STERYL GLUCOSIDES: A GENETIC APPROACH TO DETERMINE THEIR ROLE IN CELLULOSE SYNTHESIS AND LIPID METABOLISM IN *ARABIDOPSIS*

by

DANIEL FLOYD STUCKY

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Major Professor
Dr. Kathrin Schrick

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Abstract

Steryl glucosides (SGs) are a common conjugate of sterols found in the plasma membranes of most plants and fungi, but their cellular functions remain largely unknown. Glycosylation of the C3 hydroxyl group of the sterol nucleus is catalyzed by UDP-glucose:steryl glucosyltransferase 80 (UGT80) enzymes. Two genes encoding UGT80A2 and UGT80B1 are responsible for most SG production in *Arabidopsis thaliana*, while UGT80C1 presents a putative third enzyme. In *Arabidopsis*, seed imbibition signals the epidermal seed coat cells to secrete an encapsulating mucilage that consists primarily of hydrated polysaccharides. Cellulose has been identified in the inner layer of the mucilage, providing a convenient model to study cellulose synthesis since seed mucilage is dispensable for viability and pectin and cellulose staining dyes are readily available. A reverse genetics and biochemical approach was used to characterize the role of UGT80 enzymes and their impact on cellulose synthesis in seed mucilage. *ugt80B1* mucilage was found to have elongated cellulosic rays, but no defects in pectin synthesis. A double mutant of *ugt80B1* and *mum3-1*, a mutant allele of *CELLULOSE SYNTHASE 5* (*CESA5*), displays a novel phenotype with irregular cellulose patterning and extreme shedding of the pectinaceous layer surrounding the seed coat. The observed mucilage defects may be indicative of disrupted cellulose synthesis and a mechanistic relationship between SGs and the cellulose synthase machinery. UGT80A2 and B1 demonstrate glycosylation activity with a multitude of plant sterols. The two enzymes do display some substrate specificity, however, with UGT80A2 producing the large majority of sitosterol and stigmasterol glucoside compared to B1. UGT80C1 shows little or no sterol glucosyltransferase activity *in vitro* or *in vivo* and likely has evolved a different function from the two other genes. GFP:UGT80C1 expressed either from the constitutive 35S or from its native promoter was localized to lipid droplets and possibly chloroplasts as well, creating a new perspective on the role of the protein in plant lipid metabolism. This study extends the currently limited view of SGs as ubiquitous components of the plasma membrane to active regulators of cellulose synthesis in seeds. Evidence presented here changes the perceived role of the plant conserved protein, UGT80C1, from a putative steryl glucosyltransferase enzyme to having a function in intracellular lipid droplets.

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Dedication

I dedicate this work to my mother and father for their constant support and understanding through all of my time here. You are a big part of my life even though graduate school has severely hindered the time we are able to spend together. Also, I likely would have died from malnutrition during the writing of this thesis without the Subway gift cards they provided.

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Chapter 1 - Introduction

1.1 Cellulose in higher plants

Cellulose represents the most abundant biopolymer on Earth and is found in the cell walls of plants, most algae, and some bacteria. Its primary function in nature is to provide strength and structural support crucial to the organism's survival, but the polymer also plays roles in cellular growth, cell differentiation, and stress response (Somerville et al., 2004; Geisler et al., 2008). Cellulose has become an integral part of human society with far-reaching applications including paper production, food additives, agriculture, textiles, and incorporation into various chemicals (Somerville, 2006). Despite being one of the most common and important biopolymers in the world, a complete understanding of its synthesis and deposition is still lacking. Researchers have been struggling with this question for over 60 years, but have seen only considerable progress in the past decade. The model system *Arabidopsis* has contributed a great deal towards the identification and functional characterization of gene encoding enzymes involved in plant cell wall biosynthesis (Liepman et al., 2010). A full understanding of cellulose has the potential for huge improvements in plant-based products, crop biomass output, and herbicide development. In addition, about 180 billion tons of cellulose is produced every year in nature, creating incentive for research into biofuel utilization (Delmer, 1999; Sabba and Vaughn, 1999; Carroll and Somerville, 2009).

The plant cell wall can be divided into three distinct layers: the middle lamellae, primary cell wall, and secondary cell wall. The lamella is the outermost layer composed of pectins and hemicelluloses that serve as cellular adhesion molecules. Primary cell walls surround all growing cells, while secondary cell walls are only formed in a few specialized cells, such as vessel elements, within the primary wall. Both layers contain cellulose, but the secondary cell wall is thicker and contains a second structural polymer called lignin (Keegstra, 2010). The rigid structure of cellulose is the result of repeating glucose monomers linked by β(1-4) glycosidic bonds. These long, unbranched glucan chains connect through hydrogen bonds and Van der Waals forces to form microfibrils (Endler and Persson, 2011). One microfibril is thought to consist of 36 glucan chains and can serve as docking sites for other cell wall polymers such as hemicelluloses and pectins (Mutwil et al., 2008).

1.2 The CesA complex

A number of glycosyltransferases have been identified in *Arabidopsis*, but by far the most important in relation to cellulose synthesis are the *CesA* genes, which encode the catalytic subunits of cellulose synthase. Cellulose synthase is the main cellulose synthesizing machinery in higher plants. It is a large (>500 kDa), multi-meric rosette complex that utilizes UDP-glucose as a substrate (Doblin et al., 2002). According to a widely accepted model, one rosette consists of six subunits arranged with six-fold symmetry (Endler and Persson, 2011). Each subunit contains eight transmembrane helices, potentially forming a pore for the passage of growing glucan chains out of the cell (Richmond, 2000). With six rosettes each constructed from six CesA subunits, the cellulose synthase complex is believed to synthesize 36 glucan chains simultaneously to form a complete microfibril. This model is consistent with the observed sizes of microfibrils, however, there is other evidence to suggest a microfibril contains 18 or less glucan chains (Ha et al., 1998; Delmer, 1999; Kennedy et al., 2007). The subunits of cellulose synthase are manufactured in the endoplasmic reticulum and exported to the plasma membrane by exocytosis where they are assembled (Haigler and Brown, 1986). Deposition of cellulose to the cell wall is highly organized and controlled in part by the action of microtubules (Baskin, 2001; Emons et al., 2007).

1.2.1 Challenges in studying cellulose synthesis

Biochemical methods for studying cellulose synthases in plants have traditionally been very difficult for a number of reasons. The enzyme complex is embedded in the plasma membrane and attempts to isolate the protein yield an unstable form (Bessueille and Bulone, 2008). These synthase extracts not only produce very little cellulose in the presence of UDP-glucose *in vitro*, but also produce a sister β-(1→3)-glucan called callose in its place (Okuda et al., 1993). Callose represents a small percentage of the cell wall in comparison to cellulose, but has an important role in formation of the cell plate during cell division and also in response to infection and wounding stress (Guerriero et al., 2010). Callose is normally produced by callose synthase, an enzyme complex similar to cellulose synthase and utilizes the same substrate. For a while it was even thought that callose and cellulose synthases are actually the same enzyme and synthesize callose or cellulose depending on the presence of regulatory proteins and

conformational changes in the protein complex (Delmer, 1999). However, putative genes encoding catalytic callose synthase subunits have now been identified and are genetically distinct from their cellulose counterparts indicating that the two enzyme complexes are separate entities (Cui et al., 2001; Doblin et al., 2001; Li et al., 2003). Callose synthase typically co-extracts with cellulose synthase, making a pure analysis of the protein impossible. In addition, it is difficult to distinguish cellulose synthesis from callose synthesis without rigorous linkage analysis, due to the large structural similarity of the two polymers (Bulone, 2007). It is also still unclear how *in vitro* cellulose synthase enzymes synthesize callose at a much higher rate than cellulose, although some recent methods have been successful in reducing callose output (Colombani et al., 2004; Cifuentes et al., 2010). On top of all these challenges, the conditions for synthesizing cellulose *in vitro* also need to be optimized according to plant species.

With the complications of studying cellulose synthesis in a lab setting, it is very important to select an ideal model system. Because biochemical approaches for understanding the process have fallen short, researchers have turned to molecular genetic techniques as a solution. The completion of the *Arabidopsis* genome sequence coupled with an extensive online database makes it the organism of choice for this field. The *Arabidopsis* genome contains more than 730 genes encoding putative glycosyltransferases or glycosyl hydrolases, along with several hundred more genes encoding other proteins implicated in cell wall synthesis (Henrissat et al., 2001). To the best of our knowledge, all of the cellulose synthase subunit encoding genes have been identified in *Arabidopsis* along with numerous accessory proteins involved in the process. The wealth of available information and genetic tools offered by *Arabidopsis* makes it an ideal system for studying the complex process underlying cellulose synthesis.

1.2.2 CesA components in Arabidopsis

The protein subunits that make up the cellulose synthase complex vary from tissue to tissue and whether they are producing cellulose in the primary or secondary cell wall. The *Arabidopsis* genome contains ten cellulose synthase (*CesA*) genes with an obligatory minimum of three different CesA proteins in a functional rosette. Most of the *CesA* genes were identified in *Arabidopsis* by forward genetic screens. *CesA1, CesA3*, and *CesA6* make up the CesA complex in the primary cell wall and mutants of these genes, along with other genes involved in

primary cell wall synthesis, are characterized by dwarfism, sterility, short and swollen hypocotyls, ectopic lignin accumulation, and stunted root elongation (Hauser et al., 1995; Nicol et al., 1998; Schindelman et al., 2001; Desprez et al., 2002). *CesA1* and *CesA3* mutants are also gametophytic lethal due to defects during pollen development (Persson et al., 2007a). *CesA7*, *CesA8*, and *CesA9* are members of the secondary cell wall CesA complex and knock-outs are characterized by collapsed xylem vessels (Turner and Somerville, 1997).

1.3 Key players in cellulose synthesis

Cellulose synthase lies at the core of cellulose synthesis through the elongation of glucan chains at the plasma membrane, but there are also many other components required for normal synthesis and regulation in plants. There are two enzymes in *Arabidopsis* that produce UDP-glucose, the substrate of cellulose synthase. UDP-glucose pyrophosphorylase glycosylates UTP using glucose-1-phosphate, but nothing is known about its potential involvement in cellulose synthesis. Sucrose synthase (SuSy) presents a second enzyme that can synthesize UDP-glucose from sucrose and UDP and has been hypothesized to channel UDP-glucose directly to the cellulose synthase complex (Haigler et al., 2001). SuSy serves as a sink for sucrose levels in plants and is normally found in high levels in the cytoplasm allowing the products of its catalytic activity to be easily accessed for general metabolism (Quick, 1996). However, a form of SuSy was found to be associated with the plasma membrane as well, often at sites of high cellulose polymerization (Amor et al., 1995; Babb and Haigler, 2001; Persia et al., 2008). Overexpression of SuSy genes in poplar and tobacco result in increased plant biomass and cellulose levels (Coleman et al., 2009). It should be noted, however, that the increased biomass may also be the result of other cell wall polysaccharides incorporating the UDP-glucose in place of cellulose (Seifert, 2004). Conflicting with these results, an *Arabidopsis* quadruple *sus1 sus2 sus3 sus4* mutant lacking SuSy activity in all cells except the phloem resembled the wild-type and had no decrease in cellulose levels (Barratt et al., 2009). The dispensability of SuSy led researchers to search for another source of glucose that could provide sufficient amounts of the substrate in SuSy's absence. Invertases comprise a class of enzymes that hydrolyze sucrose into glucose and fructose, making them a plausible candidate for the job. When the two main cytosolic invertase genes expressed in roots, *INV1* and *INV2*, are knocked out in *Arabidopsis*, the plants become

smaller in size with severely reduced root growth. Furthermore, the mutant phenotype can be rescued with the addition of sugar, indicating that invertases may be a primary supplier of glucose to the cell wall (Barratt et al., 2009).

Cellulose synthesis has been connected to many other proteins as well, although most of their functions and extent of involvement remain elusive. Co-immunoprecipitation and pull-downs of epitope-tagged CesA subunits have failed to identify any binding partners for the complex (Desprez et al., 2007; Atanassov et al., 2009). Perhaps the best established component, after CesA and SuSy, is KORRIGAN (KOR), a membrane-anchored endo-ß-1,4-glucanase. An *Arabidopsis* hypomorphic mutant, *kor1*, exhibits dwarfism, reduced cell elongation, and reduced cellulose content. The more severe *kor2* allele amplifies these effects in addition to transmission electron microscopy images of abnormal cell plates and incomplete cell walls (Zuo et al., 2000). COBRA is another membrane protein encoding gene strongly connected to cellulose. Schindelman demonstrated that mRNA expression of COBRA drastically increases in cells entering the zone of rapid elongation, and mutations in the gene result in reduced crystalline cellulose content in their cell walls (Schindelman et al., 2001). Furthermore, there is increasing evidence for the importance of the cytoskeleton in the synthesis and regulation of cellulose. Fluorescently labeled CesAs in *Arabidopsis* cells were observed to move through the plasma membrane along tracks of co-labeled microtubules, supporting their role in cellulose deposition (Paredez et al., 2006). In this way, microtubules allow the CesA complex to move in a controlled manner throughout the membrane of the cell and polymerize cellulose microfibrils to areas in need. A second component of the cytoskeleton, actin, was found to aid in the transport of organelles carrying CesA subunits to regions of secondary cell wall thickenings in the xylem (Wightman and Turner, 2008). Actin interacts with SuSy *in vivo,* presenting a possible mechanism for directing its localization to CesA complexes (Winter et al., 1998).

1.4 Lipids in cellulose synthesis

Proteins play a central role in cellulose synthesis, but they are not the only class of biomolecules involved. There is increasing evidence for the importance of lipids, mainly sterols and their conjugates. Sterols are a type of steroid compound with a hydroxylated C3 atom on the A ring and often an alkyl side chain at the C17 position, which varies between sterols (Fig. 1.1).

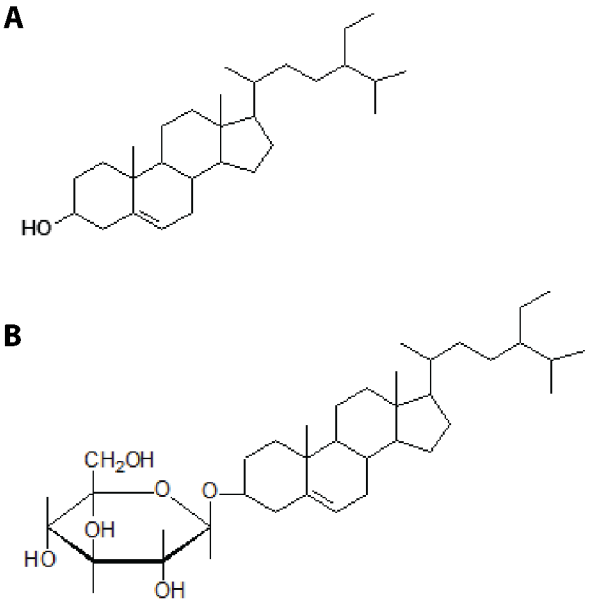
Arabidopsis and other higher plants synthesize a multitude of sterols, with sitosterol, campesterol, and stigmasterol representing a large majority of the total (Hartmann, 1998). These are synthesized at the endoplasmic reticulum and are actively transported throughout the cell, mostly to the plasma membrane (PM) where they become incorporated (Moreau et al., 1998). Campesterol is a precursor to the brassinosteroids (BRs), the only class of plant steroids known to act as hormones (Clouse, 2011; Schrick et al., 2012a). Sitosterol and stigmasterol are among the structural components of the PM and their proportions greatly influence its fluidity and permeability. However, their function at the PM was extended when Grandmougin-Ferjani et al. discovered that the sterol composition could modulate the activity of the membrane protein H+-ATPase in soybean, indicating plants may have the ability to regulate certain enzymes by altering the level of sterols in the PM (Grandmougin-Ferjani et al., 1997). Sterols were linked to the cell wall in characterization of the *Arabidopsis* sterol biosynthesis mutants, *fackel (fk)*, *cephalopod/sterol methyl transferase 1 (cph/smt1)*, and *hydra1 (hyd1),* which correspond to the enzymes sterol C-14 reductase, C-24 sterol methyltransferase, and sterol C-8,7 isomerase, respectively. These mutants exhibit abnormal sterol composition, embryogenesis patterning defects, and defects in cell division and elongation (Schrick et al., 2000; Schrick et al., 2002). Surprisingly, all three mutants were also found to have reduced cellulose content, which was confirmed with treatment of two sterol biosynthesis inhibitors, fenpropimorph and 15-azasterol. Detailed analysis revealed cell wall gaps, multiple nuclei, and irregular cell wall thickenings with lignin and callose deposition, while pectin levels appear normal (Schrick et al., 2004). BRs in *Arabidopsis* can regulate cellulose biosynthesis by upregulating expression of *CesA* genes through the interaction of the BES1 transcription factor (Xie et al., 2011). In contrast, BR deficient mutants exhibit zero to very mild decreases in cellulose accumulation, indicating the presence of an alternative, novel connection between sterols and cellulose (Schrick et al., 2004; Xie et al., 2011).

Plants synthesize a multitude of sterol conjugates of diverse structures. Steryl glucosides and acylated steryl glucosides are the most abundant free sterol derivatives in plants, but little is known about their functions (Grille et al., 2010). Of these, steryl glucosides (SGs) are the most common and are situated in the plasma membrane, representing over 30 mol % of the total lipids during freezing stress (Palta et al., 1993; Uemura and Steponkus, 1994). SGs are formed by the addition of a sugar moiety to the C3-hydroxyl of a sterol (Fig 1.1). UDP-glucose:sterol

glycosyltransferases (UGT) are responsible for catalyzing the reaction and their recent cloning has allowed the use of genetic approaches to study the biological functions of SGs (Grille et al., 2010). For a long time, SGs were not known to have any function in plants, but they have now been connected to various stress responses and also to cellulose synthesis (Webb et al., 1995; MurakamiMurofushi et al., 1997; Sakaki et al., 2001; Peng et al., 2002; Wewer et al., 2011). Their involvement with cellulose, however, is a point of debate and remains unclear and stagnant with conflicting evidence. Further research into the functions of SGs pertaining to cellulose has great potential to advance our understanding of a mechanism that has perplexed researchers for decades, yielding benefits in both crop and biofuel technology.

Figure 1.1. Sterol structure.

(A) Sitosterol and its glycosylated conjugate, (B) sitosterol glucoside. Images were modified from the AOCS Lipid Library.



