The Identification of Novel Genes Involved in the Synthesis, Secretion and Modification of Cell Wall Components in the Seed Coat of *Arabidopsis thaliana*

Andrej Adam Arsovski

Department of Biology

McGill University, Montreal

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ABSTRACT

Plant cells are encased within a complex polysaccharide wall that not only strengthens the plant, but also has key roles in plant growth, cell differentiation, and defence. The plant cell wall is comprised of a network of cellulose microfibrils interconnected by hemicelluloses; this framework is embedded in a more soluble pectin matrix. This dynamic structure is under continual modification during plant growth and development, and its synthesis and modification requires the activity of a myriad of enzymes. Recent research has provided insight into how plants manufacture and regulate the cell wall during development, but much remains unknown. The mucilage secretory cells (MSCs) of *Arabidopsis thaliana* are used as a model to discover novel genes involved in the synthesis, secretion and modification of cell wall components, particularly pectin. These cells synthesize copious amounts of pectinaceous mucilage during development and, upon hydration of the desiccated seed, this mucilage rapidly swells, bursts from the MSCs and surrounds the seed in a gelatinous capsule. The *patchy (pty)/beta-xylosidase1(bxl1)* mutant has a peculiar phenotype where mucilage release is patchy and slow, and mutant seeds are delayed in germination. Cloning of the mutant locus revealed a lesion in an encoded bifunctional β-xylosidase/α-arabinofuranosidase. Chemical and immunological analyses indicate an increase in 1,5-linked arabinans, suggesting the action of *AtBXL1* is required for the trimming of these chains to allow correct mucilage release. In addition to the study of *AtBXL1*, an enhancer/suppressor screen of the *mum4* reduced mucilage mutant was performed in order to identify novel genes involved in mucilage secretory cell differentiation. The screen identified six novel mutants named *mum enhancer (men) 1-6*. Characterization of *men mum4* double mutants revealed two distinct groups, those that produced similar amounts of mucilage to *mum4* but failed to release it (*men2, 6*), and those that produced a further reduced amount of mucilage compared to *mum4* (*men1, 3, 4, 5*). Of these, the *men4* mutant was chosen for additional study as it had a single mutant phenotype of reduced mucilage. Characterization of *men4* seed development, chemical analysis of *men4* mucilage, and the use of pectin-specific antibodies suggest that *MEN4* has a role in mucilage synthesis or secretion. Molecular mapping places the *MEN4* locus on the

upper arm on chromosome 5, away from known mucilage production or cell wall-related genes. Finally, the role of mucilage during seed germination was investigated using a series of mucilage mutants affected in either mucilage production or release upon seed hydration. Preliminary results indicate that the production of mucilage may be more important than its release in the promotion of germination.

RÉSUMÉ

Les cellules végétales sont encastrées dans une paroi de polysaccharide complexe qui non seulement renforce la plante mais aussi agit de façon cruciale dans les mécanismes de croissance, de différentiation cellulaire et de défense. La paroi des cellules végétales consiste en un réseau de microfibrilles de cellulose connectées par des hemicelluloses. Ce réseau est encastré dans une matrice de pectine plus solubilisable. Cette structure dynamique est en modification perpétuelle pendant le développement et la croissance de la plante. Ces changements et sa synthèse engage l'action d'une myriade d'enzymes. Des études récentes ont données de nouvelles perspectives sur comment les plantes produisent et régulent leur paroi cellulaire pendant le développement cependant beaucoup reste à découvrir. Les cellules sécréteuses de mucilage (MSCs) d'*Arabidopsis thaliana* sont utilisées comme modèles pour la découverte de nouveaux gène impliqués dans la synthèse, sécrétion et la modification des composants de la paroi cellulaire, particulièrement la pectine. Ces cellules synthétisent de grandes quantité de mucilage pectiné durant le développement et, après hydration de la graine disséquée, celui-ci gonfle rapidement, jaillit des MSCs entourant la graine d'une capsule gélatineuse. Le mutant *patchy (pty)/beta-xylosidase1(bxl1)* présente un phénotype particulier où le relargage est épars et lent, ces graines présentent aussi un retard dans la germination. Le clonage du locus muté a révélé une lésion dans la β-xylosidase/α-arabinofuranosidase bifonctionelle transcrite. Les analyses chimique et immunologique ont indiquées une augmentation des 1,5-linked arabinans suggérant que l'action de BXL1 est requise pour l'hydrolyse de ces chaines permettant un bon relargage du mucilage. En parallèle de cette étude, un screen des enhancers/suppresseurs du mutant au mucilage réduit *mum4* dans l'intention d'identifier des nouveaux gènes impliqués dans la différentiation ces cellules sécréteuses de mucilage. Ce screen a identifié six nouveau mutants nommés *mum enhancer* (*men*) 1-6. La caractérisation des doubles mutants *men mum4* a révélée deux groupes distincts, ceux produisant une quantité similaire de mucilage à *mum4* mais échouant à le relarguer (*men2, 6*) et ceux produisant une quantité réduite de mucilage comparée a *mum4* (*men 1, 3, 4, 5*). Dans ces derniers, le mutant *men4* a été choisi pour des études plus approfondies car il

présentait aussi un phénotype de mutant simple de synthèse réduite de mucilage. La caractérisation du développement de la graine de *men4*, l'analyse chimique du mucilage produit par celui-ci et l'utilisation d'anticorps spécifique à la pectine suggère que MEN4 joue un rôle dans la synthèse du mucilage ou sa sécrétion. La cartographie moléculaire place le locus MEN4 sur le brin haut du chromosome 5, loin des gènes connus pour la production du mucilage ou reliés a la paroi cellulaire. Finalement, le rôle du mucilage durant la germination de la graine a été examinée en utilisant une série de mutant affectés dans la production du mucilage ou son relargage après hydration. Les résultats préliminaires semblent démontrés que la production de mucilage serait plus important qu'un rôle de promotion de la germination grâce à son relargage.

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CHAPTER 1

Introduction to the plant cell wall and its components, the Arabidopsis mucilage secretory cells, and the role of mucilage in germination

INTRODUCTION

The plant cell wall is a complex structure that acts as an extracellular matrix mediating plant interaction with both the biotic and abiotic environments. This dynamic structure surrounds and protects the cell and is a distinguishing characteristic of plants. The cell wall is the fundamental determinant of cell shape and form and as such must be continuously modified during the growth and development of the organism. This continuous modification requires the hydrolysis and alteration of existing cell wall material as well as de novo synthesis and secretion of cell wall components. The cell wall is composed of cellulose microfibrils tethered together by cross-linking hemicelluloses. This fundamental framework lies embedded in a second network of matrix polysaccharides, glycoproteins, proteoglycans and various low molecular weight compounds (Fig.1.1). The main component of this matrix is pectin, thought to account for one third of primary cell wall macromolecules (Carpita and McCann 2000; Willats, McCartney et al. 2001). A number of structural models of the cell wall have been proposed that attempt to resolve the interaction between the cellulose microfibrils, hemicelluloses and pectin network (Vincken, Schols et al. 2003). There is now well established evidence that suggests that pectic polysaccharides form a covalent network. There is also data that suggest hemicelluloses such as xyloglucan have a strong affinity for cellulose and that they function to coat and tether the cellulose microfibrils. Finally there is strong indication that suggests the pectin network interacts with the various hemicelluloses (Fig.1.1). The architectural complexity of the plant cell wall suggests that a myriad of enzymes may be required for the synthesis and necessary modifications of cell wall

components during the growth and development of the plant. These enzymes are beginning to be identified and characterized in greater numbers.

This literature review begins with an examination of the composition of the cell wall including cellulose, hemicelluloses and pectins. The synthesis and modification of these components will also be covered in this section. The review will then shift focus to the mucilage secretory cells of the seed coat of *Arabidopsis thaliana* and their use as a model for studying the synthesis and secretion of cell wall components, particularly pectic polysaccharides. This section will also include an examination into the necessity to modify pectic components in order to affect the physical properties of the cell wall. The role of seed-synthesized mucilage during germination will be reviewed in the concluding section.

CELL WALL COMPONENTS

Cellulose

Cellulose is a central component in plant cell walls. In the primary cell wall, it is a vital component of the load-bearing network and, because of its physical properties, is important in determining the orientation of cell expansion. In the secondary cell wall, deposited following initial growth, cellulose is highly enriched, providing plants with the mechanical properties to stand upright, as well as constituting a major component of xylem vessels. Roughly one-third of the dry mass of many plants is cellulose (Somerville 2006).

Cellulose is a simple polymer composed of unbranched β-1,4-glucan chains. Successive glucose residues are inverted 180° and form a flat ribbon, where the repeating unit is known as cellobiose. These ribbons form parallel chains that are held together by hydrogen bonds and Van der Waals forces to form microfibrils (Nishiyama, Langan et al. 2002; Nishiyama, Sugiyama et al. 2003). Analysis of cellulose microfibrils from primary and secondary cell walls have shown the degree of polymerization to be between 500 and 15 000 (as reviewed by (Brett 2000). Electron microscopy and NMR revealed that the 8-nm-wide microfibrils derive from a six globule rosette, with each globule made up of a number of protein subunits that synthesize six to ten chains which hydrogen bond to form a 2 nm fibril. When these 2 nm fibrils assemble together, they form a microfibril (Herth 1983; Ha, Apperley et al. 1998). These microfibrils have been shown to be synthesized by cellulose synthase, an enzyme found in the plasma membrane consisting of symmetrical rosettes of six globular complexes (Kimura, Laosinchai et al. 1999). It has been proposed that each of these subunits of a rosette may synthesize six β-1,4-glucan chains, which crystallize into a 36-glucan chain microfibril (Fig.1.1)(Herth 1983). The only known components of cellulose synthase in higher plants are the CESA proteins (Pear, Kawagoe et al. 1996). With the sequencing of the Arabidopsis genome it was revealed that Arabidopsis has 10 CESA genes (Table 1.1) (Richmond 2000). Analysis of mutants with defects in the secondary cell wall revealed that three separate CESA proteins are required in the same cell at the same time (Taylor, Howells et al. 2003). *CESA4, CESA7,* and *CESA8* are required for secondary wall synthesis, while three of *CESA1, CESA2, CESA3,* and *CESA6* are needed for

primary wall synthesis (Arioli, Peng et al. 1998; Scheible, Eshed et al. 2001; Beeckman, Przemeck et al. 2002; Burn, Hocart et al. 2002; Desprez, Vernhettes et al. 2002). Mutant analyses have shown that the three CESAs involved in secondary cell wall synthesis are equally important for cellulose deposition (Turner and Somerville 1997). In contrast, mutant analysis revealed unequal contribution of the CESAs involved in primary cell wall synthesis. Missense mutations in *CESA1* and *CESA3* result in severely retarded growth phenotypes, while *CESA6* null mutations (*procuste 1-1* to *12*), only show anisotropic cell swelling (Arioli, Peng et al. 1998; Fagard, Desnos et al. 2000; Beeckman, Przemeck et al. 2002; CS, P et al. 2002). It is therefore thought that other CESA subunits may act redundantly with CESA6, thus concealing more severe phenotypes. CESA2, 5 and 9 are very similar and may be functionally redundant to CESA6. *cesa2 cesa6* double mutants are smaller, and have swollen organs compared to their single mutant parents (Persson, Paredez et al. 2007). Additionally, *cesa5 cesa6* double mutants result in severely dwarfed seedlings and their growth arrests at the rosette stage (Desprez, Juraniec et al. 2007). *CESA9* is expressed in pollen and in developing embryos, and, consistently, *cesa2 cesa6 cesa9* triple mutants were male gametophytic lethal and produced collapsed pollen. TEM revealed irregular cell walls in the mutant pollen, suggesting the lack of primary cell wall deposition results in pollen deformities (Persson, Paredez et al. 2007). While these results suggest that *CESA2, 5, 6* and *9* may functionally compensate for each other, *CESA2* and *CESA5* cDNA under the *CESA6* promoter did not rescue the *cesa6* mutant phenotype, indicating that this redundancy may be only partial (Desprez, Juraniec et al. 2007; Persson, Paredez et al. 2007). This may be due to

small but significant differences in function between these CESAs. Two other CESAs, *CESA1* and *CESA3*, when mutated, also led to male gametophytic lethality, suggesting these two genes may work together with the CESA6-like CESAs. This hypothesis was reinforced by the co-immunoprecipitation of CESA3 and CESA6, which was also the first direct biochemical evidence that the primary CESAs form complexes (Desprez, Juraniec et al. 2007).

In addition to the *CESA* genes, the synthesis of cellulose requires the action of other proteins (Table 1.1). The *KORRIGAN* (*KOR*) gene encodes a β-1,4-glucanase, which, when mutated, results in lateral organ swelling, reduced cellulose production and altered pectin composition (JR, QW et al. 2000; S, T et al. 2001). A tomato endo-β-1,4-glucanase SICe19C1 was also shown to directly interact with crystalline cellulose through a novel C-terminal carbohydrate-binding module (Urbanowicz, Catala et al. 2007). *POM1*, also known as *CHITINASE-like 1* (*CTL1*), may be involved in the production of chitin-like polymers but its function remains unclear (Zhong, Kays et al. 2002). Mutations in the *KOBITO* (*KOB*) and *COBRA* (*COB*) genes result in root and hypocotyl swelling, and reduced cellulose content, suggesting they may be involved in cellulose synthesis but their specific functions remain unclear (Table 1.1) (Schindelman, Morikami et al. 2001; Pagant, Bichet et al. 2002).

A distinguishing feature of plant cells is the presence of cortical microtubules adjacent to the plasma membrane (Shaw SL, Kamyar R et al. 2003). It has been hypothesized that the deposition of cellulose is oriented by an interaction between cellulose synthase and microtubules. The observation that the orientation of cortical

microtubules in growing cells is similar to that of cellulose microfibrils supports this idea (M and K 1963). Numerous further observations of correlations between microtubule and microfibril organization have reinforced this idea (TI 2001). Most recently, fluorescently labelled CESA3 has been used to show that the CESA complex is delivered to the plasma membrane coincident with microtubules (Gutierrez, Lindeboom et al. 2009). Additionally, this construct has been used to illustrate the localization of CESA3 to the plasma membrane, Golgi apparatus, and a novel microtubule-associated cellulose synthase compartment (MASC) (Crowell, Bischoff et al. 2009). The formation and movement of MASC was shown to depend of the dynamics of cortical microtubule array, as it has greatly reduced motility when seedlings were treated with the microtubule stabilizing drug taxol (Crowell, Bischoff et al. 2009). Treatment with cellulose synthase inhibitors and osmotic stress induced the internalization of GFP-CESA3 and GFP-CESA6 within the MASC, or similar small CESA compartments (SmaCCs as termed by Gutierrez, Lindeboom et al. 2009), comparable to what is seen in non-growing cells (Crowell, Bischoff et al. 2009; Gutierrez, Lindeboom et al. 2009). These findings provide support for the coordination of cellulose synthesis with cellular growth status and its regulation through internalization of CESA complexes (Paredez, Somerville et al. 2006). It has been postulated that the communication between the apoplast and cytoplasm is mediated by arabinogalactan proteins (AGPs), possibly through interactions between AGPs and microtubules (Driouich and Baskin 2008). The idea that microtubules and AGPs are linked was supported by the discovery that both AGPs and microtubules are altered in the abnormally swollen trichoblasts of *reb1* (Andeme-

Onzighi, Sivaguru et al. 2002). This was confirmed in studies where microtubule disruption was achieved by treatment with the AGP-disturbing Yariv reagent (Singh Sardar, Yang et al. 2006; Nguema-Ona, Bannigan et al. 2007).

During cell expansion, cellulose synthesis is a major consumer of carbon, and it is thus likely that cellulose synthesis regulation may also be linked to other aspects of carbon metabolism. In plants, UDP-D-glucose is thought to be primarily synthesized by sucrose synthase (SUSY) (CH, M et al. 2001). A form of SUSY that is associated with the plasma membrane has been observed, and sucrose supports much higher rates of cellulose synthesis (Y, CH et al. 1995).

Since the identification of the CESA genes and the completion of the Arabidopsis genome, much research has been done and significant progress made in elucidating the process of cellulose synthesis. However, many mysteries remain and further work is required in order to completely understand the synthesis of this important compound.

