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Name: Seth DeBolt

Address: University of Kentucky, N-318 Agricultural Science Center North

Telephone: (859) 257 8654

Fax: (859) 257 2934

Email: sdebo2@email.uky.edu

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Mutations in UDP-glucose:sterol-glucosyltransferase in Arabidopsis cause transparent testa phenotype and suberization defect in seeds

Seth DeBolt\textsuperscript{a, 1}, Wolf-Rüdiger Scheible\textsuperscript{b}, Kathrin Schrick\textsuperscript{c,d,e}, Manfred Auer\textsuperscript{f}, Fred Beisson\textsuperscript{g}, Volker Bischoff\textsuperscript{b}, Pierrette Bouvier-Navé\textsuperscript{h}, Andrew Carroll\textsuperscript{i}, Kian Hematyj, Yonghua Li\textsuperscript{g}, Jennifer Milne\textsuperscript{k}, Meera Nair\textsuperscript{a}, Hubert Schaller\textsuperscript{h}, Marcin Zemla\textsuperscript{j} and Chris Somerville\textsuperscript{j}

\textsuperscript{a} Department of Horticulture, University of Kentucky, Lexington, KY 40506, USA
\textsuperscript{b} Max Planck Institute of Molecular Plant Physiology, Science Park Golm, 14476 Potsdam, Germany
\textsuperscript{c} Keck Graduate Institute of Applied Life Sciences, Claremont, CA 91711, USA
\textsuperscript{d} Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA
\textsuperscript{e} Division of Biology, Kansas State University, Manhattan, KS 66506, USA
\textsuperscript{f} Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
\textsuperscript{g} Membrane Biogenesis Laboratory (UMR 5200), CNRS-University of Bordeaux 2, Bordeaux, 33076 cedex, France
\textsuperscript{h} Institute for Plant Molecular Biology, UPR CNRS 2357, 67083 Strasbourg, France
\textsuperscript{i} Department of Biology, Stanford University, CA 94305 USA
\textsuperscript{j} Energy Biosciences Institute, University of California, Berkeley, CA 94720, USA
\textsuperscript{k} Global Climate and Energy Project, Stanford University, CA 94305, USA

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\textbf{Abbreviations:} UGT80B1 and UGT80A2 (UDP-glucose:sterol glucosyltransferase), DCB (2,6-dichlorobenzonitrile), CESA (cellulose synthase A), GUS (β-glucuronidase), GFP (green fluorescent protein), ASG (acyl steryl glycosides), SG (steryl glycosides), SE (steryl esters), SSG, sitosteryl β-glycoside, FS (free sterols), DMACA (4-(Dimethylamino)-cinnamaldehyde)

\textsuperscript{1} The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors is: Seth DeBolt (sdebo2@email.uky.edu)
ABSTRACT

In higher plants, the most abundant sterol derivatives are steryl glycosides and acyl steryl glycosides. Arabidopsis contains two genes, UGT80A2 and UGT80B1, that encode UDP-glucose:sterol glycosyltransferases, enzymes that catalyze the synthesis of steryl glycosides. Lines having mutations in UGT80A2, UGT80B1, or both UGT80A2 and UGT8B1 were identified and characterized. The ugt80A2 lines were viable and exhibited relatively minor effects on plant growth. Conversely, ugt80B1 mutants displayed an array of phenotypes that were pronounced in the embryo and seed. Most notable was the finding that ugt80B1 was allelic to tt15 and displayed a transparent testa phenotype and a reduction in seed size. In addition to the role of UGT80B1 in the deposition of flavonoids, a loss of suberization of the seed was apparent in ugt80B1 by the lack of autofluorescence at the hilum region. Moreover, in ugt80B1, scanning and transmission electron microscopy reveals that the outer integument of the seed coat lost the electron dense cuticle layer at its surface and displayed altered cell morphology. Gas chromatography coupled with mass spectrometry of lipid polyester monomers confirmed a drastic decrease in aliphatic suberin and cutin-like polymers that was associated with an inability to limit tetrazolium salt uptake. The findings suggest a membrane function for steryl glycosides and acyl steryl glycosides in trafficking of lipid polyester precursors. An ancillary observation was that cellulose biosynthesis was unaffected in the double mutant, inconsistent with a predicted role for steryl glycosides in priming cellulose synthesis.
INTRODUCTION

Steryl glycosides (SG) and acyl sterol glycosides (ASG) are abundant constituents of the membranes of higher plants (Frasch and Grunwald, 1976, Warnecke and Heinz, 1994; Warnecke et al., 1997; Warnecke et al., 1999). SGs are synthesized by membrane-bound UDP-glucose:sterol glucosyltransferase (Hartmann-Bouillon and Benveniste, 1978; Ury et al., 1989; Warnecke et al., 1997) which catalyzes the glycosylation of the 3β-hydroxy group of sterols to produce a 3-β-D-glycoside. UGT80A2 has been found in the plasma membrane (PM), Golgi vesicles, the endoplasmic reticulum (ER) membrane, and occasionally the tonoplast (Hartmann-Bouillon and Benveniste, 1978; Yoshida and Uemura, 1986; Ullmann et al., 1987; Warnecke et al., 1997). It has also been reported that a UDP-glucose-dependent glucosylceramide synthase from cotton is capable of synthesizing SG in plants (Hillig et al., 2003). All plant sterols can be glycosylated, given that sterol substrates are pathway end products (Δ5-sterols in Arabidopsis) and not intermediates. The most commonly observed glycoside is glucose (Warnecke et al., 1997) but xylose (Iribarren and Pomilio, 1985), galactose, and mannose have been observed (Grunwald, 1978). Although rare in occurrence, SGs with di-, tri-, and tetraglucoside residues have also been reported (Kojima et al., 1989). SGs can be acylated, polyhydroxylated or sulfated, but ASGs with fatty acids esterified to the primary alcohol group of the carbohydrate unit are the most common modifications (Lepage, 1964).

