Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction

Miin-Feng Wu, Qing Tian* and Jason W. Reed†

In flowering plants, diploid sporophytic tissues in ovules and anthers support meiosis and subsequent haploid gametophyte development. These analogous reproductive functions suggest that common mechanisms may regulate ovule and anther development. Two Arabidopsis Auxin Response Factors, ARF6 and ARF8, regulate gynoecium and stamen development in immature flowers. Wild-type pollen grew poorly in arf6 arf8 gynoecia, correlating with ARF6 and ARF8 expression in style and transmitting tract. ARF6 and ARF8 transcripts are cleavage targets of the microRNA miR167, and overexpressing miR167 mimicked arf6 arf8 phenotypes. Mutations in the miR167 target sites of ARF6 or ARF8 caused ectopic expression of these genes in domains of both ovules and anthers where miR167 was normally present. As a result, ovule integuments had arrested growth, and anthers grew abnormally and failed to release pollen. Thus, miR167 is essential for correct patterning of gene expression, and for fertility of both ovules and anthers. The essential patterning function of miR167 contrasts with cases from animals in which miRNAs reinforce or maintain transcriptionally established gene expression patterns.

KEY WORDS: Auxin response factor, microRNA, Ovule, Anther, Arabidopsis

INTRODUCTION
Plant life cycles alternate between diploid sporophyte and haploid gametophyte phases. In flowering plants, the more prominent sporophyte supports meiosis and subsequent gametophyte development in specialized female and male organs within flowers. Ovules, the female sporophyte organs, support development of the embryo sac and growth of embryos and seeds after fertilization. Anthers, the male sporophyte organs, support the formation, development and subsequent release of pollen. Gametophyte development and successful reproduction thus require correct pattern formation of ovules and anthers.

Arabidopsis ovules initiate as filament-like structures on the flanks of carpel margin meristems at around floral stage 8 (Smyth et al., 1990). Megasporocyte mother cells, which later give rise to the female gametophyte, reside in the distal nucellus end of ovules. Proximal to the nucellus is the chalaza, where both inner and outer integuments initiate. Inner and outer integuments grow out to enclose the entire ovule as the ovule matures, and asymmetric growth of the outer integument causes the developing ovule to curve. After fertilization and embryo development, integuments form the seed coat. Ovules are connected to the placental tissues by funiculi, which supply nutrients to support ovule and seed growth (Schneitz et al., 1997; Skinner et al., 2004).

Stamen primordia initiate at floral stage 6 and form a filament that holds an anther at its distal end. Several distinct cell types in anthers are important for male gametogenesis and anther dehiscence (Goldberg et al., 1993; Smyth et al., 1990). Some of these undergo cell death or desiccation to allow dispersal of pollen grains at anthesis. Prior to anthesis, tapetum cells that coat the anther locule wall and septum cells between two anther locules are degraded. Stomium cells then break to allow pollen dispersal (Sanders et al., 1999).

Endogenous small non-coding RNAs called microRNAs (miRNAs) regulate several developmental events in Arabidopsis (Baker et al., 2005; Bao et al., 2004; Chen, 2004; Emery et al., 2003; Laufs et al., 2004; Mallory et al., 2004a; Williams et al., 2005). miRNA precursor genes (MIRs) are transcribed by RNA polymerase II in both animals and plants (Kurihara and Watanabe, 2004; Lee et al., 2004; Xie et al., 2005). DICER-LIKE 1 (DCL1), an Arabidopsis DICER RNase III family homolog, cleaves the pri-miRNA and prec-miRNA hairpin precursors to produce a miRNA:miRNA* duplex in the nucleus (Jones-Rhoade et al., 2006). This duplex is transported to the cytoplasm where the mature miRNA is incorporated into the RNA-induced silencing complex (RISC). The RISC complex then identifies target miRNAs with specificity provided by base pairing between the miRNA and the target site (Bartel, 2004).