Hemicellulose

Hemicelluloses are believed to bind the cellulose microfibrils through hydrogen bonds, both coating them and spanning the distance between adjacent microfibrils. Hemicelluloses can be divided into four main classes: xyloglucan (XG) consists of linear chains of β-1,4-D-glucose with numerous α-D-xylose residues linked at regular sites to the O-6 position of the glucan units and is predominantly found in

the primary cell wall of dicotyledonous plants. In addition, O-acetylation of glucose residues of the XG backbone, as well as sidechains of various combinations of galactose, fucose, arabinose and glucose have been observed (Jia, Cash et al. 2005). Mannans include galactomannans (GMs) and galactoglucomannans (GGMs) that have a similar three-dimensional structure to cellulose, and are important structural components of the cell wall and a source of storage polysaccharides. The specific functions of mannans in the cell wall are unclear, but they may play a role in the growth of pollen tubes and roots (Goubet, Misrahi et al. 2003; Zhu, Nam et al. 2003). Xylan, or glucoarabinoxylan (GAX), is the main hemicellulose of the secondary cell wall (but is also present in the primary cell wall), and has a backbone of β-1,4-linked xylan substituted to varying extents with side chains of α-1,2-arabinans as well as galactosyl and glucuronyl groups (Carpita and McCann 2000). Finally, mixed-linkage glucans involve an unsubstituted backbone of glycosyl residues containing both β-1,3- and β-1,4-linkages (Lerouxel, Cavalier et al. 2006).

Xyloglucan and xylan are the two main hemicelluloses in the cell walls of dicotyledonous plants (Carpita and McCann 2000). The structural similarity between the β-1,4-linked glucan chains of cellulose and the backbones of β-linked hemicellulosic polysaccharides led to the prediction that the *CELLULOSE SYNTHASE LIKE (CSL)* genes encoding family 2 glycosyltransferases (GTs) might encode glycan synthases involved in the biosynthesis of these polysaccharides (Richmond and Somerville 2001; Hazen, Scott-Craig et al. 2002). *CSL* genes encoding glycan synthases for three different hemicellulosic polysaccharides have been identified (Edwards, Dickson et al. 1999; Dhugga, Barreiro et al. 2004;

Liepman, Wilkerson et al. 2005). Guar seeds undergoing hemicellulosic deposition were used to demonstrate that a member of the *CSLA* family encodes a β-1,4-mannan synthase (Dhugga, Barreiro et al. 2004). Heterologous expression of CSL proteins was used to reveal that three *CSLA* genes from Arabidopsis encode mannan and glucomannan synthases (Liepman, Wilkerson et al. 2005). Finally, a mannose synthase and a galactosyltransferase were found to interact during the in vitro synthesis of glucomannan (Edwards, Dickson et al. 1999).

A number of the GTs that add the sidechains to these hemicelluloses also have been identified. The Arabidopsis *murus2* (*mur2*) mutant was isolated because of its fucose deficiency and was found to have a mutation in a gene that encodes a xyloglucan fucosyltransferase (Vanzin, Madson et al. 2002). The *mur3* mutant led to the identification of a xyloglucan galactosyltransferase that adds galactose onto the backbone of the same polymer (Madson, Dunand et al. 2003). This addition of a galactose was further shown to be important for the maintenance of mechanical strength during hypocotyl growth (Pena, Ryden et al. 2004). Examination of the CAZy family GT34 led to the identification of a seven-member family of candidate xyloglucan xylotransferases (Faik, Price et al. 2002). Heterologous expression and examination of their enzymatic activity revealed that two of these genes, *XXT1* and *XXT2*, encode enzymes that transfer xylose from UDP-D-xylose to acceptor substrates (Cavalier and Keegstra 2006). *xxt1* and *xxt2* single mutants exhibited subtle reductions in xyloglucan, while the double mutant completely lacked detectable xyloglucan (Cavalier, Lerouxel et al. 2008).

During cell growth, the plant cell wall must be modified in order to allow for expansion. Hemicelluloses can be hydrolyzed or realigned, allowing cellulose microfibrils to extend and separate in order to incorporate new cell wall components. The binding of xyloglucan to cellulose is known to weaken cellulose networks while simultaneously increasing their expansibility (Chanliaud, Burrows et al. 2002; Whitney, Wilson et al. 2006). It has also been observed that xyloglucans with different sidechains bind differently to the cellulose microfibrils, suggesting a role for the sidechains in cellulose binding (Whitney, Wilson et al. 2006). The modification of hemicelluloses requires the action of a battery of enzymes. Since xyloglucans are the major hemicelluloses interconnecting cellulose microfibrils, enzymes modifying this polysaccharide are essential in mediating cell growth. Xyloglucan endotransglucosylase/hydrolases (XTHs) cleave donor xyloglucan chains and rejoin the newly formed ends to acceptor chains (endotransglucosylase activity), or water (hydrolase activity). The endotransglucosylase activity results in the grafting of new chains, the hydrolase in the cleavage of xyloglucan (Rose, Braam et al. 2002). Analysis of the Arabidopsis genome has predicted the existence of at least 33 XTHs in 3 groups (Rose, Braam et al. 2002). While many in vitro studies have supplied evidence that XTHs mediate cell elongation, it was only recently shown that a decrease in *AtXTH18* mRNA resulted in reduction of root cell growth (Osato, Yokoyama et al. 2006). Two XTHs, AtXTH14 and AtXTH26, have subsequently been found to exhibit endotransglucosylase but not hydrolase activity. When exogenously applied to growing roots, both proteins resulted in reduced cell elongation (Maris, Suslov et al. 2009). In the secondary cell wall xylan hydrolysis

begins with arabinofuranosidases and galactosidases first removing side chains, an endoxylanase would then break down the backbone into xylose oligomers, and finally, a β-xylosidase releases the individual xylose monomers (Rahman, Sugitani et al. 2003; Tuncer and and Ball 2003).

Pectin

The primary cell walls of dicots are characterized by a greater relative level of pectin and less cellulose than the secondary cell wall. The pectic polysaccharides are the major fraction of non-cellulosic components in the primary cell walls of dicotyledonous species. The pectin network provides an environment for the deposition, extension, and slippage of the cellulose-hemicellulose framework required for cell growth. By limiting cell wall porosity, pectins may affect cell wall growth by regulating the access of cell wall modifying enzymes to their glucan targets (Carpita and McCann, 2000). The four main types of pectins are homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II), and xylogalacturonan (XGA) (Fig.1.2) (Somerville, Bauer et al. 2004; Mohnen 2008). Pectins are characterized by the presence of galacturonic acid. HG is made up of straight chains of α-1-4 linked galacturonic acid residues, the carboxyl groups of which can be methyl esterified to various degrees. RG I is composed of a backbone of alternating α-1-2-linked rhamnose and α-1-4- linked galacturonic acid residues, the rhamnose residues of which are frequently branched with side chains of arabinose, galactose and arabinogalactan molecules. RG II, like HG, has a backbone of α-1-4

linked galacturonic acid, which is substituted with four complex, evolutionarily-conserved sidechains containing a variety of sugars (Willats, McCartney et al. 2001; Somerville, Bauer et al. 2004; Mohnen 2008). XGA is HG whose galacturonic acid backbone residues are substituted with xylose (Fig.1.2) (Nakamura, Furuta et al. 2002).

Pectins are synthesized by pectin biosynthetic GTs which attach specific nucleotide-sugar substrates onto acceptor oligosaccharides. The synthesis of pectic polysaccharides is thought to occur in the Golgi apparatus with GTs situated in the Golgi lumen. Their substrate nucleotide sugars are either synthesized in the cytoplasm by nucleotide sugar interconversion enzymes and imported into the Golgi lumen by membrane spanning proteins, or may be synthesized within the Golgi lumen (Scheller, Doong et al. 1999; Ridley, O'Neill et al. 2001; Scheible and Pauly 2004; Mohnen 2008). Pectic polysaccharides are then sorted in to vesicular compartments and secreted to the apoplast (Zhang and Staehelin 1992). Once in the cell wall, pectins are under constant dynamic modifications in order to accommodate cell growth, elongation, signalling and division. Understanding this complex harmonization of elements is necessary in order to understand the plant cell wall and its immense ecological and economic applications.

Homogalacturonan

HG is made up of straight chains of α-1-4 linked galacturonic acid residues and can account for up to 60% of cell wall pectins (Ridley, O'Neill et al. 2001). HG galacturonic acid residues can be methyl esterified at the C-6 carboxyl residue or acetylated at the O-2 or O-3 position (Fig.1.2) (Ridley, O'Neill et al. 2001). The synthesis of the HG backbone is proposed to require the action of several HG GTs. These enzymes catalyze the transfer of D-galacturonic acid from UDP-D-galacturonic acid onto a growing stretch of HG in the lumen of the Golgi apparatus (Sterling, Quigley et al. 2001). The only functionally proven HG:GalAT is GAUT1. This protein, along with its close homolog GAUT7, is predicted to be a Golgi-localized Type-II membrane protein (Sterling, Atmodjo et al. 2006). Truncated GAUT1, but not GAUT7, expressed in human kidney cells was shown to incorporate [^{14}C]galacturonic acid into galacturonic acid accepting oligosaccharides when incubated with UDP-[^{14}C]galacturonic acid (Sterling, Atmodjo et al. 2006). The GAUT1 catalyzed elongation was in the 1,4-configuration, consistent with a role of GAUT1 as an HG:GalAT in pectin biosynthesis. GAUT1 and GAUT7 are part of a subfamily of GT family 8, which includes 15 GAUT genes and 10 GAUT-like (GATL) genes (Coutinho, Deleury et al. 2003). It has been proposed that the GAUT1 related gene family encodes GalATs required to synthesize the many varied galacturonic acid linkages of cell wall polysaccharides. Further study of this gene family is required in order discover the many unique GalAT activities that likely exist.

HG, along with other pectins, appears to be secreted in a highly methylesterified form (Li, Moscatelli et al. 1997; Lennon and Lord 2000). Following

the deposition of HG into the apoplast, pectin methylesterases (PMEs) selectively remove the methyl groups. HG, as well as other pectic polysaccharides, are methylated by pectin methyl transferases (PMTs) which catalyze the transfer of a methyl group from S-adenosyl-methionine (SAM) to the target polysaccharide (Ibar and Orellana 2007). PMT activity has been localized to the Golgi apparatus, and the import of SAM into the lumen is necessary for the methylation of HG (Goubet and Mohnen 1999; Dunkley, Hester et al. 2006; Mouille, Ralet et al. 2007). The *quasimodo-2/tumorous shoot development-2* (*qua2/tsd2*) mutants that have decreased cell adhesion and reduced levels of HG have been found to result from mutations in a putative PMT gene with sequence similarity to known methyltransferases (Kruptova, Immerzeel et al. 2007; Mouille, Ralet et al. 2007). The catalytic activity of QUA2/TSD2 has not been definitively determined, but its phenotype, which is similar to *qua1*, a putative HG GT (HG:GalAT), suggests it functions in pectin synthesis (Bouton, Leboeuf et al. 2002).

Pectin methyl esterification is believed to be strictly regulated by the plant in a developmental and tissue-specific manner (Willats, Orfila et al. 2001). Unmethylated, the C-6 of the galacturonic acid residue is negatively charged and may interact with Ca^{2+} to form a gel with other pectic molecules if more than 10 consecutive unmethylated residues are present (Liners, Letesson et al. 1989). Therefore, the porosity of the cell wall can be regulated by the esterification of HG as well as by the presence of branched pectins like RG I and RG II (Willats, McCartney et al. 2001; Willats, Orfila et al. 2001; Vincken, Schols et al. 2003; Mohnen 2008). A decrease in wall expansibility and increase in stiffness have been

correlated with a decrease in RG I arabinan and galactan sidechains and an increase in HG –calcium complexes (Stolle-Smits, Beekhuizen et al. 1999). Activities of enzymes active on RG I sidechains has been correlated with that of PMEs during pea pod development (Stolle-Smits, Beekhuizen et al. 1999). It was found that the activities of α-arabinase, β-galactanase, and PME gradually increased up to the mature stage, with β-galactanase and PME activity dramatically increasing in the senescing pods. These data suggest that the loss of RG I sidechains in combination with the de-methylation of HG, but not its degradation, contributes to the locking of cell wall components (Stolle-Smits, Beekhuizen et al. 1999). In soybean, the addition of a calcium chelator increased wall expansibility, which ceased with the addition of calcium (Ezaki, Kido et al. 2005). Calcium induced wall stiffening may therefore play a role in increased wall strength.

Rhamnogalacturonan I

RG I is composed of a backbone of alternating α-1-2-linked rhamnose and α-1-4- linked galacturonic acid residues and was originally characterized from suspension-cultured sycamore walls, and soybean soluble polysaccharides (Fig.1.2) (Lau, McNeil et al. 1985; Nakamura, Furuta et al. 2002). The rhamnose of the backbone is frequently found branched with side chains of arabinose and/or galactose (Willats, McCartney et al. 2001; Somerville, Bauer et al. 2004; Mohnen 2008). RG I is the primary component of seed coat mucilage of myxospermous species (Western, Skinner et al. 2000; Naran, Chen et al. 2008). RG I isolated from

Arabidopsis seed mucilage is largely unbranched, while RG I from potato walls appears branched at approximately half of the rhamnose residues at the C-4 position (Penfield, Meissner et al. 2001; Øbro, Harholt et al. 2004). 1,5-linked arabinose and 1,4-linked galactan chains are 4-linked to approximately half of the rhamnose residues of the RG I backbone of potato isolated primary cell wall (Lau, McNeil et al. 1985; Carpita and Gibeaut 1993). The structure of RG I sidechains, however, show considerable variation from species to species. Sugar beet and soybean RG I arabinan is 1,5-linked with terminal 3-linked arabinose residues and occasional terminal galactose residues (Sakamoto and Sakai 1995; Huisman, Brüll et al. 2001). In potato, galactan oligosaccharides have been found linked to RG I arabinan sidechains, rather than to the backbone rhamnose residues (Øbro, Harholt et al. 2004). Three types of galactan polysaccharides have been characterized as association with RG I: simple galactans, Type-I, and Type-II arabinogalactans. In soybean, galactans can reach up to approximately 45 1,4-linked galactose residues in length (Nakamura, Furuta et al. 2002). The most abundant RG I associated arabinogalactan is Type-I. It is characterized by lone interspersed 1,5-linked arabinose residues in a 1-4-linked galactan chain that has branches of multiple arabinofuranose residues or single arabinopyranose terminal residues (Huisman, Brüll et al. 2001). The post translational modification of AGP2 is the main source of Type-II arabinogalactans present in the cell wall, but some are found associated with RG I in wall extracts. Type-II arabinogalactans have a backbone of 1,3-linked galactans with branch points of 6-linked galactose up to three residues in length (An, O'Neill et al. 1994). RG I may also be decorated with single glucuronic acid and 4-

O-methyl-glucuronic acid residues. These have been isolated as 1,6- and 1,4-linked to galactose residues suggesting they may be linked to RG I, but this linkage remains to be directly shown (An, O'Neill et al. 1994).

The activity of 34 potential GTs may be required for the synthesis of the RG I backbone and its accompanying side chains (Mohnen 2008). The 1,4-rhamnosyltransferase and 1,2-galacturonosyltransferase required for the synthesis of the RG I backbone have not yet been identified. The synthesis of the galactan side chains of RG I is hypothesized to require up to 10 or more unique pectin-specific galactosyltransferases (GalTs) (Mohnen 2008). An RG I:GalT activity that elongates existing galactan branches was recovered from membrane preparations of mung bean. This GalT was shown to catalyze the addition of a galactopyranose onto 1,4-galactan acceptor molecules (Ishii, Ohnishi-Kameyama et al. 2004). Similar experiments have been carried out in potato and soybean showing that isolated GalT activity could add up to 25 galactosyl residues onto an existing galactan chain (Geshi, Jorgenson et al. 2000; Konishi, Mitome et al. 2004). In order to initiate the formation of a galactan chain, the activity of a galacosyltransferase that can initiate a linkage onto the rhamnose C-4 of the RG I backbone is required. A GalT that adds a galactosyl residue onto a rhamnose chain with a single terminal galactosyl residue was identified from potato microsomal membranes. The galactosyl transferase catalyzed the transfer of galactose residues from UDP- $^{[14C]}$galactose onto small RG I backbones with a low level of galactosylation, but not to RG I without galactosylation or with a high level of galactosylation (Geshi, Pauly et al. 2002). This suggests that the activity of this particular GalT represents the second step in RG I

galactan chain synthesis. Despite the identification of these enzyme activities, no genes encoding pectin-specific GalTs have yet been identified. The Arabidopsis *MUR3* gene encodes a potential xyloglucan galactosyltransferase in the GT47 family. Cell wall analysis of the *mur3* mutant plants revealed a significant reduction in galactose content, and their xyloglucan had reduced galactosylation (Madson, Dunand et al. 2003). This family of genes has 9 subfamilies and holds potential that some of the remaining members might be GalTs involved in RG I synthesis.