SGs have been found as abundant membrane components in many species of plants, mosses, bacteria, fungi and in some species of animals (Esders and Light, 1972; Mayberry and Smith, 1983; Murakami-Murofushi et al., 1987; Haque et al., 1996; Tabata et al., 2008), yet relatively little is known about their biological functions. Because of the importance of sterols in membrane fluidity and permeability (Warnecke and Heinz, 1994; Warnecke et al., 1999; Schaller, 2003) and the phospholipid dependence of UDP-glucose:sterol glucosyltransferase (Bouvier-Nave et al., 1984), it has been postulated that SGs may have a role in adaptation to temperature stress (Palta et al., 1993). A difference in the proportion of glycosylated versus acylated sterols were reported in two different Solanaceous species under the same cold acclimation experiment (Palta et al., 1993). In one species an increase in SG was correlated with a decrease in ASG. In contrast, the other species displayed no change in SG and ASG levels with cold acclimation.
conditioning. Hence, evidence for a role in temperature adaptation is lacking.

Understanding the processes involved in SG production has additional human importance because SGs are highly bioactive food components and laboratory mice fed SGs faithfully lead to either amyotrophic lateral sclerosis or parkinsonism pathologies (Ly et al., 2007). Similarly, consumption of seeds of the cycad palm (*Cycas micronesica*) containing high SG levels, has been linked to an unusual human neurological disorder, ALS-parkinsonism dementia complex (ALS-PDC), in studies of the people of Guam (Cruz-Aguado and Shaw, 2009). However, SG is a dominant moiety of all plant membranes and some of the most widely consumed plant products in the United States such as soybeans (*Glycine max*) have concentrations well within the dose range obtained by consumption of cycad seeds. Cholesterol glycoside is the SG most commonly identified in animal membranes and is exemplified by cases in snake epidermis cells (Abraham et al., 1987) and human fibroblast cells under heat shock (Kunimoto et al., 2000). Regarding a role for SGs in the membrane, in comparison to normal sterols, SG and ASG exchange more slowly between the monolayer halves of a bilayer, which could serve to regulate free sterol content and its distribution (Ullmann et al., 1987; Warneke et al., 1999).

A study of cellulose synthesis in herbicide-treated cotton fibers found that sitosterol β-glucoside (SSG) co-purified with cellulose fragments (Peng et al., 2002), leading to speculation that SGs act as a primer for cellulose biosynthesis in higher plants (Peng et al., 2002). In support of the hypothesis, SSG biosynthesis was reported to be pharmacologically inhibited by the known cellulose biosynthesis inhibitor 2-6-dichlorobenzonitrile (DCB) (Peng et al., 2002). However, in subsequent studies, DCB inhibition of cellulose synthesis was not reversed by the exogenous addition of SSG, and the effects of DCB on cellulose synthesis were so rapid that the turnover of SGs would need to be very fast to account for the effects of DCB on cellulose synthesis (DeBolt et al., 2007). Schrick et al. (2004) reported that sterol biosynthesis mutants *fackel*, *hydra1* and *sterol methyltransferase1/cephalopod* have reduced levels of cellulose but a specific effect on SGs was not established. Hence, a role for SGs in plant growth and development remains speculative.

Here we describe a genetic analysis of the biological roles of two isoforms of
UDP-glucose:sterol glucosyltransferase, UGT80A2 and USGT80B1, that participate in the synthesis of SG in *Arabidopsis*. UDP-glucose-dependent glucosylceramide synthase may also be capable of synthesizing SG in plants (Hillig et al., 2003), but no analysis was performed herein. We show that mutations in one of these genes, *UGT80B1*, results in a lack of flavonoid accumulation in the seed coat and that it corresponds to *transparent testa 15* (*tt15*). Analysis of *ugt80A2*, *ugt80B1* and a double mutant, suggests that glycosylation of sterols by the UGT80A2 and UGT80B1 enzymes had no measurable consequence on cellulose levels in *Arabidopsis* seed, siliques, flowers, stems, trichomes and leaves. Rather, we demonstrate that mutation of *UGT80B1* principally alters embryonic development and seed suberin accumulation and cutin formation in the seed coat leading to abnormal permeability and tetrazolium salt uptake.

