ATP-Binding Cassette Transporter G26 Is Required for Male Fertility and Pollen Exine Formation in Arabidopsis

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The highly resistant biopolymer, sporopollenin, gives the outer wall (exine) of spores and pollen grains their unparalleled strength, shielding these structures from terrestrial stresses. Despite a limited understanding of the composition of sporopollenin, it appears that the synthesis of sporopollenin occurs in the tapetum and requires the transport of one or more sporopollenin constituents to the surface of developing microspores. Here, we describe ABCG26, a member of the ATP-binding cassette (ABC) transporter superfamily, which is required for pollen exine formation in Arabidopsis (Arabidopsis thaliana). abcg26 mutants are severely reduced in fertility, with most siliques failing to produce seeds by self-fertilization and mature anthers failing to release pollen. Transmission electron microscopy analyses revealed an absence of an exine wall on abcg26-1 mutant microspores. Phenotypic abnormalities in pollen wall formation were first apparent in early uninucleate microspores as a lack of exine formation and sporopollenin deposition. Additionally, the highest levels of ABCG26 mRNA were in the tapetum, during early pollen wall formation, sporopollenin biosynthesis, and sporopollenin deposition. Accumulations resembling the trilamellar lipidic coils in the abcg11 and abcg12 mutants defective in cuticular wax export were observed in the anther locules of abcg26 mutants. A yellow fluorescent protein-ABCG26 protein was localized to the endoplasmic reticulum and plasma membrane. Our results show that ABCG26 plays a critical role in exine formation and pollen development and are consistent with a model by which ABCG26 transports sporopollenin precursors across the tapetum plasma membrane into the locule for polymerization on developing microspore walls.

The central importance of spores in plant reproduction and the inherent stresses of a terrestrial environment have led to the evolution of a toughened wall surrounding the male gametophyte in angiosperms and gymnosperms and haploid spores in nonflowering plants. Sporopollenin, a structurally robust biopolymer, gives the outer walls of spores and pollen their unparalleled strength and resistance to terrestrial stresses (Scott, 1994).

The composition of sporopollenin is thought to include phenolics and polyhydroxylated unbranched aliphatics, coupled by ester and ether linkages, which provide this biopolymer with its characteristic resistance to chemical degradation (Guilford et al., 1988; Wiermann et al., 2001; Scott et al., 2004). Through the application of solid-state NMR, aromatic, aliphatic, and oxygen functionalities have been identified in the sporopollenin biopolymer (Hemsley et al., 1994; Ahlers et al., 1999, 2000, 2003). However, despite the technologies available, our knowledge of the polymer structure of sporopollenin is far from complete.

Investigation of anther and microspore development by light microscopy and transmission electron microscopy (TEM) has improved our knowledge of pollen wall formation and provided a context for understanding the mechanism of sporopollenin formation. In Arabidopsis (Arabidopsis thaliana) anther development, 14 stages can be distinguished based on distinct cellular events (Sanders et al., 1999). In stages 1 through 4, archesporial cells of the anther primordia divide periclinally to produce inner sporogenous cells and outer parietal cells, from which all anther cell types differentiate: endothecium, middle layer, tapetum, and microspore mother cells. Microspore mother cell meiosis produces haploid microspore tetrads, which are encased in callose (β-1,3-glucan; Dong et al., 2005; Nishikawa et al., 2005). At the tetrad stage (stage 7), a microspore-derived matrix of polysaccharides known as the primexine forms between the microspore and surrounding callose, followed by the emergence of probaculae, which serve as a scaffold for sporopollenin deposition (Suzuki et al., 2008; Wilson and Zhang, 2009). After the release of microspores from callose-encased tetrads in stage 8, the sporopollenin-based exine wall forms by the deposition of sporopollenin generated in the tapetum, producing the sculptured baculae and tecta of the exine. During...
the continued maturation of microspores into tricellular pollen grains, a cellulosic and pectin-rich intine forms between the exine and microspore plasma membrane (Blackmore et al., 2007). The final component of the pollen wall, the waxy pollen coat or trypine, is deposited on and within the crevices of the mature pollen grain exine in the late stages of pollen development, coinciding with tapetum degeneration in stage 10 (Blackmore et al., 2007; Grienengerber et al., 2009).

The production of viable pollen requires precise spatial and temporal coordination of both gametophytic and sporophytic developmental and metabolic events (Blackmore et al., 2007). The sporophytic tapetum, a single cell layer encasing the anther locale, plays key functions in pollen development. It supplies nutrients, structural components, and enzymes essential to the survival and development of microspores (Blackmore et al., 2007). Specifically, the tapetum functions in the production and secretion of structural components, including sporopollenin precursors to early uninucleate microspores, and later as the source of waxy pollen coat exine sporopollenin precursors to early uninucleate microspores, suggesting that these genes are involved in sporopollenin biosynthesis in pollen wall development, three ABC transporters are required for normal male fertility, exine formation, and pollen maturation and that ABCG26 is expressed in the tapetum at the time of exine formation.

Analyses of male-sterile mutants defective in pollen wall formation, primarily in the model plant Arabidopsis, have revealed genes required for normal sporopollenin biosynthesis or deposition, including MALE STERILE2 (MS2), DEFECTIVE IN EXINE PATTERNING1 (DEX1), DEFECTIVE IN EXINE PATTERNING2 (DEX2/CYP703A2), FACELESS POLLEN1 (FLPI/WAX2/YRE/CER3), NO EXINE FORMATION1 (NEF1), TRANSIENT DEFECTIVE EXINE (TDE1), ACYL-COA SYNTHETASE5 (ACOS5), DIHYDROFLAVONOL 4-REDUCTASE-LIKE1 (DR1L), CYP704B1, LESS ADHERENT POLLEN3 (LAP3), and CALLOSE SYNTHASE5 (CALSS5/LAP1; Aarts et al., 1997; Paxson-Sowders et al., 2001; Arizumi et al., 2003, 2004, 2008; Dong et al., 2005; Nishikawa et al., 2005; Morant et al., 2007; Rowland et al., 2007; de Azevedo Souza et al., 2009; Dobrisa et al., 2009a, 2009b; Tang et al., 2009). The expression of MS2, ACOS5, CYP703A2, CYP704B1, and DR1 in the tapetum at the time of exine formation, together with acetylation-sensitive exine or the apparent absence of exine on developing microspores, suggest that these genes are involved in sporopollenin biosynthesis (Aarts et al., 1997; Morant et al., 2007; de Azevedo Souza et al., 2009; Dobrisa et al., 2009b; Tang et al., 2009). These mutants have provided clues regarding the biosynthesis of sporopollenin and have defined a key role for the tapetum as the site of sporopollenin biosynthesis in pollen wall formation.