Most plant miRNAs have high sequence complementarity to their target binding sites, allowing a straightforward prediction of the genes they regulate (Rhoade et al., 2002). In most cases, plant miRNAs shut down their target gene activities by transcript cleavage (Axtell and Bartel, 2005; Schwab et al., 2005). Overexpressing MIR precursor transcripts in transgenic plants decreased the corresponding target gene transcript levels (Schwab et al., 2005). In addition, cleavage products of computationally predicted miRNA targets have been detected in wild-type plants (Allen et al., 2005; Kasschau et al., 2003; Mallory et al., 2005; Xie et al., 2005). Nevertheless, miRNAs can act by other regulatory mechanisms, including translational inhibition and methylation-induced gene silencing (Bao et al., 2004; Bartel, 2004; Chen, 2004; Kurihara and Watanabe, 2004).

More than half of the known Arabidopsis miRNA target genes encode transcription factors, suggesting that miRNAs regulate various developmental processes (Jones-Rhoade et al., 2006). The importance of plant miRNAs is further supported by the finding that most Arabidopsis miRNA families are conserved among other
species of land plants, both vascular and, in some cases, lower plants (Axtell and Bartel, 2005; Floyd and Bowman, 2004; Reinhart et al., 2002; Rhoades et al., 2002; Sunkar et al., 2005).

Among miRNA targets are several ARF genes encoding Auxin Response Factors. ARF6 and ARF8 are targeted by miR167, whereas ARF10, ARF16 and ARF17 are targeted by miR166 (Mallory et al., 2005; Rhoades et al., 2002; Wang et al., 2005). ARF proteins bind to auxin response promoter elements and mediate gene expression responses to the plant hormone auxin (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002; Mallory et al., 2005; Tiwari et al., 2003). Different ARF proteins regulate embryogenesis, root development and floral organ formation (Hardtke and Berleth, 1998; Hardtke et al., 2004; Mallory et al., 2005; Sessions et al., 1997; Wang et al., 2005).

We previously found that ARF6 and ARF8 regulate flower maturation (Nagpal et al., 2005). Flowers of arf6 arf8 double loss-of-function mutant plants were arrested at stage 12, just before wild-type flower buds normally open. Stamens of arf6 arf8 flowers were short, and anthers did not dehisce to release pollen. The double mutant insufficiency was due to a lack of jasmonic acid (JA) production, and pollen release could be restored by spraying the flower buds with JA or its precursors. arf6 arf8 double mutant flowers were also female sterile and their stigmatic papillae did not elongate as those of wild-type flowers. Single loss-of-function arf6 or arf8 mutants had only subtly reduced fecundity, resulting from shorter stamen filaments and delayed anther dehiscence, indicating that ARF6 and ARF8 act largely redundantly.

To determine the developmental functions of miR167, we have overexpressed MIR167-coding sequences, mutated ARF6 and ARF8 to make them immune to miR167-mediated effects, and studied the expression of MIR167, ARF6 and ARF8 genes. Our results indicate that miR167 regulates the pattern of ARF6 and ARF8 expression, which is vital for both ovule and anther development.

MATERIALS AND METHODS

Plant materials and constructs
Most plants used in this work were of the Columbia (Col-0) ecotype. arf6-2, arf8-3 and arf6-2 arf8-3 mutants were isolated and described previously (Nagpal et al., 2005). The intro1 mutant (Villanueva et al., 1999) was of the Landsberg erecta ecotype.

MIR167a (At3g22886; stem-loop sequence accession number, M0000208), MIR167b (At3g63360; stem-loop accession number, M0000209), MIR167c (stem-loop accession number, M0001088) and MIR167d (stem-loop accession number, M0000975) were PCR amplified from wild-type genomic DNA using the following primers:

- MIR167a, 5'-CACCCACTTCTCCGACCTTTAACTTCTCCA-3' and 5'-TGAAGCTTAAAGGAGGACCCTTGG-3';
- MIR167b, 5'-CACTCTAGGCTTCTTTAAATCTCGGT-3' and 5'-AATCTTGAATGCTGAAACGCCAAA-3';
- MIR167c, 5'-CACTCTAATGGGGGAAAGTGGTAAGAAA-3' and 5'-TCACTTGAATGCTGAAACGCCAAA-3';
- MIR167d, 5'-CGTGCTTAGCTTCAACACA-3' and 5'-CGTGCTTAGCTTCAACACA-3'.

