Genes Genet. Syst. (2021) **96**, p. 1–7

Spatiotemporal regulation and roles of reproductive phasiRNAs in plants

Reina Komiya[1,2*]

[1]*Science and Technology Group, Okinawa Institute of Science and Technology Graduate University (OIST), Onna-son, Okinawa 904-0495, Japan*
[2]*PRESTO, Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-0012, Japan*

(Received 6 June 2021, accepted 20 August 2021; J-STAGE Advance published date: 11 November 2021)

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

Edited by Kenji Ichiyanagi
* Corresponding author. reina.komiya@oist.jp
DOI: https://doi.org/10.1266/ggs.21-00042

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 *PHAS*s, contain consensus sequences that are recognized by 22-nt miRNAs. First, these *PHAS*s 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 *21PHAS*s, 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, *21PHAS*s 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 monocot *21PHAS*s, dicot *21PHAS*s, with the miR2118 recognition motif, are mainly protein-coding genes belonging to a large gene family. Over 140 leucine-rich repeat genes (*LRR*s) that are involved in the plant defense mechanism are representative *PHAS*s in dicots (Zhai et al., 2011). The tomato miR2118 influences disease resistance by affecting the expression of *LRR*s (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 *21PHAS*s: 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 *24PHAS*s (> 100 loci), in contrast to the numerous reproductive *21PHAS*s (> 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-

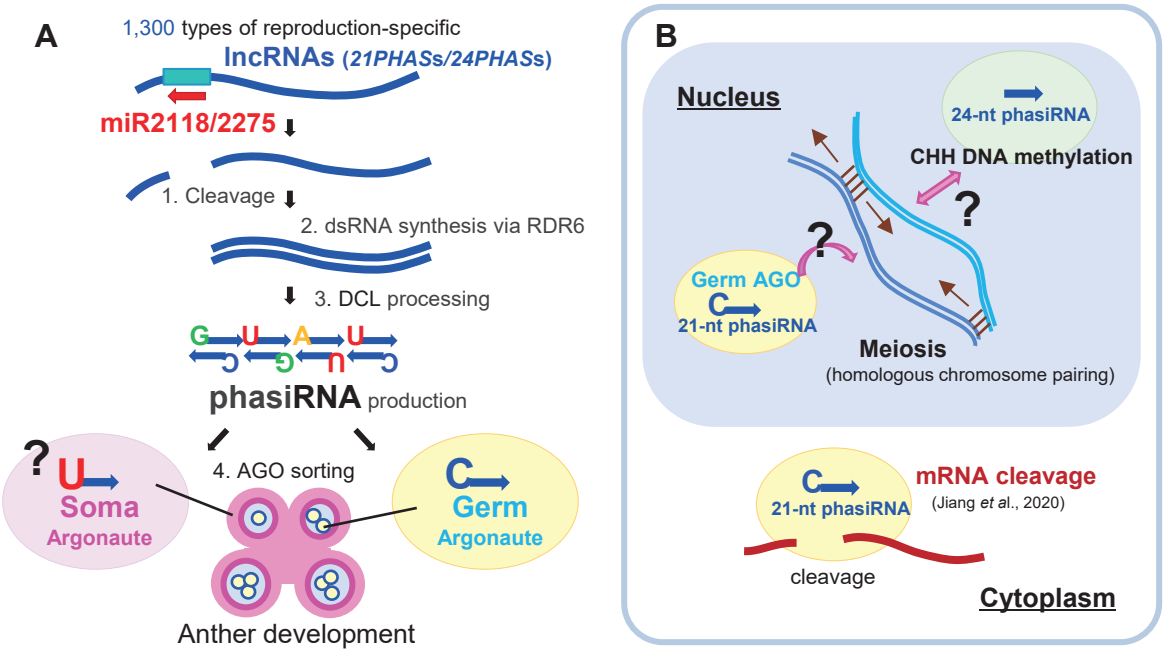


Fig. 1. Model for cell-specific phasiRNA biogenesis and roles during rice reproduction. (A) Biogenesis of reproduction-specific phasiRNAs. More than 1,300 types of long non-coding RNAs are specifically expressed in the reproductive stages. These lncRNAs/*PHAS*s are cleaved via miR2118/miR2275, and the 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.

