COPII components Sar1b and Sar1c play distinct yet interchangeable roles in pollen development

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X. L. performed most of the experiments with the assistance of S.-W.L., L.-M.G.; Y.Z. initiated and supervised the project together with S.L.; Y.Z. and S.L. secured the funding; X.L. and Y.Z. analyzed the data; Y.Z. wrote the article with contributions from all authors.

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**One-sentence summary**
The small GTPase Sar1, components of the COPII complex that mediates vesicular trafficking from ER to Golgi, participate in sporophytic and gametophytic control of pollen development in Arabidopsis, with different isoforms playing distinct or redundant roles.

**Abstract**
The development of pollen is a pre-requisite for double fertilization in angiosperms. COPII mediates anterograde transport of vesicles from the endoplasmic reticulum to Golgi. Components of the COPII complex have been reported to regulate either sporophytic or gametophytic control of pollen development. The Arabidopsis genome encodes five Sar1 isoforms, the small GTPases essential for COPII formation. By using a dominant negative (DN) approach, Sar1 isoforms were proposed to have distinct cargo specificity despite their sequence similarity. Here, we examined the function of three Sar1 isoforms through analysis of 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. The study uncovers the role of Sar1 isoforms in both sporophytic and gametophytic control of pollen development. It also suggests that distinct function of Sar1 isoforms may be caused by their distinct transcription programs.

**Key words:** COPII, anther development, tapetum, male gametophytes, fertility

**Introduction**
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; Zhu et al., 2015), as well as proteins involved in intercellular signaling, such as Receptor-like Protein Kinase 2 (RPK2), NO PRIMEXINE AND PLASMA MEMBRANE UNDULATION (NPU), the secreted small peptide CLAVATA3/ESR-RELATED19 (CLE19), ATP binding cassette (ABC) transporters ABCGs, NADPH oxidases, 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., 2017). 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)P2 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., 2017), 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 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 compromise 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 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., 2017; Ito et al., 2018). Expression of Sar1\textsuperscript{DN} compromised the vesicular transport from ER to Golgi (Takeuchi et al., 2000; Hanton et al., 2008; Zeng et al., 2015; Feng et al., 2017; 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., 2017). The expression of Sar1\textsuperscript{aDN} and Sar1\textsuperscript{bDN} had a significant difference regarding the transport of α-amylase (Hanton et al., 2008); the expression of Sar1\textsuperscript{aDN} and Sar1\textsuperscript{cDN} differed in the transport of a membrane-bound transcription factor (Zeng et al., 2015); the expression of Sar1\textsuperscript{bDN} and Sar1\textsuperscript{cDN} differed in the transport of PAT10 (Feng et al., 2017). Whether the distinction in cargo specificity is the result of functional difference of the isoforms (Zeng et al., 2015) or transcriptional specificities as being reported in mammals (Khoriaty et al., 2018) is 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 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 function 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 Figure 1). Both mutants are null mutants based on transcript analysis. Single mutants or the sar1a; sar1c double mutant grew comparably to wild type in both vegetative and reproductive stages (Supplemental Figure 1). Because there were no valid T-DNA insertion mutants of Sar1b, we generated sar1b mutants by CRISPR/Cas9 (Figure 1A). Two mutant alleles were identified from transformants, sar1b-1 and sar1b-2, both of which expressed mutated Sar1b transcripts with pre-stop codons resulted either from an insertion or from a deletion in the Sar1b coding region (Figure 1A). Vegetative growth of both sar1b mutants was comparable to that of wild type (Supplemental Figure 2). However, siliques of the homozygous sar1b mutants did not elongate (Figure 1C-D) in contrast to those of wild type (Figure 1B, 1E). Siliques of sar1b pollinated using mature wild-type anthers contained a full seed set (Figure 1H,I) unlike those of sar1b (Figure 1F, G), suggesting that sar1b is male sterile. Indeed, by Alexander staining and scanning electron micrograph (SEM) analysis of dehiscing anthers, we determined that mature sar1b anthers did not produce viable pollen (Figure 1K, L, N, O), unlike wild type (Figure 1J, 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 (Figure 2). The specification of anthers was comparable between wild type and sar1b such that both contain epidermis, endothecium, middle layer, tapetum, and microspore mother cells (Figure 2A). At anther developmental stage 6–7, i.e. when tetrads are released in pollen sac (Sanders et al., 1999), sar1b started to differ from wild type (Figure 2A-B). The tapetal cells in sar1b were generally larger than those of wild type, often containing large vacuoles (Figure 2A-B). 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 (Figure 2A-B). By contrast, microspores in sar1b pollen sacs started to degenerate as early as at stage 8–9 (Figure 2A-B). The plasma membrane (PM) of microspores in sar1b pollen sacs was detached from cell wall and at anther dehiscence, sar1b pollen sacs contained only a pile of cellular debris (Figure 2A-B).