PCR products were cloned into pENTR/D-TOPO (Invitrogen) and then introduced into pGWB3 (a kind gift from Dr Tsuyoshi Ito and Shigeo Nakagawa, Shimane University, Japan) by LR clonase (Invitrogen).

Northern blots and in situ hybridization
Total cellular RNA was isolated from pollen clusters of day-long-grown plants by Trizol reagent (Invitrogen). RNA gel blot analysis was performed as previously described (Tian et al., 2003). ARF6 (coding region position 1346-2211) and ARF8 (coding region position 1151-2106) probes were amplified from cDNA with the following primers: ARF6, 5'-CGGAGGATTGGATGCTGACCTTAC-3' and 5'-GGGATCCGAAGGGGT-TGGTGAGTTAATCTG-3'; ARF8, 5'-GGGATCCGAAGGGGT-TGGTGAGTTAATCTG-3' and 5'-GGGATCCGAAGGGGT-TGGTGAGTTAATCTG-3'.

For low molecular weight RNA, 30 μg of total cellular RNA was suspended in 20 μl loading buffer (95% formamide, 5 mM EDTA, 0.025% SDS, 0.025% bromophenol blue and 0.025% xylene cyanol FF) and separated in 15% denaturing polyacrylamide gel containing 8 M urea. Antisense miR167 (5'-TAGATCATGCTGACCCCTTCC-3') and U6 snRNA probes (5'-CTCGATTATGCCGATCTTCCG-3') were endlabeled by T4 polynucleotide kinase (New England Biolabs) in the presence of γ32P-ATP.

In situ hybridization was performed as previously described (Long and Barton, 1998). ARF6 and ARF8 fragments used in northern blots were cloned into plasmid PGEM-T (Promega). Probes were labeled by in vitro transcription with SP6 polymerase using a DIG RNA labeling kit (Roche). Wild-type and mARF6 hybridizations were done together, so as to increase comparability of results. INNER NO OUTER probe was amplified from wild-type flower cDNA using primers described by Sieber et al. (2004) and cloned into pmGEM-T (Promega).

Histology and microscopy
Flower X-gluc staining was performed as described by Sessions et al. (1999), and the concentration of potassium ferrocyanide and ferricyanide used depended on the constructs. For MIR167 promoter:GFP-GUS lines, the concentration used was 5 mM each. For ARF6 and ARF8 protein:GUS fusions, it was 0.5 mM each for ovules and 0.2 mM each for flowers.

For tracking pollen tube growth, stigmas were dusted with pollen from LAT52:GUS plants (Johnson et al., 2004). Twenty-four hours after pollination, carpel walls were removed and gynoecia were stained with X-gluc overnight at 37°C.
Ovules for DIC microscopy were fixed in 3:1 ethanol:acetic acid for 15 minutes, incubated in 70% ethanol for another 15 minutes, cleared in chlorohydrate solution (chlorohydrate:water, 8:2), and observed under DIC microscopy. Scanning electron microscopy was performed as previously described (Nagpal et al., 2005). Anthers were fixed and sectioned based on methods described by Ellis et al. (Ellis et al., 2005).

RESULTS

ARF6 and ARF8 are required to support pollen tube growth

Our previous analyses of promoter:GUS plants suggested that both ARF6 and ARF8 were expressed in multiple flower organs, but would not have revealed effects of miR167 or other regulatory elements missing from the promoter:GUS constructs. We therefore analyzed the expression patterns of ARF6 and ARF8 in wild-type flowers by in situ hybridization (Fig. 1). We also analyzed X-gluc staining patterns in plants carrying genomic translational fusions to the GUS reporter gene (Fig. 2). These gARF6:GUS and gARF8:GUS constructs were able to increase fecundity of an arf8 null mutant (data not shown), suggesting that they were partially functional (although, as discussed below, miR167-resistant versions conferred weaker phenotypes than did unfused genes). In most tissues, staining patterns of the GUS fusions were very similar to the distribution of transcripts shown by in situ hybridization.

