PERSPECTIVE

Small RNAs in angiosperm gametophytes: from epigenetics to gamete development

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The established role of various small RNA pathways in the epigenetic regulation of gene expression in the diploid sporophytic generation of flowering plants contrasts sharply with the lack of knowledge of their role in haploid gametophyte generation. Several recent studies now uncover the operation of multiple small RNA pathways in male and female gametophytes and their essential roles in genome integrity, cell specification, and, most recently, sperm cell function, as described in the May 15, 2010, issue of *Genes & Development* by Ron and colleagues (pp. 1010–1021).

Epigenetic phenomena that involve control of the chromatin state and post-transcriptional gene regulation by small RNAs have emerged as key mechanisms governing eukaryotic gene expression. Small RNA molecules and their associated regulatory pathways are thus key effectors of transcriptional and post-transcriptional gene silencing mechanisms, and continue to increase in diversity as new mechanisms are identified. Current knowledge of the diversity and functions of small RNAs in flowering plants has been driven by studies of the leafy sporophyte generation, leaving the haploid gametophyte generation relatively unexplored (Grant-Downton 2010). A suite of recent studies now reveals the operation of multiple small RNA pathways in male and female gametophytes, and uncovers their roles in genome integrity and gamete development. These advances have been driven by the combination of cell isolation procedures, high-throughput sequencing technologies, and genetic analysis. Here we present an overview of the variety of small RNA pathways in plants and the extent to which these pathways are known to contribute to the gametophyte generation of the plant life cycle.

Small RNA pathways in plants

In the plant world, small RNA pathways are divided into two main classes, depending on the type of RNAs from

which they originate: microRNAs (miRNAs) and siRNAs. miRNAs are produced from loci that generate ssRNAs that form a hairpin structure that is processed by specific enzymes like DICER-LIKE (DCL1) into 20- to 24-nucleotide (nt) small RNAs. These are loaded onto an AGO1–RNA-induced silencing complex (RISC) and guided to the target mRNA through base-pairing. The resulting action on target transcripts can then involve either endonucleolytic cleavage or translational inhibition (Brodersen et al. 2008). miRNAs have been shown to play important roles in plant development, regulating phase transition, hormone biosynthesis and signaling, pattern formation, and morphogenesis (for review, see Chen 2009).

In contrast, siRNAs are produced from longer dsRNAs that are processed into 18- to 25-nt small RNAs. Prominent in this category are siRNAs complementary to heterochromatic regions of the genome. Indeed, transcription of loci composed mainly of noncoding RNAs, transposable elements (TEs), or repeated sequences can give rise to so-called “aberrant RNAs” that form dsRNA regions or that lack normal termini. Such aberrant RNAs are copied into dsRNAs and are processed into 21- to 24-nt siRNAs. These are associated with complexes that guide DNA and histone methylation to complementary loci so that potentially damaging regions of the genome can be epigenetically or transcriptionally silenced. Other categories of siRNAs are associated with coding regions of genomes, such as *trans*-acting siRNAs (ta-siRNAs), where the first cleavage event is initiated by a miRNA. The cleaved products are then used as a matrix to produce dsRNAs that are processed into 21-nt siRNAs directing the post-transcriptional regulation of specific target transcripts. For example, small RNAs derived from Tasi-ARF loci are involved in hormonal regulation of leaf patterning, promoting abaxial identity through repression of the auxin response factors *ARF3* and *ARF4* (Adenot et al. 2006; Fahlgren et al. 2006; Garcia et al. 2006; Hunter et al. 2006).

Another class of siRNAs arise from loci producing *cis*-natural antisense transcript pairs (*cis*-NATs) that are a common feature within eukaryotic genomes (9% of *Arabidopsis* genes). It is established that the level of expression of the transcripts constituting the pair can be regulated by the action of 21- to 24-nt natural *cis*-antisense siRNAs (*cis*-nat-siRNAs) derived from the

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cleavage of the dsRNA formed by the overlap of the two transcripts (Jin et al. 2008). Although a regulatory role of nat-siRNAs has not been demonstrated in animal models, two recent studies reveal their involvement in stress-responsive gene expression in *Arabidopsis*. In response to a specific stress condition (pathogen presence or high salt), one transcript of each *cis*-NAT pair is specifically produced, whereas the second is constitutively expressed. The resulting dsRNAs give rise to specific nat-siRNAs down-regulating the constitutive transcript, thereby producing the appropriate response to stress (Borsani et al. 2005; Katiyar-Agarwal et al. 2006). These represent the first examples clearly establishing the regulatory role of this class of siRNAs in eukaryotes.

