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ARF2–ARF4 and *ARF5* are Essential for Female and Male Gametophyte Development in Arabidopsis

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Regular Paper

The plant hormone auxin plays critical roles in plant growth and development. Auxin response factors (ARFs) are a class of transcription factors which regulate auxin-mediated gene expression. While the functions of ARFs in sporophytic development have been well characterized, their functions specific to gametophytic development have not been studied extensively. In this study, Arabidopsis *ARF* genes were selectively down-regulated in gametophytes by misexpression of targeted microRNAs (*amiRARF234*, *amiRARFMP* and *MIR167a*) to silence *AtARF2–AtAEF4*, *AtARF5*, *AtARF6* and *AtARF8*. Embryo sacs in *amiRARF234*- and *amiRARFMP*-expressing plants exhibited identity defects in cells at the micropylar pole, such as formation of two cells with egg cell-like morphology, concomitant with loss of synergid marker expression and seed abortion. The pollen grains of the transgenic plants were morphologically aberrant and unviable, and the inclusions and nuclei were lost in the abnormal pollen grains. However, plants misexpressing *MIR167a* showed no obvious abnormal phenotypes in the embryo sacs and pollen grains. Overall, these results provide evidence that *AtARF2–AtARF4* and *AtARF5* play significant roles in regulating both female and male gametophyte development in Arabidopsis.

Keywords: Auxin • Auxin response factor • Gametophyte • MicroRNA.

Abbreviations: amiR, artificial microRNA; ANT, AINTEGUMENTA; ARF, auxin response factor; AUX1, AUXIN RESISTANT 1; DAPI, 4′,6-diamidino-2-phenylindole; DM, *Double marker*; DIC, differential interference contrast; ES1, Embryo Sac 1; FG, female gametophyte; GFP, green fluorescent protein; GUS, β-glucuronidase; MP, MONOPTEROS; NLS, nuclear localized signal; qRT-PCR, quantitative real-time PCR; RFP, red fluorescent protein; SEM, scanning electron microscopy; TEM, transmission electron microscopy; *TM*, *Triple marker*; WT, wild type.

Introduction

The life cycle of plants includes alternating generations of a diploid sporophyte and a highly reduced haploid gametophyte. In angiosperms, gametophytes contain both female and male gametes. The development of female and male gametophytes has been extensively investigated and reviewed (McCormick 1993, Yadegari and Drews 2004, Borg et al. 2009, Ma and Sundaresan 2010, Sundaresan and Alandete-Saez 2010, Yang et al. 2010). Gametophytes are critical for sexual reproduction. The female gametophyte (or embryo sac) is a seven-celled eight-nucleate haploid structure which develops into an embryo and endosperm after fertilization; in angiosperms, the female gametophyte is important in sexual reproductive processes, including pollen tube guidance, fertilization, induction of seed development upon fertilization and maternal control of seed development after fertilization (Yadegari and Drews 2004). In contrast, the three-celled male gametophyte (or pollen grain) plays a vital role in plant fertility and crop production by generating and delivering the male gametes into the embryo sac for double fertilization (Borg et al. 2009). Sexual reproduction thus requires correct patterning in both female and male gametophytes.

Auxin is a hormone which plays a crucial role in the entire development of plants. Auxin response factors (ARFs) are a class of transcription factors which act as key regulators of auxin-mediated gene expression. They were initially identified by their ability to bind to TGTCTC auxin response elements (AuxREs) (Ulmasov et al. 1997). The Arabidopsis genome encodes 23 ARF proteins, several of which have already been described. ARF2/MNT (MEGAINTEGUMENTA) is a repressor of cell division and organ growth (Schruff et al. 2006); *arf2* mutants exhibit pleiotropic developmental phenotypes, including large, dark green rosette leaves, delayed flowering, thick and long inflorescence, abnormal flower morphology and sterility in early formed flowers, large organ size and delayed senescence and abscission (Okushima et al. 2005a). ARF3/ETTIN determines the pattern of floral meristem and reproductive organ development in Arabidopsis; *ettin*/*arf3* mutants exhibit defects in gynoecium patterning and floral organ numbers (Sessions et al. 1997, Kelley et al. 2012, Tantikanjana and Nasrallah 2012). ARF5/MP (MONOPTEROS) promotes cell axialization and cell file formation, and establishes the vascular and body patterns in embryonic and post-embryonic development; *arf5* mutants exhibit defects in embryo patterning, vascular tissue formation,

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root growth and auxin-responsive gene expression (Przemeck et al. 1996, Hardtke and Berleth 1998, Mattsson et al. 2003, Hardtke et al. 2004). ARF6 and ARF8 regulate both stamen and gynoecium maturation; the flowers of *arf6 arf8* double mutants are arrested as infertile closed buds with short petals, short stamen filaments, undehisced anthers which did not release pollen and immature gynoecia (Nagpal et al. 2005, Wu et al. 2006). ARF8 can inhibit fruit development before fertilization occurs, where *arf8* mutants develop fruit in the absence of fertilization (parthenocarpic fruit) (Goetz et al. 2006, Goetz et al. 2007). However, insufficient information is available about the functions of ARFs in gametophyte development and the direct or indirect impact of sporophytic tissues surrounding the gametes.

Transgene-mediated gene silencing using artificial microRNAs (amiRNAs) and other small RNAs can overcome the limitations of interactions between gametophytic and sporophytic tissues and gene function redundancies through tissue-specific inducible and partial gene inactivation; these molecules can also simultaneously target sequence-related genes (Schwab et al. 2006, Ossowski et al. 2008). Gametophytic expression of a microRNA *amiR-ARFa* that simultaneously targeted nine *ARF* genes, *ARF1–ARF8* and *ARF19*, results in cell specification defects, with switch of synergids to egg cell identity (Pagnussat et al. 2009). However, it is not clear whether all the nine *ARF* genes targeted previously are required in the female gametophyte. Also, the male gametophytic functions of *ARF* genes have not been established. To demonstrate which *ARF* genes play essential roles in gametophyte development in Arabidopsis, we silenced *ARF5* using *amiRARFMP*; *ARF2*, *ARF3* and *ARF4* using *amiRARF234*; and *ARF6* and *ARF8* using *MIR167a* (Wu et al. 2006). These miRNAs were driven by the female and male gametophyte promoter *ES1* (*Embryo Sac 1*; Pagnussat et al. 2009). The pOp/LhG4 system was used to avoid gametophytic lethal events (Moore et al. 1998). The development of embryo sacs and pollen grains was carefully examined. Plants overexpressing *amiRARFMP* and *amiRARF234* exhibited defective synergid nuclei and synergid cell fate loss. In addition, the pollen grains were morphologically aberrant, with reduced viability and germination rate resulting from the loss of inclusions and nuclei.

