Review

MicroRNA activity in the *Arabidopsis* male germline

Filipe Borges[1], Patrícia A. Pereira[1], R. Keith Slotkin[2], Robert A. Martienssen[3], Jörg D. Becker[1]

1. Instituto Gulbenkian de Ciência, 2780-901 Oeiras, Portugal
2. Department of Molecular Genetics, The Ohio State University, Columbus OH 43210, USA
3. Cold Spring Harbor Laboratory, Cold Spring Harbor NY 11724, USA

Corresponding author: Jörg D. Becker, Plant Genomics Lab & Gene Expression Unit, Instituto Gulbenkian de Ciência, Rua da Quinta Grande Nº6, 2780-156 Oeiras, Portugal, e-mail: jbecker@igc.gulbenkian.pt; phone: +351-214464526, fax: +351-214407970

Date of revised version: December 13th, 2010

Tables and figures: 1 Table, 4 Figures

Running title: MicroRNAs in sperm cells

Keywords:
Arabidopsis
Male Germ Cells
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.

MiRNA activity in pollen

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

(Olmedo-Monfil et al., 2010; Slotkin et al., 2009). 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 *ARI 13/14/15*, 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).

<u>Comparative analyses of miRNAs accumulated in sperm cells</u>

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.*,

2009). 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

with AGO5.

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 spermatozoon-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 know 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

We thank Paulo Almeida for bioinformatics support and Elena Baena-González for critical reading of the manuscript. This work was supported by grants PTDC/AGR-GPL/103778/2008, PTDC/BIA-BCM/103787/2008 and PTDC/AGR-GPL/70592/2006 from Fundacão para a Ciência e a Tecnologia (FCT), Portugal. FB and PAP were supported by FCT PhD fellowships SFRH/BD/48761/2008 and SFRH/BD/63477/2009, respectively.

References

Allen E, Xie Z, Gustafson AM, Carrington JC. 2005. microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121**, 207-221.

Allen RS, Li J, Alonso-Peral MM, White RG, Gubler F, Millar AA. 2010. MicroR159 regulation of most conserved targets in Arabidopsis has negligible phenotypic effects. *Silence* **1**, 18.

Amanai M, Brahmajosyula M, Perry AC. 2006. A restricted role for sperm-borne microRNAs in mammalian fertilization. *Biology of Reproduction* **75**, 877-884.

Avendano C, Franchi A, Jones E, Oehninger S. 2009. Pregnancy-specific {beta}-1-glycoprotein 1 and human leukocyte antigen-E mRNA in human sperm: differential expression in fertile and infertile men and evidence of a possible functional role during early development. *Human Reproduction* **24**, 270-277.

Barakat A, Wall K, Leebens-Mack J, Wang YJ, Carlson JE, Depamphilis CW. 2007. Large-scale identification of microRNAs from a basal eudicot (Eschscholzia californica) and conservation in flowering plants. *Plant Journal* **51**, 991-1003.

Bayer M, Nawy T, Giglione C, Galli M, Meinnel T, Lukowitz W. 2009. Paternal control of embryonic patterning in Arabidopsis thaliana. *Science* **323**, 1485-1488.

Becker JD, Feijo JA. 2007. How many genes are needed to make a pollen tube? Lessons from transcriptomics. *Annals of Botany* **100**, 1117-1123.

Boavida LC, Becker JD, Feijo JA. 2005. The making of gametes in higher plants. *International Journal of Developmental Biology* **49**, 595-614.

Borg M, Brownfield L, Twell D. 2009. Male gametophyte development: a molecular perspective. *Journal of Experimental Botany* **60**, 1465-1478.

Borges F, Gomes G, Gardner R, Moreno N, McCormick S, Feijo JA, Becker JD. 2008. Comparative transcriptomics of Arabidopsis sperm cells. *Plant Physiology* **148**, 1168-1181.

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK. 2005. Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. *Cell* **123**, 1279-1291.

Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M, Dunoyer P, Yamamoto YY, Sieburth L, Voinnet O. 2008. Widespread translational inhibition by plant miRNAs and siRNAs. *Science* **320**, 1185-1190.

Brownfield L, Hafidh S, Borg M, Sidorova A, Mori T, Twell D. 2009. A Plant Germline-Specific Integrator of Sperm Specification and Cell Cycle Progression. *PloS Genetics* **5**, -.