Based on their putative roles in the export of extracellular polymer components, such as cutin precursors (for review, see Bird, 2008), we hypothesized that ATP-binding cassette (ABC) transporters could be required for sporopollenin export from the tapetum to the developing microspore. ABC transporters represent one of the largest protein superfamilies, known primarily for their function in substrate translocation across membranes (Theodoulou, 2000; Higgins, 2001). A wide range of transport capabilities have been discovered for plant ABC transporters, including ion, lipid, hormone, secondary metabolite, xenobiotic, and peptide transport (Theodoulou, 2000; Rea, 2007). In Arabidopsis, functions have been reported for four of the 28 ABC transporters in the G subfamily of half-size transporters: AtABCG12/WBC12/CER5 and AtABCG11/WBC11/COF1 are required for lipid export to the cuticle (Pighin et al., 2004; Bird et al., 2007; Panikashvili et al., 2007; Ukitsu et al., 2007), AtABCG19/WBC19 overexpression increases antibiotic resistance (Mentewab and Stewart, 2005), and AtABCG25 is involved in abscisic acid transport (Kuromori et al., 2010).

In this study, we identified a gene encoding an ABC transporter, ABCG26, that is tightly coexpressed with genes required for sporopollenin biosynthesis and is highly expressed in the early flower bud, supporting a role for this gene in pollen wall formation. We examined the function of ABCG26 in pollen wall development using reverse genetics and found that ABCG26 is required for normal male fertility, exine formation, and pollen maturation and that ABCG26 is expressed in the tapetum at the time of exine formation.

RESULTS

ABCG26 Is Required for Male Fertility

To test the hypothesis that ABC transporters are required for the export of sporopollenin precursors or other constituents of the exine from the tapetum to developing microspores, it was necessary to identify candidate ABC transporters out of the over 120 annotated ABC transporter genes in the Arabidopsis genome (Verrier et al., 2008). Since ACOS5 is preferentially expressed in the tapetum and encodes an acyl-CoA synthetase required for sporopollenin biosynthesis (de Azevedo Souza et al., 2009), this gene was used for coexpression analysis to identify potential Arabidopsis transporters on the PRIME database (prime.psc.riken.jp/?action=coexpression_index). The Arabidopsis ABCG26 (formerly WBC27) gene At3g13220 shows a high coefficient of coexpression in the tissue and development version 1 data set ($r^2 = 0.95$), along with several other genes involved in sporopollenin biosynthesis (de Azevedo Souza et al., 2009). ABCG26 encodes a predicted half-size ABC transporter in the G subfamily, formerly called the White-Brown Complex subfamily. The highly conserved protein architecture of ABCG26 is typical of this subfamily, where dimerization of two half-transporters produces a functional transport unit with two transmembrane domains and two ABC domains (Higgins, 2001; Verrier et al., 2008).

To test a role for ABCG26 in male fertility and pollen development, three ABCG26 T-DNA insertion lines
(SALK_062317, SAIL_318_B09, and SAIL_885_F06, named abcg26-1, abcg26-2, and abcg26-3, respectively) were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003). PCR genotyping identified plants homozygous for each ABCG26 T-DNA insertion, and sequencing confirmed insertion in the fifth exon (abcg26-1) or the 5′ untranslated region (abcg26-2, abcg26-3) of ABCG26 (Fig. 1A). Plants homozygous for each of the mutant alleles exhibited highly similar mutant phenotypes of severe impairment in male fertility (Supplemental Fig. S1), and the abcg26-1 mutant was used for further experiments.

Reverse transcription (RT)-PCR analysis revealed a strong reduction of ABCG26 mRNA in the abcg26-1 mutant relative to the wild type, suggesting that abcg26-1 is a loss-of-function allele of ABCG26 (Fig. 1B). In homozygous abcg26-1 mutant plants, most siliques failed to mature and produce seeds (Fig. 1C), and most abcg26-1 anthers appeared brown, dry, and lacked any obvious pollen grains (Fig. 1E). However, occasional abcg26-1 anthers with bulbous yellow surfaces consistent with pollen that was not shed were identified, and occasional fully fertile siliques were observed, suggesting a low rate of fertile pollen formation. Wild-type pollen applied to homozygous abcg26-1 stigmas on flowers with brown, dry anthers produced fertile progeny, demonstrating normal female fertility and strong male sterility of abcg26-1. No other morphological differences between mature wild-type and abcg26-1 plants were observed, except that abcg26-1 mutant plants flowered for a longer time and grew taller (50 ± 7 cm at maturity [mean ± SD; n = 12 plants] versus 29 ± 2 cm at maturity for the wild type [n = 12 plants]; Fig. 1C). Growth under elevated humidity did not recover fertility in abcg26-1 plants.

To confirm that the observed male-sterile phenotype is caused by the abcg26-1 T-DNA insertion mutation, we cloned ABCG26 cDNA from developing flower buds in order to complement the abcg26-1 mutant with a wild-type copy of ABCG26. However, during the isolation of the expected annotated ABCG26 cDNA, a second cDNA transcript, ABCG26.2 (At3g13220.2), was found, and sequencing revealed that it corresponded to an alternatively spliced ABCG26 variant resulting from the retention of intron 7 in the transcript (Fig. 2A). The retention of intron 7, which encodes multiple in-frame stop codons, is predicted to produce a truncated protein if it were translated.

