ARABIDOPSIS POLLEN ABORTION MUTANT 2, Encoding an Unknown Protein with Three WD40-Repeats, Is Essential for Late Pollen Mature in Arabidopsis thaliana

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Abstract

Background

In flowering plants, pollen formation is a very complex process. It is strictly regulated by various genetic factors. Some of these factors have been identified, but the regulatory mechanism concerning the process of late pollen maturing remains unknown.

Results

Previously we identified and reported an ARABIDOPSIS POLLEN ABORTION MUTANT 2 (APAM2) gene which was required for pollen formation in Arabidopsis thaliana. In this study, we further analysed the phenotype of atapam2 and the expression pattern of AtAPAM2. Mutation in AtAPAM2 drastically affected male gametophytic function, impacted the late pollen mature process, and resulted in mature pollen grains from atapam2/+ plants being shrivelled and dead. It was expressed among seedlings, roots, stems, leaves, siliques, flowers, and pollen grains. However, its expression levels in flowers and pollen grains were much higher than in other tissues. AtAPAM2 encodes an unknown protein with three WD40-repeats with localisation in plasma membrane and nucleus, and homologous proteins exist in many species.

Conclusions

We determined that AtAPAM2 may play a role in pollen formation and other development processes by interacting with other proteins, but we did not identify the protein that interacts with it.

Background

In flowering plants, the male gametophyte plays a crucial role in plant fertility and crop production by producing and transferring male gametes to embryos for double fertilisation. The male gametophyte is derived from the anther, which is the male sexual organ. Formation of the male gametophyte involves several important steps. First, reproductive cells divide and microspore mother cells form. Then, the microspore mother cells undergo meiosis and produce tetrad microspores surrounded by callose. Later, the callose is degraded by callose enzyme, releasing the haploid microspores. The individual microspores further form three-celled pollen grains, comprising a larger vegetative cell and two smaller sperm cells. The three-celled pollen grain further undergoes dehydration to form a mature pollen grain [1]. The dehydration of pollen grains is very important for maintaining maximum pollen viability so that pollen grains can tolerate various environmental stresses after release from the anther [2]. Defects in any of these steps will affect pollen formation or male gametophytic function [1, 3, 4]. In recent years, many mutants with altered processes of pollen formation have been identified in *Arabidopsis,* rice, and maize. Because of both male and female gametogenesis defects, loss-of-function of nucleoporin NUP1 in *Arabidopsis* caused fertility defects. The ovules are arrested during meiosis, and pollen grains are aborted at mitosis I [5]. Loss of *AP1G* function compromised dynamic vacuolar remodelling during pollen

development and impaired vacuolar acidification of pollen, which resulted in male gametophytic lethality due to defective pollen development [6]. A conserved glycerol–3-phosphate acyltransferase gene *OsGPAT3* plays an important role in regulating anther wall degradation and pollen exocrine formation in rice [7]. Gene *OsMS1* regulates tapetal programmed cell death and pollen exine formation in rice; it functions as a transcriptional activator and interacts with known tapetal regulatory factors through its plant homeodomain [8]. Loss-of-functionof *OsTKPR1* delays degeneration of the tapetum, reduces the lipid level of the anther cuticle, disrupts the Ubisch body and pollen exine formation, and results in complete male sterility [9]. *Abnormal Pollen Vacuolation1 (APV1)*,, a tapetum-specific gene, affects anther cuticle and pollen exine formation in maize. The *apv1* mutant was completely male sterile [10].

Previously, we isolated four gametophytic male-sterile mutants*atapam1, atapam2, atapam3* and *atapam4)*in *Arabidopsis [*11]. Mutation of *AtAPAM2 (ARABIDOPSIS POLLEN ABORTION MUTANT 2)* drastically affected male gametophytic function and the *Ds* element was inserted in the 18th intron of *At3g50590* [11]. In this study, we reported the characterisation of *atapam2. AtAPAM2* is constitutively expressed in seedlings, roots, stems, leaves, siliques, flowers, and pollen grains. It encodes an unknown protein with three WD40-repeats that localises in plasma membrane and the nucleus. Homologous proteins of AtAPAM2 exist in many species, and we speculate that this protein may play a role in pollen formation and other development processes by interacting with other proteins.

Results

The *AtAPAM2* mutation affected male gametophytic function

We crossed the *atapam2/+* plant with the *quartet1* (*qrt1)* mutant to obtain the *atapam2/+*; *qrt1/qrt1* plants for tetrad analysis. The *qrt1* mutant caused the pollen grains not to separate from the tetrad, but had little effect on growth of the pollen tube [12]. Therefore, the *atapam2/+*; *qrt1/qrt1* plants produced a tetrad consisting of two *qrt1* (representing wild type) pollen grains and two *atapam2* pollen grains. Scanning electron microscopy (SEM) showed that all four pollen grains in the tetrad from *qrt1* plants had normal morphology (Fig. 1 a), yet the *atapam2/+*; *qrt1/qrt1* plants had only two normal pollen grains (Fig. 1 b). Alexander staining (Fig. 1 c and 1 d) and 4, 6-diamidino–2-phenylindole (DAPI) staining showed the same results (Fig. 1 e–1 h). These results showed that the *AtAPAM2* mutation affected male gametophytic function.

The atapam2/+ mutant is defective in mature pollen grains

The DAPI staining was used to investigate at which developmental stage pollen became abnormal (Fig. 2). Before the 12th anther development stage, the *atapam2/+* pollen grains had a normal phenotype with wild-type pollen grains (Fig. 2 a–2 c and 2 k–2 m). When anthers developed to the 12th stage, the mutant grains were smaller than normal pollen grains in bright field image and their nuclei began to disappear

under UV-light (Fig. 2 d and 2 n). By the 13th stage, the nuclei of mutant pollen grains had disappeared completely (Fig. 2 e and 2 o).

To further determine the results of DAPI staining, we obtained transverse sections of different development stages anthers from wild-type and *atapam2/+* plants using the semi-thin section method (Fig. 3). The results showed that in the 11th stage of anther development or before, the appearance and size of pollen grains from mutants were the same as for wild type (Fig. 3 a–3 d). In the 12th stage of anther development, some pollen grains were of irregular appearance and were not as smooth as the wild type (Fig. 3 e and 3 f). Combining the results of DAPI staining with semi-thin sections, we conclude that pollen grains of *atapam2/+* mutant began to turn abnormal at the 12th anther development stage.

Next, we used transmission electron microscopy (TEM) to observe differences in pollen wall structure between wild-type and *atapam2* pollen grains (Fig. 4). Mature *atapam2/+* pollen grains had characteristic wrinkled intine (Fig. 4 c–4 e), which was smooth in wild type and closely connected to the inner side of nexine (Fig. 4 a and 4 b). Mutant pollen grains also had deformed cytoplasm (Fig. 4 c–4 e).

