Running title: AtSEC31B in pollen wall development

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Secretry COPII Protein SEC31B Is Required for Pollen Wall Development

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One-sentence Summary: Sec31B protein is crucial for pollen wall formation through participating in COPII-coated vesicle trafficking in the tapetum in Arabidopsis.

Authors Contributions
Y.G., R.L. and B.Z. conceived the project and designed the experiments. B.Z. carried out phenotypic analysis, SEM and TEM assay, CLSM imaging and protein interaction experiments. H.S. and X.L. carried out the Arabidopsis transgenic experiments. W.W. and Y. Z carried out the paraffin section and semi-section experiment. H.G. and M.Y carried out the RT-PCR analysis. X.W. and X.L. carried out the GUS staining assay. Y.G., R.L. and B.Z. wrote the manuscript.

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Abstract

The pollen wall protects pollen grains from abiotic and biotic stresses. During pollen wall development, tapetal cells play vital role by secreting proteins, signals and pollen wall material to ensure microspore development. But the regulatory mechanism underlying the secretory pathway of the tapetum is largely unknown. Here, we characterize the essential role of the Arabidopsis COPII protein Secretory31B (SEC31B) in pollen wall development and secretive activity of tapetal cells. The sporophyte-controlled atsec31b mutant exhibits severe pollen and seed abortion. TEM observation indicates the pollen exine formation in atsec31b mutant is significantly disrupted. AtSEC31B is a functional COPII protein revealed by ER exit sites (ERESs) localization, interaction with AtSEC13A and retarded ER-Golgi protein trafficking in the atsec31b mutant. A genetic tapetum-specific rescue assay indicates AtSEC31B primarily function in the tapetum. Moreover, deletion of AtSEC31B interrupted the formation of the ER-derived tapetosome and altered the location of ATP-binding cassette transporter9 (ABCG9) protein in the tapetum. Therefore, this work demonstrates AtSEC31B play a vital role in pollen wall development by regulating the secretory pathway of the tapetal cells.

Keywords: pollen wall, exine, AtSEC31B, tapetum, COPII-coated vesicle, Arabidopsis.
Introduction

Pollen cell wall is complex and robust structure surrounding the microspore cytoplasm helping to resist various severe environments and also involved in the pollination and pollen-stigma recognition. The pollen wall of mature pollen grains comprises intine and exine, plus tryphine deposited on the surface of the exine (Scott et al., 2004). Intine enriched in cellulose and hemicelluloses is comparable to the primary cell wall of somatic cells. Exine consists of inner nexine and outer sexine which contains tectum and bacula. The main composition of exine is sporopollenin derived from the tapetum, the innermost layer of the anther locule wall. More importantly, normal pollen wall development is crucial for microspore development (Scott et al., 2004; Ariizumi and Toriyama, 2011). Aberrant pollen wall formation frequently leads to severe pollen abortion.

In higher plants, during microspore development, tapetum secretes signals, enzymes and pollen wall materials to ensure successful microspore development. At the anther stage, while going through the callose wall of tetrads degenerating, tapetal cells continuously secretes enzymes, i.e., callose-degrading glucanases (Stieglitz, 1977), into the anther locule to release the microspores. Simultaneously, primexine forms outside the surface of the microspores and serves as a mould for the subsequent sporopollenin deposition. Impaired primexine formation often results in abnormal exine and abortive pollen. Many reports describe members involved in the formation of primexine, such as DEFECTIVE IN EXINE FORMATION1 (DEX1), MALE STERILE1 (MS1)/HACKLY MICROSPORE (HKM), NO PRIMEXINE AND....
PLASMA MEMBRANE UNDULATION1 (NPU1), RUPTURED POLLEN GRAIN1 (RPG1), NO EXINE FORMATION1 (NEF1), recently reported SPONGY2 (SPG2) and UNEVEN PATTERN OF EXINE 1 (UPE X1), which are all highly expressed in the tapetum and sporophytic-controlled tissues (Paxson-Sowders et al., 2001; Ariizumi et al., 2004; Ariizumi et al., 2008; Guan et al., 2008; Chang et al., 2012; Li et al., 2016; Xu et al., 2016). After the microspores are released, tapetal cells constantly synthesise precursors of sporopollenin and release them into the locule to deposit on the surface of the pollen exine. MS2, ACOS5, CYP703A2, CYP704B2 are reportedly involved in the synthesis of sporopollenin precursors in Arabidopsis (Morant et al., 2007; de Azevedo Souza et al., 2009; Dobritsa et al., 2009; Chen et al., 2011). Besides, DPW2 (Defective Pollen Wall 2) encoding an acyltransferase is required for rice pollen development (Xu et al., 2016). Then, these precursors and other structural materials are transported outside tapetal cells via lipid transfer proteins or ABCG transporters (McFarlane et al., 2010; Quilichini et al., 2010; Choi et al., 2011; Huang et al., 2013; Choi et al., 2014).

The tapetum is a temporary cell layer. As the tapetal programmed cell death proceeds, the cell contents are released into the locule, and thus, the lipids and proteins accumulated in tapetosomes and elaioplasts are deposited on the exine of the microspores, forming the trypHime. Some ABCG transporters in the tapetum are also involved in pollen coat deposition. ABCG9 and ABCG31 show high expression in the tapetum, and abcg9 abcg31 mutants mirror the immature pollen coat of wild type pollen (Choi et al., 2014). ABCG26 is required for the trafficking of polyketide and
hydroxycinnamoyl spermidines, which facilitate the formation of pollen exine in Arabidopsis (Quilichini et al., 2010; Quilichini et al., 2014). In addition, rice ABCG26 and ABCG15 play important role in male reproduction (Zhao et al., 2015). Therefore, successful pollen wall development largely relies on normal secretory activity in the tapetum. However, very little information is available about the regulatory mechanism underlying the secretive activity of tapetal cells.

Angiosperms exhibit two primary types of tapetum. One is the secretory tapetum, which releases the materials required for pollen development via locular fluid. The other is the amoeboid tapetum, which can move to and contact with microspores directly (Pacini et al., 1985). Compared with the amoeboid tapetum, the secretory tapetum may produce more pollen grains in larger anthers. Arabidopsis possesses a secretory tapetum. However, few mutants have been reported to affect the secretory pathway in Arabidopsis tapetal cells. Disruption of AtGPAT1,6 (glycerol-3-phosphate acyltransferase), which involved in the initial step of glycerolipid biosynthesis, leads to abnormal ER structure and decreased secretion in tapetal cells (Zheng et al., 2003; Li et al., 2012). Male gametogenesis impaired anthers (MIA), a P-type ATPase cation pump, may regulate the cation homeostasis of the secretory pathway compartments in the tapetum (Jakobsen et al., 2005). Moreover, deletion of Male Sterile 1 (MS1), a PHD-type transcriptional factor, also causes abnormal secretion of pollen wall material in the tapetum (Ito et al., 2007). Recently, ECHIDNA was reported to be involved in trans-Golgi network transport, and echidna showed multiple male fertility defects (Fan et al., 2014). However, the key components or factors that participate in
the secretory pathway of tapetum cells are less understood.

COPII-coated vesicle specifically mediates anterograde transport from the ER to the Golgi apparatus and plays a vital role in the early secretory pathway (Wuestehube et al., 1996). Through COPII vesicle trafficking, proteins and lipids can be transported from the ER to the Golgi and then sorted to the plasma membrane, apoplastic space and other organelles. In yeast, the initiation of COPII complex assembly starts with the recruitment of Sar1-GDP by Sec12p. Sar1p initiates recruitment of Sec23/24p and Sec13/31p from cytosol. Sec24p is responsible for cargo selecting, and Sec13/31p forms the vesicle coat (Wuestehube et al., 1996; Salama et al., 1997; Shugrue et al., 1999; Robinson et al., 2007). However, little is known regarding COPII vesicle transport in plants. Arabidopsis SEC24A is required for the maintenance of endoplasmic reticulum morphology, and deletion of AtSEC24A affects the size of sepal cells and pollen germination (Faso et al., 2009; Nakano et al., 2009; Qu et al., 2014). Knockout of both AtSEC24B and AtSEC24C leads to defective gametophyte development (Tanaka et al., 2013). More recently, AtSAR1A and AtSEC23A interacts as a pair, which is essential for protein ER export in Arabidopsis (Zeng et al., 2015). However, the function of other COPII proteins in Arabidopsis has yet to be determined.

