COPII Components Sar1b and Sar1c Play Distinct Yet Interchangeable Roles in Pollen Development[1][OPEN]

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The development of pollen is a prerequisite for double fertilization in angiosperms. Coat protein complex II (COPII) mediates anterograde transport of vesicles from the endoplasmic reticulum to the Golgi. Components of the COPII complex have been reported to regulate either sporophytic or gametophytic control of pollen development. The Arabidopsis (*Arabidopsis thaliana*) genome encodes five Sar1 isoforms, the small GTPases essential for COPII formation. By using a dominant negative approach, Sar1 isoforms were proposed to have distinct cargo specificity despite their sequence similarity. Here, we examined the functions of three Sar1 isoforms through analysis of transfer DNA insertion mutants and CRISPR/Cas9-generated mutants. We report that functional loss of *Sar1b* caused malfunction of tapetum, leading to male sterility. Ectopic expression of *Sar1c* could compensate for *Sar1b* loss of function in sporophytic control of pollen development, suggesting that they are interchangeable. Functional distinction between Sar1b and Sar1c may have resulted from their different gene transcription levels based on expression analyses. On the other hand, Sar1b and Sar1c redundantly mediate male gametophytic development such that the *sar1b;sar1c* microspores aborted at anther developmental stage 10. This study uncovers the role of Sar1 isoforms in both sporophytic and gametophytic control of pollen development. It also suggests that distinct functions of Sar1 isoforms may be caused by their distinct transcription programs.

Pollen development is synergistically controlled by sporophytic and gametophytic factors (McCormick, 2004; Chang et al., 2011). The sporophytic tapetum, adjacent to developing microspores, is critical for pollen development through its secretion at early stages and programmed cell death (PCD) at late stages (Parish and Li, 2010). Function of the tapetum is controlled by an evolutionarily conserved transcriptional cascade (Wilson and Zhang, 2009; Zhu et al., 2011, 2015) as well as proteins involved in intercellular signaling, such as RECEPTOR-LIKE PROTEIN KINASE2 (RPK2), NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION (NPU), the secreted small peptide CLAVATA3/ESR-RELATED19 (CLE19), ATP-binding cassette (ABC) transporters (ABCGs), NADPH oxidases, and PROTEIN *S*-ACYL TRANSFERASE10 (PAT10; Mizuno et al., 2007; Quilichini et al., 2010; Chang et al., 2012; Zhou et al., 2013; Choi et al., 2014; Xie et al., 2014; Wang et al., 2017b). Genes critical for the gametophytic control of pollen development (Borg et al., 2009) have also been identified. They mostly encode proteins participating in endomembrane dynamics, such as proteins mediating PI3P-PI(3,5)P_2 conversion (Whitley et al., 2009; Zhang et al., 2018), protein sorting (Van Damme et al., 2006; Backues et al., 2010; Wang et al., 2013; Feng et al., 2017b), and vesicle trafficking (Backues et al., 2010; El-Kasmi et al., 2011).

The anterograde transport of vesicles from the endoplasmic reticulum (ER) to the Golgi apparatus relies on coat protein complex II (COPII), which is composed of five cytosolic components, the coat-GTPase Sar1, Sec23, Sec24, Sec13, and Sec31 (Lord et al., 2013; Hawes et al., 2015; Brandizzi, 2018). Mutations of Arabidopsis (*Arabidopsis thaliana*) *Sec24a* cause a disrupted ER morphology (Faso et al., 2009; Nakano et al., 2009). A key role of COPII in the gametophytic or sporophytic control of pollen development was also reported. Mutations of *Sec24b* cause a reduction in pollen germination, whereas functional loss of both *Sec24b* and *Sec24c* compromises male gametophytic transmission (Tanaka et al., 2013). On the other hand, sporophytic control of pollen development, likely through modulating tapetal function, was reported for Sec31b (Zhao et al., 2016), Sec23a and Sec23d (Aboulela et al., 2018), as well as

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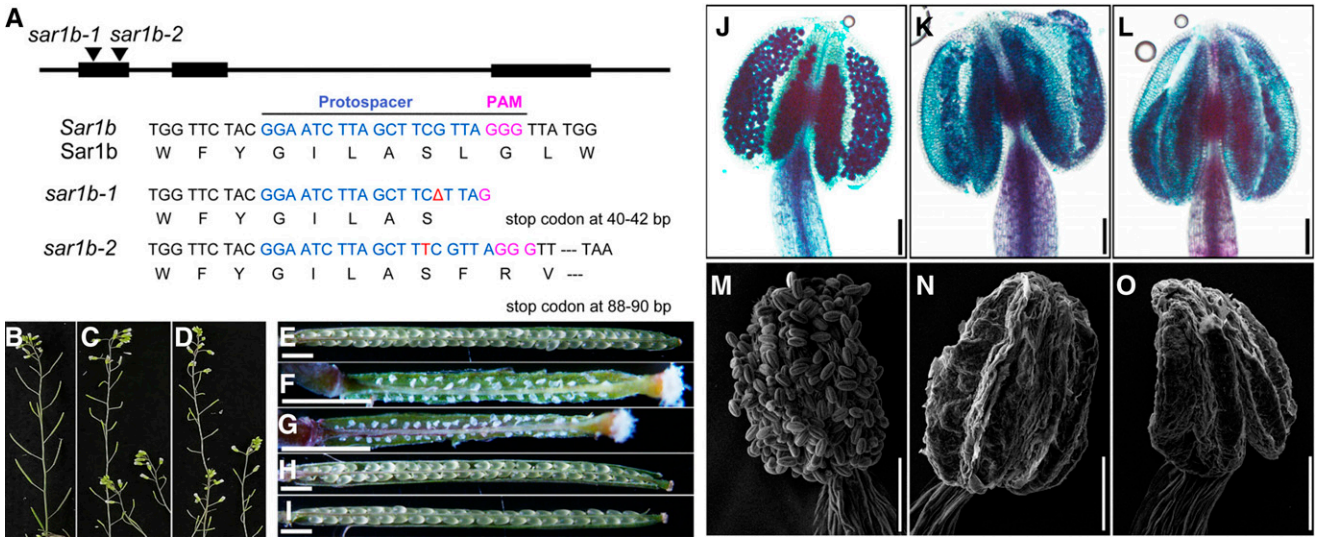

Figure 1. Functional loss of *Sar1b* results in male sterility. A, Schematic illustration of the *Sar1b* genomic locus. Triangles indicate CRISPR/Cas9-targeting sites. The mutants generated by CRISPR/Cas9, *sar1b-1* and *sar1b-2*, were the result of one nucleotide deletion or insertion, respectively. B to D, Inflorescences from wild-type (B), *sar1b-1* (C), or *sar1b-2* (D) plants. E to G, Siliques from self-pollinated wild-type (E), *sar1b-1* (F), or *sar1b-2* (G) plants. H and I, Siliques from *sar1b-1* (H) or *sar1b-2* (I) pollinated with wild-type pollen. J to O, Alexander staining (J–L) and SEM (M–O) of dehiscing anthers from wild-type (J and M), *sar1b-1* (K and N), or *sar1b-2* (L and O) plants. Bars = 1 mm (E–I) and 100 μm (J–O).

Sec24a (Conger et al., 2011). Whether the different roles of distinct isoforms are due to expression specificity or functional specificity, as proposed (Chung et al., 2016), is unclear.