Chapter 2 - Steryl glucosides influence cellulose deposition in *Arabidopsis* seed mucilage

2.1 Introduction

Steryl glucosides and cellulose synthesis

Genetic studies with *Arabidopsis* suggest that sterols and their conjugates may interact with the cellulose synthase machinery and influence deposition of the polymer in plants (Schrick et al., 2004; Schrick et al., 2012a). As the most common sterol conjugate, steryl glucosides (SGs) could play an essential role in this influence, but their function in cellulose synthesis remains unclear. SGs constitute a major component of membrane microdomains enriched in sphingolipids and sterols referred to as lipid rafts or detergent resistant membranes (DRMs), where cellulose synthase activity has also been linked (Laloi et al., 2007; Bessueille et al., 2009). DRMs are characterized by their isolation from cells which entails using the non-ionic detergent, Triton X-100, at 4°C due to their insolubility in solution compared to the rest of the membrane (Simons and Ikonen, 1997; Mongrand et al., 2004; Borner et al., 2005). However, whether DRMs represent biologically functional lipid rafts is a topic of debate in the scientific community. Studies reveal that detergent extractions can lead to artificial formation of DRMs and alter their compositions (Schuck et al., 2003; Lichtenberg et al., 2005; Tanner et al., 2011). Lipid rafts serve a crucial purpose by concentrating all the necessary proteins for a particular function, such as signal transduction, into a membrane subdomain (Leslie, 2011). SGs and their sterol precursors present in lipid rafts could function to maintain the structure and stability of transmembrane proteins, such as cellulose synthase. SGs have been identified in *Arabidopsis* DRM preparations at a 3-fold higher amount than the surrounding membrane (Laloi et al., 2007) and may play a role in proper function of the enzyme.

In the above model, SGs play an indirect role compared to their sterol counterparts. However, there is evidence that SGs could directly interact with the synthase complex. Using radiolabeled ^{14}C SGs, Peng et al. demonstrated that cotton fiber membranes synthesize sitosterol-cellodextrins (SCDs) from SGs and UDP-glucose *in vitro* (Peng et al., 2002). Furthermore, synthesis levels of SGs and SCDs were highest in membranes isolated from fibers at the stage of active secondary cell wall formation. Cellodextrins are precursors of glucan chains, so the

formation of sterol cellodextrins presents a direct link between SGs and cellulose synthesis. Upon further inspection, they found that the cellodextrins were incorporated into the glucan chains, while the SGs themselves, were not. Here, the SG serves as a primer where cellulose synthase attaches successive glucose monomers to form elongated glucan chains. From this model, it is appealing to assign KOR, a putative membrane endoglucanase, the function of cleaving SGs from the glucan chain (Zuo et al., 2000; Mølhøj et al., 2002).

Reverse genetic analysis of UGT80A2 and UGT80B1

Two UDP-glucose:sterol glucosyltransferase (UGT) genes, *UGT80A2* and *UGT80B1*, encode glucosyltransferase enzymes required for SG synthesis (DeBolt et al., 2009). T-DNA insertion mutants of *ugt80A2,B1* show significantly reduced SG production in seeds, but still maintain about 6-10% of the wild type SG levels (Schrick et al., 2012b). Interestingly, the double mutant exhibited a slow seedling growth rate and stunted cell elongation during embryogenesis, but no change in cellulose or sugar composition compared to wild type measured in *Arabidopsis* dry seeds, siliques, flowers, stems, trichomes, and leaves (DeBolt et al., 2009). However, according to the primer model, SGs interacting with cellulose synthase are recycled. Trace amounts of SGs could be sufficient to fulfill this priming function, but their role has not yet been adequately explored. Residual SG levels in the *ugt80A2,B1* mutant lead us to predict the existence of additional genes encoding enzymes with UGT function. BLAST results using UGT80A2 and UGT80B1 amino acid sequences revealed a putative UGT enzyme, designated *UGT80C1,* which will be discussed in greater detail in Chapter 3. In addition to UGT80C1, the 519 amino acid glucosylceramide synthase (GCS protein) is a second candidate enzyme encoded by At2g19880. Yeast mutants expressing heterologous cotton GCS were found to produce a small amount of SGs in addition to glucosylceramides (Hillig et al., 2003). This low SG production by GCS is reminiscent of the low levels of residual SGs found in *Arabidopsis ugt80A2,B1* double mutants, making it an ideal candidate. However glucosyltransferase activity of GCS has yet to be validated in a plant system, and needs further investigation.

UGT80A2 (At3g07020) is made up of 14 exons and 13 introns, encoding a 637 amino acid protein. The 615 amino acid UGT80B1 (At1g43620) protein shares 51.2% identity and 61.5% similarity to UGT80A2 and encompasses a genomic segment with the same number of

exons and introns. UGT80A2 has one predicted transmembrane helix according to TMpred software (Hofmann, 1993) and has been isolated from the plasma membrane, endoplasmic reticulum membrane, and golgi vesicles in plants (Hartmannbouillon and Benveniste, 1978; Yoshida and Uemura, 1986; Ullmann et al., 1987; Warnecke et al., 1997). UGT80B1 has two predicted transmembrane helices and assumed to be in the plasma membrane as well, but its localization has not been experimentally confirmed. T-DNA mutants of both *UGT80A2* and *UGT80B1* show reduced SG content in *Arabidopsis*, but sterol glycosyltransferase activity has only been verified *in vitro* using UGT80A2 with cholesterol and UDP-glucose as substrates (Warnecke et al., 1997). Glucose is the most commonly observed glycoside attached to sterols, but xylose, mannose, and galactose have also been identified (Grunwald, 1978; Iribarren and Pomilio, 1985; Warnecke et al., 1997). It remains unclear, however, whether UGT80A2 and UGT80B1 are specific to utilizing glucose or can also catalyze the glycosylation of sterols with these more rare substrates. *UGT80C1* (At5g24750) is a largely uncharacterized gene with 15 exons and 14 introns. The 520 amino acid protein has five predicted transmembrane domains and assumed to localize to the membrane, though this has yet to be determined.

Plant seed coat mucilage

When exposed to water, the seeds of *Arabidopsis*, among other higher plants, exude an encapsulating mucilage originating from the epidermal seed coat cells. The exact purpose of seed coat mucilage is uncertain, but it is speculated to have roles in germination and hydration, prevention of gas exchange, protection, and seed dispersal by attaching to substrates or animal vectors (Arsovski et al., 2009). Upon differentiation of the outer seed coat cells, mucilage is synthesized and secreted between the primary cell wall and plasma membrane along with the formation of a column-shaped cytoplasm at the center of the cell. A thick, secondary cell wall later displaces the cytoplasmic column to form a volcano-like projection called the columella (Haughn and Western, 2012). Imbibition causes the hydrophilic mucilage to expand and rupture the primary cell wall, allowing it to release from the seed coat cells and surround the seed (Western et al., 2000). The specialized seed coat cells are maternally derived and numerous transcriptional regulators have been identified to influence their differentiation, including *APETALA2, ENHANCER OF GLABRA3, GLABRA2, MYB5, MYB61, TRANSPARENT*

TESTSA8, and TRANSPARENT TESTA GLABRA1/2 (Jofuku et al., 1994; Rerie et al., 1994; Walker et al., 1999; Penfield et al., 2001; Johnson et al., 2002; Gonzalez et al., 2009; Li et al., 2009). Mutations in these genes result in abnormal seed coat cell morphology and/or mucilage production.

The mucilage is composed of a complex mixture of pectins, cellulose, hemicellulose, and proteins. Of these, pectins represent the large majority and are characterized as heterogeneous polysaccharides rich in galacturonic acid (Macquet et al., 2007). The highly substituted, branched polysaccharide, rhamnogalacturonan I (RG I), is the most prevalent pectin in *Arabidopsis* seed mucilage, consisting of an alternating backbone of (1 → 2)-α-l- rhamnose and (1 → 4)-β-d-galacturonic acid. Homogalacturonan (HG) presents a second important pectin composed of an unbranched chain of (1 → 4)-β-d- GalA residues, which are commonly methylesterified or acetylated (Haughn and Western, 2012). Cellulose has been detected in seed mucilage through the use of β-glucan binding fluorescent dyes such as pontamine scarlet (S4B) and calcofluor white, fluorescently labeled probes, and by exploitation of its birefringent properties (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). Hemicellulose is a polysaccharide similar to cellulose, but consisting of shorter chains of sugar monomers and many branches. They are found in high quantities in the primary cell wall and supposedly cross-link adjacent cellulose microfibrils to form a strong network (Obel et al., 2007; Eckardt, 2008). Xyloglucan is the most abundant hemicellulose in the primary cell wall of higher plants and this polymer was also recently detected in the mucilage and columella of *Arabidopsis* seed coat cells using immunofluorescence with a polyclonal antibody, suggesting a similar cross-linking function in mucilage (Young et al., 2008).

Seed mucilage can be separated into two domains: An outer, diffuse layer primarily constructed from unbranched RG I, and an inner, adherent layer densely packed with a complex pectin and cellulose network. The outer layer can be easily removed by shaking the seeds in water or treatment with a weak chelator. The inner layer, on the other hand, can only be separated from the seed coat by harsh agitation with a strong acid or by enzymatic digestion (Western et al., 2000; Macquet et al., 2007). This is due to cellulosic rays that radiate out from the tops of the columella and interact with a network of hemicelluloses and pectins. Unbranched RG I still comprises much of the inner mucilage layer, however, linkage analysis and immunofluorescence has also confirmed the presence of branched RG, HG, XG, arabinans,

galactans, arabinogalactans, and arabinoxylans.(Dean et al., 2007; Macquet et al., 2007; Young et al., 2008; Arsovski et al., 2009; Sullivan et al., 2011; Walker et al., 2011). The structural organization of mucilage and its adhesion to the seed is largely a result of the cellulosic rays, accounting for about 12 – 19% of the total inner domain (Macquet et al., 2007). Genetic mutants of mucilage cellulose often demonstrate nonadherent mucilage that easily falls off and structural defects. *CesA5*, for example, encodes a subunit of the CesA complex found only in epidermal cells of the seed coat and is required for the proper structuring of adherent mucilage (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). The receptor-like kinase proteins, FEI1/FEI2, and fasciclin-like GPI anchored extracellular arabinogalactan protein, SOS5, act on the same pathway to regulate cellulose synthesis in *Arabidopsis* roots (Shi et al., 2003). In addition, *fei2* and *sos5* seeds display a reduction in the cellulose rays across the inner mucilage, resulting in a loss of mucilage structure (Harpaz-Saad et al., 2011).

Seed mucilage presents an excellent model for studying secondary cell wall structure and function, despite some differences. Both are composed of cellulose, hemicellulose, pectin, and protein, though the composition differs between the two with mucilage being much more pectin rich compared to the cellulose rich secondary cell wall. However, the outer mucilage can be easily extracted from seeds with water for chemical analysis whereas, the preparation of cell walls from other tissues involves a rigorous treatment with KPO_4 and acetic acid (Zablackis et al., 1995). Furthermore, different cell types often have varying cell wall compositions. Mucilage stems from a single cell type, seed coat cells, but standard cell wall preparations contain multiple cell types even when derived from the same tissue, such as leaves (Haughn and Western, 2012). Furthermore, the seed mucilage is dispensable, allowing for reverse and forward genetic studies with no effect on the seed's viability or development, at least in a laboratory environment. Genetic manipulation of genes involved in cell wall production of other tissues, however, can have drastic consequences resulting in lethality or sterility, often making it an infeasible approach.

2.2 Materials and Methods

Plant Material and Growth Conditions

The *Arabidopsis thaliana* Columbia (Col) ecotype was used for all lines in this study.

Seeds were sown on soil and stratified at 4°C for 3 days before being moved to growth chambers. Plants were grown under continuous light at 23°C until ready for harvesting. For phenotypic analysis of growth and senescence, each tray of plants was given 500 ml of water every two days while under constant light. Images were taken 7, 14, 21, and 30 days after exposure to light. To determine seed masses, one hundred *Arabidopsis* seeds were counted and weighed as a group for each mutant background with 5 replicates. These were averaged and the mass/seed was calculated.

T-DNA insertions of UGT genes

Arabidopsis seeds containing T-DNA insertions for *UGT80A2*, *UG8T0B1*, and *UGT80C1* were obtained from ABRC Stock Center. Homozygous lines for these three alleles were crossed to obtain all possible double mutant combinations and a triple. The progeny were screened for T-DNA insertions by PCR genotyping using the primers listed in Table 2.1. *tt15* seeds were obtained from Christoph Benning and *mum3-1* seeds were a gift from Jonathan Griffiths and George Haughn. *ugt80A2,B1,C1* plants were crossed with a line homozygous for *mum3-1,* and the resulting progeny were screened for mutant combinations, particularly a *ugt80B1,mum3* line. The *mum3* allele was identified by restriction fragment length polymorphisms using the XhoI or DdeI enzymes from NEB after PCR amplification.

Cytochemical Probes

About 20 – 30 seeds were imbibed in 0.01% ruthenium red for 1.5 hours while gently shaking on an orbital shaker. Samples were washed with water and viewed with a Leica DFC295 stereo microscope equipped with a digital camera for imaging. To remove the outer mucilage, seeds were first imbibed in 0.05 M Na_2CO_3 for 10 minutes, washed with distilled water, and transferred to a 24 well plate for ruthenium red treatment as before. Removing the outer mucilage layer allows easy visualization of the rays radiating from the seed coat. 18 – 24 seeds were randomly chosen from each genetic background for quantification of ray lengths. The ten longest rays from each seed were measured using the segmented line tool in ImageJ. All data calculations and statistics in this study were carried out in Microsoft Excel software.

In order to observe differences in mucilage integrity between *ugt* mutants, seeds were subjected to mechanical stress similar to Sullivan et al. (2011). They were suspended in 800 µl of water in a 1.5 ml eppendorf tube and shaken vigorously head to tail every minute for 10 minutes. Ruthenium red staining was repeated to confirm whether the mucilage remained intact with the seed.

The techniques used to label mucilage cellulose were adapted from Willats et al. (2001a), Macquet et al. (2007), and Mendu et al. (2011). About 2.5 mg seeds were stained with 1 mg/ml calcofluor white (Sigma) for 5 minutes or with 0.01% w/v pontamine S4B (Sigma) in 100 mM NaCl for one hour. Seeds were washed 3x with distilled water and mounted on a 35 mm coverslip-bottom dish (BD Biocoat). Imaging was performed on a Zeiss 510 scanning laser confocal microscope. Calcofluor and pontamine S4B were visualized at 410 nm and 543 nm.

SEM

Arabidopsis seeds were sputter coated with a 60% gold - 40% palladium alloy using the DESK II SPUTTER/ETCH UNIT (Denton Vacuum). Images were taken using a Hitachi S-3500N scanning electron microscope fitted with a Robinson backscattered detector.

Immunochemistry

The specificities of the monoclonal antibodies and CBM used in this study have been extensively described (Willats et al., 2001b; Blake et al., 2006; Macquet et al., 2007; Young et al., 2008; Pattathil et al., 2010; Harpaz-Saad et al., 2011). The protocol for immuno-labeling of mucilage carbohydrates was adapted from previously described techniques (Blake et al., 2006; Young et al., 2008; Harpaz-Saad et al., 2011). Less than 5 mg of seeds were shaken in PBS pH 7.4 for 1 hour to first remove the outer mucilage. These were shaken again in PBS with 5% BSA for 30 min and washed in PBS. For immunolabeling, seeds were bathed in the monoclonal antibodies, CCRC-M36 or JIM5 (CarboSource; Athens, Georgia), for 1.5 hours at room temperature followed by another wash and treatment with the appropriate Alexa Fluor 488 secondary antibody (Life Technologies). CBM3a (PlantProbes; Leeds, England) labeling was completed in the same manner, but utilized a Penta-His Alexa Fluor 488 conjugate secondary antibody against it. As a negative control, immunolabeling was also carried out without primary

antibody. Lastly, seeds were counterstained with pontamine S4B in 100 mM NaCl PBS solution for 1 hour. Confocal images were taken with a 488 nm laser for antibody fluorescence and 543 nm for S4B fluorescence.

Neutral Sugar Analysis

Mucilage was extracted from 100 mg seeds by the ammonium-oxalate method (Mendu et al., 2011). Freeze dried mucilage was weighed, placed in a glass tube, and hydrolyzed with 1 mL of 2 M TFA containing 10mg/L myo-inositol (used as standard) for 3 hours at 120°C. After hydrolysis, the samples were transferred to 1.5 microcentrifuge tubes and centrifuged at high speed for 10 min at 4°C. 500 μL of the hydrolysate was transferred back to a clean glass tube and allowed to dry overnight on a needle evaporator using air. 1 mL of water was added to each dried sample to redissolve the sugars. The sample was then passed through a Chromacol 17-SF-021N 17 mm filter 0.2-μM nylon syringe filter into HPAEC-PAD vials.

Sugars were analyzed using high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA-10 anion-exchange column (4.6 by 250 mm; Dionex) using a pulsed amperometric detector (PAD; Dionex ICS 3000 system). Monosaccharides were eluted at 1 ml min−1 using water. Uronic acids were eluted using a linear saline gradient: 100 mM NaOH to 100 mM NaOH–300 mM sodium acetate over 20 min. Peak areas were calculated using the internal standard from each sample referenced to an 8 point calibration curve containing 0.5 – 100.0 mg/L of each monosaccharide and 10 mg/L of myo-inositol

2.3 Results

ugt80B1 mutants of Col ecotype exhibit transparent testa and reduced seed mass

ugt80A2, *ugt80B1*, and *ugt80C1* T-DNA insertion mutants were confirmed by PCR genotyping (Fig. 2.1), and double mutant combinations and a triple were constructed through the appropriate crosses. DeBolt et al. (2009) previously reported no change in the cellulose or neutral sugar levels of wild-type (WT) stems compared to *ugt80A2,B1* mutants. To investigate whether there is a growth defect in the triple mutant, germinating seeds were monitored for 30 days on soil under the same conditions (Fig. 2.2). However, no obvious differences were seen in

the growth or senescence for any of the genetic mutants. Seed phenotypes for each mutant combination were examined (Fig 2.1). *ugt80B1* seeds displayed a lighter seed coat, referred to as a transparent testa phenotype as reported for *tt15* (Focks et al., 1999). Furthermore, one hundred seeds were counted and weighed for each genotype to compare their masses. *ugt80B1* seeds and multiple mutants with *ugt80B1* have a mass reduction of about 30% with $p < 0.005$. This data is consistent with previously published data for *ugt80B1* allele in the WS ecotype (DeBolt et al., 2009).

Cellulosic rays exhibit abnormal patterning in ugt80B1 mutant combinations

Publicly available microarray data on the *Arabidopsis* EFP browser (Winter et al., 2007) shows up-regulated expression of all three *UGT80* genes in meristemic tissues, regions of high cellular growth and division . This is expected, as SGs are important pieces of the plasma membrane and would be incorporated into the new cells at these tissues. Interestingly, the data also reveals a significant up-regulation for *UGT80C1* in imbibed seeds and also decreases in *UGT80A2* and *UGT80B1* mRNA expression (Nakabayashi et al., 2005). These transcriptional differences, along with the previous discussed evidence, lead to the hypothesis that SGs play a role in the synthesis or deposition of cellulose in seed mucilage. Ruthenium red, a dye that binds to pectins, was used to visualize seed mucilage as shown in Figure 2.3 (Sterling, 1970). Seeds were then treated with a salt to remove the outer mucilage, allowing for improved visualization of the cellulosic rays. *ugt80A2* and *ugt80C1* seeds resembled wild-type, but *ugt80B1* mutant combinations displayed elongated rays extending from the seed coat. Quantification of the ray lengths indicated significantly longer rays in *ugt80B1*, *ugt80B1,A2* ($p < 0.001$), *ugt80B1,C1*, and *ugt80A2,B1,C1* ($p < 0.0001$), suggesting abnormal cellulose deposition (Fig. 2.4c).

