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Biogenesis and regulatory hierarchy of phased small interfering RNAs in plants

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Abstract:

Several varieties of small RNAs including microRNAs (miRNAs) and small interfering RNAs (siRNAs) are generated in plants to regulate development, genome stability, and response to adverse environments. Phased siRNA (phasiRNA) is a type of secondary siRNA that is processed from a miRNA-mediated cleavage of RNA transcripts, increasing silencing efficiency or simultaneously suppressing multiple target genes. *Trans*-acting siRNAs (ta-siRNAs) are a particular class of phasiRNA produced from non-coding transcripts that silence targets in *trans*. It was originally thought that "one hit" and "two hit" models were essential for processing distinct *TAS* precursors; however, a single hit event was recently shown to be sufficient at triggering all types of ta-siRNAs. This review discusses the findings about biogenesis, targeting modes, and regulatory networks of plant ta-siRNAs. We also summarize recent advances in the generation of other phasiRNAs and their possible biological benefits to plants.

Key words: Phased siRNA, *TAS*, one hit, NBS-LRR, DNA methylation

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Introduction

Small RNAs are short RNA molecules, 21–24 nucleotides (nt) in length, involved in the control of gene expression during development and environmental adaptation. Small RNAs in plants are categorized into two major classes according to their origin and biogenesis: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Axtell, 2013). Typically, miRNA precursors originating from stem–loop structures are processed by the RNase III enzyme Dicer-like (DCL) into 20–24 nt fragments, and subsequently loaded onto Argonaute (AGO) proteins as part of the RNA-induced silencing complexes (RISCs) to silence targets via nucleotide base-pairing (Achkar et al., 2016; Megraw et al., 2016; Wu et al., 2009). While AGO1-mediated RISCs promote 21-nt miRNA suppression by mRNA digestion and translation inhibition, AGO4-mediated epigenetic modification complexes guide 24-nt miRNAs to target DNA methylation at the transcriptional level (Wu et al., 2010).

Common features of siRNAs and miRNAs are that they share the same processes of 3′ end modification by the RNA 2′-O-methyl-transferase Hua enhancer 1 (HEN1) (Rogers and Chen, 2013). However, while siRNAs are enormously amplified by an RNA-dependent RNA polymerase (RDR) after their generation, miRNAs are not (Matzke et al., 2015). siRNAs can be divided into three subclasses according to the differences in their origin and processing enzymes: heterochromatic siRNAs (hc-siRNAs), natural antisense siRNAs (nat-siRNAs), and phased siRNAs (phasiRNAs). hc-siRNAs are 24-nt in length, transcribed by a plant-specific RNA polymerase, PolIV, and processed via DCL3 from a genomic repeat or transposon region. After amplification involving RDR2 activity, hc-siRNAs are recognized by AGO4 complexes, and recruited by PolV with a long non-coding scaffold RNA to direct DNA methylation for the maintenance of genome stability and integrity (Matzke et al., 2015). nat-siRNAs is a class of highly enriched small RNAs at the annealed pair regions of natural antisense transcripts, of which one gene is constitutively expressed while the other is inducible (Zhang et al., 2013).

In animals, transport of nascent mRNAs from nucleus towards cytoplasm can be mediated by a multimeric protein complex, namely THO/TREX. If an RNA transcript in plants is exported by the THO/TREX complex and then becomes a template targeted by an miRNA, it can form numerous secondary siRNAs in a phased pattern relative to the miRNA cleavage site; therefore, these small RNAs are termed phasiRNAs (Fei et al., 2013). Furthermore, when a non-coding transcript is sliced by a miRNA, it generates a subset of phasiRNAs known as *trans*-acting siRNAs (ta-siRNAs) that can regulate target coding genes via mRNA cleavage in *trans* (Figure 1) (Fei et al., 2013). Nowadays, the recent development of sequencing

approaches and resultant increase in the genome-wide small RNA identification has enabled more secondary siRNAs to be characterized in a head-to-tail arrangement. Canonical phasiRNAs and ta-siRNAs are readily distinguished, because when phasiRNAs are generated from non-coding transcripts and function outside of the local region, they are known as ta-siRNAs.

To date, four families of *TAS* genes with eight loci have been discovered in the *Arabidopsis* genome. miR173 triggers three *TAS1* families and one *TAS2* family of ta-siRNAs, miR390 initiates three *TAS3* families of ta-siRNAs, and miR828 induces ta-siRNAs at the *TAS4* locus (Rajagopalan et al., 2006). *TAS1*, *TAS2*, and *TAS4* only exist in a limited number of plant species; however, *TAS3* ta-siRNAs are conserved in thousands, from bryophytes to angiosperms (Xia et al., 2017). Compared with ta-siRNAs, canonical phasiRNAs are less conserved (Xia et al., 2015a). In this review, we will mainly discuss the production of ta-siRNAs and phasiRNAs, and describe that how they behave in regulatory cascades rather than other small RNA actions, which have been extensively described earlier (D'Ario et al., 2017; Li et al., 2017).

The biogenesis of phasiRNAs and ta-siRNAs

Generally, miRNAs repress targets either by direct digestion or by translation inhibition in a homology-dependent manner. However, when miRNAs of a particular length, structure, or effector features cleave RNA molecules, they can generate small RNA precursors (Fei et al., 2013). These are amplified into double-stranded RNAs (dsRNAs) by RDR6 with the binding factor SUPPRESSOR OF GENE SILENCING3 (SGS3). The dsRNAs are subsequently processed by DCL4 and DOUBLE-STRANDED RNA BINDING FACTOR4 (DRB4) into 21-nt secondary siRNAs in a "head-to-tail" phased pattern (Figure 2). While the importance of RDR6 and DCL4 is largely known in the phasiRNA biogenesis pathway, the molecular mechanism by which SGS3 and DRB4 process phasiRNA is unclear, even though a dramatic decrease of phasiRNAs is observed in their respective mutants (Yoshikawa et al., 2013). These 21-nt phasiRNAs are mainly deployed into AGO1-mediated RISCs, and then function against other mRNAs. A class of 24-nt phasiRNAs have been recently discovered during the reproductive stage in rice, and these small RNAs are processed by a DCL3 protein, DCL3b, rather than DCL4 for their biogenesis, even if their precursor dsRNAs are amplified by the SGS3 and RDR6 module as canonical 21-nt phasiRNAs (Figure 2) (Johnson et al., 2009; Komiya, 2017; Song et al., 2012a; Song et al., 2012b). Whether these 24-nt phasiRNAs are distributed into AGO4 complexes for transcription silencing or AGO1 effectors for mRNA degradation still remains elusive.

miRNA hijack action against long non-coding RNAs can lead to the production of ta-siRNAs. The biogenesis model of ta-siRNAs is a good example of how plants produce phasiRNAs. Thus far, four distinct classes of ta-siRNAs have been characterized in *Arabidopsis* which can be interpreted by two different mechanisms: "one hit" and "two hit".

