



Arabidopsis FAX1 mediated fatty acid export is required for the transcriptional regulation of anther development and pollen wall formation

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Abstract

Key Message The mutation of *FAX1* (*Fatty Acid Export 1*) disrupts ROS homeostasis and suppresses transcription activity of *DYT1-TDF1-AMS-MS188* genetic network, leading to atypical tapetum PCD and defective pollen formation in *Arabidopsis*.

Abstract Fatty acids (FAs) have multiple important biological functions and exert diverse cellular effects through modulating Reactive Oxygen Species (ROS) homeostasis. *Arabidopsis FAX1* (*Fatty Acid Export 1*) mediates the export of de novo synthesized FA from chloroplast and loss of function of *FAX1* impairs male fertility. However, mechanisms underlying the association of *FAX1*-mediated FA export with male sterility remain enigmatic. In this study, by using an integrated approach that included morphological, cytological, histological, and molecular analyses, we revealed that loss of function of *FAX1* breaks cellular FA/lipid homeostasis, which disrupts ROS homeostasis and suppresses transcriptional activation of the *DYT1-TDF1-AMS-MS188* genetic network of anther development, impairing tapetum development and pollen wall formation, and resulting in male sterility. This study provides new insights into the regulatory network for male reproduction in plants, highlighting an important role of FA export-mediated ROS homeostasis in the process.

Keywords Anther development · FAX1 · Fatty acid transport · Genetic network · ROS

Introduction

Fatty acids (FAs) have multiple important biological functions. They are not only the basic components of living organisms but also important signals and/or signal precursors that regulate the development of cells. Studies in animal confirm that, on one hand, FAs provide membrane-structured phospholipids for rapidly proliferating tumor cells, and

on the other hand, their metabolites act as signal molecules that are widely involved in signal transduction pathways in tumor cells (Pike et al. 2011). FAs may have similar functions in plant development and signal transduction, particularly in the process of anther and pollen wall development (Shi et al. 2015; Zhang et al. 2016).

The anther tapetum is a non-green tissue that contains plastids, where the de novo biosynthesis of FAs, necessary raw materials exported via various molecules including FAX1 for the formation of the main component of plant male gametophyte walls, occurs (Li et al. 2015). When FA metabolism in anther tapetum is interrupted, not only the formation of male gametophyte wall but also the tapetum programmed cell death (PCD) are seriously affected, resulting in semi- or complete male sterility (Shi et al. 2015; Xu et al. 2014). In addition, reactive oxygen species (ROS) are required for anther development and male fertility in plants (Hu et al. 2011; Xie et al. 2014; Xing and Zachgo 2008; Yi et al. 2016). However, the relationship between lipid metabolism and ROS in context of tapetum PCD and male fertility remains obscure.

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In *Arabidopsis*, the genetic network composed of transcription factors DYSFUNCTIONAL TAPETUM1 (DYT1)-DEFECTIVE in TAPETAL DEVELOPMENT FUNCTION1 (TDF1)-ABORTED MICROSPORES (AMS)-MALE STERILE 188 (MS188) plays key roles in regulating tapetum development and pollen wall formation (Zhu et al. 2011). Each transcription factor has hundreds of specific downstream genes independent of the downstream factors of this pathway, playing its function mainly through downstream transcription regulators for tapetum development. In addition, all four transcription factors have more than 530 common downstream tapetum genes (Li et al. 2017). Nevertheless, the upstream regulators or signals of the *DYT1-TDF1-AMS-MS188* pathway are poorly known.

Arabidopsis FAX1 (Fatty acid export 1), a plastid inner envelope localized protein, is recently found to function in the export of de novo synthesized FAs out of plastid and play crucial roles in biomass production and male fertility (Li et al. 2015). Loss of function of *FAX1* exhibits smaller anthers and wrinkled pollens lacking exine layer and pollen coat (Li et al. 2015). Mutation of *FAX1* delays tapetum degradation, impairs pollen wall formation, pollen viability, and alters sepal cuticular patterning and bud cuticle profiles (Liu et al. 2018). Abovementioned studies highlight multiple functions of *FAX1* on male reproductive development, however, the link between FAs export and male fertility is yet missing. In this study, we performed morphological, cytological, histological, and molecular analyses, aiming to build such a link in context of tapetum PCD and pollen wall formation. Our data revealed that *FAX1* is highly expressed in developing anthers particularly in tapetum at early stages, and that loss of function of *FAX1* breaks cellular FA/lipid homeostasis, which not only disrupts ROS homeostasis, but also suppresses transcriptional activation of the DYT1-TDF1-AMS-MS188 genetic network, leading to defective pollen wall formation and male sterility.

Materials and methods

Plant materials

Arabidopsis thaliana (L.) Heynh. Columbia-0 (Col) and mutant *fax1-2* (GABI_599E01) (Li et al. 2015) were both gifts from Professor Katrin Philipp of Ludwig Maximilians University, Germany. *Arabidopsis* seeds were vernalized for 3 days at 4 °C, and grown in chambers under long-day conditions (16 h/8 h of light/dark) at 22 °C.

GUS staining analysis

For expression analysis, *FAX1* genomic sequence was amplified from Col genomic DNA using primers

FAX1-pro-F and FAX1-pro-R containing restriction sites BamHI and BglII. The PCR product was ligated to the upstream of the GUS gene into the pCAMBIA1301 vector, which transformed to wild type *Arabidopsis* by floral dip method (Clough and Bent 1998), and transgenic plants were selected for presence of hygromycin. For GUS staining analysis, floral buds from positive transformants were incubated in a GUS staining solution (Jones-Rhoades et al. 2007) at 37 °C for 24 h, then viewed on a Leica Stereo Microscopes (M205A). Selected stained floral buds were embedded for semi-thin section analysis.

Microscopy and phenotype characterization

Plant materials for the semi-thin sections were prepared as described (Zhang et al. 2007). For SEM examination, fresh pollen grains were coated with 8 nm of gold and observed under an S-3400 N microscope (HITACHI). For TEM observation, specimens of *Arabidopsis* floral buds were prepared as described (Zhang et al. 2007) and observed under a Tecnai G2 SpiritBiotwin microscope (FEI).

TUNEL assay

The TUNEL (TdT-mediated DUTP nick end labeling) assay was modified from a previously described protocol (Vizcay-Barrena and Wilson 2006). Whole inflorescences of the wild type and *fax1-2* were fixed in fixative with 50% (v/v) anhydrous alcohol, 5% (v/v) glacial acetic acid, 3.7% (v/v) methanol and 35% (v/v) ddH₂O overnight at 4 °C. Samples were then washed with PBS buffer, followed by dehydration in a graded ethanol series, which were cleared in ethanol/histoclear (2:1, 1:1, and 1:2) for 1 h and three times in 100% histoclear for 1 h each at room temperature, embedded in paraffin wax, and sliced into 8 µm thick sections. The *in-situ* Cell Death Detection Kit (Promega, Madison, WI, USA, G3250) was used for TUNEL analysis as instructed. Sections were deparaffinized with histoclear, hydrated in a graded ethanol series, washed in 0.85% NaCl and PBS, fixed in 3.7% (v/v) methanol in PBS, and washed in PBS. Resulting samples were incubated with 20 µg/ml Proteinase K, followed by the wash in PBS, fixation in 3.7% (v/v) methanol, and the wash in PBS. Samples were then incubated with equilibration buffer for 10 min, followed by the incubation with rTdT reaction mixture for 1 h in the dark at 37 °C, which then was washed in SSC for 15 min and PBS for 15 min, and counterstained by 1 µg/ml PI (propidium iodide) and 2 µg/ml DAPI (4',6-diamidino-2-phenylindole). Signals were then detected using a confocal laser scanning microscope (Leica & TCS SP8).

Total RNA isolation and mRNA expression analysis

Total RNA was extracted using the RNAPrep pure Plant Kit (TIANGEN) from young inflorescences (approximately floral stage 1–12), leaves and stems. 1–2 µg total RNA was used for reverse transcription according to the manufacturer's instruction to synthesize cDNA (FastQuant RT Kit, TIANGEN), which was used directly as PCR templates. For qRT-PCR, oligonucleotides used for the experiments are listed in the Supplementary Table 1 online. All samples were run at least in triplicate.

Histochemical assays for ROS

Anthers of various developmental stages were incubated in NBT (Hu et al. 2011) or H₂DCF-DA (Sigma-Aldrich) staining solution (Xie et al. 2014) for ROS detection. Anthers were classified into six groups based on their sizes as described previously (Xie et al. 2014).