Results

Promoter activities of *ARF2–ARF6* and *ARF8* during female and male gametophyte development

Using promoter fusions of *ARF* genes to nuclear localized green fluorescent protein (GFP) (Rademacher et al. 2011), we carefully examined the promoter activities of *ARF2–ARF6* and *ARF8* in the embryo sacs and stamens. *ARF2* was strongly expressed in the integument and funiculus and weakly expressed in the embryo sacs (Supplementary Fig. S1A). *ARF3* was expressed in the inner integument and weakly expressed in the central cell and micropylar end (Supplementary Fig. S1B). *ARF4* was expressed in the synergids and funiculus (Supplementary Fig. S1C). *ARF5* was strongly expressed in the chalazal end and weakly expressed in the synergids (Supplementary Fig. S1D). *ARF6* and *ARF8* were both strongly expressed in the integument and weakly expressed in the embryo sacs (Supplementary Fig. S1E, F). In the stamens, promoter activities of all these *ARF* genes were detected, especially in the vascular tissues (Supplementary Fig. S2). However, none of these promoters showed activities in the pollen grains (data not shown).

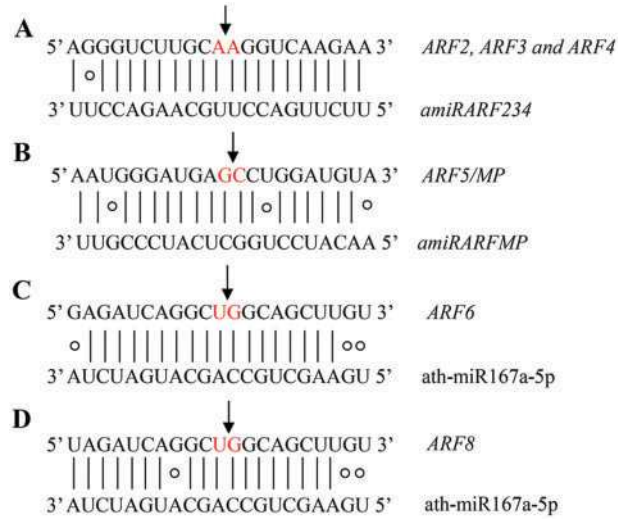

Fig. 1 Design of *amiRARF234*, *amiRARFMP* and *MIR167a*. (A) Sequences of *amiRARF234* target sites on *ARF2–ARF4* mRNA. (B) Sequences of *amiRARFMP* target sites on *ARF5* mRNA. (C) Sequences of *MIR167a* target sites on *ARF6* mRNA. (D) Sequences of *MIR167a* target sites on *ARF8* mRNA. Black arrows indicate putative cleavage sites of mRNA, and mismatched nucleotides are shown with black circles.

Silencing of *ARF* genes by amiRs could faithfully mimic *arf* mutant phenotypes

amiRARF234 and *amiRARFMP* were designed to target *AtARF2–AtARF4* and *AtARF5*, respectively (**Fig. 1A–D**; Supplementary Table S1). To check the specific silencing of designed *amiRARF234* and *amiRARFMP*, *ANTpro::LhG4*-transformed plants containing the *ANT* (*AINTEGUMENTA*) promoter which is expressed in the primordia of all above-ground organs (Schoof et al. 2000, Krizek 2009) was crossed with *Op::amiRARF234*- and *Op::amiRARFMP*-transformed lines, and the resulting F_1 progeny *ANTpro>>amiRARF234* and *ANTpro>>amiRARFMP* were morphologically analyzed. Compared with *ANTpro::LhG4* control plants, the dwarf *ANTpro>>amiRARF234* showed narrow and curled dark-green leaves, late flowering and thick inflorescences with mis-shapen floral organs (Supplementary Figs. S3B, S4B, E), which phenocopy the previously described *arf3 arf4* double mutants and *35S:miR-ARF* mutants (Pekker et al. 2005, Alvarez et al. 2006). Also the semi-dwarf *ANTpro>>amiRARFMP* showed abnormalities in inflorescences with strongly reduced flowers lacking predominantly organs of the outer whorls, and thin, sterile pistils (Supplementary Figs. S3C, S4C, F), resembling the *arf5* single mutant phenotypes (Przemeck et al. 1996).

Consistently, expressions of *AtARF2-4* and *AtARF5* in *ANTpro>>amiRARF234* and *ANTpro>>amiRARFMP* was reduced to varying degrees (Supplementary Fig. S5). These results proved that the designed *amiRARF234* and *amiRARFMP* could actually silence the target genes.

Gametophytic expression of *amiRARF234* and *amiRARFMP* results in defective synergid identities and seed abortions

To express the *ARF*-targeting microRNAs in the gametophyte, we selected the *ES1* (*At5g40260*) promoter (Yu et al. 2005). We primarily performed a detailed characterization of the *ES1* promoter activities in the Arabidopsis embryo sacs and pollen grains using both *GUS* (β-glucuronidase) and *NLS-3×GFP* reporter genes. *ES1* was exclusively expressed in the reproductive organs. Before fertilization, *ES1* was expressed in both female and male gametophytes. In the female gametophyte, the signals were weak at the female gametophyte (FG) FG0 stage (Supplementary Fig. S6A) but intensified at FG2 until FG7 (Supplementary Fig. S6B–F). This result is consistent with that of GUS staining (Supplementary Fig. S6H–K). At the FG7 stage (Supplementary Fig. S6F), *ES1* was strongly expressed in the central cell and synergids, whereas *ES1* showed relatively weaker expressions in the egg cell and antipodal cells. This result is consistent with previous microarray data on Arabidopsis female gametophyte (Wuest et al. 2010) and RNA sequencing (RNA-Seq) data on the central cell (Schmidt et al. 2012) and egg cell (unpublished data). In the male gametophyte, consistent signals were observed in the locules of the anther at stages 4–12 (Sanders et al. 1999) (Supplementary Fig. S6L–Q). When the pollen grains matured, signals were observed in the two sperm nuclei and in the vegetative nucleus (Supplementary Fig. S6G).

