ADAPTOR PROTEIN-1 complex-mediated post-Golgi trafficking is critical for pollen wall development in Arabidopsis

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Summary

- Primexine deposition is essential for the formation of pollen wall patterns and is precisely regulated by the tapetum and microspores. While tapetum- and/or microspore-localized proteins are required for primexine biosynthesis, how their trafficking is established and controlled is poorly understood.
- In Arabidopsis thaliana, AP1σ1 and AP1σ2, two genes encoding the σ subunit of the trans-Golgi network/early endosome (TGN/EE)-localized ADAPTOR PROTEIN-1 complex (AP-1), are partially redundant for plant viability, and the loss of AP1σ1 function reduces male fertility due to defective primexine formation. Here, we investigated the role of AP-1 in pollen wall formation.
- The deposition of Acyl-CoA SYNTHETASE5 (ACOS5) and type III LIPID TRANSFER PROTEINS (LTPs) secreted from the anther tapetum, which are involved in exine formation, were impaired in ap1σ1 mutants. In addition, the microspore plasma membrane (PM) protein RUPTURED POLLEN GRAIN1 (RPG1), which regulates primexine deposition, accumulated abnormally at the TGN/EE in ap1σ1 mutants. We show that AP1μ recognizes the YXXΦ motif of RPG1, thereby regulating its PM abundance through endocytic trafficking, and that loss of AP1σ1 decreases the levels of other AP-1 subunits at the TGN/EE.
- Our observations show that AP-1-mediated post-Golgi trafficking plays a vital role in pollen wall development by regulating protein transport in tapetal cells and microspores.

Introduction

The pollen wall is a unique and complex structure developed over the course of plant evolution that plays multiple roles in pollen development and double fertilization. A delicate regulatory mechanism controls the formation of the pollen wall (Blackmore et al., 2007). Angiosperm pollen walls consist of two layers: the exine and the intine (Scott et al., 2004). The exine forms from the deposition of sporopollenin in the primexine (Heslop-Harrison, 1968), enveloping microspores already covered by a callose wall at the tetrad stage. The primexine, mainly composed of polysaccharides, plays a key role in exine patterning as a template for the initial accumulation of sporopollenin (Heslop-Harrison, 1968; Li et al., 2017).

The tapetum plays a critical role in the development of the pollen wall. The Arabidopsis (Arabidopsis thaliana) tapetum is a single layer of secretory cells that surrounds the microspores and provides nutrients and materials for microspore development (Liu & Fan, 2013; Qullichini et al., 2014; Shi et al., 2015; D. Zhang et al., 2016). Specific organelles within tapetal cells including elaioplasts, which are plastids containing fatty acids and other lipids, and tapetosomes derived from the endoplasmic reticulum (ER) are important for the biosynthesis and release of lipids and other materials to form the pollen coat (Suzuki et al., 2013). In addition, proteins synthesized in the tapetum are important for the development of the pollen wall. For instance, sporopollenin is produced by Acyl-CoA SYNTHETASE5 (ACOS5) accumulating in the tapetum, while type III LIPID TRANSFER PROTEINS (LTPs) are transported from the tapetum to the pollen exine as a component (de Azevedo Souza et al., 2009; Huang et al., 2013). ACOS5 is secreted from the tapetum into the locular space (Wang et al., 2018). Similarly, type III LTPs are deposited on the surface of developing pollen grains following their translation in the ER and secretion from tapetal cells (Huang et al., 2013). Little is known about the secretory mechanisms of ACOS5 and type III LTPs in plants.

Arabidopsis RUPTURED POLLEN GRAIN1 (RPG1), a member of the MtN3/saliva domain family of sugar transporters, localizes to the plasma membrane (PM) of microspores (Guan et al., 2008; Chen et al., 2010; Sun et al., 2013). The rpg1 mutant exhibits abnormal primexine deposition and rupture of...
encoded by the functionally redundant genes \textit{ap1} type III LTPs in tapetal cells. Therefore, the regulation of pollen development is obscure.

Many PM proteins undergo constitutive endocytosis and recycling within vesicles in plant cells, including the boron exporter \textit{REQUIRES HIGH BORON1} (BOR1), the phytohormone receptor \textit{BRASSINOSTEROID INSENSITIVE1} (BRI1) and the auxin efflux transporter \textit{PIN-FORMED2} (PIN2) (Dhonukshe \textit{et al.}, 2007; Viotti \textit{et al.}, 2010). Internalized PM proteins are then sorted within the endomembrane compartment trans-Golgi network/early endosome (TGN/EE) and recycled back to the PM or transported to the vacuole for degradation (Viotti \textit{et al.}, 2010; Uemura, 2016; Reynolds \textit{et al.}, 2018). In addition to participating in endocytic trafficking, the TGN/EE functions in the post-Golgi secretory pathway (Viotti \textit{et al.}, 2010; Uemura \textit{et al.}, 2019). The TGN/EE is a central hub directing cargo proteins into distinct transport carriers, thereby determining the abundance and activity of PM proteins in plants (Rosquete \textit{et al.}, 2018; Zhang \textit{et al.}, 2019). The \textit{ADAPTOR PROTEIN-1} complex (AP-1) mediates the sorting of cargo proteins at the TGN in eukaryotes (Gravotta \textit{et al.}, 2012, 2019; Li \textit{et al.}, 2016). The heterotetrameric AP-1 complex is composed of two large subunits (\(\gamma\) and \(\beta\)), one medium subunit (\(\mu\)) and one small subunit (\(\sigma\)) (Castillon \textit{et al.}, 2018; Martzoukou \textit{et al.}, 2018). In mammalian cells, the AP-1 complex guides the intracellular trafficking of multiple membrane proteins, including membrane-associated soluble secretory protein receptors, through their interaction with the AP1\(\mu\) or AP1\(\sigma\) subunit-specific tyrosine-based YXX\(\Phi\) or dileucine-based [DE]XXXL[II] sorting motifs, respectively (Janvier \textit{et al.}, 2003; Carvajal-Gonzalez \textit{et al.}, 2012). The \(\mu\) subunit of the AP-1 complex has been functionally characterized in Arabidopsis and is crucial for various post-Golgi trafficking routes, including endocytic recycling, the secretory pathway and vacuolar trafficking (Park \textit{et al.}, 2013; Teh \textit{et al.}, 2013; J. G. Wang \textit{et al.}, 2013; Shimada \textit{et al.}, 2018; Yan \textit{et al.}, 2021). \textit{ap1\(\mu\)} mutant plants exhibit defects in growth and development, caused by the missorting of proteins, such as BRI1, the syntaxin KNOLLE, PIN2 and the receptor kinase STRUBBELIG (SUB) (Park \textit{et al.}, 2013; Teh \textit{et al.}, 2013; J. G. Wang \textit{et al.}, 2013; J. G. Wang \textit{et al.}, 2016).

