Spatiotemporal regulation and roles of reproductive phasiRNAs in plants

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Since co-suppression was discovered as a pioneer silencing phenomenon of RNA interference (RNAi) in petunia in 1990, many types of small RNAs have been identified in the RNAi pathway among various eukaryotes. In plants, a large number of 21- or 24-nucleotide (nt) phased small interfering RNAs (phasiRNAs) are produced via processing of long RNA precursors by Dicer-like proteins. However, the roles of phasiRNAs remain largely unknown. The development of imaging technology and RNA profiling has clarified the spatiotemporal regulation of phasiRNAs, and subsequently the different functions of 21-nt trans-acting phasiRNAs and 24-nt cis-regulatory phasiRNAs during male organ development. This review focuses on the biogenesis, diversification, spatiotemporal expression pattern and function of phasiRNAs in plants.

Key words: phasiRNA, microRNA, Argonaute, reproduction, plants

INTRODUCTION

Small RNAs associated with Argonaute (AGO) proteins form the RNA-induced silencing complex (RISC), the core machinery in silencing systems that are conserved in many organisms (Bohmert et al., 1998; Hannon, 2002; Czech and Hannon, 2011). RNA silencing has various functions, such as antiviral defense, transcriptional gene silencing for development, repression via transgenes and suppression of transposable elements, and is thus vital for species’ survival (Napoli et al., 1990; Castel and Martienssen, 2013; Bond and Baulcombe, 2014). In plants, small RNAs are primarily classified into microRNAs (miRNAs), repeat-associated small interfering RNAs (rasiRNAs) and phased small interfering RNAs (phasiRNAs). miRNAs are generally 21 or 22 nt long, and they mediate post-transcriptional silencing via target cleavage and translational repression (Iwakawa and Tomari, 2015). rasiRNAs are generally 24 nt long and mediate transcriptional gene silencing via chromatin-based mechanisms. In Arabidopsis thaliana, the AGO4–rasiRNA RISC causes RNA-directed DNA methylation (RdDM), suppressing numerous transposable elements and preventing their transposition (Matzke et al., 2009).

Plant phasiRNAs are generated from long RNA precursors, which are processed into 21- or 24-nt small RNAs by Dicer-like proteins (DCLs), DCL4 and DCL3b/5. The majority of phasiRNAs are 21 nt long, while other phasiRNAs are 24 nt long. Moreover, 21-nt phasiRNAs have diverged in land plants, being expressed during various developmental stages depending on the plant species. In contrast, the expression of 24-nt phasiRNAs is restricted to reproduction. Since the identification in 2009 of numerous phasiRNAs in rice reproduction (Johnson et al., 2009), a large number of phasiRNAs have been found in many land plants. However, the functions of phasiRNAs remain poorly understood. Here, I describe the diverged phasiRNAs in land plants and the spatiotemporal functions of 21-nt/24-nt phasiRNAs, especially during anther development.

BIOGENESIS OF DIVERSE phasiRNAs IN LAND PLANTS

The plant phasiRNA pathway includes four main processes: 1) cleavage of phasiRNA precursors by 22-nt miRNAs; 2) double-strand RNA (dsRNA) synthesis by RNA-dependent RNA polymerase (RDR); 3) processing by
DCLs; and 4) sorting to AGOs (Fig. 1A) (Yoshikawa et al., 2005; Johnson et al., 2009; Komiya, 2017). The most distinctive feature of the biogenesis is that the long RNA precursors of phasiRNAs, named PHASs, contain consensus sequences that are recognized by 22-nt miRNAs. First, these PHASs are cleaved at the 22-nt miRNA targeting site, leading to dsRNA synthesis via RDR6 (Song et al., 2012b). Thereafter, DCL4 or DCL3b/5 processes these dsRNAs into 21-nt or 24-nt phasiRNAs (Song et al., 2012a; Teng et al., 2020). Finally, AGOs bind to the phasiRNAs and mediate silencing (Komiya et al., 2014; Lee et al., 2021).