Confocal analysis of cellulose rays in seed mucilage

Confocal microscopy with cellulose specific dyes was performed to ascertain whether the elongated rays comprise cellulose (Fig. 2.4). Triple mutant seeds retained the elongated rays when stained with both calcofluor white and pontamine S4B after first removing the outer mucilage. To ensure the elongated cellulosic rays were not an artifact of salt treatment, seeds were also visualized with S4B directly and found to exhibit the same phenotype in all *ugt80B1*

mutant combinations, but *ugt80A2* and *ugt80C1* resembled wild-type. Finally, a second allele for *ugt80B1* called *tt15* was observed. Light microscopy analysis of ruthenium red staining verified the same phenotype for these seeds, establishing a novel function for UGT80B1 in mucilage cellulose deposition.

UGT80B1 is required for normal seed coat morphology but is dispensable for germination

The abnormal mucilage of *ugt80B1* seeds has no effect on their germination under laboratory conditions (Fig. 2.5). The seed coat cells are responsible not only for synthesizing the pectinaceous mucilage, but also for releasing it and holding it together by anchoring the cellulosic rays to their columella (Haughn and Western, 2012). Scanning electron microscopy (SEM) was used to search for any defects in seed coat morphology among the *ugt80* mutant combinations. Consistent with previous findings, *ugt80A2* and *ugt80C1* showed no differences compared to the wild-type seeds. However, *ugt80B1* mutant combinations displayed a collapsed funiculus, a stalk that attaches the ovule to the placenta, in some of the seeds. In addition, they also had reduced size and shallow columella (Robinson-Beers et al., 1992). It is not clear how the sunken funiculus ties together with the mucilage phenotype, but the abnormal cellulose deposition may impact columella morphology.

Analysis of mucilage carbohydrates in ugt80B1 seeds

Mutations in *MUCILAGE-MODIFIED 3* (*MUM3*) gene were found to be allelic to *CESA5* and display loosely held together mucilage due to a cellulose deficiency (Sullivan et al., 2011). Seeds were shaken in water to determine whether *UGT80B1* was also required for mucilage adherence to the seed coat, but very little mucilage was lost compared to the *mum3* control (Fig 2.6a). *mum3-1,ugt80B1* double mutants were constructed through the appropriate crosses and verified by PCR genotyping. Because *CESA5/MUM3* is required for cellulose production in *Arabidopsis* seed mucilage, *mum3* mutations were hypothesized to be epistatic to *UGT80B1*. This was tested by ruthenium red staining after first removing the outer mucilage with sodium carbonate (Fig 2.6). The results indicate that the double mutant contains elongated cellulosic rays, similar to the phenotype in *ugt80B1* seeds in contrast to *mum3* seeds that are

deficient in cellulose polymerization and lack the characteristic rays. Confocal analysis of pontamine S4B stained seeds confirmed the presence of cellulose containing rays in *ugt80B1,mum3* seed mucilage. However, the double mutants were not identical to *ugt80B1* and appeared to possess fewer rays overall, although this has not been quantified. In addition, the double mutant seeds exhibited abnormal surface features and shedding of a layer around the seed coat that stains positive with ruthenium red.

To reveal any discrepancies in the mucilage carbohydrate composition, two common polysaccharides were labeled using monoclonal antibodies and Alexa Fluor. Homogalacturonan (HG) and Rhamnogalacturonan I (RG-I) were monitored with JIM5 and CCRC-M36 respectively in adherent seed mucilage. The *mum3* single, *ugt80B1* single, and double mutant all showed decreased HG, but it was associated with the cellulosic rays when these could be visualized and appeared tightly wrapped around them. This observation suggests that the decreased HG levels are likely a consequence of abnormalities in cellulose structure, which prevent the HG from adhering to the seed coat, instead of a defect in HG production itself. RG-I is the most abundant pectin in *Arabidopsis* mucilage and appears relatively unaffected by the absence of CESA5 or UGT80B1 with the exception of reduced width in the adherent mucilage layer in *mum3* seeds compared to wild-type. This finding has been previously described and is associated with reduced cellulose (Sullivan et al., 2011). The immunofluorescence data is supported by neutral sugar analysis of *ugt80* seed mucilage. None of the analyzed sugars showed a significant change with the loss of *UGT80* genes, consistent with a function for steryl glucosides in mucilage structure relating to cellulose.

2.4 Discussion

No cellulose deficiencies are apparent in steryl glucoside depleted mature plants

Steryl glucosides have been linked to cellulose synthesis through their sterol counterparts as membrane components that can influence enzyme activity (Schrick et al., 2012b). They have also been proposed to act as a primer for forming glucan chains and initiate synthesis, however, there is currently no convincing evidence that solidifies their involvement by either of these mechanisms (Peng et al., 2002). The aim of this study was to elucidate the connection between SGs and cellulose through genetic and biochemical methods. In addition to the two previously

known sterol UGT80 enzymes in *Arabidopsis*, a putative third, UGT80C1, was identified for genetic analysis. Typically, factors disrupting cellulose synthesis, deposition, or regulation result in stunted growth, swollen tissues, drug tolerance, altered vascular morphology, or embryonic lethality (Somerville, 2006). None of the *ugt80* mutants, including the triple, displayed severe phenotypes during their growth or germination, indicating SGs are likely not involved in cellulose mechanisms in seedlings or mature plants. Stunted embryo growth has been observed in *ugt80A2,B1* seeds, however (DeBolt et al., 2009).

Role of steryl glucosides in seeds

Cellulose is also very prevalent in seeds and helps form the columella of seed coat cells and fibrous extensions in mucilage upon imbibition. In mucilage, cellulose promotes adhesion to the seed and provides structural integrity to the sticky secretion by acting as a scaffold for other polysaccharides to attach to or wrap around. This is why the cellulosic rays can be visualized with ruthenium red, which binds to pectins and not cellulose, even after removing the outer mucilage domain with salt treatment or vigorous shaking. Loss of cellulose in seeds is accompanied by shedding of mucilage, irregular cell uniformity, and reduced radial wall integrity (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011).

The transparent testa phenotype in *ugt80B1* seeds has been previously characterized by DeBolt et al. (2009) where a reduction in suberin and cutin production was identified by GC-MS analysis of seeds, leading to the hypothesis that SGs function in membrane transport of lipid polyester precursors for suberin and cutin production. The lack of suberin also made the seeds more permeable to salt, as they could not limit their uptake of tetrazolium salts. Transparent testa phenotypes are often the result of transcription factors or other enzymes involved in flavonoid biosynthesis, which are usually responsible for plant pigmentation (Quattrocchio et al., 1993; Debeaujon et al., 2003). The brown color of seeds is due to the presence of a class of flavonoids called proanthocyanidins and *ugt80B1* seeds were found to have reduced levels of proanthocyanidins despite the enzyme not being involved in the flavonoid pathway (Debeaujon et al., 2003). Similar to the cutin and suberin levels, the reduction in proanthocyanidin is likely the result of altered membrane composition, and may be due to disrupting proteins involved in synthesis and trafficking. This study did not address why *ugt80A2* and *ugt80B1* seeds have

reduced mass, though it appears not to be the result of a cellulose deficiency as *ugt80A2,B1* dry seeds have no change in their total cellulose content (DeBolt et al., 2009). A general defect in membrane activity and/or permeability may explain reduced growth. Embryos of these double mutants display elongation defects as well (DeBolt et al., 2009), but more research is needed to elucidate the role of SGs in development.

Abnormal cellulose patterning in ugt80B1 seed mucilage

The elongation of cellulosic rays reported here is a novel phenotype of *ugt80B1*. It is easy to visualize in imbibed seeds with ruthenium red, however, this dye is not a good representation of the presence of cellulose in itself as it binds pectins. One limitation of the dye is that it is also unable to stain very loose or disperse pectin. This is because it must bind to two adjacent polysaccharide strands instead of just one. To confirm whether the ruthenium red stained mucilage rays were comprised in part of cellulose, Pontamine S4B and calcofluor white dyes were used in conjunction with confocal microscopy. Calcofluor white has binding affinity for cellulose, arabinans, and pectic galactans while Pontamine S4B interacts with cellulose and to a much lesser degree, xyloglucans (Herth, 1980; Anderson et al., 2010). The appearance of abnormally elongated rays with both of these dyes in the mutant seeds verified the presence of cellulose and suggested a defect in the regulation or deposition of the polymer. Quantification of this defect leads to the interpretation that UGT80B1 is the major enzyme required for proper cellulose deposition in the mucilage in the UGT family. It is curious why its close genetic relative, UGT80A2, exerts no influence towards the phenotype despite similar mRNA expression patterns in seeds. Perhaps differences in substrate utilization or membrane localization could account for this. For example, UGT80B1 may be concentrated in areas of cellulose or other carbohydrate polymerization and influence the activity of associated membrane enzymes, while UGT80A2 remains spread evenly across the plasma membrane.

The main purpose of mucilage cellulose is adherence to the seed coat and structural integrity. *CESA5* encodes a protein subunit of the CesA complex found primarily in seeds. The *cesa5/mum3* allele disrupts polymerization of the cellulosic rays, allowing the mucilage to easily be detached with shaking. A similar phenotype was searched for in *ugt80B1*, but the seeds demonstrated very little difference from wild-type, indicating the elongated rays do not hinder

mucilage adherence. The reason for the abnormal seed epidermal cell morphology observed in *ugt80B1* mutants is unclear, but may be related to abnormal elongation of cellulose since these cells are responsible for its synthesis and deposition. It was previously determined that *ugt80A2,B1* whole seeds have no reduction in total cellulose (DeBolt et al., 2009) so the shallow columella observed by SEM does not reflect a decrease in cellulose, but is more likely the result of other defects that arise during embryonic development. In support of this notion, transmission electron microscopy (TEM) inspection of young, mutant seeds extracted from siliques revealed a greatly diminished electron dense cuticle layer around the seed compared to wild-type. Reduced suberin and cutin has also been confirmed by GC-MS as stated previously. Furthermore, pockets of electron dense regions were found in the cytosol of *ugt80B1* mutants, suggesting that the suberin, wax, or cutin components fail to be transported outside to the seed surface (DeBolt et al., 2009).

The phenotype of *ugt80B1,mum3* double mutants is difficult to explain. CESA5 is thought to be required for cellulose synthesis in *Arabidopsis* seed mucilage, as evidenced by the absence of rays in *cesa5* or *mum3* seeds. Despite a reduction in total number of cellulosic rays in the double mutant, a few elongated rays clearly remain present when stained with both ruthenium red and pontamine S4B, indicating that CESA5 is not strictly required their appearance. Ruthenium red also reveals a thin layer peeling off of the double mutants that is at least in part composed of pectin. However, immunofluorescence and neutral sugar analysis failed to uncover any differences in the levels of common mucilage polysaccharides for *mum3* or *ugt80B1*. To explain this result, it is proposed that the reduced cuticle layer of *ugt80B1* is deteriorated further in the double mutant and becomes completely absent. This would expose the underlying polar pectins on the surface of the seed coat, causing them to dissociate when exposed to water. In support of this hypothesis, ruthenium red staining of the seed bodies is less dense in the double mutants than the singles or wild-type, indicating there are less attached pectins to bind to. TEM and GC-MS analysis of *ugt80B1,mum3* seeds would need to be done to test this, however.

There are a number of possible explanations for the apparent elongation of cellulosic rays in *ugt80B1* seed mucilage. First, reduced SGs in the plasma membrane, particularly in lipid rafts, could influence the activity of the CesA complex. Lipid composition is important for many membrane enzymes and has been shown to have large effects on the activity of a few enzymes

such as H+-ATPase (Grandmougin-Ferjani et al., 1997). Perhaps reduced SGs in the membrane disrupt the stability of the CesA complex to make it more active or block a binding site for another protein. The membrane alteration may even influence other proteins that interact with the complex and not actually affect CesA directly at all. A second possibility is that SGs act as a sponge to control glucose levels in the seed. Here, UGT enzymes would normally glycosylate sterols to limit the glucose substrate availability for CesA and thus act as a regulator for cellulose synthesis. In this model, *ugt80* plants would have greatly reduced SG and increased free glucose content, which could drive CesA to expand the cellulosic rays in the mucilage by adding successive glucose monomers. Thirdly, SGs may play a very indirect role in cellulose deposition by disrupting membrane trafficking. This may lead to serious defects in development such as the embryo elongation defects, flavonoid pathway, and cuticle formation discussed earlier. It is quite possible that altered plasma membranes disrupt the flow of materials or signaling components involved in cellulose deposition. In addition, there is some evidence to indicate that lipid rafts associate with plasmodesmata (PD), membranous pores that traverse cell walls to connect adjacent cells (Tilsner et al., 2011). Turning off SG production could alter the lipid composition of the lipid rafts or the PD membranes themselves causing decreased PD diameters or unstable connections between cells.

Future perspective

The limited understanding of cellulose synthesis and regulation in general, makes it difficult to elucidate the roles of SGs in this complicated mechanism. It is still not known whether cellulose synthesis is increased in *ugt80B1* seeds or whether the deposition of the polymer is affected to construct longer rays. Linkage analysis as well as measuring cellulose content in imbibed wild-type and mutant seeds by an anthrone assay present appropriate experiments to address this question. Checking a second *CESA5* mutant allele such as *cesa5-1* or *cesa5-2* would also be a good idea to confirm whether the *ugt80B1,mum3* phenotype is true for any defect in the gene or specific to the *mum3* allele. In addition, the absence of SGs in the lipid membrane may affect the localization of the CesA machinery. To test this hypothesis, *CESA5*-GFP may be imaged by confocal microscopy in live wild-type and *ugt80B1* mutant seeds of developing plants to discern any discrepancies in its subcellular localization. Lastly, a forward

genetics screen could be conducted with ethylmethane sulfonate-mutagenized (EMS) *ugt80B1* and *ugt80B1,mum3* plants for mutants with enhanced or completely absent cellulosic rays in the mucilage. This would identify genes potentially required for or associated with UGT80B1's connection to cellulose deposition and help elucidate the mechanism by which it influences the polymer in seeds.

Figure 2.1. *ugt80B1* seeds have a transparent testa and reduced mass.

(A) SALK T-DNA insertion lines were obtained for each *ugt80* mutant and were used for further crosses in constructing double and triple mutants. (B) Light microscopy of wild-type (WT) and *ugt80* seeds (scale bar = 500 µm). The seed coat of *ugt80B1* is lighter in color. (C) *ugt80B1* mutant combinations have reduced mass compared to WT seeds with *p < 0.005 (n=5 per genotype).

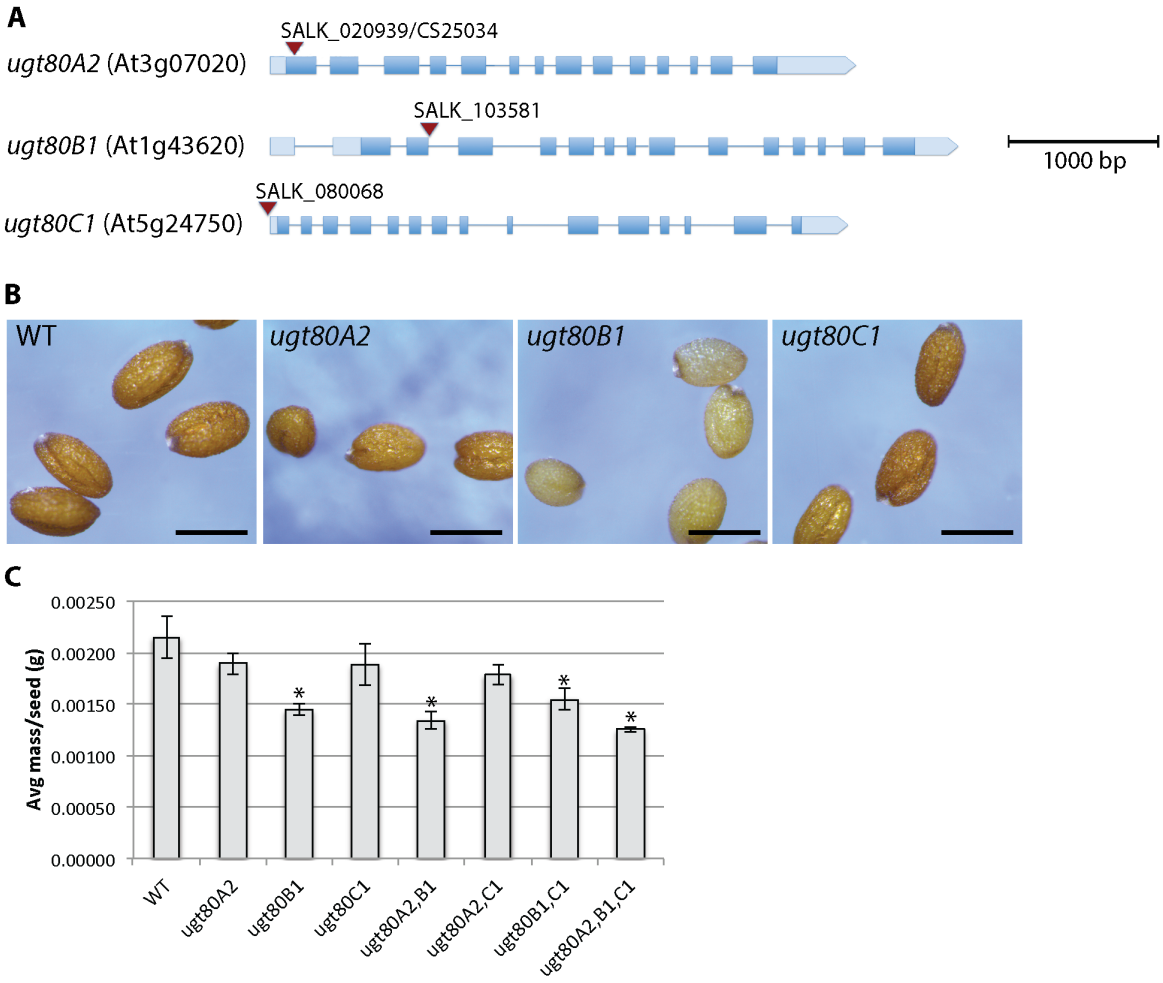


Figure 2.2. Plant growth comparison.