Carlsbecker A, Lee JY, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, Lindgren O, Moreno-Risueno MA, Vaten A, Thitamadee S, Campilho A, Sebastian J, Bowman JL, Helariutta Y, Benfey PN. 2010. Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* **465**, 316-321.

Carmell MA, Girard A, van de Kant HJ, Bourc'his D, Bestor TH, de Rooij DG, Hannon GJ. 2007. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Developmental Cell* **12**, 503-514.

Chambers C, Shuai B. 2009. Profiling microRNA expression in Arabidopsis pollen using microRNA array and real-time PCR. *BMC Plant Biology* **9**, 87.

Chellappan P, Xia J, Zhou X, Gao S, Zhang X, Coutino G, Vazquez F, Zhang W, Jin H. 2010. siRNAs from miRNA sites mediate DNA methylation of target genes. *Nucleic Acids Research*, 1-12.

Chen HM, Chen LT, Patel K, Li YH, Baulcombe DC, Wu SH. 2010. 22-Nucleotide RNAs trigger secondary siRNA biogenesis in plants. *Proceedings of the National Academy of Sciences USA* **107**, 15269-15274.

Chen X. 2004. A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. *Science* **303**, 2022-2025.

Cifuentes D, Xue H, Taylor DW, Patnode H, Mishima Y, Cheloufi S, Ma E, Mane S, Hannon GJ, Lawson ND, Wolfe SA, Giraldez AJ. 2010. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science* **328**, 1694-1698.

Cuperus JT, Carbonell A, Fahlgren N, Garcia-Ruiz H, Burke RT, Takeda A, Sullivan CM, Gilbert SD, Montgomery TA, Carrington JC. 2010. Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in Arabidopsis. *Nature Structural & Molecular Biology* **17**, 997-1003.

Dadoune JP. 2009. Spermatozoal RNAs: what about their functions? *Microscopy Research and Technique* **72**, 536-551.

Ebhardt HA, Fedynak A, Fahlman RP. 2010. Naturally occurring variations in sequence length creates microRNA isoforms that differ in argonaute effector complex specificity. *Silence* **1**, 12.

Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, Baskerville S, Hammond SM, Bartel DP, Schier AF. 2005. MicroRNAs regulate brain morphogenesis in zebrafish. *Science* **308**, 833-838.

Grant-Downton R, Hafidh S, Twell D, Dickinson HG. 2009a. Small RNA pathways are present and functional in the angiosperm male gametophyte. *Molecular Plant* **2**, 500-512.

Grant-Downton R, Le Trionnaire G, Schmid R, Rodriguez-Enriquez J, Hafidh S, Mehdi S, Twell D, Dickinson H. 2009b. MicroRNA and tasiRNA diversity in mature pollen of Arabidopsis thaliana. *BMC Genomics* **10**, 643.

Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, Ruohola-Baker H. 2005. Stem cell division is regulated by the microRNA pathway. *Nature* **435**, 974-978.

Hayashi K, Chuva de Sousa Lopes SM, Kaneda M, Tang F, Hajkova P, Lao K, O'Carroll D, Das PP, Tarakhovsky A, Miska EA, Surani MA. 2008. MicroRNA

biogenesis is required for mouse primordial germ cell development and spermatogenesis. *PLoS One* **3**, e1738.

Honys D, Twell D. 2004. Transcriptome analysis of haploid male gametophyte development in Arabidopsis. *Genome Biology* **5**, R85.

Hutvagner G, Simard MJ. 2008. Argonaute proteins: key players in RNA silencing. *Nature Reviews Molecular Cell Biology* **9**, 22-32.

Katiyar-Agarwal S, Morgan R, Dahlbeck D, Borsani O, Villegas A, Jr., Zhu JK, Staskawicz BJ, Jin H. 2006. A pathogen-inducible endogenous siRNA in plant immunity. *Proceedings of the National Academy of Sciences USA* **103**, 18002-18007.

Kedde M, Strasser MJ, Boldajipour B, Oude Vrielink JA, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Orom UA, Lund AH, Perrakis A, Raz E, Agami R. 2007. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell* **131**, 1273-1286.

Kidner CA, Martienssen RA. 2005. The role of ARGONAUTE1 (AGO1) in meristem formation and identity. *Developmental Biology* **280**, 504-517.

Knight SW, Bass BL. 2001. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science* **293**, 2269-2271.

Le Trionnaire G, Grant-Downton RT, Kourmpetli S, Dickinson HG, Twell D. 2011. Small RNA activity and function in angiosperm gametophytes. *J Exp Bot*.