Results

FAX1 is highly expressed in tapetum

Although expression of plastid *FAX1* throughout development has been presented by in silica (Li et al. 2015) and partially validated via quantitative RT-PCR (qRT-PCR) in developing flowers (Liu et al. 2018), its spatial-temporal pattern remains unrevealed experimentally. Thus, we first analyzed the spatial-temporal expression characteristics of *FAX1* in different tissues and at different stages of anther development through qRT-PCR. Although it was expressed also in stem and leaf tissues, *FAX1* was found to be highly expressed in anthers at different stages, in which the expression initiated highly before stage 6, peaked at stage 7–8, and kept relative high levels even at stage 12 (Fig. 1c). The qRT-PCR results were further verified with p1301-Pro::FAX1-GUS transgenic plants, in which GUS signals could be detected in almost all tested stages of anther development: appearing from stage 5, increasing gradually and peaking at stage 8 and stage 9, and decreasing thereafter gradually (Fig. 1a). Semi-thin sections of anthers at stage 9 showed that GUS signal of *FAX1* appeared dominantly in the tapetal cells (Fig. 1b). Abovementioned expression results established a close association of *FAX1* expression with anther development.

Deficiency of *FAX1* leads to atypical tapetum development and defective pollens

We performed detailed semi-section analysis to determine the effects of mutation of *FAX1* on anther development. The development of *fax1-2* anthers at stage 5 was similar to that of WT (Wild Type) (Fig. 2a, e), but distinct developmental defectives were observed in *fax1-2* anthers at stage 6 (Fig. 2b, f), which did not show tapetum vacuolation as WT did. During stage 7 to stage 8, tapetum, meiocyte, tetrads, and microspores in WT exhibited obvious degrees of vacuolation (Fig. 2c, d), while tapetum in *fax1-2* experienced much less vacuulations and meiocyte, tetrads, microspores in *fax1-2* were filled with cytosol (Fig. 2g, h). From stage 9 to stage 11, WT tapetal cells became condensed and microspores were angular in shape (Fig. 2i–k), but tapetal and middle layer cells in *fax1-2* were swelled, which filled the locules together with round but ununiformed microspores, and seemed to impair the release of microspores (Fig. 2m–o). At stage 12, the WT anther contained mature pollen grains and tapetal cells disappeared (Fig. 2l). However, the degrading tapetum cells in *fax1-2* were still observed, and over 80% of the round-like microspores released was lightly stained with obvious abnormal cavities (Fig. 2p), which was also observed previously (Fig. 4c in Li et al. 2015; Fig. 4 in Liu et al. 2018). Semi-section analysis result indicated that *FAX1* affects not only tapetum development but also microspore formation and release, which confirmed the previous cytological analysis result (Liu et al. 2018).

Mutation of *FAX1* interrupts lipid transport

For a more detailed ultrastructural characterization of tapetal cells of *fax1-2*, we performed transmission electron microscopy (TEM) analyses on both WT and *fax1-2* mutant anthers. At stage 6 and stage 7, different from that of WT (Fig. 3a, b), *fax1-2* tapetal cells were less vacuolated (Fig. 3e, f). At stage 8, as compared with WT (Fig. 3c), *fax1-2* mutant tapetal cells accumulated more elaioplasts (ELs) (Fig. 3g). From stage 9 to 11, when WT tapetal cells showed more ELs full of huger light colored stone-like cells (Fig. 3d, i and k), *fax1-2* exhibited, however, different ELs with dark stained regular shaped cells separated by fewer and smaller light-colored stone-like cells (Fig. 3h, m and o). This result indicated an interrupted lipid export from tapetal cells in *fax1-2*. In *Arabidopsis*, elaioplasts and tapetosomes two important organelles, are transporters of lipidic molecules from tapetum to pollen wall for pollen wall formation (Quilichini et al. 2014). At stage 12, WT tapetal cells degraded completely (Fig. 3l); however, tapetum debrides in *fax1-2* tapetal cells

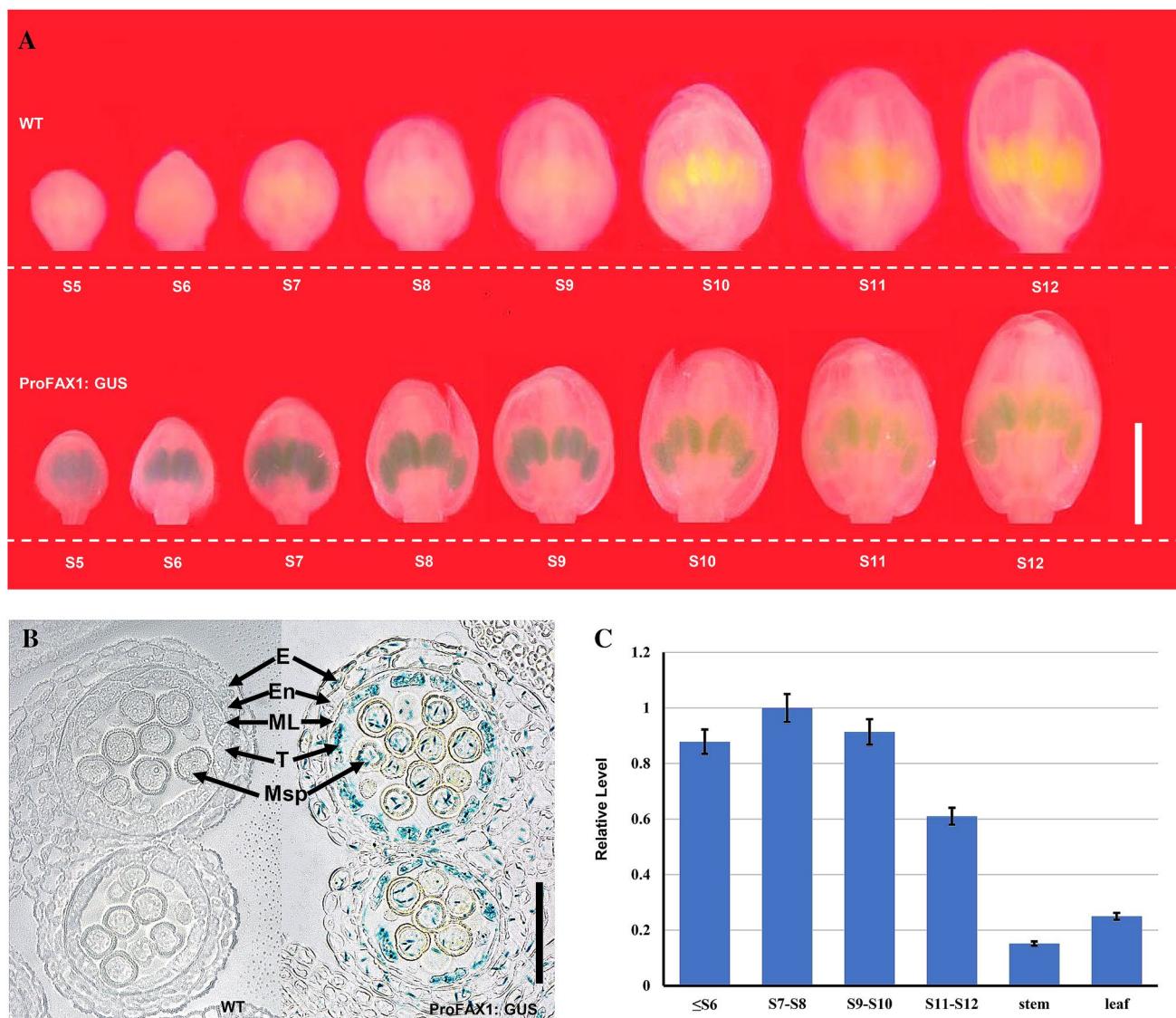


Fig. 1 Spatial-temporal expression pattern of *FAX1*. **a** GUS staining images of representative buds from ProFAX1: GUS transgenic plants at different developmental stages. Bar=0.5 mm; **b** GUS signals detected in cross section of anthers at stage 9 in transgenic plants (p1301-Pro: FAX1-GUS). E epidermis, En endothecium, ML middle

layer, T tapetum, Msp microspores; Bar=50 μ m; **c** qRT-PCR analysis of the spatial-temporal expression pattern of *FAX1*. RNA was extracted from anthers at various developmental stage, stems and leaves of wild type plant. ≤S6, Stage1–6; S7–S8, Stage 7–8; S9–S10, Stage 9–10; S11–S12, Stage11–12

still persisted, indicating delayed tapetal cell degeneration in *fax1-2* (Fig. 3p). Therefore, mutation of *FAX1* interrupted tapetal lipid export in addition to tapetal cell development and degradation.