miRNA identification in the male gametophyte

The diversity of small RNAs in plant gametophytes has just begun to be investigated, and the male gametophyte (pollen) has been preferentially studied to date. Indeed, pollen constitutes a more accessible model because of its direct release from the anther, whereas the female gametophyte (embryo sac) is embedded in sporophytic tissues. The mature pollen grain is a simple three-celled structure, which develops from a haploid microspore that first divides asymmetrically to produce a vegetative cell and a generative (or male germ) cell, which then divides once to form two sperm cells (Borg and Twell 2010). Major transcriptomic analyses have revealed complete pollen developmental expression profiles (Honys and Twell 2004; Pina et al. 2005), the sperm cell transcriptome (Borges et al. 2008), and transcriptome changes associated with pollen germination and tube growth (Wang et al. 2008; Qin et al. 2009). Together with the recent discovery of several male gametophytic regulators of cell cycle progression and differentiation (Kim et al. 2008; Brownfield et al. 2009a,b; Chen et al. 2009; Gusti et al. 2009), these studies serve as a foundation to decipher the mechanisms regulating male gamete production and delivery.

These data sets also provide a valuable resource with which to explore the presence of the different small RNA pathways during male gametophyte development. The detection of some transcripts coding for key components of miRNA (such as *AGO1* or *DCL1*) and ta-siRNA (especially *RDR6*) pathways provided a first indication of their operation in *Arabidopsis* pollen (Grant-Downton et al. 2009a). Further investigation confirmed that miRNAs are conventionally processed, since pre-miRNA, pri-miRNA, and mature miRNA could be detected for miR162 (a regulator of *DCL1* transcripts). Moreover, cleavage products of the correct size were detected for different miRNA target transcripts, such as *AGO1* (targeted by miR168) and *ARF16* and *ARF17* (miR160), confirming the function of the miRNA pathway in pollen (Fig. 1). Deep sequencing and Exiqon miRCURY LNA arrays have been used to identify more exhaustively miRNAs expressed in *Arabidopsis* pollen (Chambers and Shuai 2009; Grant-Downton et al. 2009b). Approximately 30 known miRNAs were detected, with the most abundant being miR156, suggesting a role in the repression of flowering-related

transcripts in pollen. The abundance of miR158 and miR161 further indicates that PPR protein transcripts may need to be suppressed in pollen. Additional bioinformatic analysis of 454 sequencing data identified seven new species of miRNAs, including miR2939, which is highly gametophyte-enriched and targets a gametophyte-specific F-box family transcript for cleavage (Grant-Downton et al. 2009b). Interestingly, this transcript is also cleaved in another location by miR774. This unusual double miRNA cleavage event suggests stringent post-transcriptional control of this transcript and important undiscovered roles for proteolytic degradation in gametophyte development and function (Kim et al. 2008; Liu et al. 2008; Gusti et al. 2009).

Undoubtedly, the increases in sequencing depth now available will help to uncover the complete set of gametophyte-expressed miRNAs and their overlap with the sporophyte. These initial studies suggest important roles for miRNAs in pollen development. The analysis of specific classes of miRNAs and their targets is now needed to reveal their functional significance in male gametophyte biology.

siRNAs as silencers of TEs in male and female germlines

In invertebrates and mammals, germline-specific longer small RNAs of 26–31 nt can be detected. These are called piRNAs because of their specific association with Argonaute family PIWI proteins. Their roles are to direct the silencing of transposons and repeated sequences in germ cells, thereby preserving the genomic integrity of the gametes and preventing the transmission of deleterious effects of active elements to the next generation (Kim et al. 2009). Although such longer small RNAs with potential roles in transposon silencing have not been described in plants, a recent study of *Arabidopsis* pollen small RNAs reported that 21-nt siRNAs targeting specific TEs (*Athila*) are present in sperm cells (Slotkin et al. 2009). In contrast, certain classes of TEs were shown to be reactivated and to transpose only in the vegetative nucleus, consistent with the down-regulation of the chromatin remodeling enzyme DECREASED DNA METHYLATION (DDM1) and the absence of the corresponding TE-siRNAs in pollen. Slotkin et al. (2009) thus propose a model whereby the vegetative cell acts as a germline “companion” cell to reveal the presence of intact TEs in the genome and to regulate their activity in the germline by transport of siRNAs into the sperm cells (Fig. 1). In a separate study, the loss of DDM1 from the vegetative cell has been linked to the hypermethylation of centromeric repeats and a class of retrotransposon sequences at non-CG sites (Schoft et al. 2009). Schoft et al. (2009) propose that down-regulation of DDM1 leads to demethylation of H3K27me2 and disturbed centromeric heterochromatin, allowing siRNA-directed DNA methylation (RdDM) at centromeric repeats in vegetative cell nuclei. This contrasts with the hypomethylation of some TEs in pollen and their strong non-CG methylation in the sperm cells (Slotkin et al. 2009). A more dynamic view of the distribution of siRNAs and DNA methylation during male gametophyte development