We show here that RPG1 undergoes dynamic and continuous recycling between the microspore PM and the TGN/EE during pollen development and that the sorting of intracellular RPG1 at the TGN/EE relies on its YXX\(\Phi\) motif being bound by AP-1\(\mu\). We investigated the function of the AP-1\(\sigma\) subunit, which is encoded by the functionally redundant genes \textit{AP1\(\sigma\)1} and \textit{AP1\(\sigma\)2}, and discovered that the loss of \textit{AP1\(\sigma\)1} function impairs assembly of the AP-1 complex, resulting in the inhibition of RPG1 sorting to the microspore TGN/EE and thus interfering with exine formation. Loss of \textit{AP1\(\sigma\)1} also blocked the secretion of ACOS5 and type III LTPs in tapetal cells. Therefore, the regulation of pollen wall development by the microspore and tapetum was disrupted in \textit{ap1\(\sigma\)1} mutants, providing the molecular and biochemical basis for the male sterility phenotype seen in these mutants. These findings revealed that AP-1\(\sigma\) is required for AP-1 to sort cargo proteins in microspores and tapetal cells and that AP-1-mediated vesicle transport regulates formation of the pollen wall and thus contributes to male fertility.

**Materials and Methods**

Plant materials and growth conditions

All \textit{A. thaliana} materials used in this study have been authorized, and they were all in the Columbia-0 (Col-0) background. The following transgenic lines and mutants were used in this study: \textit{ProRPG1:RPG1-GFP} (Guan \textit{et al.}, 2008), \textit{DR5:GFP} (Benková \textit{et al.}, 2003), \textit{ProAP1\(\mu\)2:AP1\(\mu\)2-RFP} (J. G. Wang \textit{et al.}, 2013) and \textit{ProVHAa1:VHAa1-RFP} (Dettmer \textit{et al.}, 2006). The T-DNA insertion lines \textit{ap1\(\sigma\)1-1} (SALK\_145719) and \textit{ap1\(\sigma\)2-1} (SALK\_049615C) were obtained from the Arabidopsis Biological Resource Center. The \textit{ProRPG1:RPG1-GFP} line was a kind gift from Z. Yang (Guan \textit{et al.}, 2008). Homozygous mutant lines were isolated and confirmed by PCR (Supporting Information Table S1). The \textit{ap1\(\sigma\)1-1 \textit{ap1\(\sigma\)2-1}} double mutant was generated by crossing the two single mutants and genotyping their F\(_2\) progeny by PCR. All plants were grown in a climate-controlled growth room (22°C: 20°C day : night temperature, 16 h : 8 h light : dark photoperiod and 80 µE s\(^{-1}\) m\(^{-2}\) light intensity).

**Phenotypic characterization**

Pictures of plant inflorescences and overall morphology were taken with a digital camera (Nikon, Tokyo, Japan). Pictures of flowers were taken using an ultradethree-dimensional microscope (VHX-600E; Keyence, Osaka, Japan). The number of seeds per plant was manually counted after all siliques had matured; the wild-type (WT) and mutant plants used for comparative analysis were sown and grown at the same time under the same growth conditions. Alexander’s staining of anthers was performed to examine the viability of pollen grains as described (Alexander, 1969). The anthers were observed under visible light, using a Zeiss Axio Imager.Z2 microscope (Zeiss) with a x20 (numerical aperture 0.8) objective, and images were taken using a digital camera attached to the microscope. Tissues of transgenic plants harbouring the \textit{ProAP1\(\sigma\)1:GUS} or \textit{ProAP1\(\sigma\)2:GUS} reporter construct were stained for β-glucuronidase (GUS) activity as described by Yu \textit{et al.} (2016) and observed under visible light using a Zeiss Axio Imager.Z2 microscope with a x20 (numerical aperture 0.8) objective. For semithin sections of anthers, whole inflorescences were collected and fixed in FAA (2% (v/v) formaldehyde, 5% (v/v) acetic acid, and 45% (v/v) ethanol) solution and embedded in Technovit 7100 resin as described by Jin \textit{et al.} (2013), and sectioned to 2 µm with a microtome (RM2245; Leica, Wetzlar, Germany). Anther sections were stained with 0.05% (w/v) toluidine blue O for 3 min and photographed on a Zeiss Axio Imager.Z2 microscope with a x100 (numerical aperture 1.4) oil-immersion objective.

For scanning electron microscopy (SEM), anthers and pollen grains were removed from fully dehiscent flowers and mounted on aluminium SEM stubs. Samples were coated with gold and...
examined by a scanning electron microscope (S-3400N; Hitachi, Tokyo, Japan) with a secondary electron detector set to 15 kV, ×1000 magnification and 12.7 mm working distance. For transmission electron microscopy (TEM), inflorescence buds at different stages were collected from Arabidopsis plants and fixed in 0.1 M phosphate buffer (pH 7.2) with an ice-cold drop of 2.5% (v/v) glutaraldehyde and embedded in freshly mixed resin as previously described (Lou et al., 2014). The samples were sectioned (70–100 nm thickness) using an EM UC7 ultramicrotome (Leica Microsystems) and stained with 2% uranyl acetate and lead citrate. Sections were observed using a transmission electron microscope (Tecnai G2 Spirit Bio-TWIN; FEI, Hillsboro, OR, USA) with a WA-Veleta camera at 120 kV, spot size of 1, 2 μm defocus, 1 s integration time and 2048×2048 pixels.

Confocal microscopy and quantification

All fluorescence images were captured using a confocal laser scanning microscope (A1R+Ti2-E; Nikon). To define the developmental stages, the anthers in which tetrads were observed under bright-field confocal microscopy were first identified as stage 7 in an inflorescence. Then, the anthers in the buds between stage 7 and stage 13 anthers (latest open flowers) in the inflorescence were sequentially defined as stages 8–12 from the inside to the outside of the inflorescence. Anthers at the tetrad stage were fixed and stained with 0.1% (w/v) aniline blue as described by Zhang et al. (2020), and callose deposition was observed under ultraviolet illumination by confocal microscopy. For live-cell imaging of green fluorescent protein (GFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP) and Chl autofluorescence, the 488, 514, 561 and 594 nm lines of the laser were used for excitation, and emission was detected by a GaAsP spectrum detector at 460–500, 496–532, 612–666 and 651–761 nm, respectively. A ×60 (numerical aperture 1.2) water-immersion objective was used for live-cell imaging. The scanning resolution of these fluorescence images was 1024×1024. For quantitative measurements of fluorescence intensities, laser power and detection gain of the confocal microscope were identical among different treatments or genotypes. To quantify the intensities of fluorescence signals at the PM or intracellular compartments, digital images of microspores were analysed using IMAGEJ software (http://rsb.info.nih.gov/ij/). The specific calculation method for the fluorescence intensity of individual cells was as described by Yan et al. (2021).