In wild-type flowers, ARF6 transcript was present in the carpel medial ridge (which later forms the transmitting tract for pollen tube growth), in placental tissues, and in young ovule primordia as they emerged (Fig. 1B,D). As integuments initiated on the flanks of ovules (ovule stage 2-II), ARF6 transcript became restricted to the ovule funiculus and the placental tissues, and was excluded from the integuments and the nucellus (Fig. 1E,F). These expression patterns persisted at least through flower stage 12, just before fertilization normally would normally occur. ARF6 transcript was also detected at a low level in the vasculature of flower stems and stamen filaments, in petals, and in nectaries (Fig. 1A,C). Consistent with the in situ hybridization data, gARF6:GUS expression was detected in the transmitting tract, the ovule funiculi and nectaries, and faintly in the stamen filaments (Fig. 2A-E).

ARF8 was expressed in a similar pattern to ARF6, with strong expression in the funiculus and placenta (Fig. 1J). ARF8 was also detected in stigmatic papillae in flowers approaching anthesis (data not shown). Similarly, gARF8:GUS was expressed in the transmitting tract, placenta, funiculi and stamen filaments (Fig. 2K,M). Stigmatic papillae expression was also detected in some strongly expressing gARF8:GUS lines (data not shown). In addition, we detected weak X-gluc staining in the style and in the valves of both gARF6:GUS and gARF8:GUS plants, but we did not detect ARF6 or ARF8 transcripts in these tissues by in situ hybridization.

Expression of ARF6 and ARF8 in style, transmitting tract and funiculus suggests that ARF6 and ARF8 may regulate fertilization rather than gametophyte development. To explore why arf6 arf8 flowers were female sterile, we pollinated wild-type and arf6 arf8 stigmas with pollen from the LAT52:GUS reporter line (Johnson et al., 2004). Whereas pollen grew efficiently in wild-type transmitting tracts and fertilized the majority of ovules, pollen tubes elongated very little in arf6 arf8 transmitting tracts (Fig. 4M). These results indicate that ARF6 and ARF8 may act within the stigma, style or transmitting tract to regulate the production of some component necessary for pollen tube germination or growth.

MIR167 genes can decrease ARF6 and ARF8 transcript levels

ARF6 and ARF8 mRNA cleavage products ending within the miR167 target site have been detected in wild-type plants (Allen et al., 2005; Jones-Rhoades and Bartel, 2004; Rhoades et al., 2002). To test whether miR167 targets only these two genes, we made transgenic plants expressing the stem-loop regions of either of the
four predicted Arabidopsis MIR167 precursor genes behind the strong Cauliflower Mosaic Virus 35S promoter (P_{35S}:MIR167a, b, c and d). Only P_{35S}:MIR167a caused twisted leaves, short inflorescences and arrested flower development, thereby fully recapitulating arf6 arf8 mature plant phenotypes (Fig. 3B,C and Table 1). We did not examine seedling or root phenotypes in these sterile plants. P_{35S}:MIR167b and P_{35S}:MIR167c caused weaker mutant phenotypes, whereas P_{35S}:MIR167d plants all appeared identical to wild-type plants (Fig. 3B,C and Table 1). The phenotypic strengths of plants expressing different MIR167 precursor genes correlated with the amount of mature miR167 produced, and with the degree of reduction of ARF6 and ARF8 transcript levels (Fig. 3D). These results confirm that miR167 can remove or destabilize ARF6 and ARF8 transcripts in vivo. No additional leaf or flower phenotype was observed in transgenic plants carrying any of the four constructs, suggesting that miR167 targets only ARF6 and ARF8 in adult leaves, inflorescences and flowers.