**RESULTS**

*Isolation of T-DNA mutations in the genes encoding UDP-glucose:sterol-glucosyltransferase*

Warnecke et al. (1999) previously reported the isolation of a cDNA coding for UDP-glucose:sterol glucosyltransferase from *Arabidopsis*. The gene they described, At3g07020, which we designate here as *UGT80A2*, encodes a 637 amino acid protein comprised of 14 exons and 13 introns. The completion of the *Arabidopsis* genome sequence revealed the existence of a second unlinked gene, At1g43620, encoding a 615 amino acid protein and corresponding to a genomic region encompassing 14 exons and 13 introns, as well as a predicted intron in the 3' untranslated region. The predicted gene product which we designate as *UGT80B1* exhibits strong sequence identity to *UGT80A2* (61.5% and 51.2% amino acid similarity and identity, respectively).

T-DNA insertion alleles for both *UGT80A2* and *UGT80B1* were identified by screening the University of Wisconsin T-DNA collection (Sussman et al., 2000). Homozygous lines carrying T-DNA mutations in *UGT80A2* and *UGT80B1* were confirmed by PCR (**Fig S1**). A double mutant homozygous for both the *ugt80* (*B1* and *A2*) mutations was obtained by crossing *ugt80A2* and *ugt80B1* homozygous plants, followed by PCR screening of the F$_2$ population for the double homozygote. The double mutant was found to be viable and was designated *ugt80A2,B1*. 


Sterol and sterol derivative analysis reveals reduced levels of SG and ASG and increased free sterols in \textit{ugt80A2,B1} mutant

Free sterols (FS), steryl esters (SE), SG and ASG were isolated from leaf tissue of wild-type, \textit{ugt80A2} and \textit{ugt80B1} mutants, and the \textit{ugt80A2,B1} double mutant. The wild-type tissues contained FS, SG and ASG, at levels that are similar to those previously described (Patterson et al., 1993). There was no difference in FS + SE content between wild-type and \textit{ugt80A2,B1} in leaf or stem tissue (Fig 1A). However, in inflorescence + silique tissue FS + SE content was 26\% higher in the \textit{ugt80A2,B1} relative to wild-type (Fig 1A). SG + ASG content decreased 5, 21 and 22-fold in leaf, stem and siliques + inflorescence tissue respectively (Fig 1B). Sterol derivatives that were SG + ASG were significantly reduced in \textit{ugt80A2} and \textit{ugt80B1} mutants (Table S1). The ratio of (SG +ASG):FS (\%) for rosette leaf tissue and ASG:SE (\%) for stem and inflorescence tissue both showed additive alteration in the sterol profile between the single and double mutant (Table S1). Thus, both \textit{UGT80A2} and \textit{UGT80B1} are required for the normal production of SG and ASG, and their gene products appear to function in a partially redundant manner in catalyzing the glycosylation of 24-alkyl-\(\Delta^5\)-sterols in \textit{Arabidopsis}.

Steryl glycoside mutant \textit{ugt80A2,B1} exhibits a slow growth phenotype and elongation defects in embryogenesis

The mature \textit{ugt80A2}, \textit{ugt80B1} and double mutant plants were viable and fully fertile. At 22\°C the growth habit of the mature plants showed no substantial radial swelling or dwarfing as was expected for a cellulose deficient mutant. Because of evidence suggesting that SG may be important in stress responses in fungi (Warnecke et al., 1999), slime molds (Murakami-Murofushi et al., 1987) and plants (Patterson et al., 1993), we grew the mutants at the seedling stage at a range of temperatures from 10-22\°C. The results indicated significantly lower seedling growth rates in the double mutant compared to wild-type, \textit{ugt80A2} and \textit{ugt80B1} single mutants at all 22\°, 15\° and 10\°C temperatures (Fig S2). We also explored the ability of the plants to acclimate to cold by measuring ion leakage and survival rates after one week acclimation at 4\°C but observed no significant difference between any of the lines and wild-type (Fig. S3). Mutant and
wild-type parental plants grown on soil at 4°C for 5 months in 24 hour light also displayed no differences in height or growth habit of the plants (data not shown).

Since double mutants displayed a slow growth phenotype during post-embryonic stages, we asked whether growth during embryogenesis was also affected. Embryonic stages of development were compared between the wild-type and double mutant (Fig 2). Although the early stages of double mutant development, from globular to young heart stages, displayed morphologies that were similar to wild-type, the late heart, torpedo, bent-cotyledon, and mature embryo stages exhibited abnormally stunted morphologies indicating a defect in cell elongation (Fig 2). Elongation of the cotyledon primordia, developing hypocotyl, and embryonic root were affected.

**Cellulose and cell wall sugar levels are not significantly altered in sterol glycoside mutants**

The cellulose priming model (Peng et al., 2002) predicts that a deficiency in SG may alter cellulose synthesis. To test this hypothesis we measured the relative amounts of crystalline glucose derived from cellulose in flowers, siliques, leaves, trichomes and stems of the double mutant. The results of these measurements indicated no significant difference in the amount of cellulose in the mutants in comparison with wild-type plants in any of our experiments (Fig 3a). Cellulose measurements were performed on leaf trichomes using birefringence and the results also failed to uncover a deficiency in cellulose content in the double mutant as did Fourier-Transform Infra-Red (FTIR) analysis (data not shown). Neutral sugar analysis showed some minor differences in the sugar profile of the cell wall (Fig 3b). Specifically, in the ugt80B1 mutant xylose, mannose and glucose content appeared greater than that of the wild-type plant and the ugt80A2 had slightly greater glucose levels than wild-type.