In the "one hit" model, a single miRNA guides target RNA transcript digestion through AGO1 RISCs, resulting in the generation of ta-siRNAs 3′ downstream of the miRNA cleavage site (Figure 2) (Felippes and Weigel, 2009; Montgomery et al., 2008b). PhasiRNAs cannot triggered by all miRNAs, but only by those with 22-nt in length or a bulge structure in the miRNA and miRNA* duplex (Chen et al., 2010; Cuperus et al., 2010; Manavella et al., 2012). Because the generation of 22-nt miRNAs usually results from an asymmetric duplex in the miRNA precursor, it is assumed that 22-nt miRNAs or those with unpaired nucleotides are the same cause for triggering secondary siRNAs (Manavella et al., 2012). Intriguingly, although miR171 is a canonical 21-nt miRNA with no asymmetric structure, it was recently shown to give rise to phasiRNAs in a *hen1* background (Zhai et al., 2013). This may be because of the presence of an additional nucleotide at the miR171 3′ terminal end in the *hen1* mutant, where small RNAs are increased uridylation (Zhai et al., 2013). This observation further suggests that miRNA with 22-nt in length is indeed important for phasiRNA biogenesis. Nonetheless, some exceptions of miRNAs also exist in the "one hit" model, for instance, miR824, a 21-nt miRNA and with no bulge structure between miRNA and miRNA*, can be loaded onto AGO1 to generate ta-siRNAs at *TAS4* loci, although the mechanistic detail is still elusive (Rajagopalan et al., 2006). Taken simply, "one hit" refers to the generation of phasiRNAs by an miRNA-mediated cleavage event.

Compared with *TAS1* and *TAS2* ta-siRNAs and most phasiRNAs, which are triggered by 22-nt miRNAs through "one hit" process, ta-siRNAs from *TAS3* loci in *Arabidopsis* are initiated by the 21-nt miR390 in a "two hit" mechanism (Figure 2) (Axtell et al., 2006). Under this process, all three *TAS3* loci have two miR390 target sites, but only the 3′ proximal site of *TAS3* can be cleaved while the 5′ site loses its cleavage ability in *Arabidopsis* because it has fewer nucleotides that match miR390 (Montgomery et al., 2008a). miR390 3′ targeting seems not to be essential for *TAS3* ta-siRNA biogenesis because phased siRNAs can still be generated if other miRNAs replace miR390 at the *TAS3* 3′ proximal site, as long as cleavage takes place (Montgomery et al., 2008a). By contrast, the *TAS3 5′* region is indispensable for triggering ta-siRNAs because a change in this miR390 binding site to anmiRNA entirely blocks secondary siRNA generation (Montgomery et al., 2008a).

Another feature of the "two hit" process is that miR390 must be recruited into AGO7 rather than an alternative AGO at the 5′ miR390 targeting site to trigger *TAS3* ta-siRNAs (Figure 2) (Axtell et al., 2006; Montgomery et al., 2008a). The AGO7–miR390 complex has been proposed to operate through a ‘‘stable’’ association, which is required for subsequent routing of *TAS3a* transcripts (Endo et al., 2013; Montgomery et al., 2008a). This may generate an intermediate aberrant poly(A)-less substrate that facilitates the amplification by RDR6 (Baeg et al., 2017). Although miR390/AGO7 RISCs appear to have an essential non-cleavable interaction at the 5′ site for *TAS3* ta-siRNA biogenesis in *Arabidopsis*, this is not consistent across all plant species because both miR390 sites are cleavable in pine and moss (Axtell et al., 2006). Moreover, both cleavable and non-cleavable configurations of miR390 and *TAS3* transcripts have been identified among distinct *TAS3* paralogs in spruce, implying that the machinery of the miR390–AGO7 module in *TAS3* transcripts of the "two-hit" model differs between different plant species (Xia et al., 2015a). It will be of interest to understand why distinct mechanisms occur at miR390 and *TAS3* 5′ binding sites.

Using a variety of artificial ta-siRNA transgenic *Arabidopsis* lines that silence SULPHUR, an ideal system to observe the bleaching phenotype, de Felippes et al. recently showed that secondary siRNAs are still generated when the 5′ proximal miR390 target site of *TAS3* is mutated to allow cleavage (de Felippes et al., 2017). This mimics what occurs in moss, whereby two cleavable events at both the 5′ and 3′ ends impair the phasing pattern of one cleavage event in the "two-hit" configuration. Furthermore, a single slicing process at the *TAS3* 5′ proximal region by miR390 was found to be sufficient to trigger ta-siRNA production, even if no cleavage occurs at the 3′ miR390 targeting region (de Felippes et al., 2017). This suggests that *TAS3* ta-siRNA biogenesis is independent of miR390 cleavage at the 3′ end. Importantly, they showed that the generation of ta-siRNAs could be achieved by a non-cleavable interaction of miR390 and the TAS3 5′ target site (de Felippes et al., 2017). Although unexpectedly, triggering ta-siRNA by an miRNA-mediated non-cleavage event is believable because ta-siRNAs from *TAS1* in *Arabidopsis* can also be initiated by miR173 in a cleavage-independent manner (de Felippes et al., 2017). Therefore, the "one-hit" model process appears to be applicable to all secondary siRNA syntheses. However, specificities on how ta-siRNAs are generated from *TAS1* and *TAS3* loci still exist; for instance, the former requires 22-nt miRNAs and AGO1-mediated RISCs, whereas the latter relies on 21-nt miRNAs and AGO7 activity. Although "one-hit" action at TAS3 is sufficient to generate ta-siRNAs, the quantity and accuracy is lower than that achieved by "two-hit" action, suggesting that the "two-hit" model has evolved to improve the efficiency of secondary siRNA identity (de Felippes et al., 2017). Thus, secondary sRNA can be effectively triggered via a

non-cleavable miRNA interaction, but the absence of cleavage causes suboptimal phasing, possibly due to ill-defined siRNA processing ends available for DCL4 action.