Plasmid construction and transgenic plants

The ProAP1σ1:GUS, ProAP1σ2:GUS and ProAP1σ1:AP1σ2-YFP constructs were generated by PCR amplification of the respective promoters and coding regions, followed by restriction digestion and ligation into transformation vectors. The following vectors were generated by Gateway cloning (Invitrogen). The full-length coding sequences of AP1β1, AP1β2, RPG1 and ACOS5 were PCR-amplified and cloned into the pDONR/zeo vector and then recombined into the ProUBQ10-GFP-GWR destination vector. The RPG1 coding sequence with two point mutations (RPG11766A/F169A) was generated using pDONR-RPG1 as a template for site-directed mutagenesis with the primers listed in Table S1. The promoters (c. 2 kb) and genomic coding sequences of AP1σ1, AP1σ2 and LTP were cloned into the pDONR/zeo vector and then recombined into the pBIB-GWR-YFP destination vector. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)-mediated editing of AP1σ1 was performed as previously described (Chen et al., 2019), and the sequence of targets and primers used are provided in Table S2. The artificial microRNA (amiRNA) construct was designed using the WMD3-Web MicroRNA Designer website. The amiRNA precursor was cloned into the pUBQ10-GWR-FLAG plasmid (Hu et al., 2018) and consequently transformed into Col-0 plants using the floral dip method. The primers used for generating the amiRNA construct are listed in Table S2.

Chemical solutions and treatments

All reagents were from Sigma-Aldrich unless otherwise specified. Dimethyl sulphoxide (DMSO) was used to dissolve brefeldin A (BFA, 50 mM stock), ES2 (40 mM stock) and cycloheximide (CHX, 50 mM stock). Unless otherwise indicated in the text, the final working concentrations were 40 μM for ES2 and 100 μM for CHX and BFA. The flower buds of stage 8 anthers were removed from the plants for subsequent chemical treatment. BFA treatment and washout were performed as previously described (C. Wang et al., 2013). After chemical treatment, the anthers in the flower buds were dissected for live-cell imaging.

Reverse transcription–quantitative PCR (RT-qPCR) assays

Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized with the SuperScript III First-Strand Synthesis System (Invitrogen). qPCR was performed with Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan) and the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a 20 μl volume consisting of 10 μl 2× SYBR qPCR mix, 10 ng cDNA and 1 μM of each gene-specific primer (Table S3). Transcript levels were normalized to UBIQUITIN7 according to a previously described method (Livak & Schmittgen, 2001).

Bimolecular fluorescence complementation (BiFC) assays

The full-length coding sequences of AP1μ2, AP1σ1 and AP1σ2 were individually cloned in-frame with the N-terminal half of the YFP sequence in the vector pEarleygate201-YN. The full-length coding sequences of RPG1, RPG11766A/F169A and the four entire cytoplasmic loops of RPG1 (C1, C2, C3 and C4) were cloned in-frame with the C-terminal half of YFP in pEarleygate202-YC. These constructs were introduced into Agrobacterium (Agrobacterium tumefaciens) strain GV3101 and coinfected into young leaves of Nicotiana benthamiana plants grown for c. 3 wk. YFP fluorescence was examined by confocal laser scanning microscopy (Nikon A1R+ Ti2-E) after 48 h. Arabidopsis CLATHRIN
HEAVY CHAIN2 (CHC2)-YN and CLATHRIN LIGHT CHAIN2 (CLC2)-YC were used as a positive control. Constructs expressing YFP-N (YN) and YFP-C (YC) were used as a negative control.

Yeast two-hybrid (Y2H) assays

A mating-based split ubiquitin Y2H system (mbSUS) (Obrdlik et al., 2004) was used to test interactions between AP1r1, AP1r2, AP1μ2, AP1γ1, AP1β1 and RPG1. Full-length coding sequences of AP1r1, AP1r2, AP1μ2, AP1γ1, AP1β1 and RPG1 were PCR-amplified and mixed with linearized pMetYCgate or pX-NubWTgate vectors to transform yeast strain THY.AP4 or THY.AP5 through in vivo DNA recombination. Diploid cells were selected on synthetic complete medium containing adenine and histidine (SC+Ad+His), and interactions were tested by spotting colonies onto synthetic defined minimal medium (SD) plates containing 0, 75, 150 or 400 μM methionine. pMetYC, pX-NubWT and ERL2 were used as negative controls, and KAT1 (as N-KAT1 and KAT-Cub) was used as a positive control.

Accession numbers

Sequence data from this paper can be found in the Arabidopsis Genome Initiative under the following accession numbers: AP1r1 (At4g35410), AP1r2 (At2g17380), AP1μ2 (At1g60780), AP1γ1 (At1g60070), AP1β1 (At1g11380), RPG1 (At5g40260), CLC2 (At2g40060), CHC2 (At3g08530), VHAA1 (At2g28520), ACOS5 (At1g62940), type III LTP (At5g62080), ARF17 (At1g77850), Cals5 (At2g13680), UBIQUITIN7 (At2g35635) and ACTIN2 (At3g18780).

Results

Loss of AP1σ reduces male fertility

To explore the function of the AP-1σ subunit in plants, we identified one T-DNA insertion mutant for each encoding gene, ap1σ1-1 and ap1σ2-1 (Fig. S1a). RT-PCR analysis showed that AP1σ1 and AP1σ2 transcripts are not expressed in their respective mutants, indicating that these may be null alleles (Fig. S1b). Mutant plants homozygous for ap1σ1-1, but not ap1σ2-1, showed a severe reduction in fertility, while plants homozygous for ap1σ1-1 and heterozygous for ap1σ2-2 (ap1σ1-1 ap1σ2-2/-+) had a lower fertility than the ap1σ1-1 single mutant, suggesting that AP1σ1 and AP1σ2 are partially redundant in reproductive growth (Fig. 1a–e). The ap1σ1-1 mutant exhibited normal vegetative growth despite having a fertility defect (Fig. S1c,d).