In this paper, we found that disruption of AtSEC31B, a putative homolog of yeast Sec31p, caused dramatically reduced viable pollen grain and seed abortion. TEM analysis indicates pollen wall development is significantly impaired. Though AtSEC31B is ubiquitously expressed, particularly strong transcriptional and protein
expression are detected in the tapetum. SEC31B driven by tapetum-specific A9 promoter could fully recover the atsec31b mutant phenotype. AtSEC31B served as a functional COPII protein revealed by subcellular location, co-immunoprecipitation (Co-IP) and FRAP assays. The alterations in the size and structure of the lipid storage organelles in the tapetum were also observed in the atsec31b mutant. Finally, we found that the distribution of ABCG9 in the tapetum is altered in atsec31b mutant. Therefore, our work suggests SEC31B is crucial for the pollen wall development and may be involved in the early secretory pathway of tapetal cells.
Results

The homozygous \textit{atsec31b} mutant shows significantly reduced male fertility

\textit{AtSEC31B} (AT3G63460) is selected from our previous proteomic analyses in germinated \textit{Arabidopsis thaliana} pollen grains (Ge et al., 2011). AtSEC31B shows 24% identity with \textit{Saccharomyces cerevisiae} SEC31p (ScSEC31p, Supplemental Fig. S1) (Robinson et al., 2007). To explore the detailed functions of AtSEC31B, we obtained a knock-out T-DNA insertion mutant, \textit{atsec31b} (SALK_103304), from the \textit{Arabidopsis} Biological Resource Centre (Fig. 1, A and B). During vegetative growth, \textit{atsec31b} mutant plants exhibit normal development (Fig. 1, C). However, in the reproductive stage, the anthers of the \textit{atsec31b} mutant become shrivelled and dark yellow, and little pollen appears in the pistil, whereas wild-type plants exhibit bright yellow anthers and abundant pollens (Fig. 1, D, top row). Most of the pollen grains in \textit{atsec31b} anthers were not stained purple by Alexander’s staining and were likely aborted (Fig. 1, D, bottom row). Moreover, \textit{atsec31b} mutant plants produced short, small siliques and showed an abortion ratio of approximately 55% (Fig. 1, E-I). This mutation is recessive, because heterozygous \textit{atsec31b/+} plants show normal growth. Artificial pollinations with excess mutant pollens on the stigma of \textit{atsec31b} could recover the abortion phenotype, indicating that decreased viable pollen number is responsible for severe seed abortion (Supplemental Fig. S2).

Next, we performed a genetic analysis of \textit{atsec31b} mutants. The progeny of the self-pollinated heterozygous plants significantly deviated from classic Mendelian segregation (Supplemental Table S1). Male gametophyte transmission efficiency is
significantly reduced to 17% in the *atsec31b* mutant, whereas the female function is 
unaﬀected (Supplemental Table S1). Therefore, the disruption of *AtSEC31B* 
dramatically impairs male fertility.

Moreover, we obtained another knock-out T-DNA insertion line, designated 
*atsec31b-n* (CS918444) from ABRC. *atsec31b-n* contains a T-DNA insertion fragment 
in the twelfth exon of *AtSEC31B* (Supplemental Fig. S3A). The phenotype of 
homozygous *atsec31b-n* is similar to *atsec31b* mutant (Supplemental Fig. S3 B and 
C). Subsequently, genetic complementation was performed. *AtSEC31B* genome 
fragment was transformed to *atsec31b* mutant. ~20 T1 transgenic lines are fully 
recovered to wild type plants. The phenotype of three independent single insertion 
lines were analyzed in detail, including seedling height, seed abortion rate, pollen 
fertility and silique length (Fig. 1, C, D, G, H, I). We selected *atsec31b* mutant for 
subsequent experiment.

The *atsec31b* mutant exhibits an abnormal pollen wall and compromised pollen 
germination and pollen tube growth

Scanning electron microscopy (SEM) was performed to examine the detailed 
morphology of mature pollen. In wild type and rescued lines, mature pollen grains 
were abundant and plump and showed a typical reticular exine pattern (Fig. 2, A, B, I, 
J). By contrast, few mature pollen grains were observed in the *atsec31b* (Fig. 2, C, D, 
E, F) and *atsec31b-n* anthers (Supplemental Fig. S3D). In the *atsec31b/+* anthers, 
mature pollen grains were comparable with wild type (Fig. 2 G and H), which implies 
that AtSEC31B function sporophytically controlling pollen development. In addition,
some remnants were attached to the mutant anthers and were assumed to be degradation products of pollen (Fig. 2, C). Thus, we carefully examined these pollen grains recognized in \textit{atsec31b} plants. They could be classified into three types (n>500): (1) grains with a normal exine, as observed in wild-type plants, accounting for 40% of the grains (Fig. 2, D, K); (2) grains with abnormal pollen coat, accounting for 25% (Fig. 2, E, K); and (3) collapsed grains with a concave and shrunken morphology without a typical exine, accounting for 35% (Fig. 2, F, K). These observations revealed more than half of the pollens in the \textit{atsec31b} mutant displayed an abnormal exine pattern (Fig. 2, K). The pollen grains stuck to the remnants of \textit{atsec31b} anthers, and only a portion of the grains could be obtained and examined.

We next performed pollen germination assays \textit{in vitro} and \textit{in vivo} to observe germination rates and tube elongation. \textit{In vitro}, only 25% (n>300) of the available \textit{atsec31b} mutant pollen grains could germinate compared with 82% (n>300) of wild-type pollen grains (Supplemental Fig. S4 A). The ratio of pollen germination in rescued lines has no significant difference with that of wild-type (Supplemental Fig. S4 A). The length of the \textit{atsec31b} mutant pollen tubes is significantly shorter than those of wild-type plants (Supplemental Fig. S4 B). \textit{In vivo}, the \textit{atsec31b} pollen tubes also grew slower than those of wild-type plants, but could reach the bottom in 24 hours after pollination (hap) (Supplemental Fig. S4 C). These results indicate that the pollen germination rate and pollen tube elongation are seriously compromised in the \textit{atsec31b} plants.

\textbf{Microspores of \textit{atsec31b} plants exhibit defective primexine development}
Next, we further explored the progress of pollen development in *atsec31b* and wild-type plants by examining transverse sections of the anthers. According to Sanders’ morphological characteristics, anther development can be divided into 14 well-ordered stages in *Arabidopsis* (Sanders et al., 1999). At anther stage 7, the anther locules were filled with tetrads wrapped in callose walls in wild-type plants (Fig. 2, L), and no morphological defects were observed in *atsec31b* plants (Fig. 2, R). At stages 8 and 9, the *atsec31b* plants (Fig. 2, S, T) appeared to be similar to the wild type (Fig. 2, M, N). The first significant developmental defects were observed at anther stage 10, in which the microspores of wild-type plants generated a significant pollen wall (Fig. 2, O), whereas the pollen wall of *atsec31b* microspores was ambiguous and incomplete (Fig. 2, U). At stage 11, the microspores of wild-type plants exhibited a typically regular exine wall (Fig. 2, P). In *atsec31b* plants, only a small amount of pollen survived (Fig. 2, V). At stage 12, pollen development was completed in wild-type plants (Fig. 2, Q), but in the locules of *atsec31b* plants, debris and few normal pollens could be observed (Fig. 2, W). Besides, the tapetum in *atsec31b* mutant exhibits normal structure and undergoes similar programmed cell death with that of wild type (Fig. 2, R-W). Thus, these observations suggest that the exine formation of microspores was remarkably impaired in the *atsec31b* mutant.

