Review

**MicroRNA activity in the Arabidopsis male germline**

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**Keywords:**
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MicroRNA
Pollen
Post-transcriptional Gene Silencing

**Abbreviations:**
SC – Sperm Cell
GC – Generative Cell
VC – Vegetative Cell
VN – Vegetative Nucleus
PMI – Pollen Mitosis I
PMII – Pollen Mitosis II
FACS – Fluorescence-Activated Cell Sorting
Abstract

Most of the core proteins involved in the microRNA (miRNA) pathway in plants have been identified and almost simultaneously hundreds of miRNA sequences processed in the *Arabidopsis* sporophyte have been discovered exploiting next-generation sequencing technologies. However, there is very limited understanding about potentially distinct mechanisms of post-transcriptional regulation between different cell lineages.

In this review we focus on the *Arabidopsis* male gametophyte (pollen), where the germline differentiates after meiosis giving rise to the male gametes. Based on comparative analysis of miRNAs identified in sperm cells by deep sequencing we discuss their possible functions along germ cell specification and beyond fertilization. In addition, 25 potentially novel miRNAs processed in sperm cells and pollen were identified, as well as enriched variations in the sequence length of known miRNAs, which might indicate sub-functionalization by association with a putative germline-specific Argonaute complex. ARGONAUTE 5 (AGO5), by close homology to AGO1 and localizing preferentially to the sperm cell cytoplasm in mature pollen, may be part of such a complex.

Introduction

Post-transcriptional gene silencing was first observed over a decade ago and since then numerous discoveries unveiled a fascinating and unexpectedly conserved cellular mechanism. In addition, several studies from plants to animals underlined distinct regulatory mechanisms that evolved throughout different cell lineages.
The miRNA pathway is an essential mechanism to regulate different biological processes. In plants it is involved in several fundamental processes such as development, response to biotic and abiotic stresses and hormone responses. These small non-coding RNAs act through cleavage of highly complementary target mRNAs, but also through translational repression (Brodersen et al., 2008; Chen, 2004). More indirectly, miRNA-guided cleavage of TAS transcripts gives rise to trans-acting siRNAs (ta-siRNA) that regulate expression of other genes (Allen et al., 2005). miRNA biogenesis depends on processing of stem-loop precursors by DICER-Like 1 (DCL1), and subsequent cleavage of target mRNAs requires binding of mature miRNAs to ARGONAUTE 1 (AGO1) (Voinnet, 2009). Additionally, some miRNAs may also associate with AGO10 promoting translational repression of specific target transcripts (Brodersen et al., 2008). Based on data generated by numerous high-throughput sRNA sequencing studies, additional types of small RNAs resulting from known precursor stem loops were identified (Chellappan et al., 2010; Vazquez et al., 2008; Zhang et al., 2010). Although processing of these miRNA-like RNAs is mostly dependent on the regular DCL1-based processing pathway (Zhang et al., 2010), it was additionally shown that the production of some long miRNAs (23-27 nt) depends on a DCL3/RDR2/Pol IV pathway (Chellappan et al., 2010).

Although miRNA activity is well characterized in the Arabidopsis sporophyte, little is known about the role of miRNAs during male gametophyte development. The haploid microspores in higher plants undergo two mitotic divisions to form the gametes, which are nourished inside the pollen vegetative cell and passively
transported to the embryo sac by the pollen tube. The male germline starts after pollen mitosis I (PMI), by asymmetric cell division in the microspore. The larger cell (Vegetative Cell - VC) arrests cell cycle progression, while the smaller cell (Generative Cell - GC) divides further in PMII to originate two identical male gametes (Sperm Cells – SC) (Boavida et al., 2005). The genetic programs driving male gametogenesis and pollen development have been studied and reported throughout the last decade mostly by means of microarray-based studies, but also with the characterization of loss-of-function mutations impairing gametophyte development (Becker and Feijo, 2007; Borg et al., 2009). These studies hold great promise towards the identification of a core transcriptional program driving plant germline differentiation and specification. However, understanding post-transcriptional regulation of gene expression in pollen and the gametes requires additional efforts.