The Arabidopsis genome encodes five Sar1 isoforms (Bassham et al., 2008; Brandizzi and Barlowe, 2013), among which three have been functionally studied through a dominant negative (DN) approach (i.e. the expression of a mutated Sar1 to deplete endogenous guanine nucleotide exchange factors; Takeuchi et al., 2000; Hanton et al., 2008; Zeng et al., 2015; Feng et al., 2017b; Ito et al., 2018). Expression of Sar1^{DN} compromised the vesicular transport from ER to Golgi (Takeuchi et al., 2000; Hanton et al., 2008; Zeng et al., 2015; Feng et al., 2017b; Ito et al., 2018). Despite the high sequence similarity of the three Sar1 isoforms, studies indicated that they have cargo specificity (Hanton et al., 2008; Zeng et al., 2015; Feng et al., 2017b). The expression of Sar1a^{DN} and Sar1b^{DN} had a significant difference regarding the transport of α-amylase (Hanton et al., 2008); the expression of Sar1a^{DN} and Sar1c^{DN} differed in the transport of a membrane-bound transcription factor (Zeng et al., 2015); and the expression of Sar1b^{DN} and Sar1c^{DN} differed in the transport of PAT10 (Feng et al., 2017b). Whether the distinction in cargo specificity is the result of functional differences of the isoforms (Zeng et al., 2015) or transcriptional specificities, as reported in mammals (Khoriaty et al., 2018), has yet to be determined.

In this study, we examined the function of Sar1 isoforms by mutant studies. We report that functional loss of *Sar1b* caused malfunction of tapetum, leading to male sterility. Ectopic expression of *Sar1c* could compensate for *Sar1b* loss of function in sporophytic control of pollen development, suggesting that they are interchangeable. Functional distinction between Sar1b and Sar1c may have resulted from their different gene transcription levels, based on expression analyses. Interestingly, Sar1b and Sar1c redundantly mediate male gametophytic development, such that the *sar1b;sar1c* microspores aborted at anther developmental stage 10. This work uncovers the role of Sar1 isoforms in both sporophytic and gametophytic control of pollen development. It also suggests that the distinct function of Sar1 isoforms may be caused by their distinct gene transcription programs.

RESULTS

Functional Loss of *Sar1b* But Not *Sar1a* and *Sar1c* Results in Male Sterility

To examine the functions of the three Sar1 isoforms in plant development, we took a reverse genetic approach. We isolated T-DNA insertion mutants of *Sar1a* and *Sar1c* (i.e. *sar1a* and *sar1c*; Supplemental Fig. S1). Both mutants are null mutants based on transcript analysis. Single mutants or the *sar1a;sar1c* double mutant grew comparably to the wild type in both vegetative and reproductive stages (Supplemental Fig. S1). Because there were no valid T-DNA insertion mutants of *Sar1b*, we generated *sar1b* mutants by CRISPR/Cas9 (Fig. 1A). Two mutant alleles were identified from transformants, *sar1b-1* and *sar1b-2*, both of which expressed mutated *Sar1b* transcripts

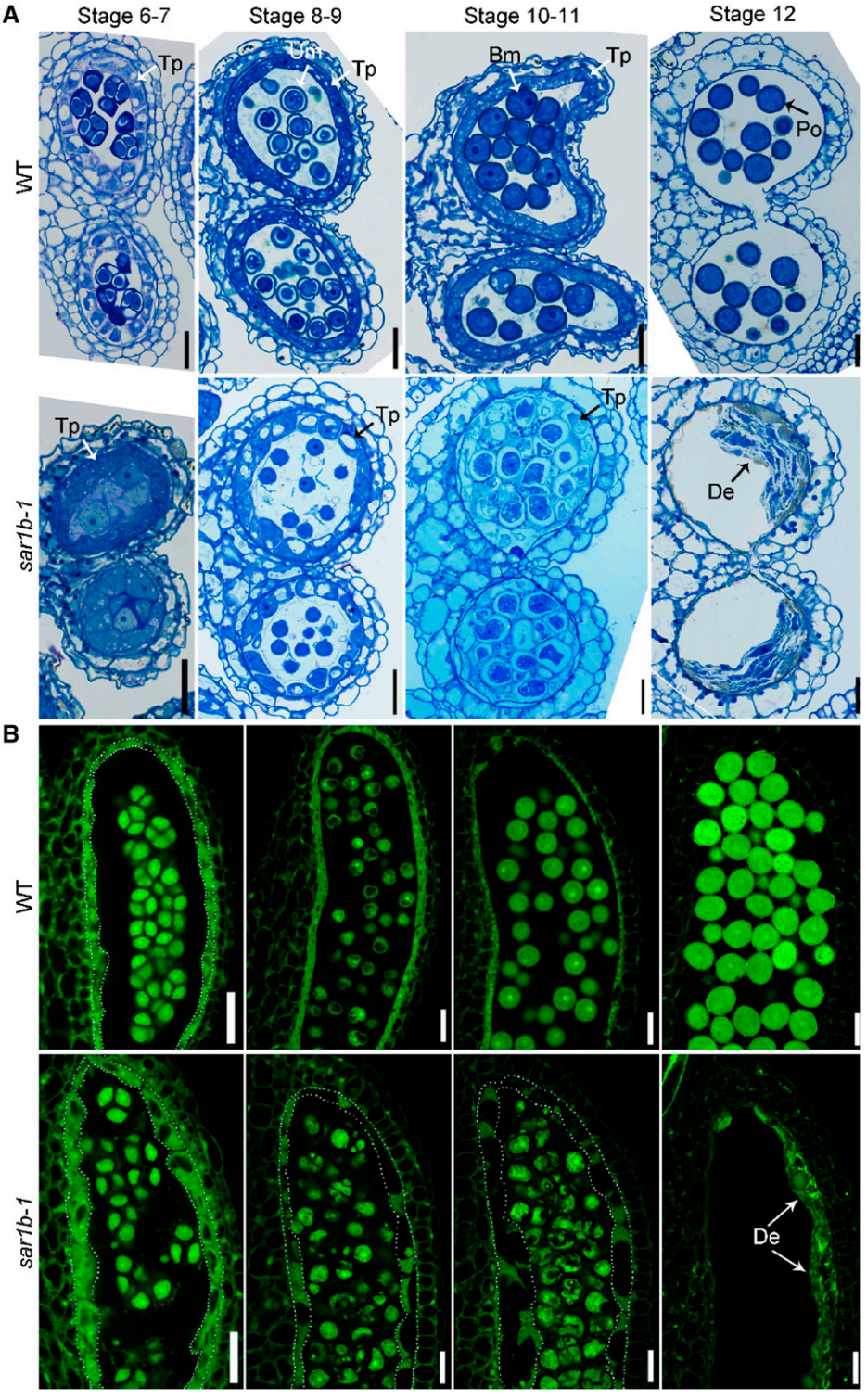

Figure 2. Functional loss of *Sar1b* compromises anther development. A and B, Transverse sections (A) or confocal laser scanning microscopy (CLSM) sections (B) of wild-type (WT) versus *sar1b-1* anthers at different anther developmental stages. Bm, Bicellular microspores; De, debris of microspores and tapetum; Po, pollen; Tp, tapetum; Um, unicellular microspores. Dotted lines in B highlight the tapetal layer. Bars = 20 μm.