Ma Z, Coruh C, Axtell MJ. 2010. Arabidopsis lyrata small RNAs: transient MIRNA and small interfering RNA loci within the Arabidopsis genus. *Plant Cell* **22**, 1090-1103.

Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD. 2008. Dicer1 is required for differentiation of the mouse male germline. *Biology of Reproduction* **79**, 696-703.

Martienssen R. 2010. Molecular biology. Small RNA makes its move. *Science* **328**, 834-835.

Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, Wu L, Li S, Zhou H, Long C, Chen S, Hannon GJ, Qi Y. 2008. Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide. *Cell* **133**, 116-127.

Mishima Y, Giraldez AJ, Takeda Y, Fujiwara T, Sakamoto H, Schier AF, Inoue K. 2006. Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. *Current Biology* **16**, 2135-2142.

Moxon S, Schwach F, Dalmay T, Maclean D, Studholme DJ, Moulton V. 2008. A toolkit for analysing large-scale plant small RNA datasets. *Bioinformatics* **24**, 2252-2253.

Nakano M, Nobuta K, Vemaraju K, Tej SS, Skogen JW, Meyers BC. 2006. Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. *Nucleic Acids Research* **34**, D731-735.

Nonomura K, Morohoshi A, Nakano M, Eiguchi M, Miyao A, Hirochika H, 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.

Olmedo-Monfil V, Duran-Figueroa N, Arteaga-Vazquez M, Demesa-Arevalo E, Autran D, Grimanelli D, Slotkin RK, Martienssen RA, Vielle-Calzada JP. 2010. Control of female gamete formation by a small RNA pathway in Arabidopsis. *Nature* **464**, 628-632.

Ostermeier GC, Goodrich RJ, Moldenhauer JS, Diamond MP, Krawetz SA. 2005. A suite of novel human spermatozoal RNAs. *Journal of Andrology* **26**, 70-74.

Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. 2004. Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature* **429**, 154.

Palatnik JF, Wollmann H, Schommer C, Schwab R, Boisbouvier J, Rodriguez R, Warthmann N, Allen E, Dezulian T, Huson D, Carrington JC, Weigel D. 2007. Sequence and expression differences underlie functional specialization of Arabidopsis microRNAs miR159 and miR319. *Developmental Cell* **13**, 115-125.

Pina C, Pinto F, Feijo JA, Becker JD. 2005. Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. *Plant Physiology* **138**, 744-756.

Rassoulzadegan M, Grandjean V, Gounon P, Vincent S, Gillot I, Cuzin F. 2006. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature* **441**, 469-474.

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. 2002. Prediction of plant microRNA targets. *Cell* **110**, 513-520.

Ron M, Saez MA, Williams LE, Fletcher JC, McCormick S. 2010. Proper regulation of a sperm-specific cis-nat-siRNA is essential for double fertilization in Arabidopsis. *Genes & Development* **24**, 1010-1021.

Slotkin RK, Vaughn M, Borges F, Tanurdzic M, Becker JD, Feijo JA, Martienssen RA. 2009. Epigenetic reprogramming and small RNA silencing of transposable elements in pollen. *Cell* **136**, 461-472.

Spielman M, Vinkenoog R, Dickinson HG, Scott RJ. 2001. The epigenetic basis of gender in flowering plants and mammals. *Trends in Genetics* **17**, 705-711.

Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, Chen J, Blelloch R. 2010. MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Current Biology* **20**, 271-277.

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

Todesco M, Rubio-Somoza I, Paz-Ares J, Weigel D. 2010. A collection of target mimics for comprehensive analysis of microRNA function in Arabidopsis thaliana. *PLoS Genetics* **6**, e1001031.

Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD. 2006. A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**, 320-324.

Vaucheret H, Vazquez F, Crete P, Bartel DP. 2004. The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes & Development* **18**, 1187-1197.

Vazquez F, Blevins T, Ailhas J, Boller T, Meins F, Jr. 2008. Evolution of Arabidopsis MIR genes generates novel microRNA classes. *Nucleic Acids Research* **36**, 6429-6438.

Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. *Cell* **136**, 669-687.

Yang JS, Maurin T, Robine N, Rasmussen KD, Jeffrey KL, Chandwani R, Papapetrou EP, Sadelain M, O'Carroll D, Lai EC. 2010. Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. *Proceedings of the National Academy of Sciences USA* **107**, 15163-15168.