This review aims to summarize recent discoveries on miRNA pathways and epigenetic reprogramming in the Arabidopsis germline, bridging novel findings with our current understanding of miRNA activity in plants. Our comparative analysis of known miRNA families and potentially novel miRNAs identified in total pollen and isolated sperm cells by deep sequencing highlights their possible functions during gamete specification and fertilization.

Small RNA pathways during male gametogenesis

Perspective from transcriptomics

Arabidopsis pollen at anthesis is tricellular, having originated from a
uninucleated microspore after two rounds of mitotic division. Honys and Twell (2004) have shown that throughout male gametogenesis there is a decrease in the number of genes expressed, going along with an enrichment of pollen-specific transcripts. Their data suggested activation of a pollen-specific transcriptional machinery, simultaneously with selective down-regulation of somatic genes as a possible result of miRNA activity. However, this early study used whole pollen and thus could not distinguish gene expression profiles between the two differentiated cell types in mature pollen grains. The understanding of general molecular functions acting in Arabidopsis SCs increased significantly over the last few years owing to SC purification methods developed for FACS, and microarray-based transcriptomics (Borges et al., 2008). Comparison of the expression profile of mature pollen grains with that of purified sperm cells clearly indicated that after PMI, the resulting VC and SCs activate distinct transcriptional programs. The vegetative cell seems to be enriched in transcripts functionally skewed towards pollen germination and tube growth (Pina et al., 2005), and expression of repeats as a result of its chromatin decondensation (Slotkin et al., 2009). At this stage the twin sperm cells initiated a long DNA replication phase that will sustain until the moment of fertilization. Concordantly, the sperm cell transcriptome shows intricate control over DNA repair, cell cycle transitions and ubiquitin-mediated protein degradation (Borges et al., 2008). Yet, these results showed that messenger RNAs accumulating in the male gametes are diverse and abundant, suggesting that the same might be true for other RNA species such as small non-coding RNAs.
Primary evidence indicating that miRNAs could be actively controlling male gametophytic development came from the study by Kidner and Martienssen (2005) in Arabidopsis. This work characterized a mutant allele of AGO1 (ago1-10) that is poorly transmitted through the male gametes. More recently, miRNAs were shown to be present and functional in mature pollen, along with expression of some of the most important genes involved in the miRNA silencing pathway such as DCL1, AGO1 and RDR6, that are consistently expressed during pollen development (Grant-Downton et al., 2009a). For details on small RNA pathways and their components see review by Le Trionnaire et al. in this issue (Le Trionnaire et al., 2011). Two independent studies reported the miRNA repertoire in Arabidopsis pollen using microarrays, qRT-PCR and pyrosequencing, resulting in the identification of approximately 33 known miRNAs (Chambers and Shuai, 2009; Grant-Downton et al., 2009b). Grant-Downton et al. (2009b) identified also 7 putative novel miRNAs, including one that targets a F-Box protein specifically expressed in pollen and co-targeted by miR774. Their work showed that ta-siRNAs derived from miR173-directed cleavage of TAS transcripts also accumulate in pollen, but intriguingly precursor TAS transcripts could not be detected. These results clearly indicate that mature miRNAs and products of miRNA-guided cleavage are present in mature pollen, but the spatial temporal regulation of miRNA activity during pollen development and in plant germ cells remained unexplored.

A potentially unique small RNA silencing pathway during germ cell differentiation
and specification

