ABCG Transporters Are Required for Suberin and Pollen Wall Extracellular Barriers in Arabidopsis

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INTRODUCTION

Effective regulation of water balance in plants requires localized extracellular barriers that control water and solute movement. We describe a clade of five Arabidopsis thaliana ABCG half-transporters that are required for synthesis of an effective suberin barrier in roots and seed coats (ABCG2, ABCG6, and ABCG20) and for synthesis of an intact pollen wall (ABCG1 and ABCG16). Seed coats of abcg2 abcg6 abcg20 triple mutant plants had increased permeability to tetrazolium red and decreased suberin content. The root system of triple mutant plants was more permeable to water and salts in a zone complementary to that affected by the Casparian strip. Suberin of mutant roots and seed coats had distorted lamellar structure and reduced proportions of aliphatic components. Root wax from the mutant was deficient in alkylhydroxycinnamate esters. These mutant plants also had few lateral roots and precocious secondary growth in primary roots. abcg1 abcg16 double mutants defective in the other two members of the clade had pollen with defects in the nexine layer of the tapetum-derived exine pollen wall and in the pollen-derived intine layer. Mutant pollen collapsed at the time of anther desiccation. These mutants reveal transport requirements for barrier synthesis as well as physiological and developmental consequences of barrier deficiency.

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have lower solute permeability than do wild-type root systems, probably as a result of their increased suberin content (Ranathunge and Schreiber, 2011).

ATP binding cassette (ABC) transporter proteins are found in both prokaryotes and eukaryotes and pump diverse substrates across membranes using energy from ATP hydrolysis (Higgins and Linton, 2004). Canonical ABC transporters have two repeats each of a transmembrane domain with about six transmembrane helices often conferring substrate specificity and a cytoplasmic ATPase domain. ABC half-transporters have just one of each domain and must form homo- or heterodimers for their transport activity. Arabidopsis has ~130 ABC transporters that fall into several clades (Sánchez-Fernández et al., 2001; Verrier et al., 2008). Among these, the ABCG clade is the largest and includes both full-length and half-length transporters.

Several plant ABCG proteins are known or suspected to contribute to synthesis of extracellular barriers. Arabidopsis DESPERADO/WBC11/ABCG11 and ABCG12/CERS are required in the shoot epidermis to transport lipid precursors for cutin and wax biosynthesis (Pighini et al., 2004; Bird et al., 2007; Luo et al., 2007; Panikashvili et al., 2007). These two half-transporters can form heterodimers, and ABCG11 can also homodimerize (McFarlane et al., 2010). ABCG11 is induced by salt, ABA, and wounding and also affects suberin deposition in roots (Panikashvili et al., 2010). ABCG13 is closely related to ABCG11 and ABCG12 and contributes to cutin formation in flowers (Panikashvili et al., 2011). The full-length transporter ABCG32/PEC1 is required for hydroxylated fatty acid incorporation in the cuticular layer of the cell wall in leaves and flowers (Bessire et al., 2011). ABCG29 exports monolignols required for lignin biosynthesis (Alejandro et al., 2012). Other ABCG transporters are required for pollen exine formation in Arabidopsis or rice (Oryza sativa; Quilichini et al., 2010; Kuromori et al., 2011; Qin et al., 2013). ABCG9 and ABCG14 can also dimerize with ABCG11, and these as well as ABCG31 affect sterol ester levels in vegetative tissues or on pollen (Le Hir et al., 2013; Choi et al., 2014). In addition, several ABCG proteins are required to transport the hormones ABA, indole-3-butryic acid, strigolactone, or trans-zeatin (Strader and Bartel, 2009; Kang et al., 2010; Kuromori et al., 2010; Ruzicka et al., 2010; Kretzschmar et al., 2012; Ko et al., 2014; Zhang et al., 2014) or to transport an unknown substrate required for symbiotic mycorrhizal arbuscule development (Zhang et al., 2010).

We have identified a clade of Arabidopsis ABCG half-transporters that are coexpressed with several known suberin biosynthesis genes. A triple mutant deficient in three of these, ABCG2, ABCG6, and ABCG20, had defective suberin lamellae structure and increased seed coat and root system permeability attributable to decreased barrier function. It also had altered root system architecture suggestive of activated stress responses. Two additional proteins in the clade, ABCG1 and ABCG16, were required for pollen wall integrity.

RESULTS

A Clade of ABCG Transporters Is Coexpressed with Suberin Biosynthesis Genes

ABCG1, ABCG2, ABCG6, ABCG16, and ABCG20 proteins form a clade of five ABCG half-transporters (Figure 1A; Supplemental Data Set 1 and Supplemental Figure 1). After transient expression in tobacco (Nicotiana benthamiana) leaves, ABCG1:GFP, ABCG2:GFP, ABCG6:GFP, ABCG16:GFP, and ABCG20:GFP protein fusions each colocalized with staining of the plasma membrane marker dye FM-64 (Supplemental Figure 2), suggesting that they act at the plasma membrane. Based on the ATTED-II and Aranet databases of global gene expression data (Lee et al., 2010; Obayashi et al., 2014) and on a translatome data set of several root and shoot tissues (Moustroph et al., 2009), ABCG2, ABCG6, ABCG16, and ABCG20 genes are coexpressed with known suberin biosynthesis genes, such as GPAT5, FAR1, FAR4, FAR5, CYP86A1/HORST, CYP86B1, and ASFT. Translatome data of different cell types in roots and shoots confirm that ABCG2, ABCG6, and ABCG20 expression are particularly enriched in root endodermis (Moustroph et al., 2009) (Supplemental Data Set 2).

X-Gluc staining of plant lines carrying PABCGx:GUS reporter fusions revealed spatial expression patterns. Five-day-old seedlings of ABCG1, ABCG2, ABCG6, and ABCG16 reporter lines had X-Gluc staining at the root-shoot junction (Supplemental Figure 3A). Fifteen-day-old seedlings carrying each of the fusions had X-Gluc staining in the endodermal layer of roots (Figure 1B). Endodermal staining only appeared starting in the maturation zone of the root above the meristem. For ABCG6 and ABCG20 reporters, X-Gluc staining was also observed in the outer periderm of roots of six-week-old soil-grown plants (Supplemental Figure 3E). All five reporter lines also had expression in anthers and/or in maturing pollen grains (Figures 1C and 1D; Supplemental Figures 3C and 3D). For ABCG1, ABCG2, and ABCG16 reporters, anther staining appeared strongest in the tapetal layer (Figure 1D). X-Gluc staining of PABCG2:GUS plants was generally weaker than the others and appeared at a later stage in anthers. PABCG1:GUS was also expressed in the shoot meristematic region, in leaf hydathodes, and in the style (Supplemental Figure 3B). Quantitative RT-PCR assays confirmed much higher expression of each of these genes in roots than in vegetative shoots (Supplemental Figure 4B). In addition, available global gene expression data indicate that all five genes are expressed in developing seed coats (Le et al., 2010; Supplemental Figure 5). These results suggest that these ABCG genes act in the maturing root endodermis, in developing anthers and pollen, and in seed coats.

Four-hour treatments of seedlings with any of several hormones increased transcript levels of some of these genes (Figure 2A). ABA increased levels of all five genes, with the greatest effect on ABCG6 and ABCG16; the auxin indole-3-acetic acid (IAA) increased ABCG6, ABCG16, and ABCG20 levels; the gibberellin GA4 increased ABCG6 level; and the auxin transport inhibitor N-1-naphthylphthalamic acid induced ABCG6. For PABCG6:GUS and PABCG16:GUS fusions, Fluridone, which inhibits ABA biosynthesis, decreased root staining, and ABA restored staining in the presence of Fluridone (Supplemental Figure 4A). These data indicate that endogenous ABA likely contributes to expression of these genes in root endodermis. In addition, wounding of leaves of 3-week-old plants induced PABCG2:GUS, PABCG6:GUS, PABCG16:GUS, and (very weakly) PABCG20:GUS within 48 h (Supplemental Figure 3F). Lastly, growth on medium with added mannitol to induce osmotic stress increased root expression of ABCG2, ABCG6, ABCG16, and ABCG20, as well as the suberin...
biosynthesis genes GPAT5 and CYP86B1 (Supplemental Figure 4C). Exogenous NaCl also increases expression in wild-type roots of ABCG1, ABCG6, ABCG16, and ABCG20 and of several known suberin biosynthesis genes (Geng et al., 2013) (Supplemental Data Set 3). Salt increased expression of some of these genes in endodermis and also in cortex or stele cells.