Zhang W, Gao S, Zhou X, Xia J, Chellappan P, Zhang X, Jin H. 2010. Multiple distinct small RNAs originate from the same microRNA precursors. *Genome Biology* **11**, R81.

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.

microRNA	Sequence	SC	Pollen	Inf	psRNA target(s)
Variations of known miRNA/miRNA*[(a)]					
miR156h	UUGACAGAAGAAAGAGAGCAC	107	24	162	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE
miR158b	UCCCCAAAUGUAGACAAAGCA	248	1099	12	AT2G46590
miR161a.1	UUGAAAGUGACUACAUCGGGGu	5605	7146	2066	PPR AT5G41170
miR162	AUCGAUAAACCUCUGCAUCCAGg	4	58	1	DICER-LIKE 1
miR165*	gGAAUGUUGUCUGGAUCGAGGA	46	102	9	-
miR167c	uUAAGCUGCCAGCAUGAUCUUGU	10	0	2	-
miR408*	ACAGGGAACAAGCAGAGCATGG	589	29	12	AT2G47020, AT4G02940, AT1G04210, AT1G17180, AT4G03950
miR773a	TTTGCTTCCAGCTTTTGTCTCC	82	524	22	MET2 AT4G14140, AT4G05390, AT4G08990
miR778*	ACAAACUCGGUGUACAUAGACcaaaccaag	116	316	2	AT5G22380, AT1G69610
miR840*	UUGUUUAGGUCCCUUAGUUUCu	21	27	9	-
miR844a	AAUGGUAAGAUUGCUUAUAAGcu	11	22	11	AT2G13720, AT5G44120
miR852	AAGAUAAGCGCCUUAGUUCUGA	42	63	15	AT5G56650, AT5G56660
miR852a	AAGAUAAGCGCCUUAGUUCUGA	32	107	7	-
miR860	ucaAUAGAUUGGACUAUGUAUAUU	17	9	10	AT5G26030, AT3G12640
miR863a	uugAGAGCAACAAGACAUAAUAAAGAG	2	11	0	-
miR870	AAUCUAAUUUGGUGUUUCUUCGaug	2	13	1	AT3G55370
Potentially novel miRNAs [(b)]					
miR2934[(c)]	CAUCCAAGGUGUUUGUAGAAA*	101	392	6	-
miR4240.2[(d)]	AUGGCUAGAGUGACUAGACCCG	23	0	10	-
miR447a.2	UAUGGAAGAAAUUGUAGUAUU	84	42	152	AT1G42630, AT1G54710
miR447c.2	CCCCUUACAAUGUCGAGUAAA	0	13	3	-
miR868.2	UCAUGUCGUAAUAGUAGUCAC	32	92	5	CMT1 AT1G80740, CMT2 AT4G19020
miR5012	UUUUACUGCUACUUGUGUUCC	6	0	3	AT1G53700, AT2G37678
miR5013	UUUGUGACAUCUAGGUGCUUU	86	317	1	AT3G60580
miR5014	UUGUACAAAUUUAAGUGUACG	15	4	9	-
miR5015.1	UUGGUGUUAUGUGUAGUCUUC	17	20	0	-
miR5015.2	UCUGUUGUUGUUGGUGUUAUG	17	38	2	AT2G38320, AT1G12860, AT2G01760, AT5G38740
miR5016	UUCUUGUGGAUUCCUUGGAAA	2	5	0	-
miR5017	UUAUACCAAAUUAAUAGCAAA	4	5	67	-
miR5018	UUAAAGCUCCACCAUGAGUCCAAU	0	5	0	-
miR5019	UGUUGGGAAAGAAAAACUCUU	6	40	2	AT3G58810, AT1G14510, AT4G19550
miR5020a	UGGAAGAAGGUGAGACUUGCA	15	53	0	-
miR5020b	AUGGCAUGAAAGAAGGUGAGA	158	711	1	-
miR5021	UGAGAAGAAGAAGAAGAAAA	0	7	0	-
miR5022	GUCAUGGGGUAUGAUCGAAUG*	17	56	0	-
miR5023	AUUGGUAGUGGAUAAGGGGGC*	0	18	0	AT5G24950
miR5024	AUGACAAGGCCAAGAUAUAACA	4	7	0	-
miR5025	ACUGUAUAUAUGUAAGUGACA	4	9	0	AT2G48010
miR5026	ACUCAUAAGAUCGUGACACGU	10	16	2	-
miR5027	ACCGGUUGGAACUUGCCUUAA	10	27	3	-
miR5028	AAUUGGGUUUAUGCUAGAGUU	92	443	8	-
miR5029	AAUGAGAGAGAACACUGCAAA*	84	288	1	AT2G30070, AT4G02900

(a) – Annotation as in ASRP: The *Arabidopsis* Small RNA Project Database (http://asrp.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 UCUUUCUGCAAACGCCUUGGA reported in Grant-Downton *et al*. (2009b), but classified in our study as ath-miR2934* (Table S1).
(d) – Processed from ath-MIR4240 (Ma *et al.*, 2010), shifted 10nt from ath-miR4240 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*

pollen

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.