with prestop codons resulting either from an insertion or a deletion in the *Sar1b* coding region (Fig. 1A). Vegetative growth of both *sar1b* mutants was comparable to that of the wild type (Supplemental Fig. S2). However, siliques of the homozygous *sar1b* mutants did not elongate (Fig. 1, C and D), in contrast to those of the wild type (Fig. 1, B and E). Siliques of *sar1b* pollinated using mature wild-type anthers contained a full seed set (Fig. 1, H and I), unlike those of *sar1b* (Fig. 1, F and G), suggesting that *sar1b* is male sterile. Indeed, by Alexander staining and scanning electron micrography (SEM) analysis of dehiscing anthers, we determined that mature *sar1b* anthers did not produce viable pollen (Fig. 1, K, L, N, and O), unlike the wild

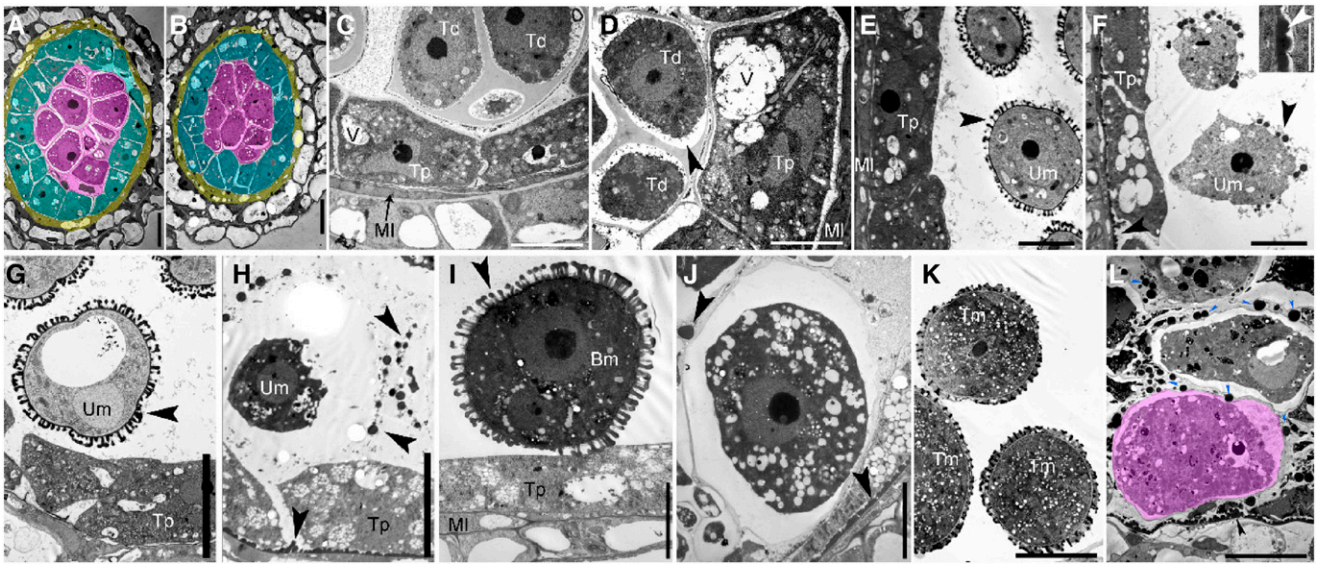

Figure 3. Functional loss of *Sar1b* compromises anther development. A and B, TEM scans of wild-type (A) or *sar1b-1* (B) anthers at developmental stage 5–6 showing the presence of different cell layers in the anther locule (pink, tetrads; blue, tapetum; yellow, middle layer). C and D, Wild-type (C) or *sar1b-1* (D) anthers at stage 6–7. The arrowhead in D points to a tetrad detached from the callose wall. E and F, Wild-type (E) or *sar1b-1* (F) anthers at stage 8–9. Arrowheads point to the deposition of sporopollenins at the surface of microspores (E) or at the interface of the tapetum and the middle layer (F inset) as well as in the pollen sac without attaching to the surface of microspores (F). G and H, Wild-type (G) or *sar1b-1* (H) anthers at stage 10. Arrowheads point to the deposition of sporopollenins at the surface of wild-type microspores (G) or at the interface of the tapetum and the middle layer as well as in the pollen sac without attaching to the surface of *sar1b-1* microspores (H). I and J, Wild-type (I) or *sar1b-1* (J) anthers at stage 11. Arrowheads point to the deposition of sporopollenins at the surface of wild-type microspores (I) or at the interface of the tapetum and the middle layer as well as in the pollen sac without attaching to the surface of *sar1b-1* microspores (J). K and L, Wild-type (K) or *sar1b-1* (L) anthers at maturation. Sporopollenin-like materials are deposited at the interface of the tapetum and the middle layer (black arrowhead) or between two degenerating microspores (blue arrowheads). One degenerating microspore is highlighted in pink. Bm, Bicellular microspores; Ml, middle layer; Td, tetrads; Tm, tricellular microspores; Tp, tapetum; Um, unicellular microspore; V, vacuole. Bars = 10 μm (A, B, G, H, K, and L), 5 μm (C–F, I, and K), and 1 μm (F inset).

type (Fig. 1, J and M), demonstrating that functional loss of *Sar1b* causes male sterility.

Sporophytic Control of Pollen Development Was Impaired in *sar1b*

To determine what caused the male sterility of *sar1b* mutants, we performed sectioning and light microscopy of developing anthers (Fig. 2). The specification of anthers was comparable between the wild type and *sar1b*, such that both contain epidermis, endothecium, middle layer, tapetum, and microspore mother cells (Fig. 2A). At anther developmental stage 6–7 (i.e. when tetrads are released in the pollen sac; Sanders et al., 1999), *sar1b* started to differ from the wild type (Fig. 2). The tapetal cells in *sar1b* were generally larger than those of the wild type, often containing large vacuoles (Fig. 2). In wild-type anthers, tapetal cells started to show signs of degeneration from anther stage 10 on and were reduced to undetectable levels, while microspores developed into mature pollen grains and were released at stage 12 upon anther dehiscence (Fig. 2). By contrast, microspores in *sar1b* pollen sacs started to degenerate as early as stage 8–9 (Fig. 2). The plasma membrane (PM) of microspores in *sar1b* pollen sacs was detached from the cell wall, and at anther dehiscence, *sar1b* pollen sacs contained only a pile of cellular debris (Fig. 2).