Mutation of *FAX1* impairs pollen wall formation

Because the mutation altered tapetal development and degradation and as well the tapetal lipid export, we further examined ultrastructure of pollens at various developmental stages using TEM. At stage 7, WT tetrad was formed with normal primexine (Fig. 4a; Supplementary Fig. 2A online)

while normally formed *fax1-2* tetrad displayed a much thinner primexine (Fig. 4h; Supplementary Fig. 2G online). At stage 8, WT microspores released from the tetrad showed distinctive probaculae structure and more prominent nexine structure on the microspore surface (Fig. 4b; Supplementary Fig. 2b online). In contrast, *fax1-2* microspores displayed a thin thread like nexine structure and randomly distributed unstructured probaculae that disconnected with the nexine (Fig. 4i; Supplementary Fig. 2H online). At stage 9, WT microspore exhibited well organized typical mature bacula with partially formed tecta (the sexine) (Fig. 4c; Supplementary Fig. 2C online), whereas *fax1-2* microspore showed

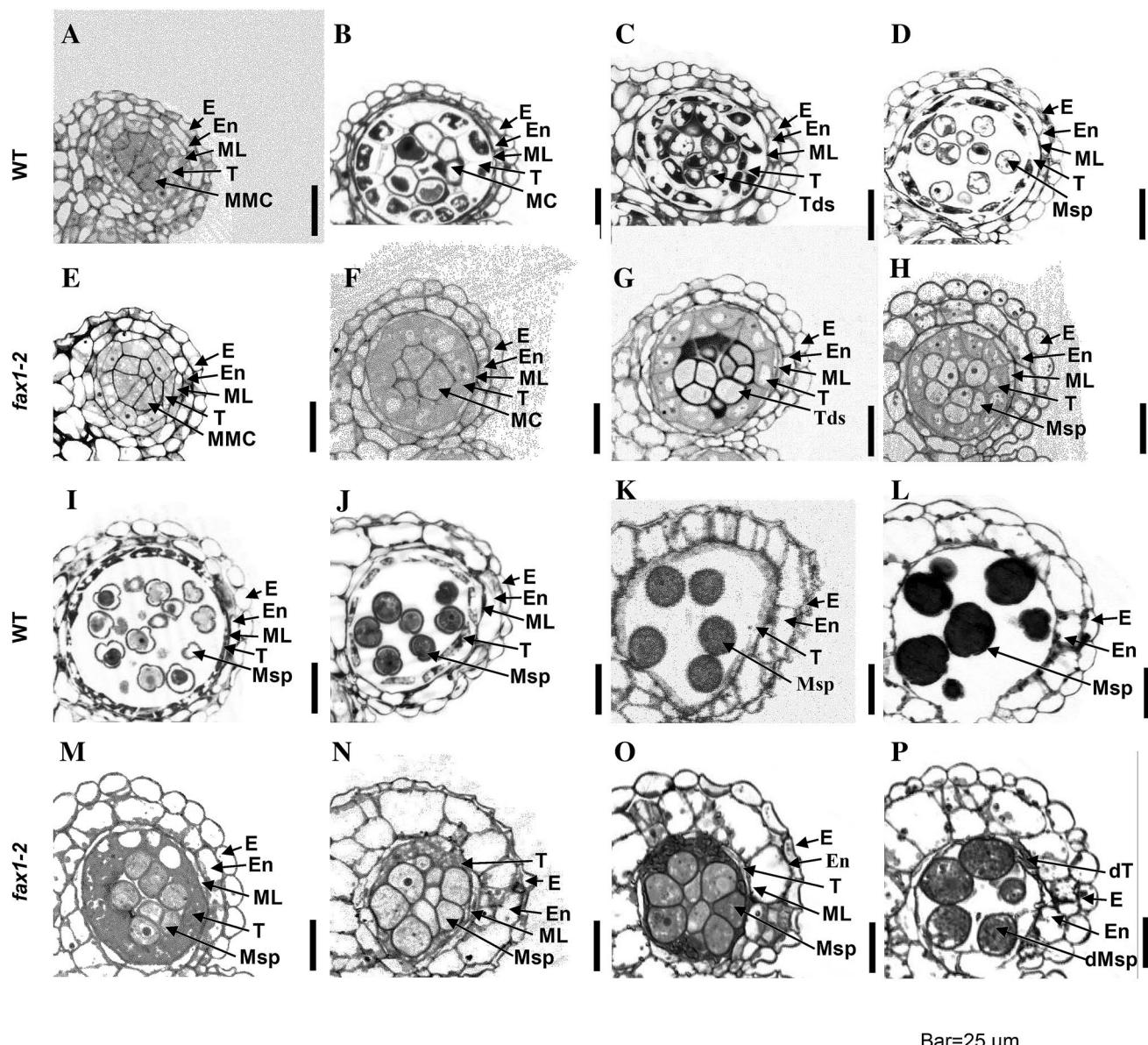


Fig. 2 Histological characterization of anther development in wild-type (**a–d** and **i–l**) and *fax1-2* (**e–h** and **m–p**) at different developmental stages. **a** and **e** Anther at stage 5; **b** and **f** Anther at stage 6; **c** and **g** Anther at stage 7; **d** and **h** Anther at stage 8; **i** and **m** Anther at stage 9; **j** and **n** Anther at stage 10; **k** and **o** Anther at stage 11; **l** and **p**

Anther at stage 12. *dMsp* degenerated microspores, *dT* degraded tapetum, *E* epidermis, *En* endothecium, *MC* meiocyte, *MMC* microspore mother cell, *ML* middle layer, *Msp* microspores, *T* tapetum, *Tds* tetrads. Bar = 25 μ m

irregular dot-shaped baculas that connected with nexine, but no sexine structures (Fig. 4j; Supplementary Fig. 2I online). At stage 10, WT pollen displayed more evident exine structure with tectum and baculum on the surface (Fig. 4d; Supplementary Fig. 2D online); in contrast, *fax1-2* pollen showed no exine structure on the surface, and even no dot-shaped baculas previously appeared (Fig. 4k; Supplementary Fig. 2J online). At stage 11, WT pollen showed complete pollen wall include nexine, intine, and exine with tectum and baculum on the surface (Fig. 4e; Supplementary Fig. 2E

online). In contrast, *fax1-2* pollen showed abnormal pollen wall consisting of only intine, nexine, and some dark stained unstructured and disorder distributed particles attached on nexine (Fig. 4L; Supplementary Fig. 2K online). At stage 12, WT pollen wall was fully completed and filled with pollen coat in the interstices of exine (Fig. 4f; Supplementary Fig. 2F online). In contrast, defective *fax1-2* pollen wall was seen to be unevenly covered with pollen coat-like materials (Fig. 4m; Supplementary Fig. 2L online). These findings indicated that mutation of *FAX1* affects many aspects

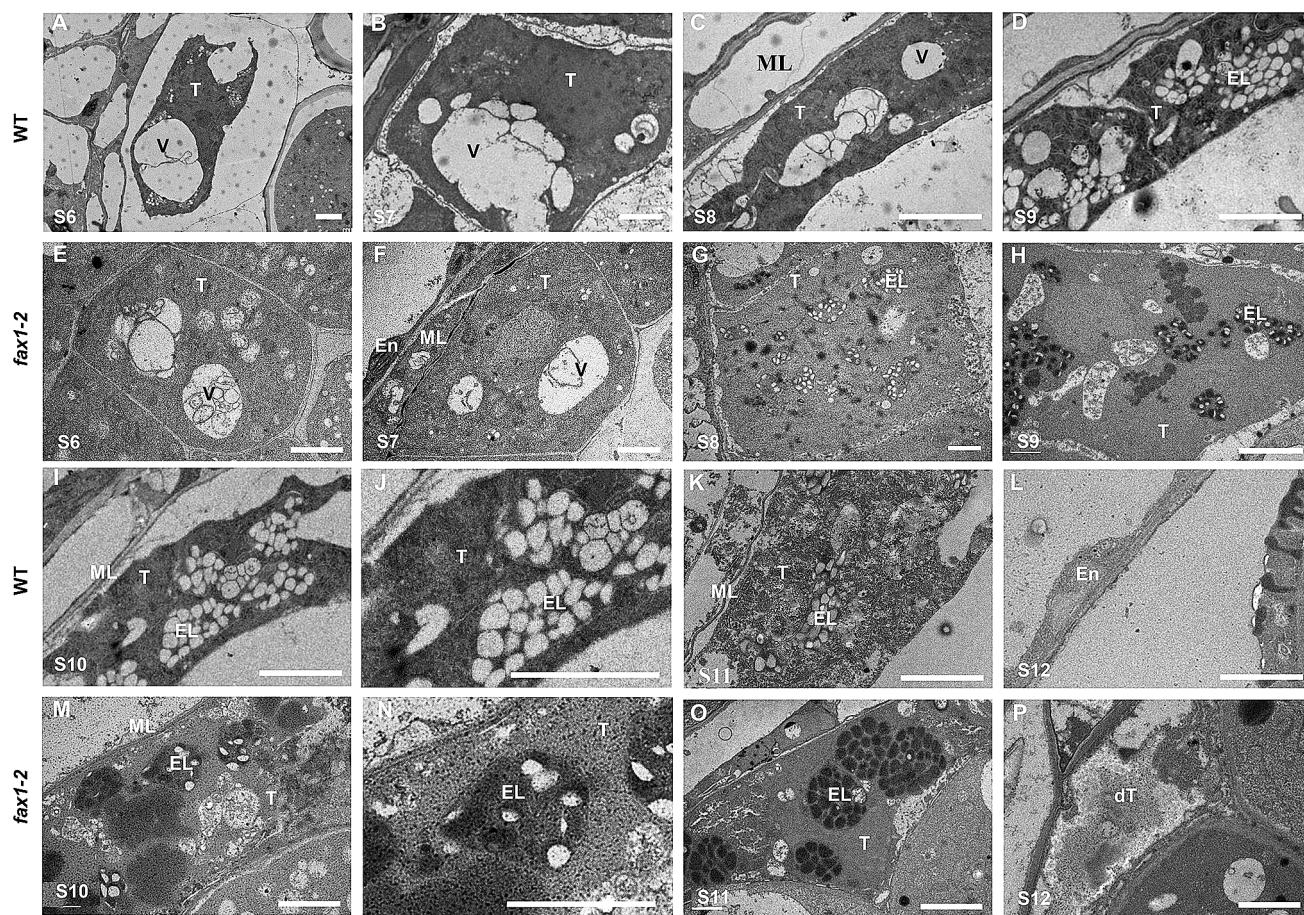


Fig. 3 Transmission electron microscopy (TEM) images of tapetal cells from wild type (**a–d** and **i–l**) and *fax1-2* (**e–h** and **m–p**) at different developmental stages. **a** and **e** Tapetal cells at stage 6; **b** and **f** Tapetal cells at stage 7; **c** and **g** Tapetal cells at stage 8; **d** and **h**