Next, transmission electron microscopy (TEM) was performed. At anther stage 7, a primexine gradually formed between the callose wall and the microspore plasma membrane in wild-type plants, which was initiated simultaneously with plasma membrane (PM) undulation (Fig. 3, A-C). In the *atsec31b* mutant plants, the PM of
the microspores undulated more lightly than in wild-type plants (Fig. 3, E-G). The
formation of primexine was completed in wild-type plants, and a regular structure of
the probaculae and protectum was gradually built and accomplished in the primexine
matrix (Fig. 3, D). However, in the \textit{atsec31b} mutant, primexine-like structures that
were significantly thinner than those of wild-type plants were deposited, and the
probaculae showed a podgy morphology without any observable protectum (Fig. 3,
H). At anther stages 8 and 9, the wild-type microspores developed a regular
lattice-patterned exine containing the tectum, the bacula and the foot layer (Fig. 3,
I-L). By contrast, the tectum was absent in \textit{atsec31b} microspores, and the baculas
appeared to be stubbier than those of wild-type microspores (Fig. 3, M-P). At stages
10 and 11, wild-type microspores showed further development (Fig. 3, Q-T), whereas
in \textit{atsec31b} microspores, the exine degenerated and was ultimately fragmented (Fig. 3,
V-W), and abnormal spherical sporopollenin aggregated around the microspores (Fig.
3, X-Y). At anther stage 12, the formation of a typical pollen wall and pollen coat was
completed in wild-type microspores (Fig. 3, U). In \textit{atsec31b} anthers, only remnants
could be observed (Fig. 3, Z). These TEM observations imply that knocking out
\textit{AtSEC31B} severely disrupted exine development. Moreover, random deposits of
sporopollenin on the locule wall (the inner surface of locule) appeared at stage 8 in
\textit{atsec31b} mutant plants (Supplemental Fig. S5).
**AtSEC31B is widely expressed and mainly functions in the tapetum**

We next investigate the expression pattern of *AtSEC31B*. RT-PCR analysis showed that *AtSEC31B* is universally expressed, predominantly in the inflorescences and root (Fig. 4, A). In *Pro*-*AtSEC31B*:GUS transgenic plants, GUS activity was detected in the stamens, pistils, young siliques, leaves, roots and cotyledons (Fig. 4, B and Supplemental Fig. S6). In cross-sections, *AtSEC31B* was expressed at a high level in all cell types of the anther before anther stage 9 (Fig. 4, C-E). However, at anther stages 9-11, GUS expression was prominently detected in the tapetum (Fig. 4, F-H), in accordance with the stage which the tapetum continuously secreted a large amount of exine materials into the anther locule. In addition, *AtSEC31B* transcripts were expressed highly in mature pollens and pollen tubes (Fig. 4, B, I, J). We also generated *Pro*-*AtSEC31B*:*AtSEC31Bc*:EGFP (enhanced green fluorescence protein), which fully complements the *atsec31b* mutant phenotype (Supplemental Fig. S7).

Consistent with GUS staining, GFP signals were detected in tapetal cells at floral stage 9 (corresponding to anther stage 5-7) (Fig. 5, A), in both the tapetum and locular fluid at floral stages 10 and 11 (Fig. 5, B, C), and in mature pollen at floral stage 12 (Fig. 5, D).

Combined with the sporophyte-controlled mutant phenotype, defective exine formation in *atsec31b* mutant and highly-expression of AtSEC31B in the tapetum (Fig. 3 and Fig. 4, C-F), we speculate AtSEC31B primarily functions in the tapetum. Then we fused *AtSEC31B* cDNA with the *Arabidopsis* A9 promoter (Paul et al., 1992), which is expressed exclusively in tapetum cells, and transformed the resulting plasmid.
into atsec1b plants. In ~20 T1 transgenic lines, A9 promoter-driven AtSEC31B nearly completely rescued the male sterile phenotype of atsec1b mutants, including the defective pollen development and pollen germination rate in vitro (Fig. 5, E-U). All of these observations suggest the crucial role of AtSEC31B in tapetal cells.

**AtSEC31B-GFP is a functional COPII component**

To demonstrate AtSEC31B is bona fide COPII protein, we further investigate the subcellular location of AtSEC31B. We crossed AtSEC31B-EGFP with stable transgenic plants expressing the ER marker HDEL-mCherry, the cis-Golgi marker Sed5/SYP31-YFP, and the trans-Golgi marker sialyl transferase ST-YFP (Chatre et al., 2005; Nelson et al., 2007; Li et al., 2009). AtSEC31B-EGFP was distributed in punctuate regions and spread along the ER network (Supplemental Fig. S8, A), whereas AtSEC31B-GFP foci were embedded in the “mouth” of the “U”-shaped Sed5-YFP signal (Supplemental Fig. S8, B). We also found that the smaller AtSEC31B-GFP foci were accumulated around the larger punctuate-like ST-YFP (Supplemental Fig. S8, C). These results indicate that AtSEC31B-GFP is closely associated with ER and cis-Golgi markers and most likely localizes to ERESs. Next, we co-expressed AtSEC31B-GFP and the ERES marker mCherry-SEC24A in tobacco leaves (Hanton et al., 2007; Wei and Wang, 2008). The AtSEC31B-GFP signals strongly overlapped with the mCherry-SEC24A signals (Fig. 6, A).

In yeast, the coat components of the COPII complex, Sec13p and Sec31p, directly interact with each other, forming a tetramer complex. AtSEC13A, a homolog of ScSEC13p in Arabidopsis, is supposed to be a subunit of COPII complex in
Arabidopsis and was reported to interact with AtSEC31B in leek inner epidermal cells (Hino et al., 2011). Thus, we next performed a bimolecular fluorescence complementation (BiFC) assay to test the interaction of AtSEC31B and AtSEC13A in Arabidopsis. YFP signals were only detected in the cytosol of protoplasts co-transformed with AtSEC13A-nY plus AtSEC31B-cY or AtSEC13A-cY plus AtSEC31B-nY (Fig. 6, B) but not in other combinations (Supplemental Fig. S9). Co-immunoprecipitation (Co-IP) was also performed using stable transgenic lines of native promoter-driven AtSEC31B-EbGFP and 7Myc-6His-AtSEC13A. We found AtSEC31B-EGFP and 7Myc-6His-AtSEC13A could co-immunoprecipitate with each other (Fig. 6, C, D). These data suggest that AtSEC31B can directly interact with AtSEC13A in vivo.

To further explore protein trafficking processes in the atsec31b mutant, we observed the subcellular localization of Golgi marker proteins in wild-type and atsec31b mutant plants. ST-YFP and Erd2-CFP are reported to be transported from the ER to Golgi via anterograde trafficking (Brandizzi et al., 2002). ST-YFP and Erd2-CFP signals were clearly retained in the ER in atsec31b plants (Fig. 6, E-H). Subsequently, fluorescence recovery after photobleaching (FRAP) was conducted to determine whether the retained signals resulted from defective ER export. The t1/2 values for both markers in atsec31b mutants were significantly longer than those in wild-type plants (Fig. 6, I, K). However, the mobile fraction in each individual test was not affected, indicating that the available mobile fraction of the marker proteins was not altered in atsec31b plants (Fig. 6, J, L). These data suggest that disruption of
AtSEC31B dramatically affects ER-Golgi vesicle trafficking. Therefore, all above
date indicates AtSEC31B-GFP is a functional COPII protein.