The phenotype of *atapam2/+* is caused by a *Ds* insertion in *AtAPAM2*

In previous study, TAIL-PCR was used to localise the mutation site in *atapam2.* Sequence analysis indicated that the *Ds* element was inserted in the 18th intron of *At3g50590 [*11]. To confirm that the *atapam2/+* mutant phenotype was caused by the *Ds* insertion in this gene, we subcloned the full-length genomic DNA fragment of *At3g50590* into the pCAMBIA1300 vector. Then, this construct was introduced into *atapam2/+* mutant plants. Transgenic lines were screened by MS culture medium containing kanamycin and hygromycin. The self-pollinated T_1 transgenic seeds were plated on MS culture medium containing kanamycin to determine the segregation ratio of *Ds.* The Kan^R:Kan^S segregation ratio was approximately 2:1 for most of the transgenic lines (19 of 25). The complemented mutant plants produced offspring of *atapam2/+* mutation homozygotes in T_2 and subsequent generations. When pollen grains from the two independent complemented lines homozygous for the *atapam2/+* mutation were observed by SEM, Alexander staining assay, and DAPI staining assay, the configuration of surface, vitality, and nuclei development were returned to the level of wild type (Fig. 5 c, 5 f, 5 i, and 5 l). At the same time, we calculated the abnormal rate of pollen grains from the two complemented lines, and found about 1.28% (8/624) and 1.42% (10/700) of pollen grains were abnormal, respectively (Fig. 5 m). These results demonstrate that the defect in male gametophytic function in *atapam2/+* plants was fully complemented by *At3g50590* full-length genomic DNA.

To validate this result, *atapam2–2 (*SAIL_1288_C09) was obtained from the seed stocks of the Arabidopsis Biological Resource Center (ABRC) germplasm stock (http://www.arabidopsis.org). The *atapam2–2* is a T-DNA insertion mutant of Columbia (Col) background. Using a T-DNA left-border primer Lba1 and a gene-specific primers (atapam2–2-F and atapam2–2-R) (Table 1) for PCR analysis, we

confirmed that T-DNA was inserted in the 13th exon (Fig. 8 a). The *atapam2–2* mutant carried a Basta^{R}-selective marker. The self-pollinated *atapam2–2/+* progeny exhibited a Basta^{R}:Basta^{S} segregation ratio of approximately 1:1 (1590:1500) (Table 2) rather than 3:1. We also used SEM to observe the surface of pollen grains from *atapam2–2/+,* and found about 50% (134/271) of pollen grains were shrivelled (data not shown). These findings are consistent with previous results concerning *atapam2/+* plants [11], and confirm that the defect in the male gametophyte in *atapam2/+* was caused by mutation in *At3g50590.*

AtAPAM2 is constitutively expressed and highly expressed in flowers and pollen grains

To understand the function of *AtAPAM2,* we used RT-PCR to assess its expression pattern. The RT-PCR analyses showed that *AtAPAM2* was ubiquitously expressed in many tissues, was constitutively expressed, and highly expressed in inflorescences (Fig. 6 a).

To further study the expression pattern of *AtAPAM2,* the promoter fragment of *AtAPAM2* was incorporated into *GUS* reporter gene and introduced into wild-type plants. In the T_2 transgenic plants harbouring this fusion, GUS staining signals were detected in seedlings, roots, leaves, inflorescences, flowers, siliques, and pollen grains (Fig. 6 b–6 h). This further demonstrated that *AtAPAM2* was expressed constitutively.

Subcellular localisation of AtAPAM2 protein

To further study the function of AtAPAM2 protein, the GFP–AtAPAM2 fusion protein under control of the 35S promoter (p35S: GFP–AtAPAM2) was constructed. This construct was transformed into leaves of *Nicotiana benthamiana* for transient expression and *Arabidopsis* wild-type plants for stable expression. Both in epidermal cells of *N. benthamiana (*Fig. 7 a–7 c) androots of *Arabidopsis (*Fig. 7 d–7 f), the GFP signals indicated AtAPAM2 localisation in plasma membrane and nucleus.

AtAPAM2 encodes a putative WD40-repeat protein and is evolutionarily conserved

The *AtAPAM2* mRNA encodes a putative WD40-repeat protein (Fig. 8 b) of 1614 aa and contained three WD40-repeat domains (Fig. 8 c). We used the entire aa sequence of AtAPAM2 to carry out a basic local alignment search tool (BLAST) search from NCBI. The results showed several proteins with >60% aa sequence similarity to AtAPAM2 in higher plants. Specifically, AtAPAM2 had 98% identity with EOA23389.1 of *Capsella rubella,* 96% with XP_002876038.1 of *Arabidopsis lyrata* subsp. *lyrata,* 77% with XP_002522312.1 of *Ricinus communis,* 76% with EOX91354.1 of *Theobroma cacao,* 76% with XP_004161728.1 of *Cucumis sativus,* 75% with XP_004232045.1 of *Solanum lycopersicum,* and 68%

with EEC84558.1 of *Oryza sativa* (Fig. 8 d). The above are WD40-repeat proteins, but of unknown function.

The AtAPAM2 has three WD40-repeats and in eukaryotes this domain usually functions as a protein–protein or protein–DNA/RNA interaction platform [12, 13]. In order to understand the function of this protein, we made use of the WD40 domain of AtAPAM2 protein to carry out a yeast two-hybrid assay, with the aim of finding the protein that interacted with it; however, this was not successful.

Discussion

In this study, a detailed characterization of mutant of the *Arabidopsis AtAPAM2* gene was performed. Genetic studies indicated that *AtAPAM2* plays an important role for male gametophyte development. The results of SEM, Alexander staining and DAPI staining from *atapam2/+; qrt1/qrt1* plants showed that the mutation affected male gametophytic function and the results of DAPI staining, semi-thin section and TEM from different development stages showed that the mutation is defective in the late pollen mature process. At the 11^{th} stage of anther development, pollen grains from *atapam2/+* mutants were normal, with two sperm cell nuclei and a vegetative nucleus, suggesting that pollen grains had finished the second mitosis process. But at the 12^{th} stage of anther development, pollen grains from *atapam2/+* mutant began to turn abnormal. During this stage, the three nuclei pollen grains completed the process of dehydration and the mutation may have affected this process.

It was also found that the *AtAPAM2* was constitutively expressed and highly expressed in flowers and pollen grains. This expression pattern is in agreement with its essential role during mature pollen. The above results showed that mutation of *AtAPAM2* affected maturation of pollen grains.

The AtAPAM2 protein was a putative WD40-repeat protein.The WD40 domain is one of the most abundant domains in eukaryotic genomes, and consists of 44–60 amino acid (aa) residues with a glycine–histidine di-peptide close to the N-terminal and a tryptophan–aspartic acid (WD) di-peptide at the C-terminal [13]. When present in a protein, the WD40 motif is typically found as several (usually 4–10) tandem repeat units and they usually function as a protein–protein or protein–DNA/RNA interaction platform [14–16]. Since *AtAPAM2* encodes a protein containing three WD40 repeat domains, we attempted to obtain other proteins that interact with it by yeast two-hybridization, but without success. We speculate that it may be because it contains two transmembrane domains, whereas traditional yeast two-hybrid is used for nuclear protein interactions.