**Promoter::GUS fusions indicate distinct but partially overlapping relative gene expression patterns for UGT80A2 and UGT80B1.**

To investigate UGT80A2 and UGT80B1 expression in more detail, we constructed transgenic plants in which the β-glucuronidase (GUS) reporter gene was placed under the control of the ~2-kb promoter regions upstream of the UGT80A2 and UGT80B1 genes.
Relative gene expression of proUGT80A2::GUS was observed in a patchy distribution in cauline leaf epidermal cells, stomata, pollen, around the base of siliques and in the stamen (Fig S4). Relative gene expression of proUGT80B1::GUS was primarily observed in leaves, seedlings, around the apical tip of cotyledons and developing seeds (Fig S5). Characterization of the GUS staining pattern in embryos revealed that expression was strongest around the apical tip of the cotyledons and at the root apex. Strong GUS expression was also apparent around the seed coat epidermal cell boundaries and in the central columella, but not in the trough (Fig S5). Taken together the results indicate that UGT80A2 and UGT80B1 mRNAs are found in distinct expression domains within the plant. UGT80B1 was uniquely expressed in the seed coat and in the cotyledons of the embryo, consistent with its mutant phenotype related to these tissues. The promoter fusion expression results for both genes are largely consistent with expression analysis performed using available microarray data for Arabidopsis (available online through The Arabidopsis Information Resource; https://3.met.genevestigator.com/).

**Mutations in UGT80A2 and USG80B1 genes result in reduced seed size, transparent testa and salt uptake phenotypes**

Visual inspection revealed a lightened seed coat hue in ugt80B1 and in the double mutant as well as a dramatic reduction in seed size compared to wild-type (Fig 4a, Fig S6). The light colored testa phenotype in the UGT80B1 mutant was consistent with being a transparent testa mutant and this was confirmed by allelism tests to transparent testa 15 (data not shown) (Focks et al., 1999). The color of seed from ugt80A2 mutants did not appear lightened to the same degree as ugt80B1, however ugt80A2 seeds displayed a measurable reduction in size (Fig 4a, Fig S6).

A series of histochemical and microscopy analyses were performed to deduce possible reasons for the increased permeability of the ugt80B1 mutant. When seeds were incubated in a solution of tetrazolium salts, ugt80B1 and the double mutant seed were found to be highly sensitive to salt uptake (Fig 4b). Wild-type and ugt80A2 seeds absorbed small amount of salt at the hilum region, but ugt80B1 seeds were unable to limit uptake and the entire embryo became stained with formazan dye (a tetrazolium reduction product) (Fig 4b). The salt-uptake phenotype of ugt80B1 mutants was rescued by
complementation with a $p35S::UGT80B1$ construct (Fig 4b). Additional salt uptake experiments were performed during the development of the embryo within the seed. Wild-type seeds restricted salt uptake during the maturation stages, whereas double mutants never developed the ability to restrict salt from penetrating the seed coat (data not shown).

Pigmentation of the seed coat was determined by the deposition of flavanoids using 4-(Dimethylamino)-cinnamaldehyde (DMACA) reagent. Consistent with the transparent testa phenotype, the most drastic reduction in DMACA staining was in $ugt80B1$ and in the double mutant (Fig 4c). The DMACA staining phenotype of $ugt80B1$ mutants was also rescued by complementation with a $p35S::UGT80B1$ construct (Fig 4c).

**Flavanol and starch analysis in the seedling reveals altered cotyledon morphology and hydathode composition in $ugt80B1$ mutants**

Seedlings were grown in the dark for three days and then stained with DPBA to reveal sinapate derivatives. In comparison to wild-type, the $ugt80B1$ mutant displayed a striking increase in sinapate derivatives (blue color) localized specifically at the apical hydathode in the cotyledon (Fig S7a). The double mutant did not show a substantial change from the $ugt80B1$ single mutant in the DPBA staining pattern at the hydathode. After two days of dark growth, wild-type seedlings exhibited blue colored sinapate derivatives that were only localized at the hydathode region whereas in $ugt80B1$ and the double mutant, almost half the cotyledon showed sinapate derivatives. Thus, it appears that the accumulation of sinapate derivatives around the hydathode of cotyledons is a natural occurrence in seedling development and that $ugt80B1$ mutants retain this pattern in a greater proportion of the cotyledon during development. The cotyledon hydathode from $ugt80A1,B2$ seedlings was stained with an iodine mixture (I2:KI) to determine whether starch accumulation is altered in this region. Reminiscent of the DPBA staining (Fig S7b), the double mutant cotyledon displayed a distinct staining pattern around the hydathode region that was markedly more pronounced than wild-type hydathode staining. High magnification imaging of the hydathode region revealed an altered morphology of cells in this region in double mutant seedlings.
Steryl glycosides are critical for normal cell morphology of the seed coat and deposition of lipid polyesters as analyzed by electron microscopy and GC-MS