To obtain better resolution of pollen development in *sar1b* anthers, we performed transmission electron micrography (TEM) analysis. Consistent with the results from sectioning and light microscopy, TEM scans of early-stage anthers showed that *sar1b* (Fig. 3B) did not differ from the wild type (Fig. 3A) in cell specification. However, starting from stage 7, when tetrads were formed normally in the wild type (Fig. 3C), tetrads in *sar1b* anthers seemed detached from the callose wall that encased them (Fig. 3D). Later on, unicellular microspores with reticular pollen coat structures, presumably sporopollenins, were developed in the wild type (Fig. 3E). By contrast, a large amount of electron-dense materials was deposited to the intercellular space between the tapetum and the middle layer in *sar1b* (Fig. 3F). There were also aggregates of electron-dense materials deposited in pollen sacs without attaching to the surface of microspores in *sar1b* (Fig. 3F). Microspores continued to develop from the late unicellular stage (Fig. 3G) to the bicellular stage (Fig. 3I) and finally to maturation (Fig. 3K), with gradual formation of exine

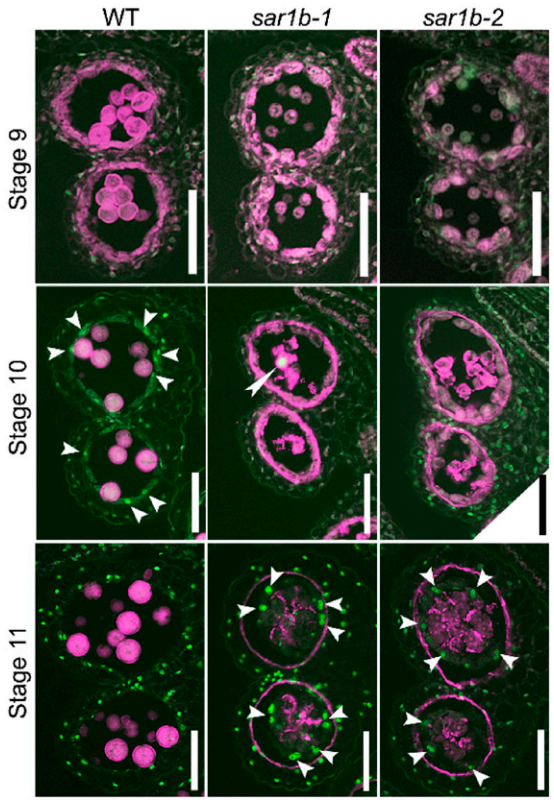

Figure 4. *Sar1b* loss of function interfered with the timing of tapetal PCD. CLSM of DNA fragmentation detected using the TUNEL assays in sections of wild-type (WT), *sar1b-1*, or *sar1b-2* anthers is shown at different stages. Images shown are merges of TUNEL-positive signals (green) and propidium iodide staining (magenta). Arrowheads indicate TUNEL-positive signals from degenerating tapetal cells or degenerating microspores. Note that the tapetal layer of *sar1b-1* or *sar1b-2* is detached from the middle layer and rescinded. Bars = 50 μm.

at the pollen surface and degeneration of the tapetal layer in the wild type. By contrast, microspores in *sar1b* anthers did not develop a reticular pollen coat structure (Fig. 3, H and J). Electron-dense materials were deposited between the middle layer and the tapetum as well as at the interface between microspores in *sar1b* (Fig. 3, H and J). During anther dehiscence, instead of producing normal pollen grains as in the wild type (Fig. 3K), *sar1b* anthers contained only cellular debris (Fig. 3L). These results support a key role of Sar1b in sporophytic control of pollen development.

Sar1b Loss of Function Interferes with the Timing of Tapetal PCD

Sporophytic control of pollen development has mostly been attributed to the function of tapetum, which is fulfilled through the secretion of enzymes and pollen coat materials at early stages and through PCD at later stages (Parish and Li, 2010). The abnormal deposition of sporopollenin-like materials in *sar1b* anthers during pollen development suggested that the early-stage function of tapetum was compromised by *Sar1b* loss of function. To determine whether the late function of tapetum (i.e. PCD) was also affected by *Sar1b* loss of function, we performed terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays that allow in situ detection of cell death (Vizcay-Barrena and Wilson, 2006; Phan et al., 2011; Xie et al., 2014). No TUNEL-positive signals were detected in the wild type or in *sar1b* before the unicellular stage (i.e. anther developmental stage 10; Fig. 4). At stage 10, TUNEL-positive signals appeared in the tapetal layer of wild-type anthers (Fig. 4). Anthers of *sar1b* at the same stage did not show TUNEL-positive signals in the tapetal layer (Fig. 4). Instead, TUNEL-positive signals occasionally appeared in developing microspores (Fig. 4), indicating microspore degeneration. At stage 11, TUNEL-positive signals were not only detected in the tapetal layer but also in other sporophytic cell layers in the wild type (Fig. 4), consistent with the following degeneration of vascular cells, septum, and stomium (Sanders et al., 1999). At this stage, TUNEL-positive signals appeared in the tapetal layer, vascular cells, and degenerating microspores of *sar1b* anthers (Fig. 4). These results suggest that Sar1b also participates in tapetal PCD.

Tapetum-Specific Expression of Sar1b Partially Rescues Anther Defects of *sar1b*

Because both early secretory function and late PCD of *sar1b* tapetum seem to be affected, we hypothesized

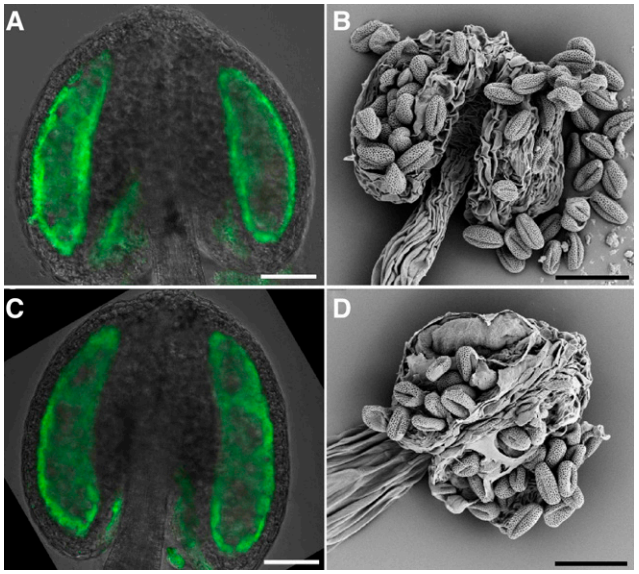

Figure 5. Tapetal expression of *Sar1b* partially complemented anther defects of *sar1b*. CLSM (A and C) or SEM (B and D) of a maturing anther from Pro_{A9}:GFP-Sar1b#1;*sar1b-1* (A and B) or Pro_{A9}:GFP-Sar1b#2;*sar1b-1* (C and D) plants is shown. CLSM scans of the two lines were taken with the same settings. Bars = 50 μm.