The WD40 proteins are involved in a variety of cellular functions. AtNEDD1 is a WD40 repeat protein, which interacts with the γ-tubulin complex and participates in microtubule organisation during cell division [17]. The *Arabidopsis* WD40 protein TRANSPARENT TESTA GLABRA1 (TTG1) regulates several developmental and biochemical pathways, including formation of hairs on leaves, stems, and roots, and production of seed mucilage and anthocyanin pigments [18]. The WD40 proteins also participate in the progress of gametophytic development. *SLOW WALKER1* (*SWA1)* encodes a protein with six WD40

repeats that localises in the nucleolus of interphase cells, which are involved in 18S ribosomal RNA biogenesis, and is essential for gametogenesis in *Arabidopsis* [19]. The *Arabidopsis* Yaozhe (YAO) is a nucleolar WD40 protein involved in embryogenesis and gametogenesis [20]. *Rice Immature Pollen1 (RIP1),*, containing five WD40 repeats, regulates late pollen development in rice [21]. The *OsLIS-L1* gene encoding the lissencephaly type–1-like protein with WD40 repeats is involved in plant height and male gametophyte formation in rice [22]. The *LuWD40–1* gene encoding a WD40 repeat protein regulates growth and pollen viability in flax (*Linum usitatissimum L.)*) [23]. The *JINGUBANG* gene encodes a putative hydrophilic protein containing seven WD40 repeats, which is a negative regulator of pollen germination and prevents pollination in moist environments [24].

Since the WD40 proteins are involved in many biological processes, including gametophyte development, the AtAPAM2 we identified is highly expressed in inflorescence and pollen grains, and mutates to affect pollen maturation. Although traditional yeast double hybridization has failed, it is possible to look for proteins that interact with it in a different way.

Conclusions

We determined that AtAPAM2 protein may play a role in pollen formation and other development processes by interacting with other proteins, but we did not identify the protein that interacts with it.

Methods

Plant materials and mutant isolation

The plants used were of Landsberg *erecta (*L*er)* and Colombia (Col) backgrounds. The L*er* and Col seeds were propagated and preserved in the laboratory of Ye De (China Agricultural University). The seeds were surface-sterilised and pre-germinated on MS-salt agar plates with or without 50 μg/mL of kanamycin (E004000, Sigma, USA) or 25 μg/mL hygromycin (Roche, Mannheim, Germany) or 10 μg/mL Basta (45520, Sigma, USA) at 22°C with a 16/8 h of light/dark cycle. The plants were grown in soil at 22°C under the same light cycle as for seed germination. The *Ds* insertion line was generated and mutants selected according to methods described previously [25]. The SALK T-DNA insertion lines were obtained from the ABRC (http://www.arabidopsis.org/).

Phenotypic characterisation of mutant

Morphological observations of pollen grains by SEM were carried out as previously described [26]. The DAPI staining was performed as previously described [27]. Pollen viability was assayed using Alexander's staining [28]. For semi-thin section analysis, developing anthers were fixed overnight in a fixation solution of 5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde (pH 7.2) and then rinsed three times with 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide for 3–4 h, and rinsed three times with 0.1 M

phosphate buffer again. The samples were dehydrated through 30 min of exposure to a series of ethanol/water mixtures (30, 50, 70, 80, 90, and 100% ethanol) and 100% epoxypropane for two times. Subsequently, samples were transferred to a 1:1 dry epoxypropane: resin mix [29] on a rotator (12 h) and infiltrated in a 1:3 dry epoxypropane:resin mix for an additional 12 h before transferring to freshly mixed resin. Following two fresh resin changes, the materials were polymerised in moulds (70°C for 8–10 h) [29]. Semi-thin sections (1.0 μm thick) were cut using a glass cutter on a Leica RM2265 microtome (Leica, Wetzlar, Germany). The sections were dyed with toluidine blue and viewed under an optical microscope (DM2500, Leica, Wetzlar, Germany). The TEM of pollen grains was performed as described previously [30].

Molecular cloning of *AtAPAM2* and complementation experiments

The full-length *AtAPAM2* genomic DNA fragment was amplified by PCR with primers AtAPAM2-C-F and AtAPAM2-C-R (Table 1). For complementation experiments, the full-length *AtAPAM2* genomic DNA fragment was subcloned into pCAMBIA1300 vector (CAMBIA, Canberra, Australia) and introduced into *atapam2/+* heterozygous plants using the *Agrobacterium tumefaciens*-mediated infiltration method. The transformant plants were selected in MS medium containing 50 mg/L of kanamycin and 25 mg/L of hygromycin. The homozygous transformant plants were selected in T_2 generations and used for further analyses.

Analysis of expression pattern

The promoter fragments of *AtAPAM2* were amplified using *LA* Taq and *Pfu* DNA polymerase (Takara, Dalian, China) with the gene-specific primers AtAPAM2-P-F and AtAPAM2-P-R (Table 1). The resulting fragments were subcloned upstream of the *GUS* reporter gene in pCAMBIA1300 Ti-derived binary vector and introduced into wild-type plants. Transformation and GUS activity analysis were carried out as described previously [25, 31].

For the RT-PCR assay, total RNAs were extracted from roots, stems, leaves, inflorescences, and siliques of 5-week-old wild-type and 10-day-old transformant seedlings, respectively, using an RNAprep pure plant kit (Tiangen, Beijing, China) according to the manufacturer's instructions. First-strand cDNA was synthesised from 1 μg of total RNA using the M-MLV reverse transcriptase from Invitrogen (CA, USA) according to the manufacturer's instructions. The gene-specific primers AtAPAM2-RT-F and AtAPAM2-RT-R were used for PCR reactions. *TUBULIN8* expression level was applied as the internal control by including the specific primers TUBULIN-F and TUBULIN-R (Table 1).

Subcellular localisation assay of the AtAPAM2 protein

The AtAPAM2 cDNA fragment was amplified by PCR using the primers AtAPAM2GFP-F and AtAPAM2GFP-R (Table 1) and then cloned into the modified pCAMBIA1300 vector to generate the fusion protein-expressing cassette p35S:GFP–AtAPAM2. The resulting construct was transformed into leaves of *N. benthamiana* for transient expression and *Arabidopsis* wild-type plants for stable expression. The GFP signals were observed under a Leica DM2500 microscope as previously described [32- 34].

Abbreviations

DAPI: 4',6-Diamidino–2-Phenylindole; *SEM:* Scanning Electron Microscope;

TEM: Transmission Electron Microscope; *GUS:* β-Glucuronidase;

RT-PCR: Reverse transcription-Polymerase Chain Reaction;

GFP: Green Fluorescent Protein; *BLAST:* Basic Local Alignment Search Tool;

TAIL-PCR: Thermal Asymmetric Interlaced PCR.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' Contributions

The work presented here was carried out in collaboration among all the authors. ZM, YL, and GZ performed the experiments, analysed data, and contributed to writing the manuscript. HY designed the project, analysed data, wrote the manuscript, and obtained funds to support the project. DY contributed to performing the experiment and manuscript writing. XZ and LC helped to perform the experiments. All authors have read and approved the final manuscript.