To better understand the fertility defects of ap1σ mutants, we examined their anthers and noted that ap1σ1-1 and ap1σ1-1 ap1σ2-2/-+ stamens have fewer visible pollen grains when compared to WT, ap1σ2-1 and ap1σ1-1 ap1σ2-2/-+ plants (Fig. 1h–l). Furthermore, Alexander’s staining revealed fewer viable pollen grains in anthers from ap1σ1-1 and ap1σ1-1 ap1σ2-2/-+ plants relative to the WT (Fig. 1o–s). To confirm that the pollen phenotype is specific to the loss of AP1σ1, we generated an additional knockout mutant (ap1σ1-2) and knockdown lines (amiR-AP1σ1) for AP1σ1 by CRISPR/Cas9-mediated genome editing or an amiRNA-based silencing approach, respectively (Fig. S2a). The ap1σ1-2 mutant harboured a 286 bp deletion between +382 and +667 bp downstream of the translation start site (Fig. S2b). RT-qPCR results showed that the expression levels of AP1σ1 in flowers was significantly lower in four independent amiR-AP1σ1 transgenic lines (amiR-AP1σ1-1–4), compared to the WT (P < 0.0001, Fig. S2c). As shown in Fig. S2(a–d), plants from the ap1σ1-2 mutant and amiR-AP1σ1 transgenic lines also displayed fewer pollen grains and lower fertility compared to the WT. We detected no significant differences in pistil or stamen length between WT and ap1σ1-1 mutant plants (Fig. S1e–l). In addition, siliques with normal seed set were produced when we pollinated ap1σ1-1-1 stigmas with WT pollen (Fig. S1m), indicating that female reproductive development is not affected in ap1σ1-1 mutants. Together, these data suggested that the sterility of ap1σ1 mutants is caused by pollen defects.

AP1σ1 and AP1σ2 share c. 97% sequence identity (Fig. S3a). In addition, staining for GUS activity in transgenic lines harbouring transcriptional reporters driving GUS expression revealed that AP1σ1 and AP1σ2 have similar expression patterns and are widely expressed in Arabidopsis tissues (Fig. S3b–i). However, AP1σ1 appeared to be more highly expressed than AP1σ2 in anthers, as evidenced by the intensity of GUS staining (Fig. S3b–i). The introduction of transgenes encoding either AP1σ1 or AP1σ2 fused to YFP and driven by the AP1σ1 native promoter substantially rescued the male sterility phenotype of the ap1σ1-1 mutant (Figs 1f,g,m,n,t,u,v, S2c), confirming the redundant functions of AP1σ1 and AP1σ2 in pollen development.

Pollen exine defects in ap1σ1 mutants

We also generated anther cross-sections to compare anther development between the WT and the ap1σ1-1 mutant. Anther development follows 14 well-defined stages in Arabidopsis (Sanders et al., 1999). Anther development in ap1σ1-1 was similar to that of the WT from stage 1 to stage 8 (Fig. 2a,b,g,h). However, by stage 9, the microspores of WT plants generated the basal exine wall and became vacuolated (Fig. 2c), whereas microspores of ap1σ1-1 plants appeared shrunken and fragmented (Fig. 2i). During stages 10–12, the microspores of the WT gradually became mature and were released from the locules following anther dehiscence (Fig. 2d–f), while the microspores of ap1σ1-1 plants had mostly degenerated (Fig. 2j–l).

To investigate the mechanism of pollen degeneration in ap1σ1-1, we used SEM to observe the ultrastructure of pollen grains in both WT and ap1σ1-1 mutant plants. WT pollen grains were plump and covered with reticulate exine (Fig. 3a). By contrast, we observed shrunken pollen grains and abnormal exine pattern formation in ap1σ1-1 anthers (Fig. 3b). We used TEM to clarify the details of abnormal exine development of ap1σ1-1 pollen and discovered that primexine deposition is impaired in ap1σ1-1 microspores at stage 7 (Fig. 3b,i). In the following development stage, WT pollen grains successfully formed a complete pollen wall (Fig. 3c–g), whereas ap1σ1-1 pollen grains were...
Loss of AP-1σ function reduces fertility. (a–g) Representative images of the main inflorescences of WT Col-0 (a), ap1σ1 mutants (b–e) and two complementation lines (f, g). (h–n) Flowers of WT (h), ap1σ1 mutants (i–l) and two complementation lines (m, n). (o–u) Alexander’s staining of mature anthers of WT (o), ap1σ1 mutants (p–s) and two complementation lines (t, u). (v) Representative silique images from WT, ap1σ1 mutants and two complementation lines (left), and mean seed number per plant (right, n = 45 plants). Data are shown as means ± SD. Statistical analyses were performed by pairwise comparison to the WT or ap1σ1-1 (Student’s t-test, ***, P < 0.0001). Dots represent single data points. Bars: (a–g) 1 cm; (h–n) 200 μm; (o–u) 100 μm; (v) 1 mm.
mostly ruptured, with no exine pattern and only remnants of exine visible (Fig. 3j–n). These results indicated that the reduced male fertility in \( \text{ap1}_1 \) is due to failed primexine development.

To elucidate the function of AP-1\(_r\) in pollen wall development, we analysed the activity of the \( \text{AP1}_r1 \) and \( \text{AP1}_r2 \) promoters in more detail with the same GUS reporter lines mentioned above. We observed expression of \( \text{AP1}_r1 \) and \( \text{AP1}_r2 \) in the tapetum and microspores, as determined from anther cross-sections, and \( \text{AP1}_r2 \) was more weakly expressed than \( \text{AP1}_r1 \) (Fig. S4a–l). Consistent with this observation, our RT-qPCR results revealed that the expression levels of \( \text{AP1}_r1 \) are much higher than those of \( \text{AP1}_r2 \) in anthers from stage 7 to stage 12 (Fig. S4m). These data suggested that AP-1\(_\sigma\) plays a critical role in primexine development in the tapetum and microspores.

Secretion of type III LTPs and ACOS5 is impaired in \( \text{ap1}_1 \) mutant tapetum

Various components of the pollen wall are produced by tapetal cells and subsequently transported out to form the primexine (Blackmore et al., 2007; Liu & Fan, 2013; Ma et al., 2021). To determine whether loss of AP1\(_\sigma1\) affects the function of tapetal cells, we assessed the transport capacity of type III LTP (At5g62080) and ACOS5 in the \( \text{ap1}_1 \) mutant. Consistent

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**Fig. 2** \( \text{ap1}_1 \) mutant plants exhibit defects in anther development. (a–l) Semithin cross-sections illustrating anther development in wild-type (WT) (a–f) and \( \text{ap1}_1 \) mutants (g–l), from stage 7 to stage 12. DPG, degenerated pollen grain; E, epidermis; En, endothecium; ML, middle layer; MSP, microspore; PG, pollen grain; T, tapetum; Tds, tetrads. Bar, 10 \( \mu \)m.