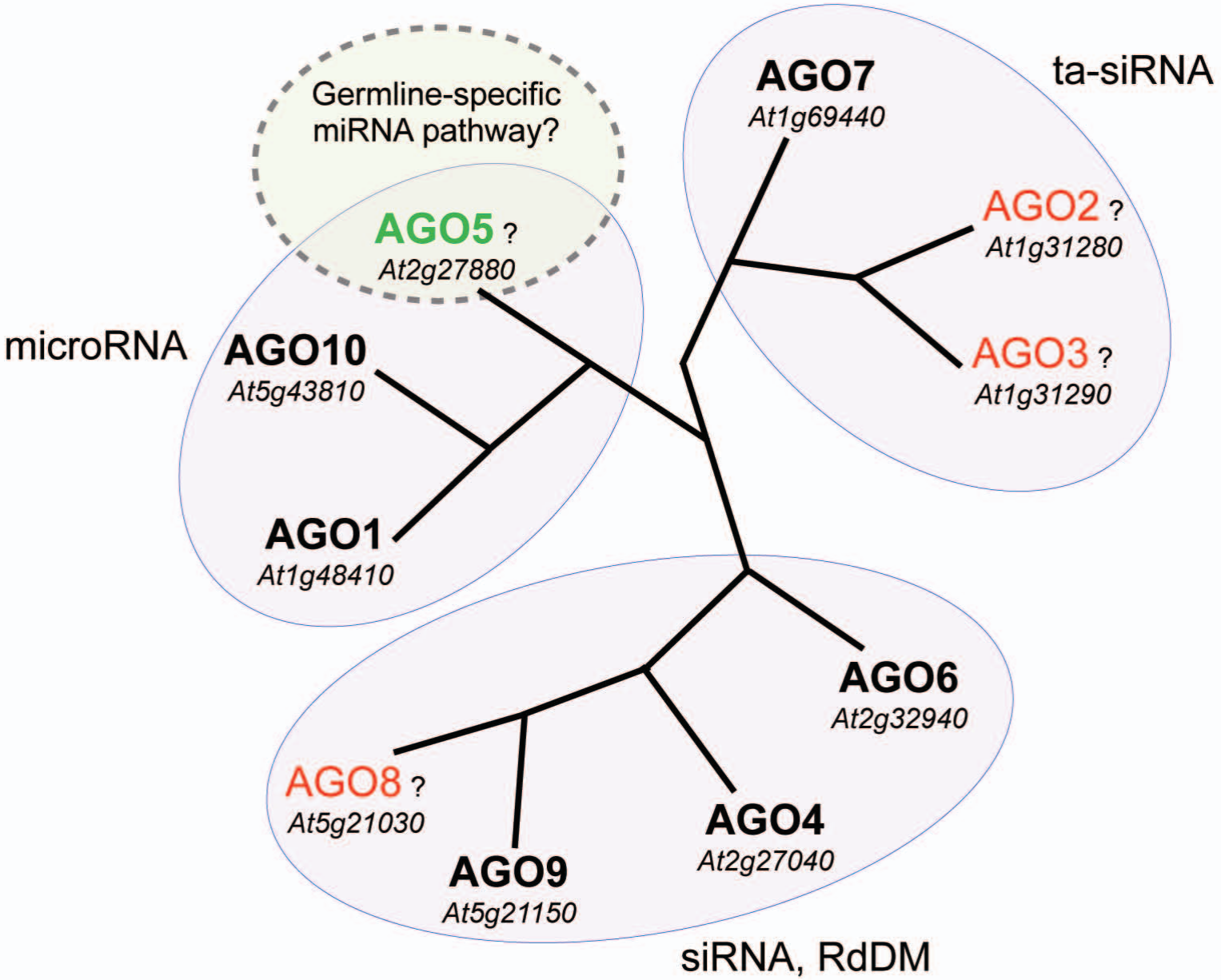

Germline-specific
miRNA pathway?
AGO5 ?
At2g27880
AGO7
At1g69440
ta-siRNA
AGO2 ?
At1g31280
AGO3 ?
At1g31290
microRNA
AGO10
At5g43810
AGO1
At1g48410
AGO6
At2g32940
AGO8 ?
At5g21030
AGO4
At2g27040
AGO9
At5g21150
siRNA, RdDM

