RESEARCH ARTICLE

Fine regulation of ARF17 for anther development and pollen formation

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Abstract

Background: In Arabidopsis, the tapetum and microsporocytes are critical for pollen formation. Previous studies have shown that ARF17 is expressed in microsporocytes and tetrads and directly regulates tetrad wall synthesis for pollen formation. ARF17 is the direct target of miR160, and promoterARF17::5mARF17 (5mARF17/WT) transgenic plants, which have five silent mutations within the miR160-complementary domain, are sterile.

Results: Here, we found that ARF17 is also expressed in the tapetum, which was defective in arf17 mutants. Compared with arf17 mutants, 5mARF17/WT plants had abnormal tapetal cells and tetrads but were less vacuolated in the tapetum. Immunocytochemical assays showed that the ARF17 protein over-accumulated in tapetum, microsporocytes and tetrads of 5mARF17/WT plants at early anther stages, but its expression pattern was not affected during anther development. 5mARF17 driven by its native promoter did not rescue the arf17 male-sterile phenotype. The expression of 5mARF17 driven by the tapetum-specific promoter A9 led to a defective tapetum and male sterility in transgenic plants. These results suggest that the overexpression of ARF17 in the tapetum and microsporocytes of 5mARF17/WT plants leads to male sterility. Microarray data revealed that an abundance of genes involved in transcription and translation are ectopically expressed in 5mARF17/WT plants.

Conclusions: Our work shows that ARF17 plays an essential role in anther development and pollen formation, and ARF17 expression under miR160 regulation is critical for its function during anther development.

Keywords: Anther, Tapetum, Male sterility, ARF17, 5mARF17

Background

In flowering plants, male reproductive processes occur in the stamen. After meiosis, haploid microspores further develop into pollen grains within the locules. Each locule contains four somatic layers that surround microsporocytes/microspores/pollen [1]. The pollen wall is typically composed of an exine and intine. The innermost layer of locule sporophytic tissue, the tapetum, is in direct contact with the microspores to provide necessary nutrition and pollen wall materials for pollen grain maturation [2, 3]. The deposition pattern of the exine is determined by callose formation during the tetrad stage [4, 5].

Auxin plays an important role in plant male reproductive development [6, 7]. Both auxin biosynthesis and transportation are involved in pollen development [8–11]. Auxin response factors (ARFs) respond to auxin; ARFs can be targeted to auxin response elements of downstream genes and can function as transcriptional activators or repressors [12, 13]. Several ARFs are involved in anther development and pollen formation. ARF1 and ARF2 regulate flowering time and dehiscence [14], and ARF6 and ARF8 facilitate gynoecium and stamen development [15, 16]. Loss of function of ARF17 directly affects the expression of CALLOSE SYNTHASE 5 (CALSS), which is critical for callose synthesis. arf17 mutants show a thin callose wall and an abnormal exine pattern, resulting in male sterility [4, 17].

MicroRNAs (miRNAs) are 21 nucleotides long and are negative regulators of gene expression in both plants and animals [18]. Most plant miRNAs have the function of cleaving target mRNAs that have a perfect or nearly
perfect complementary sequence [19]. In Arabidopsis, miR167 regulates ARF6 and ARF8 expression for ovule and anther development [15]. miR160 is complementary to the transcripts of ARF10, ARF16 and ARF17. Changing 5 bases of the miR160 recognition site without altering the amino acid sequence of the ARF17 protein (5mARF17) prevents miR160-directed ARF17 cleavage [20]. Promoter-ARF17::5mARF17 transgenic plants (5mARF17/WT) are sterile [20]. However, the details and mechanism of the effects of 5mARF17 on plant fertility are not clear.

Here, we show that ARF17 is important not only for microsporocyte/tetrad development but also for tapetum development. ARF17 protein was over-accumulated in the tapetum and microsporocytes, and this over-accumulation led to defects in 5mARF17/WT plants. Therefore, fine regulation of ARF17 is critical for tapetum development and pollen wall formation.