Figure 1. Small RNA pathways in *Arabidopsis* pollen. In the past few years, several studies have demonstrated the role of different small RNA pathways in the *Arabidopsis* male gametophyte. (1) Molecular and deep sequencing analyses first demonstrated that miRNAs were detectable and conventionally processed in mature pollen, suggesting the full functionality of the pathway (Grant-Downton et al. 2009a,b). (2) These data also allowed the identification transcripts showing ta-siRNA features. (3) A further study highlights the critical role of siRNA pathways in silencing TEs in germ cells. Slotkin et al. (2009) suggested that the reactivation of TEs in the vegetative cell (associated with the absence of the chromatin remodeling protein DDM1) could serve as a template for the production of TE-siRNAs. Slotkin et al. (2009) propose that these siRNAs constitute a signal that could direct DNA methylation (indicated on the figure by an asterisk [*]) at the complementary loci sperm (DDM1 being present in sperm cells). (4) A recent

discovery by Ron et al. (2010) further demonstrates a developmental role for the *cis-nat-siRNA* pathway in pollen. Indeed, they demonstrate that the KPL/ARI14 NAT pair gives rise to the production of KPL *cis-nat-siRNAs*, triggering ARI14 degradation. ARI14 control by an siRNA pathway is thought to be a key requirement for proper fertilization, since *kpl* mutants (unable to give rise to the production of the siRNAs) show failed and “single sperm” fertilization events.

will help to resolve the uncertainties regarding potential siRNA transport and the epigenetic control of different classes of transposon sequences.

An exciting parallel discovery is that the ARGONAUTE 9 (AGO9) protein is necessary to silence TEs in the female gametophyte (Olmedo-Monfil et al. 2010). AGO9 is expressed in sporophytic companion cells surrounding the female gametophyte (but not in the female germ cell lineage) and interacts preferentially with 24-nt siRNAs derived from TEs. Both studies suggest that TEs reactivated in specific accessory cells of both male and female gametophytes enhance siRNA-triggered silencing of TEs in germline cells that is reminiscent of the role of piRNAs in transposon silencing in animals (Mosher and Melnyk 2010). Olmedo-Monfil et al. (2010) also demonstrate that gametophyte precursors are incorrectly specified in ovules of *ago9* and several siRNA pathway mutants (*rdr2*, *rdr6*, *sgs3*, *dcl3*, and *nripd1a/nripd1b*), resulting in the presence of two female gametophytes in a single ovule. This reveals an important non-cell-autonomous role for a 24-nt siRNA biosynthetic pathway in the restriction of cell fate in the young ovule (Fig. 2). This raises the possibility that some maternal siRNA sequences found in the endosperm (Mosher et al. 2009) and 24-nt siRNAs found in pollen (Slotkin et al. 2009) that resemble AGO9-interacting sRNAs may also constitute a similar non-cell-autonomous silencing signal (Olmedo-Monfil et al. 2010). The mechanisms involved in the cell–cell transmission of this type of siRNA signal, and whether TE suppression and restriction of cell fate are directly linked, nevertheless remain to be determined.

Regulatory functions for ta-siRNAs and *cis-nat-siRNAs* in gametophyte development?

Small RNAs and, specifically, siRNAs seem to play a key role in the epigenetic control of gametic development, yet little is known about the putative role of siRNAs in regulating the level of expression of specific transcripts during pollen development. A first indication has been given by Grant-Downton et al. (2009b) in their identification of ta-siRNA features in small RNA populations from *Arabidopsis* pollen (Fig. 1). Analysis of 454 sequencing data identified phased siRNAs for TAS1 and TAS2 transcripts, suggesting the function of this pathway in mature pollen and a regulatory role for ta-siRNAs in pollen development. In the May 15, 2010, issue of *Genes & Development*, Ron et al. (2010) demonstrate that the misregulation of a specific *cis-nat-siRNA* pair in *Arabidopsis* sperm cells results in impaired fertilization (Fig. 1). They show that, in wild-type plants, the overlapping transcripts from the KOKOPELLI (KPL)/ARIADNE14 (ARI14) *cis-NAT* pair exhibit inverse expression patterns during pollen development. This suggested that this pair could give rise to the production of nat-siRNAs originating from KPL transcripts directing down-regulation of ARI14 transcripts in sperm. Analysis of *kpl* mutants confirmed the overexpression of ARI14 transcripts in pollen, which was correlated with impaired fertilization involving single fertilization of either the egg or the central cell, leading to seed abortion. Ron et al. (2010) further demonstrated that a KPL/ARI14 nat-siRNA was generated only in sperm cells and was responsible for