Figure 1

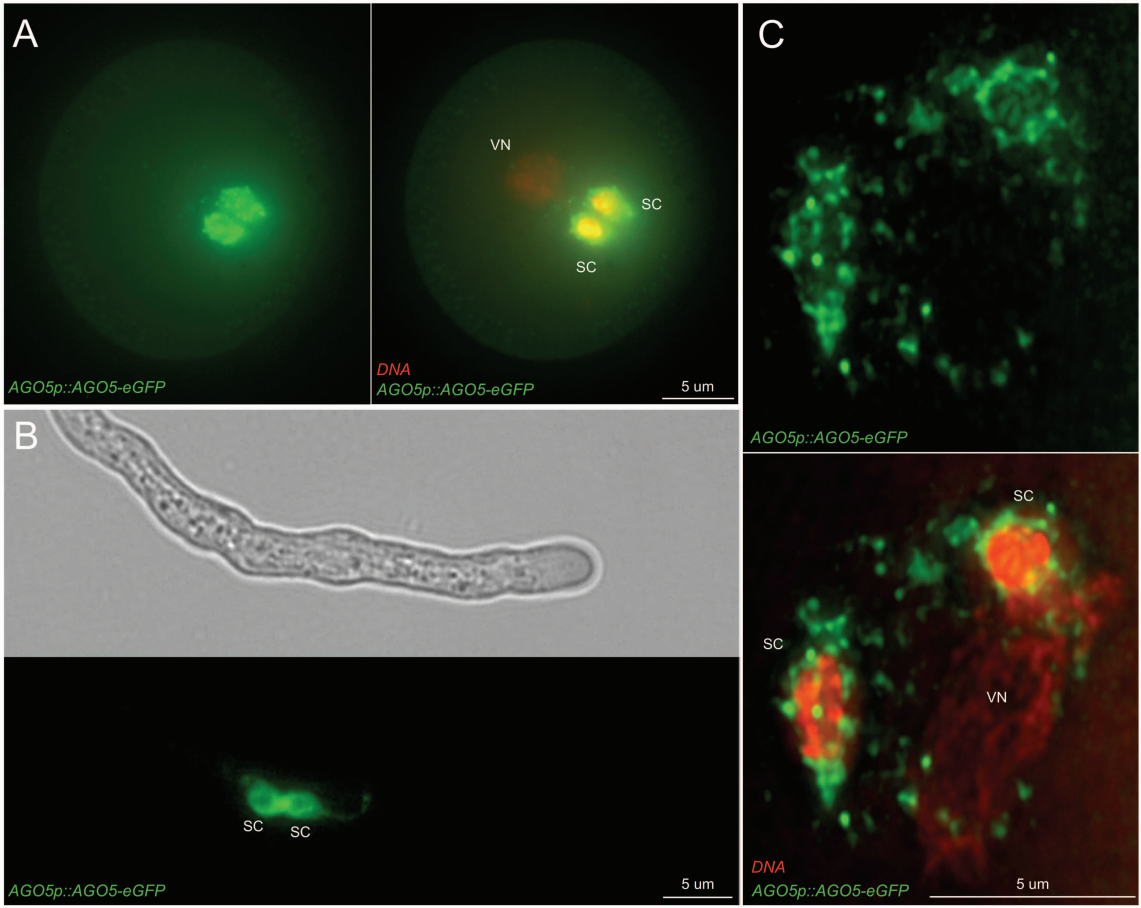

A
VN
SC
SC
AGO5p::AGO5-eGFP
DNA
AGO5p::AGO5-eGFP
5 um
B
SC
SC
AGO5p::AGO5-eGFP
5 um
C
AGO5p::AGO5-eGFP
SC
SC
VN
DNA
AGO5p::AGO5-eGFP
5 um