After processing by miRNA, the poly(A)-less mRNA or non-coding RNA may function as a substrate for RDR6 to synthesize a complementary strand, and successively amplify the template of secondary siRNAs. However, it remains unclear that why the precursors of ta-siRNAs and other phasiRNAs are specifically amplified by RDR6 rather than other RDR proteins. It is possible that RDR6 discrimination of secondary siRNA precursors is assisted by the plant-specific coiled-coiled protein SGS3, which has been proposed to play a role in the stabilization of primary siRNAs and cleavage products (Yoshikawa et al., 2013; Yoshikawa et al., 2005).

Intriguingly, some *TAS* primary transcripts, such as *TAS2*, has been observed with several short open reading frames, which can be translated and associate with *TAS* transcript fragments in polysome fractions (Yoshikawa et al., 2016). These form large complexes including SGS3, miRNA-programmed RISCs, and ribosomes (Yoshikawa et al., 2016). While it is uncertain that how these complexes are formed, modified, and stabilized, they can affect the RDR6 amplification efficiency and resultant ta-siRNA accumulations.

A recent finding shows that miRNAs and ta-siRNA precursors are associated with membrane-bound polysomes at the endoplasmic reticulum, where they can be recruited by AGO1 to exert their endonuclease activities (Li et al., 2016). In an AGO1 loss-of-function mutant, the amount of ta-siRNAs and phasiRNAs was dramatically reduced because the triggering of miRNAs did not correctly associate with the membranes (Li et al., 2016). The location of the AGO complex is not important only , but AGO1 slicer activity is also required.Plants with defective AGO1 slicer activity cannot produce secondary siRNAs in a phased pattern at *TAS* loci, although their abundance is not obviously reduced (Arribas-Hernandez et al., 2016). This finding is consistent with the "update one-hit" model that the interaction between miRNA and target mRNA rather than the cleavage, is essential for secondary sRNAs formation at *TAS* loci, even though the cleavage event seems to be crucial for their phasing pattern.

Once generated, long non-coding transcripts are often sliced into small RNAs by DCL proteins. DCL4 is the major DCL protein that recognizes transcripts produced by RDR6 and

dices them into 21-nt fragments in a phased manner (Yoshikawa et al., 2005). DCL2 and DCL3 may also be involved in ta-siRNA and phasiRNA enhancement, because they can process sRNAs similarlywhen DCL4 is compromised (Gasciolli et al., 2005; Henderson et al., 2006).

After processing by DCL proteins, ta-siRNAs and phasiRNAs are transported into the cytoplasm and sorted into different AGO complexes based on their 5′ terminal nucleotide (Mi et al., 2008). Although most ta-siRNAs enter into AGO2 in *Arabidopsis* because of their 5′ adenine residue, their roles with AGO2 are not clear (Mi et al., 2008; Montgomery et al., 2008a). By contrast, although only a small proportion of ta-siRNAs and phasiRNAs are loaded into AGO1, their biological importance is much obvious. Most phasiRNAs slice their target mRNAs in *cis*, while ta-siRNAs cleave their target transcripts in *trans* (Fei et al., 2013). It is unclear whether ta-siRNA and phasiRNA can repress targets via translation repression like miRNAs so far, but ta-siRNAs are known with capacity to direct DNA methylation like hc-siRNAs (Wu et al., 2012). The biological relevance of ta-siRNA-guided DNA methylation is still vague, because *TAS* expression is unchanged in the *ago4* mutant which shows reduced methylation at *TAS* loci, and no ta-siRNA target genes have been found methylated in *trans* yet (Wu, 2013; Wu et al., 2012).

Intestinally, the methylation levelis not reduced in *PolIV*, *DCL3* and *RDR2* knock-out mutants, suggesting the involvement of 21-nt rather than 24-nt hc-siRNAs in *TAS* DNA methylation (Wu et al., 2012). Another unexpected observation is that ta-siRNA-mediated methylation is not compromised by the loss of DCL4 activity, even if most ta-siRNAs are absent in the *dcl4* mutant, indicating that secondary siRNAs generated by DCL4 are not required for *TAS* loci methylation (Wu et al., 2012). Additionally, the methylation is consistent at the *TAS3a* locus in the *dcl2/3/4* triple mutant, indicating that small RNAs generated by DCL1 are responsible for this process (Wu et al., 2012). Meanwhile, DNA methylation guided by ta-siRNAs is inhibited in the *ago4* and *polV* mutant, reminiscent of hc-siRNA-directed DNA methylation, suggesting a similar downstream pathway of DNA methylation by ta-siRNAs and hc-siRNAs (Wu et al., 2012). Based on these observations, two possible functioning pathways involving ta-siRNA have been proposed. In the first, ta-siRNA precursors are amplified by RDR6 and processed by DCL4, then recruited into AGO1 to guide target mRNA cleavage (Figure 2). Alternatively, ta-siRNA precursors may be directly processed by DCL1 to form small RNAs that are hierarchically loaded into AGO4 to mediate DNA methylation, but it is still unknown whether these *TAS* sRNAs are expressed in a phasing pattern (Figure 2). It is interesting to know in

the future how DCL4 and DCL1 cooperate in tasiRNAs biogenesis for differential action pathways.

Regulatory cascade of ta-siRNAs, phasiRNAs and their targets

ta-siRNAs and targets

While miRNAs can regulate diverse targets, ta-siRNAs and phasiRNAs often target gene families. To date, four *TAS* families have been characterized in *Arabidopsis thaliana*, and the physiological functions of three of them have been described.