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Tables

Table 1. The primers used in this study

Primers	Sequences
AtAPAM2-C-F	5'-TACAAAGAAATGGACTAACTC-3'
AtAPAM2-C-R	5'-CTGTCAAGAACGATGAAC-3'
AtAPAM2-P-F	5'- gctgcagctcttaaagggaagatttgtga-3'
AtAPAM2-P-R	5'-ctctagagaacttctgaaagacaaaatac-3'
AtAPAM2-RT-F	5'-GAGCCTGCTGCTATGGAATC-3'
AtAPAM2-RT-R	5'-TAGCTGACTCTGGCCCTTGT-3'
TUBULIN-F	5'-CTTCGTATTTGGTCAATCCGGTGC-3'
TUBULIN-R	5'-GAACATGGCTGAGGCTGTCAAGTA-3'
atapam2-2-F	5'-CCAGAGTCAGCTAACAATTTC-3'
atapam2-2-R	5'-CTTCTGTCTCAAACATGAAT-3'
AtAPAM2GFP-F	5’-GGGGTACCGAGAGCAGAAGTTATGGAGTGGGCAACGGTGCAGCAT-3'
AtAPAM2GFP-R	5’-ACGCGTCGACTTAGATGTTGATTAACACCAACGGTGA-3’

Table 2 Genetic analysis of *atapam2-2/+* mutant

Crosses (Female×Male)	$Basta^R$	$Basta^S$	$Basta^R$：$Basta^S$	TEf	TEm
atapam2-2/+×*atapam2-2/+*	1590	1500	1.06	NA	NA
atapam2-2/+×WT	851	900	0.95	100	NA
WT×*atapam2-2/+*	0	2108	0	NA	0

$Basta^R$: Basta-resistant seedings; $Basta^S$: Basta-sensitive seedings; WT: Wild-Type seedings;

NA: Not applicable; TEf: Transmission efficienly of *atapam2-2/+* female gametophyte; TEm: Transmission efficienly of *atapam2-2/+* male gametophyte; TE=$Basta^R$：$Basta^S$×100%.

Figures

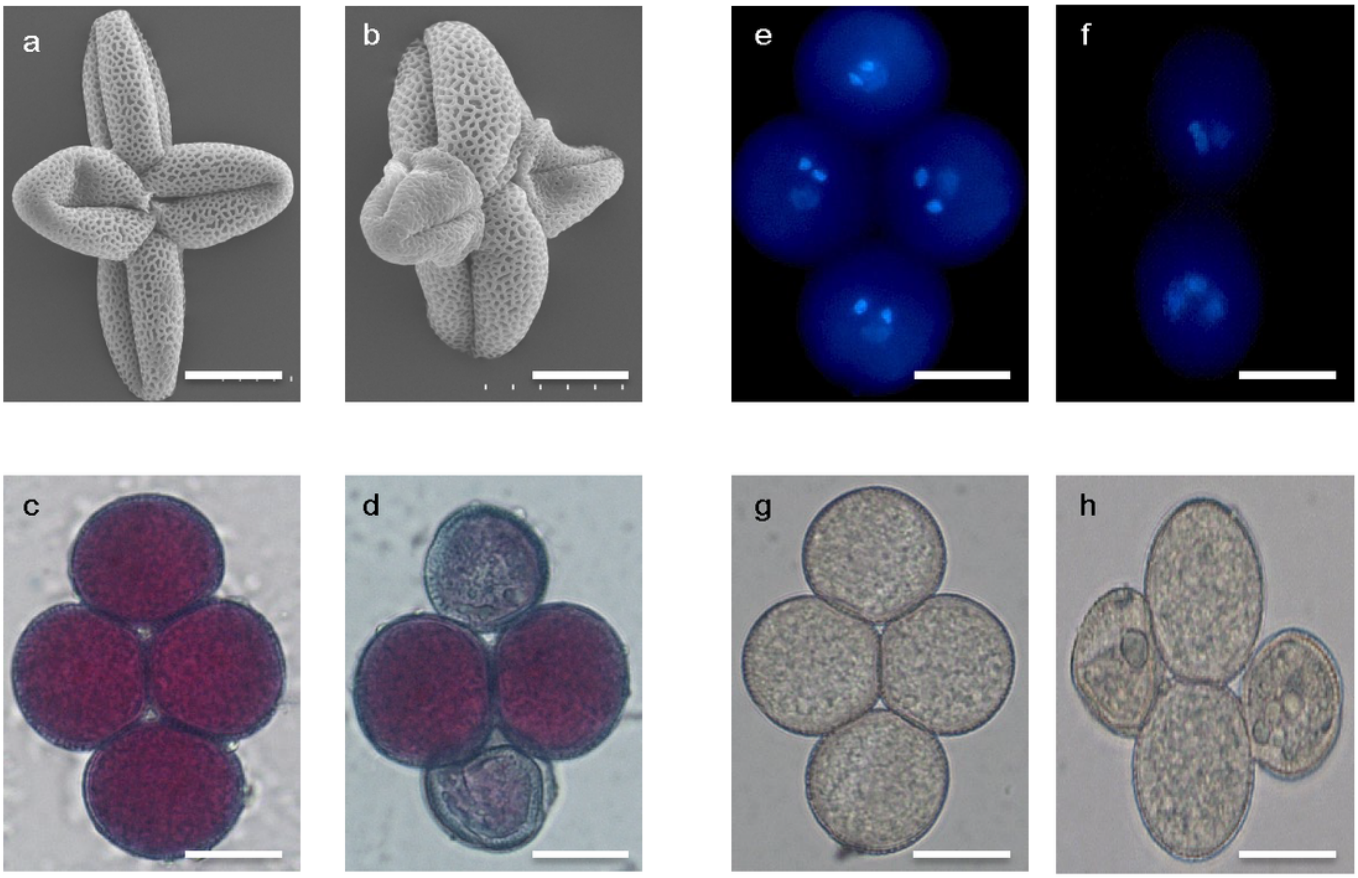

Figure 1

The phenotype of atapam2/+; qrt1/qrt1 pollen grains. a Scanning electron microscopy of pollen from qrt1/qrt1. b Scanning electron microscopy of pollen from atapam2/+; qrt1/qrt1. c Alexander staining of pollen from qrt1/qrt1. d Alexander staining of pollen from atapam2/+; qrt1/qrt1. e DAPI staining of pollen from qrt1/qrt1. f DAPI staining of pollen from atapam2/+; qrt1/qrt1. g and h Bright fields of e and f. Bars = 10 μm.