**Results**

**ARF17 is essential for tapetum development**

Previous investigations have shown that ARF17 directly regulates the expression of CALSS for tetrad wall formation. In arf17 mutants, tetrad walls are much thinner than those of wild type (WT), and the pollen wall pattern is defective [17]. In this work, we found that tapetum development was also defective in arf17 mutants and was abnormally vacuolated during development from stage 7 to stage 9 (Fig. 1a, b). Previous investigations have shown that the ARF17-GFP signal is localized in microsporocytes, microspores and mature pollen instead of tapetal cells [17]. Here, we rescanned ARF17-GFP signals using confocal laser scanning microscopy in pARF17::ARF17-GFP/arf17 complementary plants (T3 generation) in which ARF17-GFP fully restored the arf17 male-sterile phenotype. The ARF17-GFP signal was detected in tapetal cells at stage 5, but then the signal weakened from stage 6 to 7 (Fig. 1c). The localization of ARF17-GFP in tapetal cells was consistent with the defective tapetum in the arf17 mutants. These results suggest that ARF17 is also essential for tapetum development.

**5mARF17/WT plants are defective in both the tapetum and microsporocytes**

ARF17 is among the miR160 targets in Arabidopsis, and promoter-ARF17::5mARF17 (5mARF17/WT) transgenic plants are sterile [20, 21]. To better understand the role of ARF17 in male reproduction, we performed the same 5mARF17 construction and obtained 5mARF17/WT transgenic plants, which showed complete sterility as previously described (Additional file 1: Figure S1a-c [20]). Alexander staining showed no mature pollen in
the 5mARF17/WT plants, which was similar to observations in arf17 mutants (Fig. 2a-c). Reciprocal crosses with WT indicated that female fertility was not affected. Half of the F1 generation of SmARF17/WT plants pollinated with WT showed the male-sterile phenotype. PCR demonstrated that all these male-sterile plants contained the SmARF17 fragment and that none of the fertile plants contained this fragment (Additional file 1: Figure S1d). These results suggest that SmARF17 acts as a dominant gene for male sterility. Semi-thin sections showed no significant defects in anther development in the SmARF17/WT plants before stage 6. Tetrads formed normally in the SmARF17/WT plants at stage 7 (Fig. 2d), but the callose cell wall was much thinner than that in the WT plants (Additional file 1: Figure S1e-g). Tapetum development was also abnormal in the SmARF17/WT plants (Fig. 2d) but was less vacuolated than that in the arf17 mutants (Fig. 1a). At stage 8, individual microspores were observed in the SmARF17/WT plants (Fig. 2d). Transmission electron microscopy (TEM) revealed that the exine of the SmARF17/WT plants was abnormal (Fig. 2e). After stage 8, the microspores became vacuolated and then degraded in the SmARF17 plants (Fig. 2d). Therefore, both the tapetum and tetrad were defective in the SmARF17/WT plants, which led to pollen rupture and male sterility.

SmARF17 does not affect the expression of native ARF17

The phenotype of the SmARF17/WT plants regarding male development was similar to that of the arf17 mutants, except the tapetal cells (Fig. 1, Additional file 1: Figure S1a-c, Fig. 2d and e). To determine whether the SmARF17 transgene affected the expression of the native ARF17, SmARF17/WT plants were pollinated with pollen.
from ARF17-GFP/arf17 plants to obtain ARF17-GFP/5mARF17 plants. Because ARF17-GFP can complement the arf17 phenotype, the ARF17-GFP signal represents the expression of native ARF17. In the 5mARF17/WT plants, no GFP signals were observed (Fig. 3a). In the ARF17-GFP/5mARF17 plants, the ARF17-GFP signals were detected in microsporocytes, tetrads and microspores (Fig. 3b). The expression pattern of ARF17-GFP in the ARF17-GFP/5mARF17 plants was similar to that in the ARF17-GFP/arf17 plants (Fig. 1c), which represented the native expression. Thus, the 5mARF17 transgene did not affect the expression of the native ARF17.