Both *TAS1* and *TAS2* are targeted by miR173. They regulate a gene family encoding the Pentatricopeptide repeat (PPR) protein, which are widespread in dozens of plant species (Chen et al., 2007; Felippes and Weigel, 2009). Since the biological relevance of *TAS1*,*TAS2* and their *PPR* targets has not been revealed, it is unclear why such PPR-encoding mRNAs need to be sliced by *TAS1* and *TAS2* ta-siRNAs. However, the fact that *TAS1* mediate plant thermo-tolerance through regulation of several heat stress transcription factors has been validated recently (Li et al., 2014a). Exposure of plants to heat shock quickly represses *TAS1* ta-siRNAs, resulting in an increase of *HEATINDUCED TAS1 TARGET1* (*HTT1*) and *HTT2* for heat tolerance enhancement (Li et al., 2014a).

Almost all plant developmental processes can be affected by auxin, an endogenous plant hormone. The auxin signaling pathway is eventually integrated to regulate DNA-binding auxin transcription response factors (ARFs). The plant ARF family members can be targeted by miRNAs and *TAS3* ta-siRNAs (Yamamuro et al., 2016). The ta-siRNA-ARF module is one of the most conserved plant small RNA-target regulatory circuits, and has been observed from simple organisms, including liverworts and ferns, to monocots and eudicots (Xia et al., 2017). Moreover, the initial 5′ adenosine of miR390 and the central region of the miR390/miR390* duplex has been found conserved and responsible for the interaction with AGO7 (Endo et al., 2013). This interaction occurs late during the specification of seed plants, which may reflect the complexity of the evolutionary path of the miR390-*TAS3-ARF* pathway in land plants. Another two relatively high-conserved regions of the miR390 precursors locate in either 5′ or 3′ lower stem of the miR390 hairpin structure, but their biological significance is still an enigma (Xia et al., 2017). Additionally, the number of *TAS3* loci is species dependent, being able to range from two to hundreds (Xia et al., 2015a; Xia et al., 2017). This diversity is

suggestive of a long evolutionary history for the miR390-*TAS3-ARF* cascade in distinct land plant lineages.

The expressions of at least two *ARF* genes are consistently regulated by *TAS3* ta-siRNAs in different plant species (Xia et al., 2017). Interestingly, the variability in the nucleotide sequence of the *TAS3* ta-siRNA target sites in *ARF* genes is much lower than that of other positions, even if they are encoding conserved protein structure domains (Xia et al., 2017). This suggests that the *TAS3* ta-siRNA target site is under a higher selective force than the conserved protein-encoding regions. It is unclear why plants evolved such a complex system. Perhaps this regulatory mechanism could be more accurate than regulation via protein-encoding domains over a long-term evolutionary history.

The critical functions of the ta-siRNA-ARF module is to modulate leaf morphology, developmental transitions, flower and root architecture, embryo development, responses to biotic and abiotic stresses, and phytohormone crosstalk and these functions were characterized in *A. thaliana* several years ago (Table 1) (Adenot et al., 2006; Fahlgren et al., 2006; Marin et al., 2010; Matsui et al., 2014; Xu et al., 2007; Yoon et al., 2010; Yoshikawa et al., 2005). Recent studies have revealed that this module is also essential in adaptation to extreme environments via auxin networks in several other plant species (e.g., *Medicago truncatula*, *Lotus japonicus*, *Zea mays*, *Dimocarpus longan Lour*., and *Pyrus serotina*) (Bai et al., 2016; Dotto et al., 2014; Li et al., 2014b; Lin et al., 2015; Zhou et al., 2013). Particularly, the ta-siRNA-ARF module in the moss *Physcomitrella patens* contributes to auxin and nitrogen sensitivities, and this implies a cooption of regulatory networks by ta-siRNAs and targets during the evolution of lower plants (Xia et al., 2016). Intriguingly, *TAS3* ta-siRNAs are variable and have been indentified more potential targets other than ARFs in *Bruguieragy mnorrhiza* (Wen et al., 2016), suggesting an important role of ta-siRNA and their regulatory networks in plant stress adaptations. Consistent with these implications, several AP2 transcription factors can be targeted by *TAS3* ta-siRNAs in bryophytes , (Axtell et al., 2007; Xia et al., 2017).

In *A. thaliana*, a non-coding transcript generated from the *TAS4* locus between the *MYB* and *PROTEIN PHOSPHATASE 2A SUBUNIT A2* (*PP2A*) genes is processed by miR828, resulting in an accumulation of 21-nt ta-siRNAs, which further target other *MYB* transcripts for degradation in response to sugars (Luo et al., 2012; Rajagopalan et al., 2006). In cotton, fiber development is regulated by two homoeologous *MYB* genes, and one of them is targeted by

miR828 and miR858 to generate 21-nt phasiRNAs. Interestingly, the regions targeted by miR828 are *TAS4* orthologs in *A. thaliana* and cotton (Guan et al., 2014). Thus, miR828 can target coding and non-coding transcripts, and thereby generates *cis*- and *trans* siRNAs and phasiRNAs in different species, respectively, and both of them can influence trichome development (Table 1).

TAS5 was firstly described in tomato. A putative miR482 homologous precursor (i.e. sly-MIR482d) with a asymmetric bulge miRNA/miRNA* duplex was identified as targeting tomato *Bacterial Spot Disease Resistance Protein 4* (*Bs4*) mRNAs. Thus, secondary siRNAs can be induced at *Bs4*, resulting in a self-regulated expression (Li et al., 2012). However, in a strict sense, these secondary siRNAs are phasiRNAs rather than *bona fide* ta-siRNAs, because they are not generated from non-coding transcripts and are not functional in *trans*.