Constructs containing either the major or minor splice variant of ABCG26 cDNA driven by the cauliflower mosaic virus 35S promoter were generated and transformed into the homozygous abcg26-1 mutant line. The presence of each construct, as well as the T-DNA insertion in the T1 progeny of homozygous abcg26-1 mutant plants, was confirmed by PCR. Figure 3 shows the fertility phenotypes of T1 progeny relative to those of wild-type ecotype Columbia (Col-0) and abcg26-1 plants, assayed by silique development and seed set. Although occasional fertile siliques were observed in abcg26-1 mutant plants, very few fully developed siliques were observed, and clear fertility differences were observed between multiple mutant and wild-type individuals (85% of siliques on wild-type plants were classified as large, indicative of full fertility, whereas 1% of siliques on abcg26-1 plants were large; Fig. 3). When assayed in a similar manner, T1 progeny from multiple lines expressing cDNA for

![Figure 1. Identification of Arabidopsis ABCG26 T-DNA insertion alleles. A, Diagrammatic representation of ABCG26 with T-DNA insertions in exon 5 (abcg26-1, SALK_062317) and the 5′ untranslated region (abcg26-2, SAIL_318_B09; abcg26-3, SAIL_885_F06). Black boxes represent exons, and the positions of T-DNA insertions are indicated by triangles. Arrowheads labeled P8 to P11 represent primer positions used in B. B, RT-PCR detection of ABCG26 transcripts in wild-type (Col-0) and abcg26-1 flowers. ACTIN2 expression was monitored as a control. C, Mature wild-type (left) and abcg26-1 mutant (right) plant morphology. Terminal siliques are shown (insets). D and E, Mature wild-type and abcg26-1 mutant flowers, respectively. Bars = 200 μm.](https://example.com/figure1.png)
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Figure 2. Flower preferred expression of ABCG26 splice variants. A, Diagrammatic representation of ABCG26 splice variants ABCG26.1 (At3g13220.1) and ABCG26.2 (At3g13220.2). Boxes and lines represent exons and introns, respectively. B, Expression of ABCG26 splice variants in various organs, assayed by quantitative RT-PCR. Expressed levels in the measured plant organs are shown as fold change relative to ABCG26.1 flower expression (set at 100) and were normalized using ACTIN2 as a reference gene. FLWR, Flower; 7D, 7-d-old seedling; YS, young stem; YL, young leaf; MS, mature stem; ML, mature leaf; MR, mature root. Error bars represent SD of three technical replicates; biological replication gave similar results.

To determine whether ABCG26 is expressed in sporophytic or gametophytic tissues, we used in situ hybridization. Figure 4 shows that ABCG26 transcripts were transiently localized to tapetum cells in developing anthers. Tapetum-localized expression was first observed at stage 6 (Sanders et al., 1999), when microspore mother cells became apparent in locules. Maximum expression was observed at stages 7 and 8, corresponding to tetrad formation and free microspore release, respectively, and expression declined at stages 9 and 10 and disappeared by stage 11 in conjunction with tapetum degeneration. Relative to the sense RNA hybridization controls (shown for stages 8 and 11; Fig. 4), some ABCG26 signal also appeared in developing microspores. The timing of tapetum-localized ABCG26 expression is similar to that of ACS5, which encodes a key enzyme in sporopollenin biosynthesis, and coincides with the onset of exine formation and sporopollenin biosynthesis (de Azevedo Souza et al., 2009), suggesting that ABCG26 could be involved in this process.

Microspore Development Is Impaired in abcg26-1

To identify the stage at which pollen development is affected in the abcg26-1 mutant, we used light microscopy to compare anther and microspore development in wild-type (Col-0) and abcg26-1 flower buds using the stages defined by Sanders et al. (1999; Fig. 5). In all early stages, including the completion of meiosis in stage 7, both wild-type and abcg26-1 microspore tetrads were encased by callose and appeared normal (Fig. 5, A and B). However, by stage 9, characterized by the release of free microspores and formation of the exine wall, abcg26-1 anthers differed significantly from the wild type. Figure 5D shows that free microspores in abcg26-1 exhibited signs of incipient degeneration. In stages 10 to 11, the degeneration of the tapetum and formation of tricellular mature pollen proceeded in wild-type anthers (Fig. 5E), while pollen grains in the abcg26-1 mutant generally lacked cytoplasm and exhibited misshapen morphologies (Fig. 5F). By stage 12, all abcg26-1 mutant anthers observed in multiple sections were devoid of pollen grains, while abundant mature pollen was evident in wild-type anthers (Fig. 5, G and H). Despite impaired microspore development in abcg26-1, no additional changes in anther morphology were observed in the tapetum or integuments, and growth and expansion proceeded as in wild-type plants. These data suggest that impaired microspore...
development occurs in the abcg26-1 mutant at the developmental stage, when sporopollenin deposition and exine formation normally occur.

Pollen Wall and Tapetum Development in abcg26-1

The observed degeneration of microspores at the time of pollen wall formation indicated that defects in pollen wall and exine formation could be the primary cause of pollen abortion and male infertility in abcg26-1 mutants. To examine pollen wall and tapetum morphology at higher resolution in the abcg26-1 mutant relative to wild-type plants, scanning electron microscopy (SEM) and TEM were used. The surface of wild-type pollen, viewed with SEM, exhibited a characteristic reticulate exine (Fig. 6). In contrast, SEM revealed that abcg26-1 anthers occasionally released small amounts of pollen or pollen-like structures with smooth surfaces and collapsed morphology (Fig. 6B). This loss of normal pollen cell wall morphology and collapse of the microspores suggests a severe pollen wall defect, but since occasional fully fertile siliques were observed in the mutant, low numbers of fertile pollen grains were likely formed.