In the first process of biogenesis of 21-nt phasiRNAs, most 21PHASs, which are 21-nt phasiRNA precursors, are targeted by microRNA2118 (miR2118), which is widely conserved in land plants (Johnson et al., 2009; Fei et al., 2015; Xia et al., 2015). Furthermore, in monocots, 21PHASs are comprised of 1,300–2,000 types of long non-coding RNAs (lncRNAs) that are specifically enriched during reproduction. These reproduction-specific lncRNAs contain the consensus sequences targeted by miR2118, and their other regions are mostly unique sequences (Komiya et al., 2014). In contrast to monocots 21PHASs, dicot 21PHASs, with the miR2118 recognition motif, are mainly protein-coding genes belonging to a large gene family. Over 140 leucine-rich repeat genes (LRRs) that are involved in the plant defense mechanism are representative PHASs in dicots (Zhai et al., 2011). The tomato miR2118 influences disease resistance by affecting the expression of LRRs (Canto-Pastor et al., 2019). The deletion of rice miR2118 family members leads to partial sterility caused by abnormal anther development (Araki et al., 2020). Thus, the functional differentiation of miR2118 between dicots and monocots is linked to different kinds of 21PHASs: protein-coding genes in dicots and reproductive lncRNAs in monocots.

The 24-nt phasiRNAs are identified as reproduction-specific small RNAs derived from a much smaller number of lncRNAs called 24PHASs (> 100 loci), in contrast to the numerous reproductive 21PHASs (> 1,300 loci). The 24-nt reproductive phasiRNA pathway mostly involves cleavage by the 22-nt miR2275 and processing by DCL3b/5, which differs from the 21-nt phasiRNA biogenesis via miR2118 cleavage and DCL4 processing (Johnson et al., 2009; Song et al., 2012a; Teng et al., 2020). Small RNA profiling in anthers of grasses revealed the tempo-

Fig. 1. Model for cell-specific phasiRNA biogenesis and roles during rice reproduction. (A) Biogenesis of reproduction-specific phasiRNAs. More than 1,300 types of long non-coding RNAs are specifically expressed in the reproductive stages. These lncRNAs/PHASs are cleaved via miR2118/miR2275, and the cleaved lncRNAs are processed into 21- or 24-nt reproduction-specific phasiRNAs via DCL4 or DCL3b/5. The 21-nt uracil (U)-rich phasiRNAs are mainly miR2118-dependent somatic phasiRNAs, whereas the 21-nt 1st cytosine (C)-phasiRNAs are loaded onto a germ cell-specific Argonaute protein. Site-specific phasiRNAs and AGOs are crucial for anther development. (B) Roles of 21- and 24-nt phasiRNAs in meiocytes. The 21-nt germ cell-specific phasiRNAs, which are trans-acting small RNAs, cleave target RNAs. In contrast, the mel1 mutants of the germ cell-specific AGO that interacts with 21-nt 1st C-phasiRNAs show defects in pairing during prophase I of meiosis, suggesting that 21-nt 1st C-phasiRNAs function in the nucleus. Additionally, CHH DNA methylation at 24PHAS loci is upregulated in a 24-nt phasiRNA-dependent manner during this meiotic stage. The 21-nt reproductive phasiRNAs have functions in both the cytoplasm and the nucleus during anther development, while the 24-nt meiotic phasiRNAs have epigenetic functions in the nucleus.
eral expression of phasiRNAs, including that of 21-nt premeiotic phasiRNAs and 24-nt meiotic phasiRNAs, during anther development (Zhai et al., 2015). The 24-nt meiotic phasiRNAs are mainly detected in monocots, although several 21-nt/24-nt phasiRNAs are specifically expressed during reproduction in dicots, such as rose, strawberry and columbine (Pokhrel et al., 2021a, 2021b). Therefore, 21-nt phasiRNAs are widely generated in land plants during the developmental process or defense response, and differ from 24-nt phasiRNAs, which are restricted to the reproductive stage mainly in monocots.