Moss is considered to carry three *TAS6* genes because some non-coding transcripts can give rise to secondary siRNAs following slicing by miR156 and miR529 (Arif et al., 2012; Cho et al., 2012). All three *TAS6* genes are adjacent to *TAS3* loci. Even though ta-siRNAs generated from the *TAS6a* and *TAS6b* have variable sequences (Cho et al., 2012), they are likely to be the same ta-siRNA family with the same ancestor, because both of their precursors can be sliced by miR156 and their loci are close to *TAS3* (Cho et al., 2012). Furthermore, *TAS6a* and *TAS3a* are sharing a single primary transcript, and only separated by a small central intron (Cho et al., 2012). Thus, it is reasonable that the actions of miR156 and miR390 may be linked in biogenesis of *TAS6a* and *TAS3a* ta-siRNA in moss. This is also supported by a fact that tasiARF can accumulate in *MIM156* plants, in which miR156 and *TAS6* activity is blocked (Cho et al., 2012). It is interesting to explore more crosstalk of miRNAs in generation of different ta-siRNA classes.

To date, *TAS7–TAS10* has been only predicted in grapevine (Table 1) (Zhang et al., 2012). However, because little is known about their initiator miRNA and how they are induced, it is unclear whether they are *bona fide* ta-siRNAs and how they function in grapevine. An independent class of siRNAs designated *TAS9* and *TAS10* ta-siRNAs has been reported in a tomato fruit small RNA dataset, when plants are undergoing low temperature stresses, but how they are processed still remains unknown (Zuo et al., 2017). Further identification of *TAS7-TAS10* homologs in other plant species may help to clarify their biogenesis and behaviors.

TAS-like siRNAs and targets

In addition to *TAS* transcripts, two other long non-coding RNAs as a substrate that produce secondary siRNAs by miRNAs have been elucidated in rice (Johnson et al., 2009). Previous research revealed that miR2118 and miR2275 are complementary with and slicing twonon-coding transcripts, producing 21-nt and 24-nt secondary siRNAs, respectively (Song et al., 2012a). while miR2118-induced phasiRNAs that are dependent on DCL4, those generated by miR2275 require DCL3b activity for biogenesis (Song et al., 2012a). Although it is unknown whether these secondary siRNAs are *trans*- or *cis*-acting to targets, they may have important regulatory roles affecting propagation since they preferentially expressed in reproductive tissues (Johnson et al., 2009).

Photoperiod-sensitive male sterility (PSMS), a gene locus responsible for initiation of two-line hybrid rice breeding has been recently discovered related to *TAS*-like siRNAs (Ding et al., 2012; Fan et al., 2016; Zhou et al., 2012). The photopheriod-sensitive genic male sterility 1 (Pms1) locus encodes a long-noncoding RNA *PMS1T* that was preferentially expressed in young panicles and targeted by miR2118, giving rise to 21-nt phasiRNAs. A single nucleotide polymorphism in PMS1T nearby the miR2118 recognition site has been found to affect rice fertility, because the accumulation of secondary siRNAs is altered accordingly (Fan et al., 2016). The targets and functioning modes of these phasiRNAs still remains unclear, but they have been shown incorporated into MEIOSIS ARRESTED AT LEPTOTENE1 (MEL1), a rice germ cell-specific AGO protein, during meiosis (Komiya et al., 2014; Nonomura et al., 2007). MEL1 can associate with siRNAs from over 700 long non-coding RNAs, which are potential targets of miR2118 (Komiya et al., 2014).

In rice, MULTIPLE SPOROCYTES1 (MSP1) and its cofactor TDL1a directly influence the number of sporocytes and anther development (Zhang and Yang, 2014). When *MSP1* and *TDL1a* are mutated, the precursor and mature 24-nt secondary siRNAs generated by miR2275 are almost completely disappeared (Fei et al., 2016), suggesting that MSP1 and TDL1a affect male gamete formation via regulation of miR2275-produced *TAS*-like siRNAs . It has been observed that the spatial and temporal expressions of miR2275-produced *TAS*-like siRNAsare overlapped with those of *OsAGO1d*, *OsAGO2b*, and *OsAGO18* , suggesting that these phasiRNAs may be acting through these three AGO proteins (Fei et al., 2016). Furthermore, miR2218 and miR2275 were also recently suggested to be involved in the dynamic biogenesis of meiotic phasiRNAs in maize in a cell-type–dependent manner (Zhai et al., 2015). Therefore, a thorough characterization of the molecular basis and functions of ta-siRNA–like secondary siRNAs involving miR2218 and miR2275 in monocots

may be helpful for establishing male sterile germplasm for hybrid crop breeding (Qin et al., 2014).

Another*TAS*-like non-coding transcript, which is targeted by a 22-nt miR4392, can generate secondary siRNAs that are predicted to cleave transposable elements in soybean (Arikit et al., 2014). Because they are preferentially accumulated in anthers, they may have a specific function related to soybean propagation.

Consequently, it seems that miRNA-mediated ta-siRNA biogenesis is a general regulatory mechanism in control of reproductive development in diverse crops.

other phasiRNAs and targets

In addition to non-coding transcripts, miRNA-triggered secondary siRNAs can be produced from protein-coding gene families To date, genes encoding nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins have been shown to be the largest gene family targeted by miRNAs to generate phasiRNAs. Three 22-nt *M. truncatula* miRNAs, including miR2275, miR2109, and miR2118 were the first class of miRNAs determined to be responsible for these phasiRNAs (Table 1) (Zhai et al., 2011). In soybean, another leguminous plant, multiple phasiRNAs-generating loci (*PHAS* loci) overlapping with the *NBS-LRR* genes are targeted by miR482, miR1507, and miR1510, and are preferentially expressed in nodules (Zhai et al., 2011). This implies that a phasiRNA-induced post-transcriptional modification may be essential in developing nodules. Interestingly, although the miR2109 sequence is conserved in both soybean and *M. truncatula*, it can only initiate phasiRNAs in *M. truncatula* (Zhai et al., 2011). The reason behind this is the difference in length of miR2109 as it is 21-nt in soybean but, 22-nt in *M. truncatul*. Thus, the same miRNA differing in length can have different functions thtough distinct functioning mechanisms.

So far, phasiRNAs generated at NBS-LRR conserved motifs have been detected in diverse plant species, including spruce, grapevine, poplar, cotton, A*rabidopsis* and citrus (Boccara et al., 2014; Kallman et al., 2013; Wu et al., 2015) suggesting that ancient small RNAs limited *NBS-LRR* expressions in these species. However, this phenomenon has not been observed in rice, perhaps because of the specific variations in defense mechanisms in monocots (Fei et al., 2013).