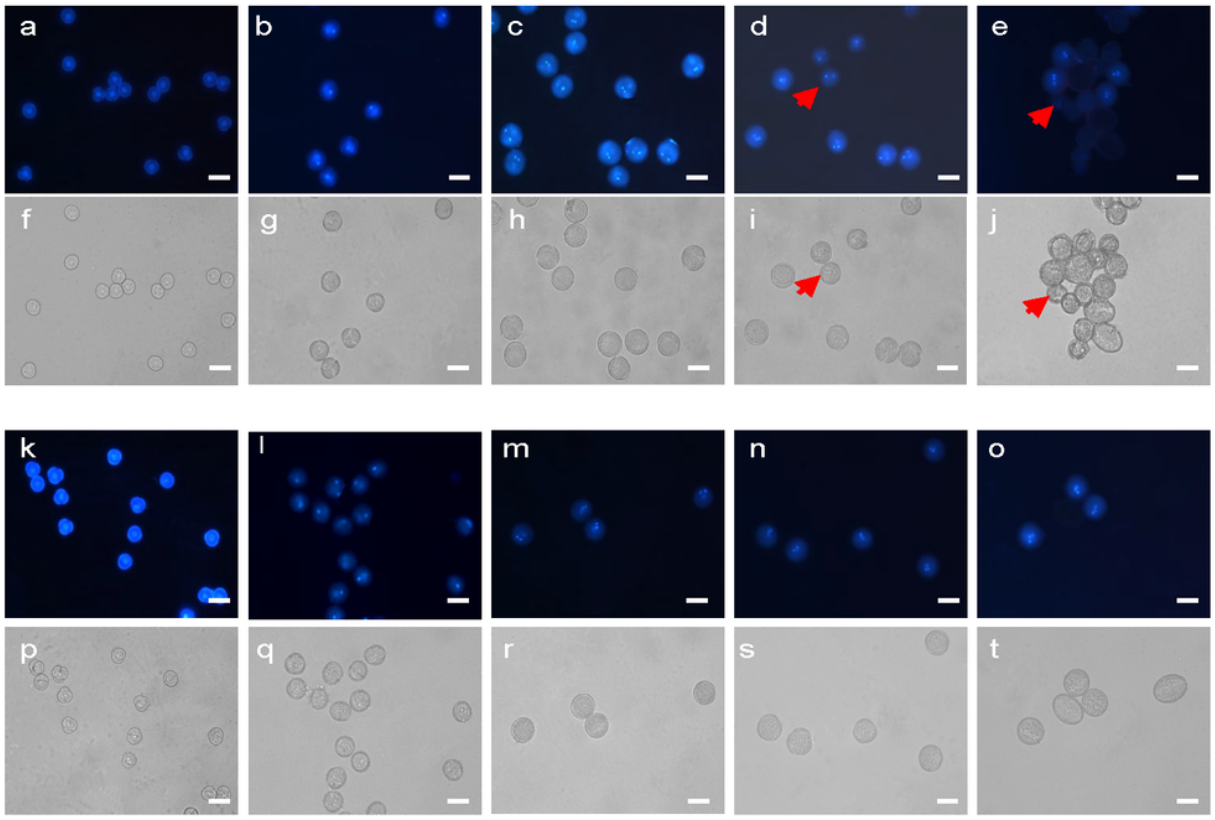

Figure 2

DAPI staining of different stages of pollen development. a, f, k, p Uninucleate pollen grains. b, g, l, q Binucleate pollen grains. c, h, m, r Early trinucleate pollen grains. d, i, n, s Middle trinucleate pollen grains. e, j, o, t Late trinucleate pollen grains. a–e DAPI staining of different stages of pollen grains from atapam2/+. f–j Bright fields of a–e. k–o DAPI staining of different stages of pollen grains from wild type. p–t Bright fields of k–o. Red arrowheads indicate abnormal pollen grains. Bars = 100 μm.

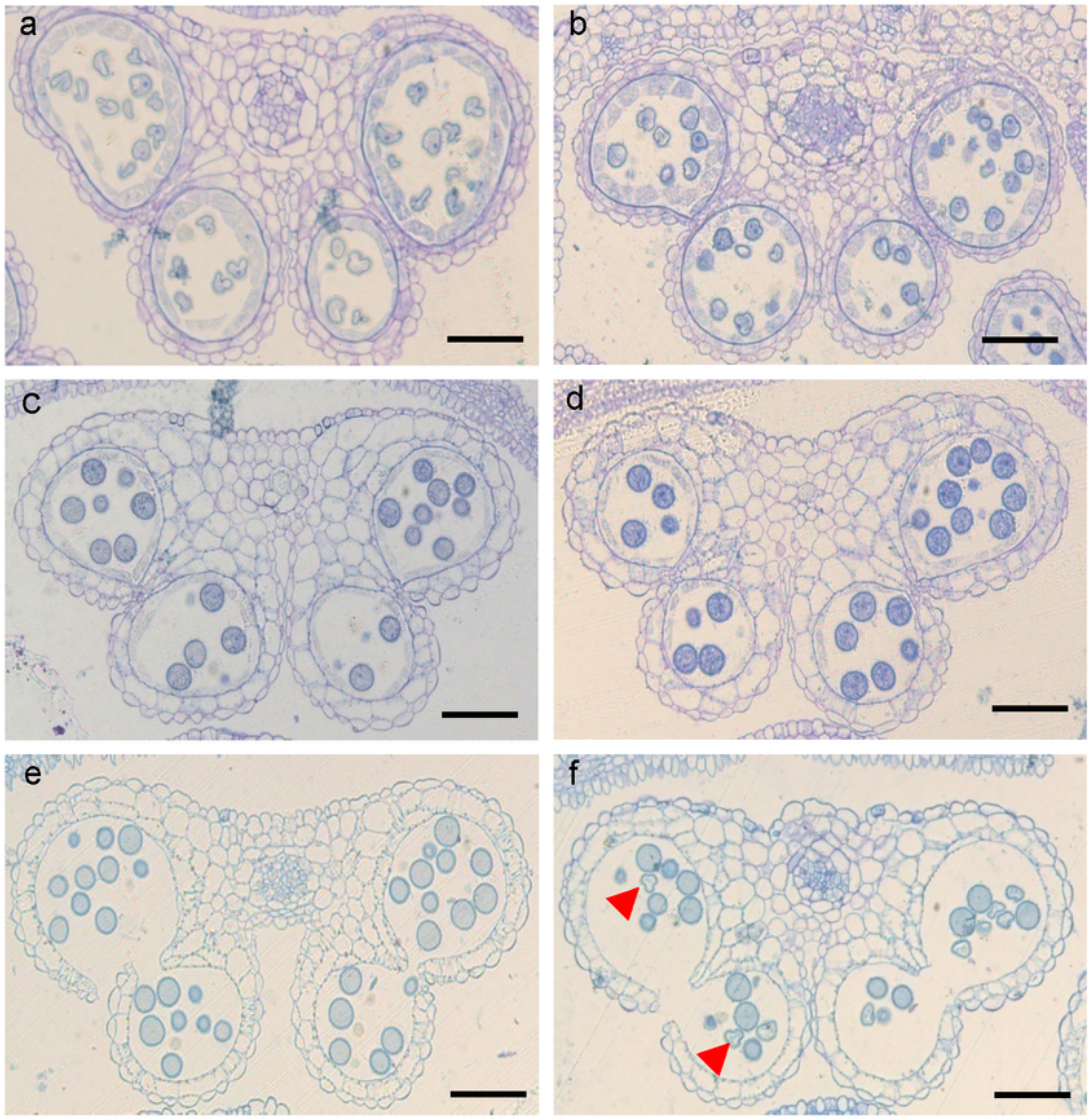

Figure 3

Semi-thin section images of anthers in wild-type and atapam2/+ mutant. a, c, e Wild-type plants. b, d, f atapam2/+ plants. a, b Stage 10 of anther development. c, d Stage 11 of anther development. e, f Stage 12 of anther development. Red arrowheads indicate the atapam2/+ mutant pollen grains. Bars = 50 μm.