**ROLE AND PATHWAY OF tasiRNAs IN ARABIDOPSIS**

Trans-acting siRNAs (tasiRNAs) belong to the 21-nt phasiRNAs, and their biogenesis and function have been elucidated in *Arabidopsis* (Schwab et al., 2009; Liu et al., 2020b). tasiRNA precursors include eight types of IncRNAs (TAS1a, TAS1b, TAS1c, TAS2, TAS3a, TAS3b, TAS3c and TAS4), which are enriched at the vegetative stages (Howell et al., 2007). TASs are cleaved via the 22-nt miR173/miR390, which interacts with two AGOs, AtAGO1 and AtAGO7, to form RISCs (Allen et al., 2005; Montgomery et al., 2008). SUPPRESSOR OF GENE SILENCING 3 (SGS3), a dsRNA-binding protein, is required for TAS cleavage via these RISCs (Peragine et al., 2004). The cleaved TASs are processed in a DCL4-dependent manner into 21-nt tasiRNAs (Yoshikawa et al., 2005), which are loaded onto AtAGO1/AtAGO7 and ARF (Liu et al., 2020b). The SGS3–AGO–miRNA complexes also mediate ribosome stalling in *Arabidopsis* (Iwakawa et al., 2021). Accordingly, elucidation of the functional relationship between ribosome pausing and phasiRNA production may explain how numerous phasiRNAs are produced simultaneously at specific developmental stages.

**SPATIOTEMPORAL REGULATION OF REPRODUCTIVE phasiRNAs AND ARGONAUTE PROTEINS DURING ANther DEVELOPMENT**

The 21-nt/24-nt reproductive phasiRNAs are enriched in the anther, a major part of the male organ that contains germ cells and the somatic anther wall. The development of the somatic cell layer synchronizes with that of the germ cells in the anther. Therefore, defects of somatic anther wall development cause the sterility of pollen (Nonomura et al., 2003; Fu et al., 2014; Zhang and Yang, 2014; Ono et al., 2018). In addition to elucidating the temporal regulation of the 21-nt premeiotic phasiRNAs and 24-nt meiotic phasiRNAs, evaluating phasiRNA expression at specific sites is essential for understanding the functions of 21-nt and 24-nt phasiRNAs in anther development. Table 1 shows their spatiotemporal expression, summarizing small RNA profiling and fluorescence *in situ* hybridization (FISH or ISH) data by classifying anther development into five stages. Stage 1 is the premeiotic stage, in which the four layers of the anther wall, epidermis, endothecium, middle layer and tapetum, are formed, surrounding the pollen mother cells. Stages 2 to 4 are the meiotic stages. In stage 2, meiotic chromosome association is initiated in the meiocytes; in stage 3, homologous chromosome pairing is completed in the pollen mother cells; in stage 4, the homologous chromosomes are segregated in the meiocytes. During these meiotic stages, the middle layer degenerates. In stage 5, programmed cell death (PCD) occurs in the tapetum layers, meiosis is completed, and microsperos are formed. Following stage 5, the tapetum decays and the microsperos undergo mitosis to generate bicallecular pollen.

mir2118 family members are highly expressed in the outer layer, the epidermis, at stage 1 (premeiosis) in rice (Table 1) (Zhai et al., 2015; Ta et al., 2016; Araki et al., 2020). mir2118 mutant rice exhibits both male and female partial sterility associated with somatic abnormalities during anther wall development. Three-dimensional (3D) structure imaging of whole anthers, used to study the internal structure of the anthers, indicated that the abnormal anther development of mir2118 mutants is caused by defects in the elongation of epidermal cells (Araki et al., 2020). Spatially restricted mir2118 expression coincides with the defects in the outer layer of the anthers of mir2118 mutants. In addition to the defects of mir2118 mutant epidermis at premeiosis, there is also a failure in the maturation of the anther wall, specifically the middle layer and tapetum, at post-meiosis (stage 5) (Araki et al., 2020). Thus, mir2118 is required for anther wall development, specifically from the outer layers around premeiosis to the inner layers at post-meiosis. Moreover, the peak expression of 21-nt rice phasiRNAs corresponds to that of miR2118 at stage 2 (early meiosis) (Jiang et al., 2020). In maize, the levels of 21-nt phasiRNAs increase at stage 1, and they are highly abundant mainly in the tapetum inner layers during meiostic stages 2 and 3 (Table 1) (Zhai et al., 2015). Taken together with the expression profile, the site specificity of miR2118/21-nt phasiRNAs shifts from outer to inner layers in the anther wall, suggesting that cell-to-cell movement of miR2118 and 21-nt phasiRNAs is vital for successful development of this structure. Furthermore, strawberry 21-nt phasiRNAs are enriched around the pollen mother cells and meiocytes in addition to the tapetum (stage 1–3) (Pokhrel et al., 2021a). Litchi 24-nt phasiRNAs and miR2275 are highly expressed in the tapetum and around meiocytes (stage 2; Table 1) (Xia et al., 2019). Mobility and non-cell-autonomous regulation between soma and germ may be important issues for...
uncovering the roles of reproductive phasiRNAs.