Arabidopsis ugt80 mutants were grown under continuous light for 30 days and monitored for differences in growth and senescence. No obvious defects among the mutants were observed in comparing mutants to wild-type (WT).

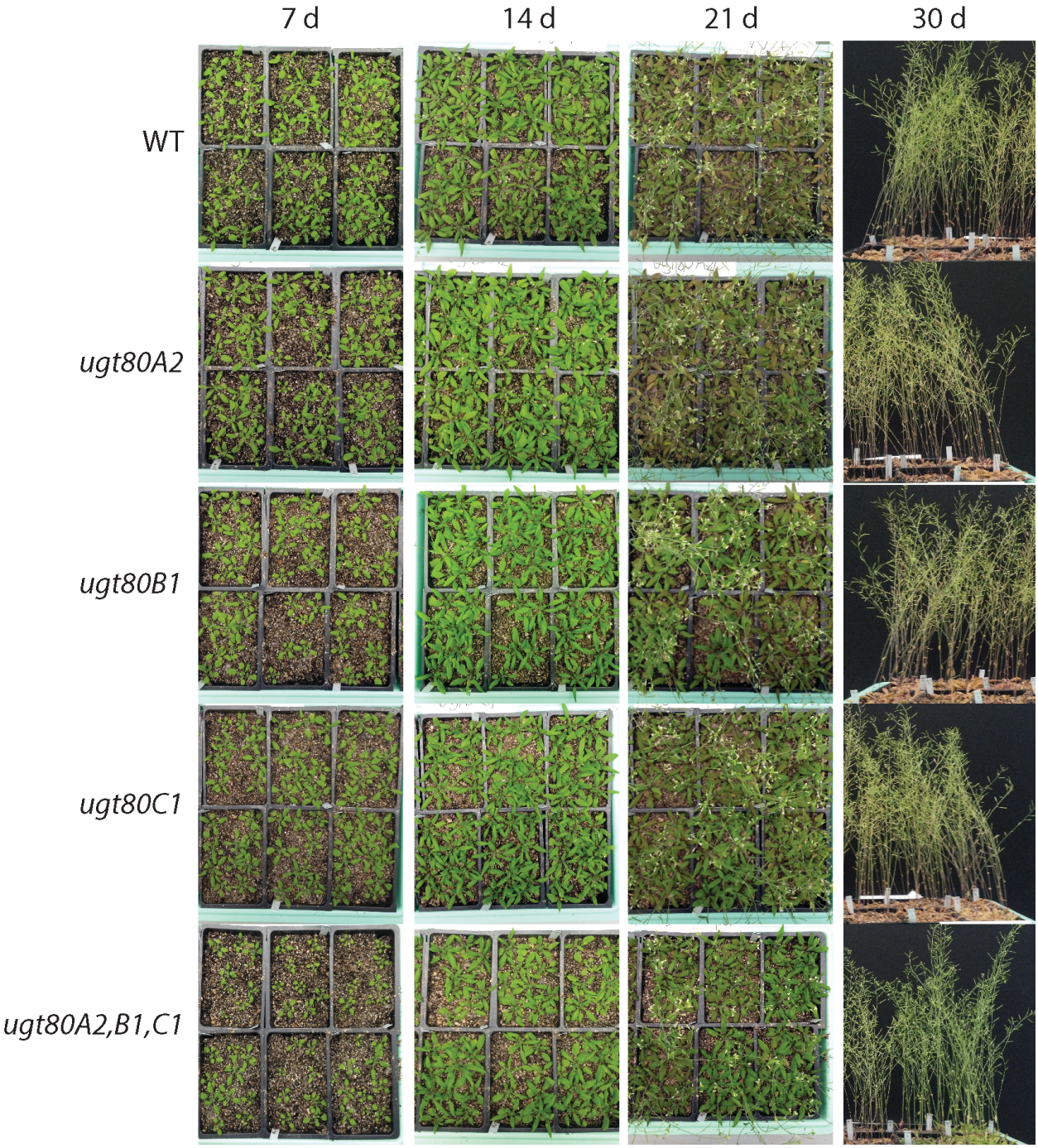


Figure 2.3. Light microscopy of *ugt80* seeds after treatment with 0.05 M Na_2CO_3 and staining with 0.01% ruthenium red.

(A) Wild-type (WT) seeds retain very little mucilage and display short, uniform rays across the surface. (B) *ugt80A2* (D) *ugt80C1* and (F) *ugt80A2,C1* seeds resemble WT. (C,E,G,H) Abnormal patterning of seed mucilage rays is observed in *ugt80B1* mutant combinations. Black scale bars represent 125 μm.

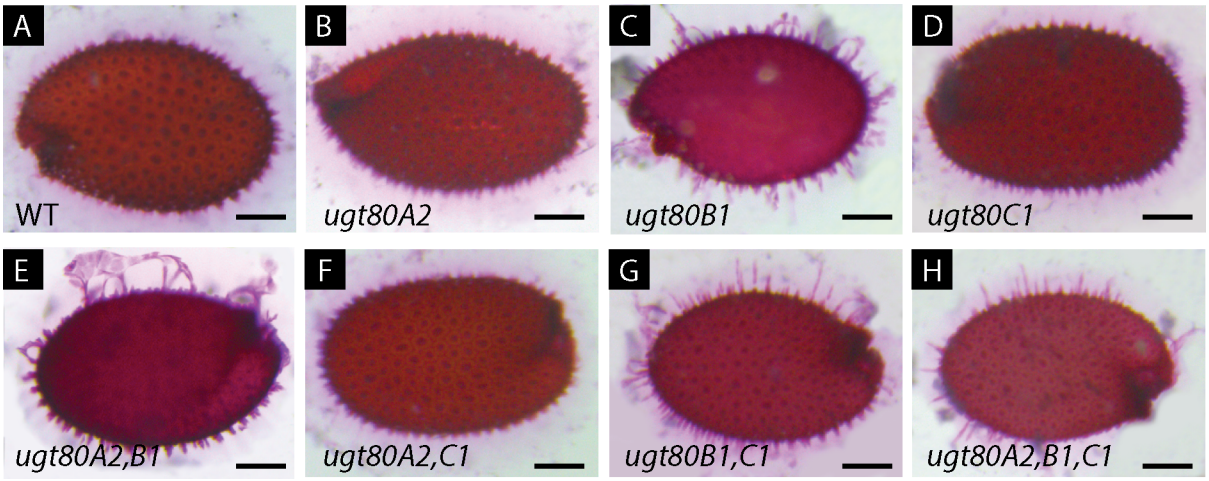


Figure 2.4. *UGT80B1* is required for proper patterning of cellulosic rays in seed mucilage. (A) Confocal laser scanning microscopy of adherent seed mucilage in wild-type (WT) and *ugt80* triple mutant seeds. Seeds were treated in 100 mM NaCl and stained with either calcofluor white (green) or pontamine S4B (red). Arrows indicate elongated cellulosic rays in the mutant. (B) *ugt80B1* mutant seeds stained with S4B and no NaCl treatment retain elongated ray phenotype, indicating the phenotype is not an artifact of treatment with salt. White scale bars represent 125 μm. (C) Comparison of cellulosic ray length in WT and *ugt* seeds. All *ugt80B1* mutant combinations show significant elongation compared to WT. Standard deviations are indicated for the ten longest rays per seed (n = 24 seeds). *$p < 0.001$ and **$p < 0.0001$. (D) Ruthenium red staining of a WT and *ugt80B1* seed imbibed in Na_2CO_3. *tt15*, a second mutant allele for the *UGT80B1* locus in the Col background, displays a similar mucilage ray phenotype as seen in the SALK_103581 allele. Scale bars represent 250 μm.

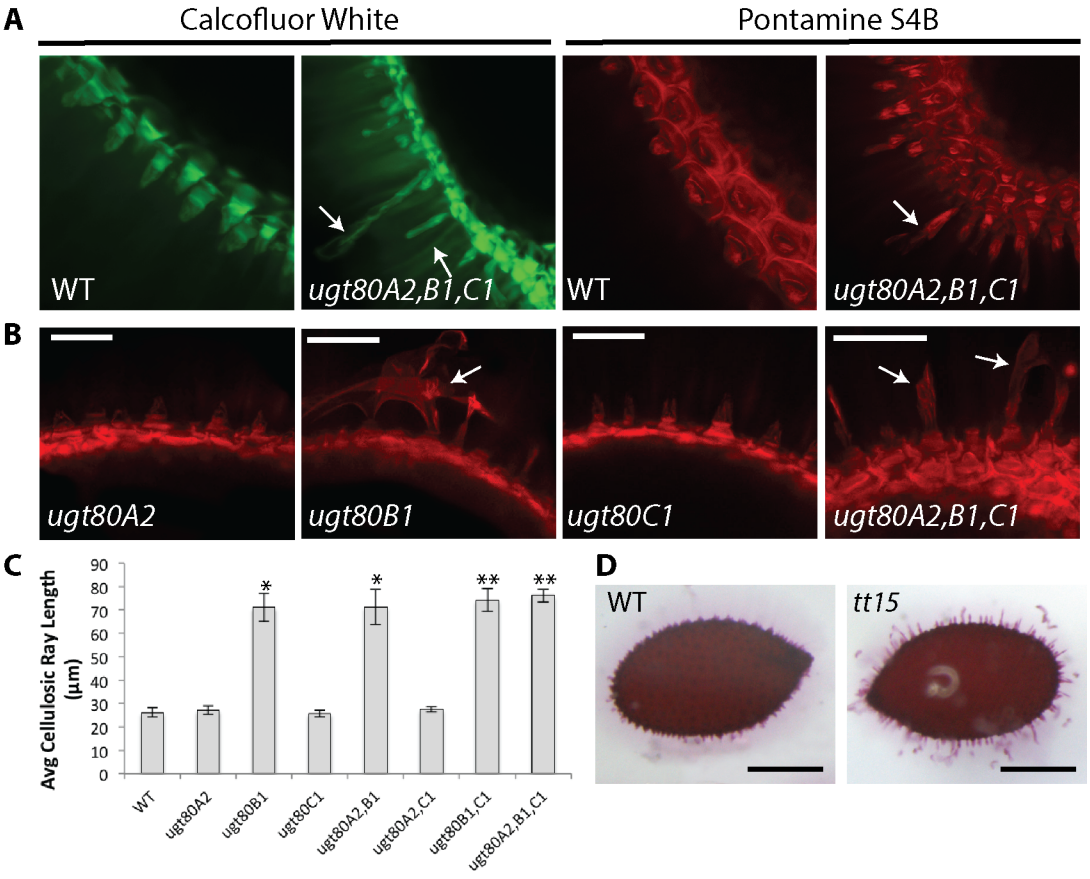
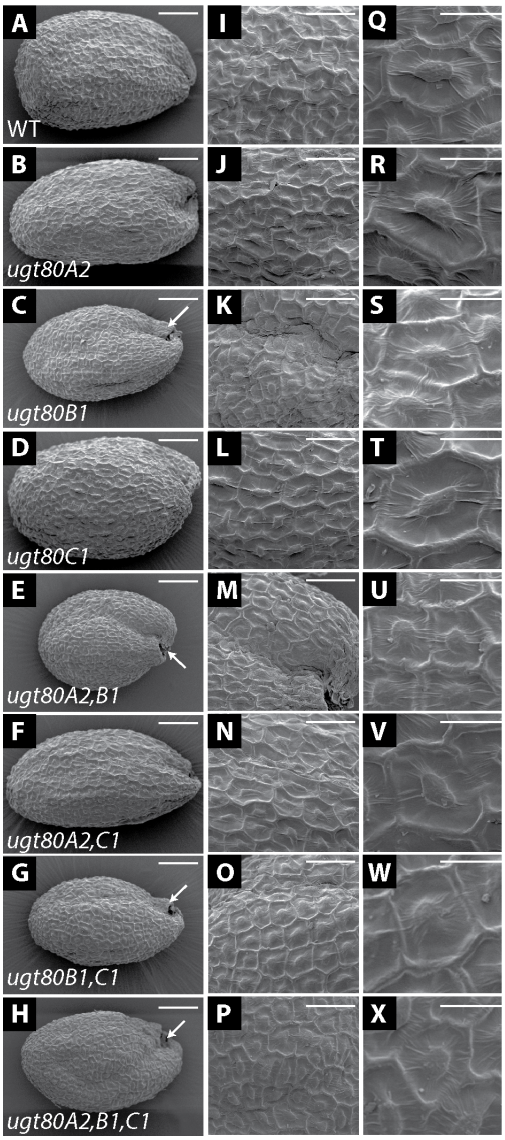


Figure 2.5. Scanning electron micrography of seed coats and germination efficiency.
(A-H) Whole seeds, (I-P) seed coat details, and (Q-X) single mucilage secretory cells with the columella in the center are shown. Wild-type (WT) seeds have uniform cells with well-formed columella and cell walls, while *ugt80B1* mutant combinations exhibit shallow columella and a collapsed region near the funiculus (indicated by arrows). *ugt80A2* and *C1* mutant combinations display no apparent differences in comparison to WT seeds. Left column scale bars = 100 μm, middle = 50 μm, and right column = 25 μm. (Y) Germination efficiency was quantified after 4 days of light exposure and no significant differences were found among *ugt80* mutants (n=60).



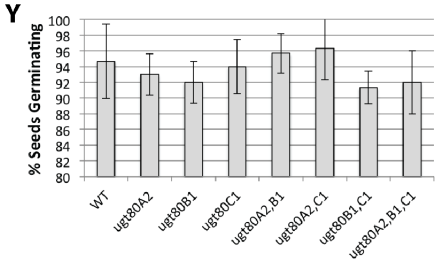


Figure 2.6. Cellulose and pectin labeling of adherent mucilage released from *ugt80B1* and *ugt80B1,mum3* seeds.

(A) *ugt80B1* mucilage remains adhesive to the seed coat after shaking in water. Seeds were stained with ruthenium red (B) and pontamine S4B (C) after Na_2CO_3 treatment. Immuno-labeling of (D) homogalacturonan (JIM5) and (E) rhamnogalacturonan I (CCRM-36) with the specified monoclonal antibodies is shown. Cellulose was stained with S4B after antibody treatment and visualized with confocal microscopy. Negative controls for confocal analysis were treated without primary antibody to account for background and autofluorescence, but very little was seen. Scale bars represent 500 μm (B), 200 μm (C,E) and 50 μm (D). (F) Mucilage from wild-type (WT), *ugt80B1*, and *ugt80A2,B1,C1* seeds was extracted and neutral sugar levels were quantified (n=3). (G) A zoomed version of (F). GlcA = Glucuronic Acid, GalA = Galacturonic Acid.

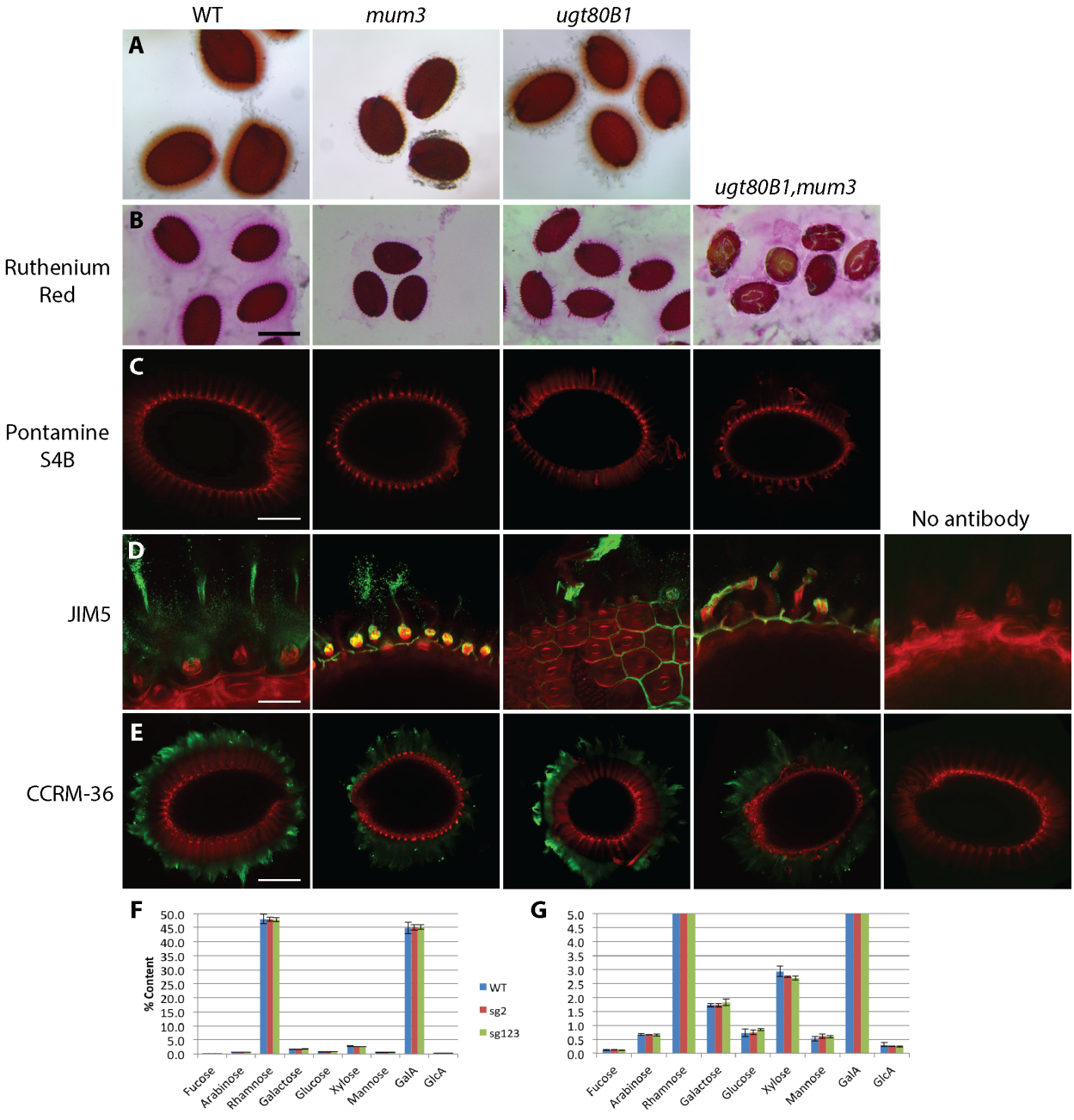

WT
mum3
ugt80B1
ugt80B1,mum3
A
B
Ruthenium Red
C
Pontamine S4B
No antibody
D
JIM5
E
CCRM-36
F
% Content
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
WT
sg2
sg123
Fucose
Arabinose
Rhamnose
Galactose
Glucose
Xylose
Mannose
GalA
GlcA
G
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fucose
Arabinose
Rhamnose
Galactose
Glucose
Xylose
Mannose
GalA
GlcA

Table 2.1. PCR primers for genotyping of plants.