**Fig. 3** Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis of pollen wall development in the wild-type (WT) and the \( \text{ap1}_1 \) mutant. (a, h) SEM analysis of dehiscent anthers in the WT (a) and \( \text{ap1}_1 \) (h). (b–g) TEM analysis of microspore development in the WT from stage 7 to stage 12. (i–n) TEM analysis of microspore development in \( \text{ap1}_1 \) from stage 7 to stage 12. CW, callose wall; DEx, defective exine; DPG, degenerated pollen grain; In, intine; MSP, microspore; Ne, nexine; PC, pollen coat; Pe, primexine; RMsp, ruptured microspore; Se, sexine. Bars: (a, h) 10 \( \mu \)m; (b–g, i–n) 1 \( \mu \)m.
with a previous report, live-cell imaging showed that LTP-YFP (fusion protein between At5g62080 and YFP) specifically accumulates in tapetal layers at stage 7 before becoming gradually distributed to anther locules during stages 8 and 9, finally being deposited on the surface of pollen grains at stage 10 (Huang et al., 2013; Fig. 4a–d). By contrast, we failed to observe LTP-YFP fluorescence in ap1σ1-1 locules during pollen development at stage 8 and later (Fig. 4e–h), indicating that loss of AP1σ1 blocks the type III LTP secretion pathway in tapetal cells, which is dependent on TGN/EE function (Huang et al., 2013). Similarly, GFP-ACOS5 (fusion protein between GFP and ACOS5) was transported to anther locules from stage 7 to stage 10 in WT plants (Wang et al., 2018; Fig. 4i–l), whereas we detected no GFP-ACOS5 fluorescence in anther locules during stages 8–10 in the ap1σ1-1 mutant (Fig. 4m–p). Although trafficking from the tapetum to anther locule in the ap1σ1-1 mutant was blocked, the subcellular distribution of LTP-YFP and GFP-ACOS5 in ap1σ1-1 tapetal cells did not change relative to that in the WT (Fig. S5). Furthermore, RT-qPCR analysis showed that the transcript levels of the LTP-YFP and GFP-ACOS5 transgenes in the ap1σ1-1 mutant are similar to those in the WT in anthers from stage 7 to stage 10 (Fig. S6). These results demonstrated that loss of AP1σ1 function impairs transport of type III LTPs and ACOS5 from tapetal cells to locules via the secretion pathway.

To further explore the effect of AP1σ1 absence on tapetum function, we performed TEM analysis of ap1σ1-1 tapetal cells during stages 7–10 (Fig. S7). We observed the generation of the unique tapetum lipid-accumulating organelles, elaioplasts and tapetosomes, at stage 8 in WT plants, while their emergence was delayed to stage 9 in the ap1σ1-1 mutant (Fig. S7), suggesting that AP1σ1 may function in the development of tapetal cells.

Post-Golgi trafficking of RPG1 is impaired in ap1σ1 mutants

The reduced male fertility phenotype of ap1σ1-1 was similar to that of the rpg1 mutant, raising the possibility that AP1σ1 might be required for RPG1 function at the microspore. To test this hypothesis, we examined the distribution of RPG1-GFP (Guan et al., 2008) encoded by a transgene whose expression was driven by the RPG1 promoter in the microspores of ap1σ1-1 and ap1σ2-1 mutants. We observed a strong alteration in the localization of RPG1-GFP in ap1σ1-1 stage 8 microspores, but not ap1σ2-1 microspores (Fig. 5a). We detected a marked intracellular accumulation of RPG1-GFP signal in subcellular structures in ap1σ1-1 that are not seen in the WT (Fig. 5a). These RPG1-GFP-labelled subcellular structures in ap1σ1-1 microspores overlapped with the TGN/EE compartment marker protein VHAa1-RFP (fusion protein between vacuolar H+-ATPase a1 and RFP; Dettmer et al., 2006) (Fig. 5b).
To identify the transport pathway from which the RPG1-GFP foci originated at the ap1σ1-1 mutant, we used the post-Golgi trafficking inhibitor BFA and the protein biosynthesis inhibitor CHX to test whether RPG1-GFP enters the endosome of microspores through endocytosis. RPG1-GFP accumulated in BFA bodies after treatment with BFA and CHX in WT microspores (Fig. 6a,b). Following BFA washout, RPG1-GFP-labelled BFA bodies disappeared, suggesting that these intracellular RPG1-GFP foci are recycled back to the PM (Fig. 6c,d). Similarly, we observed RPG1-GFP-labelled bodies in the cytoplasm of WT microspores treated with ES2, an inhibitor of exocytosis (C. Zhang et al., 2016; Fig. 6j,k). To examine whether AP1σ1 was required for the recycling of RPG1-GFP, we measured the ratios of intracellular to PM RPG1-GFP fluorescent signal intensities after BFA removal for 60 and 120 min in WT and ap1σ1-1 microspores (Fig. 6a–i). Following BFA washout, the entrapped RPG1-GFP in BFA bodies recycled back to the PM, resulting in a decrease of intracellular signal intensity and an increase of PM signal intensity. Quantitative image analysis showed that the recycling of RPG1-GFP is significantly reduced in the ap1σ1-1 mutant relative to the WT (P<0.0001, Fig. 6i). Together, these data demonstrated that internalized RPG1-GFP recycling from the TGN/EE to the PM in microspores depends on AP1σ1. Besides internalized RPG1-GFP, trafficking of newly synthesized RPG1-GFP that is transported to the PM from the TGN/EE is probably disrupted in ap1σ1-1 mutant microspores.