Thus the *ES1pro::LhG4*-transformed line containing the *ES1* promoter was crossed with *Op::amiRARF234-*, *Op::amiRARFMP-* and *Op::MIR167a*-transformed lines (each at least 10 lines), respectively, and the resulting F_1 progeny lines *ANTpro>>amiRARF234*, *ANTpro>>amiRARFMP* and *ANTpro>>MIR167a* were morphologically analyzed. Since each group of amiRARF-down-regulated plant lines showed quite similar phenotypes, one line in each group was used for further research. Using differential interference contrast (DIC) microscopy, we determined the number of normal [wild-type (WT) embryo sacs] and abnormal embryo sacs (defective embryo sac identity and arrested embryo sac) before fertilization (FG6 and FG7). The results showed that 25.8% and 32.2% of the embryo sacs in the *ES1pro::LhG4/+*; *Op::amiRARF234/+* and *ES1pro::LhG4/+*; *Op::amiRARFMP/+* plants exhibited defective micropylar end identities, respectively. These ratios are considerably higher than that in the control *ES1pro::LhG4/+* plants (9.0%) (**Table 1**). Some embryo sacs showed arrested development at the FG1 or FG2 stage, but the proportions were very low, showing no obvious differences compared with the control plants (2.5% and 4.3% vs. 3.9%). In addition, the embryo sacs of the *ES1pro::LhG4/+*; *Op::MIR167a/+* plants did not differ considerably from those of the control *ES1pro::LhG4/+* plants. Thus, the *ES1pro::LhG4/+*; *Op::MIR167a/+* plants were not used in further investigations. Both *ES1pro::LhG4/+*; *Op::amiRARF234/+* and *ES1pro::LhG4/+*; *Op::amiRARFMP/+* plants showed defective embryo sacs, which were characterized by the presence of synergid nuclei toward the chalazal end of the embryo sacs (**Fig. 2A–C**) or two egg cell-like structures (**Fig. 2D**). The egg cell and central cell nuclei showed normal positions, indicating that their development was unaffected. Synergids play essential roles in pollen tube growth, pollen tube discharge and gamete fusion (Punwani and Drews 2007). Displacement of the synergids may render the egg cell dysfunctional, leading to abnormal fertilization and seed abortion (Pagnussat et al. 2007, Pagnussat et al. 2009). Therefore, we further examined the siliques of the transgenic plants. Proportions of seed abortion corresponded well with the percentage of abnormal embryo sacs before fertilization (**Table 1**; Supplementary Fig S7), indicating that the defective synergids resulted in seed abortion.

Synergid fate was affected in embryo sacs with defective micropylar end identity

To investigate further whether the micropylar end identity defects in the embryo sacs affect cell fate, we introduced cell type-specific markers into the plants with down-regulated *ARF* genes. The *TM* (*Triple marker*) line [nuclear-localized GFP signal in synergids, nuclear-localized red gluorescent protein (RFP) signal in the egg cell, and cytoplasm-localized GFP signal in the central cell] was used as a synergid-specific marker showing strong nuclear-localized GFP signals (**Fig. 3A**). As shown in **Table 2**, 49.0% of the embryo sacs in the *ES1pro::LhG4/+*; *TMs/+* plants were GFP positive and showed a WT expression pattern resulting from the segregation of the GFP reporter. In contrast, only 37.9% and 40.0% of the embryo sacs in the *ES1pro::LhG4/+*; *Op::amiRARF234/+*; *TMs/+* and *ES1pro::LhG4/+*; *Op::amiRARFMP/+*; *TMs/+* plants were GFP positive. This result suggests that the synergid cell identity was partially lost. In some cases, synergid-specific marker signals were completely lost in the two synergids (**Fig. 3D**); in some embryo sacs, the synergid-specific marker signal was normal in one synergid but weak or lost in the other synergid (**Fig. 3B, C**). In all of these cases, the central cell- and egg cell-specific markers were apparently unaffected. For a safe conclusion, we further introduced *DM* (*Double marker*) (Yuan et al. 2016) to label only the central cell with cytoplasmic GFP and the egg cell with nuclear-localized RFP (Supplementary Fig. S8). Approximately 50% of the embryo sacs in the *ES1pro::LhG4/+*; *Op::amiRARF234/+* (*Op::amiRARFMP/+*); *DMs/+* plants exhibited positive GFP and RFP signals, which did not differ significantly from that in the *ES1pro::LhG4/+*; *DMs/+* plants (**Table 3**). Moreover, no cell exhibited the signal characters of the other cell types, suggesting that the identities of the egg and central cells were not altered and that the synergids were not transformed into either egg or central cells.

Pollen grains were morphologically aberrant and unviable in *ARF2–ARF4-* and *ARF5*-down-regulated plants

The *ES1* promoter was strongly expressed in the male gametophyte; thus, we determined the pollen grain development in

Table 1 Statistical analysis of embryo sacs and seeds from plants separately carrying the *Op::amiRARF234*, *Op::amiRARFMP* and *Op::MIR167a* constructs crossed with *ES1pro::LhG4* plants

Plants studied	Before fertilization (FG6 and FG7)				Total	*P*-value	After fertilization (silique check)		Total	*P*-value
	No. of normal embryo sacs	No. of abnormal embryo sacs		Total			No. of normal seeds	No. of abnormal seeds		
		No. of identity defect embryo sacs	No. of arrested embryo sacs							
ES1pro::LhG4/+	203 (87.1%)	21 (9.0%)	9 (3.9%)	30 (12.9%)	233	0.124	664 (94.6%)	38 (5.4%)	702	0.177
ES1pro::LhG4/+; Op::amiRARF234/+	172 (71.7%)	62 (25.8%)	6 (2.5%)	68 (28.3%)	240	0.233	532 (78.8%)	143 (21.2%)	675	0.021
ES1pro::LhG4/+; Op::amiRARFMP/+	132 (63.5%)	67 (32.2%)	9 (4.3%)	76 (36.5%)	208	0.0001	692 (71.7%)	273 (28.3%)	965	0.017
ES1pro::LhG4/+; Op::MIR167a/+	108 (85.0%)	12 (9.5%)	7 (5.5%)	19 (15.0%)	127	0.008	628 (93.0%)	47 (7.0%)	675	$2.25\ e^{-27}$

Among the embryo sacs, 25% were predicted to inherit both constructs of the crossed plants. *ES1pro::LhG4* plants served as control.