Finally, the synthesis of 1,5-linked arabinans is thought to involve the action of as many as 18 arabinosyltransferases (AraTs) (Mohnen 2008). 1,5-AraT activity was observed when mung bean hypocotyl Golgi membranes were incubated with UDP-arabinofuranose and exogenous 1,5-arabinooligosaccharides. 1,5-Arabinofuranosidase cleavage and NMR structure analysis determined the products to be 1,5-linked (Konishi, Ono et al. 2006). The complex branches of the RG I arabinan side chains also require the action of a 1,3-AraT. Such AraT activity was identified in mung bean, but the Golgi-enriched membranes incorporated donor residues onto arabinofuranooligos very inefficiently (Ishii, Konishi et al. 2005). In Arabidopsis, the T-DNA insertion mutant *arad1* has reduced cell wall arabinan, suggesting that *ARAD1* encodes an AraT. ARAD1 is a GT47 GT that has 7 additional homologs in the Arabidopsis genome (Harholdt, Jensen et al. 2006).

Rhamnogalacturonan II

RG II has a complex structure composed of four evolutionarily conserved sidechains containing many sugars on a stretch of HG backbone (Fig.1.2) (Willats, McCartney et al. 2001; Somerville, Bauer et al. 2004; Mohnen 2008). Despite its complexity, the high conservation of RG II structure across land plants suggests it must play an essential role in wall function (O'Neill, Ishii et al. 2004). Using in vitro NMR, RG II molecules have been shown to self-associate, forming dimers via boron diester bonds (O'Neill, Warrenfeltz et al. 1996). The importance of RG II borate crosslinking has been implicated in the generation of new growth, meristem development, and root growth (Blevins and Lukaszewski 1998). The Arabidopsis *bor1-1* mutant is boron deficient and the mutant plants are dwarfed with stems that do not elongate and exhibit a loss of apical dominance (Noguchi, Yasumori et al. 1997). Phenotypically similar is the Arabidopsis *mur1-1* mutant that is deficient in the production of L-fucose. The loss of this essential RG II component is caused by a lesion in GDP-mannose-4,6-dehydratase, an enzyme that synthesizes GDP-L-fucose, the substrate for addition of fucose into wall polysaccharides. In WT plants, 95% of RG II is found in the dimer form, while this is down to 50% in the *mur1-1* mutant, suggesting the fucose residues on RG II sidechains play a role in boron crosslinking (Bonin, Potter et al. 1997). The *nolac-H18* mutant has a T-DNA insertion in the *NpGUT1* gene in tobacco. *NpGUT1* encodes a putative glucuronosyltrasferase, which, when mutated, fails to incorporate a glucuronic acid residue and its corresponding galactose branch into RG II. This results in a callus with loosely attached cells (Iwai, Ishii et al. 2001). Together these mutants demonstrate that RG

II boron complex disruption results in plants that have compromised cell adhesion and decreased cell wall expansibility.

Xylogalacturonan

Xylogalacturonan is HG substituted with xylose residues at the C-3 of the galacturonic acid backbone residues (Fig.1.2) (Nakamura, Furuta et al. 2002). XGA isolated from Arabidopsis is primarily substituted with single xylose residues. Rarely, a substitution of two xylose residues may be found. XGA accounts for 2.5%, 7%, and 6% (W/W) of the pectin fraction from stem, young leaves, and mature leaves respectively, while no XGA was detected in pectin extracts from the seed and roots (Zandleven, Sørensen et al. 2007). A putative XGA xylosyltransferase has been identified in Arabidopsis. The *xylogalacturonan deficient1* (*xgd1*) mutant had significantly reduced pectic XGA levels compared to wild type. When heterologously expressed, XGD1 was shown to catalyze the transfer of xylose from UDP-xylose onto oligogalacturonide acceptors, confirming that XGD1 is a XGA xylosyltransferase (Jensen, Sorensen et al. 2008).

Expanding our knowledge of how pectin structure is modified and regulated in response to environmental stimuli and during cell growth is essential in understanding the role of these molecules in plant biology. The activities of only a handful of GTs involved in pectin synthesis have been characterized and fewer genes have been identified. The mucilage secretory cells of the Arabidopsis seed coat

provide a new model for the study of both the synthesis and secretion of pectin, as well as aspects of the mechanical alteration of the primary cell wall.

ARABIDOPSIS MUCILAGE SECRETORY CELLS AND THE CELL WALL

The mucilage secretory cells (MSCs) of the Arabidopsis seed coat comprise a new model for the study of pectin synthesis, its regulation, and the modification of the primary cell wall (Haughn and Chaudhury 2005; Western 2006). In some species of plants, including Arabidopsis, the seed coat epidermis undergoes a differentiation process which results in the production of a pectinaceous substance known as mucilage (Grubert 1981; Boesewinkel and Bouman 1995). In Arabidopsis, the epidermal cells of the outer ovule integument differentiate through a complex process into specialized cells that produce mucilage between the primary cell wall and plasma membrane. The growth and differentiation of the MSCs is triggered by pollination (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). The initial growth of the MSCs is driven by vacuolar expansion, which is followed by the synthesis and secretion of large quantities of pectinaceous mucilage. The polar secretion of large quantities of mucilage to a ring-shaped pocket on the apical cell face results in a cytoplasmic column in the centre of the cell. A secondary cell wall is then deposited over the cytoplasmic column to form a cellulosic columella (Fig.1.3) (Western, Skinner et al. 2000). Eventually the cell undergoes apoptosis and desiccation. Upon wetting, the hydrophilic mucilage

rapidly expands, ruptures the primary cell wall and surrounds the seed in a gel-like capsule (Western, Skinner et al. 2000). Staining of Arabidopsis mucilage has revealed the presence of two distinct layers, an inner, dense layer tightly associated with the seed, and an outer, diffuse, water-soluble layer. Both layers have been shown to be comprised primarily of unbranched RG I, with homogalacturonan, cellulose, arabinans, galactans and xyloglucans in the inner layer (Western, Skinner et al. 2000; Penfield, Meissner et al. 2001; Willats, McCartney et al. 2001; Western, Young et al. 2004; Macquet, Ralet et al. 2007; Young, McFarlane et al. 2008). The ease of identifying changes in pectin quantity and structure in the mucilage, as well the dispensability of mucilage under laboratory conditions, make the MSCs an ideal model for identifying genes involved in pectin production and modification (Western, Skinner et al. 2000; Western, Burn et al. 2001).

Pectin Synthesis in the MSCs

The first genes found to be important for MSCs development and mucilage production were *TRANSPARENT TESTA GLABRA1 (TTG1), GLABRA2 (GL2),* and *APETALA2 (AP2)* (Koornneef 1981; Jofuku, den Boer et al. 1994; Rerie, Feldmann et al. 1994). *TTG1* and *GL2* encode a WD40 repeat protein and a homeobox transcription factor, respectively, while *AP2* encodes a transcription factor of the AP2 family. *ttg1* and *gl2* mutant seeds produce less mucilage and their MSCs have flattened columellae, while *ap2* mutants lack differentiation past the growth phase of both the MSCs and underlying palisade layer of the seed coat

(Koornneef 1981; Penfield, Meissner et al. 2001; Western, Burn et al. 2001). Subsequently, two more transcription factors were identified with mutant phenotypes similar to *ttg1* and *gl2*. *MYB61* encodes an R2R3 MYB transcription factor expressed in the vasculature and the seed coat, while *TTG2* encodes a WRKY transcription factor that acts downstream of *TTG1* in several pathways (Fig.1.4) (Penfield, Meissner et al. 2001; Johnson, Kolevski et al. 2002).

A targeted screen for mucilage mutants identified a set of novel genes with various roles in MSC development, *MUCILAGE-MODIFIED 1—5 (MUM1—5*). Of these, the *mum4* mutant had a similar mucilage phenotype to the above-mentioned transcription factor mutants. Cloning of *MUM4* (aka *RHM2*) revealed that it encoded a UDP-L-rhamnose synthase involved in the conversion of UDP-D-glucose to UDP-L-rhamnose, and consequently, the synthesis of RG I, the main pectic component of mucilage (Fig.1.3) (Usadel, Kuchinsky et al. 2004; Western, Young et al. 2004; Oka, Nemoto et al. 2007). Accordingly, *mum4* mutants exhibit a significant drop in rhamnose and galacturonic acid (Usadel, Kuchinsky et al. 2004; Western, Young et al. 2004). Though expressed throughout the plant, *MUM4* was found to be specifically up-regulated in developing seeds at the time of mucilage production. This up-regulation is mediated by *GL2,* which is in turn regulated by *AP2* and *TTG1* in developing seeds (Fig.1.4) (Western, Young et al. 2004). TTG1 is a transcription factor with a key role in multiple aspects of epidermal cell differentiation, ranging from MSCs to leaf trichomes to root hairs to stomata. TTG1 has been demonstrated to work in a complex with a specific combination of MYB class and bHLH class transcription factors to specify a particular epidermal cell fate

(Guimil and Dunand 2007; Martin and Glover 2007). With regards to seeds, TTG1 forms a complex with the bHLH proteins ENHANCER OF GLABRA3 (EGL3) and TRANSPARENT TESTA8 (TT8) to up-regulate *GL2* (Zhang, Gonzalez et al. 2003; Western, Young et al. 2004). The same TTG1 complex also regulates *TTG2* independently from AP2 in a pathway parallel to *GL2* in mucilage production (Zhang, Gonzalez et al. 2003; Western, Young et al. 2004). A third, independent pathway for mucilage synthesis exists under the control of *MYB61* (Fig.1.4) (Penfield, Meissner et al. 2001; Western, Young et al. 2004).

While the bHLH proteins that form a part of the TTG1 complex had been identified as EGL3 and TT8, the particular MYB or MYBs that act with TTG1 to control outer seed coat differentiation remained unknown until recently. Early candidates *MYB61* and *MYB23* were shown to function in a genetic pathway distinct from TTG1 and to not affect seed development, respectively (Stracke, Werber et al. 2001; Western, Young et al. 2004; Kirik, Lee et al. 2005). Additionally, MYB23 does not interact with TT8 and is believed to act primarily in trichome development (Kirik, Lee et al. 2005; Matsui, Hiratsu et al. 2005). Recently it has been shown that *MYB5* is expressed in the endothelial testa cell layers of the developing seed (Gonzalez, Mendenhall et al. 2009). The *myb5* mutant was observed to have reduced columellae as well as patchy mucilage staining when stained with ruthenium red, indicating its involvement in MSC development and mucilage production (Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009). Qualitative RT-PCR, promoter-reporter constructs and microarray analysis indicate that *GL2, TTG2* and *TT8* transcripts are reduced in the *myb5* mutant, suggesting that MYB5 acts to regulate

these factors in the seed epidermis (MSCs) and/or the pigmented seed endothelium, presumably as part of the TTG1 complex (Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009). Yeast two hybrid experiments showing interaction between MYB5 and EGL3, and MYB5 and TT8, further supports a regulatory role for MYB5 as part of the seed coat TTG1 complex (Zimmermann, Heim et al. 2004). Additionally, double mutant analysis suggests that *MYB5* may be acting redundantly with the *TT2* MYB factor in controlling the transition of the MSC from a non-distinct ovule surface cell to a highly differentiated state (Fig.1.4) (Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009).

Cell Biology of Pectin Secretion in the MSCs

Pectin biosynthesis is a Golgi-mediated process as initially shown by autoradiography (Northcote and Pickett-Heaps 1966). More recent antibody work done primarily in root cap and tip-growing cells has expanded our understanding of the complexity of the Golgi apparatus and its role in pectin synthesis, though much remains unknown (Staehelin, Giddings et al. 1990; Zhang and Staehelin 1992; Staehelin and Moore 1995). However, unlike root cap and tip-growing cell types, the MSC presents a model system where pectin secretion occurs in copious amounts during a discrete developmental period and is targeted to a specific domain of the apoplast. Light microscopy of developing MSCs shows mucilage synthesis peaking at approximately 7 days post anthesis (dpa), and concluding by 9dpa with secondary cell wall deposition having begun by that time (Western, Skinner et al. 2000; Young,

McFarlane et al. 2008). The monoclonal antibody CCRC-M36 has been used to label hydrated extruded mucilage from mature seeds and well as within sections of developing seeds (McFarlane, Young et al. 2008; Young, McFarlane et al. 2008). Its epitope is believed to reside somewhere on the backbone of RG I, making it a useful probe for the pectin of mucilage (McFarlane, Young et al. 2008; Young, McFarlane et al. 2008). At 4dpa, prior to mucilage synthesis, there is little RG I and thus not much label in the MSC with CCRC-M36. However, at 7dpa, the newly synthesized mucilage surrounding the columella is brightly labelled (McFarlane, Young et al. 2008; Young, McFarlane et al. 2008). When developing seeds were incubated with CCRC-M36 combined with gold-conjugated secondary antibodies and examined by TEM, the antibody was found to label the mucilage pocket as well as 70% of Golgi stacks visible at 7dpa. At 9dpa, 0% of Golgi stacks are labelled. This suggests that the RG I-associated epitope of CCRC-M36 is present in the Golgi at 7dpa, during the time of copious mucilage production, but not at later stages when secondary cell wall synthesis is predominant (Young, McFarlane et al. 2008). The high number of stacks that colocalize with CCRC-M36 during the synthesis stage indicates that mucilage production occurs simultaneously in most of the Golgi stacks throughout the MSC.

High-resolution TEM of developing seed coat cells showed the number of Golgi stacks to double from 4dpa to 7dpa, points in MSC development corresponding to pre- and post-mucilage synthesis, respectively. Golgi stack morphology also changes from 4dpa to 7dpa, with the 7dpa cells having Golgi stacks with shorter diameter and swollen *cis* cisternae. Observation of Golgi-localized GFP (ST-GFP), as well as TEM counts of labelled Golgi stacks, indicated that these

structures are evenly distributed around the cell without clustering at the site of mucilage deposition. This more closely represents Golgi distribution in diffusely growing cells than other polar systems like tip-growing root hairs and pollen tubes, where Golgi concentrate at the site of secretion. Following mucilage production, the RG I-rich pectin needs to be packaged at the trans-face of the Golgi. This was reflected by an increase in number of apparent trans-Golgi networks and associated large vesicles during mucilage synthesis (Young, McFarlane et al. 2008).

In *mum4* mutants, which exhibit a drastic decrease in synthesized mucilage, the doubling of Golgi stacks during peak mucilage synthesis was still observed, but the Golgi morphology appeared distinctly different (Young, McFarlane et al. 2008). As mentioned previously, mucilage-producing Golgi stacks had short cisternae with compressed lumens and swollen margins. In contrast, the *mum4* Golgi had longer cisternae with thinner fenestrated margins, a characteristic of wild type Golgi stacks prior to mucilage synthesis (Young, McFarlane et al. 2008). It has been proposed that the bulk of biosynthetic enzymes passing through a Golgi stack are kept in the flattened central portion of the stack, while polysaccharide chains are pushed out forming the swollen margins of the cisternae (Staehelin, Giddings et al. 1990). This could explain the thinner fenestrated margins observed in the *mum4* Golgi stacks. Therefore, while the *mum4* mutant experiences an increase in Golgi stack abundance at a time when mucilage production is at maximum, the decrease in mucilage production is reflected in the observed change in Golgi stack morphology. Additionally, the fact that the Golgi stack distribution is the same in the *mum4* mutant as in the wild type suggests that Golgi stack proliferation may not be a direct

consequence of polysaccharide production but, rather, may be activated by developmental signals during the cells' maturation.