5mARF17 cannot rescue the sterility of arf17

The 5mARF17 transgene encodes the same amino acid sequence as does ARF17 [20]. To investigate whether 5mARF17 fulfilled the same function as ARF17, we crossed 5mARF17/WT plants with pollen from a heterozygous ARF17/arf17 plant. The F1 plants had the 5mARF17 transgene in the ARF17/arf17 background and were further crossed with an ARF17/arf17 plant to obtain a plant with the 5mARF17 transgene and an arf17 background (5mARF17/arf17) (Fig. 4a, Additional file 2: Figure S2a and b). We found that the 5mARF17/arf17 plants remained male sterile (Fig. 4b). The results of qRT-PCR showed that the expression level of ARF17 in the 5mARF17/arf17 plants was higher than that in the WT (Fig. 4c). Semi-thin sections of the anthers of the 5mARF17/arf17 plants showed similar defects in the tapetum and microspores to those in the 5mARF17/WT plants (Figs. 2d and 4d). However, the tapetum showed less vacuolation than did the arf17 mutant (Fig. 1a). Therefore, the 5mARF17 transgene could partly rescue the tapetum development rather than recover male fertility in the arf17 mutants. In our previous work, the same promoter of ARF17 used to construct pARF17::ARF17 complemented the arf17 mutant phenotype [17]. These results indicate that the escape of ARF17 expression from miR160 regulation leads to developmental defects in the anthers as well as male sterility.

ARF17 protein over-accumulates in the tapetum and microsporocytes of 5mARF17/WT plants

In a previous study, miR160 failed to cleave 5mARF17 mRNA, and ARF17/5mARF17 mRNA over-accumulated in 5mARF17/WT plants [20]. To determine whether ARF17 protein also over-accumulated in the 5mARF17/WT plants, we constructed a promoterARF17::5mARF17-GFP construct and then introduced it into a WT plant to obtain 5mARF17-GFP/WT transgenic plants. The 5mARF17-GFP/WT plants also showed a male-sterile phenotype and a similar segregation ratio as that of the 5mARF17/WT plants (Additional file 3: Figure S3a-c). Confocal laser scanning microscopy was used to investigate GFP signals. During anther stages 5 to 7, 5mARF17-GFP protein was expressed in the microsporocytes, microspores and tapetum. However, the GFP signals in the 5mARF17-GFP/WT plants were much more diffuse than those of the ARF17-GFP plants.
Defects in the tapetum and microsporocytes could lead to the diffuse pattern of the ARF17-GFP signal in the 5mARF17-GFP/WT plants. To confirm whether ARF17 over-accumulated in the 5mARF17/WT plants, an immunohistochemical assay was employed using a GFP antibody to detect the accumulation of both 5mARF17-GFP in the 5mARF17/WT plants and ARF17-GFP in the ARF17-GFP/arf17 plants. In the ARF17-GFP/arf17 plants, the ARF17-GFP signal was observed in microsporocytes, tapetum, tetrads and microsperes from stage 5 to stage 9 (Fig. 5b). In the 5mARF17-GFP/WT plants, 5mARF17-GFP proteins were more highly accumulated in the tapetum and microsporocytes than were the ARF17-GFP proteins in the ARF17-GFP/arf17 plants at stage 5. At stages 6–8, 5mARF17-GFP proteins continued to be expressed in the tapetum and microsperes. Then, we harvested young buds (anther stage 5) from both 5mARF17-GFP/WT and WT plants and investigated the expression of ARF17 via qRT-PCR. The results of the qRT-PCR show that the expression of ARF17 in the 5mARF17-GFP/WT plants was higher than that in the WT plants (Additional file 4: Figure S4c). The GFP signal in the 5mARF17-GFP/WT transgenic plants were male sterile (Fig. 6a, b), and the segregation ratio was similar to that of 5mARF17/WT plants (Additional file 4: Figure S4a). The results of qRT-PCR showed that the expression of ARF17 in the pA9::5mARF17-GFP/WT plants was higher than that in the WT plants (Additional file 4: Figure S4c). The GFP signal in the pA9::5mARF17-GFP/WT plants was restricted to the tapetum (Fig. 6c). Semi-thin sections showed the defects in tapetum development and pollen formation of the pA9::5mARF17-GFP/WT plants; these defects were similar to those of 5mARF17/WT plants (Figs. 6d and 6d). These results show that the over-accumulation of

Transgenic plants containing 5mARF17 driven by the tapetum-specific promoter A9 are male sterile

Both the tapetum and microsporocytes contribute to pollen formation. To determine whether the overexpression of ARF17 in the tapetum leads to microspore abortion and male sterility, we constructed a promoter-A9::5mARF17-GFP construct and introduced it into a WT plant. The A9 promoter can drive gene expression specifically in the tapetum from anther stages 5 to 9 [22, 23]. We found that the promoterA9::5mARF17-GFP/WT transgenic plants were male sterile (Fig. 6a, b), and the segregation ratio was similar to that of 5mARF17/WT plants (Additional file 4: Figure S4a).
ARF17 in the tapetum was sufficient to lead to tapetum defects and plant sterility.