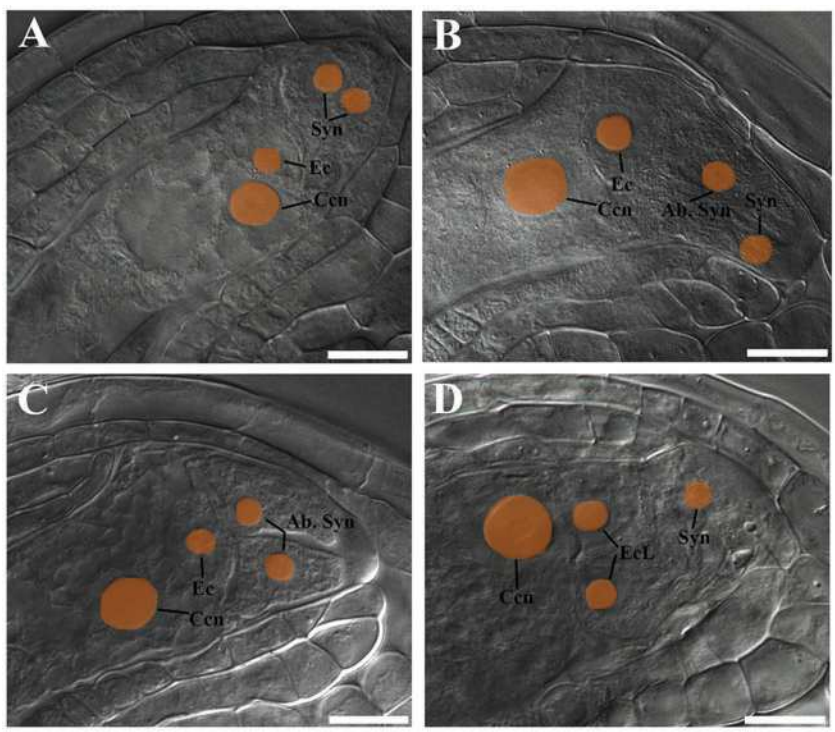

Fig. 2 DIC image of embryo sac identity in *ES1pro::LhG4* plants and plants with artificial miRNA-down-regulated *ARF* genes. (A) Normal nuclei positions in the *ES1pro::LhG4* mature embryo sac. (B) Mature embryo sac showing an abnormal synergid, with the nucleus located toward the chalazal end of the embryo sac. (C) Mature embryo sac showing two abnormal synergids, with two nuclei located toward the chalazal end of the embryo sac. (D) Embryo sac exhibiting two egg cell-like cells. Ccn, central cell nucleus; Ec, egg cell; Syn, synergid; Ab. Syn, abnormal synergid; EcL, egg cell-like cell. Scale bar = 20 μm.

plants with down-regulated *ARF* genes. Alexander stain was used to examine pollen grain viability and 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei of pollen grains. The *amiRARF234*- and *amiRARFMP*-expressing plants both exhibited morphologically aberrant pollen grains. In contrast, apparent deformities were lacking in the *MIR167a*-misexpressing plants. The results of Alexander staining revealed that a large proportion of the pollen grains were unviable (**Fig. 4A–C**) and exhibited aberrant phenotypes (i.e. surface indentation and irregular shape) (**Fig. 4D–G**). In addition, DAPI staining revealed that the three nuclei were completely lost or partially lost in these morphologically aberrant pollen

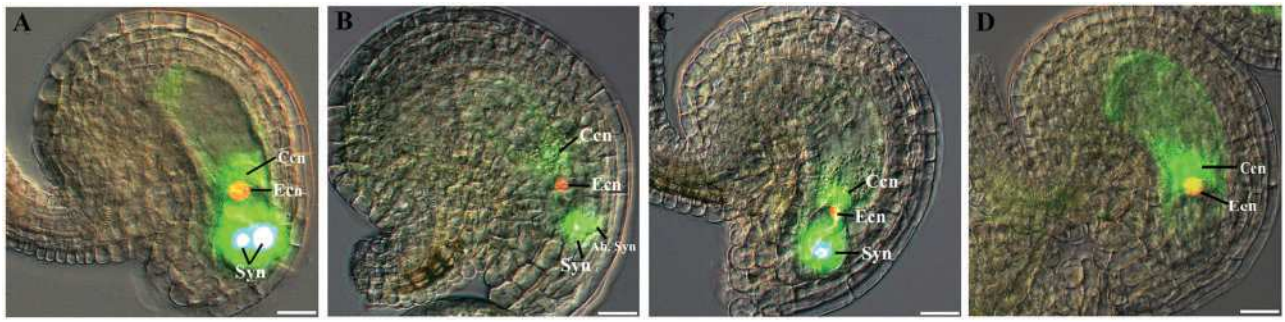

Fig. 3 Cell type-specific marker expression indicated by *TM*. The central cell is marked with the cytoplasmic GFP signal, synergids are marked with the nuclear-localized GFP signal and the egg cell nucleus is marked with nuclear-localized RFP signal. (A) Normal expression of cell type-specific markers in *ES1pro::LhG4*. (B) Abnormal synergid-specific marker with strong signal in one synergid and weak signal in the other synergid. (C) Abnormal synergid-specific maker with signal localized in one synergid only. (D) Abnormal expression of cell type-specific markers; synergid marker signals were lost. No significant difference was found between the central cell- and egg cell-specific markers in (B–D) and (A). Ccn, central cell nucleus; Ec, egg cell; Syn, synergid; Ab. Syn, abnormal synergid. Scale bars = 20 μm.

Table 2 Statistical analysis of synergid cell marker expressions in embryo sacs of *ES1pro >>amiRARFMP* and *ES1pro >>amiRARF234* plants crossed with the *TM* line

Plants studied	Synergid cell marker expression		Total	*P*-value
	No. of embryo sacs with positive GFP	No. of embryo sacs with negative GFP		
ES1pro::LhG4/+; TMs/+	121 (49.0%)	126 (51.0%)	247	0.750
ES1pro::LhG4/+; TMs/+; Op::amiRARF234/+	108 (37.9%)	177 (62.1%)	285	0.903
ES1pro::LhG4/+; TMs/+; Op::amiRARFMP/+	95 (40.0%)	142 (60%)	237	0.421

Of the total embryo sacs, 50% were expected to be positive GFP with a WT expression pattern, resulting from the segregation of the GFP reporter; 25% were predicted to overexpress *amiRARFMP* or *amiRARF234* driven by the *ES1* promoter; and 12.5% were predicted to overexpress *amiRARFMP* or *amiRARF234* and to show GFP signals.

grains (**Fig. 4H, I**). Meanwhile, the aberrant pollen grains failed to germinate in vitro (**Fig. 5A, B**), and the *ES1pro::LhG4/+; Op::amiRARFMP/+* and *ES1pro::LhG4/+; Op::amiRARF234/+* plants only reached 40.4% and 48.0% germination rates; these rates are 39.2% and 31.6% lower than those in the control *ES1pro::LhG4* plants (**Fig. 5C**). Subsequently, floral buds were fixed and embedded in paraffin, and transverse sections were stained with ferroalumen–hematoxylin for microscopy. At floral developmental stage 13 (Smyth et al. 1990), a large proportion of the aberrant pollen grains were found in the anther chambers of the *amiRARF234-* and *amiRARFMP*-expressing plants, whereas pollen grains were normal in the control *ES1pro::LhG4* plants (Supplementary Fig. S9A-C). However, no obvious difference was observed for the anther development process.