ral 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*

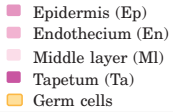
Trans-acting siRNAs (tasiRNAs) belong to the 21-nt phasiRNAs, and their biogenesis and function have been elucidated in *Arabidopsis* (Schwab et al., 2009; Liu et al., 2020b). tasiRNA precursors include eight types of lncRNAs (*TAS1a*, *TAS1b*, *TAS1c*, *TAS2*, *TAS3a*, *TAS3b*, *TAS3c* and *TAS4*), which are enriched at the vegetative stages (Howell et al., 2007). *TAS*s 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 *TAS*s are processed in a DCL4-dependent manner into 21-nt tasiRNAs (Yoshikawa et al., 2005), which are loaded onto AtAGO1/AtAGO7 and induce silencing through the cleavage of target mRNAs, such as those encoding the transcription factors MYB 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 microspores are formed. Following stage 5, the tapetum decays and the microspores undergo mitosis to generate bicellular 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 meiotic 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

Table 1. Spatiotemporal regulation of phasiRNA biogenesis in anther development

Stage	stage 1	stage 2–3	stage 4	stage 5	
Germ development	Premeiosis	Early meisosis	Meiotic division	Microspore	
Soma development	4 layers (Ep, En, Ml, Ta)	Ml differentiation	Ml differentiation	Ta PCD	
Anther locule					
Rice					**Reference**
miR2118 ISH	Ep (+++)	–	–	–	Ta et al., 2016; Araki et al., 2020
mir2118 mutant	Ep developmental defect	(Normal pairing in meiocyte)		Ml & Ta developmental defect	Araki et al., 2020
21-phasiRNA qPCR	+	+++	+/++	+/++	Araki et al., 2020
21-phasiRNA Hseq	+	+++	+	+	Jiang et al., 2020
(Function)		Target cleavage (Germ cells)			Jiang et al., 2020; Zhang et al., 2021
24-phasiRNA Hseq	–	+	+/++	+++	Jiang et al., 2020
(Function)		CHH DNA methylation			Liu et al., 2020a; Zhang et al., 2021
AGO1b ISH	Anther wall (+)	En & Ml (+)	N.D.	N.D.	Araki et al., 2020
AGO1d ISH	Anther wall (+++)	Anther wall (+++)	N.D.	N.D.	Araki et al., 2020
MEL1 ISH	PMC (+++)	N.D.	N.D.	N.D.	Nonomura et al., 2007
MEL1 protein	Binding to 21-nt C-phasiRNAs	Cytoplasm (+++), Nucleus (+)	N.D.	N.D.	Komiya et al., 2014
mel1 mutant	Vacuolation in PMC	Defect of pairing in meiocyte			Nonomura et al., 2007; Komiya et al., 2014
Maize					**Reference**
miR2118 ISH	Ep (+++)	Ep (+++)	–	Ep (+)	Zhai et al., 2015
21-phasiRNA ISH	Anther wall & PMC (++)	Ta & Meiocyte (+++)	Ta & Meiocyte (+++)	–	Zhai et al., 2015
21-phasiRNAs Hseq	+++	++	+	–	Zhai et al., 2015
miR2275 ISH	Ta & Meiocyte (+)	Ta & Meiocyte (+++)	–	–	Zhai et al., 2015
24-phasiRNA ISH	Ta & PMC (+++)	Ta & Meiocyte (+++)	Ta & Meiocyte (++)	Ta & Microspore (+)	Zhai et al., 2015
24-phasiRNA Hseq	+	+++	+++	++	Zhai et al., 2015
MAGO1/2 protein	Cytoplasm in Soma and PMC	N.D.	Cytoplasm in Ta and meiocyte	Cytoplasm in microspore	Lee et al., 2021
mago1/2 mutant				Heat-dependent sterility	Lee et al., 2021
Strawberry, litchi, rose, columbine (dicots)					**Reference**
miR11308 FISH	Ta (+++)	Ta (+++)	Ta (++)	Ta (++)	Pokhrel et al., 2021a
21-phasiRNA FISH	Cytoplasm in Ta & PMC (+++)	N.D.	Peripheral meiocyte (+)	N.D.	Pokhrel et al., 2021a
21-phasiRNA Hseq	+++	++	N.D.	N.D.	Pokhrel et al., 2021a
miR2275 Hseq	+	+	+++	+++	Pokhrel et al., 2021b
24-phasiRNA FISH	Ta & PMC (+)	Ta & Meiocyte (+++)	N.D.	N.D.	Xia et al., 2019
24-phasiRNA Hseq	+	+	+++	+++	Pokhrel et al., 2021b

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 extreamly low level, N.D.: no data.