Tapetal cells at stage 9; **i**, **j**, **m** and **n** Tapetal cells at stage 10; **k** and **o** Tapetal cells at stage 11; **l** and **p** Tapetal cells at stage 12. *dT* degraded tapetum, *El* elaioplast, *En* endothecium, *ML* middle layer, *T* tapetal cell, *V* vacuole. Bar = 2 μ m

of pollen wall formation, from the early stage of primexine formation, the mid stage of baculum and tectum formation, to the late stage of pollen coat formation, ultimately leading to defective pollen wall and collapsed pollen (Fig. 4g, n).

Loss of function of *FAX1* causes atypical tapetum PCD

To further investigate whether the tapetum PCD is affected in the *fax1-2* mutant, we performed TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assays on 8- μ m transverse sections of wild type and *fax1-2* paraffin-embedded anthers. We did not observe any distinct TUNEL signals in wild type tapetums at stage 6 and stage 8, (Fig. 5a, b) while at the same stages strong positive TUNEL signals were detected in *fax1-2* tapetum (Fig. 5e, f, arrows). Obvious TUNEL signals were detected in wild type tapetum at stage 10 (Fig. 5c, arrows), which vanished at stage 12 (Fig. 5d), consistent with a previous study (Vizcay-Barrena

and Wilson 2006). In contrast, strong TUNEL signals were still detected in the degraded tapetum debris of *fax1-2* at stage 12 (Fig. 5h, arrow). These findings implied an early initiated but long time persisted tapetum PCD process in *fax1-2*.

Mutation of *FAX1* affects expression levels of some known genes encoding lipid metabolism enzymes involved in pollen wall formation

To explain the observed *fax1-2* phenotype at the molecular level, we examined expression patterns of many lipid metabolism associated genes that known to be involved in pollen wall formation in *fax1* (Li et al. 2015). These genes included *DEX1* (Paxson-Sowders et al. 2001), *ACBP4*, *ACBP5* and *ACBP6* (Chen et al. 2008; Xiao et al. 2008), *MS2* (Chen et al. 2011), *ACOS5* (Xie et al. 2017), *CYP703A2* (Morant et al. 2007), *CYP704B1* (Dobritsa et al. 2009), *ABCG26* (Quilichini et al. 2014), *ABCG9* and *ABCG31* (Choi et al. 2014),

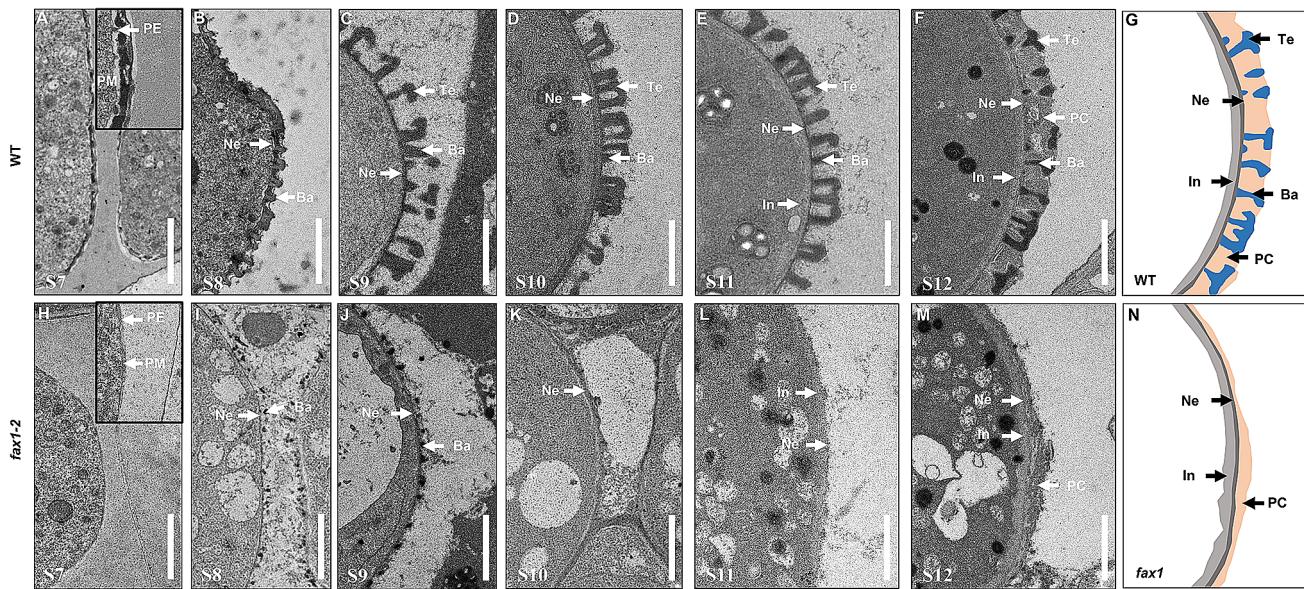


Fig. 4 Transmission electron microscopy (TEM) images of pollen wall structures from the wild type (**a–f**) and *fax1-2* (**h–m**) at different developmental stages and diagrams of mature pollen wall structures of wild type (**g**) and *fax1-2* (**n**). **a** and **h** Primexine structures at stage 7; **b** and **i** Nexine and baculum structures at stage 8; **c** and **j** Baculum

structures at stage 9; **d** and **k** Baculum and tectum structures at stage 10; **e** and **l** Exine structures at stage 11; **f** and **m** Exine structures at stage 12; **g** and **n** Diagram of mature pollen wall structures. *Ba* baculum, *In* intine, *PC* pollen coat, *PE* primexine, *PM* plasma membrane, *Ne* nexine, *Te* tectum. Bar = 2 μ m

ABCG1 and *ABCG16* (Yadav et al. 2014). In silica analysis data indicated that not all expression levels of them were significantly down-regulated in *fax1* (Supplementary Fig. 3 online), which was further confirmed by qRT-PCR (Fig. 6). In which, only were expression levels of *ACBP5*, *CYP704B1*, *ACOS5*, *ABCG26*, and *ABCG31* significantly downregulated in *fax1-2*. This result indicated an obvious inconsistency between the observed *fax1-2* phenotype and the tested transcriptional changes in genes encoding lipid metabolism enzymes associated with pollen wall formation. Nevertheless, besides those involved in lipid biosynthesis (*MS2* and *ACOS5*), modification (*CYP703A2* and *CYP704B1*), and transport (*ABCG26* and *ABCG31*), others genes could be transcriptionally affected by mutation of *FAX1*.

Mutation of *FAX1* significantly downregulates expression of several key transcriptional regulators of anther development

To answer how does *FAX1*, a FA transporter, affect anther development, pollen formation, and expression of relevant genes, we further examined the transcriptional changes of these key transcription factors in the DYT1-TDF1-AMS-MS188 network in *fax1-2* mutant using qRT-PCR. The result showed that expression levels of all those key transcription factors were significantly down-regulated in *fax1-2*, and that except *MS188*, all others were significantly downregulated at very early stages (Fig. 7a), which strongly suggested a

likely link between *FAX1* and the DYT1-TDF1-AMS-MS188 regulatory network of anther development. In addition, we further compared transcriptomic data of *fax1* with those of *dyt1*, *tdf1*, *ams* and *ms188* (Li et al. 2017) and found that transcriptome of *fax1* shares 26% (610), 25.9% (560), 21.7% (445) and 17.5% (328) identical differential expressed genes with those of *dyt1*, *tdf1*, *ams* and *ms188*, respectively. In contrast, specifically regulated genes by *DYT1*, *TDF1*, and *AMS* were only 2.3% (50), 5.4% (115), and 5.5% (117), respectively (Fig. 7b and Supplementary Fig. 4 online). Data mentioned above indicated that *FAX1* affects anther and pollen development likely via its transcriptional regulation of core transcriptional network of tapetum development, thus affecting pollen wall formation.