Since the sporopollenin-based exine of pollen grains is deposited by stage 10 of anther development, the cell structure of the developing anthers and tapetum was examined using TEM focusing on this stage. Uninucleate microspores of chemically fixed abcg26-1 anthers at stage 10 exhibited thin walls and apparently defective exine deposition, as compared with wild-type microspores that had pollen walls with a typical thick reticulate exine (Fig. 6, C and D). At higher magnification, tectum and bacula structures of wild-type exine deposited on the underlying nexine I were obvious in wild-type microspores (Fig. 6E). In contrast, walls of abcg26-1 microspores were devoid of baculae and tectum, containing instead a thin, amorphous defective wall layer (Fig. 6F). No differences in the tapetum of abcg26-1 anthers compared with wild-type
tapetum cells at the equivalent developmental stages were observed in chemically fixed material. Interestingly, coil-shaped electron-dense inclusions were consistently observed in abcg26-1 locules but were never observed in the locules of wild-type anthers (Fig. 6, E and F).

From light microscopy analyses, pollen development showed signs of disruption in abcg26-1 early free uninucleate microspores, in coordination with the onset of exine formation. In the images obtained after chemical fixation, however, a clearly delineated primexine was not observed in wild-type plants. To improve the ultrastructural preservation of microspores and facilitate high-resolution imaging of both primexine and exine analysis in developing wild-type microspores, we used high-pressure freezing (HPF) and freeze substitution to preserve stamens during anther development. At stage 5, both wild-type and abcg26-1 mutant microspore mother cells were surrounded by a uniform matrix between the microspore primary cell wall and the plasma membrane (Fig. 7, A and B). By stage 7, tetrads encased in callose were present in both the wild-type and abcg26-1 and appeared identical, showing primexine and probacula deposition between the plasma membrane and callose wall (Fig. 7, C and D). Shortly after their release from tetrads at stage 8, however, abcg26-1 early uninucleate microspores failed to develop well-defined baculae, which were obvious in wild-type microspores at the same stage (Fig. 7, E and F). The nexine I layer in wild-type and abcg26-1 mutant anthers was observed as a faint electron-translucent layer immediately outside the plasma membrane in these HPF-fixed specimens (Fig. 7, E–F). At the late unicellular stage (stage 9), wild-type microspores had a nearly complete pollen wall composed of nexine I, bacula, and tectum (Fig. 7G). However, abcg26-1 microspores at the same stage lacked any defined bacula and tectum structure (Fig. 7H); instead, ill-defined electron-dense deposits were observed on the surface surrounding the nexine I layer. The absence of defects in primexine but visible defects in exine formation in the abcg26-1 mutant demonstrate that contributions of primexine from the microspore were not affected by ABCG26 mutation, while the deposition of tapetum-derived sporopollenin was severely affected.

No differences in abcg26-1 tapetum cell ultrastructure, relative to wild-type tapetum cells, were observed in TEM samples (Supplemental Fig. S3). In stages 5 through 9, wild-type and abcg26-1 tapetum cells were binucleate, cytoplasmically dense with numerous vesicles, and contained no inclusions. However, as initially observed in TEM micrographs of chemically fixed anthers (Fig. 6, D and F), HPF-fixed abcg26-1 anther locules after stage 7 (tetrade stage) consistently contained electron-dense inclusions (commonly between one and 10 inclusions per locule per section) that were never observed in wild-type anther locules (Fig. 8). These structures were often in the shape of rings or flattened rings (Fig. 8, A–C) and at higher magnification (Fig. 8, D–F) appeared as coils with two laminar surfaces.

YFP:ABCG26 Is Localized at the Plasma Membrane

To test the subcellular localization of ABCG26, we generated a yellow fluorescent protein (YFP):ABCG26.1 N-terminal fusion under the control of a double 35S promoter and used an Arabidopsis protoplast transient expression system to monitor YFP localization. Such N-terminal fusions to ABCG transporters have been shown to retain activity (Pighin et al., 2004; Bird et al., 2007). Observation of multiple protoplasts expressing the construct showed differing levels of transgene expression, as monitored by YFP fluorescence (Fig. 9). In protoplasts expressing YFP at low levels, fluorescence was localized to the periphery of protoplasts (Fig. 9B), where it colocalized with FM4-64, a styrl dye known to insert in the plasma membrane (Bolte et al., 2004; Fig. 9, C and D). In transformed protoplasts that expressed the YFP at higher levels, fluorescence was also observed at the nuclear envelope and in trans-
vacuolar strands, in a pattern consistent with localization of some of the protein in the endoplasmic reticulum (Fig. 9, E and F). When combined with the in situ hybridization data, the predominantly plasma membrane localization suggests that ABCG26 functions at the plasma membrane of tapetum cells.

**DISCUSSION**

In this study, we identified a gene encoding an ABC transporter, ABCG26, that is required for normal male fertility and pollen development in Arabidopsis. Recently, Xu et al. (2010) reported that the ABORTED MICROSPORES (AMS) basic helix-loop-helix transcription factor is required for microspore and tapetum development and used microarray analysis of an *ams* mutant to identify ABCG26 (formerly WBC27) as an AMS downstream target. In agreement with our findings, Xu et al. (2010) reported that the ABCG26 mutant (*abcg26-1* allele) exhibits altered male fertility. Here, we explored the underlying mechanism of decreased male fertility in *abcg26* loss-of-function mutants by following pollen development from the callose-encased tetrad stage to the onset of dramatic defective exine formation following microspore release. In concert with the absence of an exine on the surface of *abcg26-1* free microspores, mature anthers were nearly devoid of mature pollen grains, and the reduced male fertility phenotype was confirmed quan-

**Figure 5.** Microspore development in wild-type (Col-0) and *abcg26-1* mutant anthers. Anther developmental stages are according to Sanders et al. (1999). Images in the left panels (A, C, E, and G) show wild-type anthers and those in the right panels (B, D, F, and H) show *abcg26-1* anthers. A and B, Microspore development appeared identical in stage 7 wild-type and *abcg26-1* anthers, when a callose wall encases tetrads of microspores. C and D, Following the release of free microspores from tetrads at stage 8, *abcg26-1* microspores showed signs of degradation not observed in wild-type microspores. E and F, In stages 10 and 11, characterized by tapetum degeneration, severe abnormalities in *abcg26-1* microspores were visible in comparison with wild-type microspores. G and H, At stage 12, pollen grains were not observed in *abcg26-1* mutant anthers, whereas tricellular pollen grains were present in wild-type anthers. Bars = 50 μm.