Name	Sequence (5'-….-3')	Gene
USG1_020939_LP	TGG AGA CCT TTT GCG TGT AAG	*UGT80A2*
USG1_020939_RP	CTT AGC AGC ATC CTC AGC AAG	*UGT80A2*
SG2_103_R	ATT GTG TGG GTT TAG GCA GTG	*UGT80B1*
SG2_103_L	TGG GTA GAG CCA TTT CAT TTG	*UGT80B1*
LBb1.3	ATT TTG CCG ATT TCG GAA C	*UGT80A2/B1*
SGT3_080_R	TGG TTT GTT GTG AAG TGA TCG	*UGT80C1*
SGT3_080_L	TGG GTA GAG CCA TTT CAT TTG	*UGT80C1*
LBb1	GCG TGG ACC GCT TGC TGC AAC T	*UGT80C1*
CESA5_CAPS_XhoI_F	ATGCGATCAGTAACGGATATGACTCG**A**	*MUM3/CESA5*
mum3-1_CAPS_DdeI_F	ATGCGATCAGTAACGGATATGACTC**T**T	*MUM3/CESA5*
CESA5_CAPS_4600_R	ATCAAAGGCAGTCCAAGCCACATATC	*MUM3/CESA5*

***Bold** letters indicate base changes to create a restriction enzyme site in the resulting amplicon.

Chapter 3 - Genetic and biochemical characterization of UGT80C1

3.1 Introduction

Plants modify their cell wall composition

In order for cell growth and division to occur, plants must continually modify their cell walls (Somerville et al., 2004). Exposure to environmental stresses such as temperature, drought, salt, and pathogens add pressure on the plant to adapt their cell wall composition. In order for this cycle to occur, plants must receive signals from their environment that alter the expression of relevant genes. For example, in addition to providing structural functions, cell walls serve as a barrier to pathogens. Evidence for a glycan-activated signal transduction pathway occurs through the defensive response of uninfected plants to exogenous application of cell wall fragments (Vorwerk et al., 2004).

Transgenic mutants of *CESA4* and *CESA8* result in enhanced resistance to infection by the pathogenic bacterium, *Ralstonia solanacearum*, and the necrotrophic fungus *Plectosphaerella cucumerina* (Hernandez-Blanco et al., 2007). Both of these CESA subunits are required for proper secondary cell wall formation, raising a possible mechanism where plants can modulate their secondary cell wall in response to biotic stress (Taylor et al., 1999; Taylor et al., 2000; Persson et al., 2007b). Furthermore, *Populus* trees exposed to tension stress exhibit up-regulated *CesA* (*PtCesA*) expression along with accumulated cellulose and reduced lignin levels in cell walls of various tissues (Wu et al., 2000). Taken together, plants modify their cell walls in response to environmental stress, often through the increase or decrease of cellulose by *CESA* regulation. The intermolecular components involved in these mechanisms, however, remain unknown and may involve a lipid or sterol intermediate.

Lipids have been shown to have a major role in stress response by not only adapting the fluidity and permeability of their membranes, but also through signaling pathways usually involving brassinosteroids (Asami et al., 2005; Wang et al., 2006). In addition, genetic assays in *Arabidopsis* revealed three novel sterol biosynthesis mutants with defects in cell wall extension and expansion: *fackel* (*fk*), *cephalopod/ sterol methyltransferase 1* (*cph/smt1*), and *hydra1* (*hyd1*) (Lindsey et al., 2003). All three mutants also showed a deficiency in cellulose deposition, as discussed earlier, while sugar and pectin levels were unaffected, indicating sterols

play a direct role in the biosynthesis of cellulose (Schrick et al., 2004). SGs were found to have no effect on cellulose deposition aside from seed mucilage in Chapter 2, but it is possible that sterols and/or SGs play a more prominent role in regulating the activity of cellulose synthase only during periods of stress. *ugt80A2,B1 Arabidopsis* mutants are not altered in cold susceptibility compared to wild type, but sensitivities to other stresses are not reported (DeBolt et al., 2009). An understanding of UGT regulation and SG production in stressed plants can clarify their relationship to cellulose synthesis and provide insight into how plants adapt to changing environments.

The enigmatic role of UGT80C1

Little is currently known about UGT80C1 (At5g24750), an uncharacterized putative UDP-Glucose:sterol glucosyltransferase from *Arabidopsis*. In comparison to *UGT80A2* and UGT80B1, UGT80C1 is slightly smaller, encoding a 520 amino acid protein over a span of 15 exons and 14 introns. Protein sequence alignments show a 38% similarity and 23% identity with UGT80A2 and a 39% similarity and 21% identity with UGT80B1. Although UGT80A2/B1 are the closest genetic relatives to UGT80C1, their similarity is relatively low. UGT80C1 may have a unique sub-cellular localization, substrate utilization, be expressed in different tissues and/or phases of development, or even have a novel function compared to UGT80A2 and B1. TMpred software predicts the presence of five transmembrane domains in UGT80C1, making a lipid membrane association quite plausible (Hofmann, 1993). Microarray data shows upregulation of *UGT80C1* in imbibed seeds and meristematic tissue, which are regions of high cellular division and growth in plants (Nakabayashi et al., 2005)

Lipid droplets

The preliminary characterization of the putative UGT80C1 enzyme has led to the investigation of lipid droplets in *Arabidopsis thaliana*. All currently studied eukaryotic cells and a few prokaryotic cells contain small, cytoplasmic organelles called lipid droplets (Packter and Olukoshi, 1995). The existence of lipid droplets has been known for centuries when van Leeuwenhoek first observed them in milk through his primitive microscope in 1674 (Kernohan and Lepherd, 1969). It has only been in the last 10 years, however, that our understanding of the

function and composition of lipid droplets has made significant advances, due to increased interest from their implications in various metabolic diseases and commercial potential as food supplements, hormones, medicines, and biofuels. The protein and lipid composition of lipid droplets remains relatively well conserved from bacteria to humans and they are primarily responsible for energy storage in the form of neutral lipids. Triacylglycerols (TAGs) and sterol esters (SEs) represent the vast majority of the core, but other lipid derivatives have also been identified and can often vary between cell and tissue types (Goodman, 2008). The neutral lipid core is surrounded by a phospholipid monolayer with the hydrophobic acyl tails directed inward and embedded with specialized proteins (Tauchi-Sato et al., 2002). In *Arabidopsis* seeds, around 79% of a lipid droplet's protein content entails oleosins. These proteins range from 16 kDa to 24 kDa and contain a highly conserved hydrophobic domain that interacts with the lipids (Jolivet et al., 2004). They are thought to function in stabilizing the lipid droplets, preventing their aggregation, and possibly facilitating lipase binding to control metabolism in plants (Huang, 1996; Jolivet et al., 2004). Animals have similar structural proteins called adipophilin and perilipin, which are highly expressed in adipocytes (Than et al., 2003; Liu et al., 2004). In addition, an extensive proteomics study of *Arabidopsis* lipid droplets using nano-chromatography-mass spectrometry identified caleosins (similar to oleosins), a 11-β-hydroxysteroid dehydrogenase-like protein, a putative aquaporin, a glycosylphosphatidylinositol-anchored protein with no known function, and a calcium binding homologue designated ATS1 (Jolivet et al., 2004). To date, these are the only known cytosolic lipid droplet proteins in plants, though the list is expected to grow as new and more powerful genetic and biochemical techniques develop. However, there appear to be fewer lipid body proteins in plants in comparison to the hundreds of proteins that have been identified in mammalian lipid droplets. Plants lack most of the functional proteins found in mammals, such as metabolic and membrane trafficking enzymes, suggesting that energy storage and lipid homeostasis are the only functions of the lipid organelles in plants (Zehmer et al., 2009; Yang et al., 2012).

In congruence with this observation, lipid droplets are most abundant in the seeds where they provide necessary energy for germination. Afterwards, the presence of droplets greatly declines presumably due to their lipolysis for cell metabolism; however, they are still synthesized in low quantities in nonseed tissues (Murphy, 2001). For example, defects in the *Arabidopsis CGI-58* homologue, an enzyme that normally hydrolyzes triglycerides, result in the

hyperaccumulation of cytosolic lipid droplets in mature leaves (James et al., 2010). A similar effect is seen in mammals, known as Chanarin-Dorfman syndrome (Lass et al., 2006). Lipid droplets are first synthesized in the endoplasmic reticulum in all eukaryotic cells, but little else of their synthesis, degradation, and regulation is known in plants (Hsieh and Huang, 2005). Understanding the connection between lipid droplets and UGT80C1 could lead to benefits in both human nutrition and diesel fuel development.

3.2 Materials and Methods

DNA constructs

UGT80C1 and *GCS* (Glucosylceramide Synthase) cDNA were PCR amplified using the primers listed in Table 3.1. *UGT80C1* cDNA from the Ler ecotype was obtained from a cDNA library isolated from silique tissue. A Col cDNA clone (GSLTFB67ZA04) was also obtained from the French Plant Genomic Resource Center. *GCS* Col cDNA was provided by a cDNA clone from Ming Chen and Edgar Cahoon. The amplicons were cloned into the TOPO entry vector using the topoisomerase I enzyme and pENTR Directional TOPO Cloning Kit (Life Technologies). From the entry vector, the genes could easily be moved into a number of different expression destination vectors by LR recombination using Invitrogen's LR Clonase II Enzyme Mix. pENTR /D-TOPO entry clones for *UGT80A2* and *UGT80B1* were obtained from the SALK Library and Seth DeBolt, respectively.

UGT80C1 cDNA was also gateway cloned into the EGFP fusion destination vector, pK7WGF2. The UGT80C1 native promoter (~1.5 kb) was amplified from *Arabidopsis* Col genomic DNA with the following primers: UGT80C1_pro_F_SalI (5'-GAAATGTCGACAATTATCTATAGTCAGTA-CTG-3') and UGT80C1_pro_R_SpeI (5'-CGATAGCTTTCCTTCTCACTAGTTCCTC-3'). The amplicons were digested with SalI and SpeI enzymes and cloned into pK7WGF2 in place of the cauliflower mosaic virus 35S promoter. The construct was transformed into *Agrobacterium tumefaciens* strain GV3101 and used to transform *ugt80C1* plants by the floral dip method (Clough and Bent, 1998).

UGT80C1 sub-cellular localization

The protocol for visualizing lipid droplets closely resembles that described in James et al. (2010). Five-day-old seedlings expressing GFP were fixed in 50 mM PIPES pH 7.0 with 4% wt/vol paraformaldehyde for one hour. After washings, the seedlings were bathed in 4 μg/ml Nile red (Sigma) for 15 minutes on a rotational shaker and washed again. Nile red was imaged using laser scanning confocal microscopy with excitation at 543 nm using a He/Ne laser and emission at 568 nm, while GFP tagged UGT80C1 was excited at 488 nm with an argon laser and detected with a 505 - 530 nm band pass. The two images were merged to determine co-localization. Imaging with BODIPY 493/508 (Life Technologies) was prepared similarly to Nile red with excitation at 493 nm and emission detected with a band pass filter of 530 – 600 nm.

Lipid analysis of seeds

About 20 mg of wild-type and *ugt80C1* seeds were imbibed on moist filter paper for 0, 1, 6, 12, and 24 hours before heating in 75°C isopropanol with 0.01% BHT for 15 minutes. Total lipids were extracted from imbibed seeds as previously described (Schrick et al., 2012b). SG, phospholipid, and TAG content were analyzed for differences between wild-type and *ugt80C1* and also changes over the imbibition period using ESI-MS/MS. SG quantities were calculated based on PG(24:0) and PG(40:0) internal standards, TAG quantities were based on TAG(17:1) standard, and phospholipids were based on a PC/PE internal standard. Dry seed lipids were extracted similarly to imbibed seeds.

RT-PCR

Seed material from wild-type and *ugt80C1* was imbibed as before and frozen in liquid nitrogen. Seeds were ground with mortor and pestle and RNA extractions were done using the Ambion RNAqueous kit with plant aid solution followed by elimination of genomic DNA contamination using the Ambion TURBO DNA-free kit (Life Technologies). Transcript abundance was estimated for UGT80A2, UGT80B1, and UGT80C1 by using the gene-specific primers given in Table 3.1. RT-PCR was performed with iTaq universal SYBR green supermix (Bio-Rad) and 50 ng total RNA under the following conditions: 95°C for 3 min and 39 cycles of

95°C for 10 s and 56°C or 58°C for 30 s depending on the annealing temperature of the primers used. Actin related protein 6 (At3g33520) was used as a reference gene for data normalization.

Yeast UGT deletion and cloning of sterol glucosyltransferases

The *Saccharomyces cerevisiae* YLR189C knockout strain from Thermo Scientific contains a KanMX marker in place of the native *UGT51* ORF. Genomic DNA was isolated from a YLR189C culture and the cassette was amplified using primers flanking ~200 bp on either side of the *UGT51* ORF: YLR189C_KanMX_F (5'-CCCTGCTTACACCAGGGTTTATC-3') and YLR189C_KanMX_R (5'- GCACAACATACGGTACGTTTAGG-3'). The resulting fragment was used for homologous recombination following a standard LiAc transformation of the yeast protein expression strain, Y258 (Li et al., 2010). Positive knockouts were confirmed following selection on G418 plates and the presence of a PCR product of the expected length using the two previously mentioned flanking primers along with two primers within the KanMX module: KanB (5'-CTGCAGCGAGGAGCCGTAAT-3) and KanC (5'-TGATTTTGATGACGAGCGTAAT-3').

cDNAs for *UGT80A2*, *UGT80B1*, and *UGT80C1* TOPO entry clones were cloned by LR recombination into the pYES-DEST52 gateway destination vector, which contains the upstream *GAL1* promoter. All sequences were confirmed using the gene specific primers listed in Table 2.1 and then transformed into the Y258 *ugt51* mutant yeast. Candidate transformants were first identified on –URA dropout plates and confirmed after sequencing the isolated plasmids with the T7 primer.

Functional expression of UGT80 genes in S. cerevisiae and enzyme assay

An enzyme assay for sterol glucosyltransferase activity using expression in *E. coli* has been reported previously (Warnecke et al., 1997). A method for expression in *S. cerevisiae* was developed in this study. 200 ml yeast cultures containing the pYES-DEST52 plasmid with *UGT80A2* (NP_566297), *UGT80B1* (NP_175027), *UGT80C1* (NP_568452), or no gene cDNA at all were grown at 30°C to an OD_{600} ~0.700 in synthetic –URA dropout mix (Sigma) with 20% raffinose. Protein expression was induced with addition of 3X yeast peptone media with 20%

galactose. Cultures were incubated for 6 hours before harvesting by centrifugation at 5000 rpm at 4°C for 10 minutes.

Pellets were washed once with distilled water, resuspended in lysis buffer (20 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, 0.3 M $(NH_4)_2SO_4$) and lysed using a Mini Beadbeater (Biospec Products) with 3x30 second cycles at max rpm. Lipid membrane fractions were separated by ultracentrifuging the samples at 24000 rpm for 1.5 hours. The crude microsome pellet was resuspended in 0.5 ml lysis buffer. Assay mixtures consisted of 80 µl yeast lysate with 10 µl of either 4 mM cholesterol (C8667, Sigma) or a plant sterol mix (~4 mM cholesterol (C8667, Sigma), campesterol, brassicasterol, β-sitosterol (Sigma, S5753), and stigmasterol (Sigma, S2424). Three different substrates were used for each UGT80 enzyme: 3.6 µl of 10 mM UDP-glucose (CalBiochem, 670120), UDP-galactose (CalBiochem, 670111), and UDP-glucuronic acid (U6751, Sigma). Each reaction contained 100 µl total and was incubated at 30°C for 2 hours before quenching with 0.9ml 0.45% NaCl. Lipid extractions were performed as previously described (Bligh and Dyer, 1959). Samples were analyzed for SG production using ESI-MS/MS as before.

A similar enzyme assay was also conducted in *E. coli* using Col UGT80A2 and Ler UGT80C1 enzymes. A glucosylceramide synthase (GCS) enzyme containing a one base insertion at amino acid #364 results in a truncated 382 amino acid version of the protein and was used as a negative control. pENTR /D-TOPO clones were moved into the pDEST17 *E. coli* expression gateway destination vector by LR recombination. 12.5 ml cultures were treated with 0.4 mM IPTG AT 37°C for 3 hours to induce protein expression. Enzyme activity was determined by incubating with cholesterol and UDP-glucose as previously described (Warnecke et al., 1997).

Stress treatments on seedlings and seeds

All stress experiments were performed using half-strength Murashige and Skoog (MS) salts, 1% sucrose, and 0.8% plant agar except where specified. After stratification at 4°C for at least two days, seedlings were grown under continuous light under various conditions. Heat stress samples were immediately incubated at 45°C for 3 hours and then 22°C for five days (Wang et al., 2006). For zinc and osmotic stress, seedlings were grown in the presence of 1 mM

$ZnCl_2$ for 10 days (Wang et al., 2010; Xu et al., 2010) or 300 mM sorbitol for 5 days (Donaldson et al., 2004). Mucilage dehydration was performed by first imbibing seeds in water for 10 minutes followed by a 24 hour incubation at 30°C before being sown on water agar plates. Root lengths were measured using the segmented line tool in ImageJ software and germination was defined as emergence of the radicle from the seed (Schneider et al., 2012).

3.3 Results

UGT80C1 is conserved in higher plants

Amino acid BLAST searches reveal only one other gene in the *Arabidopsis* genome related to *UGT80A2* and *UGT80B1*, the putative steryl glucosyltransferase *UGT80C1*. Steryl glucosides are implicated in proper membrane structure and fluidity in nearly all plants. From this function, it is hypothesized to find high evolutionary conservation of UGT proteins across the genomes of multiple species. The evolutionary history of UGT80 proteins was investigated for comparison (Fig. 3.1). All three proteins are conserved among embryophytes. However, only A2 and B1 were found to have high sequence similarity to fungi as well. Very low similarity was seen in bacteria for all three enzymes and is likely due to an ancient common ancestor. The conservation of UGT80C1 to embryophytes suggests it has biological significance specialized to plants, and perhaps diverged from its sister *UGT* genes during the early evolution of land plants. N-glycosylation sites were identified in all three UGT80 protein sequences by SIB myHits (Hau et al., 2007), suggesting UGT80C1 is a glycosyltransferase enzyme, despite its low protein sequence similarity to UGT80A2 and B1.

Steryl glucoside depletion in ugt80 seeds

SG levels were measured in *ugt80* mutant seeds to examine whether there was an additive decrease in the triple mutant (Fig. 3.2). However, *ugt80C1* combinations had no reduction in SGs compared to wild-type seeds, indicating the protein likely does not have, or has very little, sterol glycosylation activity in seeds. Interestingly, *ugt80A2* seeds lost about 75% of their SG content while *ugt80B1* seeds had only about a 30% reduction. The double mutant retained only about 6% of the wild-type level of SGs, consistent with previously observed data (DeBolt et al.,

2009). UGT80A2 accounts for the large majority of stigmasterol and sitosterol glucoside production in seeds while UG8T0B1 appears to glycosylate most of the cholesterol, indicating the A2 and B1 enzymes do have substrate preferences. Brassicasterol and campesterol glucosides are only slightly less prevalent in *ugt80A2* seeds compared to *ugt80B1*.