To obtain better resolution of pollen development in sar1b anthers, we performed transmission electron micrograph (TEM) analysis. Consistent with the results from sectioning and light microscopy, TEMs of early stage anthers showed that sar1b (Figure 3B) did not differ from wild type (Figure 3A) in cell specification. However, starting from stage 7 when tetrads were formed normally in wild type (Figure 3C), tetrads in sar1b anthers seemed detached from the callose wall that encased them (Figure 3D). Later on, unicellular microspores with reticular pollen coat structures, presumably sporopollenins, were developed in wild type (Figure 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 (Figure 3F). There were also aggregates of electron-dense materials deposited in pollen sacs without attaching to the surface of microspores in sar1b (Figure 3F). Microspores continued to develop from the late unicellular stage (Figure 3G) to the bicellular stage (Figure 3I) and finally to maturation (Figure 3K) with gradual formation of exine at the pollen surface and degeneration of the tapetal layer in wild type. By contrast, microspores in sar1b anthers did not develop a reticular pollen coat structure (Figure 3H,J). Electron-dense materials were deposited between the middle layer and the
Figure 2. Functional loss of Sar1b compromised anther development.
(A-B) Transverse sections (A) or confocal laser scanning microscopic (CLSM) sections
(B) of wild type 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.

During anther dehiscence, instead of producing normal pollen grains as in wild type
(Figure 3K), sar1b anthers contained only cellular debris (Figure 3L). These results
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 programmed cell death (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 support a key role of Sar1b in sporophytic control of pollen development.
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 wild type or in sar1b before the unicellular stage, i.e. anther developmental stage 10 (Figure 4). At stage 10, TUNEL-positive signals appeared in the tapetal layer of wild-type anthers (Figure 4). Anthers of sar1b at the same stage did not show TUNEL-positive signals in the tapetal layer (Figure 4). Instead, TUNEL-positive signals occasionally appeared in developing microspores (Figure 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 wild type (Figure 4), consistent with the following degeneration of vascular cells, septum, and stomium.
At this stage, TUNEL-positive signals appeared in the tapetal layer, vascular cells, and degenerating microspores of *sar1b* anthers (Figure 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 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 *ProAs*, a tapetal-specific promoter (Wang et al., 2017). CLSM of anthers from the *ProAs*:GFP-**Sar1b**;*sar1b* transgenic plants showed clear fluorescence signals in the tapetal layer (Figure 5A,C). Examination of dehiscing anthers by SEM analysis showed the production of pollen grains at the dehiscing anthers of the *ProAs*:GFP-**Sar1b**;*sar1b* plants (Figure 5B,D). These results suggest that tapetal expression of Sar1b at least partially rescues 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 (Figure 6A-C). Among the three *Sar1* isoforms examined, anthers of the *ProSar1b*:GUS plants showed substantially stronger GUS signals in tapetum (Figure 6B) than those of *ProSar1a*:GUS (Figure 6A) or *ProSar1c*:GUS (Figure 6C).

By reverse transcription quantitative PCR (RT-qPCR), we verified the significant transcriptional difference between *Sar1b* and *Sar1c* (Figure 7A). To determine whether the functional distinction of Sar1b was resulted from transcriptional differences, as reported for mammalian Sec23A and Sec23B (Khoriyat et al., 2018), we examined the ability of engineered expression of *Sar1c* versus *Sar1b* to complement the anther developmental defects of *sar1b* by generating *ProUBQ10*:**Sar1b**;*sar1b* and *ProUBQ10*:**Sar1c**;*sar1b* transgenic plants (Figure 7A). Ectopic expression of Sar1c fully restored male fertility of *sar1b* (Figure 7D,E,H,I) comparable to *Sar1b* (Figure 7B,C,F,G) based on alexander staining (Figure 7B-E) and SEM
analyses of dehiscing anthers (Figure 7F-I). Through RT-qPCR analysis, we confirmed that the rescue of sar1b male sterility by Sar1c was consistent with its significantly elevated transcript level (Figure 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.