**Figure 6.** Pollen wall structure in wild-type (Col-0) and *abcg26-1* mutant plants. A and B, Scanning electron micrographs of pollen from wild-type (A) and *abcg26-1* mutant (B) plants. C to F, Transmission electron micrographs of chemically fixed sections taken from wild-type (C and E) and *abcg26-1* mutant (D and F) anthers between stages 9 and 10 of anther development (late unicellular stage). C and D, Low-magnification images showing tapetum cells, locules, and free microspores. E and F, High-magnification images showing pollen wall ultrastructure and locular inclusions (unlabeled arrows), which were only observed in the *abcg26-1* mutant. Ba, Bacula; DEx, defective exine; Ex, exine; Lo, locule; M, microspore; Ne I, nexine I; T, tapetum; Te, tectum. Bars = 5 μm (A–D) and 100 nm (E and F).
The transient tapetum-localized expression of ABCG26 during postmeiotic stages of pollen development, together with the tight coexpression of ABCG26 with genes required for sporopollenin biosynthesis, demonstrate that ABCG26 is required for exine formation on the microspore surface and suggest that ABCG26 could be involved in sporopollenin precursor export from tapetum cells into anther locules. This work further highlights the critical role played by the exine in microspore and pollen function, since the profound exine defects observed in the abcg26-1 mutant correlate with severely compromised pollen grain development and male fertility.

A number of Arabidopsis mutants that exhibit varying degrees of fertility and impaired sporopollenin biosynthesis and defective exine formation have been described (Aarts et al., 1997; Ariizumi et al., 2003; Morant et al., 2007; de Azevedo Souza et al., 2009; Dobritsa et al., 2009b; Tang et al., 2009). ABCG26 is unique as the only transporter described thus far and differs from previously characterized defective exine mutants that affect sporopollenin biosynthetic enzymes or transcription factors required to coordinate tapetum developmental and biosynthetic pathways. Thus, our analysis of ABCG26 function provides new insights into the mechanisms underlying pollen wall deposition.

The abcg26-1 Mutant Has Partial Arabidopsis Male Fertility

In the abcg26-1 mutant, fertility was strongly reduced: most anthers failed to produce mature pollen and appeared brown and dry (Fig. 1, C and E) and most siliques failed to produce seeds (Fig. 3A). However, partial fertility occasionally arose late in development, visible as seed-containing siliques (Fig. 3A). Occasional abcg26-1 anthers were observed that did not produce visible pollen but exhibited bulbous surface protrusions, suggesting the production of pollen grains unable to disperse from the anther. When viewed by SEM, pollen-like structures with smooth surfaces were observed in some manually dissected abcg26-1 anthers, consistent with the hypothesis that some pollen grains with thin defective exine survive to maturity in some mature abcg26-1 anthers (Fig. 6). It is possible that sufficient defective exine is present on the surface of such developing microspores to permit pollen grain maturation.

Similar low levels of fertility are reported for the ms2 and cyp703a2 mutants that exhibit defective exine and sporopollenin deposition (Aarts et al., 1997; Morant et al., 2007), while acos5 and drl1 mutants are completely male sterile (de Azevedo Souza et al., 2009; Tang et al., 2009). The reasons underlying this variation in fertility among mutants with apparently similar exine defects remain unclear, but it could result from the chemically distinct composition of the defective exine.

Function of ABCG26 in the Tapetum

As microspores progress through defined stages of development, the formation of the pollen wall requires contributions from the gametophytic and sporophytic
anther tissues (Blackmore et al., 2007). In situ hybridization revealed ABCG26 expression in the tapetum and microspore at stages of microspore development associated with the synthesis and deposition of the pollen exine, specifically at the tetrad and early uninucleate stages. Supplemental Figure S4 illustrates this pattern of expression relative to other events in microspore and pollen development, which closely resembles the tapetum-specific expression of a number of genes encoding enzymes and proteins required for sporopollenin biosynthesis (Aarts et al., 1997; Morant et al., 2007; de Azevedo Souza et al., 2009; Dobritsa et al., 2009b). This pattern is consistent with a role for ABCG26 in the export of sporopollenin precursors or other components required for exine formation, rather than of other pollen wall components, from the tapetum. The detection of ABCG26 transcript in tetrad and early uninucleate microspores indicates that an additional role for ABCG26 in the export of primexine components from developing microspores is possible. However, the lack of a detectable primexine phenotype in abcg26-1 mutants suggests that the primary function of AGBC26 is not in the gametophyte.

The dual expression of ABCG26 primarily in the tapetum at the time of sporopollenin biosynthesis as well as expression in the developing microspores at the time of primexine formation led us to examine the function of ABCG26 in these stages of development by thorough phenotypic analysis of the abcg26-1 mutant. Using light and electron microscopy, signs of microspore degeneration and reduced pollen wall staining were first visible in abcg26-1 free microspores (stage 8), in contrast to wild-type microspores at the equivalent stage. At later stages of anther development, more pronounced defects in microspore development in the mutant were obvious, and by stage 12, pollen grains were absent in the abcg26-1 mutant. These data suggest that microspore development failed in the abcg26-1 mutant as a result of defective pollen wall formation.