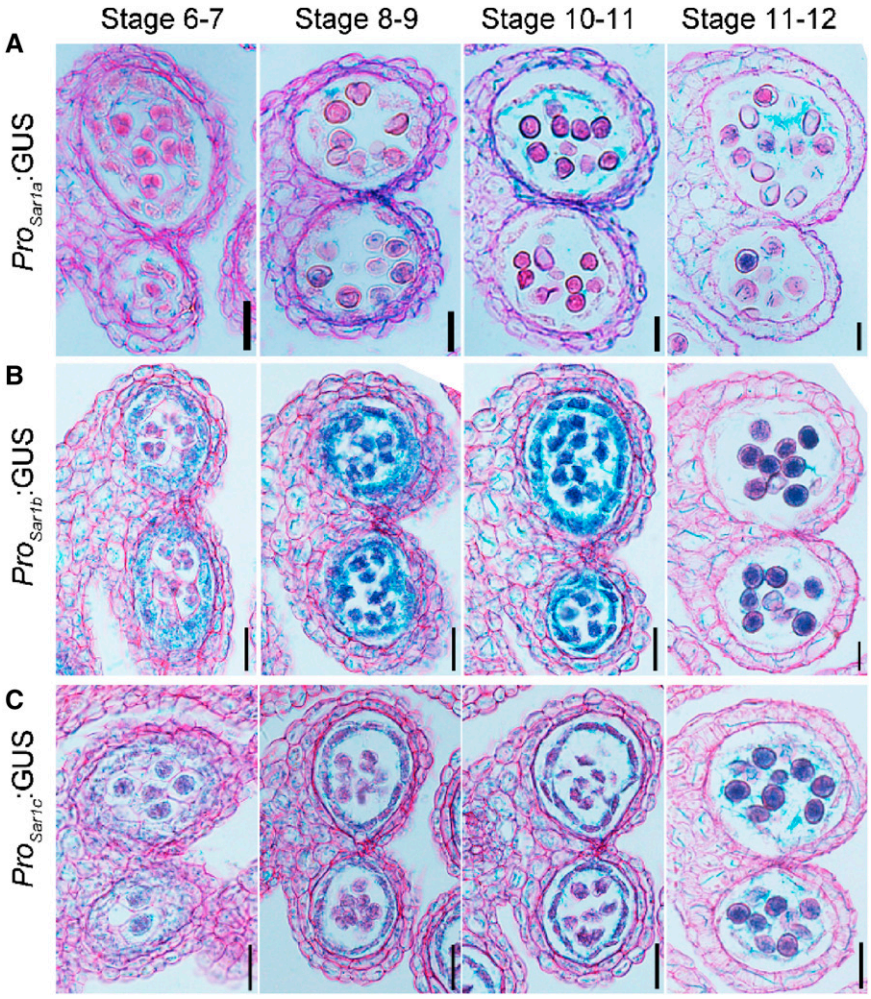

Figure 6. The expression of *Sar1* isoforms in developing anthers. Representative histochemical GUS staining of anther transverse sections from *Pro*$_{Sar1a}$:GUS (A), *Pro*$_{Sar1b}$:GUS (B), or *Pro*$_{Sar1c}$:GUS (C) transgenic plants is shown. Bars = 20 μm.

that the role of Sar1b in sporophytic control of pollen development was fulfilled through its expression in tapetum. To test this hypothesis, we expressed *Sar1b* under the control of *Pro*$_{A9}$, a tapetum-specific promoter (Wang et al., 2017a). CLSM of anthers from *Pro*$_{A9}$:GFP-Sar1b;*sar1b* transgenic plants showed clear fluorescence signals in the tapetal layer (Fig. 5, A and C). Examination of dehiscing anthers by SEM analysis showed the production of pollen grains at the dehiscing anthers of *Pro*$_{A9}$:GFP-Sar1b;*sar1b* plants (Fig. 5, B and D). These results suggest that tapetal expression of Sar1b at least partially rescues the pollen defects of *sar1b*.

Sar1c Can Replace Sar1b in Sporophytic Control of Pollen Development

To determine what underlies the distinct function of Sar1b from that of other isoforms in sporophytic control of pollen development, we first examined their respective gene expression in anthers by generating and analyzing corresponding promoter:GUS reporter lines (Fig. 6). Among the three *Sar1* isoforms examined, anthers of the *Pro*$_{Sar1b}$:GUS plants showed substantially stronger GUS signals in tapetum (Fig. 6B) than those of *Pro*$_{Sar1a}$:GUS (Fig. 6A) or *Pro*$_{Sar1c}$:GUS (Fig. 6C).

By reverse transcription quantitative PCR (RT-qPCR), we verified the significant transcriptional difference between *Sar1b* and *Sar1c* (Fig. 7A). To determine whether the functional distinction of Sar1b resulted from transcriptional differences, as reported for mammalian Sec23A and Sec23B (Khoriaty et al., 2018), we examined the ability of engineered expression of *Sar1c* versus *Sar1b* to complement the anther developmental defects of *sar1b* by generating *Pro*$_{UBQ10}$:Sar1b;*sar1b* and *Pro*$_{UBQ10}$:Sar1c;*sar1b* transgenic plants (Fig. 7A). Ectopic expression of Sar1c fully restored male fertility of *sar1b* (Fig. 7, D, E, H, and I) comparable to *Sar1b* (Fig. 7, B, C, F, and G), based on Alexander staining (Fig. 7, B–E) and SEM (Fig. 7, F–I) analyses of dehiscing anthers. Through RT-qPCR analysis, we confirmed that the rescue of *sar1b* male sterility by Sar1c was consistent with its significantly elevated transcript level (Fig. 7A). These results suggested that, although functionally distinct due to their respective transcriptional programs, Sar1b and Sar1c are interchangeable in sporophytic control of pollen development.

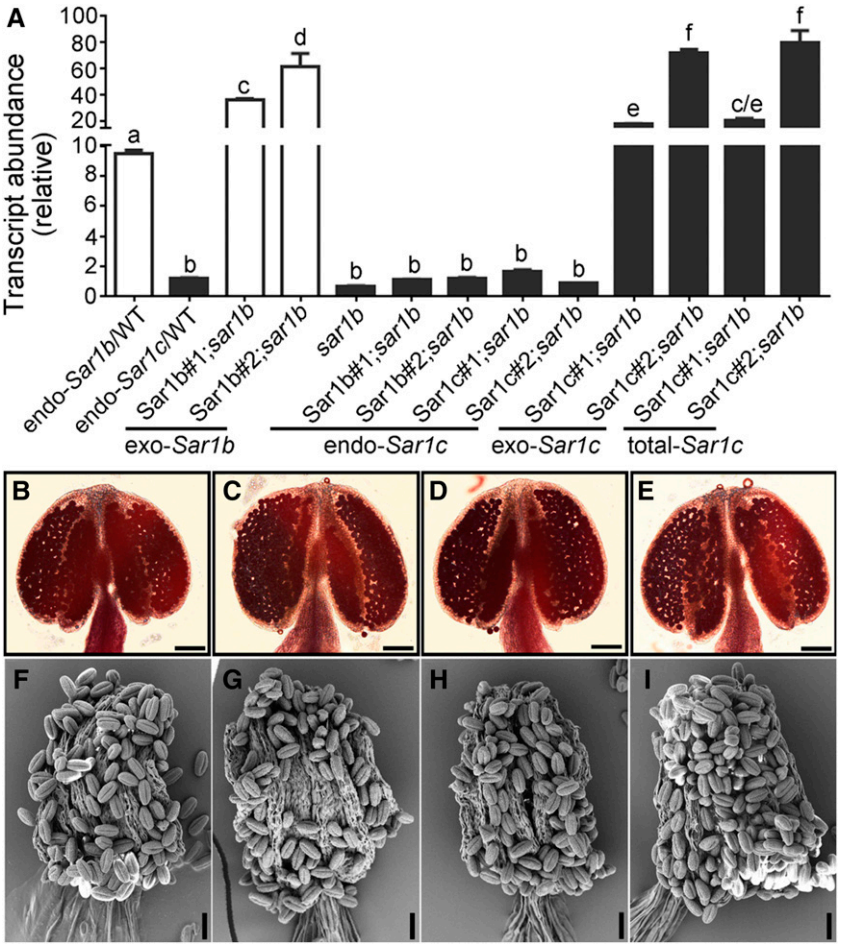

Figure 7. Ectopic expression of *Sar1c* suppresses male sterility of *sar1b*. A, Transcript abundance of *Sar1b* or *Sar1c* in the wild type (WT), *sar1b*, two lines of Pro_{UBQ10}:GFP-Sar1b;*sar1b-2* (Sar1b;*sar1b*), or two lines of Pro_{UBQ10}:GFP-Sar1c;*sar1b-2* (Sar1c;*sar1b*) by RT-qPCR. Endogenous (endo) or ectopic (GFP-fused) transcripts were analyzed in appropriate genotypes. Total *Sar1c* indicates the combined transcripts of endogenous *Sar1c* and *GFP-Sar1c*. Values are means ± SD ($n = 3$). Different letters indicate significantly different groups (one-way ANOVA, Tukey's multiple comparisons test, $P < 0.05$). RNAs were extracted from inflorescences. Three biological replicates were conducted with similar results. B to E, Alexander staining of a mature anther from two lines of Pro_{UBQ10}:GFP-Sar1b;*sar1b-2* (B and C) or two lines of Pro_{UBQ10}:GFP-Sar1c;*sar1b-2* (D and E). F to I, SEM scans of a dehiscing anther from two lines of Pro_{UBQ10}:GFP-Sar1b;*sar1b-2* (F and G) or two lines of Pro_{UBQ10}:GFP-Sar1c;*sar1b-2* (H and I). Bars = 100 μm (B–E) and 30 μm (F–I).