Scanning electron microscopy (SEM) was applied to ascertain possible alterations in the morphology of cells within the seed epidermis (Fig 5a-d). Severe defects in cell morphology were evident in double mutant seed (Fig 5b-d). In addition to smaller seed size, approximately one third of seed displayed a sunken region extending from the hilum (Fig 5b). Further examination by transmission electron microscopy (TEM) was performed to visualize the ultrastructure of the seed coat in the mutants. Strikingly, the electron-dense outer layer covering the wild-type seed coat was found to be absent in ugt80B1 and double mutant (Fig 5e and 5f). To understand the changes in development, the same analysis was applied at the developmental stage when wild-type seeds can first repel tetrazolium salts. At this stage wild-type seed already displayed an electron-dense cuticle layer covering the seed coat, and were beginning to form columella. By contrast, the cuticle layer was greatly diminished in the double mutant, and the columella were less prominent (Fig 5d). The cellular morphology in the mutant was strikingly different from wild-type: Aberrant dispersed electron-dense regions that are observed in the cytosol may represent an abnormal accumulation of suberin, wax or cutin that failed to be transported to the outer surface of the seed. The failure to form columella was consistent with the SEM data (Fig 5d) and indicates that this defect arose during embryonic development (Fig 2).

Next, we examined the hilum region of the wild-type seed compared with mutant by autofluorescence analysis using a broad spectrum UV light source, since autofluorescence around the hilum reflects accumulation of suberin (Beisson et al., 2007). The results indicate that the hilum region of both the ugt80B1 and double mutant exhibit reduced suberization, which appears as a bright autofluorescent signal in ugt80A2 and wild-type seed (Fig 5g and 5h). Consistent with these observations, analysis of lipid polyester monomers from seeds of the ugt80B1 mutant in comparison to wild-type showed an overall 50% reduction in total aliphatics (Fig 6). The vast majority of the 45 polyester monomers identified were significantly reduced in the ugt80A2,B1 mutant, including the C22 and C24 very long-chain ω-hydroxy fatty acids typical of suberin. The reduction in polyester monomers was however not equivalent. For example, the C24 ω-
hydroxy fatty acid was reduced two-fold and the C24 α,ω-diacid remained almost unchanged although both monomers are known to be mostly localized to the outer integument of the seed coat (Molina et al. 2008). Interestingly, the 9, 10, 18-trihydroxyoleate cutin monomer, which is largely specific to the embryo (Molina et al. 2006), was 60% reduced. This observation, together with the defects apparent in the cutin-like electron-dense layer of the seed coat surface (Fig 5F) indicate that the deposition of cutin polymer is also affected.

DISCUSSION

Here we used a reverse genetic approach to explore the functions of UDP-glucose:sterol-glucosyltransferase in plants. T-DNA mutations in UGT80A2 (At3g07020) and UGT80B1 (At1g43620) were identified and characterized in addition to the corresponding double mutant. Sterol derivatives SG + ASG were significantly reduced in ugt80A2 and ugt80B1 single mutants (Table S1). Hence, both UGT80A2 and UGT80B1 function in catalyzing the glycosylation of 24-alkyl-Δ^5-sterols in Arabidopsis. Moreover, FS + SE increased 26% in ugt80A2,B1 silique + inflorescence tissue relative to wild-type. In the same tissue SG + ASG content decreased 22-fold in ugt80A2,B1 relative to wild-type (Fig 1). These data implied a feedback relationship between the FS + SE content relative to SG + ASG in certain tissues. Phenotypic examination revealed that ugt80B1 caused the most notable phenotypes. Based on allelism tests, ugt80B1 corresponds to the previously unannotated transparent testa-15 mutant (Focks et al., 1999). HPLC analysis of flavanols and condensed tannins performed independently showed aberrant condensed tannin synthesis in ugt80B1 (Isabelle Debeaujon, personal communication), confirming staining results. The accumulation of suberin around the hilum region of the wild-type Arabidopsis seed, where the funiculus dissociates the seed from the parent, was ablated in the ugt80B1 mutant (Fig 5g and 5h). Reduction in the amount of suberin observed visually by autofluorescence was confirmed by GC-MS analysis (Fig 6). But moreover, GC-MS analysis also revealed that many of the structurally diverse lipid polyesters such as cutin and aliphatic suberin were reduced in ugt80A2,B1. Thus, we propose a membrane function for SG and ASG in the trafficking of lipid polyester precursors to form suberin and cutin in the seed.
Other transparent testa mutants have been characterized by forward genetics in *Arabidopsis* and these fall broadly into two categories: transcription factors and genes catalytically involved in the flavanoid biosynthesis pathway (Debeaujon et al., 2003). An atypical mutation leading to a transparent testa phenotype was found to map in a gene for plasma membrane H+-ATPase (AHA10) (Baxter et al., 2005). This mutation caused a 100-fold decrease in proanthocyanidins (PA) in seed endothelial cells. The deficiency in PA in *aha10* mutants was correlated with a defect in biogenesis of a large central vacuole (Baxter et al., 2005), yet the reason for the lack of PA accumulation was not clear. Hence, like *ugt80B1*, the *aha10* mutant appears to be distinct from other transparent testa mutants that ablate catalytic functions within the flavanoid pathway. *aha10* and *ugt80B1* may represent a novel class of mutants that indirectly cause a transparent testa phenotype due to membrane-related defects. In studies with animal cells, the gradient of SG and ASG content has been correlated with membrane thickness and the sorting of membrane proteins based on the lengths of the transmembrane domains (Bretscher and Munro, 1993). Alteration of the sterols in the membrane may result in aberrant behavior of an assortment of membrane proteins. In this scenario, alterations in the physical properties of the membrane dynamics such as reduced rate of exchange of SG and ASG between the monolayer halves of a bilayer may affect some aspect of flavanoid synthesis, such as transport or accumulation. Among proteins directly involved in flavanoid biosynthesis, chalcone synthase and chalcone isomerase are thought to be membrane anchored (Jez et al., 2000). Also, as yet unidentified proteins are presumably involved in the transport of flavanoids into vacuoles.