While some of the key proteins required for miRNA processing in *Arabidopsis* seem to be expressed in pollen and sperm cells, many other genes involved in small RNA pathways are either not expressed or depleted (Borges et al., 2008). In contrast, expression of particular genes associated with small RNA activity and DNA methylation such as *AGO9, MET1*, and *DDM1*, as well as *AGO5*, are highly enriched in sperm cells in comparison with total pollen and sporophytic tissues (Borges *et al.*, 2008; Slotkin *et al.*, 2009), suggesting that distinct genetic and epigenetic mechanisms might be established preferentially in the germline. *AGO1* and to some extent *AGO10*, are the main regulators of miRNA-directed target cleavage in *Arabidopsis* (Brodersen *et al.*, 2008; Vaucheret *et al.*, 2004), but both seem to be down-regulated in the sperm cells. While AGO9 has been recently implicated in siRNA-mediated transposon inactivation (Olmedo-Monfil *et al.*, 2010), AGO5 is a closely related homolog of AGO1 and AGO10 (Fig. 1) and could thus be involved in miRNA activity in the gametes. As predicted by the sperm transcriptome data, *AGO5* is preferentially accumulated in the sperm cell cytoplasm in mature pollen and growing pollen tubes (Fig. 2, supplemental Videos S1 and S2).

The closest homolog in rice (*MEL1*) was shown to be required for correct progression through meiosis (Nonomura *et al.*, 2007), but the biological function of AtAGO5 and OsMEL1 and their role in the small RNAs pathways remain to be elucidated. In addition to the role of Argonaute proteins in small RNA-directed DNA methylation of heterochromatic regions, and miRNA-guided slicing and translational repression of target transcripts, it was recently shown that the catalytic
activity of the mammalian Argonaute2 is responsible for a Dicer-independent
pathway of miRNA processing (Cifuentes et al., 2010; Yang et al., 2010). As
Argonaute proteins are widely conserved among eukaryotes, it might be worthwhile
to investigate whether Argonaute proteins in plants could also process miRNA stem-
loop precursors.

Small RNA activity in germ cells has been studied in more detail in animals,
leading to the discovery of divergent mechanisms of post-transcriptional regulation
between the soma and the germline (Kedde et al., 2007; Mishima et al., 2006).
General loss of miRNAs caused by Dicer mutations have been shown to impair
germline maintenance in Caenorhabditis elegans and Drosophila (Hatfield et al.,
2005; Knight and Bass, 2001), and differentiation defects in mouse germ cells
(Hayashi et al., 2008; Maatouk et al., 2008), but apparently miRNAs are not
required for germ cell proliferation in Zebrafish (Giraldez et al., 2005).

Evidence for small RNA silencing acting in Arabidopsis sperm cells

Epigenetic silencing of transposable elements

Germ cells developed specific mechanisms associated to chromatin
remodeling and resetting of epigenetic marks that must be properly established
before being transmitted to the next generation. For example, animals have a germ
cell-specific class of small RNAs termed piRNAs (Piwi-interacting RNAs),
associated with TE silencing and heterochromatin formation (Carmell et al., 2007;
Vagin et al., 2006). Plants lack piRNAs and Piwi proteins, but alternatively,
particular TE silencing mechanisms are also specifically established in the germline
A first insight into small RNA species in *Arabidopsis* sperm cells resulted from studying transposable element (TE) activity in pollen (Slotkin *et al.*, 2009). It was shown that transposons are expressed and transpose in the vegetative nucleus, concordantly with down-regulation of DECREASED DNA METHYLATION 1 (DDM1) and gain of certain classes of TE-derived siRNAs. However, the same 21-nt siRNAs are also abundantly accumulated in the sperm cells where the corresponding TE loci are highly methylated and transcriptionally silenced (Slotkin *et al.*, 2009). These results strongly suggest an active communication between the VC and SCs, reviving earlier observations of a cytoplasmic tail that remains after pollen mitosis, connecting the gametes directly to the VN to engender the Male Germ Unit (for review see McCue *et al.*, this issue).