**The development of elaioplasts and tapetosomes in the tapetum was impaired in**
the *atsec31b* mutants

We next examined whether the disruption of AtSEC31B would result in any
defects in the morphology (specially ER, Golgi and other organelles) of the tapetum
by TEM, though no obvious defect in tapetal cells could be detected in *atsec31b*
mutant by light microscope (Fig. 2). At anther stages 7-9, both in wild-type and
mutant plants, the tapetal cells exhibited large stacks of ER, Golgi, and many plastids
and in the peripheral region (Fig. 7, A, B, D, E). At stages 8 and 9, the tapetal cells
developed two main organelles, the plastid-derived elaioplasts and ER-derived
tapetosomes, which could release lipids and other materials on the surface of the
pollen-forming pollen coat (Suzuki et al., 2013). The elaioplasts of wild-type tapetal
cells contained many electron-translucent lipid plastoglobules at stage 9 (Fig. 7, C).
However, in *atsec31b* plants, the differentiated elaioplasts were first observed in
*atsec31b* plants at anther stage 10, which contained fewer, smaller plastoglobules than
wild-type plants (Fig. 7, F, J). Additionally, tapetosomes were observed in wild-type
tapetal cells (Fig. 7, G) but were not found in contemporary *atsec31b* plants (Fig. 7, J).
At anther stage 11, the elaioplasts and tapetosomes of wild-type tapetal cells enlarged
and underwent further development (Fig. 7, H, I). By contrast, except for a few,
smaller elaioplasts, the *atsec31b* tapetum contained obscure, malformed tapetosomes,
with a fragmental interior structure and no obvious boundaries (Fig. 7, K, L). These
results indicate that the formation of elaioplasts and tapetosomes in the \textit{atsec31b} tapetum is notably impaired, which may have potential effects on pollen coat deposition.

**The expression of sGFP:ABCG9 protein is altered in the \textit{atsec31b} mutant**

To find potential candidate proteins that are transported by AtSEC31B-mediated COPII vesicle trafficking, we screened nearly all proteins reported to participate in pollen wall development. ABCG9 was selected for further investigation because sGFP:ABCG9 localizes to the plasma membrane (PM) of tapetal cells and is specifically expressed at anther stage 10 (Choi et al., 2014). ABCG9 and ABCG31 also harbour several potential ER export signal sites, such as the di-acid motifs and di-basic motifs in the C-terminus of ABCG9 (Supplemental Table S2). Moreover, the PM localization of sGFP:ABCG9 could accumulated in “BFA compartments” in the tapetal cells (Supplemental Fig. S10) after BFA treatment, which inhibits transport events between the ER and Golgi stacks (Nebenführ et al., 2002). These imply that sGFP: ABCG9 is very likely transported from the ER to the Golgi. In addition, the pollen coat of \textit{atsec31b} mature pollen grains also harboured numerous vesicular, linear and electron-lucent structures (Fig. 8, A-C), which is similar to that of \textit{abcg9 abcg31} plants as previous reported (Choi et al., 2014).Then, we examined the distribution of sGFP:ABCG9 in wild-type and \textit{atsec31b} plants by laser confocal scanning microscopy (Fig. 8, D-I). In wild type background, the signal of sGFP:ABCG9 is clearly detected in plasma membrane in the tapetal cells in master gain 550, which is consistent with previous reported (Fig. 8, D). While in the same
condition, the signal of sGFP:ABCG9 could not be detected in the atsec31b (Fig. 8, G).

In master gain 670, the signal of sGFP:ABCG9 still could not be detected in the atsec31b (Fig. 8, H). In master gain 770, the signal of sGFP:ABCG9 was weakly detected in the plasma membrane of tapetum in the atsec31b (Fig. 8, I). Therefore, the expression of sGFP:ABCG9 in the atsec31b background was remarkably decreased compared with that in wild-type plants.
Discussion

The Arabidopsis tapetum is of the secretory type, and its position is maintained during microspore development (Liu and Fan, 2013). The secretory role played by the tapetum in microspore development is widely recognized, but the molecular mechanism of the secretory pathway in tapetal cells is largely unknown. Here, we found that AtSEC31B plays a crucial role in pollen wall formation by regulating the secretory pathway in the tapetum in Arabidopsis.

AtSEC31B is a putative orthologue of ScSEC31p. We provide several lines of evidence indicating that AtSEC31B is a functional COPII protein. First, AtSEC31B is targeted to ERESs (Fig. 6, A). ERESs are the ER region at which the COPII complex assembles (Marti et al., 2010). Second, the interaction of AtSEC31B and AtSEC13s has been established by previous studies, but these studies were performed in either a heterogeneous background or in vitro (Hino et al., 2011; Takagi et al., 2013). Here, we performed co-immunoprecipitation and BiFC analyses to demonstrate the interaction of AtSEC31B and AtSEC13A in vivo (Fig. 6, B-D). Third, FRAP assay demonstrate disruption of AtSEC31B leads to retarded protein trafficking between the ER and Golgi (Fig. 6, E-L). Therefore, AtSEC31B is implicated as an important functional COPII component.

The only homozygous atsec31b mutant exhibits significantly reduced viable pollens and severe seed abortion (Fig. 1, D, F and Fig. 2, C-H), suggesting that male sterile phenotype of atsec31b is under sporophytic control. The abnormal selfing segregation ratio, the decreased male transmission efficiency (Supplemental Table S1)
and compromised pollen germination rate in heterozygous plants (Supplemental Fig. S4, A) indicate ATSEC31B also function in male gametophyte. Therefore, ATSEC31B function both in sporophyte and gametophyte. We also notice that pollen tubes grow dramatically slowly in \textit{atsec31b} mutant in 4 and 10 hap, but could reach the bottom of pistil in 24 hap (Supplemental Fig. S4, C). Excessive pollination in \textit{atsec31b} self-cross plant could fully recover the abortion phenotype (Supplemental Fig. S2). Therefore, the deceased pollen grain in pistil is responsible for severe pod abortion. Though 40\% of pollen grains in \textit{atsec31b} mutant anther exhibits normal by SEM analysis, most of them stick to pollen debris in the anther locule and could not be successfully released to the pistil.

Numerous works demonstrate the tapetum plays an important role in exine formation of microspores (Ting et al., 1998; Ariizumi and Toriyama, 2011). In \textit{atsec31b} mutants, exine development was dramatically impaired (Fig. 3), which is controlled by sporophyte. These results imply abnormal functioning of the sporophytic tapetum in \textit{atsec31b} mutant. Besides, we found that \textit{AtSEC31B} is universally expressed, but particularly strong GUS and AtSEC31B-EGFP signals were detected in tapetal cells (Fig. 4 and Fig. 5). Furthermore, we use \textit{AtSEC31B} driven by the tapetum-specific \textit{A9} promoter successful rescue the \textit{atsec31b} phenotype (Fig. 5, E-U). Thus, AtSEC31B primarily functions in tapetal cells sporophytically.

However, the morphology and PCD process of tapetum in mutant is similar to that of wild-type plants (Fig. 2). By TEM analysis, the mutant tapetum also exhibit normal ER and Golgi, different from point mutation in \textit{SEC24A} which result in
aberrant ER structure. However, the development of tapetosomes is dramatically
defective in atsec31b mutants (Fig. 7). Tapetosomes are unique organelles found in
tapetal cells; they accumulate lipid materials released to the locule forming the pollen
coat (Piffanelli et al., 1998; Hsieh et al., 2003; Hsieh and Huang, 2007; Shi et al.,
2015). Tapetosomes are derived from the ER, but the mechanism of their formation is
still unclear (Boavida and McCormick, 2007). The fragmented shape of the
tapetosomes may result from some inconspicuous alteration of the tapetal ER.
Alternatively, AtSEC31 may mediate some important type of cargo export which may
be required for normal tapetosome formation. And also we cannot exclude that
impaired tapetosomes development is the indirect results from the loss function of
AtSEC31B. How COPII-coated vesicle transport is involved in tapetosome formation
require further investigation.

Moreover, the development of elaioplast in the tapetal cells in the atsec31b
mutant is also significantly retarded compared with that of wild-type plants. As
reported, elaioplast in tapetum is derived from proplastids(Piffanelli et al., 1998).
Recent bioinformatics analysis and experimental evidence supported that vesicle
transport also exist in different kinds of plastid including proplastids(Lindquist et al.,
2016). COPII related proteins may mediate vesicle trafficking in chloroplasts
(Lindquist et al., 2016). For instance, the chloroplast protein CPSAR1, which show
similarity with AtSar1 protein in cytoplasm, and CPRabA5e may participate in
thylakoid biogenesis and vesicle trafficking in Arabidopsis (Garcia et al., 2010; Khan
et al., 2013; Karim et al., 2014; Karim and Aronsson, 2014; Lindquist and Aronsson,
2014). But whether other COPII proteins are involved in vesicle traffic in chloroplasts is still unknown (Lindquist et al., 2016). Therefore, one possible explanation for the retarded development of elaioplast in the atsec31b is that the disruption of ATSEC31B leads to the less efficiency of COPII protein transport in proplastid and then the differentiation from proplastid to elaioplast is postponed eventually in atsec31b mutant. However, this question needs further investigation.