Identification and overexpression of up-regulated genes in SmARF17/WT plants

Our previous work demonstrated that ARF17 functions as a transcriptional activator [17]. To identify the affected genes in SmARF17/WT plants, we performed a microarray analysis using young buds from the SmARF17/WT and WT plants. A total of 755 genes were up-regulated in SmARF17 plants compared with WT plants (Additional file 5: Table S3). Based on the BAR information (http://bar.utoronto.ca), 220 (29.14%) genes are ectopically expressed, and 339 (44.90%) are overexpressed in SmARF17/WT plants (anther stage 5–7) (Additional file 5: Table S3). PANTHER (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org) [24] analysis indicated that 316 known genes are overexpressed in SmARF17/WT buds (Fig. 6a; Additional file 6: Table S4), with 77 of the 316 genes associated with nucleic acid binding (24.4%). In this group, DNA helicase, RNA binding protein, ribosomal protein and histones were highly enriched (Additional file 6: Table S4). These results indicate that overexpression of ARF17 may lead to alterations of normal gene expression, resulting in disruption of anther and tapetum development.

To test whether the expression of some of the up-regulated genes in the SmARF17/WT plants affect anther development and pollen formation, we fused some of the genes to CaMV35S or A9 promoters, and the
resulting constructs were introduced into WT plants. AT1G48640 encodes a transmembrane amino acid transporter family gene that is involved in root development [25]. 12/12 transgenic lines with over-expression of AT1G48640 were fertile in promoterA9::AT1G48640/WT transgenic plants (Additional file 7: Figure S6B, Fig. 7b), but their pollen grains adhered together (Fig. 7d, e), and scanning electron microscopy (SEM) showed defects in pollen wall structure in those plants (Fig. 7j, k).

Zinc-finger protein 1 (ZF1) functions as a transcriptional repressor and is involved in the inhibition of plant growth under abiotic stress conditions [25–27]. 8/8 transgenic lines with over-expression of ZF1 showed reduced fertility in CaMV35s::ZF1/WT plants (Additional file 7: Figure S6b, Fig. 7f and g). Some pollen was aborted and adhered together in the CaMV35s::ZF1/WT plants (Fig. 7h, i), and SEM showed that pollen wall formation was also defective in these plants (Fig. 7j, l).

**Discussion**

**ARF17 is important for both tapetum and microsporocyte development**

ARF17 is a member of the ARF family in *Arabidopsis*. In arf17 mutants, all pollen is ruptured, and plants show male sterility [17]. During anther development, the microsporocyte/tetrad determines the pollen wall pattern, whereas tapetal cells provide both nutrition and pollen wall materials for pollen formation. ARF17 is expressed in microsporocytes and tetrads during anther development and directly regulates the expression of CALS5 for both tetrad wall synthesis and exine pattern formation [17]. In this work, ARF17 expression was also detected in the tapetum based on the ARF17-GFP signal and immunocytochemical assay results (Figs. 1c and 5b). The ARF17-GFP signals were detected in the tapetum at an early stage for a short period. In addition, the signal was much weaker than that in the microsporocytes/tetrads (Fig. 1c), with the weaker signal the primary reason that the expression was not detected in the tapetum in previous studies [17]. However, the short expression period was important for the development of the tapetum, with the tapetum clearly defective in the arf17 mutants (Fig. 1a). After stage 4, a key genetic pathway of DYT1-TDF1-AMS is important for tapetum development and pollen wall formation [28–31]. DYT1 is the earliest transcription factor required for tapetum development and is initially detected at approximately stage 5 [28]. In the arf17 mutants, DYT1, TDF1, and AMS expression was not significantly affected (Additional file 8: Figure S5a), and in dyt1 and tdf1 mutants, ARF17 transcription was not dramatically affected (Additional file 8: Figure S5b). Therefore, ARF17 plays a critical role in early tapetum development and may be independent of the DYT1-TDF1-AMS pathway.