Inclusions and nuclei were lost in abnormal pollen grains

Pollen grains were examined via SEM (scanning electron microscopy) and TEM (transmission electron microscopy) to elucidate the mechanism of their unviability. A large proportion of pollen grains in *amiRARF234-* and *amiRARFMP*-expressing plants were morphologically abnormal (**Fig. 6A, B**), i.e. shrunken, sunken or even collapsed (**Fig. 6C–H**). Only 64.4% and 63.9% of the pollen grains were normal in the *ES1pro::LhG4/+; Op::amiRARFMP/+* and *ES1pro::LhG4/+; Op::amiRARF234/+* plants compared with 94.6% in the control plant *ES1pro::LhG4* (**Fig. 6I**). This finding is consistent with the result of Alexander staining and in vitro pollen grain germination ratios. However, the exine of the transgenic abnormal pollen grains was similar to that of the control pollen grains, indicating that the development of the pollen exine was unaffected and abnormalities might be caused by some intrinsic factors. TEM images of the abnormal pollen grains showed that the nuclei were absent and the inclusions (i.e. lipid body, starch grain and plastid) were partially lost and were replaced by large vacuoles (**Fig. 7A–D**). These observations suggest that the unviability of the abnormal pollen grains was caused by the loss of inclusions and nuclei.

Discussion

The role of auxin signaling in initiation and progression of female and male gametophyte development has been previously described (Cecchetti et al. 2008, Pagnussat et al. 2009). Genes involved in auxin biosynthesis, auxin influx and auxin efflux pathways are expressed during female and male gametophyte development (Dal Bosco et al. 2012a, Dal Bosco et al. 2012b, Panoli et al. 2015). As key regulators of auxin-mediated gene expression, ARF transcription factors function in various developmental stages of multiple plant organs. Loss-of-function *arf* mutants can be used to investigate the functions of *ARF* genes in plant organ formation and patterning. However, the role of *ARF* genes in gamete development has not been extensively investigated because of the considerable sporophytic

Table 3 Statistical analysis of central cell and egg cell marker expression in embryo sacs of *ES1pro>>amiRARFMP* and *ES1pro>>amiRARF234* plants crossed with the *DM* line

Plants studied	Cell marker expression						Total
	Central cell marker expression		*P*-value	Egg cell marker expression		*P*-value	
	No. of embryo sacs with positive YFP	No. of embryo sacs with negative YFP		No. of embryo sacs with positive RFP	No. of embryo sacs with negative RFP		
ES1pro::LhG4/+; DMs/+	116 (46.0%)	136 (54.0%)	0.208	120 (47.6%)	132 (52.4%)	0.450	252
ES1pro::LhG4/+;DMs/+; Op::amiRARF234/+;	124 (47.1%)	139 (52.9%)	0.001	129 (49.0%)	134 (51.0%)	0.0001	263
ES1pro::LhG4/+; DMs/+; Op::amiRARFMP/+;	119 (50.2%)	118 (49.8%)	5.38 e^{-05}	115 (48.5%)	122 (51.5%)	0.0005	237

Of the total embryo sacs, 50% were expected to be positive for yellow fluoresscent protein (YFP) and RFP with a WT expression pattern resulting from the segregation of the GFP reporter; 25% were predicted to overexpress *amiRARFMP* or *amiRARF234* driven by the *ES1* promoter; and 12.5% were predicted to overexpress *amiRARFMP* or *amiRARF234* and to show YFP and RFP signals.

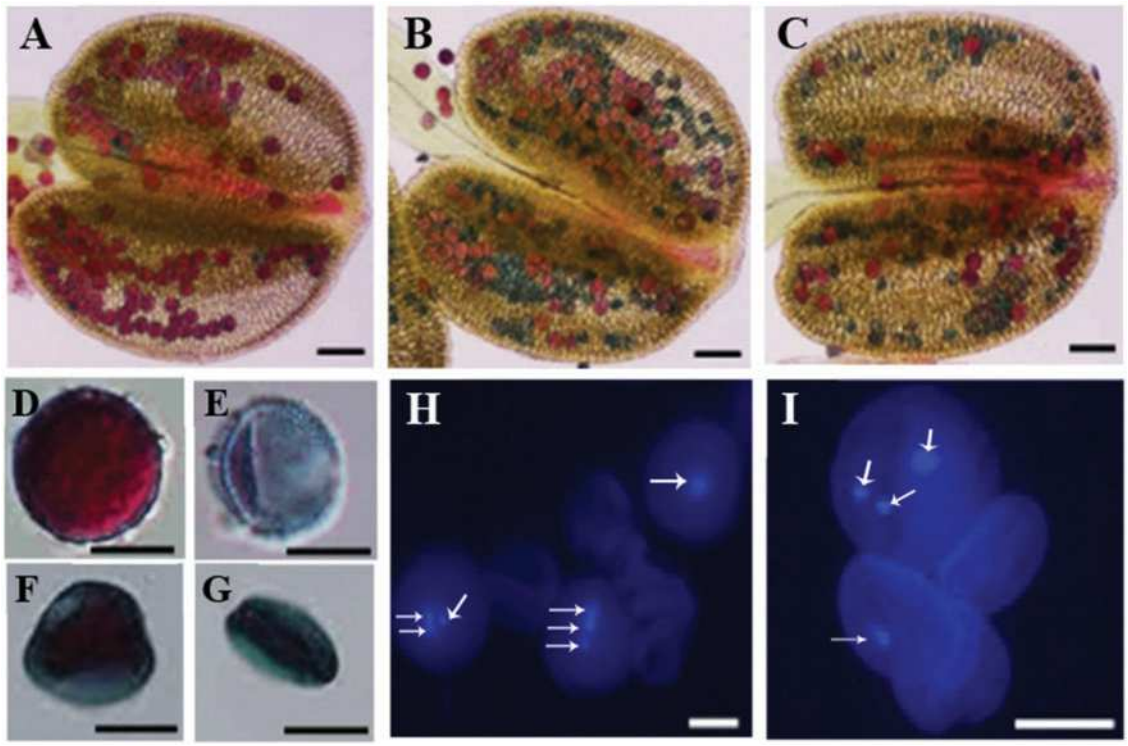

Fig. 4 Alexander staining and DAPI staining of pollen grains. (A) Alexander staining of normal viable pollen grains in the anthers of *ES1pro::LhG4* plants. Alexander staining of abnormal pollen grains in the anthers of *ES1pro>>amiRARFMP* plants (B) and *ES1pro>>amiRARF234* plants (C). Zoomed images of normal (D) and abnormal (E–G) pollen grains with Alexander stain. (H and I) DAPI stain of normal and abnormal pollen grains. Scale bars = 50 μm (A–C) and 20 μm (D–I).

effects on the gametes in single or multiple *arf* mutants. *arf2* mutants exhibit reduced self-fertility resulting from the failure of the floral bud to open (Schruff et al. 2006). In general, *ett/arf3* mutants produce flowers with narrow gynoecia, exposed smaller ovules and aberrant or uninitiated medial stamen primordia (Sessions et al. 1997). *arf3 arf4* double mutants produce misshapen flowers or flowers with reduced gynoecium and stamens (Pekker et al. 2005). *arf5* mutants produce flowers with sterile female and male parts (Przemeck et al. 1996). *arf6* and *arf8* single mutants exhibit delayed stamen development and decreased fecundity, whereas *arf6 arf8* double mutants exhibit complete block in flowering (Nagpal et al. 2005). In the present study, we used the female and male gametophyte promoter *ES1* to silence specific *ARF* genes through *amiRARF* and *MIR167a*, and we obtained transgenic plants with obvious abnormal embryo sacs and pollen grains. These plants were used to reveal the regulatory functions of *ARF* genes during gametophyte development in Arabidopsis.