Taken together, the overlapping expression patterns and similar ABA, mannitol, salt, and wound inducibility of these ABCG genes with known suberin biosynthesis genes suggest that they might contribute to suberin deposition.

**ABCG2, ABCG6, and ABCG20 Contribute to Suberin Function**

To determine physiological functions of these transporters, we characterized effects of two T-DNA insertion alleles in each gene (Supplemental Figure 6A). abcg1-1, abcg2-1, and the abcg16 and abcg20 alleles each had insertions that interrupt the protein coding sequence and are therefore expected to be null alleles. Of the alleles that did not interrupt the coding sequence, the two ABCG6 alleles had insertions in the promoter or 5′ untranslated region (UTR) and had extremely low expression levels (Supplemental Figure 6B). Insertion abcg1-2 fell in the 5′ UTR, and abcg2-2 fell in the 3′ UTR and these each reduced transcript level to ~20% of the wild-type level (Supplemental Figure 6B). As described below, the abcg1-1 and abcg1-2 mutations each conferred similar phenotypes in combination with abcg16 mutations, suggesting that both are strong alleles, whereas the abcg2-2 allele caused weaker phenotypes than did abcg2-1. Thus, most of these mutations are likely to be null except for abcg2-2, which may be hypomorphic. As single mutants did not have noticeable phenotypes, we generated several double and triple mutants. We describe in detail
phenotypes caused by combinations of mutations within each of the two subclades (1) ABCG2, ABCG6, and ABCG20, and (2) ABCG1 and ABCG16.

The abc2-1 abc6-1 abc20-1 triple mutant had several phenotypes in common with previously described suberin-deficient mutants. It produced darkly colored seeds that had increased permeability to the cationic dye tetrazolium red (Figures 3A and 3B; Supplemental Figure 7A). Although abc2-1, abc6-1, and abc20-1 single mutant seeds each had similar seed coat permeability as the wild type in this assay, all three double mutant combinations had increased permeability, with seeds of abc2-1 abc20-1 staining almost as darkly as did abc2-1 abc6-1 abc20-1 seeds (Figure 3A). Triple mutant seeds were also more sensitive than were wild-type seeds to inhibition of germination by ABA (Figure 3C). A genomic ABCG2 gene construct could rescue the seed coat permeability defect of the abc2-1 abc6-1 abc20-1 triple mutant, as well as root phenotypes described below (Supplemental Figure 8). A second independent triple mutant using alternate alleles, abc2-2 abc6-2 abc20-2, also had increased seed coat permeability (Supplemental Figure 7B), although this phenotype was weaker than for the abc2-1 abc6-1 abc20-1 triple mutant, probably because the abc2-2 allele is not null. For further analyses, we focused on the abc2-1 abc6-1 abc20-1 triple mutant.

Root systems of the abc2-1 abc6-1 abc20-1 triple mutant were more permeable to water than were wild-type root systems. Both hydrostatic hydraulic conductivity ($L_{p,os}$, driven by a hydrostatic pressure gradient) and osmotic hydraulic conductivity ($L_{p,os}$, driven by an osmotic pressure gradient) were about 2-fold higher in mutant than in wild-type root systems (Figure 4A). The $L_{p,os}/L_{p,os}$ ratios were close to one for both genotypes, indicating a relatively greater symplastic than apoplastic water flow, as for the horst/cyp86a1 suberin-deficient mutant (Ranathunge and Schreiber, 2011). Similarly, solute permeability ($P_{NaCl}$), driven by a hydrostatic pressure gradient, and osmotic hydraulic conductivity ($L_{p,os}$, driven by an osmotic pressure gradient) were about 2-fold higher in mutant than in wild-type root systems (Figure 4A). The $L_{p,os}/L_{p,os}$ ratios were close to one for both genotypes, indicating a relatively greater symplastic than apoplastic water flow, as for the horst/cyp86a1 suberin-deficient mutant (Ranathunge and Schreiber, 2011). Similarly, solute permeability ($P_{NaCl}$) was 2.5-fold higher in abc2-1 abc6-1 abc20-1 triple mutant roots than in wild-type roots (Figure 4B).

The Casparian Strip and Suberin Mediate Endodermal Permeability in Different Portions of the Root

The endodermis synthesizes both suberin and a Casparian strip composed of lignin, which serves as a barrier to apoplastic solute movement (Steudle and Peterson, 1998). The Casparian strip appeared intact in abc2-1 abc6-1 abc20-1 roots by TEM (Figure 4C). We assessed whether the Casparian strip was functional in our plants by assaying root permeability to propidium iodide (PI). Similarly to previous reports (Naseer et al., 2012; Hosmani et al., 2013), PI could enter the central stele of the young portion of 7-d-old wild-type roots up to ~13 cells above the root meristem and could enter the central stele of the Casparian-strip-deficient esb1-1 mutant up to ~26 cells above the meristem (Figures 4D to 4F). In these two cases, the stele in the older parts of the root 30 or more cells above the meristem did not stain with PI. Similarly as in wild-type roots, PI was blocked from entering the stele of abc2-1 abc6-1 abc20-1 roots starting 13 cells above the meristem (Figures 4D and 4F). However, in older portions of abc2-1 abc6-1 abc20-1 roots above 37 cells above the meristem, PI stained the endodermis and central stele (Figure 4E). Similarly, the suberin-deficient plants gpat5-2 and P_CASPT_CDEF1 (Beisson et al., 2007; Naseer et al., 2012) had an intact barrier to PI movement in the young part of the root but were deficient in excluding PI from the stele of the older part of the root (Supplemental Figure 9A). In the absence of PI, no red fluorescence was observed in the central stele of...
**abcg2-1 abcg6-1 abcg20-1 Plants Produce Suberin with Altered Structure**

These physiological phenotypes indicate that *abcg2-1 abcg6-1 abcg20-1* roots have decreased suberin barrier function. However, roots of 10-d-old *abcg2-1 abcg6-1 abcg20-1* seedlings had higher levels of staining with the fluorescent suberin stain Fluorol Yellow than did wild-type roots. Wild-type roots had background cell wall fluorescence or cross-staining of xylem, but only minimal putative Fluorol Yellow suberin staining at the ends of mature endodermal cells. Consistent with the inducibility of suberin biosynthesis genes by mannitol, wild-type root Fluorol Yellow staining was stronger around endodermal cells of plants grown on medium supplemented with mannitol (Figure 3D). In contrast, *abcg2-1 abcg6-1 abcg20-1* triple mutant roots had intense Fluorol Yellow staining along the entire length of the mature endodermis, both in the absence and presence of mannitol (Figure 3D). *abcg2-2 abcg6-2 abcg20-2* endodermis also had higher Fluorol Yellow staining than did wild-type endodermis, although this was less dramatic than in the *abcg2-1 abcg6-1 abcg20-1* triple mutant (Supplemental Figure 7C). Mature portions of *abcg2-1 abcg6-1 abcg20-1* triple mutant roots also stained more strongly than did wild-type roots with Sudan Red, another suberin stain (Supplemental Figure 4D). The endodermis and stele of *abcg2-1 abcg6-1 abcg20-1* triple mutant roots also had greater autofluorescence (Figure 4E; Supplemental Figure 8) and greater staining with the lignin stain phloroglucinol (Figure 3E). The periderm of 1-month-old triple mutant roots stained more strongly with phloroglucinol than did wild-type roots (Figure 3F). This may indicate increased accumulation of aromatic lignin and/or suberin components in the stele and periderm of mutant roots.

Increased suberin deposition in the *abcg2-1 abcg6-1 abcg20-1* mutant root was surprising in light of the increased permeability of mutant roots and seed coats. We therefore analyzed the structure and composition of suberin in the *abcg2-1 abcg6-1 abcg20-1* triple mutant. TEM of the mature zone of 10-d-old wild-type seedling roots (1.5 mm below the root-shoot junction) revealed continuous suberin lamellae on the internal face of endodermal cells between the plasma membrane and primary cell wall. These had alternating light- and dark-staining bands, with adjacent dark bands spaced ~10 to 12 nm apart (Figures 5E and 5G). In contrast, mutant endodermal cells were surrounded by light-staining material that only occasionally had a few short and discontinuous dark lamellae (Figures 5F and 5H). This layer in the mutant often appeared thicker than the suberin lamellae around wild-type endodermal cells. In the mutant, similar light-staining material was also present adjacent to pericycle cells interior to the endodermal cells, suggesting that the mutant may have begun to form periderm characteristic of mature roots (Gambetta et al., 2013). Lastly, the middle lamella between endodermis and pericycle cells appeared darker in the mutant than in the wild type.