As mentioned above, miR482 induces phasiRNAs and mediates tomato bacterial spot disease resistance (Table 1) (Shivaprasad et al., 2012). Likewise, excessive production of miR482e in potato plants compromises the NBS-LRR protein activity, resulting in plants hypersensitive to *Verticillium dahliae* infection (Yang et al., 2015). Similarly, in barley, miR9863 mediates the cleavage of *Mla1* alleles, which encode NBS-LRR protein receptors, and induces phasiRNAs underlying immunity against the powdery mildew fungus (Liu et al., 2014). These results suggest that when plants are attacked by pathogens, they would up-regulate *NBS-LRR* expressions by suppressing the miRNA-mediated gene silencing pathway. The phasiRNA–NBS-LRR protein regulatory module prevents the constitutive NBS-LRR expressions, which is deleterious for plant growth.

PPR is another large gene family that can be targeted by miRNAs to trigger phasiRNAs expanding post-transcriptional regulatory activities. In addition to being regulated by ta-siRNAs, a subset of *PPR* transcripts can be directly targeted by miR7122, a 22-nt miRNA, to initiate phasiRNA biogenesis (Xia et al., 2013). Intriguingly, in apple and grapevine, *PPR* phasiRNA production can be induced at non-coding RNA transcripts by miR7122, which is similar to *TAS*-like secondary siRNAs (Xia et al., 2013). Furthermore, in soybean and *M. truncatula*, *PPR* phasiRNA can be processed by a secondary siRNAs cascade , which are produced by miR1509-mediated cleavage (Xia et al., 2013). Thus, one, two, and three layers of phasiRNA productions can give rise to an elaborate regulatory hierarchy for multiple *PPR* regulations. Additionally, miR4376,22-nt miRNA, regulates the expression of *ACA10*, an auto-inhibited Ca^{2+}-ATPase gene through phasiRNAs to control tomato reproductive development (Wang et al., 2011). According to sequence similarities, miR7122 may have evolved from miR4376 (Xia et al., 2013). Meanwhile, the miR4376 and miR390 sequences are also similar, which is suggestive of an evolutionary relationship among miR390-miR4376-miR7122 (Xia et al., 2013). However, these three miRNA families regulate distinct genes to be involved in different biological processes, for instance, miR390 regulates *ARFs* via *TAS3*-derived ta-siRNAs and miR4376 mediates cleavage of Ca^{2+}-ATPase transcripts, while miR7122 regulates *PPR* expressions. These findings suggest that target sequence variations may cause evolution of miR390 to miR4376 and miR7122.

MYB transcription factor genes are the third richest source of miRNA-mediated phasiRNAs. On one hand, as mentioned above, *A. thaliana TAS4* non-coding RNA transcripts are targeted by miR828 to generate ta-siRNAs that down-regulate *MYB* genes (Allen et al., 2005; Rajagopalan et al., 2006). However, on the other hand, miR828 and miR858 in apple, soybean, and cotton, can directly target some *MYB* motif transcriptsto generate phasiRNAs

that further prevent the accumulation of MYB transcription factors (Guan et al., 2014; Xia et al., 2012; Zhai et al., 2011). This process has been found with consequences for secondary metabolism, seed development, and fiber growth in apple, soybean, and cotton plants, respectively (Guan et al., 2014; Xia et al., 2012).

F-box is one of the largest gene family in plants, which encode the substrate-discriminating subunits of the SCF (i.e., S-phase kinase-associated protein1–Cullin–F Box) ubiquitin ligases, and can affect numerous biological processes. One-third of the strawberry *F-box* genes have been illustrated to be targeted by a 22-nt miRFBX cluster, resulting in the generation of a set of phasiRNAs (Table 1) (Xia et al., 2015b). The miRFBX7–F-box–phasiRNA network has been observed only in wild diploid strawberry plants, suggesting that it can regulate strawberry-specific processes (Xia et al., 2015b). Consistent with this notion, the miRFBX precursor transcripts are highly enriched in the cortex and pith tissues of the receptacle, a unique part of the strawberry flower that develops rapidly to produce the characteristic fruit shape (Xia et al., 2015b).

In addition to gene families, some low-copy genes are also able to produce phasiRNAs when their transcripts are being cleaved by a 22-nt miRNA. For example, the transcripts of *TRANSPORT INHIBITOR RESPONSE* (*TIR*) and *AUXIN SIGNALING F-BOX* (*AFB*) genes are repressed by miR393, which mediates *A. thaliana* innate immunity through an enhancement of auxin response (Navarro et al., 2006). Interestingly, a couple of secondary siRNAs have recently been identified at *TIR/AFB* transcript sites cleaved by miR393, and these siRNAs are found very low in *dcl1*, *dcl4*, *rdr6*, *sgs3*, *ago1*, and *mir393b* mutants (Si-Ammour et al., 2011), implying that the processing of these siRNAs is dependent on a canonical phasiRNA biogenesis routine. In addition, two protein-encoding gene transcriptswere found sliced by these *TIR/AFB* secondary siRNAs in *trans*, suggesting that these phasiRNAs are functional, even though their involvement in auxin signaling are still unknown (Si-Ammour et al., 2011). Similar as *TIR/AFB*, a limited number of *NAC* (*NAM, ATAF and CUC*) domain transcription factor transcripts are reported yielding phasiRNAs in citrus and litchi by miR3954, and their roles has been implicated in flowering regulation (Liu et al., 2017; Ma et al., 2017), further providing evidence that phasiRNAs from low-copy genes are also important for plant development.