Pagnussat et al. (2009) proposed a model whereby female gamete specification was dependent on auxin. Furthermore, Panoli et al. (2015) studied the expression patterns of *AUX1* (*AUXIN RESISTANT 1*) with *AUX1pro::AUX1-GFP* and found that AUX1 was first detected in the micropylar end from the FG4 stage, thereby causing the polarized localization of AUX1 in

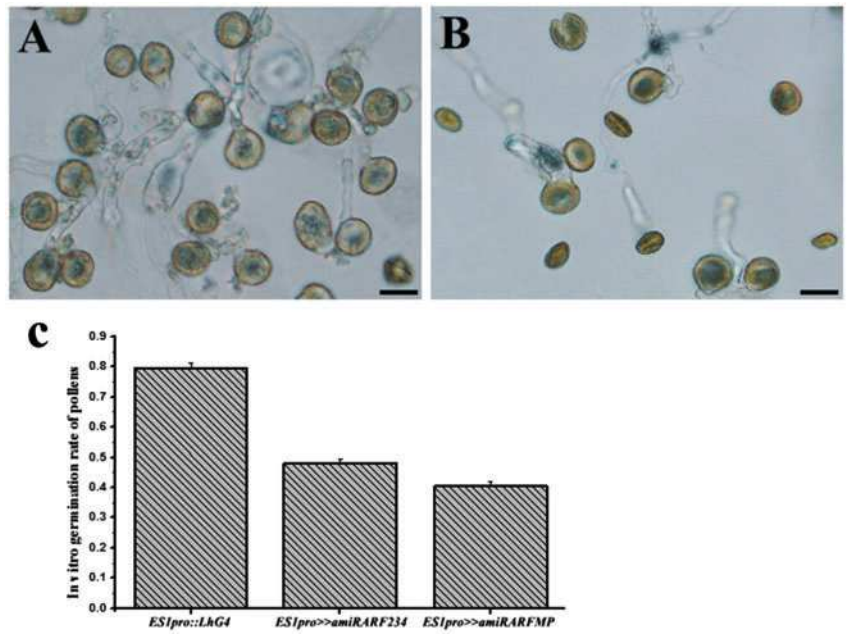

Fig. 5 In vitro germination of pollen grains. (A) Normal pollen tube growth of the pollen grains in *ES1pro::LhG4* plants. (B) Aberrant pollen grains without pollen tube growth in *ES1pro >>amiRARFMP* and *ES1pro >>amiRARF234* plants. (C) Comparison of the germination ratios of the pollen grains in *ES1pro::LhG4* plants and plants with down-regulated *ARF* genes.

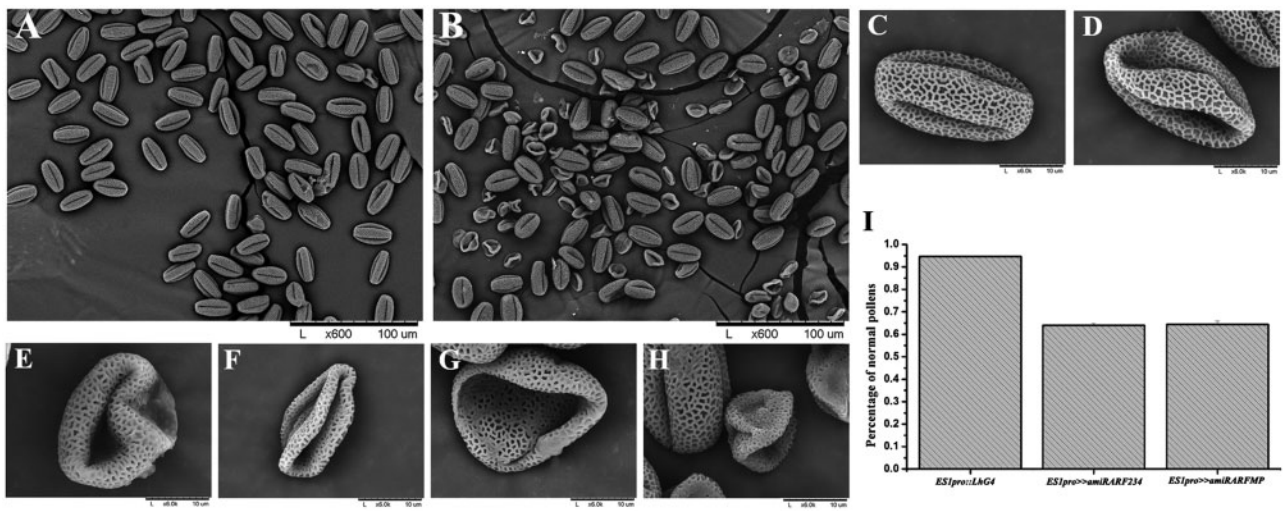

Fig. 6 Scanning electron micrographs of pollen grains. (A) Normal pollen grains in *ES1pro::LhG4* plants. (B) Morphologically abnormal pollen grains in *ES1pro >>amiRARFMP* and *ES1pro >>amiRARF234* plants. Individual view of single pollen grain with normal (C) and abnormal (D–H) morphologies. (I) Comparison of the percentage of morphologically normal pollen grains in *ES1pro::LhG4* plants and plants with down-regulated *ARF* genes.

the micropylar end. Here we showed *ARF* expression in the micropylar end, and *amiRARFMP*- and *amiRARF234*-expressing lines exhibited defective synergid identities, suggesting the involvement of auxin signals in female gametophyte establishment. However, the exact regulation pathways need further research.