Roots from mature soil-grown wild-type plants that had undergone secondary growth had suberin surrounding the outermost layer of periderm cells at the surface (Supplemental Figures 10C and 10E). The lamellae had similar spacing as those outside endodermis of younger roots (Supplemental Figure 10G). Suberin lamellae could also be distinguished in periderm of *abcg2-1 abcg6-1 abcg20-1* triple mutant roots, but the dark-staining bands
Figure 4. Root Permeability of Col, esb1-1, and abcg2-1 abcg6-1 abcg20-1 Mutants to PI and to Water and Solutes.

(A) and (B) Hydrostatic and osmotic hydraulic conductivity (A) and salt permeability (B) of Col and abcg2-1 abcg6-1 abcg20-1 mutant root systems. Hydroponically grown roots were used to measure the hydraulic conductivity or salt permeability by root pressure probe at room temperature. Values are means of means (n = 6) ± sd.

(C) TEM of Casparian strip (CS) in Col and abcg2-1 abcg6-1 abcg20-1 roots sectioned 2 mm above the root tip. Bar = 0.5 µm.

(E) and (F) Autofluorescence and PI staining images showing the permeability of Col and mutant roots after staining at 15-25 cells above the meristem. Bar = 50 µm.
were lighter than those in wild-type roots (Supplemental Figures 10F and 10H). Thus, suberin in the mutant had similarly altered appearance in both the young root endodermis and the mature root periderm. Seed coats of the abcg2-1 abcg6-1 abcg20-1 triple mutant had similarly decreased lamellar structure (Supplemental Figure 11). As discussed below, the mutant root periderm cells were also smaller than those of wild-type roots (Supplemental Figure 10D). Together with the physiological assays above, these results suggest that suberin of the triple mutant is deficient in components that confer lamellar structure and barrier properties.

**abcg2-1 abcg6-1 abcg20-1 Plants Produce Altered Suberin Polyester and Root Wax**

We analyzed the abundance of suberin polyester monomers extracted from root periderm of 7-week-old wild-type and triple mutant plants (Figure 6A; Supplemental Figure 12) and from roots of 10-d-old plants, which had not yet undergone significant periderm development and therefore contained mostly endodermal suberin (Supplemental Figure 13). Yields from periderm of wild-type 7-week-old plants were ~6-fold higher than those from endodermis of 10-d-old plants. Consistent with the microscopy and Fluorol Yellow staining results, total suberin aliphatic monomer content per dry weight was ~70% higher in roots of 10-d-old seedlings of abcg2-1 abcg6-1 abcg20-1 than in wild-type roots and ~3-fold higher in mutant periderm from 7-week-old plants (Figure 6A, inset; Supplemental Figure 13, inset). Compared with suberin from wild-type root periderm, suberin from mutant root periderm had 2-fold or lower relative proportions of 20:0 and 22:0 fatty acids and 22:0 fatty alcohol (1-OH 22:0) and a 33% relative decrease in 18:1 ω-hydroxy fatty acid (Figure 6A). (A 40% decrease in ferulate was not significant at P < 0.05.) When normalized to dry weight, only 22:0 fatty acid and 22:0 fatty alcohol were lower in mutant than wild-type samples (Supplemental Figure 12A). Conversely, suberin from mutant root periderm had 2-fold or higher molar proportions of 24:0 fatty acid, 18:0, 20:0, and 22:0 dicarboxylic acids, and 20:0 hydroxy fatty acid. Suberin from 10-d-old triple mutant seedling roots had decreased proportions of ferulate, 22:0 fatty acid, 18:0 and 22:0 fatty alcohols, and 18:2 dicarboxylic acid as well as higher proportions of 18:1 and 22:0 dicarboxylic acids and 20:0 and 22:0 hydroxy fatty acids (Supplemental Figure 13).

We also analyzed periderm suberin composition from roots of 7-week-old single and double mutant plants (Supplemental Figure 14) and from 10-d-old double mutant plants (Supplemental Figure 13). The single and double mutants generally had weaker suberin phenotypes than the triple mutant. Of the single mutants, abcg2-1 had the largest defect, with lower levels of fatty alcohols than the wild type. The double mutants generally had intermediate levels of most components, with the abcg2-1 abcg6-1 and abcg2-1 abcg20-1 double mutants having larger differences from the wild type than the abcg6-1 abcg20-1 double mutant. Curiously, in 10-d-old plant roots, the abcg2-1 abcg6-1 and abcg2-1 abcg20-1 double mutants had proportions of 18:0 and other fatty alcohols that were higher than were recovered from either wild-type or triple mutant roots (Supplemental Figure 13).

In contrast to mutant roots, total suberin polyester monomer content in abcg2-1 abcg6-1 abcg20-1 seeds was less than half that of wild-type seeds (Figure 6B, inset). This result suggests that the apparent compensatory increase in suberin biosynthesis seen in mutant roots does not occur in seeds. On a molar ratio basis, suberin from mutant seed had 2-fold or lower amounts of ferulate, 16:0 and 24:0 hydroxy fatty acids, and 22:0 fatty alcohol compared with suberin from wild-type seeds and smaller decreases in 22:0 hydroxy fatty acid and 18:0 fatty alcohol (Figure 6B). However, when normalized to seed weight, suberin from mutant seeds also had lower levels of comuramate, 24:0 fatty acid, 18:2 and 24:0 dicarboxylic acids, and 18:1, 18:2, and 22:0 hydroxy fatty acids (Supplemental Figure 12B). Overall, the most consistent differences from the wild type shared among root and seed coat abcg2-1 abcg6-1 abcg20-1 suberin were reduced proportions of ferulate, 22:0 alcohol, and 22:0 fatty acid, and increased proportions of saturated 18:0-22:0 dicarboxylic acids.

Root waxes are soluble lipids that can be isolated from roots by a quick chloroform extraction (Li et al., 2007; Kosma et al., 2012). These compounds are not polymerized and may include suberin precursors. We analyzed waxes from roots of 7-week-old wild-type and abcg2-1 abcg6-1 abcg20-1 mutant plants (Figure 7). In these samples, aromatic acids are present as esters with fatty alcohols. Strikingly, 20:0 and 22:0 alkyl comuramates, 18:0, 20:0, and 22:0 alkyl ferulates, and 18:0, 20:0, and 22:0 alkyl cinnamates were all substantially decreased in the triple mutant roots. The largest effects were on alkyl ferulates and alkyl cinnamates, most of which were not detected in waxes from the triple mutant. In addition, 20:0 fatty acid and 20:0 fatty alcohol were decreased in the mutant, as well as 29:2 sterol and β C24 monoyacyl glycerol. Conversely, several compounds were increased, most notably 18:0 fatty alcohol and, to a lesser extent, 22:0 fatty alcohol. The increased free fatty alcohol content in mutant root wax contrasts with results of suberin monomer analyses and suggest that much of the fatty alcohols recovered from suberin may be incorporated as alkyl ferulates.

**abcg2-1 abcg6-1 abcg20-1 Roots Have Altered Expression of Suberin Biosynthesis Genes**

To explore the basis for the increased production and altered composition of suberin in abcg2-1 abcg6-1 abcg20-1 roots, we...
assayed expression of several suberin biosynthesis genes. Mutant roots had higher transcript levels of GPAT5, CYP86A1, CYP86B1, and ASFT than did wild-type roots (Figure 2B), suggesting that increased expression of these and other biosynthetic genes accounts for higher suberin deposition in the triple mutant. Products of these genes synthesize hydroxy fatty acids (CYP86A1 and CYP86B1) and esterify long-chain fatty acids (GPAT5) and ferulate (ASFT). In contrast, transcripts of FAR1 and possibly also FAR4 were decreased in abcg2-1 abcg6-1 abcg20-1 mutant roots (Figure 2B). FAR1 and FAR4 synthesize primary fatty alcohols that are incorporated into suberin (Domergue et al., 2010; Vishwanath et al., 2013), and decreased expression of FAR1 and FAR4 may therefore explain the decreased fatty alcohol contents in the mutant suberin (Figure 6A). In addition, ABCG16 was overexpressed in abcg2-1 abcg6-1 abcg20-1 roots (Figure 2B), suggesting that it may compensate in part for loss of ABCG2, ABCG6, and ABCG20.