**Overexpression of ARF17 in 5mARF17/WT plants leads to defective microsporocytes and tapetum**

Previous studies have shown that 5mARF17/WT plants are sterile [20]. Reciprocal crosses with wild-type plants...
showed that the female parts are not affected in the 5mARF17/WT plants (Additional file 1: Figure S1d). The 5mARF17/WT plants had no mature pollen inside anther locules, and both the tapetum and microsporocytes/tetrads were defective (Fig. 2a-e). Overall, the cellular defects of anther development were similar between the arf17 and 5mARF17/WT plants. However, the defective tapetum development in the arf17 mutants is more severe than that in the 5mARF17/WT plants after stage 6 (Additional file 7: Figure S6a). In the arf17 mutants, the loss of ARF17 function led to pollen rupture and male sterility. In 5mARF17/WT plants, the transgene 5mARF17 did not affect the expression of the native ARF17 (Fig. 3a and b). Although 5mARF17 encodes the same protein as does ARF17, it could not complement the male sterility of arf17 mutants (Fig. 4b). Hence, the

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**Fig. 7** Up-regulated genes in a 5mARF17/WT plant. **a** Panther Go-Slim protein classes of up-regulated genes in 5mARF17/WT plants compared with those in WT. The total hits included 316 genes. The percentage of each term is the clustered gene number of the total protein class hits: calcium binding (5 genes), cell adhesion molecule (3 genes), chaperone (13 genes), cytoskeletal protein (22 genes), defense immunity protein (1 gene), enzyme modulator (12 genes), hydrolase (30 genes), isomerase (5 genes), ligase (15 genes), lyase (6 genes), membrane traffic protein (6 genes), nucleic acid binding (77 genes), oxidoreductase (20 genes), receptor (7 genes), signaling molecule (8 genes), storage protein (3 genes), structural protein (1 gene), transcription factor (18 genes), transfer/carryer protein (11 genes), transferase (26 genes), transmembrane receptor regulatory/adaptor protein (1 gene), and transporter (26 genes). **b** The phenotypes of promoterA9::AT1G48640 and CAMV35s::ZF1 plants. **b** Quantitative RT-PCR analysis of AT1G48640 expression in WT, 5mARF17/WT and promoterA9::AT1G48640/WT buds. **c** A 35-day-old promoterA9::AT1G48640/WT plant. **d** and **e** Alexander’s staining of the anthers and pollen grains of a promoterA9::AT1G48640/WT plant. Bar = 20 μm. **f** Quantitative RT-PCR analysis of ZF1 expression in WT, 5mARF17/WT and CAMV35s::ZF1/WT buds. **g** A 35-day-old CAMV35s::ZF1/WT plant. Bar = 2 cm. **h** and **i** Alexander’s staining of the anthers and pollen grains of a CAMV35s::ZF1/WT plant. Bar = 20 μm. **j** Scanning electron microscopy (SEM) showing the pollen wall structures of a WT plant (**j**), a promoterA9::AT1G48640/WT plant (**k**) and a CAMV35s::ZF1/WT plant (**l**). Bars = 5 μm. The levels of AT1G48640 and ZF1 were normalized to those of tubulin and compared with those of WT. Error bars indicate SD and were calculated from three biological replicates.
mechanisms that lead to the male sterility of both 5mARF17/WT and arf17 plants are different. In the 5mARF17/WT plants, ARF17 protein over-accumulated in the tapetum and microsporocytes (Fig. 5a, b). The tapetum-specific over-accumulation of ARF17 was sufficient to cause male sterility, which was similar to that observed for the 5mARF17/WT plants (Fig. 6a-d). Therefore, the overexpression of ARF17 in the tapetum of the 5mARF17/WT plants prevents normal tapetum function. The microsporocytes/tetrad are also defective in the 5mARF17/WT plants. It is likely that the overexpression of ARF17 in the microsporocytes/tetrad of the 5mARF17/WT plants leads to its male sterility. ARF17 directly regulates the expression of CALS5, which is needed for tetrad wall formation. In the arf17 mutants, the expression of CALS5 is significantly reduced [17]. The 5mARF17/WT plants also show defective tetrad walls and reduced expression of CALS5 (Additional file 1: Figure S1e-h). It is likely that these phenomena are a side effect of defective microsporocyte/tetrad development in the 5mARF17/WT plants. The results of the microarray analysis showed that 5mARF17 activates the ectopic expression of many genes in the anthers of the 5mARF17/WT plants. Overexpression of two of these genes, AT1G48640 and ZF1, slightly affected pollen formation and plant fertility (Fig. 7b-l). Therefore, the male sterility of the 5mARF17/WT plants resulted from the ectopic expression and overexpression of these genes, including AT1G48640 and ZF1.