AP-1 mediates RPG1 sorting at the TGN/EE during microsporogenesis

To elucidate the molecular basis for AP-1 sorting of RPG1 at the TGN/EE, we performed BiFC and Y2H assays to determine the potential for interaction between AP-1 and RPG1. Adaptor protein complexes generally sort cargo proteins either via recognition by the μ subunit of tyrosine-based sorting motifs (YXXΦ) or via recognition by the σ subunit of dileucine-based sorting motifs ([DE][XXXL][LI]) (Ohno et al., 1995; Kelly et al., 2008; Liu et al., 2020). An analysis of the cytoplasmic domains of RPG1 revealed one tyrosine-based sorting signal, 166YMPF169, in the third cytoplasmic loop (Fig. 7a). BiFC assays demonstrated that full-length RPG1 interacted with AP1μ2, which required the third entire cytoplasmic domain of RPG1 (Fig. 7b). Y2H assays further supported the interaction of RPG1 with AP1μ2, as well as the subunits AP1σ1, AP1σ2, AP1γ1 and AP1β1 (Fig. 7c).

To test whether 166YMPF169 was a canonical YXXΦ sorting motif, we generated a variant of RPG1 carrying a Y166A/F169A double mutation. We observed that AP1μ2 interacts much more weakly with the RPG1Y166A/F169A mutant compared to WT RPG1 by both BiFC and Y2H experiments (Fig. 7b,c). By contrast, the Y166A, F169A variant did not affect the interaction between AP1σ1/2 and RPG1 (Fig. 7b,c), indicating that 166YMPF169 is not a critical site for AP1σ1/2 binding to RPG1. Overexpression of GFP-RPG1 in ap1σ1-1 significantly increased the number of viable pollen grains and enhanced fertility, whereas overexpression of GFP-RPG1Y166A/F169A in the ap1σ1-1 mutant background resulted in a male fertility phenotype that was intermediate between that of the WT and that of GFP-RPG1 overexpression lines in ap1σ1-1 (P<0.0001, Fig. S8a–j). We also analysed the subcellular localization of GFP-RPG1 and GFP-RPG1Y166A/F169A in the WT and ap1σ1-1 mutant. Similar to the observed mislocalization of GFP-RPG1 in the ap1σ1-1 mutant, GFP-RPG1Y166A/F169A mislocalized in WT plants and displayed a strong intracellular signal in ap1σ1-1 plants (Fig. S8k–n). Quantitative image analysis of microspores showed that the PM levels of GFP-RPG1 and GFP-RPG1Y166A/F169A are lower in ap1σ1-1 than in the WT (Fig. S8o), indicating that AP1σ1 is
necessary for maintaining a sufficient amount of RPG1 at the microspore PM. Together, these results suggested that the putative YXXΦ sorting motif site is functional and critical for AP-1 recognition of RPG1 at the TGN/EE and for regulating RPG1 abundance at the PM during pollen development.

AP1σ1 is required for AP-1 complex function in microspores

Based on the above results, we hypothesized that loss of AP1σ1 function disrupts the sorting of RPG1 due to impaired AP-1 function at the TGN/EE. We previously showed that deletion of one ADAPTOR PROTEIN-2 complex (AP-2) subunit affects the recruitment of other subunits to the PM, thus affecting the function of AP-2-mediated endocytosis (C. Wang et al., 2016). Therefore, we examined the subcellular distribution of AP1σ1 and AP1μ in microspores. Live-cell imaging showed that AP1σ1-YFP and AP1σ2-YFP almost completely colocalize with AP1μ2-RFP at the TGN/EE compartments, as expected (Fig. S9). These observations therefore indicated that AP1σ1, AP1σ2 and AP1μ2 are recruited to assemble the AP-1 complex at the TGN/EE of microspores.

Next, we explored the consequences of removing the AP-1σ subunit on the distribution of other AP-1 subunits in...
Quantitative analysis of the TGN/EE-associated fluorescence signal in the \( \text{ap1r1-1} \) mutant showed that loss of AP1\( \text{r1} \) causes a significant reduction in AP1\( \text{l2} \)-RFP, GFP-AP1c\( \text{1} \) and GFP-AP1b\( \text{1} \) intensities at the TGN/EE (\( P < 0.0001 \), Fig. 8a–i). By contrast, absence of AP1\( \text{r1} \) had no effect on the distribution of the TGN/EE-resident marker protein VHAa1-RFP (Fig. 8j–l), indicating that loss of AP1\( \text{r1} \) specifically affects the localization of AP-1 subunits but not the distribution of other TGN/EE markers. These results suggested that the AP-1\( \text{r1} \) subunit is required for the localization of other AP-1 subunits at the TGN/EE in the microspore. However, it remains unclear whether loss of AP-1\( \text{r1} \) prevented the recruitment of other AP-1 subunits and/or reduced the stability of the AP-1 complex, thus indirectly affecting the function of AP-1 at the TGN/EE.

**Defects in auxin-mediated callose biosynthesis in ap1σ1-1 mutants**

The plant hormone auxin is important for pollen development (Cecchetti et al., 2008; Yao et al., 2018), and polar auxin transport in anther filaments and pollen grains depends on the auxin efflux carrier PIN1 (Feng et al., 2006). The transcription factor AUXIN RESPONSE FACTOR17 (ARF17) controls callose biosynthesis by regulating the expression of CALLOSE SYNTHASE5 (CALS5) and plays an important role in primexine formation (Yang et al., 2013; B. Wang et al., 2017). AP1\( \text{μ2} \) is necessary for dynamic auxin distribution in Arabidopsis (J. G. Wang et al., 2013). Therefore, we introduced the auxin reporter construct \( \text{DR5}:\text{GFP} \) into the \( \text{ap1σ1-1} \) mutant and determined the resulting GFP fluorescence pattern in WT and \( \text{ap1σ1-1} \) microspores at stage 8. Quantitative analysis of the TGN/EE-associated fluorescence signal in the \( \text{ap1σ1-1} \) mutant showed that loss of AP1\( \text{σ1} \) causes a significant reduction in AP1\( \text{μ2} \)-RFP, GFP-AP1\( \text{γ1} \) and GFP-AP1\( \text{β1} \) intensities at the TGN/EE (\( P < 0.0001 \), Fig. 8a–i). By contrast, absence of AP1\( \text{σ1} \) had no effect on the distribution of the TGN/EE-resident marker protein VHAa1-RFP (Fig. 8j–l), indicating that loss of AP1\( \text{σ1} \) specifically affects the localization of AP-1 subunits but not the distribution of other TGN/EE markers. These results suggested that the AP-1\( \text{σ1} \) subunit is required for the localization of other AP-1 subunits at the TGN/EE in the microspore. However, it remains unclear whether loss of AP-1\( \text{σ1} \) prevented the recruitment of other AP-1 subunits and/or reduced the stability of the AP-1 complex, thus indirectly affecting the function of AP-1 at the TGN/EE.
anthers. With the development of anthers, we detected strong GFP fluorescence in WT anthers from stage 9 to stage 12 (Fig. 9a–e). However, in contrast to the WT, the GFP fluorescence signal in \( ap1 \)r1-1 anthers was very weak from stage 9 to stage 11, falling below the detection limit in \( ap1 \)r1-1 stage 12 anthers (Fig. 9f–j), suggesting that auxin distribution in anthers is compromised in the \( ap1 \)r1-1 mutant.