The germination rates *in vitro* of the *ES1pro::LhG4*/+; *Op::amiRARFMP*/+, *ES1pro::LhG4*/+; *Op::amiRARF234*/+ and control *ES1pro::LhG4* plants were 40.4, 48.0 and 79.6%, respectively. In addition, the *ES1pro::LhG4*/+; *Op::amiRARFMP*/+, *ES1pro::LhG4*/+; *Op::amiRARF234*/+ and *ES1pro::LhG4* plants produced 64.4, 63.9 and 94.6% normal pollen grains, respectively. This non-conformity to Mendel's law suggests that the pollen grains were sporophytically affected in these *ARF*-down-regulated plants. A precise expression pattern of *ES1* during anther development was studied through RNA *in situ* hybridization. The *ES1* transcript was initially detected at anther development stage 4, and then the signal significantly increased in tapetal cells and microsporocytes. Weak *ES1* expression was still detectable in the tapetum and microspores after stage 7,

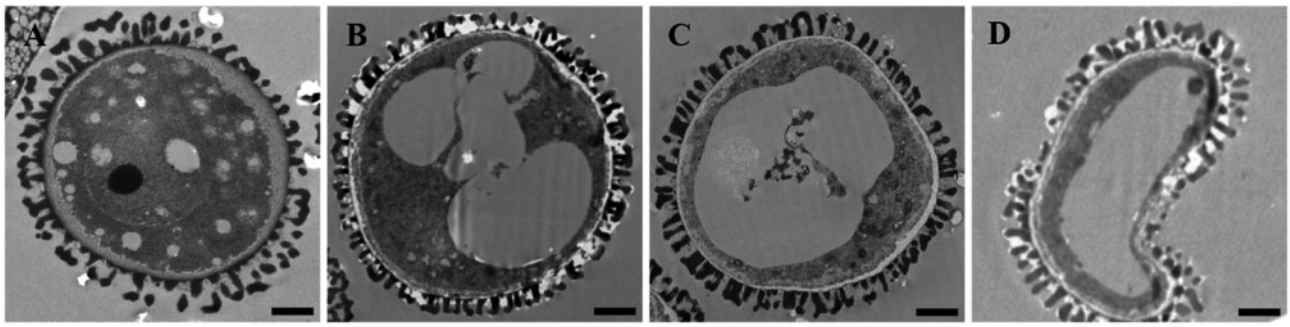

Fig. 7 Transmission electron micrographs of pollen grain development. (A) Morphologically normal pollen grain. (B) Morphologically abnormal pollen grains with nuclei absent and inclusions partially lost and replaced by large vacuoles. Scale bars = 2 μm.

whereas *ES1* transcript was detectable in the pollen grains only after complete degeneration of the tapetum at stage 12 (Guan et al. 2008). This finding indicates that *ES1* is also active in the tapetum. Actually, strong auxin signals were detected using *DR5* in the tapetum and in the degenerating tapetum cells, whereas only faint signals were detected in the pollen grains (Feng et al. 2006, Cecchetti et al. 2008). In our study, promoter activities of *ARF2–ARF5* in developing stamens revealed obvious signals in vascular tissues, while expression patterns in more precise tapetum structure are still waiting to be verified. However, traces of *ARF2–ARF5* expression in the stamen and tapetum were reported (Sessions et al. 1997, Hardtke et al. 2004, Okushima et al. 2005a, Pekker et al. 2005, Schruff et al. 2006). The tapetum has significant functions in microsporogenesis and pollen grain development. Thus, we speculated that *ARF2–ARF4* and *ARF5* regulate pollen grain development *via* the tapetum, although the mechanisms of regulation require further research.

No abnormal phenotypes were observed in the female and male gametophytes of the *MIR167a*-misexpressing transgenic plants. However, this result does not necessarily suggest that *ARF6* and *ARF8* are not required in embryo sac and pollen grain development. Since *ARF6* and *ARF8* are targets of *MIR167* and it has also been shown that *MIR167a*, *MIR167b* and *MIR167c* are expressed in the ovules and anthers (Wu et al. 2006), thus restricting the normal accumulation of *ARF6* and *ARF8* transcripts in WT plants, this may also be the case in female and male gametophytes. When *ARF6* and *ARF8* are mutated to be resistant to targeting by *MIR167*, ARF6 and ARF8 proteins are expressed in the ovules, and ARF8 protein signals are evident in the anthers; in addition, *MIR167*-immune *mARF6* and *mARF8* flowers become sterile (Panoli et al. 2015). However, the 35S promoter used in this study could not exclude sporophytic effects on gametophyte development. Employing the *ES1* promoter combined with a transactivation system to drive *MIR167*-immune *mARF6* and *mARF8* misexpression in female and male gametophytes may help illuminate the possible roles of *ARF6* and *ARF8* in gametogenesis.

Auxin is critical for gynoecium and stamen development, and auxin distribution and flow have been systematically observed using the auxin reporter *DR5* (Feng et al. 2006, Larsson et al. 2013). ARFs are important auxin response transcription factors involved in various biological functions of auxin. On the basis of sequence alignment and phylogenetic analysis, the 23 *ARF* genes in Arabidopsis can be grouped into three classes, I, II and III; class I contains the greatest number of members and can be further divided into three subclasses, Ia, Ib and Ic (Okushima et al. 2005b). Only the *ARF* genes of clades Ia, Ic and II have been primarily researched. *ARF* genes in the same branch with high sequence similarities often show overlapping and redundant functions in certain organs or tissues. *ARF6* and *ARF8* gene dosages quantitatively regulate stamen and gynoecium maturation, respectively (Nagpal et al. 2005, Wu et al. 2006). *ARF7* and *ARF19* exhibit redundant functions in lateral root formation, agravitropic hypocotyls and roots, and in apical dominance in aerial portions (Larsson et al. 2013). *ARF3* and *ARF4* are redundantly required for the specification of abaxial cell types (Pekker et al. 2005). Meanwhile, *ARF1* is partially redundant with *ARF2* (Ellis et al. 2005). In the present study, *amiRARF234*- and *amiRARFMP*-expressing lines showed similar embryo sac and pollen grain defects, suggesting the overlapping and non-redundant functions between *ARF5* and *ARF2–ARF4*. In addition, *ARF2–ARF4* are targets of tasiR-ARF (Williams et al. 2005), and *amiRARF234* was designed to silence *ARF2–ARF4*, but whether these three genes perform overlapping and redundant roles in female and male gametophyte development remains to be elucidated.