It has been accepted that the undulation of the microspore plasma membrane and primexine formation play vital roles in pollen exine formation (Ariizumi and Toriyama, 2011). The pollen coat is also vital for pollen adhesion and hydration in the stigma (Ariizumi and Toriyama, 2011). We found that primexine formation and pollen coat deposition are both significantly disrupted in the atsec31b mutant (Fig. 3 and Fig. 8, A-C). Through TEM observations, we found that the exine formation is significantly impaired in atsec31b mutant, which ultimately causes pollen abortion. The AtSEC31B mutant exhibited several changes in the process of the pollen exine formation: (1) the plasma membrane undulation was not evident and was less invaginated than wild type; (2) the sporopollenin was randomly deposited on a very thin primexine; (3) some of the microspores could form thick baculae and exine-like structures; and (4) the pollen protoplasts were separated from the aberrant exine and eventually degenerated (Fig. 3).

Plasma membrane undulation occurs in the tetrad stage, which has been found in many species, including Arabidopsis, and is considered to determine the final exine patterning in different species (Anger and Weber, 2006; Ariizumi and Toriyama, 2011).
The primexine deposited between the plasma membrane and the callose wall serves as an ideal sporopollenin receptor (Gabarayeva and Grigorjeva, 2004; Ariizumi and Toriyama, 2011). Besides, the processes of plasma membrane undulation and primexine deposition are closely related. Abnormal primexine formation often leads to severely defective pollen exine development (Ariizumi and Toriyama, 2011). Therefore, the defective primexine formation in the atsec31b mutant may be the primary and main cause of following defective exine formation, defective pollen coat, even the collapsed pollens, though we could not exclude the possibility that AtSEC31B mediates the transporting events of unidentified proteins or members which are involved in the subsequent development of pollen grains and pollen coat.

The mutant phenotype in atsec31b is clearly distinguishable from the reported primexine-defective mutants. In no primexine and plasma membrane undulation1 (npu1), auxin response factor17 (arf17) and defective in exine formation1 (dex1) mutants, the undulation of the plasma membrane is not observed (Paxson-Sowders et al., 2001; Chang et al., 2012; Ma et al., 2013, 2013). Unlike npu1 and arf17 mutants, which have no primexine formation, the dex1 mutant has a thin primexine similar to that of atsec31b mutant (Paxson-Sowders et al., 2001; Chang et al., 2012; Ma et al., 2013). However, sporopollenin never anchors on the plasma membrane, and no exine-like structure is formed in dex1 mutant (Paxson-Sowders et al., 2001). Similar to atsec31b, the undulation of the plasma membrane is also reduced in no exine formation1 (nef1), but nef1 displays coarse primexine and no sporopollenin deposition (Ariizumi et al., 2004). In contrast to other primexine-defective mutants, hackly
microspore (hkm)/male sterile1 (ms1), and ruptured pollen grain1 (rpg1) mutants have more evident undulated plasma membranes but also result in thin primexine deposition (Ariizumi et al., 2005; Guan et al., 2008). Moreover, transient defective exine 1 (tde1) and exine formation defect (efd) mutants exhibit normal plasma membrane undulation but still produce thin and no primexine deposition, respectively (Ariizumi et al., 2008; Hu et al., 2014). However, except for edf and arf17 mutants, which lack the information about sporophytically or gametophytically controlled mutant phenotype (Yang et al., 2013; Hu et al., 2014), all of the published primexine mutants phenotype are under sporophytic control. AtSEC31B also function sporophytically in the primexine formation, which support that proper primexine deposition is likely dependant on sporophytic tissue (Ariizumi and Toriyama, 2011).

The impaired exine formation and pollen coat deposition in atsec31b may result from the mis-location or less efficient trafficking of several of the reported or other unidentified proteins that participate in primexine formation and pollen coat deposition. The primexine is matrix largely composed of neutral and acidic polysaccharides, cellulose and proteins (Ariizumi and Toriyama, 2011). More recently, SPONGY2 and UPEX1, which are glycosyltransferase participated in the biosynthesis xylan backbone and galactosylation of arabinogalactan proteins (AGPs), respectively, play roles in primexine formation sporophytically (Li et al., 2016). Moreover, the trafficking of cell wall polysaccharides and glyproteins largely depends on the secretory pathway in plant (Kim and Brandizzi, 2016). Many enzymes involved in the synthesis and modification of cell wall polysaccharides and glyproteins (eg. AGPs)
are likely transported to via COPII-coated vesicles. Besides, DEX1 and RPG1 are predicted to be membrane-associated proteins and NPU1 is located in the plasma membrane in Arabidopsis protoplasts (Guan et al., 2008; Chang et al., 2012; Ma et al., 2013). These proteins are all likely transported to membrane organelles through COPII-coated vesicle trafficking. Although a lack of specific antibodies and XFP-fused protein which could fully rescue transgenic lines, the actual subcellular locations of these proteins are still needed to be confirmed.

AtABCG9 and AtABCG31 are reported to be involved in pollen coat deposition (Choi et al., 2014). We found atsec31b plants show similar pollen coat defects to that of abcg9 abcg31 plants (Fig. 8, A-C). sGFP:ABCG9 is localized in the PM of tapetal cells and harbours several potential ER export signal sites, and also accumulates in the BFA compartment following BFA treatment, which suggested sGFP:ABCG9 is very likely transported via the ER-Golgi interface (Supplemental Table S2 and Supplemental Fig. S10). sGFP-ABCG9 is expressed in tapetal cells exclusively in anther stage 10 (Choi et al., 2014), and AtSEC31B-EGFP appears from floral stages 9 to 11 (Fig. 5, A-D), which suggests that these proteins may present overlapping expression. However, in atsec31b mutant background, the whole fluorescence intensity of sGFP:ABCG9 is dramatically reduced compared to that of wild-type plants(Fig. 8, D-I). sGFP:ABCG9 signal is weakly detected in plasma membrane of tapetum and ER retention is not determined in the atsec31b. It is hypothesized that after blocking of the anterograde transporting from ER to Golgi, the premature ABCG9 may be originally over-accumulated in ER and subsequently excessive.
sGFP:ABCG9 proteins in ER may trigger ER stress. ER stress response may attenuate
the sGFP:ABCG9 translation to release the stress of ER, or the sGFP:ABCG9 may be
subjected to proteasome associated degradation (Wan and Jiang, 2015) (Pety de
Thozee and Ghislain, 2006; Kakoi et al., 2013), which also need further investigation.
Moreover, identification of other more candidates also needs to be explored.

Taken together, our findings show that AtSEC31B, as a functional COPII protein
in tapetal cells, may mediate the membrane trafficking of many crucial proteins (eg.
membranes proteins, glyproteins including AGPs, and enzymes involved in
biosynthesis and modification cell wall polysaccharides, etc), ultimately ensuring
normal pollen exine development in Arabidopsis (Supplemental Fig. S11). This work
characterizes a key factor participating in pollen wall development and also providing
regulation mechanism of secretive activity in tapetal cells at the molecular level.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

T-DNA insertion lines in background ecotype Col-0 were supplied by
Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org/) at
Ohio State University, U.S.A. Seeds were sterilized (1 min in 75% ethanol, two rinses
in sterile water, 12 min in 2.5% bleach, and four rinses in sterile water) and planted on
growth medium (Murashige and Skoog basal salt mixture, 4.33 grams per liter
supplied with 0.75% Agar, purchased from PhytoTechnology Laboratories), then were
cold treated for 3~5 days. The medium plates were then transferred to a growth chamber (PERCIVAL) at 22°C and 50% humidity under a long photoperiod (16 hr of light / 8 hr of dark) for ~14 days prior to plant in the soil.

