. 2007). In the grasses, a member of the miR482 superfamily, miR2118, has a distinct and specialized role in reproductive tissues, as a trigger of 21-nt phasiRNAs from long noncoding RNAs instead of from *NLR* transcripts (Song et al. 2012; Zhai et al. 2015). This spatially restricted pattern of miR2118 is inconsistent with its role in *R*-gene regulation, seen in most eudicots, in which *NLR*-derived phasiRNAs are observed in vegetative tissues (Arikit et al. 2014; Li et al. 2012; Shivaprasad et al. 2012; Zhai et al. 2011). Do the grasses lack miRNA-mediated regulation of *R* genes? Apparently not, as miR9863 was recently identified in both barley and wheat and was shown to target *MLA* genes, a class of coiled coil (CC)-type *NLR* genes (Liu et al. 2014). Interestingly, the 22-nt variant of miR9863 more efficiently suppresses *MLA1* than 21-nt miR9863, presumably via the *cis* activity of *MLA* phasiRNAs (Liu et al. 2014). Coupling the sequencing of ever more plant genomes with detailed molecular studies of plant defenses, we are sure to learn more about the diversity and roles of *NLR*-targeting miRNAs.

Transcripts of other types of genes resembling *R* genes other than *NB-LRR* genes are also regulated by miRNAs that trigger phasiRNAs. These genes encode RLPs targeted by miR6022 and miR6023 (Li et al. 2012) and receptor-like kinases (RLKs) targeted by miR396 (Arikit et al. 2014); both RLPs and RLKs include LRRs. Though the genomic copy number of LRR-encoding genes is similar to that of *NB-LRR* genes in at least 12 plant genomes (Wang et al. 2011), a much smaller number and proportion of miRNAs or phasiRNAs regulate these receptor-like genes. For example, in soybean, only 25 of the approximately 600 RLK genes generate phasiRNAs versus 208 of 319 *NB-LRR* genes (Arikit et al. 2014). While the role in plant immunity of miRNA regulation of these *RLK* or *RLP* genes is not yet clear, the parallels to *NB-LRR* genes are intriguing.

Despite a growing number of studies, we still lack a clear and incontrovertible understanding of the functional importance of the role of miRNAs and phasiRNAs in *R*-gene regulatory networks. Yet, clues are starting to emerge. For example, a recent study demonstrated that tomato miR482 and miR5300, the latter a member of the miR482/2118 superfamily, target four *R* genes that play a role in disease resistance to the wilt fungus *Fusarium oxysporum* (Ouyang et al. 2014). Individual knockdowns in tomato of these four *R* genes via virus-induced gene silencing rendered a resistant cultivar susceptible to *F. oxysporum* (Ouyang et al. 2014). Combining this study and the work by Shivaprasad et al. (2012), the miR482 superfamily has a demonstrated role to suppress a wide range of *R* genes that confer resistance to viral, bacterial, and fungal pathogens. Earlier work in *Medicago* spp. demonstrating a handful of miRNAs can trigger phasiRNAs from more than 100 targets, resulting in phasiRNAs with an even more greatly expanded set of related targets, led to hypothesis that these miRNAs are 'master regulators' of the *NLR* family of *R* genes. But the basis for the variation across plants in the extent of this regulatory network is puzzling.

Since phasiRNAs function as negative regulators of *NLR* genes, loss-of-function mutants in the phasiRNA biogenesis pathway should exhibit enhanced ETI-based resistance to some pathogens. Indeed, consistent with this, *Arabidopsis* mutants of both *rdr6* and miR472 (a variant of the miR482 family found in *Arabidopsis*) displayed enhanced ETI mediated by RPS5 to the *P. syringae* DC3000 strain carrying AvrPphB (Boccara et al. 2014). These results suggest that phasiRNA biogenesis from *NLR* genes may negatively regulate ETI. Boccara et al. (2014) identified a number of *CC-NB-LRR* genes targeted by *NLR*-derived phasiRNAs resulting from miR472 activity, showing that these miR472-triggered phasiRNAs act in *cis* and *trans* to suppress disease resistance genes until necessary, constituting an ETI enhancement switch (Boccara et al. 2014). Surprisingly, this study also showed that RDR6 negatively regulates PTI, because the expression levels of *WRKY22, WRKY29*, and *FRK1* (PAMP-responsive genes) were significantly higher in *rdr6* compared with wild type (Boccara et al. 2014). In addition, increased callose deposition was observed in *rdr6*. These boosted PTI responses likely contributed to enhanced resistance against the pathogen *P. syringae* in *rdr6*, as quantified by bacterial titer (Boccara et al. 2014). Interestingly, it was also observed that *RDR6* expression decreased rapidly upon flg22 treatment (Boccara et al. 2014). Therefore, it is likely that plants swiftly inhibit the RDR6-mediated RNA silencing pathway to strengthen host immune responses when sensing PAMPs. This suggests that there is likely an underlying signaling pathway mediated by PRR that downregulates *RDR6* expression; the components of this pathway and the molecular mechanism by which RDR6 suppresses PTI responses remain to be determined.