The sterol content of plant membranes has been observed to change in response to environmental conditions and it has been suggested that alterations in the sterol composition of the plasma membrane may play a role in the cold acclimation process (Patterson et al., 1993). Moreover, gene expression data from available microarray experiments (Genome Cluster Database) suggest that *UGT80B1* transcripts are slightly up-regulated by cold stress. The mutants described here provided an opportunity to test this hypothesis with respect to SG and ASG. We investigated the adaptive response to temperature stress but were unable to detect a significant difference between mutants and wild-type. Thus, although sterol content of membranes in *Arabidopsis* was modulated in
part by SG and ASG synthesis, loss of these membrane components did not appear to adversely affect growth or viability at low temperatures. The slightly reduced growth rate of the mutants at all temperatures indicates that steryl glycosides are important for growth and development, as might be expected from their widespread presence in plants. However, this role appears to be beneficial rather than crucial.

A significant motivation for the isolation of the *ugt80A2* and *ugt80B1* mutants was to test the postulate that SG functions in cellulose biosynthesis. The elongation defects of double mutant embryos (Fig 2) suggested possible defective cell wall biogenesis. Peng et al. (2002) observed the tight association of SSG with an amorphous form of cellulose and speculated that SSG may be a primer for the initiation of cellulose. We tested the hypothesis by measuring cellulose content in leaves, stems, roots, flowers, siliques and trichomes of the double mutant but found no significant differences between mutant and wild-type in cellulose content, and only minor alterations in the content of the sugars that comprise the other cell wall polysaccharides (Fig 3a and 3b). Therefore, if SSG biosynthesis by UGT80A2 or UGT80B1 has a role in cellulose biosynthesis, it was a dispensable role or was fulfilled by residual levels of SSG. The low residual level of SG in the double mutant also prevented a definitive answer to the question of whether plants require SG for viability and/or cellulose synthesis since it is possible that plants require only a trace amount of SSG. A possible source of residual SSG may arise from the activity of glucosylceramide synthase, which Hillig et al. (2003) demonstrated was capable of catalyzing the glycosylation of sterols *in vitro*.

Uptake of tetrazolium salt occurred far more readily in *ugt80B1* mutants suggesting less control of seed coat permeability (Fig 4b). A plausible explanation for the salt uptake phenotype in the *ugt80B1* mutant may be a direct result of reduced seed coat suberization in the hilum region (Fig 4g and 4h) and/or reduced cuticle formation at the surface of the outer integument. Cutin-like monomers are also known to be present in the inner integument and in the embryo (Molina et al., 2006; Molina et al., 2008). Lipid polyesters (cutin, aliphatic suberin) are structurally related biopolymers which are involved in controlling water relations (Pollard et al., 2008) and seem to have conserved features in their mechanisms of biosynthesis and export to the cell wall (Li et al. 2007). However, there was no obvious explanation as to why the reduction in SG and ASG
impacted hilum suberization as well as seed coat and embryo cuticle formation. Thus, we speculate that SG and ASG are necessary membrane components required for efficient lipid polyester precursor trafficking or export into the apoplast in plant seeds.

METHODS

Plant material and growth conditions

All Arabidopsis lines used in this study were of the Wassilewskija (WS)-O ecotype. Seeds were surface sterilized using 30% bleach solution and stratified for 3 days in 0.15% agar at 4°C. For phenotypic analysis and growth assays plants were exposed to light for 1 h and grown in either continuous light (200 mmol/m²/s) or complete darkness at 22°C on plates containing 0.5X Murashige and Skoog (MS) mineral salts (Sigma, St. Louis, MO) and 1% agar.

Identification of T-DNA insertions in UGT80A2 and UGT80B1

Homozygous T-DNA insertion mutations in both UGT80A2 and UGT80B1 were identified by screening the Wisconsin T-DNA collection by PCR as described in (Sussman et al., 2000). Screening primers for UGT80A2 result in amplification of 440 and 581 bp products in mutant and wild-type, respectively, with T-DNA primers JL214 5’-GCTGCGGACATCTACATTTTTT -3’, UGT80A2H245rev 5’-CTTCCCTGCAGAGATTTTGTCA-3’, and UGT80A2H245con 5’-ACGCATACGCAAATTTCGAGATA. Screening primers for UGT80B1 result in 595 and 455 bp mutant and wild-type products using T-DNA primer JL214 5’-GCTGCGGACATCTACATTTTTT -3’, UGT80B1rev 5’-ATTGGCATTGAGAAAGGTTAGAG-3’, and UGT80B1 con 5’-AGAATTGTGAAGTGGGTGATGG. A 55°C annealing temperature was applied. Homozygous lines for ugt80A2 and ugt80B1 were crossed and F2 progeny were screened for homozygous T-DNA insertions in both alleles.