**miR167-immune mARF6 and mARF8 flowers are sterile**

To elucidate the developmental function of miR167, we introduced eight translationally silent mutations into miR167 target sites in both ARF6 and ARF8 coding sequences, in the context of their normal 5’ and 3’ flanking sequences (Fig. 3A, mARF6 and mARF8), and transformed these constructs into wild-type plants. These mutations disrupted base pairing between miR167 and its target site, and should therefore render mARF6 and mARF8 transcripts immune to miR167-mediated turnover. Corresponding wild-type genomic constructs (gARF6 and gARF8) increased fecundity of the loss-of-function mutants (Nagpal et al., 2005) (data not shown), indicating that these genomic constructs were functional. mARF6 and mARF8 T1 plants had the same spectrum of phenotypes (Fig. 4, see also Fig. S1 in the supplementary material), supporting our previous conclusion that ARF6 and ARF8 have similar activities (Nagpal et al., 2005). We focus here on our phenotypic studies of mARF6 plants.

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**Table 1. Summary of P_{35S}:MIR167 T1 plant phenotypes**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Predicted sequence*</th>
<th>Strong (arf6 arf8-like)</th>
<th>Medium</th>
<th>Weak</th>
<th>No phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIR167a</td>
<td>5’-UGAAGCGCAGCAUGAUCAU-3’</td>
<td>100</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MIR167b</td>
<td>5’-UGAAGCGCAGCAUGAUCAU-3’</td>
<td>0</td>
<td>3</td>
<td>101</td>
<td>8</td>
</tr>
<tr>
<td>MIR167c</td>
<td>5’-UGAAGCGCAGCAUGAUCAU-3’</td>
<td>0</td>
<td>0</td>
<td>91</td>
<td>7</td>
</tr>
<tr>
<td>MIR167d</td>
<td>5’-UGAAGCGCAGCAUGAUCAUGG-3’</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>109</td>
</tr>
</tbody>
</table>

The severity of phenotypes of mARF6 plants correlated with the level of mARF6 transcript being expressed (Fig. 4A). mARF6-I transgenic plants with the highest mARF6 levels (12 out of 63 T1 plants) had small leaves and sterile flowers (Fig. 4A,B; see also Fig. S2 in the supplementary material). mARF6-II plants, with mARF6 levels higher than wild-type plants but lower than mARF-I plants (36/63), had slightly smaller leaves than wild-type plants and sterile flowers (Fig. 4A,B; Fig. S2 in the supplementary material). mARF6-III plants (15/63), with similar ARF6 levels to wild-type plants, had leaves similar in size to those of mARF6-II or wild-type plants, but did produce seeds (Fig. 4A,B; Fig. S2 in the supplementary material). However, mARF6-III seeds were small and could not germinate. As described below, embryos in these seeds were arrested.

Wild-type plants transformed with genomic ARF6 or ARF8 constructs, or expressing the wild-type ARF6-coding sequence behind the CaMV 35S promoter (P35S::ARF6) had fertile flowers despite having ARF6 or ARF8 transcript levels similar to or higher than those of mARF6-II or mARF6-III plants (Fig. 4A). A small proportion (less than 5%) of P35S::ARF6, gARF6 and gARF8 plants also had small leaves. Thus, whereas an elevated ARF6 expression level inhibited leaf growth, only a loss of mIR167 regulation caused flowers to be sterile.

mIR167 regulates ovule development

Female sterility in mARF6 plants arose from defects in ovule development. Early stage 2-IV ovules from mARF6-II plants had indistinguishable morphology from wild-type ovules, with inner and outer integuments initiated properly on ovule flanks (Fig. 4C,G). However, whereas wild-type outer integuments grew to encase the entire nucellus (Fig. 4D,E), mARF6-II outer integuments only grew slightly (Fig. 4H,I). In mARF6-I ovules, both inner and outer integuments and the nucellus were developmentally arrested (Fig. 4K). In mARF6-III ovules, outer integuments extended farther than in mARF6-II ovules, but they nevertheless failed to envelop the nucellus completely (Fig. 4L). In contrast to these effects on integument growth, cell morphology and arrangement in funiculi of mARF6 ovules appeared normal (Fig. 4E,I).