Along with siRNA movement between neighboring cells, several lines of evidence indicate now that miRNAs are also on the move (Martienssen, 2010). It was recently discovered that 21-nt miRNAs produced in roots cells can move into adjacent cells possibly as a miRNA duplex, and regulate expression of target genes post-transcriptionally (Carlsbecker *et al.*, 2010). Therefore it is possible that miRNAs in *Arabidopsis* pollen could also move between VC and SCs, as predicted for TE-derived siRNAs. Indeed, by expressing artificial miRNAs (amiRNA) against GFP under the control of the vegetative cell-specific promoter LAT52, it was possible to target GFP expression in the sperm cells (Slotkin *et al.*, 2009). Future studies should help understanding the functional purpose and extent of an intercellular cross-talk during pollen development and tube growth.
Natural cis-antisense siRNAs regulating expression of endogenous genes

Natural cis-antisense siRNAs (cis-nat-siRNAs) were recently described in Arabidopsis sperm cells, as playing a crucial role for double fertilization. These siRNAs are produced from the overlapping region of KPL and ARI14 transcripts, and were shown to be involved in ARI14 down-regulation (Ron et al., 2010). The authors proposed an interesting model in which ARI14 lost its E3 ubiquitin ligase activity due to aminoacid mutations along with gene duplication of ARI13/14/15, thus requiring that ARI14 is efficiently silenced post-transcriptionally to avoid competing with ARI13 protein that is also expressed in sperm. In addition, this study showed that KPL-ARI14 nat-siRNAs are processed by DCL1, and dependent on a RDR2/SGS3/Pol IV pathway, thus correlating with other previously described nat-siRNAs (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). siRNAs matching to the KPL transcript region were detected by co-expressing KPL and ARI14 ectopically, as well as other 21-nt siRNAs derived from the overlapping region. However, only one 21-nt siRNA that could target ARI14 was detected in the sperm cell sRNA dataset, originating from the KPL-ARI14 overlap region but outside the predicted area (Ron et al., 2010). The fact that other siRNAs were not detected may be explained by their low abundance. Alternatively, this could be a process that occurs earlier during pollen development, thus explaining why ARI14 transcripts are absent from sperm cells at the mature pollen stage (Borges et al., 2008).

Comparative analyses of miRNAs accumulated in sperm cells

Several miRNA families were identified in the previously reported small
RNA datasets of purified sperm cells and pollen by Illumina sequencing (Slotkin et al., 2009). From the 2,540,585 signatures sequenced in pollen and 1,925,202 in sperm cells, 283,561 and 256,787 sequences matched to known Arabidopsis miRNAs in pollen and sperm, respectively. In total, this corresponds to 75 known miRNA families expressed in pollen, and 83 in sperm cells (Fig. 3). The list of most known miRNAs in these two datasets is publicly available in the SBS database (Nakano et al., 2006). Comparing Illumina data with the recently available dataset obtained by 454 sequencing of pollen small RNAs (Grant-Downton et al., 2009b) shows that out of the 31 different miRNA families in mature pollen detected by 454, only miR776 was not detected in the Illumina pollen sample (Fig. 3). A summary of normalized reads matching to the annotated form of known miRNAs in sperm cells and pollen datasets in comparison with that of inflorescence is presented as supplementary Table S1.

Expression of most miRNA families seems to be distinct between total pollen and purified sperm cells (Fig. 4A), which was expected considering their different transcriptomes and cell fate (Borges et al., 2008). miR159a is particularly interesting as it is more than 5-fold enriched in sperm cells. Keeping in mind that miRNAs highly abundant in sperm cells should be identified in pollen samples despite a dilution effect, the significantly lower abundance of miR159a in pollen suggests that it could be sperm-specific. miR159 was predicted to be involved in the regulation of several transcripts belonging to the MYB family of transcription factors, including DUO1 (Palatnik et al., 2007), a germ cell-specific transcription factor that is responsible for expression of several germline genes (Brownfield et al.,
miR159-guided cleavage of *DUO1* transcripts was detected in mature pollen (Grant-Downton *et al.*, 2009a), but recent work could show that depletion of miR159 in a *miR159abc* triple mutant does not seem to impair pollen development and fertilization (Allen *et al.*, 2010), suggesting that post-transcriptional regulation of gene expression by miR159 is either not essential in pollen and the germline, or its closely related miR319 can compensate, to a certain extent, miR159 absence.

This work has further shown that miR159 activity is essential during plant development, but uniquely controlling expression of the anther-specific *MYB33* and *MYB65* (Allen *et al.*, 2010).