To investigate the effect of disturbed auxin distribution on pollen development, we analysed the peripheral callose wall of tetrads in the WT and the \( ap1 \)r1-1 mutant. Aniline blue staining showed that the thickness of the callose wall in \( ap1 \)r1-1 mutants is reduced compared to that in the WT (Fig. 9k,l). Consistent with this observation, loss of AP1r1 function was accompanied by lower ARF17 and CALS5 expression in floral buds, as determined by RT-qPCR (Fig. 9m,n). These results suggested that AP1r1 is required for the regulation of callose biosynthesis by auxin during pollen development.

**Discussion**

**AP-1-mediated post-Golgi trafficking in plants**

The AP-1 complex is involved in various post-Golgi trafficking pathways, such as sorting cargo proteins in biosynthetic and recycling routes in Madin-Darby canine kidney cells (Castillou *et al.*, 2018; Gravotta *et al.*, 2019), sorting secretory vesicles in *Aspergillus nidulans* (Martzoukou *et al.*, 2018), sorting mannose 6-phosphate receptors from immature secretory granules in pancreatic β cells (Klumperman *et al.*, 1998) and mediating mannose 6-phosphate receptors from endosomes to the TGN in mouse epithelial cells (Meyer *et al.*, 2000).

In plants, the μ subunit of the AP-1 complex is required for the transport of BRI1, KNOLLE, PIN2 and SUB (Park *et al.*, 2013; Teh *et al.*, 2013; J. G. Wang *et al.*, 2013; J. G. Wang *et al.*, 2016). Arabidopsis \( ap1 \)μ2 mutants display defects in secretory
and vacuolar trafficking (Park et al., 2013), as well as impaired endocytic recycling in root cells (J. G. Wang et al., 2013). Likewise, genetic inactivation of the AP-1γ subunit leads to mistargeting of tonoplast proteins (Wang et al., 2014; J. G. Wang et al., 2017). Here, we showed that loss of AP-1γ impairs secretion of type III LTPs and ACOS5 in tapetal cells (Fig. 4) and disrupts the recycling of RPG1 in microspores (Fig. 6). Unlike for RPG1-GFP, we observed no significant accumulation of GFP-ACOS5 or LTP-YFP at the TGN/EE in AP1γ1-deficient cells (Figs 5, S5). Potential reasons for the inability of GFP-ACOS5 and LTP-YFP to be retained at the TGN/EE in AP1γ1-deficient cells include disruption of AP-1 function preventing their transport from the ER to the TGN/EE or may lead these proteins to enter the incorrect vesicle transport pathway from the TGN/EE. Our findings provide novel insight into the regulation of AP-1-mediated post-Golgi trafficking in plant fertility. However, whether the trafficking of proteins to the vacuole for their degradation is affected in ap1γ microspores and in which vesicle transport pathway AP-1 participates remain to be investigated. Although AP-1-mediated trafficking is closely related to clathrin (Gall et al., 2002; Castillon et al., 2018; Martzoukou et al., 2018; Gravotta et al., 2019; Yan et al., 2021), an evolutionarily conserved vesicle coat protein (Robinson, 2015; Kaksonen & Roux, 2018), there is no direct evidence in plants that AP-1-mediated vesicles at the TGN/EE are clathrin-coated.

We showed that trafficking of RPG1, type III LTPs and ACOS5 is impaired in the ap1γ1-1 mutant during primexine formation, but it is likely that other cargo proteins ferried by AP-1 also contribute to the fertility defects seen in the ap1γ1-1 mutant. The patterns of the auxin reporter DR5:GFP and callose staining showed that auxin distribution in anthers and the callose wall of microspores is reduced in ap1γ1-1 compared to the WT (Fig. 9). These results suggest that AP-1 may modulate auxin flow in filaments, possibly by regulating the polarity of the PIN family of auxin transporters. However, the localization of PIN1 in ovules and roots is independent of AP1γ2 (J. G. Wang et al., 2016). The functional relationship between AP-1 and auxin in regulating pollen wall pattern formation thus warrants further investigation.

Fig. 9 Analysis of auxin-regulated pollen development in the wild-type (WT) and the ap1γ1-1 mutant. (a–j) Representative pattern from the auxin reporter DR5:GFP in anthers of the WT (a–e) and ap1γ1-1 (f–j) from stage 8 to stage 12. Green, DR5:GFP fluorescence; red, Chl autofluorescence. (k, l) Aniline blue staining of callose walls of the WT and ap1γ1-1. (m, n) Relative transcript levels of ARF17 and CALS5 in WT and ap1γ1-1 floral buds, as determined by RT-qPCR and normalized to UBIQUITIN7. Data are shown as means (±SD) from three independent experiments. Dots represent single data points. *** P < 0.0001 (Student’s t-test; compared to the WT). Bars: (a–j) 50 μm; (k, l) 10 μm.
Mechanism of AP-1 sorting cargo proteins at the TGN/EE

The AP-1 complex selects specific newly synthesized proteins and internalized proteins into distinct vesicles at the TGN and transports them to respective destinations (Guo et al., 2014; Tan & Gleeson, 2019). The specific motifs displayed by cargo proteins are critical for their interaction with adaptor proteins to ensure accurate protein sorting (Guo et al., 2014; Tan & Gleeson, 2019). AP-1 subunits can identify specific cargo proteins containing various sorting motifs. In mammalian cells, the best characterized sorting signals are the tyrosine-based motif YXXΦ and the dileucine-based motif [DE][XXX][LI], which are recognized by the AP-1 μ and γ/σ subunits, respectively (Janvier et al., 2003; Touz et al., 2004; Kyttälä et al., 2005; Rybakin et al., 2006; Doray et al., 2007; Noviello et al., 2008; Carvajal-Gonzalez et al., 2012; Baltes et al., 2014).