**Phylogenic and Sequence Analysis**

The amino acid sequences were aligned using the ClustalX2 program ([http://www.clustal.org/](http://www.clustal.org/)) (Sievers et al., 2011) with default settings. Using MEGA6.06 software ([http://www.megasoftware.net/](http://www.megasoftware.net/)) (Tamura et al., 2013), a rectangular phylogenetic tree was constructed with full-length sequences by the neighbor-joining method with 1000 replicates for bootstrap and default settings for other options.

Potential domains, motifs and phosphorylation sites were predicted by SMART ([http://smart.embl-heidelberg.de/](http://smart.embl-heidelberg.de/)) (Schultz et al., 1998; Letunic et al., 2014), epestfind ([http://emboss.bioinformatics.nl/](http://emboss.bioinformatics.nl/)) (Kyte and Doolittle, 1982; Rogers et al., 1986; Rechsteiner et al., 1987; Rechsteiner and Rogers, 1996) and CBS Prediction Server ([http://www.cbs.dtu.dk/services/](http://www.cbs.dtu.dk/services/)) (Blom et al., 1999), respectively.

**Genetic Methods**

Transgenic plants were generated via *Agrobacterium tumefaciens* (strain GV3101) -mediated flower-dip transformation (Clough and Bent, 1998), and selected on MS solid medium containing 25 mg/L hygromycin B (CALBIOCHEM) for segregation screening.

To determine plant genotypes, leaf genomic DNA was extracted via a rapid prep method as described with some modification (Edwards et al., 1991).

PCR genotyping was performed using three primers, one primer annealing to the
T-DNA insertion site (LBb1.3), and a pair of primers designed to amplify the fragment of DNA spanning the insertion site, which were obtained from Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/).

Outcrosses were conducted by applying pollens from newly dehiscing flowers onto the stigmas of flowers that had been surgically emasculated one day ago just prior to dehiscence (Cole et al., 2005).

**Total RNA Extraction and Reverse Transcription Reaction**

The total RNAs were extracted using RNAiso Plus (TaKaRa) as described by the supplier. The first-strand cDNAs were synthesized from 1 μg of total RNA by a Prime Script RT reagent Kit (TaKaRa) according to the manufacturer’s instructions.

**SEM, Cross-section and TEM**

For scanning electron microscopy, the newly opened flowers were dissected under dissection microscope and the dehiscent anthers were mouted on stubs over carbon double-sided tape (NISSHIN EM Co., Ltd. TOKYO) and coated with gold particles using a sputter coater. Specimens were observed with a scanning electron microscope (S-4800 field emission scanning electron microscope, HITACHI).

For cross-section, wild-type and *atsee31a-3/-* mutant inflorescences were fixed in FAA and embedded in Spurr’s resin as described (Sanders et al., 1999) and sectioned (2~3 μm) with a microtome (LKB Ultratome V, LKB, Bromma, Sweden). Anther sections were stained in 1% toluidine blue. Bright-field photographs of the cross-sections were taken using a Zeiss microscope.

For ultrastructural analysis, *Arabidopsis* floral buds were fixed in 4%
glutaraldehyde on ice, rinsed in 0.1 M PBS, and post-fixed in 1% OsO4 (dissolved in 0.1 M PBS). Flowers were embedded in Spurr’s resin as described in cross-section procedure. Ultrathin sections (80 nm) were cut using a diamond knife on a Leica Ultracut ultramicrotome. Sections were double stained with saturated uranyl acetate and lead citrate and examined with a transmission electron microscope (H-7650, HITACHI).

**Alexander’s Staining, FDA Staining and DAPI Staining**

Pollens were photographed with a Zeiss digital camera. Flowers and plants images were taken by a Leica dissection microscope. Pollen viability was determined using Alexander solution (Alexander, 1969) and fluorescein diacetate (FDA) (Heslop-Harrison and Heslop-Harrison, 1970; Pinney and Polito, 1989). 4’,6-diamidino-2-phenylindole (DAPI) staining was performed as described (Ross et al., 1996).

**GUS Staining**

Tissues of *ProAtSEC31B:GUS* plants were stained in a solution of 1mg/ml X-gluc (GOLDBO COM, USA), 10 mM EDTA·2Na, 0.5 mM K₃FeC₆N₆, 0.5 mM K₄Fe(CN)₆·3H₂O, 50 mM NaH₂PO₄, 50 mM Na₂HPO₄, 0.1% (v/v) Triton X-100 under 37 °C after treated in 90% pre-cooling acetone. After GUS staining, chlorophyll was removed using absolute ethanol and the tissues were treated with 2% HCl (v/v) in 20% methanol (v/v) and 7% NaOH (w/v) in 60% ethanol (v/v) (Yang et al., 1999).

For cross-section after GUS staining, the inflorescences were fixed in FAA on
ice, then transferred to 4 °C for overnight, embedded in paraplast (Pathologic, Songon), sectioned at 15 μm thickness, and observed under a Zeiss microscope (Wang et al., 2013).

**Pollen Germination in vitro and in vivo**

Pollen germination *in vitro* was performed as described (Boavida and McCormick, 2007) except under 28 °C. The aniline blue staining of pollen tubes *in vivo* was conducted as described (Ishiguro et al., 2001). The pollinated pistils were collected 4, 10 and 24 hap and continued the procedure. The stained pistils were observed and photographed using a Zeiss LSM-710 confocal microscope (Zeiss, Germany).

**Protoplast Transformation**

Protoplast preparation was performed as described (Yoo et al., 2007; Li et al., 2009). 20 μg per construction were transformed into 100 μl protoplast suspension. After cultured under 22 °C for ~22 hr, fluorescent images were taken using Zeiss LSM-710.

**CLSM and Subcellular Localization**

All confocal images were captured using a Zeiss 710 Confocal Laser Scanning Microscope (Germany) with a 40× or 63× water-immersion objective.

For subcellular of AtSEC31B-GFP in transformed inflorescences, the buds were dissected under a stereoscopic microscope, then the anthers at different developmental stages were picked out, immersed in ddH₂O, and captured on a confocal microscope. GFP and chlorophyll signals in samples were detected using the settings as described...
For co-localization, HDEL-mCherry, Sed5-YFP and ST-YFP were introduced into wild-type plants. The T1 generations selected by hygromycin B were crossed with rescue lines expressed AtSEC31B-GFP, then the progeny seeds were cultured vertically under darkness for 7 days. Cotyledon cells were analyzed for colocalization with HDEL-mCherry, and hypocotyl cells for colocalization with Sed5-YFP or ST-YFP. Fluorescent imaging was performed using line switching mode. GFP and mCherry in samples were excited with the 488-nm argon laser line and 543-nm HeNe laser line, respectively, and steps were taken for subtracting background noise. For imaging coexpression of GFP and YFP, settings matched the previous report to avoid channel crosstalk (Brandizzi et al., 2002).

For subcellular colocalization between AtSEC31B and other COPII components, GFP and mCherry signals were detected as described above. For BiFC assay, YFP and chlorophyll were excited with the 514-nm argon laser line, and emission was detected at 519-580 nm and 630-697 nm, respectively.

Post-capture processing were performed using ZEN 2010B SP1 software and Adobe Photoshop CS3.