Mutation of *FAX1* changes ROS homeostasis along anther development

To explore the molecular mechanism underlying *FAX1*'s function in the regulation of key transcriptional factors in the genetic network of DYT1-TDF1-AMS-MS188, we further examined the ROS metabolism in *fax1-2*. As shown in Fig. 8, there was a clear dynamic change in ROS levels along the WT anther development; it could be detected from stage 6 to stage 7, which increased gradually, peaked at stage 8 and stage 9, and then declined quickly afterwards to undetectable level (Fig. 8a, b). In *fax1-2*, ROS levels were significantly higher even at stage 6 and stage 7, which showed a similar

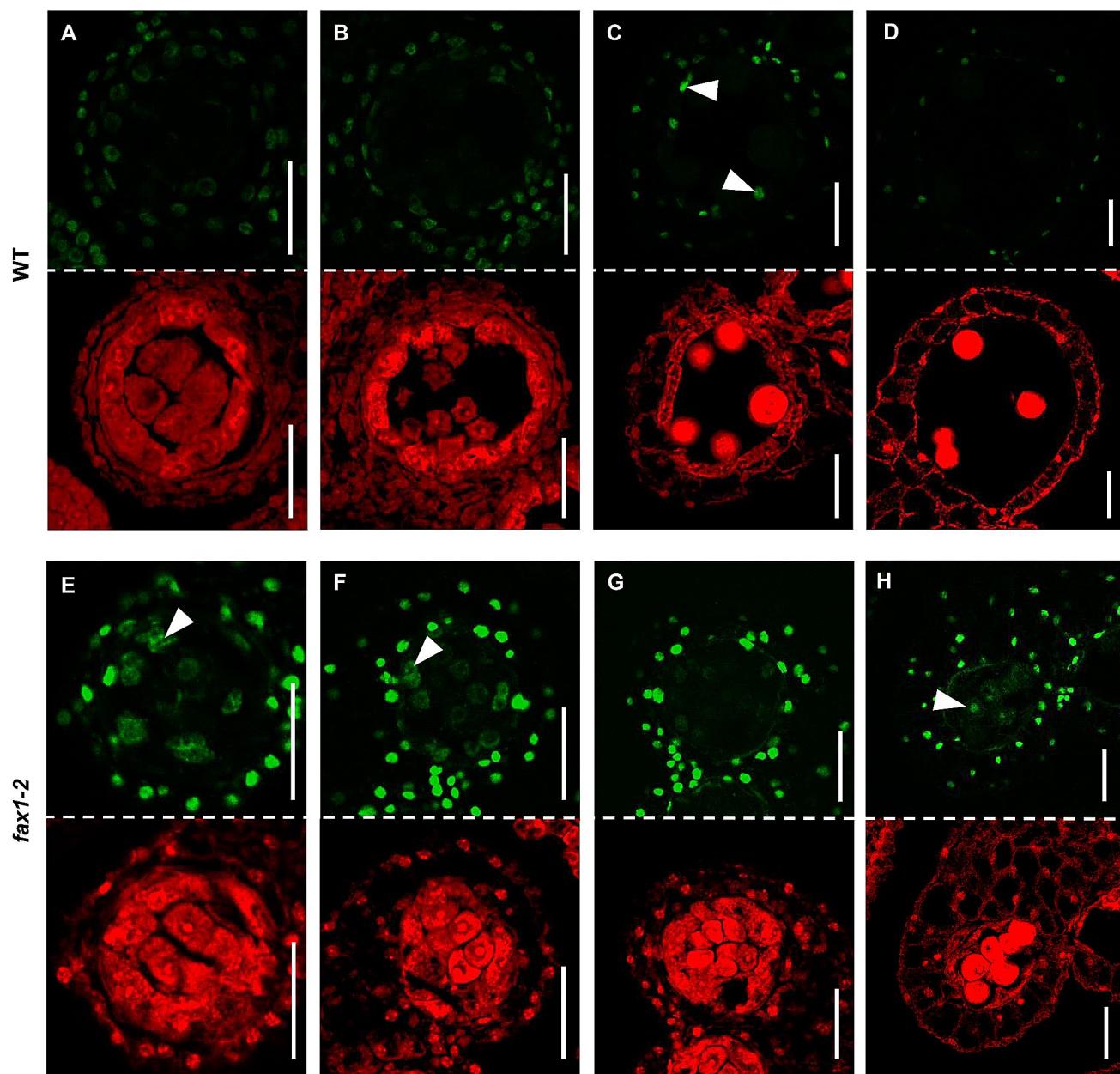


Fig. 5 Tapetal PCD kinetics along anther development in wild type (**a–d**) and *fax1-2* (**e–h**) anthers revealed by TUNEL assays. **a** and **e** Fluorescence detected in cross-sections of anthers at stage 6; **b** and **f** Fluorescence detected in cross-sections of anthers at stage 8; **c** and **g** Fluorescence detected in cross-sections of anthers at stage 10; **d**

and **h** Fluorescence detected in cross-sections of anthers at stage 12. Green fluorescence indicates TUNEL-positive signals while red fluorescence indicates propidium iodide (PI) staining. Lower panel, transmission images; Upper panel, fluorescence images. Bars = 25 μ m

increase trend, but peaked later at stage 9 and stage 10, and declined afterwards gradually (Fig. 8a, b). This result implied that FAX1 is likely a negative regulator of ROS along anther development.

Changes in the expression of several ROS scavenging genes were further examined in *fax1-2*. These genes included *PRX9*, *PRX40*, *ROXY1*, *ROXY2*, *RHD2/RBOHC* and *RBOHE*, and expression levels of *PRX9*, *PRX40*, *ROXY2*,

and *RBOHE* were significantly downregulated in *fax1-2* in stage 1–6 (Fig. 8c). This result together with the TUNEL result (Fig. 5) indicated that loss of function of *FAX1* promoted and persisted the ROS production and tapetum PCD by downregulating key genetic network elements of anther development and ROS scavenging genes.

In summary, this study further expanded understanding of *FAX1*'s role in reproductive production in plant. *FAX1*

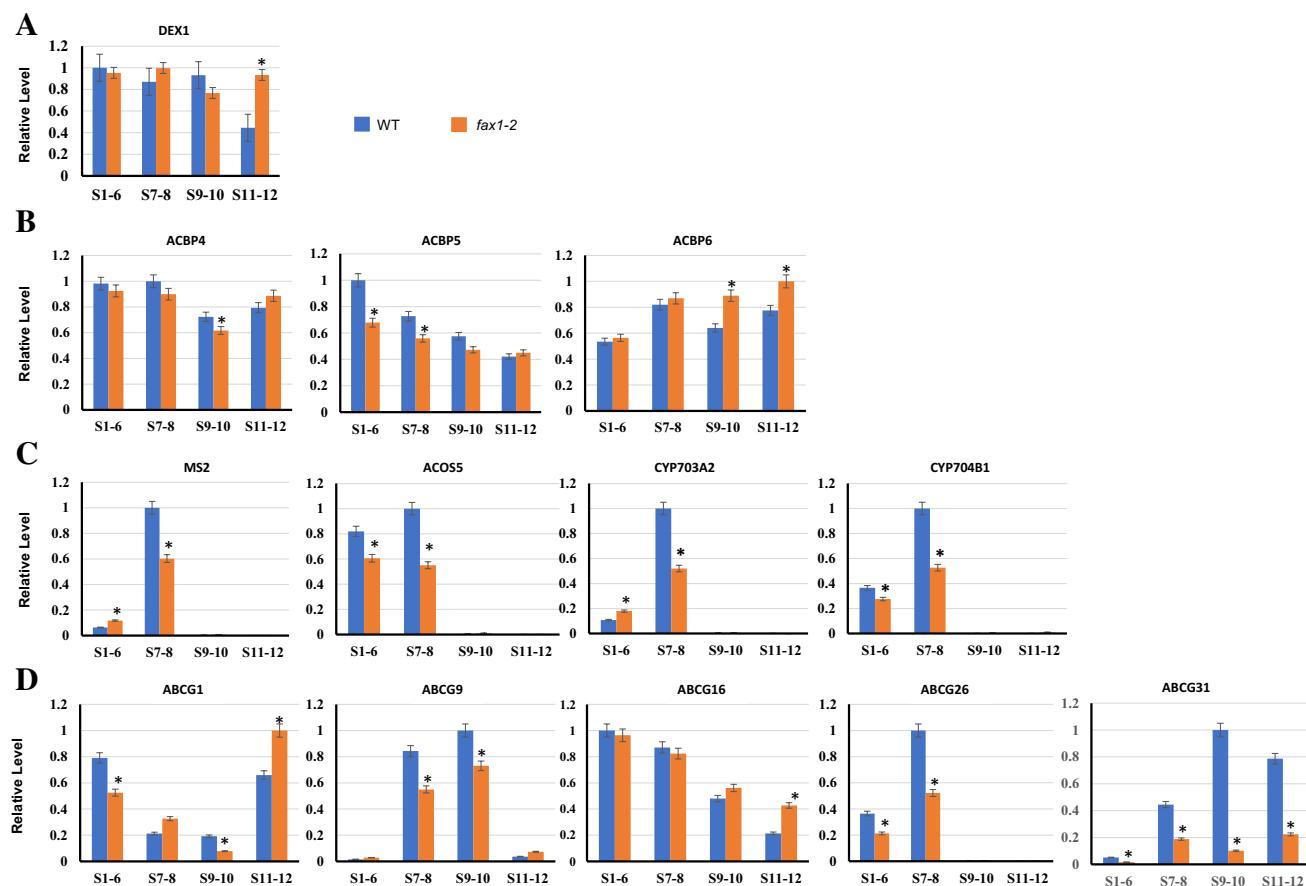


Fig. 6 qRT-PCR analysis of known genes involved in pollen wall formation in wild type and *fax1-2* flower tissues. ABCG ATP Binding Cassette G, ACBP Acyl-CoA-binding Protein, ACOS5 Acyl-CoA Synthetase 5, CYP cytochrome P450, DEX1 defective in exine for-