The plant cytoskeleton and the deposition of the cell wall components have been closely linked (Turner and Somerville 1997; Taylor, Scheible et al. 1999; Geitmann and Emons 2000; Persson, Hosmer Caffall et al. 2007). Mucilage production has been inhibited in secretory cell types such as root cap cells by treatment with actin inhibitors, while microtubule disruption has had no impact on mucilage production (Shannon and Steer 1984). Latrunculin-B mediated actin depolymerisation has also been shown to inhibit the internalization of various pectins in root cells of *Zea mays,* highlighting yet another cell wall related-role for this cytoskeletal component (Baluska, Hlavacka et al. 2002). As discussed above, cortical microtubules have been shown to align parallel to cellulose microfibrils in the cell wall (Wasteneys 2000; Baluska, Samaj et al. 2003; Laporte, Vetter et al. 2003; Paredez, Somerville et al. 2006). It been suggested that the interaction of cortical microtubules with cellulose synthase complexes is necessary in order to ensure the deposition of adequately long and strong microfibrils (Wasteneys and Galway 2003; Wasteneys and Fujita 2006). Most recently, it has been shown that the secretion of cellulose synthase complexes to the plasma membrane is regulated by the pausing of Golgi bodies on cortical microtubules indicating the interlinked relationship between the cytoskeleton and cell wall synthesis and modification (Crowell, Bischoff et al. 2009; Gutierrez, Lindeboom et al. 2009).

In a recent examination of cytoskeletal involvement in MSC pectin secretion, TEM revealed that microtubules are present in significantly greater numbers along

the mucilage domain than in either the basal-lateral or apical domains of MSCs during mucilage synthesis. Confocal images of fluorescently labelled microtubules showed that microtubules line the entire mucilage pocket (Fig.1.3) (McFarlane, Young et al. 2008). Tracking mucilage production with TEM using the CCRC-M36 antibody revealed electron dense vesicles carrying the RG I antigen amongst the microtubules surrounding the mucilage domain. In contrast, anti-actin immunofluorescence showed actin microfilaments throughout the cytoplasm with no apparent concentration around the mucilage pocket domain (McFarlane, Young et al. 2008). To examine whether microtubules were involved in targeting secretory vesicles to the plasma membrane in MSCs, microtubules were disrupted in the *mor1-1* mutant, whose microtubules lose organization when the culture temperature is raised to 29°C (Whittington, Vugrek et al. 2001; Kawamura, Himmelspach et al. 2006). TEM of the Golgi stacks and vesicles in *mor1-1* mutants at the restrictive temperature did not reveal a change in the distribution, size or shape of these organelles. Similarly, CCRC-M36 labelling revealed normal mucilage synthesis and deposition in the mutant. However, when seeds developed at the restrictive temperature were assayed for mucilage release, 40% had impaired or no release while only 22% of wild type seeds displayed abnormal release (McFarlane, Young et al. 2008). This suggests that, while the role of microtubules in guiding mucilage containing vesicles to the deposition site remains enigmatic, microtubules may indeed play a role in mucilage release. To clarify the role of cytoskeleton in mucilage secretion, further investigation is required. To do this, it will be necessary to address the technical

challenges that currently prevent the use of cytoskeletal inhibitors, specifically the difficulty of inhibitor infiltration into the MSCs (McFarlane, Young et al. 2008).

Cell Wall Modification and MSCs

The plant cell wall is a dynamic structure whose constant modification is necessary in order for plant cells to grow and divide. Several recent studies have revealed the MSCs as a burgeoning model for identifying genes involved in cell wall modification. The first identified mutants affected in mucilage release were *mum1* and *mum2,* whose unexpected phenotypes suggested that mucilage release was not a spontaneous, physical process, as previously believed (Western, Burn et al. 2001). In addition to *MUM1* and *MUM2*, *AtSBT1.7* has recently been shown to play a role in mucilage release (Western, Burn et al. 2001; Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007; Rautengarten, Usadel et al. 2008). While *MUM1* remains to be cloned, *MUM2* and *AtSBT1*.7 appear to be involved in structural modifications of either the primary cell wall and/or mucilage necessary for proper mucilage release.

mum2 mutant seed are distinguished by a complete lack of extruded mucilage when hydrated. However, sectioning and staining of developing seeds with the polychromatic dye toluidine blue showed *mum2* seeds to be indistinguishable from wild type with respect to mucilage deposition and columella formation (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). An analysis of the monosaccharide composition of whole seeds without prior mucilage extraction confirmed identical rhamnose and galacturonic acid levels between the *mum2* mutant and wild type

(Western, Burn et al. 2001; Dean, Zheng et al. 2007). Together these data suggest that the defect in *mum2* seed is the ability to extrude mucilage, rather than mucilage production. Even when effectively eliminating the primary cell wall by sectioning embedded seeds, *mum2* mucilage failed to expand when in direct contact with water, suggesting that the *mum2* mucilage may be specifically affected in its ability to hydrate and expand. Cloning of the *MUM2* gene revealed an encoded β-galactosidase whose action was significantly reduced in the mutant (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). Linkage analysis of extracted *mum2* mucilage showed increases in galactose, arabinose and glucose. This, together with an observed increase in RG I sidechain branch points, suggests that the lack of a hydrolyzing β-galactosidase in the mutant results in altered RG I that contains more arabinan side chains and terminal galactose residues than in wild type mucilage (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). As a result, the hydration and expansion properties of the *mum2* mutant mucilage could be affected such that the mucilage fails to break the primary cell wall upon imbibition (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). Though these data present a strong case for the necessity of mucilage modification for release, the possibility of concomitant cell wall modification could not be eliminated.

Similar to *mum2* mutants, *atsbt1.7* mutants both lack mucilage release upon hydration and appear to have no developmental differences compared with wild type seeds in terms of mucilage production (Rautengarten, Usadel et al. 2008). Unlike *mum2*, however, pre-treatment of seeds with the chelator EDTA leads to mucilage release by *atsbt1.7* mutants (Rautengarten, Usadel et al. 2008). Monosaccharide

analysis of the whole seed revealed no gross compositional changes between the mutant and wild type seeds, however, the *atsbt1.7* mucilage did reveal a 25% decrease in methylation (Rautengarten, Usadel et al. 2008). As discussed above, PMEs demethylate esterified HG in extracellular spaces to produce required cell wall porosity and rigidity, and have been shown to be expressed during seed development (Micheli 2001; Willats, McCartney et al. 2001; Louvet, Cavel et al. 2006). In wild type siliques, PME activity was found to increase from 4 to 10dpa and then suddenly drop. However, in *atsbt1.7* mutants, this decrease was not seen, suggesting prolonged PME activity (Rautengarten, Usadel et al. 2008). This increased PME activity was reflected through altered staining of mutant seed coats with the antibodies JIM5 and JIM7, which label HG with a low and high level of methyl esterification, respectively (Willats, Limberg et al. 2000; Willats, McCartney et al. 2001; Willats, Orfila et al. 2001; Macquet, Ralet et al. 2007; Rautengarten, Usadel et al. 2008). Extruded wild type mucilage had intense JIM5 and JIM7 staining resembling streaks originating at the columella, while *atsbt1.7* seeds showed weak JIM5 and JIM7 signal stemming from the columella but strong JIM5 signal from the cell wall (Rautengarten, Usadel et al. 2008). These data suggest the serine protease encoded by *AtSBT1.7* may be directly or indirectly inactivating a PME in wild type seeds, and resulting in prolonged PME activity in the mutant. This may mean that a certain degree of esterfication is required in developing MSCs, either in the mucilage to allow for proper swelling, or in the primary cell wall to promote breakage upon hydration.

The discovery and characterization of *MUM2* and *AtSBT1.7* have provided a glimpse into the active and complex pectin modifications occurring during MSC

development. Though it remains to be elucidated whether the activities of these enzymes are primarily required in the cell wall or mucilage, it is not difficult to imagine them working in concert in both the cell wall and mucilage in order to bring about the optimal conditions for mucilage release. *MUM2* modifies RG I, the main component of mucilage, by removing branches which could interact and physically compact the mucilage and/or the cell wall. This compacting of the mucilage is likely required in order to maximize the volume of mucilage that can occupy the space in the MSC, and, as a result mucilage may be secreted having already been highly branched during synthesis in the Golgi apparatus. Following its deposition, the activity of MUM2 may be just one of many steps in a cascade required to sufficiently modify the RG I component of mucilage for release. For example, increase of arabinan sidechains in *mum2* mucilage may suggest that the action of *MUM2* is required in order for other modifying enzymes to reach their targets. The activity of ATSBT1.7 may either directly or indirectly inactivate (a) certain PME(s) by selective proteolysis at the correct time during seed development. Resulting changes in esterification could then, in turn, affect the porosity of the mucilage, granting or preventing access to other modification enzymes that would culminate in the chemical and mechanical properties required for proper mucilage hydration and expansion. Similarly, these enzymes could be affecting the cell wall, weakening it sufficiently allowing the expanding mucilage to break through.

MUCILAGE AND GERMINATION

The seed is the dispersal unit of the plant; it is able to survive the period between embryo maturation and the establishment of the next generation with the emergence of the seedling. Consequently the survival of higher plant life depends on the longevity and germination capacity of seeds. To ensure complete germination over time and prevent premature germination, the seed enters a dormant state, where the seed is unable to germinate even under favourable conditions (reviewed in (Bewley 1997; Finch-Savage and Leubner-Metzger 2006). In order to examine the effects of environmental conditions and applied chemicals on seed dormancy and germination, numerous studies have been performed (Bewley 1997; Finch-Savage and Leubner-Metzger 2006; Holdsworth, Bentsink et al. 2008). However, still very little is known how the seed is maintained in a dormant state and how the embryo breaks this imposed state to emerge from the seed and complete germination (Bewley 1997; Holdsworth, Bentsink et al. 2008).

Germination has been defined to include events that begin with the initial uptake of water by the dry seed and terminate with the elongation of the embryonic axis. The visible sign that germination is complete is the penetration of the radicle through both the endosperm (a maternal nutritive tissue directly surrounding the embryo) and the protective seed coat or testa (Bewley and Black 1994). Water uptake surrounding germination involves three phases. The first phase is the rapid initial uptake, which results in temporary perturbations of membranes, leading to an immediate leakage of solutes and low molecular weight metabolites into the imbibition solution. The second phase is the plateau phase, which has no or little

obvious water uptake, during which mitochondria are repaired and synthesized, and proteins are made from newly synthesized mRNA. The third phase occurs after germination proper, and involves the further uptake of water necessary for cell elongation and division of the expanding embryo/seedling (Schopfer and Plachy 1984; Bewley 1997; Manz, Muller et al. 2005). Physically, germination involves two stages: the first stage is the breakage of the testa, which is followed by the rupture of the endosperm. Following endosperm rupture, the radicle emerges and germination is complete (Hepher and Roberts 1985; Leubner-Metzger, Frundt et al. 1995; Leubner-Metzger 2003; Liu, Koizuka et al. 2005). While much data exists on the genes and factors acting during dormancy and germination, most of these specifically involve hormone synthesis and signalling (Bentsink and Koornneef 2009).

Mutants with altered seed coats can have reduced seed dormancy (Debeaujon, Leon-Kloosterziel et al. 2000). The seed coat protects the embryo against environmental agents, and plays an important role in embryo nutrition during development (Debeaujon, Lepiniec et al. 2007). The seed coat and endosperm are thought to restrict germination through mechanical restriction to radicle protrusion, water or oxygen impermeability, or by producing germination inhibiting compounds. Seed coat mutants affected in flavonoid pigmentation, including the *transparent testa* (*tt*) and *transparent testa glabra* (*ttg*) mutants, range in colour from yellow to pale brown and exhibit reduced dormancy. This suggests that phenolic compounds accumulated within the seed coat may play a role in the regulation of germination (Debeaujon, Leon-Kloosterziel et al. 2000; Debeaujon, Peeters et al. 2001).

Mutants in testa structure also demonstrate decreased dormancy. The *aberrant testa shape* (*ats*) mutant is mutated in the *KANADI 4* gene and produces a single integument instead of the two present in wild type seed coats (Leon-Kloosterziel, Keijzer et al. 1994; Messmer McAbee, Hall et al. 2006). *ap2* mutants have heart shaped seeds that fail to complete differentiation of the outer two layers of the seed coat (MSCs and sub epidermal palisade layer) and exhibit slightly reduced dormancy (Jofuku, den Boer et al. 1994; Debeaujon, Leon-Kloosterziel et al. 2000; Western, Burn et al. 2001).

Due to the hydrophilic nature of mucilage, it has been suggested that mucilage may aid in germination by hydrating the seed (Grubert 1981; Boesewinkel and Bouman 1995). A number of roles for mucilage pertaining to the initial stage of water uptake may be possible. Arabidopsis mutant seeds that synthesize a reduced amount of mucilage (*myb61, ttg1, gl2*) have been shown to have altered germination rates on media with reduced water potential (simulated by increasing concentrations of polyethylene glycol) (Penfield, Meissner et al. 2001). Similarly, *atsbt1.7* seeds that make mucilage but fail in its release have also shown germination delays on polyethylene glycol (Rautengarten, Usadel et al. 2008). Seeds of *Lesquerella* species were found to release more mucilage on dryer substrates and this increase of mucilage thickness correlated with an increase in germination (Fitch 2007). This suggests that if water absorption is impeded, the imbibition period of the seed may be prolonged and germination delayed. The amount and composition of mucilage may affect the rate of water absorption, and, consequently, seed hydration and

germination. The exact role of mucilage in germination, however, remains to be determined.

RESEARCH GOALS

As outlined above, the MSC model provides new opportunities for insight into the complexities of cell wall synthesis and polysaccharide modification, with particular focus on pectins. The main goal of my research was to identify and study novel genes involved in the synthesis, secretion and modification of cell wall components in MSCs. Based on the hypothesis that the study of mutants for a specific process reveals the role of the affected genes in that process, my thesis work specifically involved:

(A) The characterization of the *patchy* mutant and its affected gene (*AtBXL1*) (described in Chapter Two). *patchy* mutants make normal quantities of pectinaceous mucilage, but exhibit abnormal mucilage release upon imbibition. Chemical and immunological analysis of *patchy* mucilage revealed an increase in arabinan. *patchy* mutants were found to be affected in the *AtBXL1* gene that encodes a bi-functional β-xylosidase/α-arabinofuranosidase. Based on the results of my study, it can be concluded that the action of this enzyme is required for the modification of RG I in either the mucilage, cell wall, or both in order for the hydrated mucilage to be released.

(B) A genetic enhancer screen of the previously known *mum4* mutant to identify novel genes involved in the synthesis or secretion of mucilage (described in

Chapter Three). The screen identified six novel mutants named *mum enhancer (men) 1-6*. Characterization of *men mum4* double mutants revealed two distinct groups. The 'release' group produced similar amounts of mucilage to *mum4* but failed to release it (*men2, 6*). The 'synthesis' group produced a further reduced amount of mucilage compared to *mum4* (*men1, 3, 4, 5*). These results suggests these genes may represent novel genes involved in either the synthesis or secretion of pectinaceous polysaccharides, or the modification of the cell wall/mucilage required for mucilage release.

(C) Characterization and mapping of the *men4* mutant and the examination of the role of mucilage in germination (described in Chapter Four). The *men4* mutant from the enhancer screen was chosen for further study due to its single mutant, reduced mucilage phenotype. Characterization of *men4* seed development, chemical analysis of *men4* mucilage, and the use of pectin-specific antibodies suggest that *MEN4* has a role in mucilage synthesis or secretion. Molecular mapping places the *MEN4* locus on the upper arm on chromosome 5, away from known mucilage production or cell wall-related genes. Finally, the role of mucilage during seed germination was investigated using a series of mucilage mutants affected in either mucilage production or release upon seed hydration. Results from germination tests under water limiting conditions suggest that the production of mucilage may be more important than its release in the promotion of germination.