Sar1b and Sar1c Redundantly Regulate Male Gametophytic Development

Developing microspores of both Pro_{Sar1b}:GUS and Pro_{Sar1c}:GUS plants showed clear GUS signals (Fig. 6, B and C), implying a role of Sar1b and Sar1c in male gametophytes. To test this possibility, we crossed *sar1b* and *sar1c* by using *sar1c* as the pollen donor to pollinate *sar1b* pistils. No *sar1b;sar1c* double mutant was obtained by screening over 400 F2 progeny, indicating gametophytic transmission defects of *sar1b;sar1c*. Indeed, the *sar1b;sar1c* male gametophytes could not be transmitted based on reciprocal crosses and segregation analysis (male transmission of *sar1b;sar1c* versus *sar1c*: 0:48). These results suggest that, in addition to sporophytic control of pollen development by Sar1b, Sar1b and Sar1c redundantly mediate male gametophytic function.

To determine which processes were affected by Sar1b and Sar1c loss of function to cause male gametophytic lethality, we examined pollen development of the *sar1b/+;sar1c* plants. The *sar1b;sar1c/+* plants were excluded from these analyses because plants of this genotype do not produce pollen due to sporophytic defects. The *sar1b/+;sar1c* plants were comparable to wild-type plants in vegetative and reproductive growth (Supplemental Fig. S3). By Alexander staining (Fig. 8, A–H and Q), 4′,6-diamino-phenylindole (DAPI) staining (Fig. 8, I–L and R), and SEM (Fig. 8, M–P and S) analyses, we found that approximately 50% of pollen produced by the *sar1b-1/+;sar1c* plants (Fig. 8, D, H, L, and P) were aborted, whereas the wild type (Fig. 8, A, E, I, and M) or either single mutant, *sar1b-1/+* (Fig. 8, B, F, J, and N) or *sar1c* (Fig. 8, C, G, K, and O), contained almost 100% normal pollen grains at maturation (Fig. 8, Q–S). These results suggest that Sar1b and Sar1c play redundant roles in gametophytic control of pollen development.

Functional Loss of Sar1b and Sar1c Causes the Arrest of Pollen Development at Pollen Mitosis I

To determine the exact defects that occurred in *sar1b;sar1c* male gametophytes, we performed sectioning and light microscopy of developing *sar1b/+;sar1c* anthers. There was no tapetal abnormality in *sar1b/+;sar1c* anthers (Fig. 9), which differs from that in *sar1b* (Figs. 2 and 3) and consistent with the fact that gametophytic but not sporophytic defects resulted in pollen abortion in *sar1b/+;sar1c*. Tetrads released in pollen

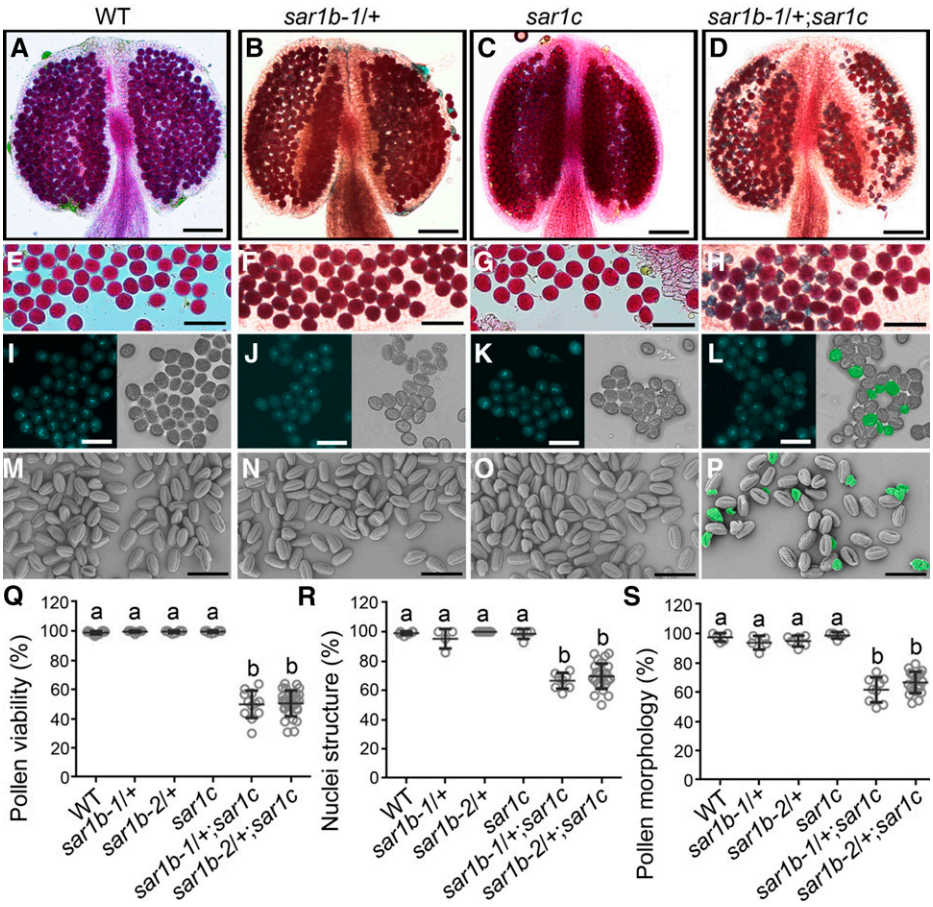

Figure 8. Functional loss of both *Sar1b* and *Sar1c* resulted in defective male gametophytic development. A to H, Alexander staining of a mature anther (A–D) or dehiscing anther (E–H) from the wild type (WT; A and E), *sar1b-1/+* (B and F), *sar1c* (C and G), or *sar1b-1/+;sar1c* (D and H). I to L, DAPI staining of mature pollen grains from the wild type (I), *sar1b-1/+* (J), *sar1c* (K), or *sar1b-1/+;sar1c* (L). M to P, SEM scans of mature pollen grains from the wild type (M), *sar1b-1/+* (N), *sar1c* (O), or *sar1b-1/+;sar1c* (P). Aborted pollen grains are highlighted in green in L and P. Q to S, Quantification of pollen viability by Alexander staining (Q), DAPI staining (R), or SEM (S). Results shown are means ± SE ($n > 5$). Different letters indicate significantly different groups (one-way ANOVA, Tukey's multiple comparison test, $P < 0.05$). Bars = 100 μm (A–D) and 50 μm (E–P).

sacs of *sar1b/+;sar1c* were comparable to those from the wild type (Fig. 9). Defective microspore development in *sar1b/+;sar1c* appeared at anther developmental stage 10 (Sanders et al., 1999), when the wild type contained unicellular microspores, whereas a portion of microspores in *sar1b/+;sar1c* pollen sacs showed the detachment of PM from the pollen coat or a large central vacuole with dense materials (Fig. 9). Later on, wild-type microspores transitioned from bicellular to tricellular mature pollen (Fig. 9). By contrast, a portion of microspores in *sar1b/+;sar1c* pollen sacs showed degenerating cytoplasm and finally collapsed (Fig. 9). These results demonstrate that Sar1b and Sar1c redundantly mediate gametophytic control of pollen development.