Plant genes encoding components of small RNA pathway can also be served as phasiRNA-producing loci if the corresponding transcripts are cleaved by miRNAs. The *SGS3*

orthologs in leguminous plants including soybean and *M. truncatula* were predicted to be miR2118 targets (Zhai et al., 2011). Unexpectedly, *SGS3* phasiRNAs can be detected in soybean, but not in *M. truncatula* (Fei et al., 2013). This may be because additional factors required for *SGS3* phasiRNA biogenesis exist in soybean, but are lacking in *M. truncatula*. Unlike *SGS3* transcripts, which are targeted by the same miRNA, *DCL2* transcripts are targeted by distinct miRNAs in different leguminous species (Fei et al., 2013). For example, miR1507 and miR1515 can initiate the generation of *DCL2* phasiRNAs in *M. truncatula* and soybean, respectively, (Table 1) (Fei et al., 2013). Collectively, the production of phasiRNAs from *DCL2* and *SGS3* sequences suggests an important mechanism responsible for exquisite control of small RNA biogenesis and activities in legumes, reminiscent of feedback-based regulation of *DCL1* and *AGO1* expression by miR162 and miR168 in *A. thaliana,* respectively.

Considering the rapid increases in plant genome and transcriptome data, it is certain that more phasiRNAs will be characterized in near future. Nevertheless, illustration of phasiRNA biological significance should also be paid attention onward and speed up the pace.

Conclusions and remarks

Over the last decade, ta-siRNAs were first demonstrated to be involved in *A. thaliana* phase transitions (Peragine et al., 2004; Vazquez et al., 2004). Subsequently, additional ta-siRNAs have been identified and the genetic requirements underlying their processing have been elucidated. Meanwhile, two models for ta-siRNA biogenesis had been proposed, although it is still unclear if they can explain the generation of all secondary siRNAs. A special AGO7 complex required for *TAS3* ta-siRNA biogenesis was discovered that made researchers interested in how miRNAs induce the generation of secondary siRNAs. One of the major breakthroughs involved the discovery of 22-nt miRNAs essential for phasiRNA biogenesis, although the bulge structures of miRNA and miRNA* may also be required for this process. Since then, researchers have uncovered numerous phasiRNAs in diverse plants, suggesting a conserved biogenesis and related regulatory cascade among plant species.

Several issues regarding ta-siRNAs and phasiRNAs need to be elucidated. For example, it is unclear how 22-nt small RNAs generate secondary siRNAs. Perhaps, only 22-nt small RNAs including miRNAs and siRNAs can link AGO1 with RDR6, SGS3, or other unidentified proteins at secondary siRNA precursors to precisely slice transcripts in a phased pattern. To address this issue, clarifying the crystal structures of the central components associated with 22-nt small RNAs is necessary. Twenty two-nt small RNAs are generally considered essential

determinants of phasiRNA biogenesis, but with exceptions. For example, miR390, which is a canonical 21-nt miRNA, can guide *TAS3* transcripts to ta-siRNAs during a process mediated by AGO7 rather than other AGO-mediated RISCs. Thus, future studies should investigate how AGO7 competes with and replaces AGO1 to interact with miR390 to induce the formation of *TAS3* ta-siRNA. Furthermore, the interaction between specific miRNAs and ta-siRNA precursors rather than a cleavage event is sufficient to initiate the production of phasiRNAs. This implies that phasiRNA biogenesis may be more complex than previously considered. Therefore, the components responsible for the interactions between miRNAs and phasiRNA precursors need to be isolated. Furthermore, only a limited number of gene families, low-copy genes, and non-coding RNAs have been shown processed into secondary siRNAs by miRNAs, thus further study is required to reveal how miRNAs recognize specific targets for phasiRNA biogenesis. In addition to this, ta-siRNAs are able to mediate DNA methylations at their own loci through an alternative RdDM pathway. However, this epigenetic process seems to be unrelated to the production of ta-siRNAs and their precursors. Therefore, the effects of ta-siRNA-mediated DNA methylation and whether *trans*-active ta-siRNAs modify targets remain to be determined.

The CRISPR-Cas9 system and gene silencing techniques are the two most efficient options for generating mutants for investigating gene functions. However, a loss-of-function mutant cannot be generated by gene editing if the mutation causes lethality. Moreover, the CRISPR-cas9 approach is inappropriate for silencing a specific alternative splicing isoform, but alternatively, the application of an artificial miRNA is suitable (Qin et al., 2017; Wu et al., 2013). Thus, small RNA-mediated gene silencing is still an important platform for reverse genetic studies on plant gene functions as well as crop traits improvement. A single *TAS* locus can be engineered to produce artificial ta-siRNAs, which can increase the efficiency of one or multiple target genes silencing (Baykal et al., 2016; Carbonell et al., 2014). Artificial phasiRNAs are currently being used to rapidly generate plants that are resistant to viruses and viroids, suggesting ta-siRNAs may be useful for improving important crops (Carbonell and Daros, 2017; Chen et al., 2016).

In conclusion, the discovery of plant ta-siRNAs and phasiRNAs has contributed greatly to the advances that have been made in the research of RNA-mediated gene silencing. Because ta-siRNAs have also been predicted in small RNA dataset of human brain (Liu et al., 2015), it is interesting to determine whether ta-siRNAs and phasiRNAs are active beyond plant species.

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Figure 1. Diagram representing the scope of plant secondary siRNAs.

Figure 2. Biogenesis and activity hierarchy of ta-siRNAs and phasiRNAs.

The ta-siRNA and phasiRNA precursors are transcribed by Pol II from non-coding loci and protein-encoding genes, respectively. They are subsequently exported to the ARGONAUTE catalytic center for miRNA-mediated cleavage after trafficked by the THO/TREX complex. If the cleavable miRNA is 22-nt long or contains a bulge structure with miRNA*, it fits the “one-hit” model, in which the miRNA has a single target site and slices the target via AGO1. If the cleavable miRNA is a 21-nt canonical miRNA, it fits the “two-hit” model, in which the miRNA has two target sites, but only one can be cleaved even though both interact with the miRNA in AGO7. An alternative “updated one-hit” model has recently been described, in which an interaction between the miRNA and the target sequence is sufficient to induce the production of secondary siRNA by AGO1 or AGO7, even in the absence of cleavage events. Fragments produced by the miRNA-AGO complex are then protected and amplified by RDR6 and SGS3 to form double-stranded RNAs, which are subsequently processed by DCL-like proteins. Processing by DCL4 results in the generation of 21-nt siRNAs that are recruited by the AGO1 complex to mediate the cleavage of target mRNA or induce the production of secondary siRNAs. Processing by DCL1 also leads to the generation of 21-nt siRNAs, but these are incorporated into AGO4 clade proteins to mediate the methylation at their own DNA loci. Processing by DCL3 family proteins (e.g., DCL3b) results in the production of 24-nt siRNAs that may associate with AGO1 to cleave target sequences or with AGO4 for DNA methylation. Whether *trans*-active ta-siRNAs and phasiRNAs can guide DNA methylation remains unknown.