Previous studies have demonstrated the improved ultrastructural preservation and elimination of chemical fixation artifacts in tissues preserved by HPF followed by freeze substitution (Kiss and Staehelin, 1995; Paxson-Sowders et al., 2001). We used this technique to examine the ultrastructure of abcg26-1 pollen at multiple stages of pollen development in anthers so that the precise appearance of pollen wall defects could be determined. When HPF-fixed anthers were examined using TEM, it became clear that the abcg26-1 loss-of-function mutant had normal primexine but displayed defects in exine formation that were first apparent in early uninucleate microspores (Fig. 7). The absence of defective primexine on the abcg26-1 mutant tetrads suggests that the function, if any, of ABCG26 in the gametophytic generation is not in primexine formation. The first pollen wall defects observed were in exine wall formation on early uninucleate microspores, suggesting that microspore development failed due to the lack of sporophytic contributions from the tapetum (Fig. 7, F and H). The similarity of the abcg26-1 mutant phenotype to sporopollenin biosynthetic mutants, such as ms2, cyp703a2, acos5, and drt1 (Aarts et al., 1997; Morant et al., 2007; de Azevedo Souza et al., 2009; Tang et al., 2009), is consistent with a function for ABCG26 in the export of sporopollenin or other exine components from the tapetum.

The preferential localization of YFP:ABCG26 to the plasma membrane (Fig. 9) further supports a role for ABCG26 in the export of sporopollenin or other components required for exine formation from tapetum cells into the locule. Endoplasmic reticulum localization observed for YFP:ABCG26 in some protoplasts could be due to high levels of transgene expression, resulting in an overabundance of ABCG26 and leading to impaired trafficking to the plasma membrane.
ternatively, ABCG26 trafficking to the plasma membrane could require a partner ABCG, as demonstrated for the human ABCG5/ABCG8 heterodimer sterol transporter, where one half-transporter partner is retained in the endoplasmic reticulum when its partner is mutated (Graf et al., 2003).

Locular Inclusions in abc26-1

Since ABCG26 transporter activity appears to be required for the export of tapetum-derived sporopollenin or other components required for exine formation, we examined the tapetum of the abc26-1 mutant for signs of accumulation due to such defective export. No differences in the structure of the tapetum in abc26-1 anthers were observed when compared with wild-type tapetum cells at the equivalent developmental stages in chemically fixed and HPF-fixed specimens (Supplemental Fig. S3). This suggests that, if the absence of ABCG26 transport results in the accumulation of exine components in the tapetum, such components are either not visible by electron microscopy or were extracted in the fixation process. It is also possible that such components do not accumulate due to rapid catabolic breakdown in tapetum cells. Unexpectedly, however, abc26-1 locules after the tetrad stage contained coiled inclusions observed in both chemically fixed (Fig. 8, A and D) and HPF-fixed (Fig. 8, B, C, E, and F) tissue.

Sporopollenin-like accumulations have been previously described in locules of Arabidopsis pollen development mutants, including dex1, nef1, tde1, and cal5 (Paxson-Sowders et al., 2001; Ariizumi et al., 2004, 2008; Dong et al., 2005; Nishikawa et al., 2005). The accumulations in all of these mutants except abc26-1 appear as homogenous, electron-dense globular aggregations, interpreted as sporopollenin-like debris. The unique coiled structure of the abc26-1 locule accumulations suggests that they differ in composition from previously described material. One possibility is that abc26-1 locule accumulations represent a component of the pollen exine that normally copolymerizes with sporopollenin constituents that may be exported by ABCG26 but fails to do so in the absence of ABCG26-exported constituents in abc26-1 mutant locules. Although the composition of the coiled locular inclusions observed in abc26-1 is unknown, they resemble the trilamellar lipidic inclusions observed in the cytoplasm of mutant stem epidermal cells of two previously characterized ABC transporter mutants, abc11 and abc12, defective in cuticular wax export (Pighin et al., 2004; Bird et al., 2007).

Potential ABCG26 Substrates

Defects in abc26-1 microspores are in the structural component of the exine, primarily composed of sporopollenin. Based on a number of chemical analyses, sporopollenin is thought to be a copolymer of simple phenolic compounds and polyhydroxylated unbranched aliphatics, coupled by ester and ether linkages, which provide this biopolymer with its characteristic resistance to chemical degradation (Guilford et al., 1988; Wiermann et al., 2001; Scott et al., 2004). The lack of knowledge regarding the specific constituents of sporopollenin poses challenges in hypothesizing the chemical nature of the cargo that could be transported by ABCG26 from the tapetum into the locule. Recently, genetic analyses have identified a small number of genes encoding enzymes required for the biosynthesis of sporopollenin. Among these, Ms2, CYP703A2, DRL1, ACOS5, and CYP704B2 appear to be involved in generating and modifying fatty acid-derived components of sporopollenin and are specifically expressed in the tapetum with similar developmental timing to ABCG26. According to the recent model for sporopollenin biosynthesis proposed by de Azevedo Souza et al. (2009), the ACOS5 enzyme produces a fatty acyl-CoA that is a central
intermediate required for the synthesis of sporopollenin constituents that is further modified by other tapetum-expressed enzymes. One attractive hypothesis is that ABCG26 substrate(s) include such ACOS5-derived aliphatic sporopollenin constituents and that after ABCG26-mediated secretion into the locule, they are further transported by an unknown mechanism to the surface of developing microspores for polymerization into the pollen exine. Transport of such molecules is consistent with the function of related Arabidopsis ABCG11 and ABCG12 proteins in the secretion of lipid constituents of cuticular wax (Pighin et al., 2004; Bird et al., 2007) and human ABCG transporters in lipid transport of lipids and steroids (van Meer et al., 2006; Velamakanni et al., 2007). However, alternative hypotheses are possible. For example, ABCG26 could transport a tapetum-generated signaling molecule required for the coordination of exine formation or for transport of an unknown component of the exine required for sporopollenin precursor polymerization and/or exine assembly on the developing microspore wall.