A separate study showed varying roles of RDR6, Dicer-like 4 (DCL4), and the DCL4-interacting protein DRB4 (DOUBLE-STRANDED RNA BINDING PROTEIN 4) in work focused on the *Arabidopsis* NB-LRR known as hypersensitive response to TCV (HRT) that confers resistance to *Turnip crinkle virus* (Zhu et al. 2013). These authors characterized a requirement for RDR6, DCL4, and DRB4 for HRT-mediated resistance and demonstrated a strong yet not well-described mechanistic connection between the pathway that produces secondary siRNAs and NB-LRR-mediated defenses. A large body of earlier work has focused on the roles of RNA interference in antiviral defenses (Pumplin and Voinnet 2013) rather than the *R* gene–mediated processes that are our focus. In the future, it will be informative to replicate assays of phasiRNA function by reducing or eliminating RDR6 activity in species with even more extensive sets of *NLR*-targeting miRNAs and phasiRNAs than *Arabidopsis*.

The evolutionary origin of *R*-gene-targeting miRNAs.

The two major domains in NLR proteins, the NBS and the LRR, are found as components of one R protein (the prototypical *NLR*) only in land plants (Yue et al. 2012). Just two and four *NLR* genes, perhaps representative of early evolved *R* genes, are encoded in the genomes of the lycophyte *Selaginella moellendorffii* or the moss *Physcomitrella patens*, respectively. In the hundreds of millions of years since those species emerged, substantial genetic diversification and expansion occurred, yielding hundreds of *NLR* genes in gymnosperm and angiosperm genomes (Yue et al. 2012). Amplification of *NLR*-targeting miRNAs may have occurred apace. In the gymnosperm Norway spruce, tens of miRNAs target *NLR* genes (Xia et al. 2015). One group of these, the miR482/2118 family, is found in many plant species; its target is typically the highly conserved region coding for the P-loop motif within the NBS domain. The presence of *MIR482/2118* can be traced back at least to gymnosperms. There are approximately 23 *MIR482/2118* members in the spruce genome, several of which have extensive sequence identity of the precursors to *NLR* genes, suggesting the origin of the miRNA via gene duplication from its target (Xia et al. 2015). Yet this miRNA family continues to diversify, evidenced by lineage-specific members, such as miR1510, found in the legumes (Zhao et al. 2015).

The coordinated evolutionary emergence of *NLR* genes and the miRNAs that target *NLR* genes may reflect a need to balance *NLR* function and diversity with *NLR* suppression. To speculate on the evolutionary process, we could imagine that plants first evolved *NLR* genes to suppress pathogens of increasing sophistication. This may have led to high genomic copy numbers of *NLR* genes through gene duplication,

improving plant defenses against diverse pathogens in a gene-for-gene manner. Yet *NLR* genes come with costs (Tian et al. 2003). To limit fitness costs and to regulate (perhaps coordinately) the expression of diverse and numerous *NLR* genes, miRNAs emerged from *NLR* fragments (Xia et al. 2015). Over an evolutionary time period, *NLR* sequences diversify, with conservation limited to discrete regions like the encoded P-loop; only those miRNAs targeting these conserved regions, like miR482/2118, are maintained.

Interestingly, in gymnosperms—in which miR482/2118 may have first appeared—this miRNA family has dual functions. It targets numerous long, noncoding (lnc)RNAs to instigate biogenesis of reproductive phasiRNAs (as in grasses), and it targets hundreds of *NLR* genes to trigger the production of phasiRNAs (as in many eudicots and perhaps some monocots). As described above and reviewed previously (Fei et al. 2013), *NLR*-derived phasiRNAs perhaps act to broadly regulate defenses or play roles in minimizing the fitness costs of *R* genes, while the function of reproductive phasiRNAs from lncRNAs in grasses remains a mystery. It is unlikely that they exert any role related to disease defense due to their strict temporal and spatial specificity to the development of reproductive tissues, especially anthers (Zhai et al. 2015).