DISCUSSION

We report here that Sar1s, and, by inference, COPII-mediated ER-to-Golgi trafficking, are essential for both sporophytic and gametophytic control of pollen development. We show that Sar1b is essential for sporophytic male fertility. It has been widely accepted that sporopollenin components of the pollen exine, electron-dense materials in TEM images, are transported via vesicles before being deposited to developing microspores. AGCG26, whose mutation compromised sporophytic pollen development (Quilichini et al., 2010), is involved in the trafficking of polyketides, major constituents of sporopollenins (Quilichini et al., 2014). Aside from this, the trafficking route involved remains poorly understood. Functional loss of *Sar1b* caused abnormal deposition of electron-dense materials to the interface between the tapetum and the middle layer in addition to the pollen sacs instead of the surface of microspores (Fig. 3). Similar defects have been observed when Arabidopsis Sec31B or two isoforms of Arabidopsis Sec23a and Sec23d were mutated (Zhao et al., 2016; Aboulela et al., 2018), which are also components of the COPII complex (Brandizzi, 2018). These results suggest that COPII is not absolutely required for the secretion of sporopollenins. Rather, COPII may be involved in the targeted delivery of sporopollenins to developing microspores, whose regulatory mechanisms are to be explored.

In addition to the secretory function of tapetum, our results also supported a role of Sar1b in the late function of tapetum (i.e. PCD; Fig. 4). It is unclear whether the delayed tapetal PCD in *sar1b* was a direct result of compromised COPII function or indirect due to early defects. However, previous studies showed that MONENSIN SENSITIVITY1 (MON1)/CALCIUM CAFFEINE ZINC SENSITIVITY1 (CCZ1)-mediated Rab7 activation

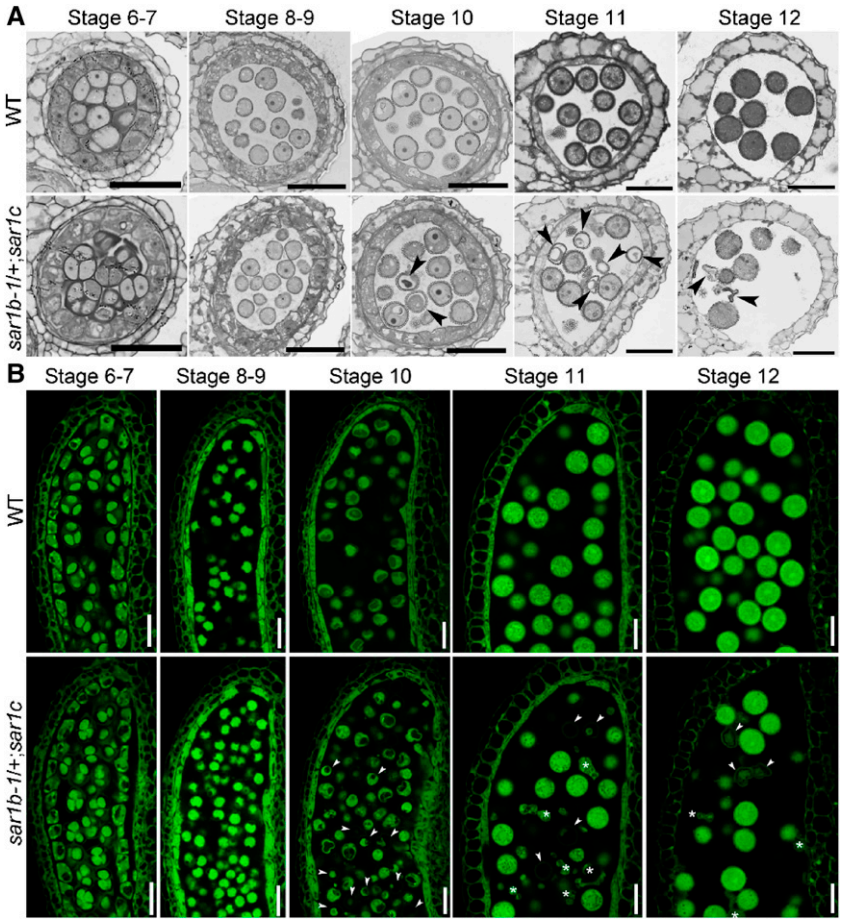

Figure 9. Functional loss of both *Sar1b* and *Sar1c* compromised male gametophytic development. Semithin transverse sections (A) or CLSM scans (B) of anthers at stage 6–7 (tetrad stage), at stage 8–9 (early unicellular stage), at stage 10 (later unicellular stage), at stage 11 (bicellular stage), or at stage 12 (tricellular stage) from wild-type (WT) or *sar1b-1/+;sar1c* plants. Arrowheads indicate defective microspores. Asterisks indicate debris of degenerating microspores. Bars = 20 μm.

regulates tapetal PCD (Cui et al., 2017). MON1/CCZ1-mediated Rab7 activation is critical for vacuolar trafficking (Cui et al., 2014), whereas COPII-mediated ER-to-Golgi trafficking is the starting point of vacuolar trafficking (Brandizzi and Barlowe, 2013). In fact, Arabidopsis CEP1, a KDEL-tailed Cys protease whose maturation requires its delivery to vacuoles presumably from the ER via COPII, is a key executor in tapetal PCD (Zhang et al., 2014). Thus, a direct role of Sar1b in tapetal PCD, by mediating the trafficking of key regulators such as CEP1, could not be excluded.

Although Sar1b expressed specifically in tapetum partially complemented the sporophytic male sterility of *sar1b* (Fig. 5), it was to a lesser extent than that driven by the constitutive Pro_{UBQ10} promoter (Fig. 7), indicating that Sar1b expressed in other cells of the developing anthers also participates in tapetum function. In fact, tapetum hypertrophy was reported for the *rpk2* mutants, in which the middle layer was not differentiated from inner secondary parietal cells (Mizuno et al., 2007), suggesting a connection between different sporophytic cell layers during anther development.

We believe that the distinct yet interchangeable functions of Sar1b and Sar1c in sporophytic control of pollen development are due to different transcription programs. Indeed, significantly elevated expression of *Sar1c* restored the male fertility of *sar1b* (Fig. 7). On the other hand, both *Sar1b* and *Sar1c* showed strong expression in male gametophytes (Fig. 6). Consistently, male gametophytic development requires both Sar1b and Sar1c (Figs. 8 and 9). Microspores of the *sar1b;sar1c* genotype failed to go through pollen mitosis I, resulting in pollen abortion (Fig. 9). Similar male gametophytic defects were known for mutations of a few other regulators mediating vesicular trafficking to or from the Golgi apparatus: other COPII components such as Sec23 (Tanaka et al., 2013), TPLATE components (Van Damme et al., 2006), Adaptor Protein Complex-1 (Wang et al., 2013; Feng et al., 2017a), Dynamin-Related Protein2 (Backues et al., 2010), and the SNARE protein Sec22 (El-Kasmi et al., 2011).