Given the promiscuity in substrates transported by many ABC transporters (Higgins, 2001; Yazaki, 2006) and the potential for both ABCG26 homodimeric and heterodimeric transporters in the tapetum plasma membrane, ABCG26 could participate in the transport of a suite of related substrates generated by a tapetum-localized sporopollenin precursor biosynthetic pathway. The abcg26-1 mutant provides a potential tool for gaining novel insights into the composition of sporopollenin precursors that are exported to the locule, since it may be possible to use biochemical profiling approaches to identify unpolymerized sporopollenin precursors that accumulate in the anther tapetum cells in the absence of functional ABCG26.

**MATERIALS AND METHODS**

**Mutant Isolation**

Arabidopsis (Arabidopsis thaliana) lines with a T-DNA insertion in ABCG26 were identified at The Arabidopsis Information Resource and obtained from the Arabidopsis Biological Resource Center (SALK_002317, SAIL_318B9, and SAIL_885_F06). Genomic DNA was isolated from the plants, and homozygous individuals were identified using PCR and primers P1 to P3 for abcg26-1, P4 to P6 for abcg26-2, and P5 to P7 for abcg26-3 (Supplemental Table S1). To test ABCG26 expression levels in the mutant (abcg26-1), intron-spanning primer sets upstream (P9 and P10) and downstream (P11 and P12) of the T-DNA insertion site were used to amplify cDNA by PCR (Supplemental Table S1). PCR was performed with Taq polymerase (Finnzymes) in a 20-μL reaction under the following conditions: 95°C for 2 min, 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, and finally 72°C for 10 min. The amplified genomic DNA fragments from the homozygous lines were gel purified and sequenced to confirm the site of T-DNA insertion. Since the homozygous abcg26-1 mutants retained some partial fertility, it was possible to collect small amounts of homozygous mutant seed.

**Plant Growth**

Wild-type (Col-0) and abcg26 mutant Arabidopsis seeds were germinated on Murashige and Skoog medium plates at pH 5.7. Seeds were imbibed at 4°C in the dark, grown at 28°C under continuous light for 1 week, transplanted to soil (Sunshine Mix 4; Sungrow Horticulture), and raised to maturity at 20°C in 18 h of light and 6 h of dark. Measurements of plant height were taken at the onset of senescence after height growth had ceased, and variance was expressed as SD of 12 plants measured.

**Quantitative RT-PCR Analysis**

Arabidopsis total RNA was isolated from the specified tissues frozen in liquid nitrogen and ground to a fine powder, and DNase-treated RNA was extracted using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad) following the manufacturer’s instructions. The quality of the RNA samples was assessed by visual inspection of the rRNA bands on a 1% agarose gel. RNA was quantified spectrophotometrically, and 2.0 μg of RNA was used to generate cDNA by Omniscript Reverse Transcriptase (Qiagen) as described by the manufacturer. Standard curves were generated for each primer pair using flower cDNA dilutions to obtain the following primer efficiencies: 101.5% (r² = 0.944) for primers P12 and P13, producing a 134-bp ABCG26.1 amplicon; 116.2% (r² = 0.978) for primers P14 and P13, producing a 191-bp ABCG26.2 amplicon; and 91.1% (r² = 0.979) for primers P15 and P16, producing the ACTIN2 amplicon (Supplemental Table S1). For the quantitative RT-PCR analysis of Arabidopsis gene expression, 10 ng of cDNA was incubated with 100 μl of SYBR Green Supermix (Bio-Rad) and 300 μM of each forward and reverse primer (Supplemental Table S1) in a total volume of 20 μL. After an initial denaturation step at 95°C for 3 min, 40 cycles at 95°C for 10 s, 53°C for 10 s, and 72°C for 20 s were carried out, followed by a melting curve ranging from 94°C to 53°C. Quantitative RT-PCR products were run on a gel and sequenced to confirm that single correct amplicons were amplified. Two biological replicates were carried out and gave similar results. Representative data are shown for three technical replicates of one biological replicate in Figure 2B, with SD shown for technical replicates. A t test comparing ABCG26.1 and ABCG26.2 expression levels indicated a significant difference (P < 0.001). Threshold cycles (Ct) were adjusted manually, and CT values were subtracted from those of the flower sample (used as the reference tissue) to generate a ΔCT value. Fold changes were calculated from the ΔCT values and normalized by dividing the corresponding ΔCT value for the ACTIN2 control gene.

**In Situ Hybridization**

Wild-type Arabidopsis (Col-0) unopened flower buds were fixed in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde under vacuum, dehydrated in an ethanol series, and stained with 0.1% eosin. Samples were then passed through a xylene-ethanol series and embedded in Paraplast (Sigma) with four changes of Paraplast. The sections were sectioned at 8 μm thickness using a microtome and mounted on precharged slides (Probe-On; Fisher Scientific). Sections from developing flower buds at multiple stages of development were selected for hybridization to sense and antisense probes.

For sense and antisense ABCG26 probe synthesis, an 822-bp DNA template corresponding to the ABCG26 region spanning exons 3 and 4 was PCR amplified from plasmid DNA containing the cloned full-length cDNA using gene-specific forward and reverse primers (Supplemental Table S1; P17–P20) and Taq polymerase (Finnzymes) at 49°C annealing and 68°C extension for 40 cycles. A T7 polymerase-binding site was incorporated into the forward primer for sense probe amplification and in the reverse primer for antisense probe amplification. Digoxigenin (Roche)-labeled probes were transcribed off the template using T7 polymerase (Roche) following the manufacturer’s instructions. Reactions were stopped using EDTA and precipitated in ethanol and LiCl. Probes were shortened to 200-bp fragments by limited carbonate hydrolysis at 60°C and quantified by dot blot dilutions compared with known samples on Hybrid XL membranes (GE Healthcare).