MiRNA families such as miR156 and miR158 have isoforms in which the 5’ terminal nucleotide is a Cytosine (miR156g and miR158b, respectively). Such isoforms were predicted to be associated preferentially with AGO5 (Mi *et al.*, 2008; Takeda *et al.*, 2008), which correlates with the fact that miR156g and miR158b levels are higher in pollen and sperm than in sporophytic tissues (Table S1).

However, it is intriguing that miR156a-f is so highly enriched in pollen and sperm (Fig. 4A), as the entire family is predicted to target the plant-specific SPL gene family of transcription factors (Rhoades *et al.*, 2002). This possibly suggests that the same miRNA family may have distinct activity by association with specific AGO complexes. *SPL* genes are known to regulate several developmental transitions like flowering and shoot development, but their importance in pollen and germline differentiation remains unexplored. Another example of a miRNA with a 5’ Cytosine is miR845a, which is also highly enriched in pollen and sperm, but its targets are still unknown. Both miR845a and 845b are almost undetectable in
inflorescence tissue (Table S1), which suggest that they must be preferentially processed in sperm cells and pollen.

Novel miRNAs and natural variations in sequence length of known miRNA families

To screen for potentially new miRNAs in the sperm cell and pollen sRNA datasets, the miRCat tool available online within the UEA plant sRNA toolkit (Moxon et al., 2008) was used. Based on correspondence with genomic loci that could encode stem-loop precursors, 25 small RNA sequences were identified as potential novel miRNAs. These results, including a number of predicted target transcripts for the novel miRNAs found, are presented in table 1 and extended in supplemental Table S2. Moreover, some of the miRNA sequences discovered seem to be isoforms of known miRNA families with variations in sequence length (Table 1). This is the case of miR158b that was detected with an additional Uracil at the 5’ nucleotide terminus. Interestingly, this isoform is more abundant in pollen and sperm cells than the normal annotated version (Fig. 4B). The biological meaning for such variations in miRNA processing is not yet fully understood, but it was recently reported that single nucleotide extensions at the 5’ terminus of known miRNAs can lead to incorporation into different Argonaute complexes (Ebhardt et al., 2010). For this reason a sperm-enriched miR156h isoform with an extension at the 5’ end and higher abundance than the known 20-nt isoform deserves closer attention (Fig. 4B). This variation in natural sequence length of miR156h was analyzed by Ebhardt et al. (2010), and curiously they observed that the known 20-nt version of miR156h is loaded into AGO1, while the miR156h plus 5’ U isoform seems to associate mainly
Isoforms of known miRNA families with nucleotide extensions at the 3’ terminus were also identified in the sperm and pollen datasets. For example, miR773 is annotated as a 21-nt miRNA and seems to be low abundant in both total pollen and sperm cells. However, a 22-nt miR773 with a C extension at the 3’ terminus is more abundant (Table 1), suggesting in this case activation of the recently discovered mechanism for triggering siRNA production from target transcripts (Chen et al., 2010; Cuperus et al., 2010). These studies showed that certain miRNAs are capable of triggering siRNA and ta-siRNAs production only if presented to the target transcript in a 22-nt form, but the biological significance of this mechanism remains controversial. Analyzing the abundance of all possible isoforms of known miRNA families from 19 to 24-nt in sequence length in the sperm and pollen datasets highlights other possible siRNA triggers (supplemental Table S3). Among these are the 22-nt miRNA isoforms miR173, miR393, miR447, miR771, miR773, miR825*, miR167d and miR828 (Fig. 4C) previously discussed (Chen et al., 2010; Cuperus et al., 2010). Interestingly miR773, targeting the DNA methyltransferase MET2, is more abundant in pollen, while miR771 that was confirmed to function as siRNA trigger (Chen et al., 2010), is enriched in sperm cells, but the targets remain unknown. Other miRNA families that could potentially function as siRNA triggers (i.e. more abundant 22-nt isoform) are miR776, miR777, miR840, miR845b, miR848a, miR852, miR853 and miR2936 (Fig. 4C). miR845 is highly enriched in pollen and sperm cells, and very low abundant in inflorescence tissue, suggesting preferential expression in the male gametophyte. This is a plant-specific and recently
evolved miRNA family (Barakat et al., 2007), for which a biological function remains obscure.