mation1, MS2 male sterility 2. Results are presented by means \pm SD ($N=3$). Asterisks indicate a significant difference between mutant and WT (Student's t-test: $p<0.05$)

mediated export of FAs from plastid is indispensable for the expression of all tested *DYI-TDF1-AMS-MS188* transcription factors and ROS scavenging associated genes, disruption of *FAX1* mediated FAs transport, thus, impairs ROS homeostasis and transcriptional regulatory activity of anther development, resulting in defective tapetum development, pollen wall formation, and male fertility (Fig. 9).

Discussion

FAs are building-blocks for the majority of cellular lipids, which are not only essential for membrane function, but also necessary for plant growth, development, and response to environmental stresses (Li-Beisson et al. 2013). They are also important energy substances, and precursors of some signaling molecules. In plant reproductive development, FAs play indispensable roles in anther and pollen wall formation (Shi et al. 2015; Zhang et al. 2016), and mutations of genes involved in FA metabolism often result in male fertility due

to abnormal anther and pollen development. Regarding to lipid transport, mutations of *FAX1* (Li et al. 2015), ABCG transporters (Choi et al. 2011; Zhao et al. 2016) and others such as type III lipid transfer protein (Huang et al. 2013) all show defectiveness in male reproduction, indicating a close association of lipid transport and male fertility. However, molecular mechanisms underlying the effect of lipid transport on male fertility remain poorly understood. *FAX1* is a plastid localized protein, functioning mainly to export plastid generated FAs. The involvement of *FAX1* in male fertility was first revealed by Li et al. (2015) based on segregation results and limited electron microscopy observations, in which loss of function of *FAX1* impairs male gametophyte transmission and male sporophyte development particularly the assembly of exine and pollen coat. Subsequently, our work using a combined cytological, molecular and chemical analysis together with reciprocal cross and electron microscopy observation confirmed the role of *FAX1* in male fertility, in which *FAX1* is found to be highly expressed in the early stages of flower development, and mutation of *FAX1*

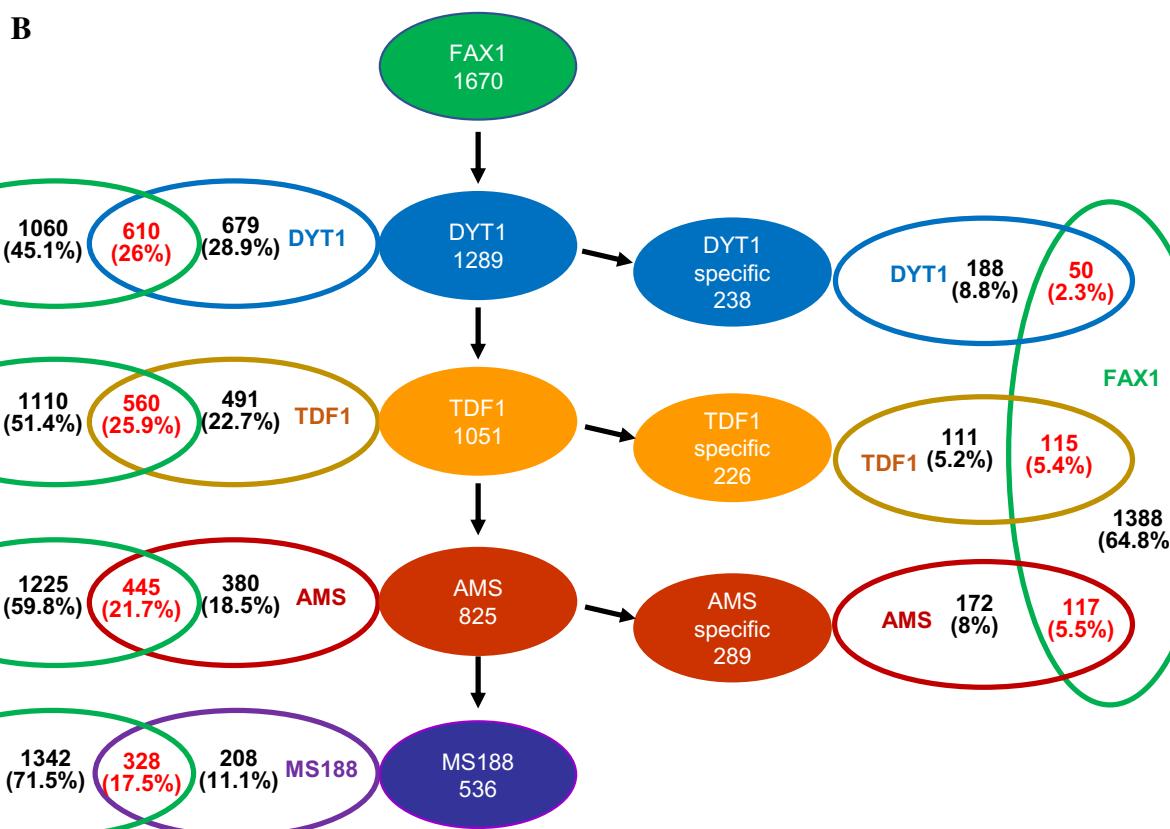
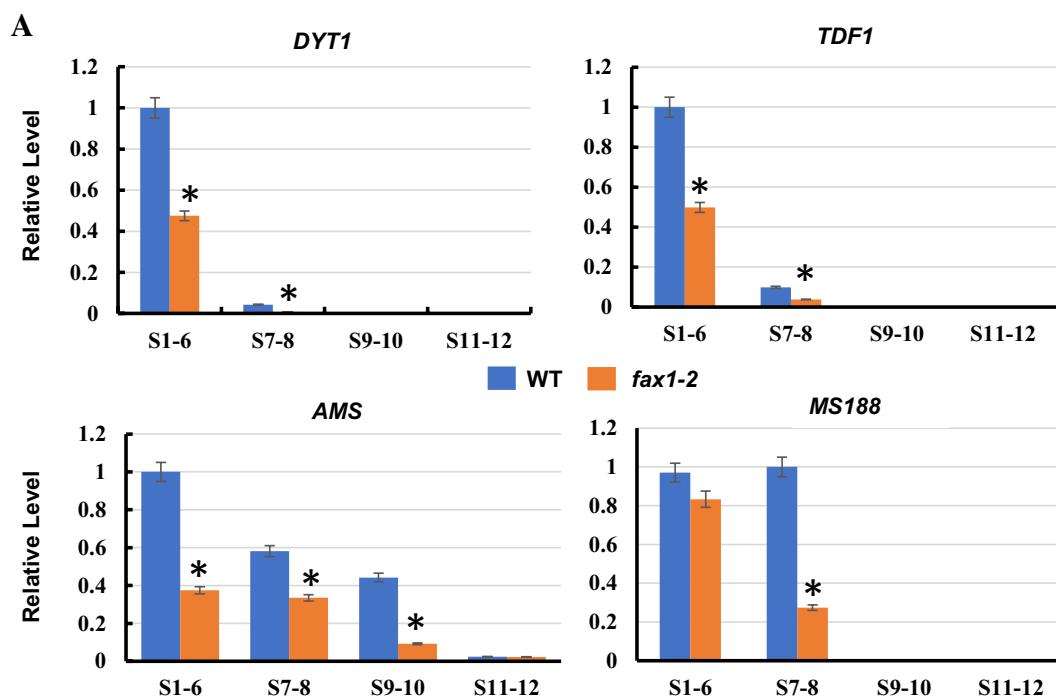


Fig. 7 Expression analysis of transcription factors in the *DYT1-TDF1-AMS-MS188* genetic network of anther development in wild type and *fax1-2* flower tissues. **a** qRT-PCR analysis of *DYT1*, *TDF1*, *AMS*, and *MS188*. Results represent averages \pm SD ($n=3$). Asterisks indicate a significant difference between mutant and WT (Student's t test: $p<0.05$). **b** In silico transcription analysis of *DYT1-TDF1-AMS-MS188* transcription factors and *FAX1* regarding anther development (Li et al. 2017). *AMS* aborted microspores, *DYT1* dysfunctional tapetum 1, *MS188* male sterility 188, *TDF1* defective in tapetal development and function 1