Increased suberin biosynthesis gene expression raises the possibility that ABA responses might be increased in abcg2-1 abcg6-1 abcg20-1 roots. However, several genes that encode proteins in ABA biosynthesis (NCED1, NCED3, NCED9, ABA2, ABA3, and AAO3) or ABA catabolism (CYP707A4), as well as several ABA-responsive genes (RAB18, COR47, RD29B, MYB96, and ABI5), were either expressed at wild-type levels or underexpressed in abcg2-1 abcg6-1 abcg20-1 roots (Figure 2C). These

Figure 5. Structure of Col and abcg2-1 abcg6-1 abcg20-1 Roots.
Ten-day-old seedlings grown on vertically oriented plates were fixed, and the mature region of roots 1 to 1.5 mm below the root-shoot junction were sectioned. C, cortex; X, xylem; PC, pericycle; E, endodermis; ML, middle lamella; S, suberin. (A) and (B) Light microscope images of Col (A) and abcg2-1 abcg6-1 abcg20-1 (B) root transverse sections stained with Toluidine blue. Bars = 10 µm. (C) and (D) TEM of Col and abcg2-1 abcg6-1 abcg20-1 root central stele. Boxed regions indicate regions shown in (E) and (F). Bars = 5 µm. (E) and (F) TEM of Col and abcg2-1 abcg6-1 abcg20-1 endodermal cells. Bars = 0.5 µm. (G) and (H) TEM of Col and abcg2-1 abcg6-1 abcg20-1 suberin lamellae at higher magnification. Arrows indicate suberin layers. Bars = 100 nm.
results suggest that mutant roots do not have a sustained increase in ABA response. P<sub>abcg2-1 abcg6-1 abcg20</sub> seedlings, which have decreased ABA response specifically in the endodermis (Duan et al., 2013), had increased Fluorol Yellow staining around the endodermis after growth on mannitol, although this appeared weaker than the effect in wild-type seedlings (Supplemental Figure 4E; Figure 3D). These results suggest that ABA may contribute to induction of suberin biosynthesis but that signals other than ABA can also stimulate suberin biosynthesis under osmotic stress or in the mutant roots.

**Altered Growth of abc2-1 abcg6-1 abcg20-1 Plants**

Microscopy of maturing roots of 10-d-old seedlings revealed that the central stele of abc2-1 abcg6-1 abcg20-1 roots was
broader and had more cells than did the stele of wild-type roots and that the triple mutant also had slightly deformed epidermal and cortical cells (Figures 5A to 5D), suggesting that secondary growth had begun precociously in the mutant. In mature roots of soil-grown plants, mutant periderm had smaller cells that appeared less regularly packed than corresponding cells in wild-type plants, and many small cells appeared to be sloughing off from the root surface (Supplemental Figures 10A, 10B, 10E, and 10F). Cells interior to the periderm also appeared less organized than in the wild type. These observations suggest that the phellogen cells, which give rise to periderm, may have continued to divide at a stage when wild-type phellogen cells had differentiated and that the mutant cells may be less adherent than wild-type cells.

The abcg2-1 abcg6-1 abcg20-1 seedlings also had fewer emerged lateral roots and fewer visible lateral root primordia than did wild-type seedlings (Figures 8A and 8B; Supplemental Figures 15B and 15C). Single and double mutants had intermediate numbers of lateral roots (Supplemental Figure 15B). abcg2-2 abcg6-2 abcg20-2 triple mutants, as well as the suberin-deficient mutants gpata5-1, asft-1, and cyp86a1-2, also had fewer lateral roots than did wild-type plants, although they had more lateral roots than did abc2-1 abc6-1 abcg20-1 seedlings (Supplemental Figure 15B and D). Although abc2-1 abc6-1 abcg20-1 mutant plants had fewer lateral roots than did wild-type plants, they often had adventitious roots that grew from the base of the hypocotyl, and these adventitious roots often grew as long as the primary root (Figures 8A and 8C; Supplemental Figure 15A). When incorporated into the growth medium, exogenous IAA increased the numbers of emerged lateral roots in both wild-type and triple mutant seedlings, to similar numbers at the highest concentration tested (Figure 8B), although under these conditions these lateral roots remained quite short (Supplemental Figures 15E and 15F). Although cotyledons and leaves initially appeared similar in wild-type and abc2-1 abc6-1 abcg20-1 seedlings, after growth for more than 7 d, triple mutant plants also had smaller leaves than did wild-type plants (Supplemental Figures 15A and 16).

Altered water balance in abc2-1 abc6-1 abcg20-1 plants might cause the changes seen in nonendodermal root cell types, such as the stele and pericycle, and the decreased shoot growth. We assessed the responses of wild-type and abc2-1 abc6-1 abcg20-1 plants to decreased water availability by growing them on medium with added mannitol. Mannitol is known to decrease lateral root formation or elongation (Xiong et al., 2006; Macgregor et al., 2008; Zolla et al., 2010). Under our conditions, mannitol decreased both root elongation and shoot growth (Figures 8D and 8E). For root and possibly leaf growth, the abc2-1 abc6-1 abcg20-1 mutant appeared more sensitive to mannitol, indicating that suberin biosynthesis in wild-type plants can contribute to effective osmotic stress tolerance.

ABCG1 and ABCG16 Contribute to Pollen Wall Integrity

ABCG1 and ABCG16 comprise a sister clade to ABCG2, ABCG6, and ABCG20 (Figure 1A). abc1 abc16 double mutants with either of two independent allelic combinations, as well as the
The triple mutant, had decreased male fecundity. More than 90% of mature pollen grains of abcg1
abcg16 plants appeared shriveled (Supplemental Table 1), and abcg1 abcg16 and abcg1-1 abcg16-1 abcg20-1 anthers released only a small amount of pollen, most of which was shriveled (Figures 9A and 9E; Supplemental Figures 17A and 17B). At least some of the uncollapsed pollen was apparently viable, as the mutants could be propagated by self-pollination. Among progeny of self-pollinated ABCG1/abcg1-2 ABCG16/abcg16-2 double heterozygotes, the abcg1-2 abcg16-2 and other mutant genotypes were recovered at frequencies consistent with a sporophytic defect (Supplemental Table 2). Moreover, sesquimutants homozygous for either abcg1-2 or abcg16-2 and heterozygous for the other mutation produced over 80% uncollapsed pollen, a proportion much higher than the 50% that would be expected for a gametophytic effect (Supplemental Table 1).

We analyzed abcg1-1 abcg16-1 abcg20-1 pollen in more detail. Mutant pollen grains appeared outwardly normal until approximately
one to two flowers before flower opening (flower stage 12), at which point they collapsed (Figures 9E, 10H, and 10J; Supplemental Figure 17C). TEM analyses revealed that at flower stage 11, before the pollen collapse, the cytoplasm of abcg1-1 abcg16-1 abcg20-1 pollen had shrunk so that the plasma membrane retracted away from the cell wall (Figure 10B). This shrinkage may have occurred during pollen development or during fixation for TEM but was not seen in wild-type pollen (Figure 10A).

Pollen is surrounded by an intine cell wall layer made by the pollen grain and an exine layer rich in the polymer sporopollenin made by the tapetum of the anther (Blackmore et al., 2007). The exine layer has an inner component called nexe or endexine and an outer layer called sexine or ektexine. Developing pollen of the abcg1-1 abcg16-1 abcg20-1 and abcg1-2 abcg16-2 mutants stained more brightly with Auromine O than did wild-type pollen, indicating that the sexine layer is present (Figure 9E; Supplemental Figure 17). Moreover, TEM of developing pollen grains revealed that abcg1-1 abcg16-1 abcg20-1 triple mutant pollen had the characteristic structure of the sexine layer consisting of columns of opaque material (Figures 10A to 10F). At flower stage 12, when the mutant pollen had collapsed, these columns appeared more densely packed than in wild-type pollen (Figures 10G to 10J).