Suppressors of RNA silencing interfere with miRNA-mediated regulation of PTI and ETI.

Host small RNAs and the RNA biogenesis machinery have well-described roles in plant disease resistance and plant-microbe interactions (Katiyar-Agarwal and Jin 2010; Peláez and Sanchez 2013). During an evolutionary 'arms race' between pathogens and their plant hosts, the secretion of suppressors of RNA silencing to promote host susceptibility has proven an effective strategy (Pumplin and Voinnet 2013). The first bacterial suppressor of RNA silencing (BSR), AvrPtoB from *P. syringae*, was demonstrated to suppress the transcription of *MIR393*, enhancing PTI via modulation of hormone signaling (Navarro et al. 2008) (Fig. 1). In contrast, the BSR AvrPto does not alter *MIR* transcription, suggesting perhaps an inhibitory role in pri-miRNA processing, while the effector HopT1-1 was shown instead to interrupt translational repression mediated by miRNAs (Navarro et al. 2008). A study in tomato showed that miR482 levels decreased upon infection by *P. syringae*, suggesting that BSR may interfere with either pri-miR482 transcription or processing (Shivaprasad et al. 2012) (Fig. 1). Levels of a control miRNA with no known role in defenses (miR168) were not impacted upon pathogen infection, suggesting a specific inhibition of miR482 (Shivaprasad et al. 2012). Alternatively, plant recognition of effectors could result in transcriptional inhibition of *MIR482*; however, it is unlikely that PAMP-mediated signaling causes miR482 reduction, because infection by a *P. syringae hrcC* mutant (mentioned above) also reduced miR482 levels (Shivaprasad et al. 2012). Intriguingly, the decrease in mature miR482 was also produced by inoculation with the fungal pathogen *F. oxysporum* in a resistant but not susceptible tomato cultivar (Ouyang et al. 2014), suggesting a possible pathway for miR482 suppression. Hence, it is possible that resistant host genotypes have evolved to recognize pathogen effectors and reduce levels of miR482 and miR482-triggered phasiRNAs, thereby increasing levels of *R* genes and enhancing ETI (Fig. 1). Alternatively, some effectors may activate transcription of *NLR*-targeting miRNAs to attenuate ETI. For example, strains of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* secrete transcription activator-like (TAL) effectors to specifically upregulate *HEN1*, encoding a methyltransferase that stabilizes small RNAs via 3′ methylation in rice (Li et al. 2005; Moscou and Bogdanove 2009). Presumably these effectors induce disease susceptibility via *HEN1* activation, although the connection between the stabilization of host miRNAs or siRNAs and defenses is not yet clear. In summary, pathogens have evolved a variety of suppressors of RNA silencing that act in diverse ways to enhance plant susceptibility.

Perspectives and outlook.

Current data indicates that plant miRNAs together with the phasiRNAs they trigger are important regulators of plant immunity in both PTI and ETI. As described above, miR393 plays a role in enhancing PTI by repressing auxin signaling, while miR398 functions in PTI in both *Arabidopsis* and rice (Li et al. 2010, 2014). Yet more remains to be explored, such as how the miR398b-mediated RNA silencing pathway affects pathogen resistance. The most extensive, yet still poorly understood set of miRNAs is miR482/2118 and other miRNAs that generate phasiRNAs from *NLR* genes; these miRNAs appear to be regulators of ETI in plants by suppression of *R* genes, presumably as master regulators—a small number of miRNAs regulating an enormous family of genes. PhasiRNAs may attenuate plant immunity either in *trans*, by targeting other *R* genes or genes in other families, or in *cis*, to target the genes that generate the phasiRNAs, thereby enhancing the suppression efficiency of the miRNA. Or another way to think about this is that relief of small RNA suppression could boost plant immunity. As mentioned above, RDR6, a key protein in the biogenesis of phasiRNAs, plays a role in resistance responses, although with an underlying mechanism that remains unclear. The miR482/2118 family is a conserved miRNA family regulating *NLR* genes in a wide range of plant species, and it is probably the most complex family in a large set of miRNAs that target different regions of *NLR* genes. Interestingly, in addition to the miR482/2118 family in dicots, the miR9863 family seems to be restricted to members of family *Triticeae* (Liu et al. 2014). Therefore, a question that needs to be solved is whether and how other monocots that lack *NLR*-targeting miRNAs regulate immunity at the level of *NLR* transcripts.

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