Scanning electron microscopy

All imaging was performed on a Quanta 200 (FEI Company, Hillsboro, OR) scanning electron microscope fit with a 1000 mm GSED detector. Whole seed specimens
were mounted in cryo gel (Ted Pella, Inc., Redding, CA) on a temperature controlled stage set at 1º C and pressure was maintained at 652 Pa. Image annotation and linear contrast optimization was performed in Adobe Illustrator CS2.

**Transmission electron microscopy**

For analysis of mature seeds, seeds were dried after release from siliques. The seeds were placed at 4°C for 24 hours in a 10 μM solution of ABA to hydrate the seeds without inducing germination. Young seeds were extracted from immature siliques and were exposed to tetrazolium salts. Wild-type seeds that resisted staining with the salts and mutant seeds of an identical timepoint were selected for further EM processing. All fixation and embedding steps were performed through microwave processing. Samples were fixed in glutaraldehyde and dehydrated with acetone, infiltrated with a resin acetone mixture in a gradient increasing the amount of resin by 10% each step with an incubation time of 1.5 minutes per step, and polymerized overnight at 55°C, followed by sectioning, mounting, and staining with 2% uranyl acetate in methanol and imaging with a Tecnai 12 TEM (FEI, Hillsboro, Oregon).

**Histochemical analysis**

*Tetrazolium salt uptake.* Ability of seeds to uptake salt was tested by placing whole seeds in an aqueous solution of 1% (w/v) tetrazolium red (2,3,5-triphenyltetrazolium) at 30°C for 4-24 h. Seeds were removed from the solution and imaged by light microscopy.

*Ruthenium red.* Ruthenium red (Sigma, St. Louis, MO) was dissolved in water at a concentration of 0.03% (w/v) and seeds from wild-type and mutants were imbibed in this solution for 30 min at 25 °C. Seeds were removed from the imbibition solution and observed under a light microscope (Debeaujon et al., 2001; Debeaujon et al., 2003).

*DPBA.* Seedlings were stained for 15 min using saturated (0.25%, w/v) diphenylboric acid-2-aminoethyl ester (DPBA) with 0.005% (v/v) Triton X-100 and were visualized with an epifluorescent microscope equipped with an FITC filter (excitation 450-490 nm, suppression LP 515 nm).

*DMACA.* Seeds were stained with 4-(Dimethylamino)-cinnamaldehyde (DMACA) reagent (2% [w/v] DMACA in 3 M HCl/50% [w/v] methanol) for one week, and then
washed three times with 70% (v/v) ethanol. The stained pools were then examined using light microscopy.

_Ultraviolet induced fluorescence analysis of suberin localized at the seed hilum._ Seeds from wild-type WS-O, _ugt80A2, ugt80B1_ and the double mutant were examined under UV illumination for analysis of characteristic autofluorescence in the region of the seed hilum, reflecting suberin deposition (Beisson et al., 2007). Individual specimens were visualized under UV fluorescence using 10-40X objectives on a compound microscope (Leitz DMRB, Leica, Deerfield, IL).

**Sterol analysis**

Sterols and sterol conjugates steryl s (steryl esters (SE), SG and ASG) were isolated from wild-type, _ugt80A2, ugt80B1_ and double mutant plant tissues. Briefly, the dried plant material was ground with a blender in a mixture of dichloromethane/methanol (2:1, v/v). Metabolites were extracted under reflux at 70°C. The dried residue was separated by TLC (Merck F254 0.25 mm thickness silica plates) using dichloromethane/methanol/water (85:15:0.5, v/v/v) as developing solvent (one run) and authentic standards as mobility references. SE, free sterols (FS), SG and ASG were scraped off the plates. The dried residues of SE were saponified in methanolic KOH (6%) under reflux at 90°C for one hour. The dried residues of SG or ASG were submitted to an acidic hydrolysis in an ethanolic solution of sulfuric acid (1%). Sterols were extracted from hydrolysates after addition of half a volume of water with three times one volume of n-hexane. Dried residues were subjected to an acetylation reaction for 1 hour at 70°C with a mixture of pyridine/acetic anhydride/toluene (1:1:1, v/v/v). After evaporation of the reagents, steryl acetates were resolved as one band in a TLC using dichloromethane as developing solvent. Steryl acetates were analyzed and quantified in GC-FID using cholesterol as an internal standard. Structures were confirmed by GC-MS.

**Cell wall preparation and analysis**

Alditol acetate derivatives of the neutral sugars were measured on ball-milled (2 h) four-week-old primary stem tissue. Cellulose contents were measured colorimetrically
and total uronic acid content was determined by gas chromatography using 500 mg (dry weight) of ball-milled material as described (Blumenkranz and Asboe-Hanson, 1973; McCann et al., 1997).