FIGURES

Figure 1.1 The primary cell wall

Cellulose microfibrils are synthesized at the plasma membrane by the cellulose synthase complex. Hemicelluloses and pectins are synthesized in the Golgi apparatus and secreted to apoplast by vesicles. Hemicelluloses span the cellulose microfibrils. The main hemicellulose in many plant species is xyloglucan (blue), while hemicelluloses such as arabinoxylans (grey) and mannans (yellow) are found in lesser amounts. The main pectic polysaccharides are rhamnogalacturonan I, homogalacturonan, rhamnogalacturonan II and xylogalacturonan. Pectin domains are believed to be covalently and non-covalently linked together and can bind to xyloglucan covalently or non-covalently (adapted from Cosgrove 2005).

Figure 1.1

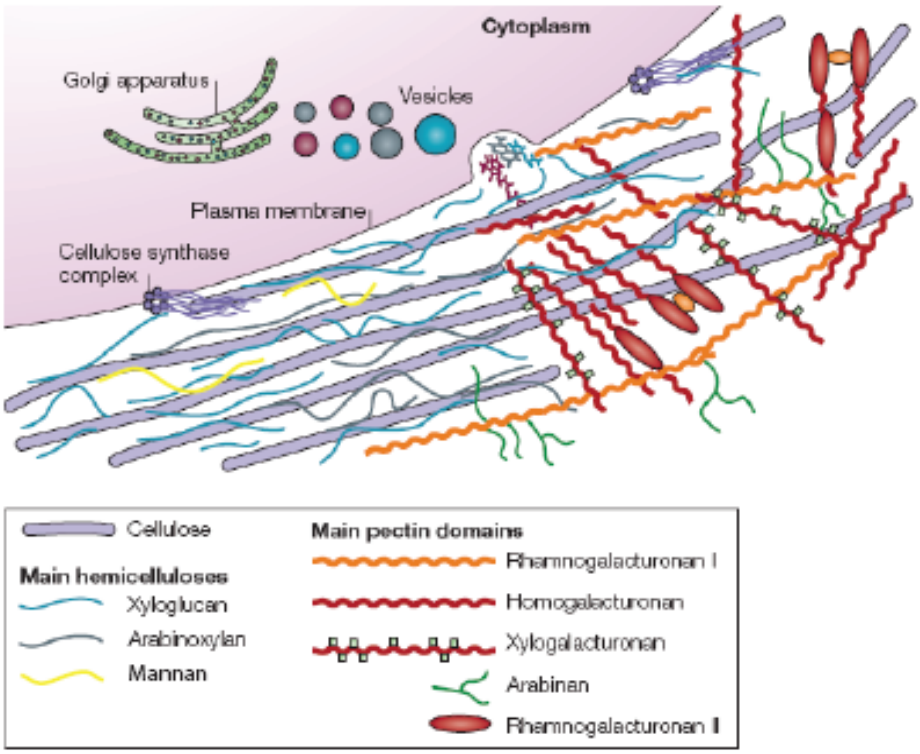

Cytoplasm
Golgi apparatus
Vesicles
Plasma membrane
Cellulose synthase complex
Cellulose
Main hemicelluloses
Xyloglucan
Arabinoxylan
Mannan
Main pectin domains
Rhamnogalacturonan I
Homogalacturonan
Xylogalacturonan
Arabinan
Rhamnogalacturonan II

Table 1.1 Genes involved in cellulose synthesis

(Updated from Somerville 2006)

Table 1.1

Gene	**Mutation**	**Phenotype**	**Gene ID**
CESA1	*rsw1*	Root swelling (conditional), embryo lethal	At4g32410
CESA2		Slight decrease in length of dark grown hypocotyls	At4g39350
CESA3	*ixr1, cev1, eli*	Isoxaben resistance, disease resistance, enhanced lignin	At5g05170
CESA4	*irx5*	Irregular xylem	At5g44030
CESA5		No clear cellulose deficient phenotype	At5g09870
CESA6	*prc, ixr2*	Cellulose defect in primary cell wall, isoxaben resistant	At5g64740
CESA7	*irx3, fra5*	Irregular xylem	At5g17420
CESA8	*irx1*	Irregular xylem	At4g18780
CESA9		Male gametophytic lethal when mutated with *CESA6* and *CESA2*, no single mutant phenotype	At2g21770
CESA10			At2g25540
KOBITO	*kob, eld1, abi8*	Dwarfed, cell elongation defects	At3g08550
KOR	*irx2, rsw2, lit, acw1*	Irregular xylem, root swelling, hypocotyl swelling	At5g49720
FRA1	*fra1*	Fragile fiber, altered orientation of cellulose microfibrils	At5g47820
FRA2	*fra2, bot, frc2, ktn1, ftr, erb3*	Fragile fiber, cytoskeletal defects	At1g80350
COBRA	*cob*	Radially expanding roots	At5g60920

Figure 1.2 The structure of the main types of pectins

Schematic representation showing the structure of the four main type of pectins: homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II), and xylogalacturonan (XGA). HG is unbranched with methyl and acetyl groups on its galacturonic acid residues. Note the galactan and arabinan side chains on RG I. RG II contains complex, conserved side chains. XGA is HG with xylose residues on its galacturonic acid backbone. Relative quantities are not represented. (Adapted from Mohnen 2008)

Figure 1.2

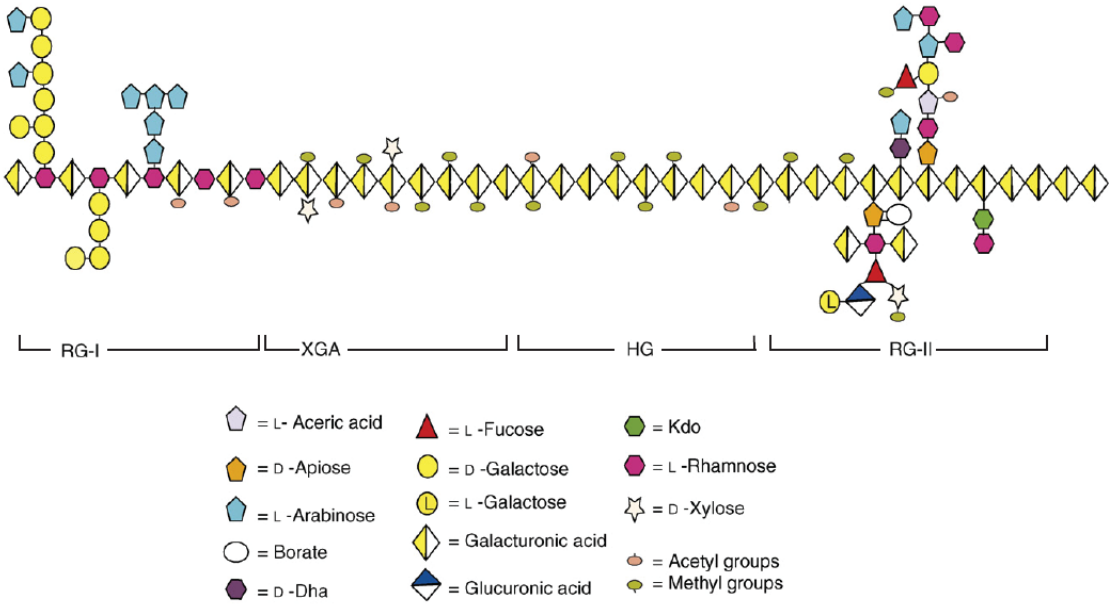

RG-I
XGA
HG
RG-II
= L- Aceric acid
= D -Apiose
= L -Arabinose
= Borate
= D -Dha
= L -Fucose
= D -Galactose
= L -Galactose
= Galacturonic acid
= Glucuronic acid
= Kdo
= L -Rhamnose
= D -Xylose
= Acetyl groups
= Methyl groups

Figure 1.3 The Arabidopsis mucilage secretory cell during mucilage synthesis

At approximately 7dpa, pectin synthesis begins in the MSC. At this stage, the Golgi apparatus and derivative secretory vesicles (maroon) are distributed throughout the cytoplasm. Nucleotide sugar interconversion enzymes embedded in the Golgi membrane or in the cytoplasm synthesize NDP-sugars, which are then transported into the Golgi lumen (sugar transporters not shown). A rhamnose synthase (MUM4/RHM2) is shown synthesizing UDP-L-rhamnose. As yet unidentified GTs in the Golgi lumen assemble pectins (RG I shown). These polysaccharides are packaged into vesicles and secreted into the extracellular space as part of the mucilage. The apoplastic, mucilage-containing pocket (pink) forms a ring around cytoplasm in the apical portion of the cell, leading to a volcano-shaped cytoplasm (grey) constrained to a central column and basal portion of the cell. Hydrolytic enzymes (BXL1 and MUM2) modify RG I sidechains in the apoplast. It is possible that this modification occurs in the mucilage and the cell wall (blue), facilitating mucilage release.

Figure 1.3

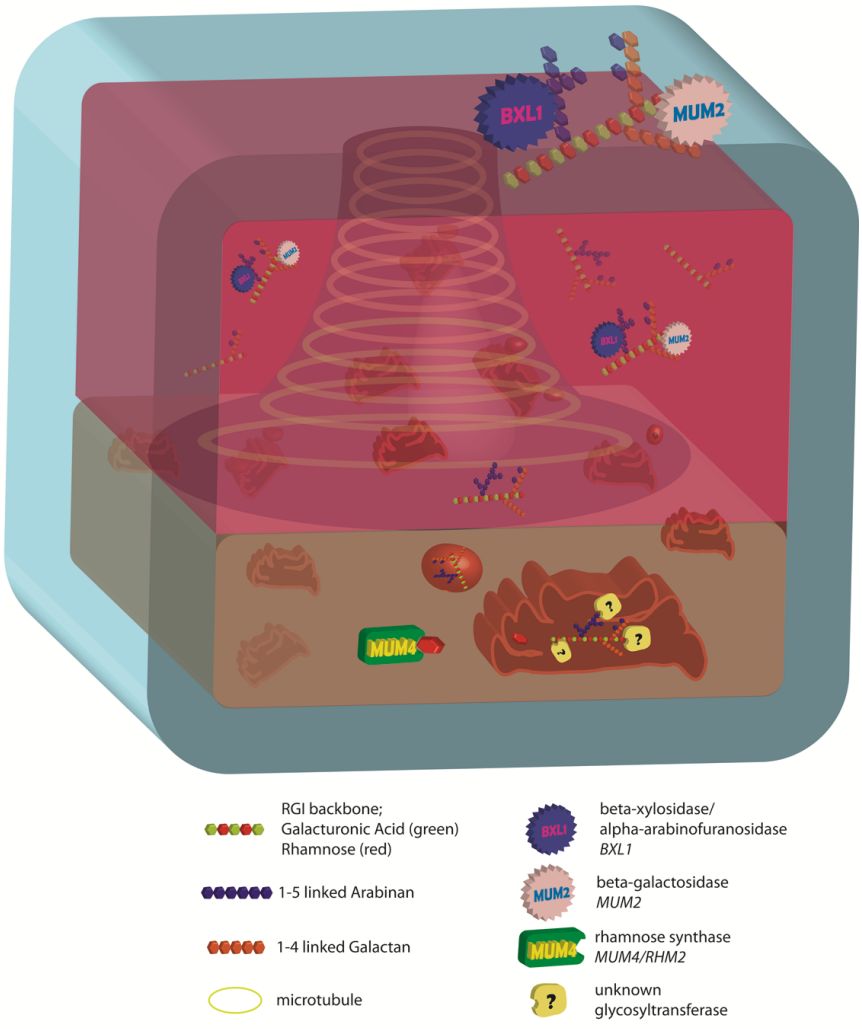
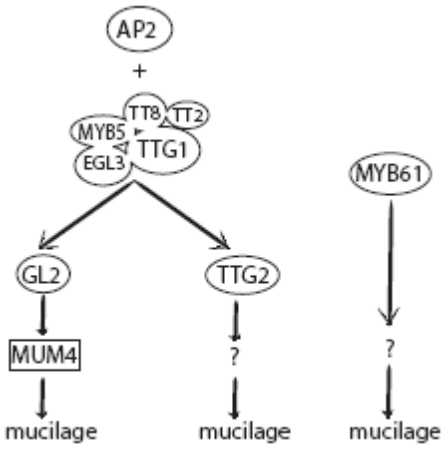

BXL1
MUM2
MUM4
?
RGI backbone;
Galacturonic Acid (green)
Rhamnose (red)
1-5 linked Arabinan
1-4 linked Galactan
microtubule
beta-xylosidase/
alpha-arabinofuranosidase
BXL1
beta-galactosidase
MUM2
rhamnose synthase
MUM4/RHM2
unknown
glycosyltransferase

Figure 1.4 Proposed regulatory pathway of mucilage production.

During MSC development, both AP2 and TTG1 regulate mucilage production through the up-regulation of downstream transcription factors (GL2 and TTG2). TTG1 acts as a complex with the bHLH proteins EGL3 and TT8, and the R2R3 MYBs MYB5 and TT2. GL2 up-regulates the rhamnose synthase MUM4, while the downstream targets of TTG2 remain unknown. A third, independent pathway of mucilage production exists under the control of MYB61 (updated from Western, Young et al. 2004)

Figure 1.4


AP2
+
TT8
TT2
MYB5
EGL3
TTG1
MYB61
GL2
TTG2
MUM4
?
?
mucilage
mucilage
mucilage

Link between Chapter 1 and 2

Chapter 1 introduced the plant cell wall and described its various components. It provided an overview of the current knowledge concerning their synthesis, deposition and modification. It also focused on the mucilage secretory cells and their role as a model for the study of cell wall components, in particular pectins. These cells synthesize copious quantities of pectinaceous mucilage that is released upon seed hydration and surrounds the seed in a gelatinous capsule. Mutants affected in the synthesis or release of mucilage allow for the identification of novel genes involved in the synthesis, secretion, or modification of this pectinaceous mucilage. Additionally, mutants affected in mucilage release may lead to the identification of genes whose products are required to modify the cell wall of these cells, allowing the mucilage to emerge and surround the seed. **Chapter 2** presents the characterization and analysis of the *patchy (pty)/beta-xylosidase (bxl1)* mutant which releases mucilage in distinct patches around the seed. The *PTY/BXL1* gene encodes a bi-functional β-xylosidase/α-arabinofuranosidase involved in the modification of side chains on the pectin RG I in the mucilage and/or the cell wall, and its action is required for proper mucilage release. This work has been published in the scientific journal Plant Physiology. The full citation for the published report is Arsovski AA, Popma TM, Haughn GW, Carpita NC, McCann MC, Western TL (2009) *AtBXL1* Encodes a Bifunctional β-D-Xylosidase/α-L-Arabinofuranosidase Required for Pectic Arabinan Modification in Arabidopsis Mucilage Secretory Cells. Plant Physiology 150(3): 1219-1234.

Chapter 2

AtBXL1 encodes a bifunctional β-D-xylosidase/α-L-arabinofuranosidase required for pectic arabinan modification in *Arabidopsis thaliana* mucilage secretory cells

ABSTRACT

Following pollination, the epidermal cells of the *Arabidopsis thaliana* ovule undergo a complex differentiation process that includes the synthesis and polar secretion of pectinaceous mucilage followed by the production of a secondary cell wall. Wetting of mature seeds leads to the rapid bursting of these mucilage secretory cells to release a hydrophilic gel that surrounds the seed and is believed to aid in seed hydration and germination. A novel mutant is identified where mucilage release is both patchy and slow, and whose seeds display delayed germination. While developmental analysis of mutant seeds reveals no change in mucilage secretory cell morphology, changes in monosaccharide quantities are detected, suggesting the mucilage release defect results from altered mucilage composition. Plasmid rescue and cloning of the mutant locus revealed a T-DNA insertion in *AtBXL1*, which encodes a putative bifunctional β-D-xylosidase/α-L-arabinofuranosidase that has been implicated as a β-D-xylosidase acting during vascular development. Chemical and immunological analyses of mucilage extracted from *bxl1* mutant seeds, and antibody staining of developing seed coats reveal an increase in 1,5-linked arabinans suggesting that BXL1 is acting as an α-L-arabinofuranosidase in the seed coat. This implication is supported by the ability to rescue mucilage release through treatment of *bxl1* seeds with exogenous α-L-arabinofuranosidases. Together, these results suggest that trimming of rhamnogalacturonan I arabinan side chains is required for correct mucilage release, and reveal a new role for BXL1 as an α-L-arabinofuranosidase acting in seed coat development.