Expression of ARF17 is precisely regulated during anther development

ARF17 is among the targets of miR160 [20, 32], and the knockout of ARF17 leads to male sterility [17]. In this work, we demonstrated that without miR160 control in 5mARF17/WT plants, ARF17 was overexpressed, which led to male sterility. Therefore, the expression level of ARF17 is critical for pollen development. The expression pattern of 5mARF17-GFP was apparently similar to that of ARF17-GFP (Fig. 5b). Therefore, the ARF17 promoter determined the cell and tissue specificity of ARF17 expression, whereas miR160 controlled the level of ARF17 expression. In Arabidopsis, three precursors, including miR160a, miR160b and miR160c, can produce miR160 [20]. In humans, the TRISTETRAPROLINE (TTP) protein is a member of the RNA-induced silencing complex (RISC) [33, 34]. AtTTP is an ortholog of hTTP in Arabidopsis and is expressed in microsporocytes, tetrads and tapetal cells, and AtTTP expression highly overlapped with that of ARF17. Overexpression of AtTTP decreases the level of mature miR160, whereas the expression of ARF17 increases it [35]. Thus, AtTTP is involved in miR160 maturation for the fine regulation of ARF17 for pollen formation during anther development.

Conclusion

ARF17 is the target of miR160, and 5mARF17/WT plants show male sterility as do arf17 plants. In this study, we showed that ARF17 plays an essential role in anther development and pollen formation. Without miR160 regulation, the expression pattern of ARF17 in the anthers is not affected, but its expression level is significantly elevated in 5mARF17/WT plants. The overexpression of ARF17 in 5mARF17 plants leads to defects in pollen formation and plant sterility.

Methods

Plant materials and growth conditions

In this study, Arabidopsis (Arabidopsis thaliana) wild-type (WT), transgenic and mutant plants in the Col-0 ecotype background were used. arf17 mutants and 5mARF17/WT plants were preserved in the laboratory of Z.N. Yang. Seeds were sown on vermiculite and allowed to imbibe for 2 to 3 days. Plants were grown under conditions of 16 h light/8 h darkness in a growth chamber for approximately 22 days.

Microscopy

The plants were imaged with a Cyber Shot T-20 digital camera (Sony). Alexander staining solution was prepared as described [23]. The anthers were stained for approximately 2–12 h at room temperature (RT, 22 °C). For semi-thin sections, the flower buds were fixed and embedded in Spurr’s resin. Semi-thin sections were prepared by cutting the buds to a thickness of 1 μm followed by incubation in a 0.01% toluidine blue/sodium borate solution for 5–10 min at 42 °C, after which the sections were washed with water. The sections were observed with an Olympus BX51 microscope under bright-field microscopy.

Fluorescence microscopy

For callose staining, anthers at the tetrad stage were squeezed onto a slide and stained with toluidine blue as described previously [17]. GFP signals in the anthers of ARF17-GFP/arf17, 5mARF17-GFP/WT, and ARF17-GFP/5mARF17 plants were observed using a Carl Zeiss confocal laser scanning microscope (LSM 5 PASCAL).

Electron microscopy

For TEM examination, flower buds were fixed in 0.1 M phosphate buffer (pH 7.2) with 2.5% glutaraldehyde (v/v) and then washed several times with PBS (pH 7.4), followed by dehydration with ethyl alcohol and replacement by propylene epoxide. The samples were embedded in Spurr’s resin and polymerized for 48 h at 60 °C as described previously [23, 30]. Then, TEM microscopy (JEOL, Japan) was used to observe the slides. For SEM observations, fresh pollen grains were coated with 8 nm...
of gold and observed under a JSM-840 microscope (JEOL) [23].