Table 1. Known plant ta-siRNA and phasiRNA loci.

Table 1 Known Ta-siRNAs and PhasiRNAs loci in Plants

Precursor name	Biogenesis model	Initiate miRNA name and length	Biological relevance	Family of plants	References
TAS1	Non-Coding	miR173 22-nt	Heat tolerance	Eudicots	(Li et al., 2014)
TAS2	Non-Coding	miR173 22-nt	Unknown	Eudicots	(Yoshikawa et al., 2016)
TAS3	Non-Coding	miR390 21-nt	Auxin signaling	Land plants	(Xia et al., 2017)
TAS4	Non-Coding	miR828 22-nt	Trichome development	Arabidopsis	(Guan et al., 2014)
TAS5	Coding	miR482 22-nt	Disease resistance	Solanaceae	(Li et al., 2012)
TAS6	Non-Coding	miR156 21-nt miR529 21-nt	Targets a zinc finger protein; Unknown	Moss	(Arif et al., 2012)
TAS7	Non-Coding	vvimiRNA828a 22-nt vviTAS7-primoRNA2 21-nt	Unknown	Grapevine	(Zhang et al., 2012)
TAS8	Non-Coding	vviTAS8-primoRNA1 21-nt vviTAS8-primoRNAA2 21-nt	Unknown	Grapevine	(Zhang et al., 2012)
vviTAS9	Non-Coding	vviTAS9-primoRNA 21-nt	Unknown	Grapevine	(Zhang et al., 2012)
vviTAS10	Non-Coding	vviTAS9-primoRNA1,2,5 22-nt	Unknown	Grapevine	(Zhang et al., 2012)

		vviTAS9-primoRNA3,4,6 22-nt			
Sly-TAS9	Non-Coding	miR9470-3p 22-nt	Chilling response	Solanaceae	(Zuo et al., 2017)
Sly-TAS10	Non-Coding	miR00093 21-nt miR00105 21-nt	Chilling response	Solanaceae	(Zuo et al., 2017)
unnamed	Non-Coding	miR2118 22-nt	Reproduction development	Rice and maize	(Fan et al., 2016)
unnamed	Non-Coding	miR2275 22-nt	Reproduction development	Rice and maize	(Zhai et al., 2015)
unnamed	Non-Coding	miR4392 22-nt	Reproduction development	Soybean	(Arikit et al., 2014)
Cs1g09600 **Cs1g09635**	Non-Coding	miR3954 22-nt	Flowering time	Citrus	(Liu et al., 2017)
NBS-LRR	Coding	miR1507 22-nt miR2109 22-nt miR2118 22-nt	Disease resistance	Medicago	(Cakir et al., 2016)
NBS-LRR	Coding	miR482 22-nt miR1507 22-nt	Nodule development	Soybean	(Zhai et al., 2011)

		miR1510 22-nt			
NBS-LRR	Coding	miR472 22-nt miR482 22-nt	Disease resistance	Citrus	(Wu et al., 2015)
Mla1	Coding	miR9863 22-nt	Disease resistance	Barley	(Liu et al., 2014)
Ca2+-ATPase	Coding	MiR4376 22-nt	Reproductive growth	Solanaceae	(Wang et al., 2011)
MYB	Coding	miR828 22-nt miR858 22-nt	Secondary metabolism, seed development and fiber growth	Apple, soybean and cotton	(Xia et al., 2012)
F-box	Coding	MiRFBX 22-nt	Shape fruit	Strawberry	(Xia et al., 2015)
TIR/AFB	Coding	miR393 22-nt	Unknown	Arabidopsis	(Wong et al., 2014)
NAC	Coding	miR3954 22-nt	Flowering time	Citrus and litchi	(Liu et al., 2017)
ARF	Coding	miR167 22-nt	Unknown	litchi	(Ma et al., 2017)
PPR	Coding	miR7122 22-nt	Unknown	Solanaceae	(Xia et al., 2013)
SGS3	Coding	miR2118 22-nt	Small RNA biogenesis	Goybean	(Zhai et al., 2011)
DCL2	Coding	miR1507 22-nt miR1515 22-nt	Small RNA biogenesis	Legumes	(Xia et al., 2012)

Provided only one most recent and relevant reference for each TAS and PHAS loci due to the space limitation here.

Figure 1

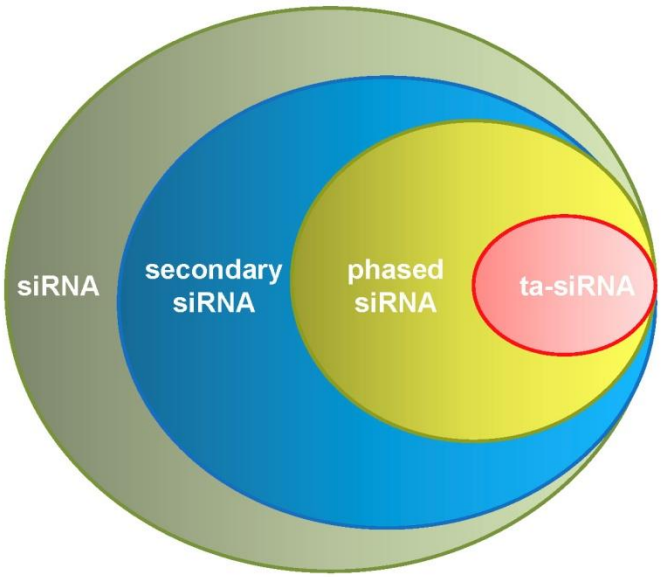

Figure 2