INTRODUCTION

Differentiation of ovule integuments after pollination establishes a number of specialized cell types, including, in some species, the creation of a mucilaginous seed coat epidermis (myxospermy) (Esau 1977; Fahn 1982). Upon seed hydration, the epidermal cells of myxospermous seeds burst to release a hydrophilic, polysaccharide gel that has been suggested to play multiple roles, including promotion of seed hydration and prevention of desiccation during germination (Fahn, 1982). Mucilages are primarily composed of pectins, a complex, heterogeneous set of acidic polysaccharides that also compose the matrix of dicot primary cell walls (Fahn 1979; Grubert 1981; Roberts 1990; Carpita and Gibeaut 1993; McCann, Bush et al. 2001). The pectin rhamnogalacturonan I (RG I) is predominant in a number of seed mucilages (Naran, Chen et al. 2008) and is comprised of a backbone of alternating (1→2)-α-L-Rha and 1,4-β-D-GalA. This backbone can be substituted with at least three different types of side chains on the Rha residues: arabinans, galactans and type I arabinogalactans. Arabinans consist of 1,5-α-L-Ara with occasional 1,3- and (1→2)-α-L-Ara branchpoints, galactans are unbranched chains of 1,4-β-D-Gal, and type I arabinogalactans are 1,4-β-D-galactans that can be decorated with terminal Ara residues(Ridley, O'Neill et al. 2001; Willats, McCartney et al. 2001; Mohnen 2008).

The *Arabidopsis thaliana* (Arabidopsis) seed coat mucilage secretory cells (MSCs) undergo a complex differentiation process including mucilage synthesis and secondary cell wall production that makes them an excellent model for understanding the developmental regulation of cell wall polysaccharide synthesis (Haughn and Chaudhury 2005; Western 2006). Following pollination, these cells

undergo a phase of growth mediated by vacuolar expansion, which is succeeded by the biosynthesis and secretion of large quantities of pectinaceous mucilage to the upper tangential corners of the cell. This targeted secretion leads to the establishment of a volcano-shaped cytoplasm topped by a ring-shaped mucilage pocket, all subtending the primary cell wall. A secondary cell wall (columella) is then laid down interior to the mucilage pocket, filling in most of the cytoplasm, followed by cell death and desiccation (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000; Young, McFarlane et al. 2008). Seed hydration leads to almost instantaneous mucilage release through the breakage of the outer primary cell wall and the formation of a gel capsule around the seed that has been demonstrated to aid germination (Western, Skinner et al. 2000; Penfield, Meissner et al. 2001). Staining of Arabidopsis mucilage has revealed the presence of two distinct layers: an outer, diffuse, water-soluble layer and an inner, dense layer that is strongly associated with the seed. Both layers have been demonstrated to be predominantly comprised of unbranched RG I, with smaller quantities of cellulose, homogalacturonan, arabinans and galactans found in the inner adherent layer (Western, Skinner et al. 2000; Penfield, Meissner et al. 2001; Willats, McCartney et al. 2001; Western, Young et al. 2004; Macquet, Ralet et al. 2007; Naran, Chen et al. 2008; Young, McFarlane et al. 2008; Arsovski, Popma et al. 2009)

A number of genes required for mucilage production and MSC differentiation have been identified. These include the developmental regulator *APETALA2 (AP2)*, the epidermal cell differentiation factors *TRANSPARENT TESTA GLABRA1 (TTG1), TTG2, TRANSPARENT TESTA2 (TT2), TT8,*

ENHANCER OF GLABRA3, GLABRA2 (GL2) and *MYB5,* and *MYB61*, all of which encode transcription factors and play roles in multiple developmental processes beyond seed coat differentiation (Koornneef 1981; Jofuku, den Boer et al. 1994; Rerie, Feldmann et al. 1994; Penfield, Meissner et al. 2001; Johnson, Kolevski et al. 2002; Zhang, Gonzalez et al. 2003; Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009). A screen for mutants affected in mucilage extrusion led to the identification of the *MUCILAGE-MODIFIED* genes (*MUM1—5*)(Western, Burn et al. 2001). *mum4* mutants make a reduced amount of mucilage, *mum3* and *mum5* appear to be affected in mucilage composition, while *mum1* and *mum2* mutants are defective in mucilage release upon seed hydration. *MUM4* encodes a UDP-L-Rha synthase (RHAMNOSE SYNTHASE2 [RHM2]), an enzyme required for the synthesis of RG I, the primary pectin found in Arabidopsis seed mucilage (Usadel, Kuchinsky et al. 2004; Western, Young et al. 2004; Oka, Nemoto et al. 2007). Analysis of *MUM4/RHM2* transcripts in mutants of the MSC-related transcription factors revealed that *MUM4* is specifically up-regulated by *GL2*, which works downstream of *TTG1* and *AP2* (Western, Young et al. 2004). In contrast, *MUM2* encodes a β-galactosidase that modifies mucilage RG I side chains to allow correct hydration properties (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). Recently, a subtilisin-like serine protease, AtSBT1.7, also was found to affect mucilage release, possibly through a role in the regulation of cell wall modification enzymes acting in MSCs (Rautengarten, Usadel et al. 2008).

In this paper, we describe a novel MSC mutant named *patchy* that demonstrates a slow and stochastic mucilage release. Our results reveal that *patchy*

mutants are defective in the bifunctional β-D-xylosidase/α-L-arabinofuranosidase BXL1, and have an increased proportion of 1,5-α-L-arabinan in both their extracted mucilage and seed coat cell walls. These data suggest that BXL1 acts as an α-L-arabinofuranosidase in differentiating MSCs and such modification of the pectin structure is required for primary cell wall disruption and mucilage release.

MATERIALS AND METHODS

Plant material and growth conditions

Lines of *Arabidopsis thaliana* used were *bxl1-1* (WS ecotype; CS16299), *bxl1-2, bxl1-3* (Salk_012090 [CS16300] and 054483 [CS16301], Arabidopsis Biological Resource Center, Columbus, OH), *ap2-1, ttg1-1* (L*er* ecotype; ABRC), *myb61-1* (Col-0; gift from Michael Bevan, John Innes Centre, Norwich, UK) and *mum4-1* (Col-2) (Western, Young et al. 2004). Plants were grown and flowers staged as in (Western, Burn et al. 2001).

Microscopy

Developing seeds were staged and prepared for brightfield and scanning electron microscopy as described in Western, Burn et al. (2001). Ruthenium red staining was performed with 0.01% (w/v) ruthenium red with pre-hydration in either water or 0.05 M EDTA, as indicated.

Germination tests

Seeds were either plated dry, or pre-treated by shaking for 90 min in water or 0.05 M EDTA and plated in 0.1% (w/v) agarose after rinsing. Seeds were stratified at 4°C for 72 hrs and incubated at 22 °C under 16 hr light: 8 hr dark, following which they were counted every day for six days and germination was scored by the presence of open green cotyledons.

Cloning of *AtBXL1* via plasmid rescue

Plasmid Rescue: DNA isolated from *patchy* mutants was digested with *Sal*I, *Eco*RI or *Bgl*II, ligated and transformed into *Escherichia coli*. Plasmid DNA isolated from the resulting colonies was sequenced to identify the genomic region flanking the T-DNA insertion using T-DNA right border and left border primers (Ponce, Quesada et al. 1998).

Molecular Complementation: A 8.1 *Pml*I/*Pst*I fragment of BAC K7J8, including At5g49360 plus 2.4 kb upstream and 1.2 kb downstream sequences was cloned into pGREEN0229 (Hellens et al., 2000) to give the PTYg construct. *patchy* plants were transformed with PTYg or the empty vector as in (Clough and Bent 1998). Transformants were selected by germinating seeds on plates containing 25µg/ml glufosinate, and putative transformants were verified by PCR.

Genetic Complementation: *patchy* mutants were crossed to Salk_012090 (*bxl1-2*) and Salk_054483 (*bxl1-3*), whose identities as At5g49360 mutants were verified through sequencing of the site of T-DNA insertion and use of RT-PCR to demonstrate reduced transcription and/or transcript truncation.

Qualitative and real time RT-PCR

RNA was isolated as in Western, Burn et al. (2004), or with a modified RNeasy plant mini protocol: two siliques were ground in liquid nitrogen, resuspended in 600 µl RLT-PVP40 (540 µl RLT + 60 µl 10% [w/v] polyvinylpyrrolidone) plus 10 µl β-mercaptoethanol per ml buffer, and processed according to the manufacturer's instructions (Qiagen). One microgram samples of total RNA were treated with DNaseI and transcribed with SuperScript II Reverse Transcriptase using an oligo-dT primer according to the manufacturer's instructions (Invitrogen).

PCR for *AtBXL1* RT-PCR (Fig. 2.4B) was performed for 30 cycles using primers At5g49360 p15/16, tests for truncated transcripts were performed with At5g49360 p 11/8, and *AtBXL2* RT-PCR was done with BXL2 p1/p2 (see Table S2.3 for primer sequences). Real-time PCR was performed with an iCycler iQ Real-Time PCR System using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). PCR conditions were 95ºC for 10 min, 40 cycles of 30 s at 95ºC and 1 min at 55ºC. Transcript levels were normalized against *GAPC*. Primers used were At5g49360 p3/p4 and RT-GAPCp5/p6.

Chemical analyses

To quantify sugars in crude mucilage extracts, 50 mg of intact seeds were incubated in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 hrs at 30 ºC. No significant difference in mass was observed between WS and *bxl1-1* seed (100 counted seed; WS = 1.5 ± 0.1 mg; *bxl1* = 1.6 ± 0.0 mg; n = 3). One µmole of myo-inositol was added to the supernatant and samples were precipitated with 5 volumes

ethanol, directly hydrolyzed with 2 M trifluroacetic acid and derivatized to alditol acetates (see below). For seedling cell walls, seedlings were dark-treated for 48 hrs prior to harvest (150-200 mg of fresh weight) and alcohol-insoluble residues were prepared by grinding tissue in N_2 (l) plus 1% SDS (w/v), followed by extensive washing in alternating hot (80°C) water and 50% ethanol (60°C) with vacuum filtration.

For determination of monosaccharide ratios including GalA, five independent samples of 250 mg of seeds were extracted sequentially with 0.2% ammonium oxalate, 0.2 N and 2 N sodium hydroxide for 1 h each with vigorous shaking at 37°C. Both sodium hydroxide extractions contained 3 mg/ml sodium borohydride to prevent end-degradation and were neutralized with acetic acid. The supernatants for each extraction were filtered through a glass fibre filter, dialyzed and freeze dried. Carboxyl reduction was performed as in Kim and Carpita (1992), as modified in Carpita and McCann (1996). Derivatization to alditol acetates was performed as in Gibeault and Carpita (1991). Linkage analysis through per-*O*-methylation was also performed as in Gibeault and Carpita (1991), with inferences on linkage structure as described in Carpita and Shea (1989).

Protein isolation and enzyme assays

Preparation of Protein Extract: 7 DPA WS and *bxl1-1* siliques were ground in liquid nitrogen and extracted with 500 µl 25 mM MOPS pH 7.0, 0.5 mM Pefabloc and centrifuged 10 min at 1000 *g*. The supernatant was collected and this constituted the soluble fraction. This was repeated three times and the fractions pooled. The

remaining cell wall fraction was extracted with 2.5 ml 25 mM MOPS pH 7.0, 200 mM $CaCl_2$ during 1 hour of vigorous shaking at 4°C. The tube was then centrifuged for 10 min at 1000 *g* and the supernatant (cell wall fraction) was recovered. The cell wall fraction was salt purified on a PD-10 column (GE Healthcare), according to the manufacturer's instructions.

Cation Exchange Chromatography: The cell wall fraction was equilibrated in 25 mM sodium acetate buffer (pH 5.0) containing 5% glycerol (v/v) and 0.015% Triton X-100 (v/v), and loaded on a HiTrap-FF SP-Sepharose column (GE Healthcare). The proteins were eluted with the same buffer, first alone and then with a 0.0-0.5 M NaCl continuous gradient. One-millilitre fractions were collected and 100-200 µl assayed from each fraction for α-L-arabinofuranosidase and β-D-galactosidase activity. Arabinofuranosidase activity was equalized to galactosidase activity in each fraction.

Enzyme Activity: The reaction mixture contained 2 mM PNP-α-L-arabinofuranose or PNP-β-D-galactopyranoside (Sigma-Aldrich), 0.1 M acetate buffer (pH 5.0) and 100-200 µl protein extract in a total volume of 0.5 ml. The reaction was carried out at 37°C for 90 min and stopped by the addition of 0.5 ml of 0.4 M sodium bicarbonate to the assay mixture. Concentration of the resulting PNP was determined spectrophotometrically at 405 nm, and its amount estimated from a calibration curve.

Immunoblotting and immunofluorescence

For immunoblotting, 75 mg of seed were shaken in 0.05 M EDTA for 90 min at 37 °C. Extracts were concentrated by evaporation and resuspended in 100 µl of PBS pH 7.4, and 4 µl of concentrated mucilage spotted on nitrocellulose membranes as 2 x 2

μl aliquots. Hybridization was performed as described in (Willats, McCartney et al. 2001) with the following modifications: membranes were blocked in antibody solution for 1 hr, followed by incubation in primary antibody (1:10 [v/v] dilution of CCRC-M36 or LM6) for 90 min. Alkaline phosphatase conjugated secondary antibodies (anti-mouse and anti-rat, respectively; Invitrogen) were diluted 1:1000 (v/v) and detected using the BCIP/NBT-Purple liquid substrate (Sigma-Aldrich), with the reaction stopped by rinsing with water. CCRC-M36, an antibody specific for RG I was obtained from CarboSource (University of Georgia, Athens; http://cell.ccrc.uga.edu/;carbosource/CSS_home.html) (Young, McFarlane et al. 2008), while LM6 is specific to 1,5-linked arabinans (Willats, McCartney et al. 2001) and was obtained from PlantProbes (University of Leeds, Leeds; http://www.plantprobes.net).

For immunofluorescence on developing seeds, seeds were dissected from 7 and 9 DPA siliques and fixed for 2 hr in 4% (v/v) formaldehyde (freshly prepared from paraformaldehyde) in 50 mM PIPES (pH 7.0). Samples were rinsed, dehydrated through an ethanol series and embedded in LR White resin. Embedded samples were sectioned to 0.5 μm, affixed to slides with poly-L-lysine, and subjected to antibody detection as described in (Young, McFarlane et al. 2008), except primary antibodies were used full strength and secondary antibodies were diluted as described below for whole seed samples. Whole seed immunofluorescence was performed as in Young, McFarlane et al. (2008). Primary antibodies (1:20 [v/v]) were detected with a 1:100 (v/v) dilution of Alexfluor 488 conjugated goat anti-mouse (CCRC-M36) or goat anti-rat (LM6) secondary antibodies (Molecular Probes, Invitrogen). Seeds were

counterstained with 0.2 μg/ml propidium iodide in 50 mM phosphate buffer pH 7.4 to visualize the outer cell wall. Treatments without primary antibody were included to test for non-specific staining, and all seeds were mounted in 1:100 (v/v) India ink in 90% (v/v) glycerol in water to confirm the presence of released mucilage. Immunofluorescence samples were observed with a Zeiss Meta 510 LSM confocal microscope.

Seed treatment with exogenous enzymes

Pichia pastoris clones for three inducible, secreted, recombinant α-L-arabinofuranosidases (AN1571, AN7908 and AN80401) and one β-D-xylosidase (AN2359) were obtained from the Fungal Genetic Stock Center (FGSC, www.fgsc.net) (Bauer, Vasu et al. 2006). Methanol treatment was used to induce secretion of recombinant enzymes into the medium. Cultures were then centrifuged and the enzymes were purified by affinity to their His tag from the supernatant as described in (Bauer, Vasu et al. 2005), with the exception that proteins were affinity isolated in a batch method (rather than in a column) using 1 ml of 50% Ni-NTA His Bind Slurry (EMD Biosciences) and 4 ml Ni-NTA Bind Buffer (Buffer A, EMD Biosciences). Protein quantitation and activity assays were carried out using PNP-glycosides as described above. For seed treatment, 10 units (defined as the amount of enzyme that would release 10 nM PNP/μg protein in 1 hr) of each enzyme were used to treat seed in 800 μl water for 90 min at 37 °C. Seeds were then rinsed with water and stained with 0.01% ruthenium red with shaking for 60 min.