These ovule integument defects affected both pollen tube guidance to the ovule and embryo development. Wild-type pollen tubes grew normally in transmitting tracts of mARF6-II gynoecia (Fig. 4M). However, only a small proportion of mARF6 ovules (12%, n=195) were fertilized by wild-type pollen (Fig. 4F,J), whereas 84% (n=70) of gARF6 ovules were fertilized. Moreover, fertilized mARF6 ovules still failed to support embryo development. Seven days after pollination, gARF6 embryos had developed to mid-torpedo stage (Fig. 4N), whereas embryos on mARF6 plants were developmentally arrested at the four-cell stage (Fig. 4O). Embryos formed in self-fertilized mARF6-III flowers also developed only to the four-cell stage. Similarly, absence of the outer integument in the inner no outer-1 (ino-1) mutant, which is deficient in a member of the YABBY gene family (Villanueva et al., 1999), caused reduced fertilization efficiency and arrested embryo development (data not shown). Thus, a primary defect in integument growth accounts for female sterility.

To determine whether altered distribution of ARF6 and ARF8 transcripts could account for these phenotypes, we examined ARF6 and ARF8 expression patterns in flowers of mARF6-II and mARF8-II plants by in situ hybridization (Fig. 1). As a second method, we also compared X-gluc staining patterns in plants carrying mIR167-insensitive translational GUS fusions (mARF6:GUS, mARF8:GUS) with the staining patterns of the gARF6:GUS and gARF8:GUS plants described above (Fig. 2). In some strongly staining mARF6:GUS lines, a subset of ovules had reduced outer integument growth similar to mARF6-III ovules (Fig. 2N), suggesting that these constructs were partially functional. However, most mARF6:GUS and mARF8:GUS plants had fertile flowers, and these reporter constructs thereby revealed expression patterns largely independently of effects of the mARF6 or mARF8 mutations on ovule or anther development.
Consistent with northern blot results, ARF6 expression in mARF6-II ovules appeared stronger in tissues where ARF6 was expressed in wild-type ovules (Fig. 1E-H-I, Fig. 2C-E-H-J). Moreover, mARF6 (Fig. 1H-I) and mARF6:GUS (Fig. 2H-J) expression also appeared in the integuments and nucellus. In stage 4-I ovules, staining of mARF6:GUS persisted most strongly in the chalazal domain of the mature ovule, but decreased in the tips of the integuments (Fig. 2I). In mARF8 ovules, the expression of ARF8 expanded only into the integuments and not into the nucellus (Fig. 1K), suggesting that the expanded expression of ARF8 into the integument region might be sufficient to arrest outer integument growth. Similarly, mARF8:GUS was expressed in both funiculi and ovules (Fig. 2L,N).

INO was expressed in outer integuments of ovules, and ino mutations also caused arrested outer integument growth (Villanueva et al., 1999). However, mARF6 ovules had a normal INO expression pattern, and ino-1 ovules had a normal ARF6 expression pattern (see Fig. S3 in the supplementary material), suggesting that mARF6 affects integument growth independently of the INO pathway.