Sar1b and Sar1c redundantly regulate male gametophytic development

Developing microspores of both ProSar1b:GUS and ProSar1c:GUS plants showed clear GUS signals (Figure 6B-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 four hundreds of the F2 progenies, 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 v.s. 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 in vegetative and reproductive growth (Supplemental Figure 3). By alexander staining (Figure 8A-H,Q), DAPI staining (Figure 8I-L,R), and SEM (Figure 8M-P,S) analyses, we found that approximately 50% pollen produced by the sar1b-1/+;sar1c plants (Figure 8D,H,L,P) were aborted whereas the wild type (Figure 8A,E,I,M) or either single mutant, i.e. sar1b-1/+ (Figure 8B,F,J,N) or sar1c (Figure 8C,G,K,O) contained almost 100% normal pollen grains at maturation (Figure 8Q-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 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 (Figure 9A-B), which differs from that in sar1b (Figure 2 and Figure 3) and consistent with the fact that gametophytic but not sporophytic defect resulted in pollen abortion in sar1b/+;sar1c. Tetrads released in pollen sacs of sar1b/+;sar1c were comparable to those from wild type (Figure 9A-B). Defective microspore development in sar1b/+;sar1c appeared at anther developmental stage 10 (Sanders et al., 1999) when 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 (Figure 9A-B). Later on, wild-type microspores transitioned from bicellular to tricellular mature pollen (Figure 9A-B). By contrast, a portion of microspores in sar1b/+;sar1c pollen sacs showed degenerating cytoplasm and finally collapsed (Figure 9A-B). 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

Figure 9. Functional loss of both Sar1b and Sar1c compromised male gametophytic development. (A-B) Semi-thin transverse sections (A) or CLSM (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 from the sar1b-1/+;sar1c plants. Arrowheads point to defective microspores. Asterisks indicate debris of degenerating microspores. Bars = 20 μm.
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 (Figure 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 (Figure 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 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 cysteine protease whose
maturation requires its delivery to vacuoles presumably from 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
sporophytic male sterility of sar1b (Figure 5), it was to a lesser extent than that
driven by the constitutive ProUBQ10 promoter (Figure 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 receptor-like protein
kinase 2 (rpk2) mutants, in which 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 function of Sar1b and Sar1c in sporophytic control of pollen development is due to different transcription programs. Indeed, significantly elevated expression of Sar1c restored male fertility of sar1b (Figure 7). On the other hand, both Sar1b and Sar1c showed strong expression in male gametophytes (Figure 6). Consistently, male gametophytic development requires both Sar1b and Sar1c (Figure 8; Figure 9). Microspores of the sar1b;sar1c genotype failed to go through pollen mitosis I, resulting in pollen abortion (Figure 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 (AP-1) (Wang et al., 2013; Feng et al., 2017), Dynamin-related protein 2 (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 plasma membrane (PM), to the tonoplast, or 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., 2017), and NPU (Chang et al., 2012). On the other hand, vacuolar ATPases (V-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 mis-targeting 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 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 half-strength MS medium supplemented with 30 µg/ml Basta salts (Sigma-Aldrich) or 25 µg/ml 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 and 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 primer pairs: ZP4300/ZP7597 for endogenous Sar1a, ZP4302/ZP4303 for endogenous Sar1c, ZP12/ZP4303 for ectopic Sar1c, ZP1648/ZP930 for Sar1b. Primers to amplify ACTIN2 were as previously described (Zhou et al., 2013).

RT-qPCR reactions were 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., 2017). Primers for GAPDH and ACTIN2 in RT-qPCR analysis were as previously described (Zhou et al., 2013). All primers are listed in Supplemental Table 1.

**DNA manipulation**

The respective promoters for Sar1a, Sar1b, and Sar1c were cloned with the following primer pairs: ZP5320/ZP5321 for ProSar1a, ZP5322/ZP5323 for ProSar1b, and ZP5324/ZP5325 for ProSar1c. ProSar1a comprises a 542-bp sequence upstream of the Sar1a start codon; ProSar1b comprises a 1,580-bp sequence upstream of the Sar1b
start codon; ProSar1c 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 ProSar1a:GUS, ProSar1b:GUS, or ProSar1c:GUS.
ProAB was amplified with ZP10372/ZP10373 as reported previously (Wang et al.,
2017).

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 ProUBQ10:GFP-GW (Feng et al., 2017) to generate the expression vectors
ProUBQ10:GFP-Sar1a, ProUBQ10:GFP-Sar1b, and ProUBQ10: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/CRI\SPRsearch.html) and was incorporated
into forward and reverse PCR primers. The Sar1b-CRISPR cassette was generated by
PCR amplifications from pCBC-DT1T2 with the primers
ZP7138/ZP7139/ZP7140/ZP7141. The PCR products were digested with BsaI and
inserted into pHSE401, resulting in pHSE401-Sar1b. Primers are listed in
Supplemental Table 1.

Phenotype analysis
Phenotype analysis of pollen development, including Alexander staining, DAPI
staining, or SEM analyses, were performed as previously described (Li et al., 2013;
Feng et al., 2016). Semi-thin sections and TEM analysis of developing anthers were
performed as previously described (Xie et al., 2014; Feng et al., 2017; Zhang et al.,
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 were performed as previously described (Zhang et al., 2018).