Plasmid construction and identification of transgenic plants
The promoterARF17:5mARF17 genomic region was cloned using ARF17 primers (ProARF17-F/R) and (5mARF17g-F/R) to complement the wild type (WT) in accordance with Mallory identification [20]. The promoterA9:SmARF17-GFP fragment was constructed with the A9 promoter (ProA9-F/R) and the SmARF17 genomic region to complement the WT. For GFP fusion, the SmARF17 genomic fragment without a stop codon was cloned into a modified GFP-pCAMBIA1300 vector using primers (5mARF17g-F/5mARF17g-GFP-R) to complement the WT. The promoterA9:AT1G48640 fragment was constructed with the A9 promoter (ProA9-F/R) and the AT1G48640 CDS (4864-F/4864-R) region transferred into the WT. The CaMV35ZFI fragment was constructed with the CAMV35 promoter (35 s-F/35 s-R) and the ZFI CDS (ZFI1-F/ZFI1-R) region introduced into the WT. These fragments were amplified using KOD polymerase (Takara Biotechnology) and cloned into the pCAMBIA1300 and GFP-pCAMBIA1300 vectors (CAMBIA). The fragments were verified by sequencing. The plasmids were transformed into Agrobacterium tumefaciens (GV3101) and screened using 50 mg/ml kanamycin, 40 mg/ml gentamicin and 50 mg/ml rifampicin. The bacteria containing the plasmid constructs were introduced into the flower buds. The transgenic plants were selected using 20 mg/l hygromycin. The primer sequences are listed in Additional file 9: Table S1.

Immunohistochemical staining
For immunohistochemical staining, flower buds were fixed in formaldehyde/acetic acid for 1 day, dehydrated in an ethanol gradient and embedded in wax. Sections that were 8 mm thick were prepared using a rotary microtome (MR2; RMC). The sections were incubated in boiled citrate buffer (pH 6.4) for 10–15 min after rehydration and then cooled to RT. The slides were washed twice in PBS (pH 7.4) for 5 min. Then, the sections were blocked with 5% BSA in PBS for 30 min to 1 h. Rabbit anti-6 × GFP antibodies (Thermo Scientific) were diluted 1:100 in PBS (pH 7.4), and the slides were then incubated at 4 °C overnight. Then, the slides were washed three times in Tris-buffered saline solution (TBS; pH 7.4) for 5 min and incubated with anti-rabbit-AP antibodies diluted 1:200 in TBS (pH 7.4) for 1 h at RT. The slides were washed three times in TBS for 5 min, and BCIP/NBT solution (CWBiO) was used for colorimetric detection at RT.

Quantitative RT-PCR
Total RNA was extracted from the flower buds of the WT, transgenic and mutant plants using a TRIzol kit (Invitrogen). In accordance with the manufacturer’s instructions (Toyobo), first-strand complementary DNA (cDNA) was synthesized. Quantitative RT-PCR was performed using an ABI PRISM 7300 detection system (Applied Biosystems) with SYBR Green I master mix (Toyobo). The primers ARF17qRT-F/R were used to detect the expression level of ARF17. The relative expression levels were calculated according to the cycle numbers. The qRT-PCR results are shown as the relative expression levels normalized to those of tubulin. The positive control was the tubulin gene (TUB-F/R), and three replicates were performed for each experiment. The relevant primer sequences are listed in Additional file 10: Table S2.

Microarrays
Microarrays were performed according to a previously described procedure [30]. Young buds collected from WT and SmARF17/WT plants were immediately frozen in liquid nitrogen. A Low-RNA-Input Linear Amplification Kit (Agilent Technologies) was used to amplify and label the total RNA. 5-(3-Aminoallyl)-UTP (Ambion), Cy3 NHS ester (GE Healthcare Biosciences) and Cy5 NHS ester (GE Healthcare Biosciences) were applied following the manufacturers’ instructions. The labeled cRNA was purified using an RNeasy Mini Kit (Qiagen). According to the manufacturer’s instructions, each 44-K Arabidopsis oligo microarray slide was hybridized with 825 ng of Cy3-labeled cRNA and 825 ng of Cy5-labeled cRNA using a gene expression hybridization kit (Agilent) in a hybridization oven (Agilent). The slides were scanned using an Agilent Microarray Scanner (Agilent) and Feature Extraction software 10.7 (Agilent) with the default settings. Three biological replicates of independently grown materials were used. The raw data were normalized with a locally weighted scatter plot smoothing (Lowess) algorithm using Gene Spring Software 11.0 (Agilent).