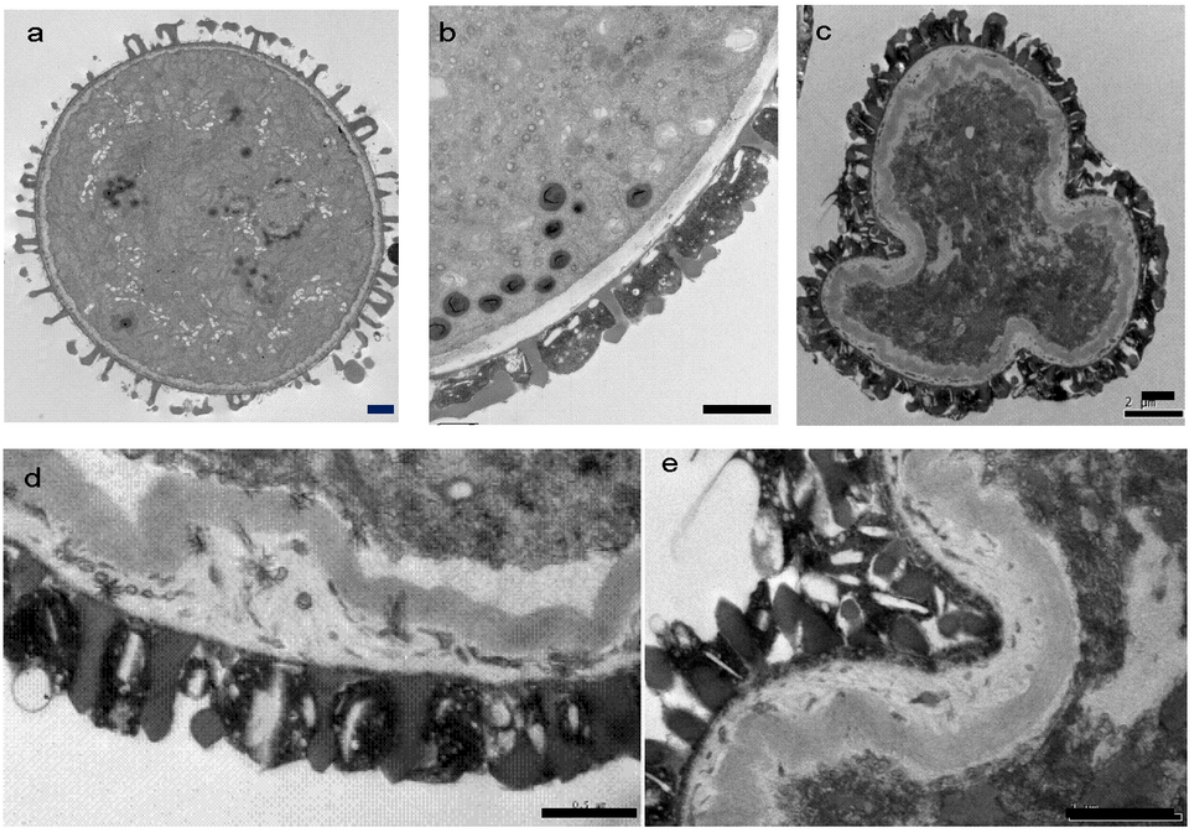

Figure 4

Transmission electron microscopy images of pollen grains from wild-type and atapam2/+. a, b Wild-type. c–e atapam2/+. Bars = 1 μm.