Inheritance of parental miRNAs

A very exciting question is if miRNAs accumulated in sperm cells could be delivered to egg and central cell upon double fertilization, to play a role in the development of early zygote and endosperm, respectively. A similar question is being addressed in the animal field, originating from the discovery of miRNAs and siRNAs in human spermatozoa (Ostermeier et al., 2005). In mice, miRNAs delivered to the oocyte can be detected up to the 2-cell stage (Amanai et al., 2006), but miRNA function in general seems to play no role during oocyte maturation and preimplantation development (Suh et al., 2010).

While the function of miRNAs delivered at fertilization is still a matter of debate (Dadoune, 2009), there is evidence that paternal mRNAs are transported to oocytes (Ostermeier et al., 2004) and that some have a function. For spermatozoa-derived PSG1 and HLA-E mRNA a possible functional role during early embryo development was demonstrated in human (Avendano et al., 2009), while in mice the Kit transcript delivered by spermatozoa seems to act as an epigenetic modifier of early embryo development (Rassoulzadegan et al., 2006). In plants, the strongest indication for delivery and function of sperm-derived mRNAs upon fertilization to date came from the study on the YDA signaling pathway during embryonic patterning and the characterization of the SHORT SUSPENSOR (SSP) protein. It was found that SSP transcripts accumulate in Arabidopsis sperm cells, but are
translationally repressed before fertilization, to be translated only during early zygotic development (Bayer et al., 2009). It is possible that in plants paternal miRNAs are also delivered at fertilization, as they could play important functions as signaling molecules, possibly triggering early zygotic patterning and endosperm development. miRNAs would certainly provide an efficient mechanism of reprogramming in the early zygote, before the gene expression program that defines the maternal to zygotic transition is established. In the light of the parental conflict theory (Spielman et al., 2001) it is also possible that paternally-inherited miRNAs could contribute as an immediate mechanism to regulate the maternally expressed inhibitors of embryo growth. However, small RNA sequencing or analysis of egg cells and early zygotes will be needed to determine if miRNAs accumulated in SCs are indeed inherited by the zygote. Moreover, the use of artificial miRNA target mimics (Todesco et al., 2010) to knock down specific miRNA families in sperm cells should clarify if their potential inheritance is of importance.

Conclusions

Post-transcriptional gene silencing involves miRNA activity to eliminate transcripts of previous developmental processes, while modulating expression of active genes. In the recent years, small RNA sequencing technologies allowed a robust temporal and spatial profiling of miRNAs processed in several species, tissues and cell types, leading to the discovery of an ancient and widespread mechanism to control gene expression. Consequently, we can assume that we now know the basis of miRNA processing and activity in eukaryotes; however, we are
just taking the first steps towards understanding its origins and evolution.

The male gametophyte of flowering plants constitutes a unique system to analyze specialized small RNA pathways and small RNA trafficking with cellular resolution. Post-meiotic pollen development in particular is an exciting biological process in plants for its structural simplicity and fast transitions over two mitotic divisions, which are coupled with germline differentiation and specification. Small RNA sequencing data has provided evidence that miRNAs are abundant and diverse in *Arabidopsis* sperm cells, and that after germ cell differentiation along PMI, the sperm cells and neighboring vegetative cell activate distinct miRNA pathways that correlate with different cell fate and gene expression profiles. However, there are still several open questions. It is intriguing that certain miRNAs seem to be processed in an unusual manner in pollen and sperm cells in comparison with what is generally observed in sporophytic tissues. The observation that AGO5 is likely to be part of the unique miRNA-induced silencing complex established in the male germline, supports the idea that distinct and rather unknown cellular pathways might exist. These results should provide useful information for future studies, as a baseline comparison to dissect the many hypotheses discussed.