COPII participates in the transport of proteins from ER to Golgi, where they go through different routes to their final destinations. Mutations of the COPII components, as in the case of Sar1s, would in turn affect the targeting of many proteins to extracellular space, to the PM, to the tonoplast, or to other endomembrane organelles. Several PM-associated proteins and secreted peptides have been reported to play key roles in sporophytic control of pollen development, such as several ABCGs (Quilichini et al., 2010; Choi et al., 2014), CLE19 (Wang et al., 2017b), and NPU (Chang et al., 2012). On the other hand, vacuolar ATPases associated with the tonoplast and the trans-Golgi network/early

endosome are critical for male gametophytic development (Dettmer et al., 2005; Strompen et al., 2005). Although it will be difficult, if at all possible, to pinpoint the cargo protein(s) of COPII for which mistargeting contributes to the observed male sterility, our results strongly support a key role of COPII-mediated trafficking in both sporophytic and gametophytic control of pollen development.

MATERIALS AND METHODS

Plant Materials

The T-DNA insertion lines *sar1a* (SALK_048796) and *sar1c* (CS878604/SAIL_1221_F10) were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Arabidopsis (*Arabidopsis thaliana*) Columbia-0 ecotype was used as the wild type. Plants were grown as previously described (Zhou et al., 2013). Stable transgenic plants were selected on one-half-strength Murashige and Skoog medium supplemented with 30 μg mL^{-1} Basta salts (Sigma-Aldrich) or 25 μg mL^{-1} hygromycin (Roche).

PCR, RT-PCR, RNA Extraction, and RT-qPCR

Genotyping PCR of *sar1a* and *sar1c* was performed with the following primer pairs: ZP4300/ZP4301 for *Sar1a*, ZP1/ZP4301 for *sar1a*, ZP5743/ZP4303 for *Sar1c*, and ZP5743/ZP4 for *sar1c*. Genotypes of the *sar1b* mutants were determined by sequencing.

Total RNAs were isolated by using a Qiagen RNeasy plant mini kit according to the manufacturer's instructions. Oligo(dT)-primed cDNAs were synthesized by using SuperScript III reverse transcriptase with on-column DNase digestion (Invitrogen). For RT-PCR analysis of complementation lines, the endogenous or ectopic *Sar1a* and *Sar1c* and ectopic *Sar1b* were amplified with the following primer pairs: ZP4300/ZP7597 for endogenous *Sar1a*, ZP4302/ZP4303 for endogenous *Sar1c*, ZP12/ZP4303 for ectopic *Sar1c*, and ZP1648/ZP930 for *Sar1b*. Primers to amplify *ACTIN2* were as previously described (Zhou et al., 2013).

RT-qPCR was performed with the Bio-Rad CFX96 real-time system using SYBR Green real-time PCR master mix (Toyobo) as previously described (Zhou et al., 2013). Primers used in RT-qPCR analysis of *Sar1a*, *Sar1c*, and *Sar1b* were as previously described (Feng et al., 2017b). Primers for *GAPDH* and *ACTIN2* in RT-qPCR analysis were as previously described (Zhou et al., 2013). All primers are listed in Supplemental Table S1.

DNA Manipulation

The respective promoters for *Sar1a*, *Sar1b*, and *Sar1c* were cloned with the following primer pairs: ZP5320/ZP5321 for Pro_{Sar1a}, ZP5322/ZP5323 for Pro_{Sar1b}, and ZP5324/ZP5325 for Pro_{Sar1c}. Pro_{Sar1a} comprises a 542-bp sequence upstream of the *Sar1a* start codon; Pro_{Sar1b} comprises a 1,580-bp sequence upstream of the *Sar1b* start codon; and Pro_{Sar1c} comprises a 3,003-bp sequence upstream of the *Sar1c* start codon. The promoter sequences were introduced into the destination vector GW:GUS (Zhou et al., 2013) to generate Pro_{Sar1a}:GUS, Pro_{Sar1b}:GUS, or Pro_{Sar1c}:GUS. Pro_{A9} was amplified with ZP10372/ZP10373 as reported previously (Wang et al., 2017a).

Full-length genomic sequences of *Sar1a*, *Sar1b*, and *Sar1c* were cloned by using the following primer pairs: ZP4300/ZP7597 for *Sar1a*, ZP929/ZP930 for *Sar1b*, and ZP4302/ZP7598 for *Sar1c*. Entry vectors were generated in the pENTR/D/TOPO vector (Invitrogen). The entry vectors were used in LR reactions with the destination vector Pro_{UBQ10}:GFP-GW (Feng et al., 2017b) to generate the expression vectors Pro_{UBQ10}:GFP-Sar1a, Pro_{UBQ10}:GFP-Sar1b, and Pro_{UBQ10}:GFP-GW.

The CRISPR construct used to generate the *sar1b* mutants was as previously described (Xing et al., 2014). Briefly, the target site for *Sar1b* was selected using an online bioinformatics tool (http://www.genome.arizona.edu/crispr/CRISPRsearch.html) and was incorporated into forward and reverse PCR primers. The Sar1b-CRISPR cassette was generated by PCR amplification from pCBC-DT1T2 with the primers ZP7138/ZP7139/ZP7140/ZP7141. The PCR products were digested with *Bsa*I and inserted into pHSE401, resulting in pHSE401-Sar1b. Primers are listed in Supplemental Table S1.

PCR amplifications were performed using Phusion hot-start high-fidelity DNA polymerase with the annealing temperature and extension times recommended by the manufacturer (Finnzyme). Entry vectors were sequenced, and sequences were analyzed using VectorNTI (Invitrogen). The Bioneer PCR purification kit and the Bioneer Spin miniprep kit were used for PCR product recovery and plasmid DNA extraction, respectively. All primers are listed in Supplemental Table S1.

Phenotype Analysis

Phenotype analyses of pollen development, including Alexander staining, DAPI staining, or SEM analysis, were performed as previously described (Li et al., 2013; Feng et al., 2016). Semithin sections and TEM analysis of developing anthers were performed as previously described (Xie et al., 2014; Feng et al., 2017a; Zhang et al., 2018). Histochemical GUS analysis was performed as previously described (Li et al., 2013; Feng et al., 2018). TUNEL assays were performed as previously described (Xie et al., 2014). CLSM of developing anthers was performed as previously described (Zhang et al., 2018).

Microscopy

Fluorescent images were captured using a Zeiss LSM 880 CLSM device with a 40/1.3 oil objective. Fluorescence was detected using a 488-nm argon laser/BP 505 to 550 filter for GFP.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: At1g09180 for *Sar1a*, At1g56330 for *Sar1b*, and At4g02080 for *Sar1c*.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Mutations at *Sar1a* or *Sar1c* did not affect plant growth or fertility.

Supplemental Figure S2. Vegetative growth is not affected by *Sar1b* loss of function.

Supplemental Figure S3. Vegetative growth of *sar1b/+;sar1c* is comparable to that of the wild type.

Supplemental Table S1. Oligonucleotides used in this study.

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