Additional files

Additional file 1: Figure S1. The phenotype, segregation analysis and expression of CALSS in SmARF17/WT plants. A-C A 35-day-old wild-type plant (WT) (A); SmARF17/WT plant (B) and arf17 mutant (C). Bars = 2 cm. (D) Transmission efficiency of SmARF17/WT mutants. TE, female transmission efficiency; TEM, male transmission efficiency; NA, no application. E-G Aniline blue staining of the callose from a WT plant (E); a 5mARF17/WT plant (F) and an arf17 mutant (G). Bars = 20 μm. (H) Quantitative RT-PCR analysis of CALSS expression in WT, SmARF17 and arf17 buds. The level of CALSS was normalized to that of tubulin and compared with that of WT. Error bars indicate SD and were calculated from three biological replicates. (TIFF 5996 kb)

Additional file 2: Figure S2. Identification of sequences in a SmARF17/arf17 plant. A Genomic PCR analysis of a SmARF17/arf17 plant background. B Clone sequencing of 5 bases in SmARF17/arf17 plants. (TIFF 4304 kb)
Additional file 3: Figure S3. Phenotype and segregation analyses of SmARF17-GFP/WT plant. A and B A 35-day-old SmARF17-GFP/WT plant (A) and ARF17-GFP/SmARF17 plant (B). Bars = 2 cm. (CI) Transmission efficiency of a SmARF17-GFP/WT plant. TE; female transmission efficiency; TEma; male transmission efficiency; NA; no application. (TIFF 1132 kb)

Additional file 4: Figure S4. Transmission efficiency of a pA9::SmARF17-GFP/WT plant. A Transmission efficiency of a promoterA9::SmARF17-GFP/WT (pA9::SmARF17-GFP/WT) plant. B Quantitative RT-PCR analysis of ARF17 expression in WT and SmARF17/WT buds at stage 5. C Quantitative RT-PCR analysis of ARF17 expression in WT and pA9::SmARF17-GFP/WT buds. The level of ARF17 was normalized to that of tubulin and compared with that of WT. Error bars indicate SD and were calculated from three biological replicates. TE; female transmission efficiency; TEma; male transmission efficiency; NA; no application. (TIFF 491 kb)

Additional file 5: Table S3. List of up-regulated genes in SmARF17/WT that are typically expressed or absent in WT buds. (XLSX 65 kb)

Additional file 6: Table S4. Protein classification of up-regulated genes by PANTHER. (XLS 116 kb)

Additional file 7: Figure S5. Semi-thin sections of anthers and PCR analysis. A A semi-thin sections of anthers in WT, arf17 and SmARF17/WT plants from stage 5 to 9. Bar = 20 μm. B Identification of transgenic sequences in WT, promoterArf17; AT1G48640/WT and CAMV1SS:ZF4/WT. DMsp, degenerated microspore; MC, meiotic cell; MMC, microspore mother cell; Msp, microspore; T, tapetum; Tds, tetrads. (TIFF 23830 kb)

Additional file 8: Figure S5. Relative expression of tapetum- and pollen-formation genes in SmARF17/WT and arf17 plants. A A Quantitative RT-PCR analysis of the expression levels of genes involved in tapetum and pollen development. B Quantitative RT-PCR analysis of ARF17 expression in dty1 and tdf1 mutants. The levels of DTY1, TDF1, TEK, CDKG1, RP51, M5188, M51, ACCOX, and ARF17 were normalized to those of tubulin and compared with those of WT. Error bars indicate SD and were calculated from three biological replicates. (TIFF 937 kb)

Additional file 9: Table S1. List of primers used in clones and identification. (XLS 21 kb)

Additional file 10: Table S2. List of primers used in qRT-PCR. (XLS 22 kb)

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Availability of data and materials
The data supporting the results of this article are included within the article and its additional files. The gene and promoter sequences of ARF17 were deposited in TAIR (https://www.arabidopsis.org) under accession number AT1G77850.

Authors’ contributions
ZXL and ZNY analyzed the experiments; XZF and ZXY conceived the original screening and research plans; ZNY supervised the experiments; BW performed most of the experiments; YYH, SQ; and JXZ were involved in performing the experiments; BW and XZF designed the experiments, analyzed the data and wrote the article with contributions from JSX and XZY; and ZNY reviewed and edited the writing. All authors read and approved the final manuscript.

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