Water absorption measurements

A modified Bowmann capillary apparatus was set up as described by (Cui 2001), using 2-3 mm glass tubing connecting a 1 ml serological pipet and a 15 ml sintered glass funnel. Water was added from the pipet end until it reached the sintered glass. Filter paper was placed on top of the sintered glass and allowed to equilibrate. 2-5 mg of the dialyzed, freeze-dried mucilage fractions described above were placed on the saturated filter paper and the level of water in the pipet determined every 5 min for 30 min, followed by every 15 min up to 2 hrs.

Isolation of double mutants

F2 seeds were first screened for visual phenotypes (*ap2-1* heart shape seeds, *ttg1-1* yellow seeds) and/or aberrant of mucilage release in ruthenium red dye (*ap2-1, ttg1-1, myb61-1, mum4-1*). Candidate plants were genotyped for the *bxl1-1* T-DNA insertion using PCR with At5g49360 p8/p11 (Table S2.3) and T-DNA LB primer iPCR-LB (Ponce, Quesada et al. 1998). Putative *bxl1-1 myb61-1* and *bxl1-1 mum4-1* double mutants, which lack non-seed coat phenotypes, had their *myb61-1* and *mum4-1* genotypes verified using PCR. The *myb61-1* dSPM insertion was confirmed using MYB61 p1/p2 and dSPM11 (Table S2.3) (Penfield, Meissner et al. 2001). The *mum4-1* point mutation leads to the addition of a new *Mse*I site, which can be detected by digesting the PCR products from At1g53500 p1/p8 (Table S2.3) (Western, Young et al. 2004).

RESULTS

patchy mutants exhibit patchy and delayed mucilage release

To identify genes required for the synthesis and extrusion of seed coat mucilage, pools of T-DNA insertion lines (Feldmann 1991) were screened for the presence of mucilage when hydrated by staining with the pectin dye ruthenium red. With this treatment, wild-type seeds are surrounded by a thick, pink-staining capsule of mucilage (Fig. 2.1A). One novel mutant identified with this screen demonstrated a "patchy" mucilage release phenotype, in that mucilage release occurred only from random patches of seed coat epidermal cells, rather than from all cells as in wild-type seeds (Fig. 2.1, A versus B). Backcrosses revealed this phenotype is due to a mutation at a single locus (223 wild type: 84 mutant; $\chi^2 = 0.9131$; $P > 0.1$, $df = 1$), which we named *PATCHY* to reflect the mucilage release phenotype.

To quantify more precisely the differences in extruded mucilage between wild type and *patchy* mutants, the amount of mucilage release was determined. In analyzed samples of seeds, 88.0 to 95.7% of wild-type seeds were completely surrounded by mucilage, while only 2.7 to 17.3% of *patchy* seeds had complete mucilage envelopes (Table 2.1). Further, when a particular line was stained in ruthenium red without agitation and pre-hydration in water, it was found that the degree of mucilage release dropped sharply, suggesting that mechanical agitation can aid release for these mutants (Table 2.1). Timing of mucilage release was determined by filming seed

hydration in the presence of ruthenium red dye (Fig. 2.2). With wild-type seeds, bulging of cells is first seen within a few seconds, following which a diffuse cloudy layer forms around the seed that intensifies in staining over time. A second, denser layer of mucilage close to the seed becomes stained later (Fig. 2.2A) (Western, Skinner et al. 2000). Wild-type seeds consistently released their mucilage in under 1 min (n=8 seeds, all of which released), while those *patchy* seeds that released any mucilage could take from 3.5 min to 95 min, with an average release time of 26.3 min (SE = 5.6 min; n=22 seeds that released out of 83 total seeds) (Fig. 2.2B).

patchy mutants appear to undergo normal seed coat development, but have an altered mucilage composition

Seeds making a severely reduced quantity of mucilage (e.g. *mum4/rhm2* mutants) demonstrate morphological changes at the cellular level as well as being defective in mucilage release (Western, Burn et al. 2001; Western, Young et al. 2004). Specifically, when observed with SEM, the columella found in the centre of the MSCs is reduced in prominence in comparison to wild-type columellae (Fig. S2.1, A versus B). This change in columella shape is accompanied by reduced intensity of mucilage staining with toluidine blue and smaller mucilage pockets. Sectioning and toluidine blue staining of developing *patchy* mutant seeds, however, shows similar mucilage staining, mucilage pocket size and columella shape compared with wild type (Fig. 2.1, E to L). This resemblance to wild type is also observed when SEM is used to visualize the cell surface details (Fig. S2.1, C versus D).

A threshold quantity of mucilage could be present in *patchy* mutants, making them look morphologically normal, but interfering with mucilage release in the stochastic manner observed. Alternately, mucilage hydration properties could be affected as in *mum2* mutants (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). Mucilage release can be induced in some reduced mucilage mutants by treatment with the heavy metal chelators such as EDTA. Chelator treatment is believed to reduce binding of pectin chains through calcium bridges, allowing more extensive hydration and swelling of mucilage and/or weakening of the primary cell wall. When *patchy* seeds are treated with EDTA, and their mucilage levels compared to wild-type seeds, no gross difference was detected between the thickness of wild-type and *patchy* mucilage enveloping the seeds (compare Fig.2.1, A and B with C and D).

To confirm this result, the mild chelator ammonium oxalate was used for extraction of mucilage and the crude extracts were directly hydrolyzed and derivatized to alditol acetates (Table 2.2). A slight, but non-significant drop was observed both in the quantity of Rha and in total sugars. This was reflected in a small, but significant decrease in both Fuc and Xyl. In addition, the amount of Ara increased ~1.5 fold (P-value <0.005) in *patchy* mucilage. Following mucilage extraction, the remaining seeds were also analyzed, but no significant differences in monosaccharide levels were observed (Table 2.2). These results suggest that there is a change in mucilage composition that may be responsible for the slow and patchy mucilage release observed in the *patchy* mutants.

patchy seeds show delayed germination in the absence of pre-hydration

General observation of growing *patchy* mutant plants revealed no significant vegetative differences compared to wild-type plants. A timecourse of seed germination revealed a 1 to 2 day delay of germination such that there is a roughly 40% reduction in germination in *patchy* mutants versus wild-type seeds at 3 days after plating, which becomes only 10% after 4 days (Fig.2.3). Pre-hydration of *patchy* seeds, however, by shaking for 90 min in either water or EDTA restores germination to wild-type rates (Fig. 2.3). These data suggest that the changes in the mucilage of *patchy* mutants affect germination, presumably due to the reduced ability for *patchy* seeds to attract or hold water around the seed.

patchy mutants have mutations in the gene encoding the bifunctional β-D-xylosidase/α-L-arabinofuranosidase BXL1

The *patchy* mutant was isolated from a pool of T-DNA insertional mutants (CS2497) (Feldmann 1991). Kanamycin resistance encoded by the T-DNA was found to segregate with the mutant phenotype. In the F2 of a backcross to WS, kanamycin resistance segregated with a 3:1 ratio (161 resistant:62 sensitive; χ^2 = 0.9188; $P > 0.1$, df = 1). Of the kanamycin resistant plants, one-third had a patchy mucilage phenotype (115 wild type: 48 patchy; $\chi^2 = 1.1203$; $P > 0.1$, df = 1), and 15 of these *patchy* mutants chosen at random gave rise to 100% kanamycin resistant progeny. Southern blot analysis with multiple T-DNA probes confirmed that the *patchy* mutant resulted from the insertion of a single, largely intact T-DNA of

approximately 14kb (data not shown). Using plasmid rescue, the insertion was located within the first intron of At5g49360, which encodes the putative β-D-xylosidase/α-L-arabinofuranosidase BXL1 (Goujon, Minic et al. 2003; Minic, Rihouey et al. 2004) (Fig. 2.4A). Molecular complementation was performed using a genomic clone for At5g49360 under the endogenous promoter (PTYg; 8.1 kb of BAC K7J8, including 2.4kb upstream and 1.2kb downstream sequence). *patchy* mutants transformed with PTYg were found to have wild-type mucilage (9/10 independent transformants), while pGREEN0229-transformed plants retained the patchy mucilage phenotype (11/11) (Fig. 2.5, A and B; Table S2.1). These results are consistent with the hypothesis that an insertion in *AtBXL1* is responsible for the seed coat phenotype.

Since the *patchy* mutant (renamed *bxl1-1*) has a T-DNA insertion in an intron (Fig. 2.4A), we examined the transcript level of *AtBXL1* in *bxl1-1* mutants using RT-PCR. In leaves, where *AtBXL1* is highly expressed, a strong band is found in wild-type WS leaves, while a barely detectable band is seen for *bxl1-1* mutants at a saturating cycle number (Fig. 2.4B). Real-time PCR on 7 DPA seeds suggests that there is an approximately 1000-fold decrease in transcript amount in *bxl1-1* mutants (WS threshold cycle [C_t] = 21.8 ± 0.1 SE, n = 3; *bxl1-1* C_t = 32.1 ± 0.2 SE, n = 3). Two further T-DNA insertion lines in At5g49360 were obtained from the Salk Sequence Indexed Insertion collection (Alonso et al., 2003), which we have named *bxl1-2* (Salk_012090) and *bxl1-3* (Salk_054483). *bxl1-2* has an insertion in exon 5, while the insertion in *bxl1-3* is in intron 5 (Fig. 2.4A). Both *bxl1-2* and *bxl1-3* have similar patchy mucilage release and germination phenotypes to *bxl1-1*, and do not

complement *bxl1-1* in genetic crosses, confirming they are insertions in the same gene (Table S2.1; data not shown). Real-time PCR analyses reveal a decrease in the transcripts of both new alleles (22- and 450-fold for *bxl1-2* and *bxl1-3*, respectively; Col-0 C_t = 24.1 ± 0.4 SE; *bxl1-2* C_t = 28.6 ± 0.1 SE; *bxl1-3* C_t = 32.9 ± 0.4; n = 3). A truncation of the *bxl1-3* transcript beyond the insertion site was detected with RT-PCR. The *bxl1-2* transcript detected results from read-through of a large insertion (data not shown). Since the *bxl1-2* insertion lies upstream of the predicted catalytic Glu found in GH3 family enzymes(Minic, Rihouey et al. 2004), this insertion would be expected to render the protein non-functional. Because *bxl1-1* approaches a transcriptional null, all further analyses were performed on this allele.

AtBXL1 is transcribed throughout the plant, including in differentiating seed

RT-PCR was used for an initial determination of the transcription of *AtBXL1* throughout various tissues as well as in siliques before, during and after the time of seed mucilage production (4, 7 and 10 DPA, respectively). *AtBXL1* transcripts were found in each of these tissues (Fig. 2.4B). Promoter-GUS experiments have shown that *AtBXL1* is expressed in the vasculature of the silique (Goujon, Minic et al. 2003), thus we compared the transcription of *AtBXL1* in developing seeds versus siliques using real-time PCR (Fig. 2.4C). *AtBXL1* was found to be strongly expressed in both seeds and siliques at 7 and 10 DPA, with higher transcript levels (lower C_t) for siliques versus seeds. Separation of seeds from silique was not done for 4 DPA due to the difficulty of removing seeds at this stage.

bxl1 mutants have an increase in the proportion of Ara and arabinan-type Ara linkages in their seed coat mucilage, and have decreased α-L-arabinofuranosidase activity in their siliques

Since *AtBXL1* encodes a putative β-D-xylosidase/α-L-arabinofuranosidase, the mucilage composition was investigated more closely. Mucilage was sequentially extracted from seeds using the mild chelator ammonium oxalate, 0.2 N and 2 N sodium hydroxide to create fractions consisting of loosely attached pectins, more strongly linked pectins, and strongly-linked pectins and cross-linking glycans (hemicelluloses), respectively. These extracts were extensively dialyzed to remove monosaccharide and chemical contaminants and analyzed for monosaccharide composition through carbodiimide activation and reduction with sodium borodeuteride followed by hydrolysis and derivatization to alditol acetates to allow for detection of both neutral sugars and uronic acids (Kim and Carpita 1992; Carpita, McCann et al. 1996). For all three extracts, the primary sugars were Rha and GalA, reflecting isolation of mucilage that is largely composed of RG I (Table 2.3) (Penfield, Meissner et al. 2001; Western, Young et al. 2004; Naran, Chen et al. 2008). A decrease in the proportion of these two sugars, however, was seen in fractions from the harsher extractions, indicating the extraction of increased levels of RG I containing arabinan and galactan side chains as well as cellulose and other complex polysaccharides observed in the inner adherent layer of mucilage (Macquet, Ralet et al. 2007). Comparison between the extracts for wild type and *bxl1-1* revealed increases in Ara in both ammonium oxalate and 0.2 N sodium hydroxide fractions, with concurrent decreases in these same extracts for Xyl. The statistical significance

of the ammonium oxalate Ara increase (P-value = 0.002), is consistent with our earlier ammonium oxalate extracts without carboxyl reduction. Slight changes were also observed for Glc in 0.2 N sodium hydroxide (decrease), and Xyl, Glc (increase), Man and Gal (decrease) in 2 N sodium hydroxide extracts.

To determine if more subtle changes in the chemical structure of the mucilage occur in *bxl1-1* extracts, analysis of the sugar linkages present was performed through per-*O*-methylation (Carpita and Shea 1989; Gibeaut and Carpita 1991) for all three extractions (Table 2.4). Similar to the monosaccharide analysis, few changes are observed between wild type and *bxl1-1* across the extracts. The only difference that is consistently seen across all three extractions is an increase in the ratio of 1,5-Ara (5-Ara*f*) in *bxl1-1* versus wild type mucilage, which is consistent with the increased Ara at the monosaccharide level. Further, the lack of change in the proportion of branch-point Rha residues (2,4-Rha*p*, 2,3-Rha*p*) suggests that the side chains of RG I are increased in size rather than abundance in the mutant. Several changes were observed in the 2 N sodium hydroxide extract: a decrease in both (1→2)-Rha (2-Rha*p*) and 1,4-GalA (4-GalA*p*) suggests less RG I in the mutant. A slight change in xylan structure may also be occurring as in the 2 N sodium hydroxide extraction, there is a decrease in 1,4-Xyl (4-Xyl*p*) accompanied by an increase in branchpoint Xyl residues (2,4-Xyl*p*). The level of 1,4-Man is also slightly decreased in this extraction. A similar slight change to branching of type II arabinogalactans is suggested in the 0.2 N sodium hydroxide extraction where there is a decrease in (1→6)-Gal and increase in branchpoint Gal (3,6-Gal*p*).

To determine if there were any differences in cell wall composition beyond the mucilage, alcohol insoluble cell wall material was isolated from *bxl1-1* seedlings. Fourier Transform-Infrared Spectroscopy (FT-IR) and monosaccharide analysis were performed. While the FT-IR results suggested the possibility of a slight change in pectin esterification (data not shown), no statistically significant differences were seen in the monosaccharide composition (Table S2.2), consistent with the results of (Goujon, Minic et al. 2003) for stems of antisense *BXL1* plants.