Supplementary Material

Strategy used for cloning AGO5p::AGO5-eGFP and imaging details. (.pdf)

*Table S1* (.xls): Normalized reads for known miRNAs detected in sRNA datasets of SCs and pollen, in comparison with that of inflorescence tissue.

*Table S2* (.pdf): Potentially novel miRNAs detected in the sRNA datasets of SCs and
pollen. Predicted foldback structure of stem loop precursors and chromosome locus.

Table S3 (.xls): Normalized counts of known miRNA families and newly identified miRNAs, for the different sequence contexts from 19 to 24nt and extensions in the 5’ terminal nucleotide, in the datasets of SCs, pollen and inflorescence.

Video S1 (.mov): Time-lapse imaging of AGO5-GFP in the SCs of a growing pollen tube.

Video S2 (.mov): 3D magnification of the male germ unit expressing AGO5-GFP in SCs cytoplasm and cytoplasmic tail connecting to the VN.

Acknowledgments

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Table 1 – Normalized reads for potentially novel miRNAs and isoforms with variations in the sequence length of known miRNA/miRNA* detected by miRCat tool, in sRNA datasets of Col pollen and sperm cells (SC) in comparison with inflorescence tissue (Inf). Underlined sequences indicate the mature form annotated in ASRP(a), including missing nucleotides (lower case). These variations were preferentially detected by miRCat since they are more abundant than the annotated isoforms of known miRNAs.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Sequence</th>
<th>SC</th>
<th>Pollen</th>
<th>Inf</th>
<th>psRNA target(s)</th>
</tr>
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<tbody>
<tr>
<td>Variations of known miRNA/miRNA*</td>
<td></td>
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<tr>
<td>miR156h</td>
<td>UUGACAGAGAAAGAGAGC</td>
<td>107</td>
<td>24</td>
<td>162</td>
<td>SQUAMOSA PROMOTER BINDING PROTEIN-LIKE</td>
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<td>1099</td>
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<td>12</td>
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<td>316</td>
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<td>27</td>
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<td>-</td>
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<td>miR844a</td>
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<td>11</td>
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<td>13</td>
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<td>AT3G55370</td>
</tr>
</tbody>
</table>

Potentially novel miRNAs: | |
| miR2942 | CAUCUACUUGUUGAUUAGAAA | 101 | 392 | 6 | - |
| miR4240 | AUUGUGACUAGUUGACUACCG | 25 | 0 | 10 | - |
| miR447a.2 | UUGGAAACUAGGUUGAUAU | 84 | 42 | 152 | AT1G42630, AT1G54710 |
| miR447c.2 | CCCCCUACAAUGUGAGUAAA | 0 | 13 | 3 | - |
| miR864.2 | UCAUGUGUUAUACUGGUACAC | 32 | 92 | 5 | CMT1, AT1G80740, CMT2, AT2G19020 |
| miR5012 | UUUUACUGUCAAUGUUGUCC | 6 | 0 | 3 | AT1G53700, AT2G37678 |
| miR5013 | UUGUGAACUAUUGGUCAUUC | 86 | 317 | 1 | AT3G60580 |
| miR5014 | UUGUACAAAUAAUGUGUGAC | 15 | 4 | 9 | - |
| miR5015.1 | UUGUGGUAUGUGGUUUGU | 17 | 20 | 0 | - |
| miR5015.2 | UUGUGGUAUGUGGUUUGU | 17 | 38 | 2 | AT2G38320, AT1G12860, AT2G01760, AT5G38740 |
| miR5016 | UUGUGGUAUGUGGUUUGU | 2 | 5 | 0 | - |
| miR5017 | UUAACUAAAUAAUGUGACAA | 4 | 5 | 67 | - |
| miR5018 | UUAACUAAAUAAUGUGACAA | 0 | 5 | 0 | - |
| miR5019 | UUGUGGGAAGAAAAUCCUU | 6 | 40 | 2 | AT3G58810, AT1G41510, AT4G19550 |
| miR5020a | UUGGAAAGUGUGAGCUUCA | 15 | 53 | 0 | - |
| miR5020b | AUUGGUGUGAGAAAAGAGGA | 158 | 711 | 1 | - |
| miR5021 | UUGGAGCAUGAGAAAGGAAA | 0 | 7 | 0 | - |
| miR5022 | GUCAGGGUGUAUUGGAAUG | 17 | 56 | 0 | - |
| miR5023 | AUUGUGUAGGUGUAUAGGGGGG | 0 | 18 | 0 | AT5G24950 |
| miR5024 | AT1G00010 |
| miR5025 | AT1G00010 |
| miR5026 | AT1G00010 |
| miR5027 | AT1G00010 |
| miR5028 | AT1G00010 |
| miR5029 | AT1G00010 |