Mucilage lipid compositional changes during imbibition

In a genome-wide microarray study, Nakabayashi et al. (2005) reported an upregulation of *UGT80C1* gene expression upon seed imbibition. Gene expression and lipid content was monitored in wild-type and *ug8t0C1* seeds over 24 hours of imbibition to determine whether the gene product functioned in early development after imbibition (Fig 3.3a). RT-PCR confirmed a 3-fold increase in *UGT80C1* gene expression that was not present in the *ugt80C1* seeds. About a 6 fold decrease was also observed in both *UGT80A2* and *UGT80B1* transcript levels. Loss of *UGT80C1* was not associated with a significant increase in *A2* or *B1* expression, which is characteristic of redundant genes. Despite upregulated *UGT80C1* transcription upon imbibition, no differences in the seed lipid profile were detected within 24 hours of water exposure. TAGs, and acylated SGs (ASGs) remained relatively unchanged in wild-type seeds, but the level of sitosterol glucoside and DGDG decreased after 24 hours. A small increase was also detected in MGDG within 24 hours, but the most drastic change in wild-type seeds was the composition of membrane phospholipids after just 1 hour of imbibition (Fig 3.3b,c). Phosphatidic acid (PA) is the primary polar lipid detected in dry seeds, but exhibits a 13-fold decrease after imbibition. PA is replaced by phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI), which all show a 14 – 6 fold increase after 1 hour, indicating that seeds strikingly modulate their lipid composition in response to imbibition.

Determination of UGT80 substrate utilization

All three UGT80 enzymes were expressed in yeast, crudely extracted, and mixed with a multitude of substrates to test for the production of steryl glucosides or glucuronides (Fig. 3.4). UGT80A2 and UGT80B1 were able to glycosylate all tested sterols using both UDP-glucose and UDP-galactose. Some substrate specificity was observed with the two, however. Both produce

more SGs when using glucose as opposed to galactose and thus likely prefer UDP-glucose *in vivo*. Also, both enzymes display high activity with cholesterol and brassicasterol, but only UGT80A2 can glycosylate sitosterol with high efficiency. These findings support the notion that a good deal of redundancy exists between the two proteins in plants, though some substrate preference is detected. Reduced levels of activity were detected with UGT80A2 and UGT80B1 enzymes in the presence of glucuronic acid. The enzymatic activity of UGT80C1 still remains unknown, as it was unable to interact with any of the substrates used. Ergosterol is the major sterol constituent of most fungi, including yeast. Although ergosterol was not added to any of the samples, the compound was certainly present in the reactions presumably as a constituent of the yeast microsomal fraction. Despite UGT80A2 and UGT80B1 being *Arabidopsis* proteins, the formation of ergosterol glucoside was detected at high levels in both reaction mixtures, solidifying their close evolutionary relationship to fungal sterol glucosyltransferases. However, these numbers are higher compared to the other analyzed sterols because a larger amount of ergosterol is likely present in the samples due to its abundance in yeast.

Expression of UGT80A2 and UGT80C1 in *E. coli* was consistent with the yeast results. Enzyme activity was determined as the relative quantity of cholesterol glucoside produced. UGT80A2 displays a 35-fold increase compared to the GCS negative control, while UGT80C1 has a very minor increase over GCS. The inability of UGT80C1 to efficiently glycosylate sterols *in vitro* using both a yeast and *E. coli* expression system, coupled with the observed WT SG levels in *ugt80C1 Arabidopsis* seeds, makes a strong argument for the protein to no longer be annotated as a UGT80 enzyme.

Subcellular localization of UGT80C1

Based on mutant lipid data and substrate specificity experiments, *UGT80C1* seems to be separate from its relatives and may not possess steryl glucosyltransferase activity at all. To help elucidate the protein's function, a GFP fusion was assayed for subcellular localization in *Arabidopsis* seedlings. UGT80C1-GFP under the control of the constitutive 35S promoter, exhibited a punctate spotting pattern in hypocotyls (Fig. 3.5). This fluorescence pattern is characteristic of lipid droplets in plants, can be detected by the neutral lipid binding dye, Nile red. Nile red only fluoresces when in a lipid rich, hydrophobic environment and is commonly

used for the visualization of intracellular lipid droplets. Fixed tissue showed strong overlap of the Nile red with GFP tagged UCT80C1, strongly indicating the protein localizes to lipid droplets. Plants expressing the same construct with the UGT80C1 native promoter in place of the 35S had a similar expression pattern as well. A 1.6 kb region upstream of the *UGT80C1* ATG translational start site was designated as the native promoter in this study.

To determine whether UGT80C1 influenced the relative size or abundance of lipid droplets, WT and *ugt80C1* mutant plants were stained with BODIPY 493/503, a second dye for staining lipid droplets with greater specificity than Nile red (Gocze and Freeman, 1994). No obvious differences were seen, suggesting UGT80C1 does not affect the stability or degradation of lipid droplets, but likely affects its lipid composition instead. It is noteworthy that many of the plants that were not fixed exhibited GFP expression that resembled chloroplast localization. Curiously, this was never observed when seedlings were analyzed after paraformaldehyde fixation. It is therefore possible that in live cells, UGT80C1 is also present in the membranes of chloroplasts or even components of chloroplast specific lipid droplets called plastoglobules or chromoplasts.

Stress profiling of ugt80 seedlings

A motivation for looking at *ugt80* mutants subjected to various environmental stresses was to test whether SGs are involved in cellulose synthesis under conditions of stress response. No latency in germination was observed in any *ugt80* seeds during dehydration or heat stress (Fig. 3.6). Root lengths revealed no increased sensitivity in response to osmotic or heat stress. Lastly, *ugt80* sensitivity to heavy metals was assessed by growing seedlings in the presence of excess zinc. No major differences were observed in root growth, however, seedlings double mutant for *ugt80A2* and *ugt80B1* showed extreme chlorosis, whereas single mutants and *ugt80C1* double mutants resembled wild-type with only very minor paler color. This defect is not thought to be the result of a cellulose deficiency, as no such phenotype has been described in cellulose related mutants thus far.

3.4 Discussion

Evolutionary history of UGT80C1

The function of UGT80C1 in plants was investigated further, as no connection was found between it and cellulose synthesis in Chapter 2. Whereas UGT80A2 and UGT80B1 share about 50% protein sequence identity with one another, UGT80C1 shares only about 22% with both suggesting it may have a unique function apart from its closest relatives. Higher evolutionary conservation in a gene's lineage is common among essential proteins in an organism, due to less tolerance of changes to the protein structure. The constructed phylogenetic tree showed conservation of UGT80C1 only in plants whereas UGT80A2 and B1 were also conserved in fungi (Fig 3.1). A molecular role for UGT80C1 specific to plants is consistent with a function different from UGT enzyme activity.

Lipid composition of ugt80 seeds

It is surprising that UGT80A2 is the primary producer of SGs in seeds considering *ugt80B1* seeds and not *ugt80A2* seeds display a strong phenotype in the mucilage and exhibit a transparent testa. It is possible that UGT80B1 synthesizes SGs that are more specifically localized to the relevant membrane proteins involved or perhaps the observed *ugt80B1* phenotypes are a consequence of the differential substrate specificity of the two enzymes. RT-PCR results revealed a reduction in both genes after exposure to water, which may be reflective of the hypothesis that SGs act to regulate the level of freely available glucose during mucilage cellulose synthesis. By reducing UGT80 levels, the seed is able to create a larger pool of UDP-glucose for cellulose incorporation instead of being soaked up by the sterols. The transcriptional upregulation of *UGT80C1* mRNA upon imbibition in seeds again sets it apart from *UGT80A2* and *UGT80B1*. This observation was first determined by genomic microarray experiments (Nakabayashi et al., 2005) and confirmed here using RT-PCR. In addition, *ugt80C1* dry seeds did not show any reduction in SGs, undermining its putative function as a sterol glucosyltransferase enzyme. The lipid composition was monitored after imbibition for 24 hours and failed to uncover any differences between *ugt80C1* and WT seeds. There could be a number of reasons for this. First, the correct lipid species that UGT80C1 interacts with may simply not have been scanned for in the MS analysis. For example, neutral lipids other than TAGs such as

steryl and wax esters were not measured. Also, UGT80C1 may not have any activity with lipids, but instead glycosylate a carbohydrate or even serve as a structural protein. A carbohydrate analysis of WT and *ugt80C1* seeds, similar to the neutral sugar analysis performed on *ugt80B1* seeds in Chapter 2, would be an appropriate experiment to address this question. Although UGT80C1 transcripts are increased in response to imbibition, the proteins may not be translated or become activated until the plant begins germination. Activation may entail the release of an inhibitor protein, posttranslational modifications, or proper localization. Despite no differences between the WT and *ugt80C1* seeds, MS analysis of polar lipids and TAGs did provide insight into lipid compositional changes during seed imbibition.

Seeds modulate their lipid composition in response to imbibition

Phosphatidic acid (PA) mediates signaling of abscisic acid (ABA), a plant hormone that inhibits germination (Katagiri et al., 2005). Thus, the high levels of PA seen in the WT and *ugt80C1* dry seeds signify the seeds have not yet begun germination. However, upon imbibition PA is likely catabolized into diacylglycerol by lipid phosphate phosphatase (LPP) enzymes or converted into other phospholipids, disrupting the ABA mediated repression of seed germination (Katagiri et al., 2005; Peters et al., 2010). Water exposure serves as the catalyst for eventual PA degradation, so plant seeds can ensure they will only germinate during periods of ideal growth conditions. PC, PE, and to a lesser extent PI have been shown to be the major phospholipids present in imbibed seeds in lima beans and peas (Delaroch.Ia et al., 1973). *Arabidopsis* shows a similar lipid profile as early as 1 hour after imbibition (Fig 3.2d), but importantly all three phospholipids were nearly absent in dry seeds indicating they are synthesized in response to imbibition. Presumably, this is because the newly germinating seeds will require phospholipids to incorporate into their growing plasma membranes though this has not been confirmed with radiolabeling.

Additionally, PC has previously been shown to increase in root-derived mucilage of wheat, maize, and lupin (Read et al., 2003). Furthermore, PC surficants on the root increase root hydraulic conductivity, reduce phosphate adsorption in soil, increase phosphate concentration in water solution so the roots can absorb more, and reduce net ammonium consumption and nitrate production in soil (Read et al., 2003). The ability of PC to alter the biophysical environment of

soil surrounding the root may be replicated by the mucilage in *Arabidopsis* seeds to aid germination and root growth.

UGT80A2 and UGT80B1 retain substrate specificity in vitro

The enzyme assay with UGT proteins reflects the SG data seen in the T-DNA insertion mutants. Warnecke et al. (1997) demonstrated UGT80A2 could glycosylate cholesterol using UDP-glucose *in vitro*, but it is now clear that A2 and B1 can also glycosylate a multitude of sterols using both UDP-glucose and UDP-galactose *in vitro* (Fig 3.3). This is very likely the case *in vivo* as well because cholesterol glucoside, campesterol glucoside, stigmasterol glucoside, sitosterol glucoside, and brassicasterol glucoside levels are reduced in *ugt80A2* and *ugt80B1* seeds with an additive decrease in double mutant seeds. Galacturonic acid (GA) is a sugar acid formed by the oxidation of D-galactose and represents the most abundant component of pectin in seed mucilage (Mohnen, 2008). UGT activity with GA was measured in part to determine whether UGT80C1 was able to interact with a unique substrate, and in part to see whether UGT80B1 could utilize the substrate and influence the pectin composition of *Arabidopsis* mucilage similar to its effect on the cellulosic rays. However, very little SGs or glucuronides were detected with any of the UGT proteins, indicating they are unable to interact with the substrate efficiently. No GA activity with UGT80B1 supports the carbohydrate data in Chapter 2 where no differences were detected in *ugt80B1* mucilage sugars, further confirming the protein's impact on seed mucilage is specific to cellulose and not pectin synthesis. The enzymatic activity of UGT80C1 still remains unknown as no substrate combinations were found to interact with the protein, which is consistent with the SG *in vivo* data as well.

A role for UGT80C1 in lipid droplets

The localization of UGT80C1 to lipid droplets (LDs) in *Arabidopsis* seedlings is the first insight into its function. It is not clear what its function in LDs is, but BODIPY 493/508 and Nile red staining did not reveal any differences in their abundance in the T-DNA insertion mutant. This suggests the protein does not facilitate structural stability or lipid metabolism of the droplets because under this hypothesis mutant plants would be expected to have decreased LDs as a result of their degradation. Furthermore, LDs often self-associate and fuse to form larger

globules (Wolins et al., 2006; Bostrom et al., 2007). Although still largely unconfirmed in plants, lipid droplet fusion has been shown to involve many proteins including SNARES, motor proteins, microtubules, perilipins, and FSP27 in animals (Bostrom et al., 2005; Marcinkiewicz et al., 2006; Bostrom et al., 2007; Jambunathan et al., 2011). If UGT80C1 facilitated association with other LDs, then larger LDs, or smaller LDs if C1 inhibited fusion, would be observed in *ugt80C1* plants. However, aggregation of LDs was not observed in mutants and no obvious size differences were seen between wild-type and mutant seedling LDs. Thus, the role of UGTC80C1 in LDs appears to lie elsewhere. Characterization of other *UGT80C1* mutant alleles would strengthen this argument because the *ugt80C1* allele may still produce low levels of functional protein. The T-DNA insertion for *ugt80C1* is located in the 5'-UTR and it is possible that this mutation does not completely prevent translation and the formation of an active product.

Instead, UGT80C1 may synthesize or modify the lipids present in LDs. The lipid composition of LDs has been well characterized and contains a neutral lipid core and phospholipid monolayer membrane, but no SGs have been detected (Tzen et al., 1992; Tauchi-Sato et al., 2002; Bartz et al., 2007). UGTC80C1 may regulate the TAGs or steryl esters that make up the majority of the core, though smaller amounts of DAGs and fatty acids have also been discovered presenting further possibilities (Tauchi-Sato et al., 2002). Sterol esters were never measured in this study and TAG analysis was limited to seed extractions, however, the absence of sterol glucosyltransferase activity in UGT80C1 is consistent with the absence of SGs in LDs. Also, because the amount of polar lipids present in the monolayer of the total LD content is likely much less than the total polar lipids present in an entire seed or seedling, it is possible that any discrepancies in the membrane composition of LDs would go undetected in the whole seed/seedling lipid extracts used here.

LDs are no longer assumed to be static energy deposits such as starch granules and have had an increasing number of cellular functions associated to them. They are a site for triacylglycerol breakdown, production of some steroid precursors, and are even used to form a hydrophobic barrier around pollen grains to protect them from dehydration and other stresses (Wolins et al., 2006; Hsieh and Huang, 2007; Chapman et al., 2012). LDs also act to distribute neutral lipids and phospholipids to various membrane bound organelles (Zehmer et al., 2009). Various membrane trafficking proteins have been identified in LDs and there is evidence that plant LDs associate with the ER, peroxisomes, and chloroplasts (Kessler et al., 1999; Hsieh and

Huang, 2005; Bartz et al., 2007). LDs have also been linked to endosomes and mitochondria in mammals and can traverse the cell on microtubule tracks (Sturmey et al., 2006; Liu et al., 2007; Shaw et al., 2008). It is possible that UGT80C1 plays a role in the trafficking of lipids to one or more of these organelles, although no organelle targeting sequences have been identified in it. The presence of UGT80C1 in LDs may also simply be a result of storage, sequestration, or transportation through the cell as well. Currently, this role of LDs has been best characterized in mammals with numerous examples of droplets harboring proteins from other cellular compartments such as a nuclear spliceosome activator, viral capsid components, and cytosolic and ER localized enzymes (Hope et al., 2002; Shi et al., 2002; Whitehead et al., 2004; Cho et al., 2007). There is evidence for keeping proteins inactive by sequestration, delivering them to other organelles, and storing them for later use as reviewed by Welte (2007). In mammals, LD occurrence increases in many disease states such as osteoarthritis, liver degeneration, and cartilage overproliferation (Cermelli et al., 2006). Partially unfolded proteins present a hazard to the cell if they form aggregates through their exposed hydrophobic regions. Increased LDs in these diseases may be a consequence from disrupted lipid metabolism, but they could also be a coping response of the cell to sequester hazardous misfolded proteins and other garbage until it can be degraded by the proteasome (Fujimoto and Ohsaki, 2006; Hofmann and Munro, 2006). It should not be ruled out that UGT0C1 might be an evolutionary remnant that is simply stored in LDs to prevent interference with normal cellular activity.

Steryl glucosides are important for zinc tolerance

Based on physiological studies that compared mutants to wild-type, SGs were found to have very little to no effect on heat, osmotic, and mucilage hydration stress. However, double mutants for *ugt80A2* and *ugt80B1* resulted in seedling chlorosis suggesting the lipids do a play a role in metal transport. To date, this is the only reported visible phenotype that correlates with a general reduction in SGs as opposed to a loss in UGT80B1 activity or reduction of a specific SG species. Zinc is an essential mineral in plants required at very low concentrations as it used as a cofactor for numerous enzymes and proteins. However, chlorosis is characteristic of a deficiency in iron in plants. Due to the similar ionic radii of zinc and iron, these metals are able to compete for binding in metal transporter proteins such as Iron-Regulated Transporter1/2

(IRT1/2) (Marschner, 1995). At high zinc concentrations, the metal may overwhelm the transporter proteins and prevent iron uptake into roots. In support of this, a proteomics analysis of *Arabidopsis* roots found IRT1 protein to greatly increase in response to excess zinc (Fukao et al., 2011). Iron is required for enzymes involved in the biosynthesis of protochlorophyllide, a chlorophyll precursor, whose absence leads to chlorosis (Spiller et al., 1982; Tottey et al., 2003). SG depletion may increase zinc sensitivity by altering the biophysical properties of the plasma membrane where these metal transporters are located, leading to a reduction in iron uptake.