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;

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 synapsis 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.

This work was supported by the JST PRESTO Program (Grant Number JPMJPR17Q3), the JST FORESTO Program (Grant Number JPMJFR204U), KAKENHI programs (Grant Numbers JP17H05608 and JP15H01476), the Naito Foundation and the Okinawa Institute of Science and Technology Graduate University.

REFERENCES

Allen, E., Xie, Z., Gustafson, A. M., and Carrington, J. C. (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. Cell **121**, 207–221.

Araki, S., Le, N. T., Koizumi, K., Villar-Briones, A., Nonomura, K.-I., Endo, M., Inoue, H., Saze, H., and Komiya, R. (2020) miR2118-dependent U-rich phasiRNA production in rice anther wall development. Nat. Commun. **11**, 3115.

Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M., and Benning, C. (1998) *AGO1* defines a novel locus of *Arabidopsis* controlling leaf development. Embo J. **17**, 170–180.

Bond, D. M., and Baulcombe, D. C. (2014) Small RNAs and heritable epigenetic variation in plants. Trends Cell Biol. **24**, 100–107.

Canto-Pastor, A., Santos, B. A. M. C., Valli, A. A., Summers, W., Schornack, S., and Baulcombe, D. C. (2019) Enhanced resistance to bacterial and oomycete pathogens by short tandem target mimic RNAs in tomato. Proc. Natl. Acad. Sci. USA **116**, 2755–2760.

Castel, S. E., and Martienssen, R. A. (2013) RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. Nat. Rev. Genet. **14**, 100–112.

Czech, B., and Hannon, G. J. (2011) Small RNA sorting: matchmaking for Argonautes. Nat. Rev. Genet. **12**, 19–31.

Ding, J., Shen, J., Mao, H., Xie, W., Li, X., and Zhang, Q. (2012) RNA-directed DNA methylation is involved in regulating photoperiod-sensitive male sterility in rice. Mol. Plant **5**, 1210–1216.

Fan, Y., Yang, J., Mathioni, S. M., Yu, J., Shen, J., Yang, X., Wang, L., Zhang, Q., Cai, Z., Xu, C., et al. (2016) *PMS1T*, producing phased small-interfering RNAs, regulates photoperiod-sensitive male sterility in rice. Proc. Natl. Acad. Sci. USA **113**, 15144–15149.

Fei, Q., Li, P., Teng, C., and Meyers, B. C. (2015) Secondary siRNAs from *Medicago NB-LRRs* modulated via miRNA-target interactions and their abundances. Plant J. **83**, 451–465.

Fu, Z., Yu, J., Cheng, X., Zong, X., Xu, J., Chen, M., Li, Z., Zhang, D., and Liang, W. (2014) The rice basic helix-loop-helix transcription factor TDR INTERACTING PROTEIN2 is a central switch in early anther development. Plant Cell **26**, 1512–1524.

Hannon, G. J. (2002) RNA interference. Nature **418**, 244–251.

Howell, M. D., Fahlgren, N., Chapman, E. J., Cumbie, J. S., Sullivan, C. M., Givan, S. A., Kasschau, K. D., and Carrington, J. C. (2007) Genome-wide analysis of the RNA-DEPENDENT RNA POLYMERASE6/DICER-LIKE4 pathway in *Arabidopsis* reveals dependency on miRNA- and tasiRNA-directed targeting. Plant Cell **19**, 926–942.

Iwakawa, H.-o., Lam, A. Y. W., Mine, A., Fujita, T., Kiyokawa, K., Yoshikawa, M., Takeda, A., Iwasaki, S., and Tomari, Y. (2021) Ribosome stalling caused by the Argonaute-microRNA-SGS3 complex regulates the production of secondary siRNAs in plants. Cell Rep. **35**, 109300.

Iwakawa, H.-o., and Tomari, Y. (2015) The functions of microRNAs: mRNA Decay and translational repression. Trends Cell Biol. **25**, 651–665.

Jiang, P., Lian, B., Liu, C., Fu, Z., Shen, Y., Cheng, Z., and Qi, Y. (2020) 21-nt phasiRNAs direct target mRNA cleavage in rice male germ cells. Nat. Commun. **11**, 5191.

Johnson, C., Kasprzewska, A., Tennessen, K., Fernandes, J., Nan, G.-L., Walbot, V., Sundaresan, V., Vance, V., and Bowman, L. H. (2009) Clusters and superclusters of phased small RNAs in the developing inflorescence of rice. Genome Res. **19**, 1429–1440.