(a) – Annotation as in ASRP: The Arabidopsis Small RNA Project Database (http://arsrpm.cgrb.oregonstate.edu/).
(b) – No correspondence found in ASRP database. Predicted foldback structures and chromosome loci presented in Table S2.
(c) – Potentially mature miRNA sequence processed from ath-MIR2934, more abundant in our SC and Pollen datasets than ath-miR2934 UCUCUGCAAAAGCUCUUGGA reported in Grant-Downton et al. (2009b), but classified in our study as ath-miR2934* (Table S1).
(d) – Processed from ath-MIR2440 (Ma et al., 2010), shifted 10nt from ath-miR2440 that was not detected in our SC and Pollen datasets.

* – Correspondent miRNA* sequence also detected in the sRNA dataset.
Figure Legends

**Figure 1** – *Argonaute protein family in Arabidopsis.*

Phylogenetic tree illustrating the ten Argonaute proteins in *Arabidopsis*, subdivided into the three main functional classes based on sequence homology: MiRNA-guided slicing and translational repression of target transcripts, trans-acting siRNA (ta-siRNA) activity and chromatin remodeling by siRNA-directed DNA methylation. AGO2, AGO3, AGO8 (red) and AGO5 (green) have unknown function. AGO5 is a close relative of AGO1 and AGO10, and could be involved in a novel miRNA pathway in the germline (see Fig 2); SCs-Sperm Cells. (adapted from Hutvagner and Simard, 2008)

**Figure 2** – *AGO5 expression in Arabidopsis pollen*

(A) Transgene expression of AGO5 protein using native promoter region (1000bp upstream of 5’UTR) and genomic coding sequence translationally fused to eGFP. AGO5p::AGO5-eGFP expression in mature pollen localizes preferentially in the sperm cell (SC) cytoplasm, (B) remaining during pollen tube growth. (C) A magnification of the Male Germ Unit shows that AGO5-GFP localizes in the sperm cell cytoplasm and not in the nucleus, extending through the cytoplasmic connection that links the sperm cells with the vegetative nucleus (VN). DAPI-stained DNA shows SC and VN nuclei in A and C.

**Figure 3** – *Venn diagram illustrating miRNA families detected in sperm cells and*
Overlap between known miRNA families detected in sperm cells (49) and pollen (75) by Illumina sequencing (Slotkin et al., 2009), and a 454 sequencing dataset of pollen small RNAs (31) reported by Grant-Downton et al. (2010). Numbers within parentheses represent total miRNA families identified in each data set.

Figure 4 – MiRNA families and variations in sequence length

(A) Relative abundance of known miRNAs detected in sperm cell (SC), pollen and inflorescence datasets; (B) MiRNAs 156h and 158b have isoforms of 21-nt length with nucleotide extensions in the 5’ terminus, for which the number of reads in sperm cells and pollen are higher than the annotated 20-nt isoforms. miR158a reads are presented to exemplify that these variations do not accumulate significantly for the majority of other miRNAs; (C) 22nt miRNAs that may function as siRNA triggers are differentially accumulated in sperm cell, pollen and inflorescence tissues.
Figure 1
Figure 3

SC (83) - 11

Pollen (75) - 43

Pollen 454 (31) - 29

(Grant-Dowton et al., 2009b)
Figure 4