Plant AGOs are reported to recognize the 5′-terminal nucleotide of small RNAs and to interact with them (Mi et al., 2008; Takeda et al., 2008). MEIOSIS ARRESTED AT LEPTOTENE 1 (MEL1) is a germ cell-specific AGO among 19 AGO proteins in rice (Nonomura et al., 2007; Table 1. Spatiotemporal regulation of phasiRNA biogenesis in anther development

| Stage | stage 1 | stage 2–3 | stage 4 | stage 5 |
|-------|---------|-----------|---------|---------|
| Germ development | Premeiosis | Early meiosis | Meiotic division | Microspore |
| Soma development | 4 layers (Ep, En, Ml, Ta) | MI differentiation | MI differentiation | Ta PCD |

Anther locale

| Rice | Reference |
|------|-----------|
| miR2118 ISH | Ep (+++) |
| mir2118 mutant | Ep developmental defect |
| 21-phasiRNA qPCR | | ++/++ | ++/++ |
| 21-phasiRNA Hseq | | Target cleavage (Germ cells) |
| 24-phasiRNA Hseq | | CHH DNA methylation |

| AGO1b ISH | Anther wall (+) |
| AGO1d ISH | Anther wall (+++) |
| MEL1 ISH | PMC (+++) |
| MEL1 protein | Binding to 21-nt C-phaSiRNAs |
| mel1 mutant | Vacuolation in PMC |

Maize

| Rice | Reference |
|------|-----------|
| miR2118 ISH | Ep (+++) |
| 21-phasiRNA ISH | Anther wall & PMC (+++) |
| 21-phasiRNAs Hseq | +++ |
| mir2275 ISH | Ta & Meioyte (+++) |
| 24-phasiRNA Hseq | + |
| MAGO1/2 protein | Cytoplasm in Soma and PMC |
| mago1/2 mutant | Cytoplasm in Ta and meioyte |

Strawberry, litchi, rose, columbine (dicots)

| Rice | Reference |
|------|-----------|
| miR1308 FISH | Ta (+++) |
| 21-phasiRNA FISH | Cytoplasm in Ta & PMC (+++) |
| 21-phasiRNA Hseq | +++ |
| mir2275 Hseq | + |
| 24-phasiRNA FISH | Ta & PMC (+) |

Ep: epidermis; En: endothecium; Ml: middle layer; Ta: tapetum; PMC: pollen mother cell; (F)ISH: (fluorescense) in situ hybridization; Hseq: small RNA high-throughput sequencing.
+ : low level, ++: middle level, +++: high or peak expression level, -: no signal or extremely low level, N.D.: no data.
Kapoor et al., 2008). MEL1 mainly binds to the 21-nt phasiRNAs with the first cytosine (C) at the 5’ end (Komiya et al., 2014). In contrast, rice AGO1b and AGO1d have been identified as candidates for interaction with miR2118-dependent uracil (U)-rich phasiRNAs in the anther wall, by proteome and small RNA transcriptome analyses (Fig. 1A) (Araki et al., 2020). OsAGO1b is expressed in the endothecium and middle layers at stage 2; OsAGO1d is enriched in the tapetum, suggesting a function for OsAGO1b/d in the somatic anther walls (Table 1) (Araki et al., 2020). In contrast, MEL1 is expressed in pollen mother cells at the pre-meiotic stage 1 (Table 1) (Nonomura et al., 2007). The U-rich phasiRNAs and AGO1b/d in the miR2118-dependent somatic cell development are distinct from 1st C-phasiRNAs interacting with the germ cell-specific MEL1, highlighting the site-specific differences in small RNAs and AGOs between soma and germ cells (Fig. 1A).