Whereas the sexine layer appeared intact, the narrow electron-dense nexe layer interior to the sexine layer often appeared to stain more lightly or discontinuously in developing mutant pollen than in wild-type pollen (Figures 10C to 10F). The layer appeared intact in mature collapsed mutant pollen (Figures 10I and 10J). These observations suggest that mutant pollen may have a delay in nexe formation or an altered nexe structure. As this layer is a minor component of the exine layer and difficult to isolate, we did not try to assay its chemical composition.

To assess the integrity of the intine layer, we stained wild-type and abcg1-1 abcg16-1 abcg20-1 mutant pollen with Calcofluor, which binds to oligosaccharides with β-1,3 and β-1,4 glycosyl linkages (Wood, 1980). Whereas wild-type pollen stained evenly with Calcofluor, ~80% of abcg1-1 abcg16-1 abcg20-1 mutant pollen lacked Calcofluor staining (Figures 9B to 9D). This was true of both immature pollen in anthers of stage 11 flowers and of mature pollen in anthers of stage 12 flowers just before flowers open (Smyth et al., 1990). Similarly, in TEM images, whereas an intine layer was visible in wild-type pollen grains at both immature and mature stages (Figures 10C, 10E, and 10I), this layer was not seen in the mutant pollen (Figures 10D, 10F, and 10J).

**DISCUSSION**

**ABCG Transporters Are Required for Barrier Synthesis**

Our results indicate that ABCG2, ABCG6, and ABCG20 transporters together are required to form effective suberin barriers to water and solute movement. Both roots and seed coats of mutants deficient in these transporters had increased permeability, similarly to roots and seed coats of previously described suberin-deficient mutants. The mutant roots did make suberin, but this was structurally altered, suggesting that a specific component or organization is required to form an effective seal. These mutants therefore provide insight into suberin biosynthesis and structural requirements for suberin barrier properties.

Similarly, the closely related transporters ABCG1 and ABCG16 are required for pollen wall integrity. Most pollen from abcg1 abcg16 mutant plants collapsed at flower stage 12, when wild-type pollen matures and anthers desiccate and release pollen (Wilson et al., 2011). This pollen phenotype was inherited sporophytically and may have arisen from a defect in synthesis of the inner exine layer called nexe or endexine. Strong expression of PABCG1::GUS and PABCG16::GUS fusions in the tapetum are consistent with this interpretation. In addition, the intine layer of abcg1 abcg16 abcg20 triple mutant pollen was often missing, suggesting that a sporophytic function is required for intine as well as exine synthesis (although the ABCG genes were also expressed in pollen). Other workers have also found that nexe defects lead to absence of intine formation and to shriveled pollen (Lou et al., 2014). Mutants with defects in the outer layer of exine, called sexine or ektexine, also have defective pollen that can appear shriveled or abort (Morant et al., 2007; de Azevedo Souza et al., 2009; Quilichini et al., 2010; Dobritsa et al., 2011).

While biochemical studies of exine structure and biosynthesis are challenging (Quilichini et al., 2014), further microscopy studies of early stages of abcg1 abcg16 mutant anther and pollen development starting at the free microspore stage might help to identify more definitively which wall component is affected and when the defect first appears. Although mature pollen naturally hydrates through the apertures, it may be possible to design pollen permeability assays analogous to those used to characterize effects of suberin deficiency in roots to test whether the mutant pollen has an effective seal against water loss. Alternatively, acetolysis might reveal whether the exine is structurally intact (Arizumi et al., 2003). If the mutant pollen wall is more permeable or fragile than wild-type pollen, then the root suberin and pollen wall defects may arise from defects in structurally and functionally related barrier molecules.

Both the suberin and pollen wall defects may be incomplete, as all five ABCG genes were expressed in root endodermis, in seed coats, and in developing anthers and pollen, and may therefore act partially redundantly in these tissues. While little is known of the detailed composition of nexe, suberin and sporopollenin (comprising both nexe and sexine) each contain polymerized fatty acids and aromatic compounds (Arizumi and Torigaya, 2011; Beisson et al., 2012; Nawrath et al., 2013). Moreover, lily (*Lilium longiflorum*) nexe is first synthesized as lamellae, and in some plant species mature nexe is lamellar (Dickinson and Heslop-Harrison, 1968; Weber and Ulrich, 2010). Thus, nexe may have a similar structure to suberin. However, the strong phenotypes of abcg2 abcg6 abcg20 and abcg1 abcg16 mutants, despite the overlapping expression patterns of all five genes, raise the alternative possibility that proteins in the two subclades have differing substrate preferences. Further characterizations of these transporters may reveal whether this is true and may help to elucidate the composition of the nexe layer of pollen.

**ABCG Proteins May Transport Aliphatic Polymer Precursors**

That the abcg2-1 abcg6-1 abcg20-1 mutant roots deposit substantial amounts of permeable suberin suggests that specific
chemical components or structural features of suberin underlie its barrier properties. Suberin in roots of the abcg2-1 abcg6-1 abcg20-1 mutant had decreased proportions of 20:0 and 22:0 fatty acids, 22:0 fatty alcohol, and 18:1 ω-hydroxy fatty acid, raising the possibility that the mutant is deficient in exporting fatty acids or fatty alcohols. Consistent with a possible fatty acid transport defect, rice plants mutant for OsABCG5/RCN1, encoding a putative ortholog of the entire clade of Arabidopsis ABCG proteins studied here (Yasuno et al., 2009), had root hypodermis suberin with decreased amounts of 28:0 and 30:0 fatty

Figure 9. Pollen Phenotypes of Col and abcg1-1 abcg16-1 abcg20-1 Plants.

(A) Inflorescences and open stage 13 flowers (Smyth et al., 1990).
(B) Calcofluor staining of anthers of stage 11 flowers (three or four flowers before the first open flower).
(C) Calcofluor staining of immature pollen from stage 11 flowers.
(D) Calcofluor staining of mature pollen from stage 13 flowers (the third open flower).
(E) Auramine-O staining of pollen. Flower -3 and Flower +3 indicate immature pollen from stage 11 flowers (the third bud before flower opening) and mature pollen from stage 13 flowers (the third open flower), respectively.

Images in (B) to (E) were by confocal microscopy. For the mature pollen in (E), medial and surface optical sections are shown. Bars = 20 μm in (B) and 5 μm in (C) to (E).
acids, several \( \omega \)-hydroxy acids, and C16 and C18 diacids (Shiono et al., 2014). By contrast, the Arabidopsis \( dso/abcg11 \) mutant, which is mostly affected in cutin composition, had significant reductions in some suberin components but not in C22:0 acid or alcohol (Panikashvili et al., 2010). Suberin of the \( abcg2-1 \) \( abcg6-1 \) \( abcg20-1 \) triple mutant root lacked dark lamellae normally seen in TEM images, and other plants with defects in aliphatic components of suberin also make suberin in which these dark bands are disrupted. These include tuber periderm cell walls of potato plants with silenced CYP86A33, having suberin deficient in glycercerol, C18:1 \( \omega \)-hydroxy acid, and \( \omega,\omega \)-diacid (Serra et al., 2009); root periderm cell walls of Arabidopsis cyp86A1 mutants having suberin deficient in several fatty acids, hydroxy fatty acids, and diacids (Li et al., 2007; Höfer et al., 2008; Molina et al., 2009); and Arabidopsis plants deficient in KCS20 and KCS2/DAISY, encoding 3-ketoacyl-CoA synthases required for long-chain fatty acid synthesis (Franke et al., 2009; Lee et al., 2009). Thus, both the chemical analyses and the suberin structure observed by TEM are consistent with a defect in aliphatic components. The substrate(s) that the ABCG proteins actually transport might therefore be fatty acids, fatty alcohols, and/or conjugates of one of these. Mutant seed suberin had almost normal fatty acid content but reduced levels of ferulic acid, fatty alcohols, and hydroxy fatty acids, which suggests that the ABCG proteins may transport hydroxylated precursors.