Figure 2

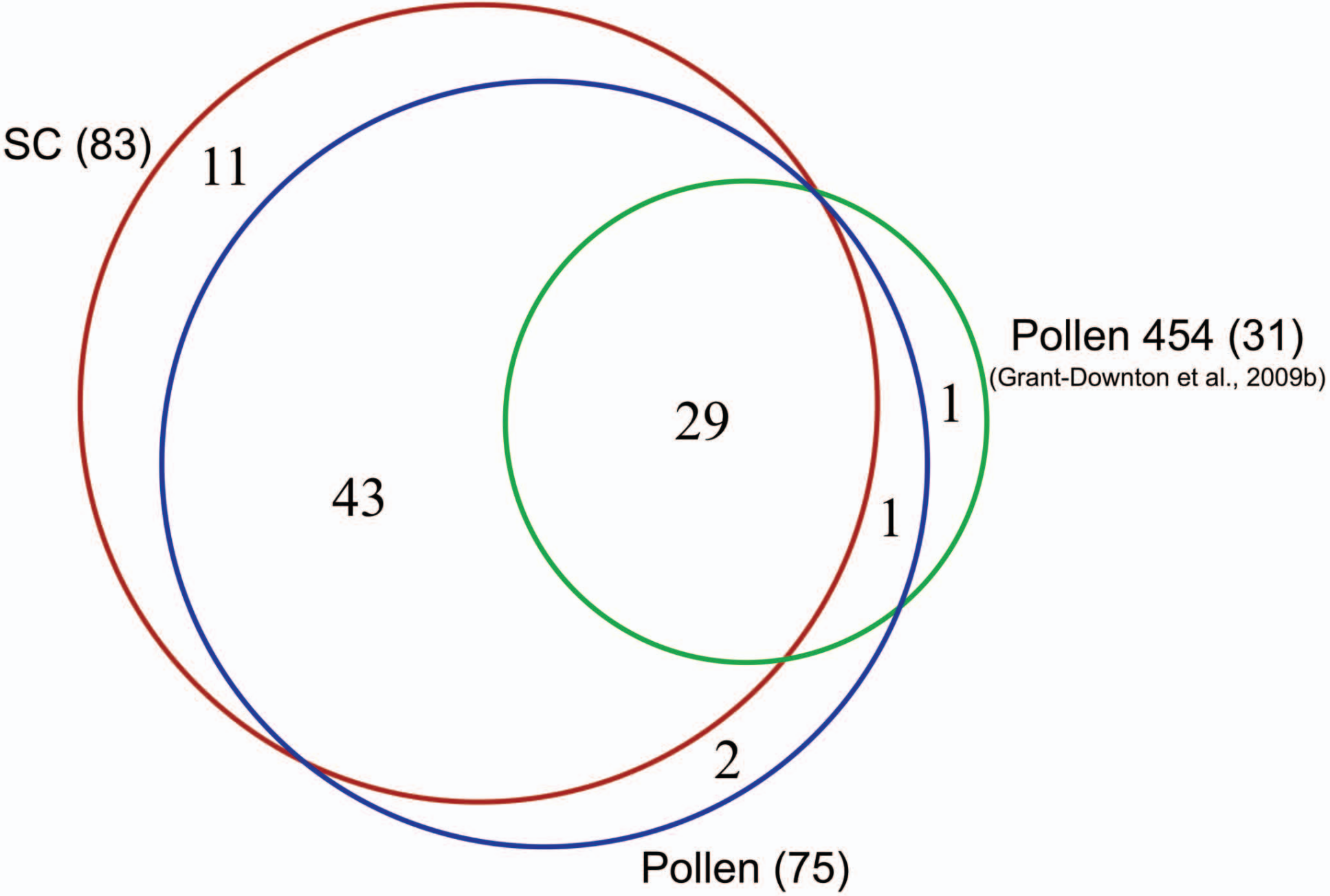
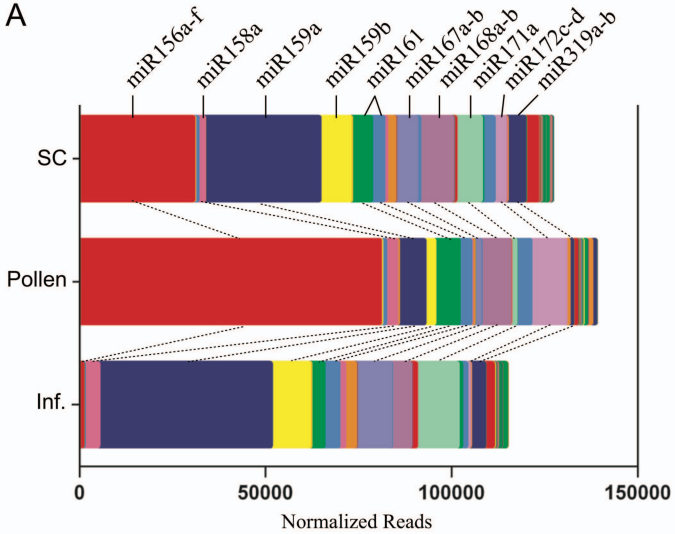