**Microscopy**

Fluorescent images were captured using a Zeiss LSM 880 confocal laser scanning microscope (CLSM) with a 40/1.3 oil objective. Fluorescence was detected using a 488-nm argon laser/BP 505–550 filter for GFP.

**Accession numbers**

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

**Supplemental Data**

The following materials are available in the online version of this article.

**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 wild type.

**Supplemental Table S1.** Oligos used in this study.

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**Figure legends**

**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, \textit{sar1b-1} and \textit{sar1b-2}, were the result of one nucleotide deletion or insertion, respectively. (B-D) Inflorescences from a wild-type (B), \textit{sar1b-1} (C), or \textit{sar1b-2} (D) plant. (E-G) A silique from self-pollinated wild-type (E), \textit{sar1b-1} (F), or \textit{sar1b-2} (G) plants. (H-I) A silique from \textit{sar1b-1} (H) or \textit{sar1b-2} (I) pollinated with wild-type pollen. (J-O) Alexander staining (J-L) and SEM (M-O) of dehiscing anthers from wild-type (J, M), \textit{sar1b-1} (K, N), or \textit{sar1b-2} (L, O) plants. Bars = 1 mm for (E-I); 100 μm for (J-O).

**Figure 2.** Functional loss of \textit{Sar1b} compromises anther development.

(A-B) Transverse sections (A) or confocal laser scanning microscopic (CLSM) sections (B) of wild-type versus \textit{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.

**Figure 3.** Functional loss of \textit{Sar1b} compromises anther development.

(A-B) Transmission electron micrographs (TEMs) of wild-type (A) or \textit{sar1b-1} anthers (B) at developmental stage 5–6 showing the presence of different cell layers in the anther locule (Pink: tetrads; blue: tapetum, yellow: middle layer). (C-D) Wild-type (C) or \textit{sar1b-1} (D) anthers at stage 6–7. Arrowhead in (D) points to a tetrad detached from the callose wall. (E-F) Wild-type (C) or \textit{sar1b-1} (D) 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-H) Wild-type (G) or \textit{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 \textit{sar1b-1} microspores (H). (I-J) Wild-type (I) or \textit{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-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 for (A, B, G, H, K, L); 5 μm for (C-F, I, K); 1 μm for (F inset).

**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, 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.

**Figure 5.** Tapetal expression of Sar1b partially complemented anther defects of sar1b. (A-D) CLSM (A, C) or SEM (B, D) of a maturing anther from ProA9:GFP-Sar1b#1;sar1b-1 (A, B) or ProA9:GFP-Sar1b#2;sar1b-1 (C, D) plants. CLSM of the two lines were taken with the same settings. Bars = 50 μm.

**Figure 6.** The expression of Sar1 isoforms in developing anthers. (A-C) Representative histochemical GUS staining of anther transverse sections from ProSar1a:GUS (A), ProSar1b:GUS (B), or ProSar1c:GUS (C) transgenic plants. Bars = 20 μm.

**Figure 7.** Ectopic expression of Sar1c suppresses male sterility of sar1b. (A) Transcript abundance of Sar1b or Sar1c in wild type (WT), sar1b, two lines of ProUBQ10:GFP-Sar1b; sar1b-2 (Sar1b; sar1b), or two lines of ProUBQ10: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-E) Alexander staining of a
mature anther from two lines of ProUBQ10::GFP-Sar1b; sar1b-2 (B-C) or two lines of
ProUBQ10::GFP-Sar1c; sar1b-2 (D-E). (F-I) SEMs of a dehiscing anther from two lines of
ProUBQ10::GFP-Sar1b; sar1b-2 (F-G) or two lines of ProUBQ10::GFP-Sar1c; sar1b-2 (H-I).
Bars = 100 μm for (B-E); 30 μm for (F-I).

Figure 8. Functional loss of both Sar1b and Sar1c resulted in defective male
gametophytic development.
(A-H) Alexander staining of a mature anther (A-D) or dehiscing anther (E-H) from wild
type (A, E), sar1b-1/+ (B, F), sar1c (C, G), or sar1b-1/+; sar1c (D, H). (I-L) DAPI staining
of mature pollen grains from wild type (I), sar1b-1/+ (J), sar1c (K), or sar1b-1/+; sar1c
(L). (M-P) SEMs of mature pollen grains from wild type (M), sar1b-1/+ (N), sar1c (O),
or sar1b-1/+; sar1c (P). Aborted pollen grains are highlighted in green in (L, P). (Q-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 for (A-D); 50 μm for (E-P).

Figure 9. Functional loss of both Sar1b and Sar1c compromised male gametophytic
development.
(A-B) Semi-thin transverse sections (A) or CLSM (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
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