**Bimolecular Fluorescence Complementation Assay**

The BiFC assay was conducted as described with modification (Hino et al., 2011). The plasmids were transformed into protoplasts using method described in ‘Protoplast Transformation’, and YFP fluorescence images were captured after cultured for ~22 hr.
Co-Immunoprecipitation

The wild-type plants stably expressed AtSEC13A-MH were crossed with
AtSEC31B-GFP/atsec31b-3 plants, and the whole plant of offsprings at about 4 weeks
were collected for co-IP analysis. Whole plants expressing both AtSEC13A-MH and
AtSEC31B-GFP were homogenized with liquid nitrogen, then 2.0 g powder were
added into 10 mL lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1%
(v/v) Triton X-100 and protease inhibitor cocktail (freshly add before use; Songon)
(Takagi et al., 2013). Homogenates were centrifuged at 20,000g for 15 min, and then
repeated twice for removing cellular debris. The supernatants were mixed with 30 μl
anti-c-Myc agarose beads (Sigma-Aldrich), and incubated on a slow rotating wheel at
4 °C for 1 hr 30 min. Then the anti-c-Myc agarose beads were transferred into a micro
bio-spin chromatography column (Bio-Rad Laboratories, Inc.), and washed with 600
μl washing buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v)
Triton X-100 for 3 times. Immunoprecipitation complexes were eluted with 50μl SDS
sample buffer, and subjected to SDS-PAGE, then to immunoblot analysis with
anti-GFP antibody (TongBio Pacific). When using AtSEC31B-GFP as bait, the
GFP-Trap A (Chromo Tek) was applied. And the procedure was according to the
described above.

Fluorescence Recovery after Photobleaching (FRAP)

FRAP analysis was conducted as previously described (Brandizzi et al., 2002).
The optimized conditions for bleaching were determined by materials fixed with 2%
paraformaldehyde. Before FRAP assay, the Golgi movement was stopped by
treatment of 25 μM Latrunculin B. Post acquisition and data analyses were performed with Zeiss AIM software.

**BFA treatment**

BFA treatment was performed as described (Brandizzi et al., 2002; Huang et al., 2013). Anthers at Stage 10 were incubated with or without 20 μg/ml BFA for 3 hr, which was prepared using DMSO and diluted by 1×PBS, while DMSO was taken as negative control. The same settings (laser power and detection gain) were used for direct comparison of the anthers before and after treatment.

**Molecular Manipulation**

For expression pattern analysis using GUS staining, a genomic DNA fragment containing nucleotide sequence from 2.2 kb upstream of the *AtSEC31B* start codon was amplified by PCR from genomic DNA of *Arabidopsis* ecotype Col plants, and cloned into *pCambia1300-Pro35S:GUS* binary vector using Pst I/Xba I, which hold GUS coding sequence behind the promoter. To generate *AtSEC31B* complementation construct, the *AtSEC31B* promoter obtained as described above was inserted into *pCambia1300-Pro35S:TerNOS* binary vector by Pst I/Xba I to take the place of 35S promoter, then three fragments of *AtSEC31B* genomic DNA, which were amplified by PCR from genomic DNA of *Arabidopsis* ecotype Col plants using specific primers, were cloned into the plasmid at the same time by the combination of Xba I/Apa I, Apa I/Sma I and Sma I/BamH I. For subcellular localization, the *AtSEC31B* promoter obtained as described above was cloned into pB35S:GFPBS-2 binary vector with enhanced GFP fused with C terminal of the target protein, and the two cDNA
fragments of *AtSEC31B* was amplified by PCR from mRNA of the inflorescences of *Arabidopsis* ecotype Col plants, then inserted into the plasmid by Xba I/Apa I and Apa I/BamH I in the meantime. For investigation on whether the *AtSEC31B* cDNA fragment driven by tapetum-specific *A9* promoter could complement the mutant phenotype, *A9* promoter fragment was cloned by specific primers from genomic DNA of wild-type plants to take the place of *AtSEC31B* promoter. For subcellular co-localization with COP II components in *Arabidopsis* protoplast, the Pro35S:Ter<sub>NOS</sub> fragment was inserted into pUC19 vector by Hind III/EcoR I, then the EGFP fragment was cloned into the plasmid by BamH I/Sac I followed by the insertion of *AtSEC31B* cDNA by Xba I/ BamH I. The cDNA fragments of COP II components including *AtSAR1*, *AtSEC13A* and *AtSEC13B*, were amplified by PCR using specific primers from mRNA of wild-type plants, and cloned into pUC19 vector which fused mCherry to the C terminal of the protein driven by 35S promoter.

The ER marker was provided by Professor Andreas Nebenführ of the University of Oklahoma Health Sciences Center originally (Nelson et al., 2007), and modified by Professor Yi-hua Zhou of Chinese Academy of Sciences (Li et al., 2009). The Golgi markers, including Sed5-YFP and ST-YFP, were provided by Professor Patrick Moreau of the University of Bordeaux (Chatre et al., 2005). The plasmids used for BiFC assay were provided by Professor Tsuyoshi Nakagawa of the Shimane University (Hino et al., 2011).

**Primers Used in This Study**

Primers and constructions used in this study are listed in Supplemental Table S3.
Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AtSEC31A (At1g18830), AtSEC31B (At3g63460), AtSAR1 (At1g56330), AtSEC13A (At3g01340), AtSEC13B (At2g30050), AtSEC24A (At3g07100), ABCG9 (At4g27420), ABCG31 (At2g29940), and ACTIN7 (At5g098103).

**Supplemental Data**

**Supplemental Figure S1.** Sequence alignment and phylogenetic analysis of SEC31 amino acid sequences from different species.

**Supplemental Figure S2.** Reciprocal cross analysis.

**Supplemental Figure S3.** Identification and phenotype analysis of atsec31b-n and wild-type plants.

**Supplemental Figure S4.** Pollen germination and pollen tube growth of the atsec31b and wild-type plants.

**Supplemental Figure S5.** Random deposition of sporopollenin on locule wall in atsec31b plants.

**Supplemental Figure S6.** Expression pattern of AtSEC31B revealed by GUS staining.

**Supplemental Figure S7.** ProAtSEC31B:AtSEC31Bc:EGFP could rescue the mutant phenotype of atsec31b.

**Supplemental Figure S8.** Subcellular localization analysis of AtSEC31B-GFP.
Supplemental Figure S9. Negative control groups of BiFC assay showing no interaction between them.

Supplemental Figure S10. The subcellular location of sGFP-AtABCG9 after BFA treatment in tapetal cells.

Supplemental Figure S11. Putative model of AtSEC31B function in the secretary pathway of the tapetal cells.

Supplemental Table S1. Transmission efficiency analysis using reciprocal crosses.

Supplemental Table S2. Potential ER export signals for AtABCG9 and AtABCG31.

Supplemental Table S3. Primers used in this study.

Supplemental Table S4. Constructs used in this study.

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We thank Professor Patrick Moreau (the University of Bordeaux) for kindly providing the ST-YFP, Sed5-YFP and Erd2-CFP plasmids, Professor Tsuyoshi Nakagawa (the Shimane University) for kindly providing the plasmids used for BiFC assay, Professor Randy W. Schekman (U.C., Berkeley) for kindly providing yeast mutant strains, RSY255 and RSY952, and Bob Lesch for helpful patient guidance on the yeast functional complementation experiment, Professor Youngsook Lee for kindly providing seeds stably expressing sGFP-ABCG9. We also thank the ABRC for providing seeds of Arabidopsis T-DNA insertion mutants.

Figure Legends
Figure 1. Disruption of AtSEC31B led to male sterile. A, Schematic representation of the T-DNA insertion in AtSEC31B. Black, gray and white closed boxes indicate the exons, introns and untranslated regions (UTR), respectively. B, RT-PCR analysis of the transcripts across the insertion sites of AtSEC31B expressed in mutant inflorescences. Primers for transcript amplification are indicated by arrows in (A), RT-FP/RP for AtSEC31B. ACTIN7 was taken as an internal control. Three biological and technical replicates were conducted. C, Comparison of vegetative and reproductive development between 40-day-old wild-type (Col-0), atsec31b mutant and Complementary (Comp) plants. The white arrowheads indicate the short siliques. Bar = 5 cm. D, Details of wild-type, atsec31b mutant and Complementary (Comp) buds. Noticed that there is less mature pollen grains on the mutant anthers and stigma (row above; bar = 200 μm). By Alexander’s staining, the anther of the wild-type plants is filled with viable, purple-stained pollen grains (purple), while that of the atsec31b mutants contains only a few viable pollen grains (row below, corresponding to each up image; bar = 100 μm).