Analysis of root waxes provided additional insight into the possible substrates of these transporters. Root waxes include compounds that may be precursors of suberin that are not yet polymerized, including several alkylhydroxycinnamates. Waxes from \( abcg2-1 \) \( abcg6-1 \) \( abcg20-1 \) mutant roots were strikingly deficient in alkyl ferulates and alkyl caffeates and to a lesser degree alkyl coumarates. Intracellular feruloyl-CoA- or caffeoyl-CoA-acyltransferases synthesize these alkylhydroxycinnamates, and defects in these enzymes affect suberin and/or wax composition in both Arabidopsis and potato (Gou et al., 2009; Molina et al., 2009; Serra et al., 2010; Kosma et al., 2012). Our data thus suggest that the ABCG proteins may transport alkylhydroxycinnamates, which might in turn cause deficiencies in suberin, fatty alcohols, and fatty acids in polymerized suberin in the mutant. This scenario suggests that a significant portion of fatty alcohol monomers in polymerized suberin may come from alkylhydroxycinnamates that are exported and polymerized, rather than from exported free fatty alcohols. Similarly, a portion of the bound ferulate in suberin is esterified to fatty alcohols (Gou et al., 2009; Molina et al., 2009; Serra et al., 2010). The \( abcg2-1 \) \( abcg6-1 \) \( abcg20-1 \) plants appear to have more severe suberin structural defects than do the asft/hht mutants, which are affected in suberin ferulate or root alkylcaffeates, respectively. It will be interesting in this regard to make higher-order mutants deficient in multiple hydroxycinnamyl-CoA-acyltransferases.

Whatever the primary transport defect may be, the increases in several dicarboxylic acids and hydroxy fatty acids in the mutant root suberin may arise from diversion of biosynthetic precursors toward these products and from compensatory induction of multiple suberin biosynthesis genes. This compensation may be less pronounced in mutant seed coats, which did not have excess suberin monomers. Alternatively, precursor metabolite pools may differ between seed coats and roots. The decreased \( FAR1 \) and \( FAR4 \) expression in the \( abcg2-1 \) \( abcg6-1 \) \( abcg20-1 \) mutant roots contrasts with the increased expression of most other suberin biosynthesis genes tested and could contribute to the reduced 22:0 fatty alcohol content of mutant suberin (Domergue et al., 2010; Vishwanath et al., 2013). \( FAR1 \) and \( FAR4 \) are induced by stress, as are several other suberin biosynthesis genes (Domergue et al., 2010; Geng et al., 2013), and their decreased expression in mutant roots could possibly reflect substrate-level negative feedback caused by decreased export of fatty alcohols in the mutant.

Additional ABCG proteins may also contribute to synthesis of suberin or other barrier polymers. Three additional closely related genes, \( ABCG17 \), \( ABCG18 \), and \( ABCG19 \) (Figure 1A; Supplemental Figure 1), are adjacent on the genome to \( ABCG16 \). These three

Figure 10. Transmission Electron Microscopy of \( abcg1-1 \) \( abcg16-1 \) \( abcg20-1 \) Pollen.

(A) to (F) Pollen from stage 11 flowers (the third bud before flower opening).

(G) to (J) Pollen from stage 12 flowers (the last closed bud before flower opening).

(A), (C), (E), (G), and (I) Wild-type pollen.

(B), (D), (F), (H), and (J) \( abcg1-1 \) \( abcg16-1 \) \( abcg20-1 \) pollen.

EX, exine; IN, intine; PM, plasma membrane. Arrows in (C) to (F) indicate the nexine layer. Bars = 2 \( \mu \text{m} \) in (A) and (B), 200 nm in (C) to (F), 5 \( \mu \text{m} \) in (G) and (H), and 0.5 \( \mu \text{m} \) in (I) and (J).
genes were not expressed in roots, but ABCG18 and ABCG19 were strongly expressed in shoot epidermis (Mustroph et al., 2009) (Supplemental Table 1), and these might contribute to cutin synthesis. ABCG10 and ABCG23, encoding ABCG proteins closely related to each other from a different clade, were also coexpressed with suberin biosynthesis genes (Supplemental Table 1). A pair of ABCG half-transporters in Medicago truncatula are closely related to the Arabidopsis clade studied here and localize to the periarbuscular membrane (Zhang et al., 2010). These may contribute to a barrier function or may transport a nutrient or signal essential for arbuscular mycorrhizal symbiosis.

Physiological Importance of Barriers

Mutants deficient in the Casparian strip lack a lignin barrier to apoplastic salt diffusion into the stele of the elongating part of the root (Naseer et al., 2012; Hosmani et al., 2013). In contrast, the abcg2-1 abcg6-1 abcg20-1 and other suberin-deficient mutants had an intact Casparian strip but allowed salt movement to the stele in the more mature zone above the elongation zone affected in the Casparian strip mutants. Thus, the two barriers are important in complementary zones of the developing root. Endodermal cells may become more permeable as they mature, for example, if they express transporters that permit transcellular movement of salts and water (Steedle and Peterson, 1998). Still older mature roots undergoing secondary growth and periderm formation lose outer cell layers including the endodermis (Dolan et al., 1993; Dolan and Roberts, 1995; Baum et al., 2002; Matsumoto-Kitano et al., 2008). Thus, the Casparian lignin strip is a temporary barrier present in the youngest portions of roots, whereas suberin is required in older portions of roots, and to some degree replaces the Casparian strip. In such older roots, suberin might also impede apoplastic water and salt movement. That the Casparian strip and suberin may have some overlapping function is suggested by the increased induction of suberin biosynthesis in Casparian-strip-deficient mutants (Naseer et al., 2012; Hosmani et al., 2013) and by the increased phloroglucinol staining and autofluorescence in the central part of abcg2-1 abcg6-1 abcg20-1 roots, indicating increased lignin or suberin aromatic subunit synthesis. It will be interesting to characterize plants deficient in both barriers and to explore the mechanisms by which plants modulate synthesis of extracellular barriers in response to drought or salt stress.

In addition to defects in extracellular barriers, abcg2-1 abcg6-1 abcg20-1 mutants had morphological phenotypes that reveal putative developmental adjustments to altered water status. Mutant roots began secondary growth and periderm formation precociously, and mutant plants had fewer lateral roots than did wild-type plants. Known suberin-deficient mutants also had fewer lateral roots, although still more than abcg2-1 abcg6-1 abcg20-1 plants. These results suggest that the lateral root defect is caused in part by suberin deficiency and that more complete suberin deficiency might reveal stronger phenotypes. Alternatively, ABCG2, ABCG6, and ABCG20 transporters may affect processes in addition to suberin biosynthesis, such as formation of other barrier compounds or export of signals.

Several possible regulatory pathways might cause these root architecture changes. Defective suberin barriers in mutant roots might lead to osmotic stress or water loss, thereby inducing responses by which wild-type plants adapt to drought conditions. Such responses might act through induction of ABA biosynthesis. However, mutant roots did not have a general increase in expression of ABA biosynthesis or response genes. Cytokinins stimulate secondary growth (Matsumoto-Kitano et al., 2008), and it is possible that mutant roots have increased cytokinin content or response. Defects in synthesis of very-long-chain fatty acids (including 20:0 and 22:0 fatty acids) and sphingolipids also cause increased cytokinin production and secondary growth (Roudier et al., 2010; Nobusawa et al., 2013), and some of the phenotypes seen in abcg2-1 abcg6-1 abcg20-1 plants might therefore arise from altered fatty acid signaling. Lastly, sucrose uptake in the shoots of plants grown in tissue culture can stimulate auxin transport to the root and lateral root production (Macgregor et al., 2008; Lilley et al., 2012); therefore, decreased sucrose availability caused by low water potential might affect lateral root production. It is likely that altered root permeability affects development of many plants. For example, rice abcg5/rcn1 mutants defective in a closely related transporter also have a defective suberin barrier and altered root and shoot architecture (Yasuno et al., 2009; Ureshi et al., 2012; Shiono et al., 2014).

METHODS

Plant Material, Growth Conditions, Phenotypic Analysis, and Hormone Treatments

All plant lines were in the Columbia (Col) ecotype. New Salk and GABI-Kat insertion lines used are summarized in Supplemental Figure 6 and were genotyped using primers listed in Supplemental Table 2. Unless otherwise stated, seeds were surface sterilized using 70% (v/v) ethanol and 0.5% (v/v) Triton X-100, plated on Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) containing 1% (w/v) Suc and 0.8% (w/v) Phyto-agar (Research Products International) adjusted to pH 5.7 using KOH, and cold stratified for 2 to 3 d at 4°C before being transferred to controlled growth chambers at 22°C with 16-h-light and 8-h-dark conditions.