**MIR167a is expressed in ovules and anthers**

The mARF6 and mARF8 expression data indicated that miR167 limits ARF6 and ARF8 transcript expression domains in ovules. To determine MIR167 expression domains, we made transgenic plants carrying approximately 2 kb promoter fragments upstream of the stem-loop sequences of MIR167a, b, c and d fused to a GFP-GUS reporter gene (P\(_{\text{MIR167a,b,c,d}}\)::GUS), and analyzed promoter activities by X-gluc staining. In ovules, P\(_{\text{MIR167a}}\)::GUS expression (Fig. 2P-R), and to a lesser degree P\(_{\text{MIR167b}}\)::GUS and P\(_{\text{MIR167c}}\)::GUS expression (Fig. 2V,X), correlated with miR167 functions revealed by mutating target sites. P\(_{\text{MIR167a}}\)::GUS expression first appeared at late ovule stage 1, in the cells from which both the inner and outer integuments would later be initiated (Fig. 2P). As both integuments enveloped the nucellus and the ovule began to grow asymmetrically, staining expanded into the entire nucellus and integuments, but was always absent from the funiculus (Fig. 2Q,R). P\(_{\text{MIR167a}}\)::GUS also stained in anthers and in sepal vasculature (Fig. 2O). P\(_{\text{MIR167a}}\)::GUS was expressed in the ovules and nectaries, but was not detected in other floral organs in the open flower (Fig. 2U), and staining in mature P\(_{\text{MIR167a}}\)::GUS ovules was restricted mostly to the tips of inner and outer integuments (Fig. 2V). P\(_{\text{MIR167a}}\)::GUS stained mainly in the stamen filaments with a trace amount of staining in the ovules (Fig. 2W,X), and P\(_{\text{MIR167a}}\)::GUS stained only in sepal and petals, but not in the internal floral organs (Fig. 2Y). In situ hybridization results have also shown that, in both Nicotiana benthamiana and Arabidopsis, miR167 is present in ovules and in anther vasculature, but not in funiculi (Valoczi et al., 2006).

**miR167 regulates anther development**

Male sterility of mARF6 and mARF8 flowers was due to indehiscent anthers (Fig. 4B). Anthers of mARF6 and mARF8 flowers appeared normal before stage 10. However, mARF6-II anthers grew to be 20% larger than wild-type anthers as a result of enlarged connective cells, without any significant increase in cell number (Fig. 5A,D). By contrast, the vascular bundles of mARF6-II anthers were smaller than those of wild-type anthers (Fig. 5B,E). In the oldest closed wild-type flower bud, anther tapetum and septum had entirely degraded, and as flowers opened stomium cells broke apart to allow the release of pollen grains (Fig. 5A). In mARF6-II anthers, traces of tapetum were present within the anther locules of the oldest closed flower bud, and the septum did not degrade so that the two anther locules did not fuse. Septum cell breakage occurred in...
mARF6-II anthers after flower opening, but the stomium still remained intact, resulting in a lack of anther dehiscence (Fig. 5D). Unlike the arf6 arf8 double mutant, spraying with JA did not restore mARF6 anther dehiscence.

Whereas wild-type ARF6 and ARF8 were expressed in stamen filaments but not in anthers (Fig. 1C; Fig. 2A,B,K), mARF6 and mARF8 transcripts were also present in anther vasculature after floral stage 9 (Fig. 1G, data not shown). P_{MIR167a}::GUS was expressed in anther primordia as they differentiated, and throughout young anthers (Fig. 2A). As anthers matured, P_{MIR167a}::GUS expression became restricted to anther connective cells (Fig. 2T). We also transformed the mARF6 construct into plants with the synthetic auxin-responsive reporter construct DR5::GUS (Ulamasov et al., 1997). In T1 plants showing mARF6-I phenotypes, we detected ectopic DR5::GUS expression in stage 13 flower anther locules, but not in vascular or connective cells (Fig. 5C,F).

**DISCUSSION**

miR167 regulates both female and male floral organ development. Loss of miR167 regulation in mARF6 and mARF8 flowers expanded the domains of ARF6 and ARF8 expression, and caused arrested ovule development and anther indehiscence. Plants that overexpressed ARF6 or ARF8 but had normal miR167 regulation were fertile, indicating that loss of miR167-regulated patterning of ARF6 and ARF8 gene expression, rather than a higher expression level, caused these phenotypes. miR167 directs ARF6 and ARF8 transcript cleavage, but might also affect ARF6 and ARF8 transcription, as it has been shown that miR165/166 decreases PHB and PHV transcription by promoting DNA methylation in the coding regions downstream of the miRNA target sites (Bao et al., 2004).