Similar to the mechanism described for AP-1 recognition of cargo proteins in animals, AP-1 μ binds to cytoplasmic-localized tyrosine-based motifs in plants. Arabidopsis VACUOLAR SORTING RECEPTOR4 (VSR4) and pea (Pisum sativum) VSR-PS1 (Happel et al., 2004; Nishimura et al., 2016). In addition, Arabidopsis AP-1 γ interacts with the dileucine-based motif of VACUOLAR ION TRANSPORTER1 (VIT1) and SECRETORY CARRIER-ASSOCIATED MEMBRANE PROTEIN1 (SCAMP1) to deliver them to the tonoplast (Wang et al., 2014). Here, our results revealed that the 166YMPF169 motif, located within one of the cytoplasmic loops of RPG1, plays an important role in the interaction of RPG1 with AP-1 μ as a sorting signal (Fig. 7). Disruption of this YXXΦ motif weakened the interaction of RPG1 with AP-1 μ and caused a mislocalization of GFP-RPG1 in the WT that is similar to that seen in the ap1-1-1 mutant (Figs 7, S8k–m). Reduced AP-1 μ localization at the TGN/EE may explain the mislocalization of RPG1 in the ap1-1-1 mutant. Nevertheless, BiFC and Y2H analysis showed that the AP-1 σ subunit also interacts with RPG1, although RPG1 lacks a clear [DE][XXX][LI] consensus motif on its cytoplasmic side (Fig. 7b,c). It is likely that as a component of the AP-1 complex, AP1σ, as well as AP1γ and AP1β, forms a contact region to carry cargo proteins (Fig. 7c). Alternatively, the AP-1 σ subunit may recognize an unknown sorting motif on RPG1 that is different from the [DE][XXX][LI] motif.

Numerous proteins are potential cargo proteins of the AP-1 complex in plants, based on their impaired trafficking and mis-targeting in ap1 μ2 mutants, such as BRI1, KNOLLE, PIN2 and SUB (Park et al., 2013; Teh et al., 2013; J. G. Wang et al., 2013; J. G. Wang et al., 2016). However, the molecular mechanism by which AP-1 sorts these proteins remains to be elucidated. Our findings demonstrate that the PM protein RPG1 is involved in pollen wall development and is a cargo protein sorted by the AP-1 complex at the TGN/EE. AP-1-mediated protein sorting in plant germ cells may thus involve a mechanism similar to that of somatic cells.

Multiple roles for AP-1 in plant development

The AP-1 complex is a cargo adaptor at the TGN and plays an essential role in protein sorting (Guo et al., 2014; Tan & Gleeson, 2019). In mammalian systems, AP-1 mediates polar transport of proteins in epithelial cells and neurons (Bonifacino, 2014). Loss of AP-1 function causes growth and development defects, including hypoactivity, lipodystrophy, impaired long-term spatial memory and even embryonic mortality in mice (Meyer et al., 2000; Glyvuk et al., 2010; Baltes et al., 2014).

Arabidopsis plants lacking AP1 μ2 function display a growth retardation phenotype (Park et al., 2013; Teh et al., 2013; J. G. Wang et al., 2013). In addition to interrupted vegetative growth, ap1–1 mutant plants exhibit abnormal development of their reproductive structures (J. G. Wang et al., 2013, 2016, 2017; Shimada et al., 2018). For example, loss of AP1 μ2 decreases pollen germination (J. G. Wang et al., 2013), impairs ovule development (J. G. Wang et al., 2016) and blocks mucilage extrusion from the seed coat (Shimada et al., 2018). In addition, loss of AP-1γ blocks pollen tube reception and leads to sterility of the female gametophyte (J. G. Wang et al., 2017). Here, we revealed a role for AP-1 in pollen wall development. Genetic interference with AP-1σ function resulted in defects in primexine formation associated with impaired RPG1 trafficking and secretion of type III LTPs and ACOS5 (Figs 3–6). Moreover, TEM analysis showed that impaired AP-1 function delays the emergence of two organelles, elaioplasts and tapetosomes, in tapetal cells associated with pollen wall formation (Fig. S7). However, how AP-1-mediated vesicle transport contributes to the formation of elaioplasts and tapetosomes is unknown. Together, these observations point to AP-1 as an important regulator for cargo transfer in plants that relies on sorting proteins at the TGN/EE and packages them into distinct transport carriers to ensure their appropriate localization and modulate their abundance.

Acknowledgements

This work was supported by grants to JP from the National Natural Science Foundation of China (nos. 91754104, 3182103008 and 31670283) and to CW from the Youth Program of National Natural Science Foundation of China (no. 31801193). We are grateful to Zhongnan Yang (College of Life and Environment Sciences, Shanghai Normal University), Cheng Zhang (College of Life and Environment Sciences, Shanghai Normal University) and Yan Zhang (College of Life Sciences, Shandong Agricultural University) for generously sharing published materials and comments and to the Arabidopsis Biological Resource Center for seed stocks. We thank Liang Peng, Yang Zhao, Liping Guan, Yahu Gao, Xia Deng and Haiyan Li (Core Facility for Life Science Research, Lanzhou University) for their technical assistance as well as Jia Li and Xiaoping Gou (School of Life Sciences, Lanzhou University) for their technical assistance as well as Jia Li and Xiaoping Gou (School of Life Sciences, Lanzhou University).

Author contributions

MX, XY, JP and CW conceived the study and designed the experiments. MX, XY, CL, QY and DT carried out the experiments. MX, XY and CW analysed the data. MX, XY, YW and CW wrote the article. MX, XY, YW, SYB and CW revised the article. MX and XY contributed equally to this work.
References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** A knockout mutant of *AP1σ1* shows reduced male fertility.

**Fig. S2** A knockout mutant of *AP1σ1* and *AP1σ1* knockdown lines shows reduced male fertility.

**Fig. S3** Amino acid sequence alignment of AP1σ1 and AP1σ2 and tissue-specific expression of *AP1σ1* and *AP1σ2* in Arabidopsis.

**Fig. S4** Expression analysis of *AP1σ1* and *AP1σ2* in anthers.

**Fig. S5** Localization of LTP-YFP and GFP-ACOS5 in *ap1σ1-1* tapetal cells.

**Fig. S6** RT-qPCR analysis of *LTP-YFP* and *GFP-ACOS5* transcript levels in *ap1σ1-1*.

**Fig. S7** Ultrastructure of tapetal cells in the WT and the *ap1σ1-1* mutant.

**Fig. S8** Overexpression of *RPG1* partially rescues the fertility defects of *ap1σ1*.

**Fig. S9** Colocalization analysis of AP1σ and AP1μ in microspores.

**Table S1** PCR primer sequences for genotyping, RT-PCR and cloning.

**Table S2** Sequences of targets and primers used in CRISPR/Cas9 editing and primers for generating amiRNA constructs.

**Table S3** Sequences of primers used for RT-qPCR.

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