Kapoor, M., Arora, R., Lama, T., Nijhawan, A., Khurana, J. P., Tyagi, A. K., and Kapoor, S. (2008) Genome-wide identification, organization and phylogenetic analysis of Dicer-like, Argonaute and RNA-dependent RNA Polymerase gene families and their expression analysis during reproductive development and stress in rice. BMC Genomics **9**, 451.

Komiya, R. (2017) Biogenesis of diverse plant phasiRNAs involves an miRNA-trigger and Dicer-processing. J. Plant Res. **130**, 17–23.

Komiya, R., Ohyanagi, H., Niihama, M., Watanabe, T., Nakano, M., Kurata, N., and Nonomura, K-I. (2014) Rice germline-specific Argonaute MEL1 protein binds to phasiRNAs generated from more than 700 lincRNAs. Plant J. **78**, 385–397.

Lee, Y.-S., Maple, R., Dürr, J., Dawson, A., Tamim, S., del Genio, C., Papareddy, R., Luo, A., Lamb, J. C., Amantia, S., et al. (2021) A transposon surveillance mechanism that safeguards plant male fertility during stress. Nat. Plants **7**, 34–41.

Liu, C., Shen, Y., Qin, B., Wen, H., Cheng, J., Mao, F., Shi, W., Tang, D., Du, G., Li, Y., et al. (2020a) *Oryza sativa* RNA-dependent RNA polymerase 6 contributes to double-strand break formation in meiosis. Plant Cell **32**, 3273–3289.

Liu, H., and Nonomura, K.-I. (2016) A wide reprogramming of histone H3 modifications during male meiosis I in rice is dependent on the Argonaute protein MEL1. J. Cell Sci. **129**, 3553–3561.

Liu, Y., Teng, C., Xia, R., and Meyers, B. C. (2020b) PhasiRNAs in plants: their biogenesis, genic sources, and roles in stress responses, development, and reproduction. Plant Cell **32**, 3059–3080.

Matzke, M., Kanno, T., Daxinger, L., Huettel, B., and Matzke, A. J. M. (2009) RNA-mediated chromatin-based silencing in plants. Curr. Opin. Cell Biol. **21**, 367–376.

Matzke, M. A., Kanno, T., and Matzke, A. J. M. (2015) RNA-directed DNA methylation: the evolution of a complex epigenetic pathway in flowering plants. Annu. Rev. Plant Biol. **66**, 243–267.

Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., et al. (2008) Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′terminal nucleotide. Cell **133**, 116–127.

Montgomery, T. A., Howell, M. D., Cuperus, J. T., Li, D., Hansen, J. E., Alexander, A. L., Chapman, E. J., Fahlgren, N., Allen, E., and Carrington, J. C. (2008) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in *TAS3 trans*-acting siRNA formation. Cell **133**, 128–141.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in *trans*. Plant Cell **2**, 279–289.

Nonomura, K.-I., Miyoshi, K., Eiguchi, M., Suzuki, T., Miyao, A., Hirochika, H., and Kurata, N. (2003) The *MSP1* gene is necessary to restrict the number of cells entering into male and

female sporogenesis and to initiate anther wall formation in rice. Plant Cell **15**, 1728–1739.

Nonomura, K.-I., Morohoshi, A., Nakano, M., Eiguchi, M., Miyao, A., Hirochika, H., and Kurata, N. (2007) A germ cell specific gene of the *ARGONAUTE* family is essential for the progression of premeiotic mitosis and meiosis during sporogenesis in rice. Plant Cell **19**, 2583–2594.

Ono, S., Liu, H., Tsuda, K., Fukai, E., Tanaka, K., Sasaki, T., and Nonomura, K.-I. (2018) EAT1 transcription factor, a non-cell-autonomous regulator of pollen production, activates meiotic small RNA biogenesis in rice anther tapetum. PLoS Genet. **14**, e1007238.

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L., and Poethig, R. S. (2004) *SGS3* and *SGS2/SDE1/RDR6* are required for juvenile development and the production of *trans*-acting siRNAs in *Arabidopsis*. Genes Dev. **18**, 2368–2379.

Pokhrel, S., Huang, K., Bélanger, S., Zhan, J., Caplan, J. L., Kramer, E. M., and Meyers, B. C. (2021a) Pre-meiotic 21-nucleotide reproductive phasiRNAs emerged in seed plants and diversified in flowering plants. Nat. Commun. **12**, 4941.