Materials and Methods

Plant materials and constructs

For the *ES1* promoter analysis, the 1,534 bp promoter region was amplified using the primers 5′-CCCAAGCTTGACCACAATAAGTGTAATG-3′ (forward) and 5′-CGCGGATCCTAAAATCGCCGTTTACAAA-3′ (reverse). This region was subsequently inserted at the *Hin*dIII and *Bam*HI restriction sites of the modified *p*CAMBIA1300 vector containing the NLS-3×eGFP-NOS terminator. The *amiRARF* sequences were designed as follows. *ARF2*, *ARF3* and *ARF4* (*amiRARF234*): GAGCACCCUAUAAUUGCGAUU; *ARF5* (*MP*) (*amiRARFMP*): UUGCCCUACUCGGU CCUACAA; and *ARF6* and *ARF8* (*MIR167a*): AUCUAG UACGACCGUCGAAGU. The backbones of *amiRARF234*, *amiRARFMP* and *MIR167a* were *AtMIR164a*, *AtMIR319a* and *AtMIR167a*, respectively (Alvarez et al. 2006, Wu et al. 2006). Synthetic miRNA was constructed using GenScript. *amiR-ARF*s were excised from *p*UC57, cloned into six OpTATA-BJ36 and subsequently cloned into the binary vector *p*MLBART (Op::amiRARF BART). The plasmid was introduced into the *Agrobacterium tumefaciens* strain GV3101 through electroporation and then transformed using the floral dip method. The forward primer used to amplify the inserted *amiR-ARF*s was MAS11 (5′-CA AGACCCTTCCTCTATATAAG-3′), and the reverse primers for *amiRARF234*,

amiRARFMP and *MIR167a* were R-234 (5′-CCGCATATATACACGCATTTGT-3′), R-MP (5′-GACTCGGTATTTGGATGAATGA-3′) and R-167 (5′-CACAACA AAGGATAAAGAAAGC-3′), respectively. In addition, the primers used to confirm the inserted *ES1pro::LhG4* were ES1-F1 (5′-TTCCCCAAGTTTAGGAGAATA TTGACTCC-3′) and LhG4-R1 (5′-ATTGCCCTTCACCGCCTGGCC-3′). Primers pairs ANT-F1 (5′-AGAAAAATGGTGTGTTCGTTGTGTA-3′) and LhG4-R1 were used to confirm the inserted *ANTpro::LhG4*.

AtARF promoter reporter lines were obtained from Professor Dolf Weijers (Rademacher et al. 2011). *TM* and *DM* reporter lines were obtained from Professor Rita Gross-Hardt. The *ANTpro::LhG4*-transformed line was obtained from Professor Yuval Eshed (Alvarez et al. 2006). The *ES1pro::GUS* reporter line and the *ES1pro::LhG4*-transformed line were stored in our laboratory (Yu et al. 2005, Pagnussat et al. 2009). All materials were in a Ler background. Plants were grown in a growth chamber in a 16 h light/8 h dark cycle at 22°C with 60% relative humidity. For the crosses, flowers of the female parent were manually emasculated 24 h before anthesis and then cross-pollinated after 24 h.

Generation of transgenic plants with down-regulated *ARF* genes

The obtained transgenic *Op::amiRARF234*, *Op::amiRARFMP* and *Op::MIR167a* lines (each at least 10 lines) were separately crossed with the *ES1pro::LhG4* line. The F_1 progeny with defective embryo sacs and seed abortion at ratios close to the expected 0.25 were used for further study. To check the specificity of *amiRARF234* and *amiRARFMP*, the transgenic *Op::amiRARF234* and*Op::amiRARFMP* plants were further separately crossed with the *ANTpro::LhG4* line, and F_1 progeny were morphologically observed.

RNA extraction and qRT-PCR analysis

Leaves of *ANTpro >>amiRARF234* and *ANTpro >>amiRARFMP* lines were sampled, with the *ANTpro::LhG4* line as control. RNA extraction, reverse transcription and quantitative real-time PCR (qRT–PCRs) were performed as previously described (Liu et al. 2013) using the primers listed in Supplementary Table S2. The *Actin7* gene was used as the reference gene. The specificity of the reactions was verified by melting curve analysis, and the products were further confirmed by agarose gel electrophoresis. The comparative $\Delta\Delta^{CT}$ method was used to calculate the relative expression levels of different genes.

Histology and microscopy

Developing flowers were dissected and incubated in GUS staining buffer as previously described (Pagnussat et al. 2007). To determine fluorescence signals, individual ovules were dissected from the pistils in 0.1 M phosphate buffer (pH 7.0) and observed under a Zeiss 2 Axioplan imaging microscope. Anthers were directly imbedded in phosphate buffer for microscopy. To prepare cleared whole-mount samples, pistils containing at least 20 ovules were dissected and cleared overnight in Hoyer's solution (Liu and Meinke 1998). The ovules were subsequently examined under DIC optics.

Anthers were dissected to release pollen grains and then incubated in various histochemical stains as previously described (Huang et al. 2009). DAPI was used to stain the nuclei of pollen grains, and Alexander stain was used to detect pollen viability.

For *in vitro* pollen germination, pollen grains were collected from newly opened flowers and then cultured in germination medium as previously described (Bou Daher et al. 2009). The number of germinating pollen grains was counted under a microscope.

For SEM, pollen grains were observed under a scanning electronic microscope XL-40 (Philips) and then palladium–gold coated using standard techniques under vacuum desiccation.

For TEM, floral buds were fixed with 2.5% glutaraldehyde (containing 0.01% Tween-20), allowed to sink to the bottom of the solution through evacuation, rinsed with 0.1 M phosphate buffer and then immersed in 1% osmic acid for 1 h. The specimens were washed again in phosphate buffer and then dehydrated through an ethanol series up to 80% ethanol. The samples were embedded in Spurr's resin, and ultrathin sections were stained with uranyl acetate and lead citrate before viewing under a JEM-1230 transmission electron microscope operating at 80 kV.

For paraffin embedding and staining, floral buds at floral development stage 13 (Smyth et al. 1990) were fixed in FAA (formalin, acetic acid, ethyl alcohol), dehydrated in an ethyl alcohol series, embedded in paraffin wax, sectioned using a Leica rotary microtome to approximately 8–10 μm thickness, and then stained with ferroalumen–hematoxylin. The stained sections were observed under a Leica microscope.

For the seed abortion analysis, immature siliques were dissected under an anatomical lens and were observed under a Leica stereoscopic microscope. Inflorescences and flowers were directly observed under a stereoscopic microscope for microscopy analysis.

Supplementary data

Supplementary data are available at PCP online.

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Z.N.L. carried out the experiment, analyzed the data, and drafted and wrote the manuscript. L.M.M. and L.J.K. performed the pollen grain germination analysis. X.L.Y. and H.R.X. performed the embryo sac analysis. X.Y.S. performed the *AtARF* gene promoter analysis. C.J., V.S. and X.L.Y. proposed and supervised the research. All authors read and approved the final manuscript.We thank Professor Dolf Weijers for sharing *AtARF* promoter reporter lines, Professor Rita Gross-Hardt for sharing *TM* and *DM* reporter lines, and Professor Yuval Eshed for sharing the *ANTpro::LhG4*-transformed line.

Disclosures

The authors have no conflicts of interest to declare.

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