Future perspective

The role of UGT80C1 in *Arabidopsis* remains elusive with many questions to be answered. In the future, the lipid composition of wild-type and *ugt80C1* LDs could be analyzed and compared by isolating them through a sucrose centrifugation gradient and analyzing the extracted lipids by mass spectrometry. It would be appropriate to isolate LDs from dry and imbibed seeds as well as seedlings, since GFP localization has been observed and *UGT80C1* exhibits elevated mRNA expression at these stages of development. The lipid analysis should also be expanded to include steryl esters and other minor lipids normally present in LDs. The evidence for a LD subcellular localization is convincing, but localization to chloroplasts needs to be confirmed. Chloroplasts contain specialized lipid droplets called plastoglobules, however, UGT80C1 does not contain a plastid signaling sequence making its relationship to the chloroplast uncertain. Chlorophyll, a pigment in chloroplasts, can be easily visualized by confocal microscopy using autofluorescence at 630 nm excitation. Chlorophyll and UGT80C1-GFP fluorescent merges would determine whether they co-localize and solidify whether the protein is connected to plastoglobules and the chloroplast. Finally, the role of SGs in metal stress response needs to be better characterized. Examining *Arabidopsis ugt80* seedling growth in the presence of other metals such as magnesium, copper, iron, cobalt, and manganese would establish whether the increased susceptibility to chlorosis in *ugt80A2,B1* double mutant seedlings is limited to zinc or can also result from other metal stress. Increased chlorosis in *ugt80* seedlings from some or all of the other metal ions would indicate that a multitude of transporter proteins, in addition to IRT1/2, are affected by the altered lipid membrane. Iron uptake into seedlings could also be measured by inductively coupled plasma atomic emission spectroscopy

to determine whether there is reduced iron content in the *ugt80A2,B1* mutant compared to wild-type seedlings. If SGs are required for efficient metal ion transport, treated mutant seedlings would be expected to contain less iron. Zinc content should also be measured in the seedlings as the mineral may hyperaccumulate in mutants and displace iron, causing iron dependent enzymes to malfunction. Such studies could extend the importance of SGs in plasma membrane trafficking.

Figure 3.1. The evolutionary history of UGT80 proteins was inferred using the Neighbor-Joining method.

(A,B) UGT80A2, B1, and C1 are conserved among higher plants (green) and mosses (blue), but UGT80A2 and B1 also share similarity with fungal proteins (red), whereas UGT80C1 is limited to land plants. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the branches. Evolutionary distances were computed using the Poisson correction method and all analyses were conducted in MEGA5 (Tamura et al., 2011). The cladogram is rooted with respect to glucosylceramide synthase (GCS) (yellow).

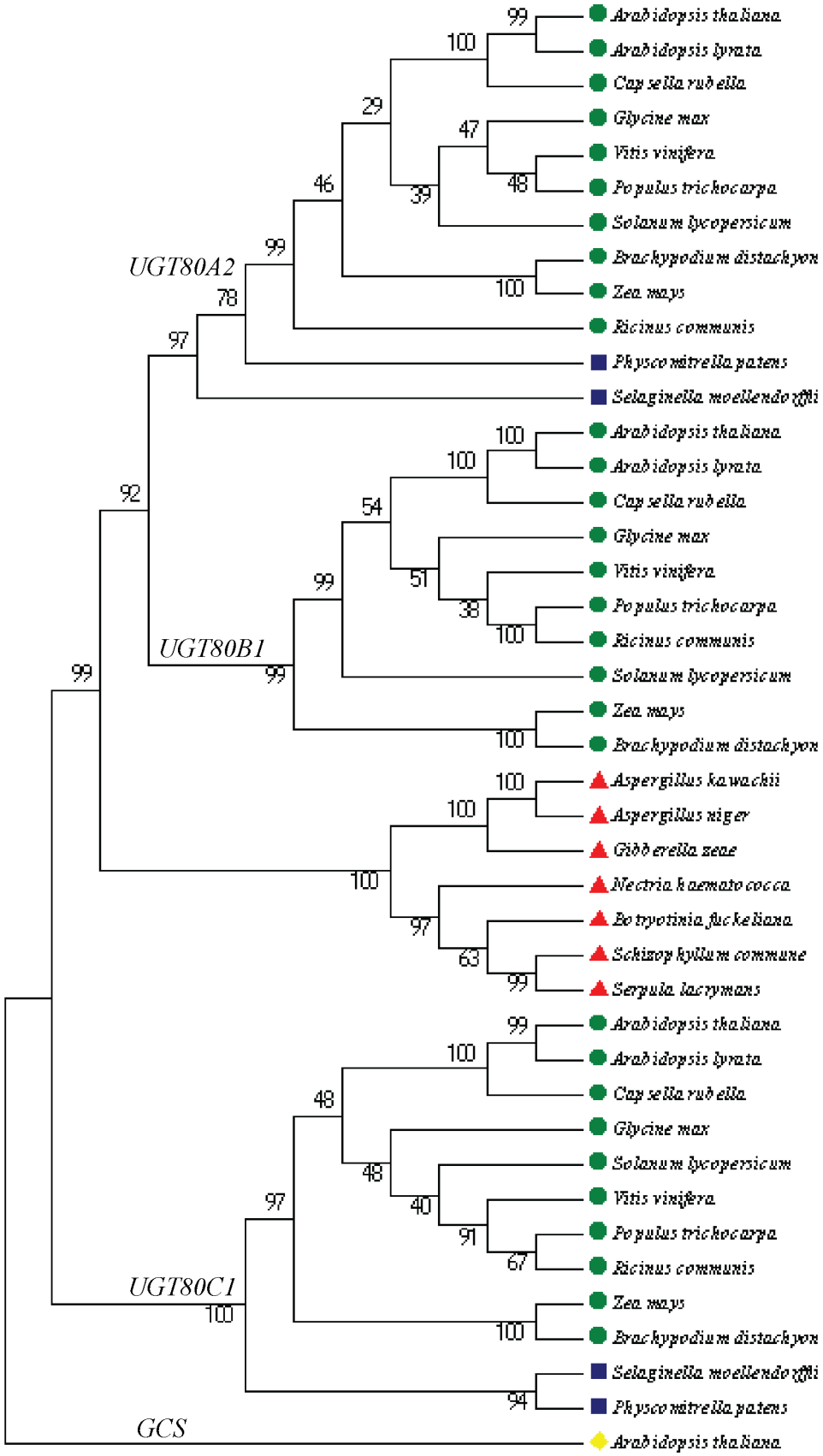

UGT80A2
UGT80B1
UGT80C1
GCS
Arabidopsis thaliana
Arabidopsis lyrata
Capsella rubella
Glycine max
Vitis vinifera
Populus trichocarpa
Solanum lycopersicum
Brachypodium distachyon
Zea mays
Ricinus communis
Physcomitrella patens
Selaginella moellendorffii
Arabidopsis thaliana
Arabidopsis lyrata
Capsella rubella
Glycine max
Vitis vinifera
Populus trichocarpa
Ricinus communis
Solanum lycopersicum
Zea mays
Brachypodium distachyon
Aspergillus kawachii
Aspergillus niger
Gibberella zeae
Nectria haematococca
Botryotinia fuckeliana
Schizophyllum commune
Serpula lacrymans
Arabidopsis thaliana
Arabidopsis lyrata
Capsella rubella
Glycine max
Solanum lycopersicum
Vitis vinifera
Populus trichocarpa
Ricinus communis
Zea mays
Brachypodium distachyon
Selaginella moellendorffii
Physcomitrella patens
Arabidopsis thaliana

Figure 3.2. SG content in *ugt80* mutant seeds.

(A) Cholesterol, (B) brassicasterol, (C) sitosterol, (D) campesterol, (E) stigmasterol, and (F) total SG content was analyzed in *Arabidopsis* seeds (n=3). *ugt80A2* and *ugt80B1* seeds have greatly reduced SG content, but *ugt80C1* resembles wild-type (WT) for all SGs analyzed. Single mutants reveal that *UG8T0A2* accounts for nearly all sitosterol and stigmasterol glucoside production in seeds, indicating the enzymes retain substrate specificity and are not completely redundant.

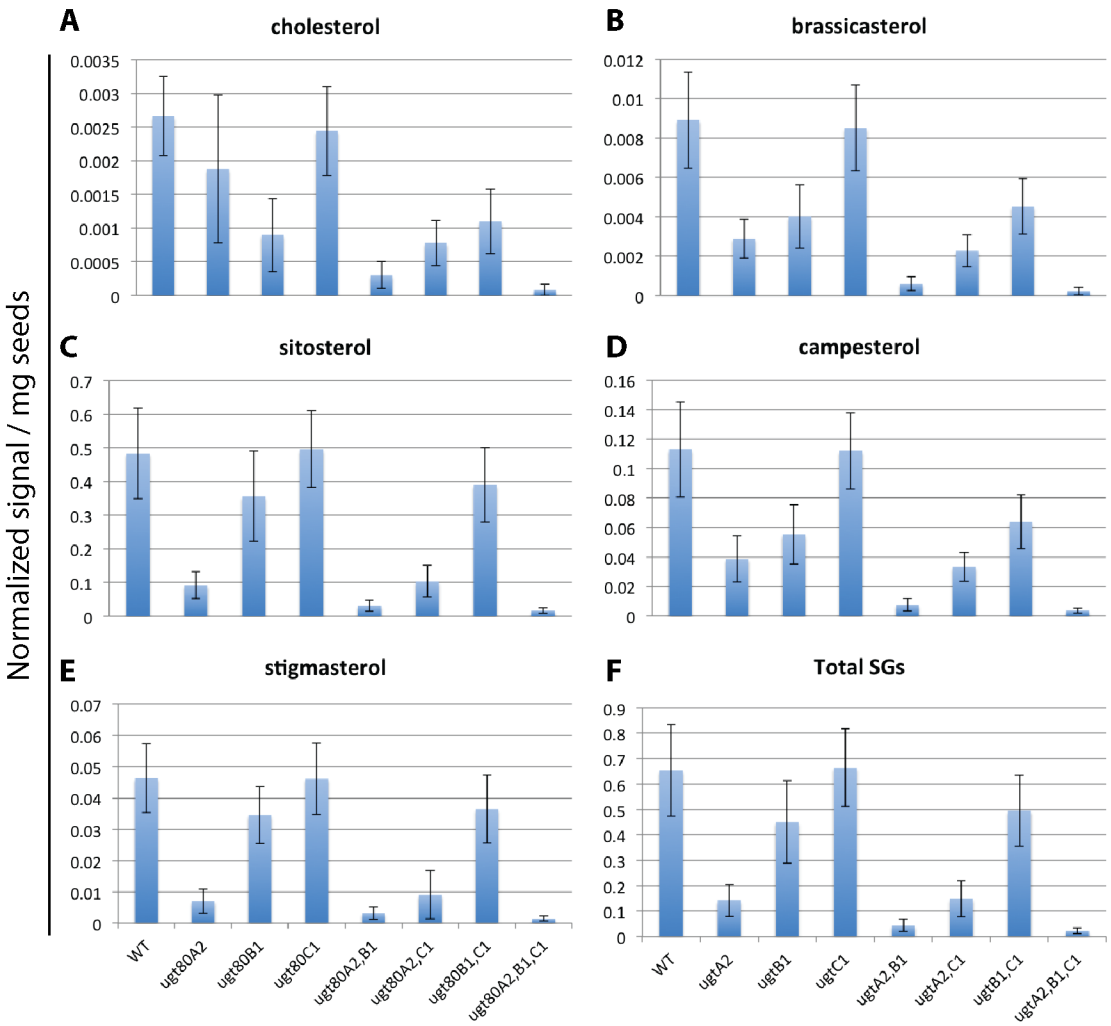


Figure 3.3. Gene expression and lipid analysis during seed imbibition.

(A) *UGT80A2*, *UGT80B1*, and *UGT80C1* mRNA expression was monitored during imbibition in wild-type (WT) (blue) and *ugt80C1* (red) seeds. *UGT80A2* and *UGT80B1* are both downregulated in response to water exposure while *UGT80C1* is upregulated 3-fold in WT after 1 hour. (B,C) TAGs, polar membrane lipids, SGs, and acylated SGs (ASGs) were measured during seed imbibition in WT (blue) and *ugt80C1* (red) seeds. No differences in lipid content were detected in the mutant compared to WT. TAGs show no change over time, however, PA levels were drastically decreased while PC, PE, and PI lipids were increased after 1 hour of imbibition. There is also a small but significant decrease in SG and DGDG as well as an increase in MGDG content after 24 hours of seed imbibition. P-values are compared between 0 and 1 hour (**$p < 0.005$) or between 0 and 24 hours (*$p < 0.02$) of imbibition for both WT and *ugt80C1* (n=3). No individual ASG species are shown.

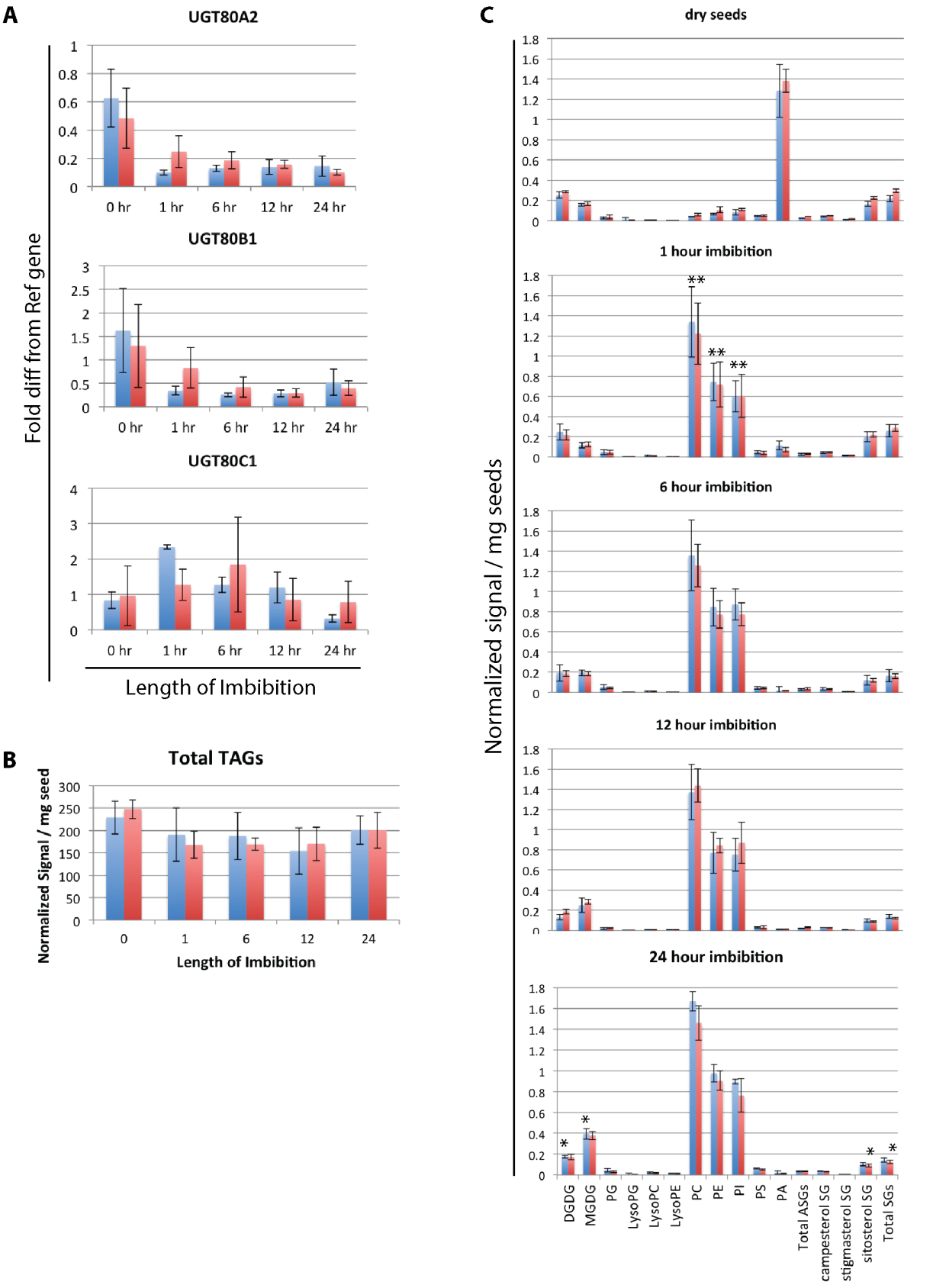

A
UGT80A2
UGT80B1
UGT80C1
Fold diff from Ref gene
0 hr
1 hr
6 hr
12 hr
24 hr
Length of Imbibition
B
Total TAGs
Normalized Signal / mg seed
Length of Imbibition
C
dry seeds
1 hour imbibition
6 hour imbibition
12 hour imbibition
24 hour imbibition
Normalized signal / mg seeds
DGDG
MGDG
PG
LysoPG
LysoPC
LysoPE
PC
PE
PI
PS
PA
Total ASGs
campesterol SG
stigmasterol SG
sitosterol SG
Total SGs

Figure 3.4. *In vitro* substrate specificity of UGT80 enzymes.

(A) UGT80A2 and UGT80B1 expressed in yeast have high glycosylation efficiency with cholesterol when utilizing UDP-glucose and UDP-galactose substrates, but very low activity with UDP-glucuronic acid (GA). Activity is determined by ESI-MS/MS analysis of cholesterol glucoside or glucuronic acid. (B) These two enzymes are also able to form brassicasterol, campesterol, stigmasterol, and sitosterol glucosides utilizing glucose and galactose, but form very little glucuronides with these sterols via GA substrate. (C) UGT80A2 and UGT80B1 displayed high activity with native yeast ergosterol when utilizing glucose and galactose UDP sugar substrates. UGT80C1 shows very weak or no activity for all substrate combinations analyzed. Yeast transformed with an empty vector serves as the negative control since those samples contain no enzyme. *$p<0.05$, **$p<0.01$ compared to empty control (n=3). (D) *E. coli* expression results resemble yeast (A,B,C). Cholesterol glucoside production was 8-fold higher with UGT80C1 compared to the GCS control, but over 35-fold lower than UGT80A2 activity. *$p < 0.01$ compared to GCS (n=3). GCS protein contains a frameshift mutation, leading to a truncated protein.