FUNCTIONS OF REPRODUCTIVE phasiRNAs
AND ENVIRONMENTAL RESPONSE IN GRASSES

The function of reproductive phasiRNAs was largely unknown until recently, owing to difficulties in distinguishing between the soma and germ in premature anthers. However, 21-nt meiocyte phasiRNAs have now been found to cleave target mRNAs during early meiosis, through the combination of phasiRNA transcriptome and degradome analyses, which detect miRNA-cleaved transcripts, from meiocytes (Jiang et al., 2020). Moreover, lines overexpressing mRNAs targeted by 21-nt phasiRNAs exhibited partial sterility with meiotic defects, suggesting that phasiRNAs cause silencing via the cleavage of targets in the cytoplasm and are required for meiosis progression and fertility (Fig. 1B) (Zhang et al., 2020).

MEL1 is essential for synopsis between homologous chromosomes during meiosis, because meiotic arrest occurs in the nucleus of mel1 at leptotene during early meiosis (stages 2 and 3) (Nonomura et al., 2007; Komiya et al., 2014; Liu and Nonomura, 2016). Interestingly, the osrdr6 mutant shows a lack of double-strand breaks during early meiosis via reductions in 21-nt small RNA levels, and increases in 24-nt small RNA levels with differential CHH DNA methylation (Liu et al., 2020a). Furthermore, 24-nt phasiRNAs influence CHH DNA methylation at the 24PHAS loci during prophase I (stage 2) in maize meiotic anthers (Zhang et al., 2021). Thus, 24-nt meiotic phasiRNAs function in cis. In Arabidopsis, CHH DNA methylation is upregulated via the 24-nt rasiRNAs in the RdDM pathway to suppress the formation of repetitive regions (Matzke et al., 2015). Therefore, CHH DNA methylation through the reproductive phasiRNAs may be a novel type of reproduction-specific regulation for maintaining normal meiosis, distinct from the polymerase IV-dependent RdDM pathway. The different functions of the 21-nt trans-acting phasiRNAs via target cleavage and the 24-nt cis-regulating phasiRNAs via CHH DNA methylation are crucial for anther development. The distinct functions of 21- and 24-nt phasiRNAs in meiocytes need further comprehensive assessment to improve our understanding of meiosis in plants.

Environmental issues are among the most important in sustainable development goals. Because plant yield is greatly influenced by environmental conditions, reproductive research that takes the external environment into consideration is indispensable for stable food output. Maize MALE-ASSOCIATED ARGONAUTE-1 and -2 (MAGO1 and MAGO2), which are categorized into the Arabidopsis AGO5 subfamily, interact with 21-nt phasiRNAs in pre-meiotic anthers. They regulate heat-induced transposons via their phosphorylation-related silencing activity to sustain male fertility, even under severe heat stress conditions (Lee et al., 2021). Maize DCL3b/5 and rice RDR6, which are involved in the biogenesis of phasiRNAs, also influence the regulation of temperature-dependent male sterility and spikelet development, respectively (Song et al., 2012b; Teng et al., 2020). In rice, polymorphisms in the 21PHAS loci (Ding et al., 2012; Fan et al., 2016) or the deletion of miR2118 family loci (Araki et al., 2020) are responsible for photoperiod-dependent sterility. Thus, phasiRNA biogenesis in grasses is strongly associated with photoperiod- or temperature-dependent reproduction. Understanding the molecular mechanisms of phasiRNAs and their regulation will allow us to develop strategies that could greatly enhance the reproductive competence of plants and provide an applied basis for ensuring stable yields under severe environmental conditions.

CONCLUSIONS AND PERSPECTIVES

Imaging technologies, including FISH and 3D structure observation, indicate the importance of the accurate spatiotemporal regulation of miRNA triggers, phasiRNAs and AGOs for reproduction, and specifically for anther development, in plants. Furthermore, germ cell-specific RNA profiling has shown that the 21-nt phasiRNAs act via target cleavage; in contrast, the 24-nt phasiRNAs mediate CHH DNA methylation, indicating epigenetic regulation. Epigenetic experimental evidence linking the phasiRNAs to meiosis should lead to a new stage in our understanding of the reproductive mechanism, because meiosis is a key event in which genetic information is inherited by the next generation. Multifaceted studies using new technologies in sequencing, imaging, proteomics, artificial intelligence and chemistry should enable pioneering novel research on such small RNAs, and elucidate the roles of numerous lncRNAs and intergenic regions that constitute the majority of the genome.
in higher organisms.

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