Pokhrel, S., Huang, K., and Meyers, B. C. (2021b) Conserved and non-conserved triggers of 24-nucleotide reproductive phasiRNAs in eudicots. Plant J. **107**, 1332–1345.

Schwab, R., Maizel, A., Ruiz-Ferrer, V., Garcia, D., Bayer, M., Crespi, M., Voinnet, O., and Martienssen, R. A. (2009) Endogenous TasiRNAs mediate non-cell autonomous effects on gene regulation in *Arabidopsis thaliana*. PLoS One **4**, e5980.

Song, X., Li, P., Zhai, J., Zhou, M., Ma, L., Liu, B., Jeong, D.-H., Nakano, M., Cao, S., Liu, C., et al. (2012a) Roles of DCL4 and DCL3b in rice phased small RNA biogenesis. Plant J. **69**, 462–474.

Song, X., Wang, D., Ma, L., Chen, Z., Li, P., Cui, X., Liu, C., Cao, S., Chu, C., Tao, Y., et al. (2012b) Rice RNA-dependent RNA polymerase 6 acts in small RNA biogenesis and spikelet development. Plant J. **71**, 378–389.

Ta, K. N., Sabot, F., Adam, H., Vigouroux, Y., De Mita, S., Ghesquiére, A., Do, N. V., Gantet, P., and Jouannic, S. (2016) *miR2118*-triggered phased siRNAs are differentially expressed during the panicle development of wild and domesticated African rice species. Rice (N Y) **9**, 10.

Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M., and Watanabe, Y. (2008) The mechanism selecting the guide strand from small RNA duplexes is different among *argonaute* proteins. Plant Cell Physiol. **49**, 493–500.

Teng, C., Zhang, H., Hammond, R., Huang, K., Meyers, B. C., and Walbot, V. (2020) *Dicer-like 5* deficiency confers temperature-sensitive male sterility in maize. Nat. Commun. **11**, 2912.

Xia, R., Chen, C., Pokhrel, S., Ma, W., Huang, K., Patel, P., Wang, F., Xu, J., Liu, Z., Li, J., et al. (2019) 24-nt reproductive phasiRNAs are broadly present in angiosperms. Nat. Commun. **10**, 627.

Xia, R., Xu, J., Arikit, S., and Meyers, B. C. (2015) Extensive families of miRNAs and *PHAS* loci in Norway spruce demonstrate the origins of complex phasiRNA networks in seed plants. Mol. Biol. Evol. **32**, 2905–2918.

Yoshikawa, M., Peragine, A., Park, M. Y., and Poethig, R. S. (2005) A pathway for the biogenesis of *trans*-acting siRNAs in *Arabidopsis*. Genes Dev. **19**, 2164–2175.

Zhai, J., Jeong, D.-H., De Paoli, E., Park, S., Rosen, B. D., Li, Y., González, A. J., Yan, Z., Kitto, S. L., Grusak, M. A., et al. (2011) MicroRNAs as master regulators of the plant *NB-LRR* defense gene family via the production of phased, *trans*-acting siRNAs. Genes Dev. **25**, 2540–2553.

Zhai, J., Zhang, H., Arikit, S., Huang, K., Nan, G.-L., Walbot, V., and Meyers, B. C. (2015) Spatiotemporally dynamic, cell-type-dependent premeiotic and meiotic phasiRNAs in maize anthers. Proc. Natl. Acad. Sci. USA **112**, 3146–3151.

Zhang, D., and Yang, L. (2014) Specification of tapetum and microsporocyte cells within the anther. Curr. Opin. Plant Biol. **17**, 49–55.

Zhang, M., Ma, X., Wang, C., Li, Q., Meyers, B. C., Springer, N. M., and Walbot, V. (2021) CHH DNA methylation increases at *24-PHAS* loci depend on 24-nt phased small interfering RNAs in maize meiotic anthers. New Phytol. **229**, 2984–2997.

Zhang, Y.-C., Lei, M.-Q., Zhou, Y.-F., Yang, Y.-W., Lian, J.-P., Yu, Y., Feng, Y.-Z., Zhou, K.-R., He, R.-R., He, H., et al. (2020) Reproductive phasiRNAs regulate reprogramming of gene expression and meiotic progression in rice. Nat. Commun. **11**, 6031.