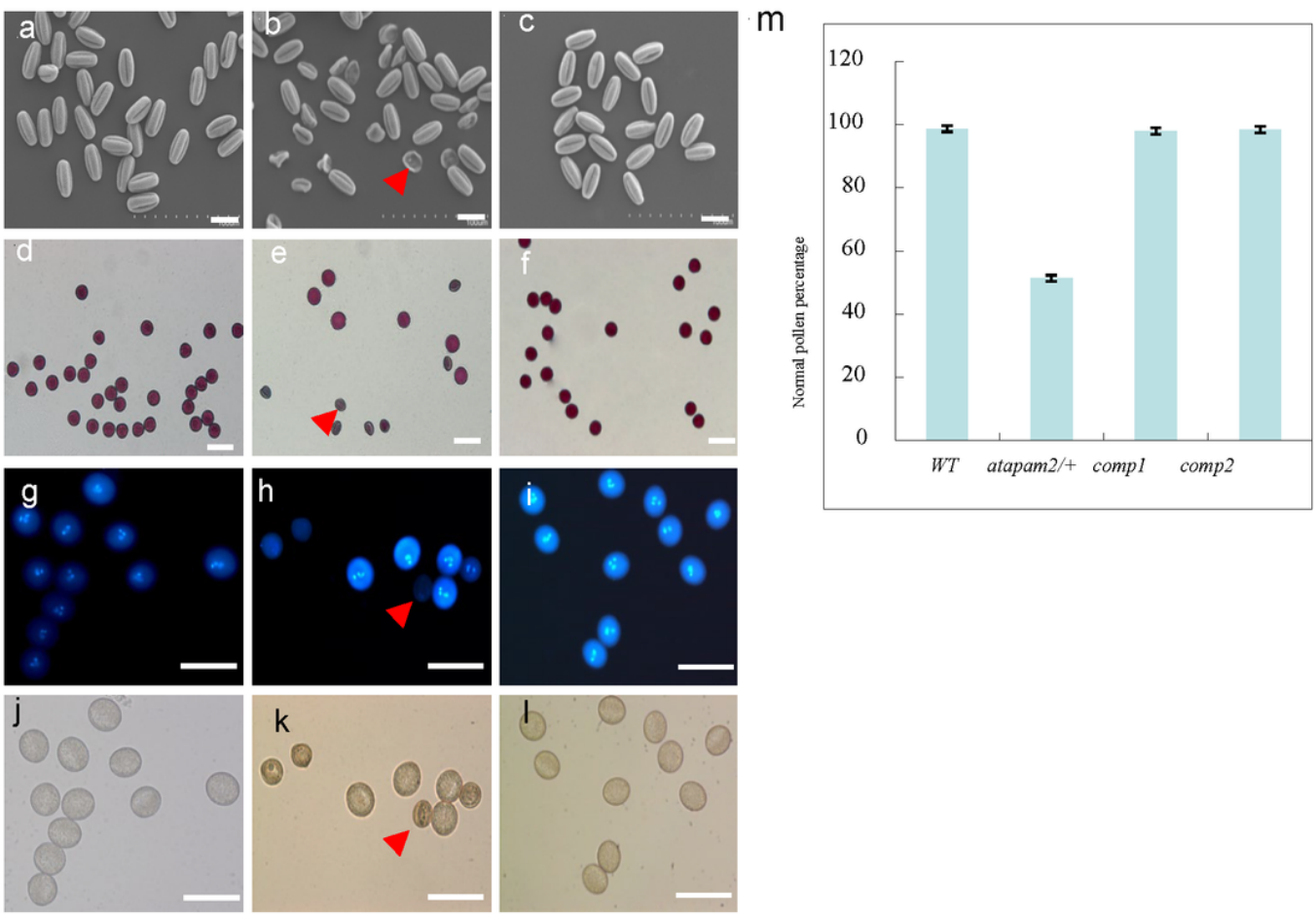

Figure 5

Characterisation of atapam2/+ mutant pollen grains and complementation plants pollen grains. a–c Scanning electron microscopy image of pollen grains from wild-type, atapam2/+ mutant, and complementation plants. d–f Alexander-stained pollen grains from wild type, atapam2/+ mutant, and complementation plants. g–i DAPI-stained pollen grains from wild type, atapam2/+ mutant, and complementation plants. j–l Light field of g–i. m Statistics of normal pollen percentage from wild type, atapam2/+ mutant, and complementation plants. Bars = 100 μm in a–f and 50 μm in g–l.

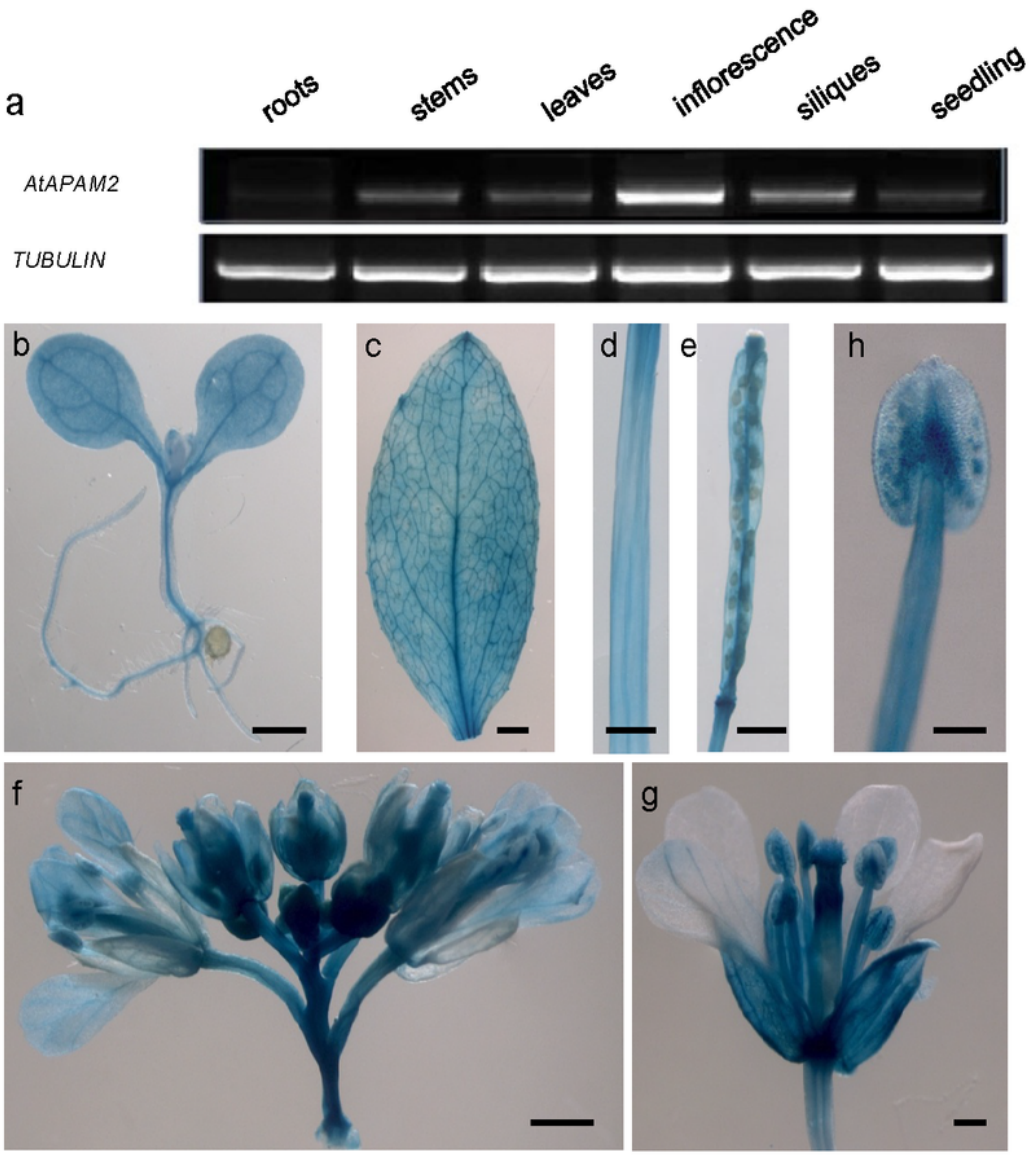

Figure 6

Expression pattern of AtAPAM2. a RT-PCR assay comparing AtAPAM2 mRNA levels in roots, stems, leaves, inflorescences, siliques, and seedlings. Expression level of TUBULIN was used as an internal control. b–h GUS activity in seedlings (b), mature leaves (c), stems (d), siliques (e), inflorescences (f), flowers (g), and anthers (h) from transgenic wild-type plants carrying the pAtAPAM2:GUS construct. Bars = 500 μm (b–f) and 100 μm (g, h).