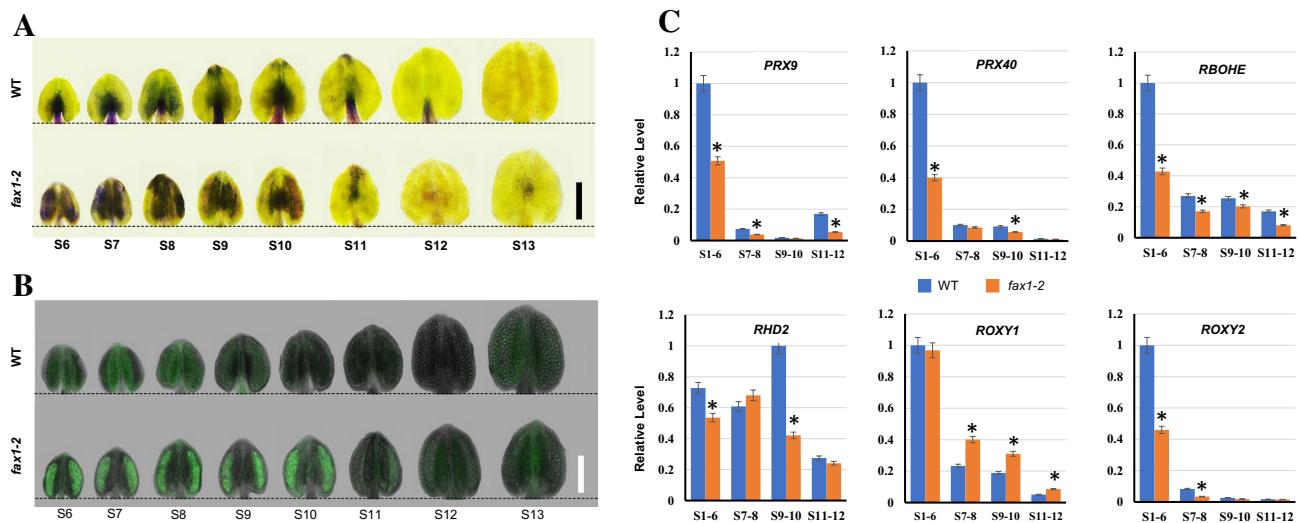


Fig. 8 ROS kinetics in anthers and qRT-PCR analysis of ROS scavenging genes in flower tissues of wild type and *fax1-2*. **a** and **b** Histological results of ROS staining by nitroblue tetrazolium (NBT) and 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA), respectively. Classification of anther groups is based on anther sizes as described (Xie et al. 2014). S6, Stage 6; S7, Stage 7; S8, Stage 8; S9, Stage 9; S10, Stage 10; S11, Stage 11; S12, Stage 12; S13,

Stage 13. Bars = 100 μ m. (C) qRT-PCR analysis of ROS scavenging genes in flower tissues of wild type and *fax1-2*. Results are presented by means \pm SD ($N=3$). Asterisks indicate a significant difference between mutant and WT (Student's *t* test: $p < 0.05$). *PRX9* Peroxidase 9, *PRX40* Peroxidase 40, *RBOHE* Respiratory-Burst Oxidase Homolog E, *RHD2* Root Hair-Defective Mutant 2, *ROXY1* CC-Type Glutaredoxin 1, *ROXY2* CC-Type Glutaredoxin 2

not only delays tapetum degradation, leading to impaired pollen wall formation and reduced pollen fertility, but also affects pollination and sepal surface patterning (Liu et al. 2018). In this study, we investigated systematically the kinetics of *FAX1* expression, ROS homeostasis, expression patterns of key enzymes involved in anther and pollen development, and expressions of each gene encoding four transcription factors in the DYT1-TDF1-AMS-MS188 genetic network of anther development, along the anther development process, using joint approaches including cytological, histochemical and expression analyses. We revealed a *FAX1* mediated regulatory network of male sterility, in which *FAX1* is vital for the export of de novo synthesized FAs out of plastid, ROS scavenging, transcriptional activation of the DYT1-TDF1-AMS-MS188 genetic network, normal tapetum development and PCD, and pollen wall formation. Therefore, this study provides new insights into the regulatory network for male reproduction in plants, highlighting an important role of FA export-mediated ROS signal in the process.

Function of a specific gene is usually associated with its spatial and temporal expression profile. Using qRT-PCR and GUS staining we demonstrated that although *FAX1* is constitutively expressed in both vegetative and reproductive organs, it is preferably and highly expressed in floral organs, specifically in the tapetum, initiating from early stage and persisting at higher levels afterwards till the complete of the anther development (Fig. 1). Our expression analysis pinpointed an essential role of *FAX1* in male reproduction.

Although the nature of the chemical composition of the pollen outer wall has not been fully elucidated, more and more experiments have confirmed that tapetum cells provides microspores and pollens with precursors including lipidic ones for the formation of pollen outer walls (Bedinger 1992; Li-Beisson et al. 2013; Piffanelli et al. 1998; Scott et al. 2004). Therefore, normal development and degradation of the tapetum is crucial for pollen wall formation (Ariizumi and Toriyama 2011; Shi et al. 2015). Because mutation of *FAX1* results in defective formation of pollen exine and pollen coat (Li et al. 2015), the development and degradation of tapetum was examined in this study. Compared with wild type, the initiation of tapetum PCD was much earlier in *fax1*, which persisted also much longer to stage 12 (Figs. 2, 3, and 5). Our results confirmed the delayed tapetum PCD previously revealed (Liu et al. 2018) and highlighted at the same time the earlier initiation of tapetum PCD in *fax1*, suggesting that disruption of FA export from plastid in tapetum induced atypical tapetum PCD, defective anther cuticle (Liu et al. 2018) and pollen wall development (Liu et al. 2018; Fig. 4) in *fax1* due directly to the loss of available FAs derived precursors (Fig. 9).

ROS are required for the induction of tapetum PCD, leading to tapetum degeneration and pollen wall formation (Fath et al. 2001; Hu et al. 2011; Mühlenbock et al. 2007; Obara et al. 2001; Qu et al. 2013). However, over accumulated high levels of ROS indiscriminately damage cellular constituents including proteins, DNAs and lipids, leading to