Figure 2. The atsec31b mutant exhibits defective microspores development. A-J, SEM observation of mature pollens and dehiscent anthers. Wild-type anther (A) contained numerous pollen grains, whereas most atsec31b pollen grains degenerated (C). B, Wild-type pollen grains with a regular reticulate exine pattern. D-F, three types of pollen grains from the anthers of atsec31b plants: normal pollens (D), plump pollens with defective pollen wall (E) and collapsed pollens (F). G and H, The atsec31b/+ plants appeared abundant and normal pollen grains, respectively. I and J,
ProAtSEC31B:AtSEC31Bg/atsec31b could complement the defective pollen
development in atsec31b mutant plants. K, Statistical analysis of three types of
pollens shown in D to F. Ab-coat, pollens with abnormal coat; collapsed, collapsed
pollens without a typical exine. For (A, C, G, I), bars = 50 μm; for (B, D, E, F), bars =
5 μm; for (H) and (J), bars = 10 μm. L-W, Locules from anther sections of wild-type
(L-Q) and atsec31b (R-W) plants are shown from anther stage 7 to12. The black
arrows in (V) indicate abnormal structures surrounding the microspores. Comp,
Complementary plants; T, tapetum; Te, tetrad; MSP, microspores; UMP, uninuclear
microspore; BCP, bicellular pollen; TCP, tricellular pollen; Re, remnants. Bars = 20
μm.

Figure 3. Ultrastructure of pollen wall development in wild-type and atsec31b
plants. The tetrads at anther stage 7 (B and F) are high magnification of the inset of (A
and E). The microspore of stage 8 (J and N), stage 9(L and P), stage 10(R and W) and
stage 11(T and Y) are high magnification of the inset of (I and M), (K and O), (Q and
V) and (S and X). White arrow and black arrowhead in (D) and (H) indicated the
primexine (Pe) and distorted primexine-like structure (PeL), respectively. CW, callose
wall; PM, plasma membrane; UPM, undulated plasma membrane; Pe, primexine; PeL,
primexine-like structure; Ve, vesicle; P, plastid; Cytop, cytoplasm; Va, vacuole; G,
Golgi body; Ba, baculae; Tc, tectum; ProBa, probaculae; ProTc, protectum; In, intine;
Mt, mitochondria; Ty, trypoline; Ex, exine; Ne, nexine; rEx, remnants of exine; rIn,
Figure 4. Expression analysis of the Arabidopsis SEC31B. A, Expression pattern of AtSEC31B in wild-type plants by RT-PCR. Total RNA was isolated from 6-week-old wild-type plants. ACTIN7 was taken as an internal control. R, root; St, stem; Ri, rosette leaf; Cl, cauline leaf; Inf, inflorescence; Sil, silique. gDNA, Genomic DNA as positive control and ddH2O as negative control also were presented. B, GUS expression profile in the inflorescence transformed with ProAtSEC31B:GUS. The internal view in the lower left corner showed GUS signal in germinating pollen tube. Bar = 1 mm. For the internal view, bar = 20 μm. C-J, Paraffin sections of anthers from plants transformed with ProAtSEC31B:GUS. GUS expression was continuously detected in tapetal cells (T) before tapetum degenerated, and expressed highly in microspore mother cells (MMC), then decreased gradually in tetrads (Tds), uninuclear microspores (UMP) and bicellular pollens (BCP), but increased in tricellular pollens (TCP) and mature pollens (MPG). Bars = 50 μm.

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analysis for (H) to (J). U, statistical analysis for (K) to (M). Each data point represents mean ± SE (n > 400). Asterisk in (U) indicates significant difference relative to wild-type (Student’s t test, **P < 0.01). For (E) to (G), bars = 100 μm; for (H) to (J), bars = 20 μm.

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**Figure 7. Ultrastructure of tapetal cells in wild-type and atsec31b plants during**
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**Figure 8. The distribution and expression of sGFP-AtABCG9 in wild-type and atsec31b mutant plants.** (A to C) TEM observation of mature pollens from wild-type (A) and atsec31b dehiscent anthers (B and C). The white arrowheads indicates the irregular structures in the pollen coat of atsec31b (B) or in the debris of atsec31b anther locule (C). The white arrows show the random deposition of sporopollenin on the locule wall of anther. (D to I) The subcellular localization pattern of sGFP-AtABCG9 in and wild-type background (D to F) and the atsec31b mutant background (G to I). For (A to C), bars = 500 nm; for (D to I), bars = 50 μm.
Figure 1. Disruption of AtSEC31B led to male sterile. A, Schematic representation of the T-DNA insertion in AtSEC31B. Black, gray and white closed boxes indicate the exons, introns and untranslated regions (UTR), respectively. B, RT-PCR analysis of the transcripts across the insertion sites of AtSEC31B expressed in mutant inflorescences. Primers for transcript amplification are indicated by arrows in (A), RT-FF/RP for AtSEC31B. ACTIN7 was taken as an internal control. Three biological and technical replicates were conducted. C, Comparison of vegetative and reproductive development between 40-day-old wild-type (Col-0), atsec31b mutant and Complementary (Comp) plants. The white arrowheads indicate the short siliques. Bar = 5 cm. D, Details of wild-type, atsec31b mutant and Complementary (Comp) buds. Noticed that there is less mature pollen grains on the mutant anthers and stigma (row above; bar = 200 μm). By Alexander’s staining, the anther of the wild-type plants is filled with viable, purple-stained pollen grains (purple), while that of the atsec31b mutants contains only a few viable pollen grains (row below, corresponding to each up image; bar = 100 μm).
Figure 2. The atsec31b mutant exhibits defective microspores development. A-J, SEM observation of mature pollens and dehiscent anthers. Wild-type anther (A) contained numerous pollen grains, whereas most atsec31b pollen grains degenerated (C). B, Wild-type pollen grains with a regular reticulate exine pattern. D-F, three types of pollen grains from the anthers of atsec31b plants: normal pollens (D), plump pollens with defective pollen wall (E) and collapsed pollens (F). G and H, The atsec31b/+ plants appeared abundant and normal pollen grains, respectively. I and J, ProAtSEC31B:AtSEC31Bg/atsec31b could complement the defective pollen development in atsec31b mutant plants. K, Statistical analysis of three types of pollens shown in D to F. Ab-coat, pollens with abnormal coat; collapsed, collapsed pollens without a typical exine. For (A, C, G, I), bars = 50 μm; for (B, D, E, F), bars = 5 μm; for (H) and (J), bars = 10 μm. L-W, Locules from anther sections of wild-type (L-Q) and atsec31b (R-W) plants are shown from anther stage 7 to 12. The black arrows in (V) indicate abnormal structures surrounding the microspores. Comp, Complementary plants; T, tapetum; Tetract; MSP, microspores; UMP, uninuclear microspore; BCP, bicellular pollen; TCP, tricellular pollen; Re, remnants. Bars = 20 μm.
Figure 3. Ultrastructure of pollen wall development in wild-type and *atsec31b* plants. The tetrads at another stage 7 (B and F) are high magnification of the inset of (A and E). The microspore of stage 8 (J and N), stage 9 (L and P), stage 10 (R and W) and stage 11 (T and Y) are high magnification of the inset of (I and M), (K and O), (Q and V) and (S and X). White arrow and black arrowhead in (D) and (H) indicated the primexine (Pe) and distorted primexine-like structure (PeL), respectively. CW, callose wall; PM, plasma membrane; UPM, undulated plasma membrane; Pe, primexine; PeL, primexine-like structure; Ve, vesicle; P, plastid; Cytop, cytoplasm; Va, vacuole; G, Golgi body; Ba, baculae; Tc, tectum; ProBa, probacaleae; ProTc, protectum; In, intine; Mt, mitochondria; Ty, tryphine; Ex, exine; Ne, nexine; rEx, remnants of exine; rIn, remnants of intine. Bars = 2 μm for (A,E,I,M,Q,V,S and X). Others, bar = 500 nm.
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