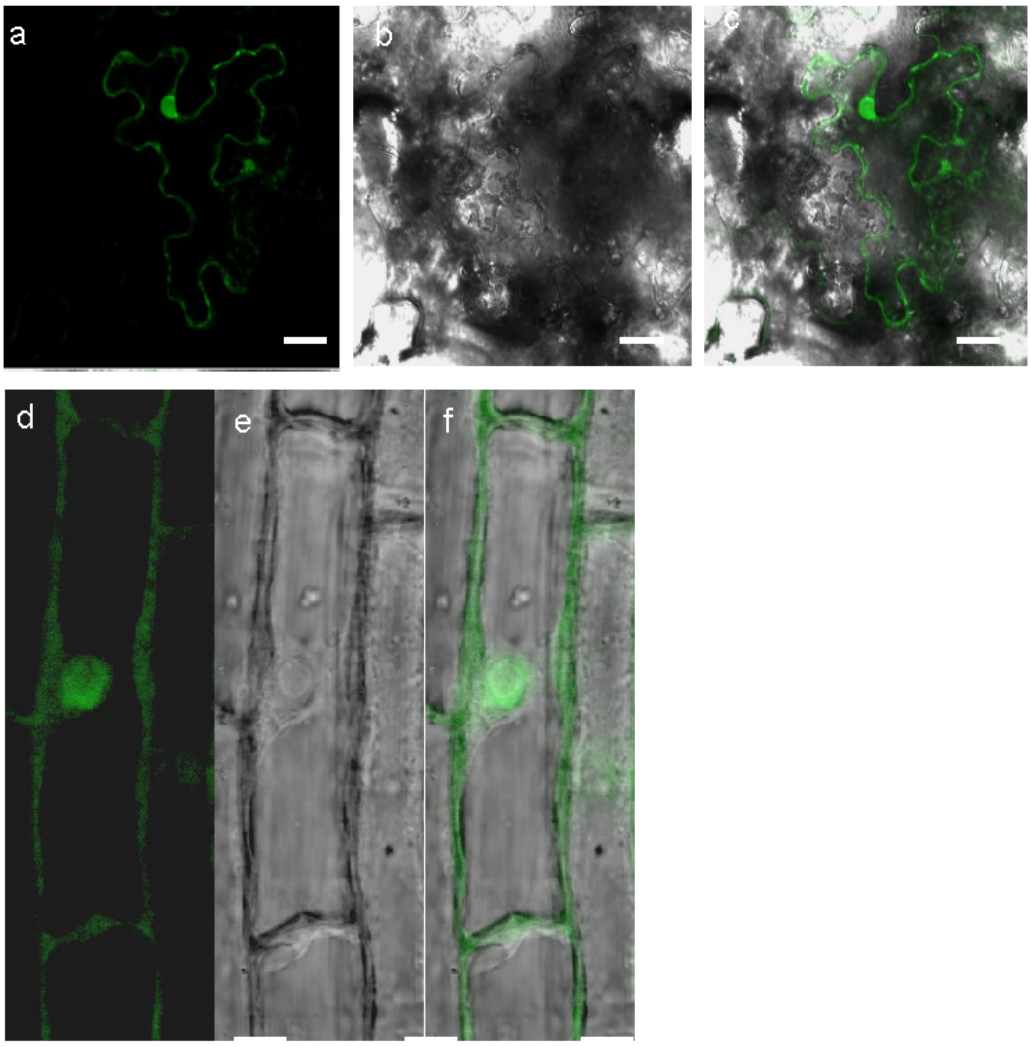

Figure 7

Subcellular localisation of AtAPAM2 protein. a–c Transient expression of p35S:GFP–AtAPAM2 in epidermal cells of Nicotiana benthamiana, showing localisation of AtAPAM2 protein in plasma membrane and nucleus. a GFP image. b Bright field image of a. c Merged image of a and b. d–f Stable expression of p35S:GFP–AtAPAM2 in roots of Arabidopsis, showing localisation of AtAPAM2 protein in

plasma membrane and nucleus. d GFP image. e Bright field image of d. f Merged image of d and e. Bars = 20 μm.

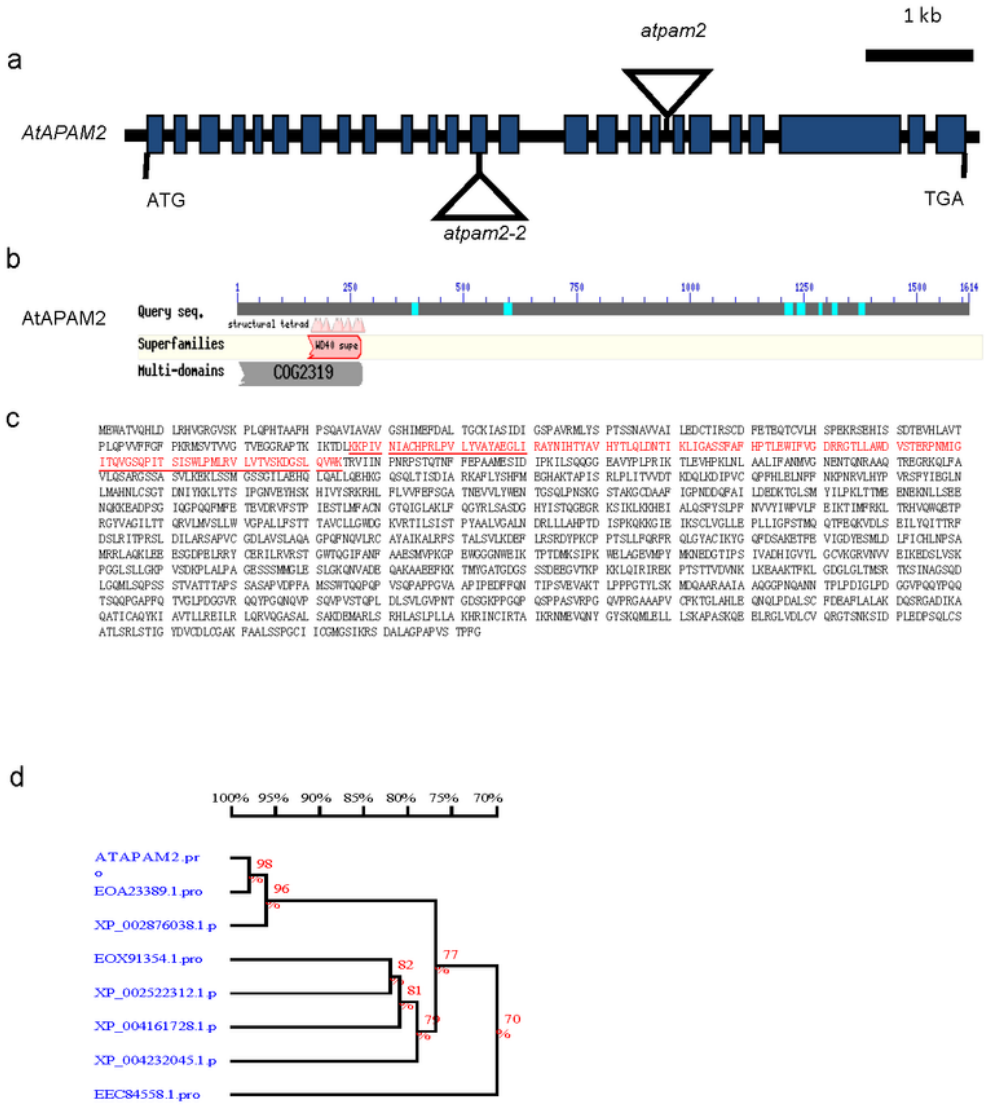

Figure 8

Molecular characterisation of the AtAPAM2 gene. a Schematic diagram of AtAPAM2 structure, showing the Ds insertion site in the atapam2/+ mutant and T-DNA insertion site in the atapam2-2 mutant. Black boxes indicate coding sequences and lines indicate introns. b Putative conserved domains of AtAPAM2

detected. c Predicted peptide sequence of AtAPAM2, showing the conserved domains. WD40 domains are shown by underline and red characters. d The sequence similarity analysis of AtAPAM2 and similar proteins from different species. AtAPAM2, Arabidopsis APAM2 protein; EOA23389.1, Capsella rubella; EEC84558.1, Oryza; XP_002522312.1, Ricinus communis; XP_004232045.1, Solanum lycopersicum; XP_004161728.1, Cucumis sativus; EOX91354.1, Theobroma cacao; XP_002876038.1, Arabidopsis lyrata subsp. Lyrata.