Samples were dehydrated through an ethanol series and subjected to hybridization at 55°C for 16 h. Both full-length and partial sense and antisense probes were used at 20 ng per slide in 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 0.5 mg of RNA, 10 μM Tris, pH 7.5, 1× mRNA EDTA, and 300 μM NaCl. The slides were washed in 2× SSC at 55°C followed by four washes in 0.2× SSC at 55°C, then 0.2× SSC at 37°C, 0.2× SSC at room temperature, and finally with phosphate-buffered saline at room temperature. The slides were covered with 1 ml of blocking buffer (1% Roche blocking agent in 100 mM maleic acid and 150 mM NaCl, pH 7.5) for 45 min at room temperature, washed in BSA solution (1% bovine serum albumin, 100 mM Tris, pH 7.5, 150 mM NaCl, and 0.3% Triton X-100), and hybridized to anti-digoxigenin antibody (Roche) at 1:1,250 dilution for 90 min. The slides were then washed in BSA solution followed by 100 μM Tris, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂. The color reaction was started by the addition of 5-bromo-
4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride color reagent (Roche), and the color was allowed to develop in the dark for 16 h before visualizing on an Olympus Provis AX-70 microscope.

Genetic Complementation of the abcg26-1 Mutant

The 2,058-bp fragment encoding ABCG26 was amplified from flower cDNA using Platinum High Fidelity Taq Polymerase (Invitrogen) with primers spanning the full-length cDNA and including a stop codon. The amplified band was gel purified and cloned into a Gateway-compatible entry vector (pcRB8/GW/TOPO; Invitrogen), and the gene was confirmed by sequencing. Additionally, a second unrelated transcript of 2,215 bp was amplified (approximately 10% of the time) that retained intron 7 and encoded premature stop codons (truncating at 2,182 bp downstream of ATG in the first stop codon). The genes were recombined into a Gateway-compatible destination binary vector (pPDG2; modified pSM-3 vector based on pCAMBIA1390 vector) using LR Clonase II enzyme mix (Invitrogen) according to the manufacturer's instructions. Agrobacterium tumefaciens LBA4404 was transformed with one of the described destination vectors and used to transform homozygous abcg26-1 plants by the floral dip method (Clough and Bent, 1998). T1 seeds were grown on Murashige and Skoog plates with 50 mg L⁻¹ hygromycin, and surviving plants were grown to maturity and allowed to self-pollinate. T2 plant genotypes were confirmed at the ABCG26 locus. PCR confirmed the presence of the transgene.

SEM Analysis

Anthers were removed from fully opened flowers and placed on aluminium SEM stubs (Ted Pella, Inc.). Samples were coated with 7-nm gold using a Cressington 208HR High Resolution Sputter Coater and examined with a Hitachi S-2600N Variable Pressure SEM device.

TEM Analysis

For chemical fixation, wild-type (Col-0) and abcg26-1 mutant flower buds were dissected from plants for immediate fixation in 4% formaldehyde and 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 6.9). Samples were postfixed in 1% osmium tetroxide, dehydrated in an ethanol series, and incubated in acetone followed by 100% propylene oxide for 30 min. Samples were gradually embedded in Spurr’s resin and polymerized at 60°C for 3 d.

For HFP followed by freeze substitution, unopened flower buds were excised from Col-0 and abcg26-1 plants, stripped of sepals and petals, and submerged in 0.2 M Suc in uncoated copper type B sample holders (Ted Pella, Inc.). Samples were frozen at high pressure with a Leica EM HPM 100 high-pressure freezer followed by freeze substitution and embedding in Spurr’s resin (Spurr, 1969) as described by McFarlane et al. (2008). Freeze substitution medium contained 2% (w/v) osmium tetroxide in acetone with 8% (v/v) dimethoxypropane.

Toluidine blue-stained thick sections (0.3–0.7 μm) were viewed by light microscopy to determine the stage of anther development as described by Sanders et al. (1999). Samples for representative stages of anther development were thin sectioned (less than 70 nm) with a Reichert Ultracut E ultramicrotome and mounted on 100-mesh 0.5% Formvar-coated grids. Sections were stained with 2% uranyl acetate in 70% (v/v) methanol for 15 min followed by Reynolds’s lead citrate for 5 to 8 min. A Hitachi H7600 TEM device was used to examine the stained samples, and images were taken with an AMT Advantage (1 megapixel) CCD camera (Advanced Microscope Technologies).

Subcellular Localization of ABCG26 in Planta

ABCG26 was PCR amplified in a 50-μL reaction from digested TOPO-A vector containing full-length ABCG26, and the resulting PCR product was separated on a 2% ethidium bromide agarose gel and purified using a Qiagen kit provided by the University of British Columbia Bioimaging Facility staff is gratefully acknowledged. We thank Dr. Stanton Gelvin (Purdue University) for providing the pE3150 vector and Dr. Shucai Wang and YuanYuan Liu (University of British Columbia) for technical assistance with protoplast transfection. We also thank Eryang Li, Yuanyuan Liu, and YuanYuan Liu (University of British Columbia) for providing the pE3150 vector and Dr. Shucai Wang and YuanYuan Liu (University of British Columbia) for technical assistance and SungSoo Kim (University of British Columbia) for technical assistance, materials, and discussions. We are grateful to Patricia Lam, Heather McFarlane, Rebecca Smith, and Mathias Schuetz (University of British Columbia) for thoughtful discussion and comments on the manuscript.

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ABCG26 Is Required for Exine Formation

593/40-nm emission filter within 15 min of staining. Images were processed with Volocity 4.3.2 (Improvision).

Supplemental Data

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

Supplemental Figure S1. Phenotypic comparison of Arabidopsis T-DNA insertion alleles.

Supplemental Figure S2. Accumulation of ABCG26.1 and ABCG26.2 transcripts in abcg26-1 mutants expressing the ABCG26.2 transgene.

Supplemental Figure S3. Transmission electron micrographs of tapetum ultrastructure in wild-type and abcg26-1 anthers.

Supplemental Figure S4. Key stages in Arabidopsis pollen wall development relative to ABCG26 expression.

Supplemental Table S1. Primer sequences used for PCR and RNA probe generation.

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