SC (83)
11
43
29
1
1
2
Pollen 454 (31)
(Grant-Downton et al., 2009b)
Pollen (75)

Figure 3



B

		Normalized Reads		
		SC	Pollen	Inf
	UGUUGACAGAAGAAAGAGAGCAC	0	0	0
	GUUGACAGAAGAAAGAGAGCAC	0	0	0
	UUGACAGAAGAAAGAGAGCAC	107	24	162
miR156h	UGACAGAAGAAAGAGAGCAC (20-nt)	34	7	45
	UGACAGAAGAAAGAGAGCACA	0	0	0
	UGACAGAAGAAAGAGAGCACAA	0	0	0
	UGACAGAAGAAAGAGAGCACAAC	0	0	0
	UGACAGAAGAAAGAGAGCACAACC	0	0	0
	UUUCCCCAAAUGUAGACAAAGCA	0	0	0
	UUCCCCAAAUGUAGACAAAGCA	0	2	0
	UCCCCAAAUGUAGACAAAGCA	248	1099	12
miR158b	CCCCAAAUGUAGACAAAGCA (20-nt)	86	464	23
	CCCCAAAUGUAGACAAAGCAA	4	13	0
	CCCCAAAUGUAGACAAAGCAAU	0	4	0
	CCCCAAAUGUAGACAAAGCAAUA	0	2	0
	CCCCAAAUGUAGACAAAGCAAUAC	0	0	0
	CUUUCCCAAAUGUAGACAAAGCA	0	0	0
	UUUCCCAAAUGUAGACAAAGCA	0	0	0
	UUCCCAAAUGUAGACAAAGCA	27	73	73
miR158a	UCCCAAAUGUAGACAAAGCA (20-nt)	1683	2907	3900
	UCCCAAAUGUAGACAAAGCAA	11	36	53
	UCCCAAAUGUAGACAAAGCAAU	2	4	7
	UCCCAAAUGUAGACAAAGCAAUA	0	0	0
	UCCCAAAUGUAGACAAAGCAAUAC	0	0	0

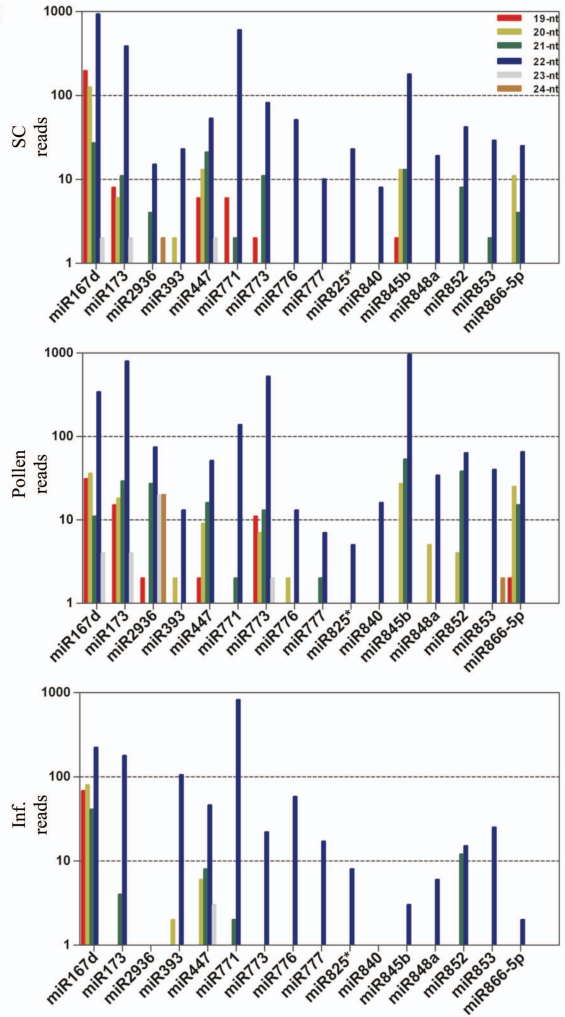


Figure 4