**Analysis of seed lipid polyesters**

Soluble lipids were removed from 250-350 mg mature seeds using the procedure described by Molina et al. (2006). The dry residue was depolymerized using acid-catalyzed methanolysis (5% v/v sulfuric acid in methanol for 2h at 85°C). C17:0 methyl ester and C15 ω-pentadecalactone were used as internal standards. Monomers were extracted with 2 volumes of dichloromethane and 1 volume of 0.9% w/v NaCl. After aqueous washing, the organic phase was dried over anhydrous sodium sulfate and evaporated under nitrogen gas. The monomers were derivatized by acetylation or sylilation and separated, identified, and quantified by gas chromatography–mass spectrometry. Splitless injection was used, and the mass spectrometer was 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. Details of chromatographic conditions and aliphatic and aromatic monomer identifications are described in Molina et al. (2006).

**Whole mounts of embryos**

Whole-mount preparations were done by clearing ovules from wild-type and mutants in chloral hydrate solution made from an 8:3:1 mixture of chloral hydrate, H2O, and glycerol. Ovules were pooled from siliques into drops of chloral hydrate solution on microscope slides, followed by 24-hour incubation at room temperature. Histological analysis and microscopy of ovules were performed with a Zeiss Axioskop 2. Digital images of embryos were captured with an AxioCam HRc with AxioVision Rel 4.3 software (Carl Zeiss GmbH, Jena, Germany). Images were processed with Adobe Photoshop 8.0 and Illustrator 11.0.0 software (Adobe Systems Inc., Mountain View, California, USA).

**Accession numbers**

UGT80A2 (AT3G07020 Genbank number AY079032), UGT80B1 (AT1G43620,
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FIGURE LEGENDS
Fig. 1. Analysis of sterols and sterol derivates in ugt80A2, B1 mutant relative to wild-type. Total sterol derivaties were quantified and compared between wild-type and mutant plants in mg per gram dry weight as A) FS + SE and B) SG + ASG. FS+SE was the sum of FS measured by GC-FID + sterols measured by GC-FID released from SE fraction after saponification. Values are the mean of three replicates and experimental analysis was duplicated; error bars indicate standard error from the mean.

Fig. 2. Elongation defects in ugt80A2,B1 double mutant embryos. Nomarski images of embryogenesis during (A,B,C) globular, (D,E) heart, (F,G) torpedo, (H,I) bent-cotyledon, and (J) mature embryo stages. Each vertical panel exhibits the identical scale so that the sizes of the mutant embryos can be directly compared with that of wild-type (top row). Deviations from the wild-type morphology are first apparent at the late heart stage (F). The ugt80A2,B1 mutant displays elongation defects in outgrowth of the cotyledon primordia (yellow dotted lines). Elongation defects along the apical-basal axis are more obvious in the torpedo, bent-cotyledon and mature stages. (J) Red dotted lines indicate shorter hypocotyl and root lengths for ugt80A2,B1 at the mature embryo stage. Bars = 50 μm.

Fig. 3. Cellulose and cell wall analysis. (A) Cellulose composition was measured for wild-type WS-O and double mutant in various tissues. (B) Cell wall neutral sugar composition of the ugt80A2 and ugt80B1 single mutants was analyzed and compared
with that of wild-type WS-O plants.

**Fig. 4.** Transparent testa phenotype of ugt80B1 and ugt80A2,B1 mutants. (A) Light microscopy to visualize seed coloration in WT, ugt80A2, ugt80B1, rescue and ugt80A2,B1 (B) Tetrazolium red uptake in WT, ugt80A2, ugt80B1, rescue and ugt80A2,B1. (C) DMACA staining shows altered flavanol composition in WT, ugt80A2, ugt80B1, rescue and ugt80A2,B1. (scale bar = 150 μm)

**Fig. 5.** Scanning and transmission electron micrographs and suberin assessment of seed phenotype indicate aberrant lipid polyester production. (A,C) Wild-type seeds exhibit uniform cell shapes and well-formed columella. (B,D) double mutant seed with a collapsed region of the seed near the funiculus. Columella exhibit aberrant morphology (scale bar A,B = 100 μm; C,D = 15 μm). (E) TEM micrograph of the outer layer of a mature wild-type seed. An electron-dense cuticle covers the outer seed coat. The integuments have collapsed into a dense brown pigment layer (arrow). (F) Micrograph of double mutant seed at maturity. The electron-dense cuticle layer is absent (arrow) (scale bar = 1μm). (G) Suberin autofluorescence in wild-type seeds by illumination under 365 nm ultra violet light compared with (H) the ugt80B1 mutant, which lacks suberin accumulation (arrows) (scale as in A,B). En – endosperm, Bpl – brown pigment layer, ow – outer cell wall, Ext – exterior, ed cyt – electron dense regions in the cytoplasm

**Fig. 6.** Lipid polyester monomers from seeds of wild-type and ugt80A2,B1 plants. The insoluble dry residue obtained after grinding and delipidation of tissues with organic solvents was depolymerized by acid-catalyzed methanalysis and aliphatic and aromatic monomers released were analyzed by GC-MS. Values are means of four replicates. Error bars denote standard deviations. DCAs, dicarboxylic acids; FAs, fatty acids; PAs, primary alcohols; br-, branched

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