Of the four predicted MIR167 genes, when overexpressed only MIR167a caused high miR167 production and arrested flower development to the same extent as in arf6 arf8 plants. DCL1 might recognize or process the stem-loop structure of MIR167a more efficiently than it does the others. In addition, miR167b and miR167c might have weaker activities toward ARF6 and ARF8 transcripts, and MIR167d may be a pseudogene that does not have activity. MIR167a is therefore most likely to be the main functional miR167 precursor gene in vivo. Consistent with this idea, P_{MIR167a}::GUS expression in ovules correlated precisely with the miR167 functions revealed in mARF6 and mARF8 plants.

In ovules, the complementary ARF6, ARF8 and miR167 expression patterns, and the arrested development of mARF6 and mARF8 integuments, indicate that miR167 functions to clear ARF6 and ARF8 transcripts from cells that will become integuments, thereby allowing integument growth. Persistence of the expression patterns at later ovule stages suggests that miR167 both establishes and maintains the correct pattern. ARF2, encoding another ARF protein, is normally expressed in the integuments and nucellus, and inhibits integument growth (Schruff et al., 2006). The ectopic ARF6 and ARF8 activity caused by blocking miR167 function may therefore activate pathways that ARF2 normally activates to restrict integument growth. Future studies may reveal the extent to which different ARF proteins have different activities, and why different ARF genes are expressed in mutually exclusive domains.

In anthers, miR167 was present in vascular cells where mARF6 and mARF8 accumulated (Valoczi et al., 2006), indicating that miR167 patterns gene expression in anthers as it does in ovules. However, although anther vasculature was altered in mARF6 and mARF8 plants, the strongest anther phenotypes were in connective cells, which grew abnormally large, and in locules, which failed to break open to release pollen and, in some cases, ectopically expressed the auxin-responsive marker DR5::GUS. mARF6 and mARF8 therefore have non-cell-autonomous effects in anthers. Anther dehiscence requires a series of desiccation events (Ishiguro et al., 2001), and excess ARF6 and ARF8 transcripts in the vasculature might increase water uptake, leading to excess connective cell expansion and preventing dehiscence.

Although miR167 accumulated in anther vasculature (Valoczi et al., 2006), we detected P_{MIR167a}::GUS expression in connective cells but not in vasculature. This difference suggests that miR167 processing or stability may differ in different cell types, or that miR167 may move between cells.

Just as ectopic mARF6 and mARF8 appear to act cell autonomously in ovules but non-cell autonomously in anthers, wild-type ARF6 and ARF8 appear to act autonomously in gynoecium transmitting tracts but non-autonomously on anthers, by affecting JA production from other tissues (Nagpal et al., 2005). Moreover, mARF6 and mARF8 restrict growth in ovules, but cause extra growth in anthers. These observations suggest that ARF6 and ARF8 may activate distinct target genes in ovules and anthers.

In *Drosophila*, microRNAs have been suggested to function to reinforce transcriptional repression patterns (Stark et al., 2005). By contrast, the function of miR167 to restrict distribution of its target transcripts is an essential patterning function that is not conferred by transcriptional controls of ARF6 and ARF8 alone. miR165/166 also affects development by excluding expression of its target transcripts from the abaxial domain of lateral organs (Juarez et al., 2004; Kidner and Martienssen, 2004; Mallory et al., 2004b). In fact, the miR165/166-insensitive phb-ld/+ mutant also has arrested outer integuments (Sieber et al., 2004), suggesting that both miR165/166 and miR167 might regulate common pathways during ovule formation.

miR167 is present in angiosperms and gymnosperms, but not in mosses, lycopods or ferns (Axtell and Bartel, 2005). Angiosperms and gymnosperms are seed plants, and form integuments around the female gametophyte that later form the seed coat. Gymnosperm male gametophytes are also surrounded by sterile cells that are similar to angiosperm anther connective cells (Gifford and Foster, 1988). The appearance of miR167 in seed plants but not in lower plants therefore suggests that regulation by miR167 could have arisen as plants evolved the formation of sporophytic structures that protect gametophytes.
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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/21/4211/DC1

References