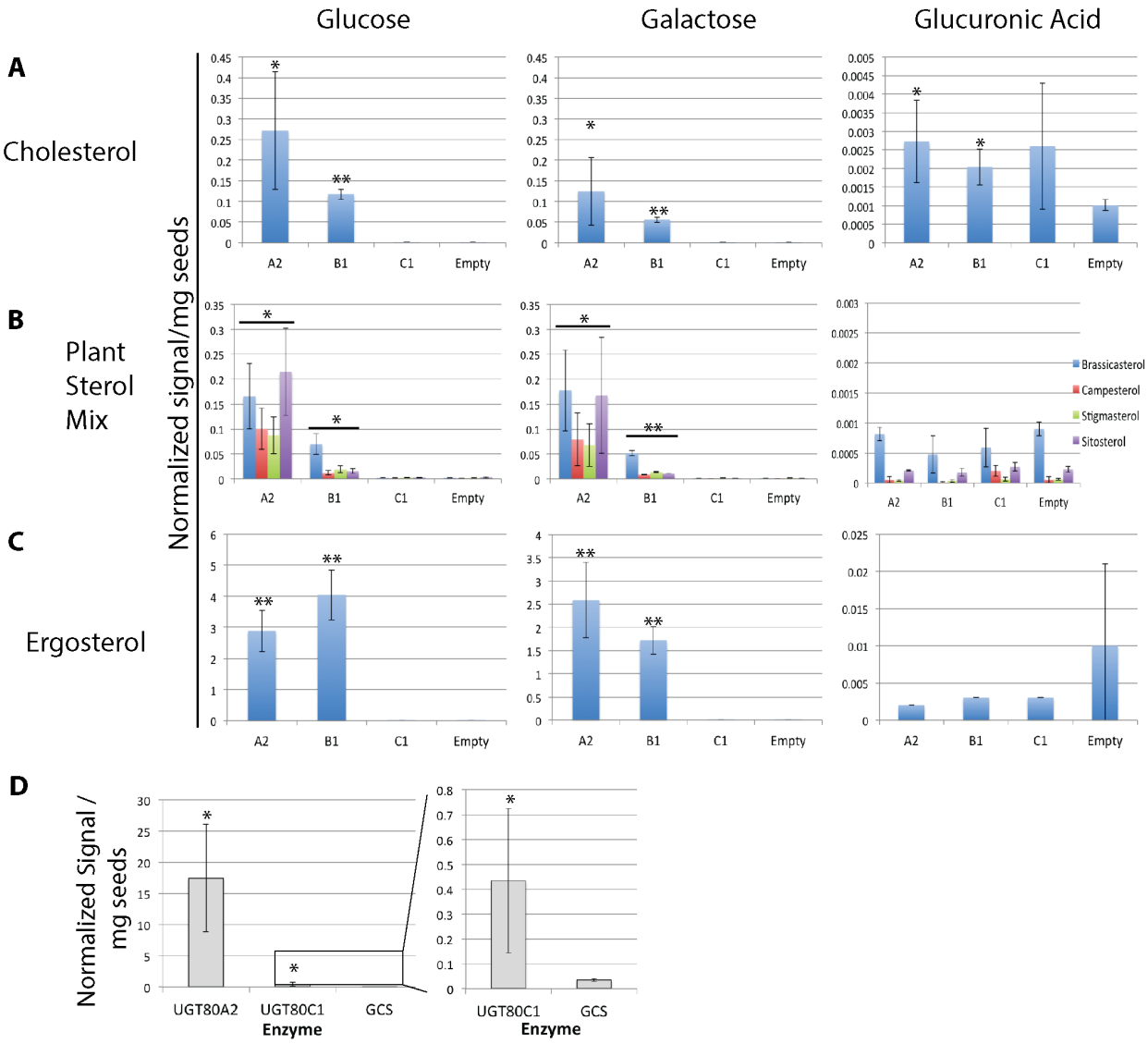

Glucose
Galactose
Glucuronic Acid
A
Cholesterol
B
Plant Sterol Mix
C
Ergosterol
Normalized signal/mg seeds
A2
B1
C1
Empty
Brassicasterol
Campesterol
Stigmasterol
Sitosterol
D
Normalized Signal / mg seeds
UGT80A2
UGT80C1
GCS
Enzyme

Figure 3.5. Subcellular localization of UGT80C1.

(A) 35S promoter driven expression of GFP tagged UGT80C1 shows punctate dots scattered across hypocotyl cells of 3-4 day-old seedlings. (B) UGT80C1 co-localizes with Nile red staining of lipid droplets and (C) behaves similarly (A) when under the control of its native promoter. (D) Lipid droplets in *ugt80C1* mutant seedlings appear like those in wild-type (WT). (E) Representative seedlings expressing GFP:UGT80C1 that display a chloroplast-like expression pattern. Tissues were not fixed in (A,C,E) but were treated with paraformaldehyde in (B,D) before imaging. White scale bar represents 50 μm for all images.

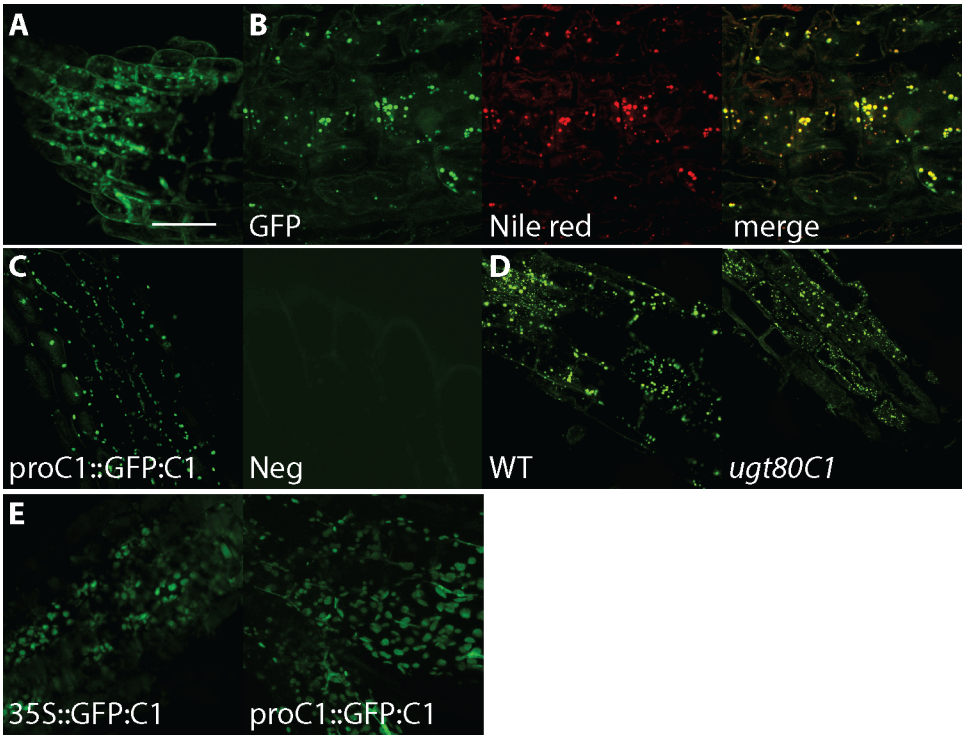
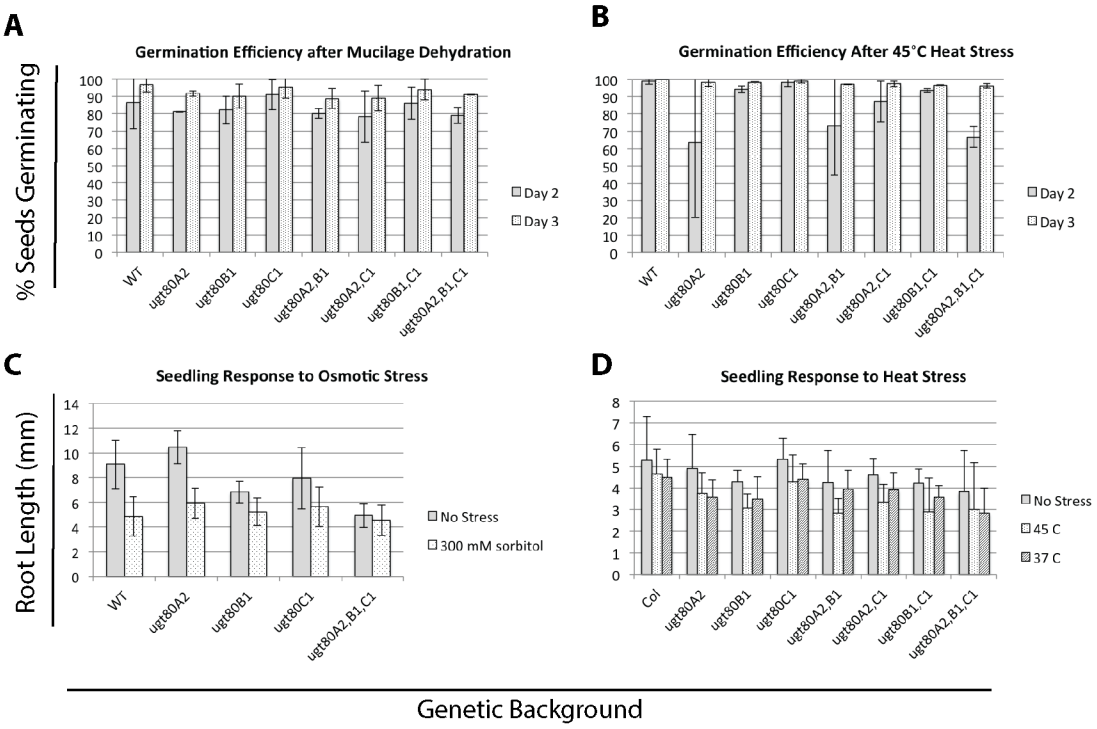
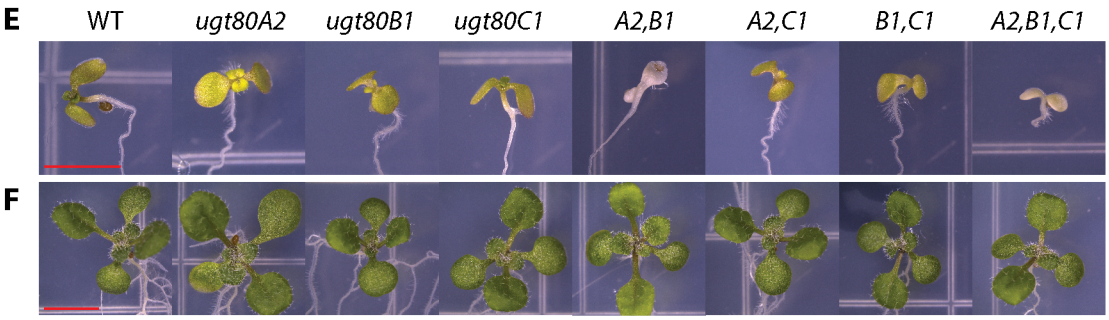


Figure 3.6. *ugt80* mutant sensitivity to stress conditions.

(A) Seeds were briefly imbibed in water and completely dried on filter paper before sowing on MS agar under continuous light. The number of days to initiate germination was recorded, but no significant differences were observed for dehydration or (B) heat stressed *ugt80* seeds compared to wild-type (WT) (n=40-50). (C) *ugt80* seedlings display slightly shorter roots after 5 days under normal conditions, but no increased sensitivity to osmotic stress (n=20-30). (D) Seedlings were also exposed to heat stress for either 3 hours at 45°C or 5 days at 37°C. Roots were measured after 5 days and no increased susceptibility was observed in mutants (n=20-30). (E) Growth in the presence of 1 mM Zn caused extreme chlorosis in *ugt80A2,B1* seedlings. (F) However, no differences were observed in absence of zinc. Images were taken 12 days after germination and red scale bars represent (E) 4 mm and (F) 5 mm. (G) Root lengths are significantly shorter in all plants upon zinc stress, but no increased sensitivity is observed in *ugt80* seedlings (n=30-50).


A
Germination Efficiency after Mucilage Dehydration
% Seeds Germinating
Day 2
Day 3
WT
ugt80A2
ugt80B1
ugt80C1
ugt80A2,B1
ugt80A2,C1
ugt80B1,C1
ugt80A2,B1,C1
B
Germination Efficiency After 45°C Heat Stress
C
Seedling Response to Osmotic Stress
Root Length (mm)
No Stress
300 mM sorbitol
D
Seedling Response to Heat Stress
Col
No Stress
45 C
37 C
Genetic Background


E
WT
ugt80A2
ugt80B1
ugt80C1
A2,B1
A2,C1
B1,C1
A2,B1,C1
F

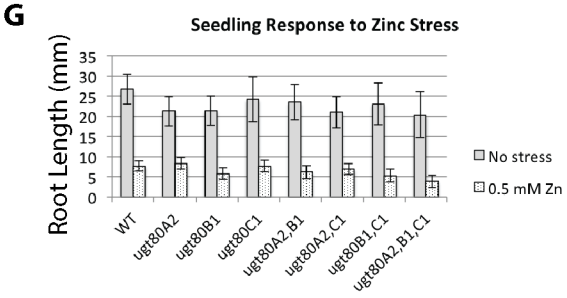

G
Seedling Response to Zinc Stress
Root Length (mm)
No stress
0.5 mM Zn
WT
ugt80A2
ugt80B1
ugt80C1
ugt80A2,B1
ugt80A2,C1
ugt80B1,C1
ugt80A2,B1,C1

Table 3.1. Primer sequences.

Name	Sequence (5'-….-3')	Gene	Atg #	fragment (bp)
TOPO Cloning				
UGT3_TOPO_R_NEW	ATCAAAATATTCGGTTGCTGCCTGAAAC	*UGT80C1*	At5g24750	
UGT3_TOPO_F_NEW	CACCATGGAGGAGGAACCGGAG	*UGT80C1*		
GCS_TOPO_F	CACCATGTCTACATTGGACTCCATTG	*GCS*	At2g19880	
GCS_TOPO_R	AATCGACTTTAGAGCTTTCTTCTACTG	*GCS*		
RT-PCR				
UGT_realtime_F_2712	TGGAGTCCTCACCTTGTACCAAAGC	*UGT80A2*	At3g07020	103
UGT_realtime_R_2896	CTGCAGGAGGTTCATAGTTGGATGC	*UGT80A2*		
UGT2_cDNA_563F	CTGATGGGCATAGGGGACTAGACCA	*UGT80B1*	At1g43620	104
UGT2_cDNA_666R	TGTCAAGGACCGAGAGTACTGAATCTCA	*UGT80B1*		
UGT3_cDNA_245F	TTCCTATCAATTCTCCTCCTGCTCTGTC	*UGT80C1*	At5g24750	87
UGT3_cDNA_331R	CCTCCAAAAACATTTTCCTCAGCG	*UGT80C1*		
ARP6_real-time_F	TAACAACTCAGGAGGACCCCA	*ARP6*	At3g33520	130
ARP6_real-time_R	CTACGACACCGAGCTGAT	*ARP6*		
Sequencing				
UGT3_Seq_R	CCATACAAGGATCCTTTCCG	*UGT80C1*		
UGT80B1_Seq_F	GGTCTCATTCCCTCTGGGC	*UGT80B1*		
C1_pro_Seq_F	GTCCAGCTCGACCAGGATG	*C1* pro		
C1_pro_Seq_R	GAAGGCGGGAAACGACAATC	*C1* pro		

Chapter 4 - Conclusions

Cellulose is an integral component of higher plants that has also become a very important polymer in industry. A greater understanding of the molecular mechanisms underlying cellulose synthesis has the potential for numerous benefits in food production and nutrition, biofuels, textiles, building materials, and other applications. The synthesis and deposition of cellulose encompasses a complex network of interacting biomolecules that have evaded the scientific community for decades. *Arabidopsis thaliana* presents an ideal model system for studying cellulose due to the availability of data resources and genetic tools. In particular, seed coat mucilage is an excellent model for studying cellulose because of its close resemblance to cell walls and dispensability for germination and development, allowing extensive genetic manipulation. Steryl glucosides (SGs) are important components of cell lipid membranes formed through the sterol glycosylation by UGT80 enzymes. This study provides the first *in vivo* evidence for a link between SGs and cellulose synthesis, and it furthermore addresses the function of the putative UGT80 enzyme, UGT80C1.

In the first data chapter (Chapter 2), no obvious deficiencies in growth and development were apparent for *ugt80* mutant plants, including the triple *ugt80A2,B1,C1* mutant. However *ugt80B1* seeds were found to have a transparent testa and reduced mass in comparison to wild-type as previously reported. A new phenotype was uncovered for imbibed seeds, which displayed abnormalities in the cellulosic rays of the mucilage. This was confirmed with a second allele and by staining with a cellulose specific dye. Cellulosic rays of *ugt80B1* mutant combinations appear almost 3-fold longer than wild-type rays when viewed under the same preparatory and confocal imaging conditions. Furthermore, the columella and cell walls of *ugt80B1* seed coat cells are shallower and some also a exhibit a collapsed funiculus. Despite these defects, the mucilage remains adhesive to the seed coat and *ugt80B1* mutants exhibit normal germination. The primary components of the mucilage are pectins such as rhamnogalacturonan I (RG-I) and homogalacturonan (HG). Immunolabeling of these two heteropolysaccharides did not reveal any disruption in their synthesis or deposition, which was confirmed by analysis of mucilage neutral sugars using high-performance anion-exchange chromatography.

The second data chapter (Chapter 3) attempts to characterize the putative UGT80C1 enzyme. Evolutionary analysis of *Arabidopsis* UGT80 amino acid sequences revealed that UGT80C1 is highly conserved specifically in land plants, while UGT80A2 conservation spans the fungi kingdom as well. The evidence presented in this study indicates that *UGT80C1* has been incorrectly classified as a UGT80 enzyme. Lipid analysis of mutant seeds shows significant decreases in SG content for both *ugt80A2* and *ugt80B1*, but *ugt80C1* SGs remain at wild-type levels. In addition, *in vitro* reactions using heterologously expressed UGT80 enzymes failed to uncover any sterol glycosylation activity for UGT80C1. In contrast, UGT80A2 and B1 reactions were able to produce SGs with cholesterol, campesterol, sitosterol, brassicasterol, stigmasterol, and even ergosterol substrates utilizing either UDP-glucose or UDP-galactose. Some substrate specificity was seen between the two, although both produce more SGs when using glucose as opposed to galactose and both enzymes had high activity with cholesterol and brassicasterol. However, only UGT80A2 can glycosylate sitosterol and stigmasterol with high efficiency, while UGT80B1 had low activity with these two sterols. Both enzymes had very little activity with glucuronic acid. The *in vitro* and *in vivo* experiments together suggest that UGT80A2 accounts for the majority of SG production in *Arabidopsis*. Seed imbibition results in a large decrease in PA and a concomitant increase in PC, PE, and PI phospholipids. RT-PCR results confirmed an upregulation of *UGT80C1* transcript expression in response to water exposure, but no significant differences were found in the lipid profiles of *ugt80C1* imbibed seeds in comparison to wild-type. *UGT80A2* and *B1* expression decreased after imbibition, which complements the small reduction of SGs detected in imbibed seeds with ESI-MS/MS. GFP:UGT80C1 was found to localize to lipid droplets in seedling hypocotyls, providing the first clue to the mysterious protein's true function. Mutant analysis of *ugt80A2,B1* suggests an enhanced sensitivity to zinc, implicating a role for SGs in stress response.

The research described here contributes to the growing pool of knowledge in the elusive mechanisms controlling cellulose synthesis in higher plants. A role for SGs in cellulose mucilage deposition and stress response is established here, as well as a better understanding of UGT80 enzyme substrate preference and activity in *Arabidopsis* that likely translates to other higher plants. A potential role for UGT80C1 in plant lipid droplets and chloroplasts is also brought forth here, setting it apart from its previously hypothesized similarity to other UGT80 enzymes. This study provides new insight to an enigmatic class of lipids and pioneers the first

steps in understanding the relationship between UGT80 enzymes, the cellulose machinery, and lipid droplets in plants.

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