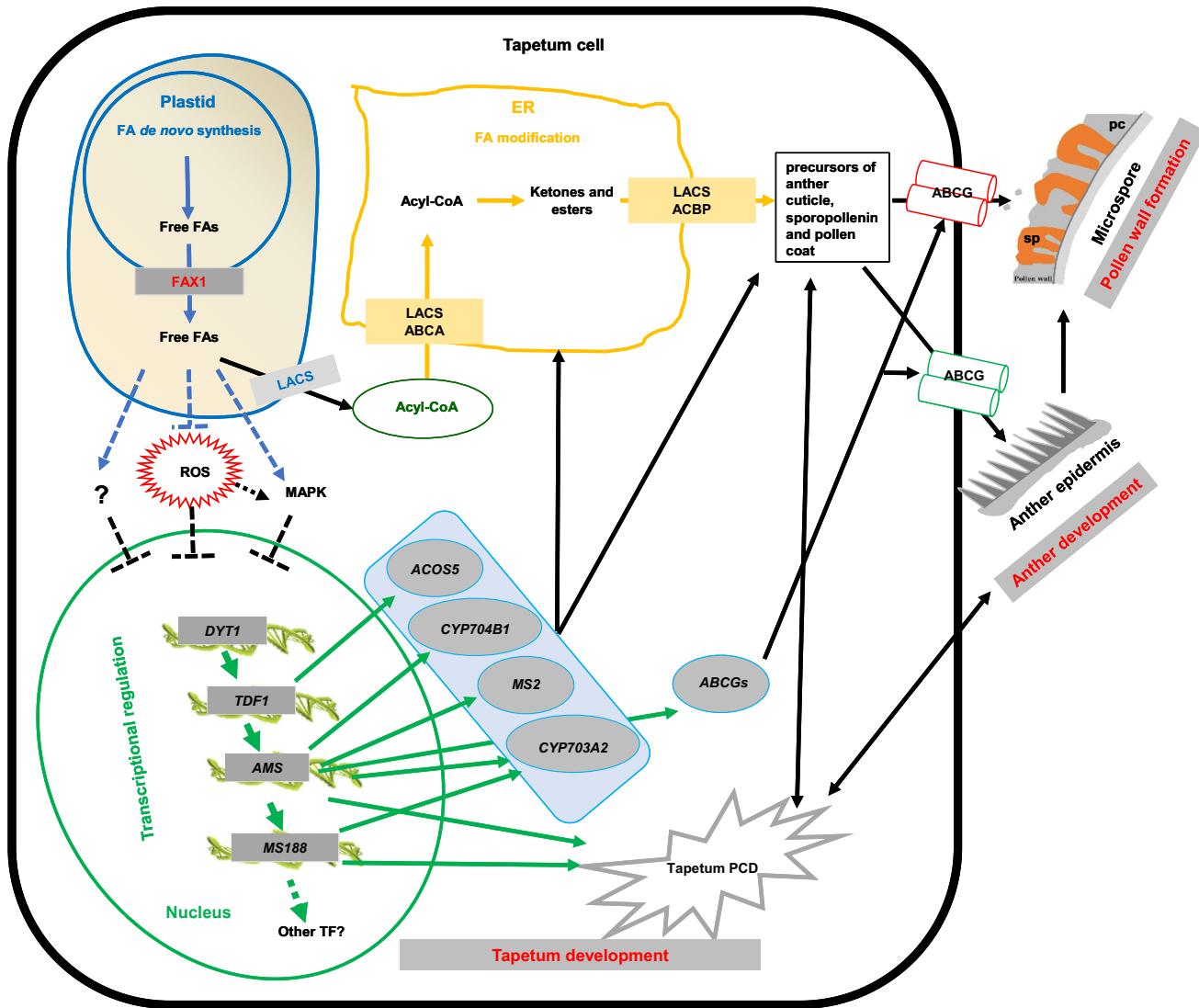


Fig. 9 A proposed model of *FAX1* mediated fatty acid export on anther development and pollen wall formation in *Arabidopsis*. In tapetum, fatty acids, exported by *FAX1* protein from plastid across inner membrane, are further exported to enter ER by plastid outer membrane localized LACS (long chain acyl CoA synthetase), where after being elongated and modified, they are exported cross ER (endoplasmic reticulum) membrane by LACS and ACBP (acyl CoA binding protein). The resulting LCFA (long chain fatty acid) and their derivatives are finally transported by ABCG transporters across PM to apoplast for the assembly of anther cuticle and pollen wall. Alternatively, lipid molecules exported by *FAX1* could affect transcriptional activities of DYT1-TDF1-AMS-MS188, their downstream targets such as *MS2*, *ACO55*, *CYP704B2*, *CYP703A2* and *ABCGs*, and tapetum PCD, thus, affecting the biosynthesis, modification and transportation and assembly of precursors for anther cuticle and pollen wall formation. The *FAX1* exported lipids could also regulate

transcriptional activation of anther development via balancing ROS homeostasis through lipid oxidation or other unknown mechanisms, either directly acting on the transcriptional activities of DYT1-TDF1-AMS-MS188 or indirectly via MAPK (mitogen activated protein kinase) pathway as reported in non-plant system. The *FAX1* exported lipids could likewise control transcriptional activation of anther development via directly binding to MAPK pathway proteins and affecting kinase activities of them on DYT1-TDF1-AMS-MS188 genetic network. Furthermore, the *FAX1* exported lipids could enter into nucleus directly or indirectly via collaboration with other signaling molecules to regulate the transcriptional activation in the nucleus. Solid and dashed lines represent previously reported results and currently implied results, respectively. Arrows and bars represent promoting and inhibiting effects, respectively. PC pollen coat, SP sporopollenin, TF transcription factor

atypical tapetum PCD and defective pollen wall formation (Van Breusegem and Dat 2006). Thus, ROS levels are tightly controlled between biosynthesis and breakdown via sophisticated but highly effective antioxidant systems. Generally,

modulation of the ROS homeostasis in cells is controlled, at least partially, at the transcriptional level (Schippers et al. 2012). Previous studies have shown that many ROS-related proteins play essential roles during tapetum and pollen wall

development (Schippers et al. 2012). For example, transcription factor MADS3 (Hu et al. 2011) and its target MT-1-4b (a ROS-scavenging protein) (Hu et al. 2011), tapetal mitochondrial protein WA352 and COX11 (its interactive protein) (Luo et al. 2013) in rice; PRX9 and PRX40 (Jacobowitz et al. 2019), ROXY1 and ROXY2 (Xing and Zachgo 2008), RHD2/RBOHC and RBOHE in *Arabidopsis* (Xie et al. 2014). *FAX1* seemed to be a new regulator of ROS homeostasis in context of tapetum PCD and pollen wall formation in *Arabidopsis*. Because mutation of *FAX1* not only initiated earlier but also persisted longer the process of tapetum PCD (Figs. 2 and 5); in addition, mutation of *FAX1* promoted the earlier accumulation of higher levels of ROS (Fig. 8). The earlier and higher accumulation of ROS in *fax1* mutant anthers could be the consequence of the downregulation of known ROS scavenging protein encoding genes, such as *PRX9*, *PRX40*, *RBOHE*, *RHD2*, and *ROXY2*, at the early stage of anther development (Fig. 8c). The atypical tapetum PCD that initiated earlier and persisted longer in *fax1* could result from the toxic effect of over accumulated ROS on relevant functional tapetum proteins in the mutant. This was supported by the significant downregulation of all four key transcription factors of the known DYT1-TDF1-AMS-MS188 genetic network of anther development in *fax1* mutant (Fig. 7). Alternatively, although ROS are generally toxic, increasing evidence implies that they are indispensable signaling molecules affecting a large number of proteins through posttranslational modifications, transcriptional changes, or activity changes (Lam, 2004; Mittler et al., 2004; Mittler et al., 2011; Neill et al., 2002; Suzuki et al., 2011; Torres and Dangl, 2005). This could explain as well, at least in part, the observed downregulation of four key transcription factors and several known lipid metabolism genes involved in male fertility (Figs. 6 and 7). Based on the expression analysis data of *FAX1* (Fig. 1), those of known genes encoding lipid metabolism enzymes involved in pollen wall formation (Fig. 6), those of the four transcription factors in the DYT1-TDF1-AMS-MS188 network in *fax1* (Fig. 7a), that of *FAX1* in mutants of each of these four transcription factors (Fig. 7b), we can assume that *FAX1* mediated FA metabolism co-occur with DYT1-TDF1-AMS-MS188 network regulated transcriptional activation as well as ROS scavenging, and that the link connecting FA/lipid homeostasis and DYT1-TDF1-AMS-MS188 transcriptional activity is likely the ROS signal.

One of the most obvious phenotype that distinguishes *fax1* from previously reported mutants of other genes associated with lipid metabolism is the altered components of elaioplasts that appeared at stage 8 (Fig. 3), which indicated a clear interruption of the export of FAs from the tapetum cells in the mutant. This phenotype is different from those inclusions observed in *cer5/atabcg12* stem epidermal cell (Pighin et al. 2004), reflecting an unique

structural feature in reproductive organs. In addition, the blocked export of FAs out of tapetum was also evidenced by another histological feature, in which the stage 11 *fax1* tapetum locule was filled with electro-dense materials enclosing pollen grains (Fig. 2), which are similar to those dark-stained materials accumulated in *osabcg26* anther locules surrounding the microspores (Zhao et al. 2015). Clearly, *FAX1* mediated export of FAs from plastids in tapetum is essential for anther development and pollen wall formation (Fig. 9). This could be the consequence of unbalanced FA homeostasis induced atypical tapetum PCD (Liu et al. 2018). Because FAs exported by *FAX1* could act as important initiators or mediators of tapetum PCD or targets for modification and destruction as they do in mammals (Agmon and Stockwell 2017). They could also serve as signaling molecules, which, in turn, could balance ROS through lipid peroxidation, and/or even interact with ROS or components of other signaling pathways, for example, mitogen-activated protein kinase (MAPK) signaling pathway (Bush and Krysan 2007; Amir et al. 2019), to affect transcriptional regulatory network of tapetum and pollen wall development. They could directly regulate gene expression in the nucleus (Agmon and Stockwell 2017). In animal, (1) specific lipid molecules can either bind directly with MAPK signaling proteins and regulate both their subcellular localizations and kinase activities or affect MAPK signaling indirectly via the regulation of endocytosis and the biophysical properties of different membrane lipids (Anderson 2006); (2) polyunsaturated FAs are reported to induce ovarian cancer cell death by activating of ROS-dependent MAP kinase signaling pathway (Tanaka et al. 2017); (3) phospholipids exist within nuclei and play regulatory roles in gene transcription (Agmon and Stockwell 2017). Currently, how lipids affect tapetum PCD, ROS scavenging, and transcriptional regulation of pollen wall formation is completely unknown, which merits further studies. In plants, reactive carbonyl species (RCSs), known as the α,β -unsaturated aldehydes and ketones derived from lipid peroxides, are regarded as mediators of ROS signals (Mano et al. 2019). Whether RCSs are involved in the *FAX1* mediated ROS signal transduction to proteins was, however, not investigated in this study.

In summary, our data from morphological, cytological, histological, and molecular studies confirmed that the disruption of the export of FAs from plastid in *fax1* anthers significantly affects ROS homeostasis and the expression of regulatory genes of anther development network, leading to atypical tapetum PCD and defective pollen wall formation (Fig. 9). This study expands the understanding of FA transport mediated regulatory network for male reproduction in plants, and points out the future research directions regarding the link of lipid molecules and/or signals with ROS in this process.

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Author contributions JX and LZ designed the research, LZ, HZ, YL and YH carried out experiments. JX, LZ and JS analyzed data and wrote the manuscript. And all authors reviewed and commented on the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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