For seed germination assays, seeds were plated on MS+Suc plates with the indicated concentrations of ABA (Sigma-Aldrich) and kept at 4°C for 3 d before being moved to growth chambers. After 5 d of incubation, the seeds whose radicle had emerged through the seed coat were scored as germinated. To assay the numbers of lateral roots in the presence or absence of IAA, seedlings were first grown for 5 d on vertically oriented plates in the absence of hormone and then transferred onto MS+Suc plates with the indicated concentrations of IAA. Lateral and adventitious roots were counted under a dissecting microscope or by staining with acetoacarmin (Booker et al., 2010). To measure root and rosette leaf lengths, scanned images of seedlings or leaves were analyzed using Image J (Abramoff et al., 2004).

Promoter Fusions and Complementation of the abcg2-1 abcg6-1 abcg20-1 Mutant

For promoter:GUS fusions, 1-kb sequences upstream of the start codon of ABCG genes were amplified using high fidelity Phusion polymerase (New England Biolabs) using primers listed in Supplemental Table 3 and then cloned into the Smal restriction site of pBl101.2 (Clontech) to make the promoter:GUS fusion reporters. For complementation, a genomic region including 1.2 kb of putative promoter and the ABCG2 coding region were amplified from genomic DNA of Col using primers listed in Supplemental Table 3 and cloned into the Smal restriction site of
pCambia2300 (http://www.cambia.org/). Positive clones were identified by PCR and verified by sequencing. The binary vector clones were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 by electroporation and selecting kanamycin and gentamicin resistance. Wild-type Columbia or *abcg2-1 abcg6-1 abcg20-1* lines segregating pCambia2300 (http://www.cambia.org/). Positive clones were identified by sequencing. The binary vector clones were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 by electroporation and selecting kanamycin and gentamicin resistance. Wild-type Columbia or *abcg2-1 abcg6-1 abcg20-1* lines segregating pCambia2300 (http://www.cambia.org/). Positive clones were identified by sequencing. The binary vector clones were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 by electroporation and selecting kanamycin and gentamicin resistance. Wild-type Columbia or

**Transgenic Expression in Tobacco for Subcellular Localization**

Gene-specific primers (Supplemental Table 3) were used to amplify the cDNAs of *ABCG1, ABCG2, ABCG6, ABCG16,* and *ABCG20* without stop codons from flower cDNA using high-fidelity Phusion Taq polymerase (Inventrogen) and were cloned in pENTR/D-Topo (Inventrogen). Clones lacking the stop codon were used for LR recombination reactions with pGWB5 (Nakagawa et al., 2007) to generate the ABCG-GFP constructs. Expression clones were transformed into *Agrobacterium tumefaciens* strain GV3101. The *Agrobacterium* clones were grown overnight at 28°C, centrifuged at 3000 rpm for a few seconds to pellet down the cells, and washed twice with water. The washed pellet was then suspended in infiltration medium containing 10 mM MgCl₂, 10 mM MES, and 100 μM acetosyringone and were kept at room temperature for 2 h before being infiltrated into the abaxial side of Nicotiana benthamiana leaves using a 1-μL syringe. Infiltrated tobacco plants were grown under long days (16 h light:8 h dark). After 48 h of incubation, leaf sections were stained with 5 μM FM4-64 (Calbiochem, EMD Millipore) and observed under a confocal microscope with excitation at 488 nm and detection at 493 to 580 nm. For Calcofluor staining, pollen grains were stained for 30 min in a drop of 1% (w/v) Calcofluor (Sigma-Aldrich) in water and observed by confocal microscopy with excitation at 405 nm and detection at 421 to 558 nm.

**Quantitative RT-PCR Analysis**

Seeding, shoot, or root tissues were frozen in liquid N₂ and total RNA was extracted using RNeasy plant mini kits (Qiagen) according to the manufacturer’s instructions. For real-time quantitative RT-PCR, cDNA was synthesized using the A3500 Reverse Transcription System (Promega) with random primers according to the manufacturer’s instructions. Total RNA of 0.1 or 1 μg was used in a 20-μL volume reaction and incubated for 1 h at 42°C. The RT reaction mixture was diluted 10-fold, and 1 μL was used as a template in 10-μL PCR reaction using the 7900 HT Fast real-time PCR system from Applied Biosystems in standard mode with SYBR Green Master Mix (Applied Biosystems) following the manufacturer’s protocol. The primers for transcript analysis were designed using the primer express software (Applied Biosystems) and are listed in Supplemental Table 3. The reactions were performed in triplicate and the products were checked by melting curve analysis. Values are averages of two or three biological replicates, normalized to the reference transcript *UBQ10*.

**Microscopy**

For Fluorol Yellow staining (Brundrett et al., 1991), seedlings were incubated in a freshly prepared solution of 0.01% (w/v) Fluorol Yellow (Santa Cruz Biotechnologies) in lactic acid (88.5%) with 1 g/mL chloral hydrate at 70°C for 1 h. The samples were washed two to three times with water and then observed under a Zeiss 710 DUO confocal laser scanning microscope (Zeiss) with yellow fluorescent protein filter with excitation at 514 nm and detection at 516 to 593 nm.

For Sudan Red staining, seedlings were incubated in a solution of Sudan Red 7B (Sigma-Aldrich) in polyethylene glycol 400:glycerol (90% PEG400:10% glycerol) as described by Brundrett et al. (1991), rinsed with water, and observed with a Leica MZ12.5 light microscope coupled to a digital camera.

For phloroglucinol staining, 7-d-old seedlings and hand sections of 1-month-old roots from soil-grown plants washed in distilled water were stained for 10 s in 1% phloroglucinol (Sigma-Aldrich) dissolved in 20% HCl (Jensen, 1962).

For Auramine-O staining (Dobritska et al., 2009), anthers were placed on a microscope slide in a drop of 0.001% (w/v) Auramine-O (diluted from a stock of 0.1% (w/v) in 50 mM Tris-HCl, pH 7.5) in 17% Suc. The anther was gently tapped with tweezers to release pollen. Pollen grains were allowed to sit in solution for 2 to 3 min before observation on a Zeiss 710 Duo Confocal microscope under fluorescein isothiocyanate settings with excitation at 488 nm and detection at 493 to 580 nm.

For Calcofluor staining, pollen grains were stained for 30 min in a drop of 1% (w/v) Calcofluor (Sigma-Aldrich) in water and observed by confocal microscopy with excitation at 405 nm and detection at 421 to 558 nm.

**Transmission Electron Microscopy**

Roots of 10-d-old seedlings were fixed in 2% glutaraldehyde 0.05 M sodium phosphate, pH 7.4, and stored at 4°C for several days before being processed. The roots were washed several times with 0.05 M sodium phosphate, pH 7.4, and then postfixed with 1% osmium tetroxide in 0.05 M sodium phosphate, pH 7.4, for 1 h at room temperature. Samples were dehydrated in a graded ethanol series (30, 50, 75, 100, and 100%), followed by two changes in propylene oxide, and infiltrated with and embedded in epoxy resin PolyBed812 (Polysciences).

Dry seeds were rehydrated in 20 μL abscisic acid in water for 24 h at 4°C. Hydrated seeds were rinsed in water briefly and fixed for 24 h in 2% paraformaldehyde/0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. To facilitate penetration of the processing reagents, the seed coats were nicked using a sharp scalpel. The seeds were postfixed with 1% osmium tetroxide/1.25% potassium ferricyanide in 0.1 M sodium cacodylate buffer for 2 h at 4°C. Dehydration and resin infiltration steps were performed using a PELEC BioWave Pro microwave oven operating at 450 W (Ted Pella). Samples were dehydrated through an ascending series of acetone (10, 30, 50, 75, 100, 100%, and 100%), and then infiltrated under vacuum in a microwave through a series of Spurr’s resin:aceton solutions (Spurr’s resin:acetone 1:2, 1:1, 2:1, and 100% Spurr’s resin). Seeds were placed into embedding capsules filled with fresh Spurr’s resin and polymerized for 16 h at 70°C.

Anthers were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.9 (Quilichini et al., 2010), and stored at 4°C for several days before processing. Following several washes in 0.05 M sodium cacodylate buffer, the samples were postfixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer for 1 h at room temperature. Samples were dehydrated in a graded ethanol series (30, 50, 75, 100, and 100%), followed by two changes in propylene oxide, and infiltrated with and embedded in Spurr’s low viscosity epoxy resin (Polysciences).