Figure 2. Role of small RNAs in cell fate restriction in the *Arabidopsis* female gametophyte. Based on comparisons between wild-type and *ago9* mutant phenotypes and the absence of AGO9 in female gametophytes and their precursors, Olmedo-Monfil et al. (2010) suggest that AGO9-associated siRNAs are produced in epidermal cells (L1) of the nucellus through a PolIV–PolV–RDR2–DCL3-dependent small RNA pathway. AGO9 complexes were found to associate preferentially with siRNAs derived from TEs, so that siRNA production is proposed to act as a non-cell-autonomous signal (through a RDR6–SGS3-dependent pathway) to restrict cell fate and TE activity. In ovules of *ago9* mutants, a subepidermal nucellar cell gives rise to a supernumerary diploid female gametophyte, and Olmedo-Monfil et al. (2010) suggest that this AGO9-driven siRNA signal normally acts to prevent the specification of a subepidermal cell as a megaspore mother cell (MMC). The MMC normally gives rise after meiosis to a fully functional female gametophyte where TEs are epigenetically silenced, but *ago9* mutants show TE reactivation in the egg and synergid cells.

ARI14 down-regulation. *KPL* encodes a protein of unknown function, while *ARI14* is a putative defective ubiquitin E3 ligase. They propose a model for regulation of fertilization by this specific nat-siRNA pair involving a putative role for *ARI14* in impairing the function of related ubiquitin E3 ligases (*ARI13/ARI15*) expressed in sperm cells. Down-regulation of *ARI14* by the nat-siRNAs would thus prevent the inhibition of *ARI13/ARI15* function, ensuring successful fertilization. Interestingly, the sperm cell-specific *KPL* gene appears to have evolved recently, as it is present in only some species of *Brassicaceae*, indicating that the regulation of *ARI14* via a *KPL/ARI14* nat-siRNA is a novel evolutionary feature. Ron et al. (2010) further highlight the general overexpression of *cis*-nat-siRNAs in animal testes, and speculate that *cis*-nat-siRNAs may be a common regulatory mechanism for gene expression in germline cells.

This study provides an intriguing example of a developmental requirement for nat-siRNAs in plant fertilization. It also raises the interesting question of the evolutionary role for these types of NAT pairs and their associated siRNAs. It is conceivable that the gain of *KPL* through insertion into the *ARI13/14/15* locus may have arisen to suppress mutations in *ARI14* (that would normally block specific E3 ligase functions in sperm cells), thereby restoring male fertility. Once established, the essential *KPL/ARI14 cis*-NAT pair would promote gamete fitness, and the preferential survival of intact *cis*-NAT genotypes could potentially contribute to population isolation and subspeciation. *KPL* may also act to promote sperm cell fertility in addition to its role in suppressing *ARI14*. This issue may be resolved by targeted knockdown of *KPL* in *ari14* mutants (or vice versa), since the juxtaposition of *KPL* and *ARI14* prevents the genetic combination of knockout alleles in germ cells. If loss of *ARI14* in a *kpl* mutant background fully restores fertility, this would exclude an independent role for *KPL* in gamete fitness.

Conclusion

The recent advances uncovering the role of multiple small RNA pathways in the haploid gametophytes of flowering plants are exemplified by the discovery of distinct siRNA pathways controlling genome integrity, cell specification and gamete function. Although this is a promising start, the functions of most small RNAs belonging to miRNA, ta-siRNA, and other *cis*-nat siRNA categories remain to be explored in the gametophytes. Deep sequencing methods now provide the means for an exhaustive description of the expression profiles of different classes of small RNAs throughout male gametophyte development. The analysis of small RNA diversity in the female gametophyte will be more challenging, but may be overcome through improvements in cellular isolation techniques and sequencing technology. These data sets will undoubtedly lead to new hypotheses of regulatory events underpinning angiosperm gametophyte development to include the contributions of small RNAs to changes in chromatin, DNA methylation, and cell differentiation, and their potential roles in *trans*-generational epigenetic inheritance.

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