For root, seed, and anther samples, 1-μm sections were cut and mounted on glass slides, stained with 1% Toluidine blue in 1% sodium borate, and viewed by light microscopy to select the region of interest for TEM. Ultrathin sections (70 nm) were cut using a diamond knife, mounted on 200 mesh copper grids (or 75 mesh Formvar-carbon coated copper grids, for seeds), and stained with 4% aqueous uranyl acetate and Reynold’s lead citrate (Reynolds, 1963). Samples were observed using a LEO EM910 transmission electron microscope operating at 80 kV (Carl Zeiss Microscopy), and digital images were acquired using a Gatan Orius SC1000 CCD digital camera with Digital Micrograph 3.11.0 (Gatan).

**Seed and Root Suberin Composition Analysis**

Arabidopsis thaliana plants were germinated and grown on soil-vermiculite-perlite (2:1:1) for 7 weeks or on MS agar medium for 10 d. Suberin analyses
were performed on roots of 10-d-old seedlings, 7-week-old plants, and mature, dry seeds following the protocol described by Molina et al. (2006) with slight modifications. Seed (100 mg per replicate), mature root (150 mg per replicate), or 10-d-old root (15 to 20 mg per replicate) samples were quenched in hot isopropanol, ground with a polytron, and thoroughly de-lipidated with a series of solvent washes: isopropanol, chloroform:methanol (2:1, v/v), and chloroform:methanol (1:2, v/v) (~12 h each). Sodium methoxide-catalyzed depolymerization was performed on delipidated dry residues, with methyl heptadecanoate and pentadecanolactone as internal standards. Samples were evaporated to dryness under N2 stream and derivatized with 100 µL each, of pyridine and acetic anhydride at 60°C for 1 h. Derivatized samples were evaporated to dryness and dissolved in heptane:toluene (1:1). Acetyl derivatives of suberin monomers were analyzed by gas chromatography-mass spectrometry in an Agilent 6890 gas chromatograph equipped with an Agilent 5975 mass spectrometer. Splitless injection was used with a HP5-MS column (J&W Scientific; 30 m × 0.25 mm × 0.25-µm film thickness) with helium carrier gas at 2 mL min⁻¹ and oven temperature programmed from 110 to 300°C at 10°C/min. Splitless injection was used and the mass spectrometer run in scan mode over 40 to 500 atomic mass units (electron impact ionization), with peaks quantified on the basis of their total ion current.

### Root Wax Analysis

Waxes were extracted from roots of 7-week-old Arabidopsis plants (grown as described above) by dipping in CHCl₃ for 1 min (Li et al., 2007). After extraction, internal standards were added to CHCl₃ extracts, and extracts were evaporated to dryness under N₂. Internal standards included heptadecanoic acid (17:0), pentadecan-1-ol (15:0), tetracosane (C24), tridecyl alcohol, and heptadecan-1-ol coumarate (Kosma et al., 2012). Samples were derivatized with 100 µL pyridine and 100 µL N,O-bis(trimethylsilyl)trifluoroacetamide at 110°C for 10 min. Derivatized samples were evaporated to dryness, dissolved in hexane, and analyzed on a Thermo Trace GC 1300 GC system coupled to an ISQ mass selective detector (EI). Gas chromatography analyses used a TG-5MS capillary column (Thermo Scientific; 30-m length, 0.25-mm i.d., and 0.25-µm film thickness) with helium carrier gas at 1.0 mL/min constant flow and temperature programmed from 150 to 325°C at 5°C/min and then held for an additional 10 min at 325°C. Split mode (5:1 ratio, 310°C injector temperature) was used and the mass spectrometer run in scan mode over 40 to 650 atomic mass units (electron impact ionization), with peaks quantified on the basis of their total ion current.

### Seed Coat and Root Permeability Assays

For seed coat permeability tests, dry seeds were incubated in the dark for 10 min (Li et al., 2007) and then washed two to three times with water before being observed under a Zeiss 710 Duo Confocal microscope (Lee et al., 2013). PI was detected with excitation at 560 nm and detection at 566 to 646 nm, and autofluorescence was detected with standard eYFP filter with excitation at 405 nm and detection at 416 to 528 nm.

### Sequence Alignment and Phylogeny

For making the phylogenetic tree, the ABCG protein sequences were submitted to http://www.phylogeny.fr/ (Dereeper et al., 2008), which uses MUSCLE for multiple sequence alignment (Edgar, 2004), PhyML for phylogeny (Guindon et al., 2005), and TreeDyn for tree drawing (Chevenet et al., 2006).

### Accession Numbers

Genes used in this work have the following Arabidopsis Genome Initiative designations: ABCG1, At2g39350; ABCG2, At2g37360; ABCG6, At5g13980; ABCG7, At3g55090; ABCG17, At3g51100; ABCG18, At3g55110; ABCG19, At3g55130; ABCG20, At3g53510; GPA5, At3g11430; FAR1, At4g15090; FARM, At3g44540; FARS, At3g44550; CYP68B1, At5g23190; CYP68A1, At5g58860; ASFT, At5g10140; MYB96, At5g62470; RAB18, At5g66400; COP4, At1g20440; NCED9, At1g78390; NCED2, At3g14440; NCED1, At3g63520; ABA1, At5g67030; ABA2, At1g52340; ABA3, At1g16540; AA03, At2g27510; and CYP707A4, At3g19270.

### Supplemental Data

The following materials are available in the online version of this article and have also been deposited in the DRYAD repository under accession number http://dx.doi.org/10.5061/dryad.1hn66.

- **Supplemental Figure 1.** Sequence Alignment of ABCG Proteins.
- **Supplemental Figure 2.** Subcellular Localization of ABCG Proteins.
- **Supplemental Figure 3.** X-Gluc Staining of Plants with P_{ABCG}GUS Reporter Genes.
- **Supplemental Figure 4.** Expression of ABCG Genes and Root Suberin Staining.
- **Supplemental Figure 5.** ABCG Expression in Seeds.
- **Supplemental Figure 6.** T-DNA Insertion Alleles of ABCG Genes.
- **Supplemental Figure 7.** Seed Coat Permeability of Suberin-Deficient Mutants and Characterization of abcg2-2 abcg6-2 abcg20-2 Phenotypes.
- **Supplemental Figure 8.** Rescue of abcg2-1 abcg6-1 abcg20-1 Triple Mutant Phenotypes by a Genomic ABCG2 Gene Construct.
- **Supplemental Figure 9.** Propidium Iodide Permeability Assays.
- **Supplemental Figure 10.** Microscopy of Mature Roots from Soil-Grown Plants.
- **Supplemental Figure 11.** TEM of Wild-Type and abcg2-1 abcg6-1 abcg20-1 Seed Coats.
- **Supplemental Figure 12.** Suberin Monomer Composition of Suberin from Wild Type and abcg2-1 abcg6-1 abcg20-1.
- **Supplemental Figure 13.** Suberin Monomer Composition of Suberin from Roots of 10-d-Old Plants.
- **Supplemental Figure 14.** Suberin Monomer Composition of Suberin from Roots of 7-Week-Old Plants of Single and Double Mutants.
- **Supplemental Figure 15.** Lateral Roots in abcg and Suberin-Deficient Mutants.
- **Supplemental Figure 16.** Rosettes of Col and abcg2-1 abcg6-1 abcg20-1 Plants Grown in Soil.
- **Supplemental Figure 17.** Phenotypes of abcg1-2 abcg16-2 Plants.
- **Supplemental Table 1.** Proportions of Collapsed Pollen from abcg1-2 and abcg16-2 Single and Double Mutant Plants.
- **Supplemental Table 2.** Segregation of Genotypes among Progeny of ABCG1/abcg1-2 ABCG6/abcg16-2 Plants.
- **Supplemental Table 3.** Primers Used in This Work.
- **Supplemental Data Set 1.** Alignments Used to Generate the Phylogeny Presented in Figure 1A.
- **Supplemental Data Set 2.** Translorme Data for Suberin Biosynthesis and ABCG Genes in Different Root and Shoot Cell Types.
**AUTHOR CONTRIBUTIONS**


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**REFERENCES**