To confirm that the changes in Ara and arabinan levels found in extracted *bxl1-1* mucilage resulted from reduced α-L-arabinofuranosidase activity in *bxl1-1* mutants, enzyme assays were undertaken. Protein was extracted from developing wild-type and *bxl1-1* siliques and fractions were collected after separation on a cation exchange column, following which α-L-arabinofuranosidase activity was assayed using PNP-α-L-Ara*f*. Two peaks of α-L-arabinofuranosidase activity were detected, one of which was strongly reduced in the *bxl1-1* mutant, confirming that *bxl1-1* mutants have decreased α-L-arabinofuranosidase activity (Fig. 2.4D). An increase in the second peak was also observed in the *bxl1-1* mutant, suggesting possible compensation by one of the other two α-L-arabinofuranosidases identified in Arabidopsis siliques (Minic, Do et al. 2006).

bxl1 mutants have an increase in, and altered distribution of, RG I arabinan side chains in their seed coat cell walls

To confirm changes existed in the levels of 1,5-linked arabinans in *bxl1-1* versus wild-type mucilage, immunoblots with extracted mucilage were performed using the arabinan-specific antibody LM6 (Willats, Marcus et al. 1998; Willats, McCartney et al. 2001) (Fig. S2.2). The CCRC-M36 antibody specific to unbranched RG I and raised to Arabidopsis mucilage was used in parallel as a control (Young, McFarlane et al. 2008). Strong binding of LM6 was observed for EDTA extracts of *bxl1-1* mucilage, while staining was only faintly visible for wild-type samples (Fig. S2.2A). Control immunoblots performed with CCRC-M36 showed roughly equal staining in wild type and *bxl1-1*, suggesting a significant increase in 1,5-linked arabinans in *bxl1-1* mucilage (Fig. S2.2A).

Staining of developing seed coats with LM6 and CCRC-M36 was also performed. In wild-type seed coats, only faint staining was detected with LM6. The only significant staining of the mucilage secretory cells was at the cell junctions on the lower face of the cells (Fig. 2.6A). By contrast, *bxl1-1* seed coats had very intense staining of all cell walls, including all cell walls of the mucilage secretory cells (Fig. 2.6B), suggesting both a general increase in arabinans in *bxl1-1* seed coats and a specific increase in arabinans in the radial and outer cell walls of the mucilage secretory cells. Similar to the immunoblot results, no significant differences were detected with CCRC-M36 staining (Fig. 2.6C,D). Using whole seed immunofluorescence of mature seeds, the increase in cell wall LM6 staining was reflected in both the intensity of LM6 stain surrounding *bxl1-1* mutant seeds and the

presence of clearly identifiable small sections of intact, hexagonal primary cell walls (Fig. S2.2D,E). These results suggest that the increase in LM6 epitopes within the wall may alter the mode of primary cell wall breakage during mucilage release in *bxl1* mutants.

The *bxl1* mucilage release defect can by rescued by treatment with exogenous α-L-arabinofuranosidases

To test the potential role for an α-L-arabinofuranosidase in mucilage release, seeds were treated with recombinant enzymes obtained from an established collection of fungal polysaccharide degrading enzymes expressed in a secreted, affinity tagged form in *Pichia pastoris* (Bauer, Vasu et al. 2006). *bxl1-1* seeds were treated with three affinity-purified recombinant arabinofuranosidases (AN1571, AN7908 and AN80401) (Bauer, Vasu et al. 2006). Treatment with all three arabinofuranosidases led to rescue of the patchy mucilage release phenotype (Fig. 2.5, D and E), while treatment with enzyme buffer and a β-D-xylosidase (AN2359) (Bauer et al., 2006) had no effect on mucilage release (Fig. 2.5, C and F). These rescue results suggest that removal of arabinans can promote mucilage release through modification of MSC walls and/or mucilage of *bxl1-1* mutants.

Genetic interactions between *AtBXL1* and known MSC-related transcription factors

To test the genetic relationship between *AtBXL1* and known MSC genes, double mutant lines were constructed between *bxl1-1* and *ap2-1, ttg1-1, myb61-1*, and *mum4-1* mutants. The double mutants *bxl1-1 ap2-1* and *bxl1-1 ttg1-1* look identical to the *ap2-1* (weak allele that shows reduced, patchy mucilage when EDTA-treated) and *ttg1-1* (no mucilage release under EDTA treatment) mutants, respectively (Fig. 2.8, A to D). These results suggest an epistatic relationship, resulting either from regulation of *AtBXL1* by *AP2* and *TTG1* or a masking of the *bxl1* phenotype by the severity of the *ap2-1* and *ttg1-1* phenotypes. By contrast, *bxl1-1 myb61-1* double mutants have an additive phenotype in which the double mutant does not release mucilage when pretreated with water (Fig. 2.8, E versus F), but releases a similar amount to the *myb61-1* parent when treated with EDTA (data not shown). Thus *AtBXL1* is acting independently from *MYB61* in the ability of seeds to release mucilage, suggesting that it is not regulated by *MYB61*. Unsurprisingly, *bxl1-1 mum4-1* double mutants also have an additive phenotype of no mucilage release when treated with EDTA (Fig. 2.8, G versus H), implying that the chelator-induced ability to release mucilage in mucilage-reduced *mum4* mutants is compromised by the loss of *AtBXL1* function. This enhancement of the *mum4-1* phenotype by *bxl1-1* suggests that the retention of mucilage release in the *ap2-1 bxl1-1* mutant, at least, may reflect true epistasis and possible regulation of *AtBXL1* by *AP2*.

Regulation of *AtBXL1* by AP2 and TTG1 was further investigated through real-time PCR of *AtBXL1* in 7 DPA seeds of *ap2-1* and *ttg-1* mutants, with only a

slight but non-significant decrease in *AtBXL1* transcript being found in *ap2-1* mutants (data not shown).

DISCUSSION

Hydration of Arabidopsis seeds leads to the breakage of the outer primary cell wall of the MSCs and the release of pectinaceous mucilage to surround the seed (Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). Our data reveal that the β-D-xylosidase/α-L-arabinofuranosidase BXL1 may play a role in mucilage release through the degradation of 1,5-linked arabinans in the mucilage and/or primary cell wall. These results suggest a requirement for the trimming of RG I side chains to allow proper swelling of the mucilage and/or weakening of the primary cell wall to enable mucilage release. The requirement for developmentally coordinated changes to arabinan and RG I side chain numbers and branching is an emerging theme in the regulation of cell wall properties during plant growth and reproduction (Willats, Steele-King et al. 1999; Fulton and Cobbett 2003; Lee, Bahn et al. 2003; Leboeuf, Thoiron et al. 2004; Pena and Carpita 2004; Minic, Do et al. 2006; Xiong, Balland-Vanney et al. 2007; Lee, Derbyshire et al. 2008).

Mucilage release requires modification of RG I side chains

bxl1 mutant slow and patchy mucilage release is correlated with a change in mucilage composition. These compositional changes appear to result in altered mucilage hydration properties, as water absorption tests of extracted mucilage suggest the speed of hydration and absorption capacity of *bxl1* mucilage are lower than those of wild-type mucilage (Fig. S2.3). Chemical analysis and immunoblot results for *bxl1* mucilage revealed an increase in Ara and 1,5-linked arabinans over

wild-type mucilage (Tables 2.3, 2.4; Fig. S2.2), suggesting that chemical modifications to the mucilage *in vivo* may be required for sufficient mucilage swelling and release. The retention of a similar number of branchpoint 2,3- and 2,4-Rha*p* residues between *bxl1* and wild-type mucilage suggest that trimming of 1,5-arabinans is occurring rather than the complete removal of the arabinan side chains from RG I polymers.

While chemical changes are observed in the extracted mucilage, a role for BXL1 in weakening of the outer primary cell wall to allow mucilage release is also supported. Mucilage release can be rescued through external treatment of *bxl1* seeds with exogenous α-L-arabinofuranosidases (Fig. 2.5, D and E), suggesting that more or larger arabinans are found in the primary cell wall in *bxl1* seeds. This is consistent with the intense staining of the cell walls of developing *bxl1* seeds hybridized with the arabinan-specific antibody LM6 (Fig. 2.6B) (Willats, Marcus et al. 1998). Failure to trim arabinans in the MSC primary cell wall may lead to wall stiffening in *bxl1*mutants, resulting in increased difficulty of cell wall breakage and the patchy mucilage release.

The exact contribution of increased cell wall strength versus reduced or slowed mucilage swelling to the altered cell wall breakage pattern seen in *bxl1* mutants is unclear. In wild-type MSCs, rapid pectin swelling is proposed to lead to rupture at the thin, radial cell walls and upward folding of the outer cell wall remnants still attached at the columella (Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). In *bxl1* mutants, either slow-building pressure due to altered mucilage and/or prolonged pressure build-up resulting from a stronger primary cell

wall could be expected to lead to outward bulging of the cell wall rather than immediate rupture. This continued bulging could eventually result in lifting of the whole surface cell wall upwards, breaking not only at the edges of the cell, but also severing connections to the columella, resulting in the hexagonal cell wall fragments observed in *bxl1* mutants (Fig. S2.2).

Mucilage release requires the activity of multiple genes

In addition to *AtBXL1*, several other genes have been demonstrated to play roles in mucilage release: *MUM1, MUM2* and *AtSBT1.7* (Western, Burn et al. 2001; Dean, Zheng et al. 2007; Rautengarten, Usadel et al. 2008). While *MUM1* is yet to be cloned, the others appear to be involved, directly or indirectly, in structural modifications of the mucilage and/or outer cell wall that appear to be necessary for proper mucilage hydration and release. Similar to *AtBXL1*, *MUM2* encodes a glycosyl hydrolase, specifically a β-galactosidase, that is believed to be involved in the degradation of RG I side chains during MSC differentiation to allow the proper swelling and release of mucilage (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). *mum2* mucilage shows abnormally high levels of arabinans as well as terminal Gal residues and type 2 arabinogalactans (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007), suggesting that MUM2 β-galactosidase activity may be required for activity of BXL1, possibly to allow access of BXL1 to its substrate. A requirement for the concerted activity of two or more glycosyl hydrolases for proper degradation of polysaccharides has been suggested previously for many enzymes, including other

bifunctional β-D-xylosidases/α-L-arabinofuranosidases (Minic and Jouanin 2006; Xiong, Balland-Vanney et al. 2007; Minic 2008).

Unlike the glycosyl hydrolases produced by *AtBXL1* and *MUM2*, the subtilisin-like serine protease encoded by *AtSBT1.7* appears to work indirectly on primary cell wall and/or mucilage structure. *sbt1.7* mutants lack mucilage release when hydrated with water, however, treatment with EDTA leads to mucilage release and the "shedding" of a sheet of intact upper primary cell walls (Rautengarten, Usadel et al. 2008). This latter phenotype is similar to, but more severe than, that seen in *bxl1* mutants and strongly points towards defects in the primary cell wall. However, as lack of mucilage release in *sbt1.7* mutants is associated with increased levels of de-methylesterified mucilage and total seed cell walls, a role for altered mucilage behaviour cannot be ruled out (Rautengarten, Usadel et al. 2008). The identification of prolonged pectin methylesterase activity in *sbt1.7* mutants suggests both that AtSBT1.7 may inactivate these enzymes *in vivo*, and that precise regulation of pectin methylesterase activity in developing MSCs is required (Rautengarten, Usadel et al. 2008).

MSC differentiation, at least at the level of mucilage synthesis, appears to be regulated by a hierarchy of transcriptional regulators, as demonstrated by the specific up-regulation of the Rha synthase gene *MUM4/RHM2* by AP2 and TTG1 via GL2 (Western, Young et al. 2004). Double mutant analysis revealed epistasis of *AP2* over *AtBXL1*, and the possibility of similar regulation of *AtBXL1* by AP2 (Fig. 2.8). This correlates with a slight decrease in *AtBXL1* transcript levels in 7 DPA seeds with real-time PCR, but should be further investigated through more specific analyses.

BXL1 acts as a bifunctional β-D-xylosidase/α-L-arabinofuranosidase *in vivo* and plays different roles in different tissues

AtBXL1 was identified by Goujon, Minic et al. 2003 as a gene encoding a β-xylosidase expressed in the vasculature, for which antisense plants with reduced β-xylosidase activity were found to have various growth defects including short siliques and curled leaf edges. Our identification of an insertional mutant in *AtBXL1* with significantly reduced transcript, however, revealed only the "patchy" release of seed coat mucilage and delayed germination. The lack of a reported mucilage defect for the antisense lines may be due either to the patchy nature of the phenotype or the poor expression of the 35S promoter in MSCs (Young, McFarlane et al. 2008). The difference in whole plant phenotypes between *bxl1* and the *AtBXL1* antisense lines may be due to the additional knockdown of *AtBXL2* in the antisense plants. *AtBXL2* is 70% identical to *AtBXL1* at the nucleotide level and its knockdown could not be ruled out by Goujon, Minic et al. 2003 using Northern blots. RT-PCR of *AtBXL2* reveals that it has a lower, but overlapping transcription pattern in most tissues where *AtBXL1* is expressed (leaves, stems, seedlings, roots, inflorescences and 4 DPA siliques; Fig. S2.2A) and BXL2 has been co-purified from stems with BXL1 in a proteomic analysis (Minic, Jamet et al. 2007). Preliminary results suggest that *bxl1 bxl2* double mutants have shortened siliques and curled leaf edges similar to that observed in the antisense lines (data not shown).

While Goujon, Minic et al. 2003 suggested that BXL1 was functioning as a β-D-xylosidase in the stems, our chemical, enzymatic and immunological analyses of *bxl1-1* mucilage and seed coat suggest that it is working as an α-L-arabinofuranosidase in seed MSCs due to the accumulation of both Ara and arabinans in *bxl1* mutants. α-L-arabinofuranosidase function correlates with the data of Minic, Rihouey et al. (2004), who isolated BXL1 enzyme (XYL1) from Arabidopsis stems and demonstrated its activity as a bifunctional β-D-xylosidase/α-L-arabinofuranosidase with a substrate preference for sugar beet 1,5-linked arabinan in *in vitro* enzyme assays. Taking both our data and those of Goujon, Minic et al. 2003, it appears that BXL1 performs two roles: that of a β-D-xylosidase and/or a bifunctional β-xylosidase/α-L-arabinofuranosidase in the remodeling of xylans in vascular development, and that of an α-L-arabinofuranosidase in the cell wall of MSCs. BXL1 belongs to glycosyl hydrolase family three (GH3), from which a number of enzymes have been characterized to have β-xylosidase (XYL4/[At]BXL4), α-L-arabinofuranosidase ([At]BXL3, PpARF2, [Hv]ARA-I) or bifunctional β-xylosidase/α-L-arabinofuranosidase activities (MsXyl1, [Hv]XYL, RsAraf1) (Lee, Hrmova et al. 2003; Minic, Rihouey et al. 2004; Tateishi, Mori et al. 2005; Kotake, Tsuchiya et al. 2006; Minic, Do et al. 2006; Xiong, Balland-Vanney et al. 2007). The bifunctional enzymes tend to have a substrate preference for arabinans *in vitro* and to be expressed in developing tissues, suggesting roles in the modification of primary cell walls rather than acting on secondary cell wall xylans (Kotake, Tsuchiya et al. 2006; Xiong, Balland-Vanney et al. 2007). The bifunctionality of these enzymes has been suggested to allow flexibility of cell wall modifications with a

limited number of enzymes. The activity of BXL1 as a β-xylosidase in stems (Goujon, Minic et al. 2003) and as an α-L-arabinofuranosidase in MSCs is the first *in vivo* demonstration of a bifunctional cell wall enzyme playing different roles in different tissues.

Regulation of RG I and arabinan side chain structure in plant growth and development

Cell walls are heterogeneous and dynamic structures that vary in composition throughout growth and development (Carpita and Gibeaut 1993; Carpita and McCann 2000; Somerville, Bauer et al. 2004; Farrokhi, Burton et al. 2006). Arabinan and galactan side chains show developmental, tissue, cell type and within-cell wall specificity in their localization (Willats, Gilmartin et al. 1999; Orfila, Seymour et al. 2001; Ridley, O'Neill et al. 2001; Willats, McCartney et al. 2001; McCartney and Knox 2002; Yves Verhertbruggen, Susan E. Marcus et al. 2009). Roles for arabinans and their modification during development have come from the localization of arabinan epitopes to meristematic and proliferating root cells in carrots as well as the transcript expression pattern of both GH3 and GH51 α-L-arabinofuranosidases and bifunctional β-D-xylosidase/α-L-arabinofuranosidases in developing roots and stems (Willats, Steele-King et al. 1999; Fulton and Cobbett 2003; Lee, Hrmova et al. 2003; Minic, Do et al. 2006; Xiong, Balland-Vanney et al. 2007). Modification of arabinan side chains, particularly debranching or trimming by α-L-arabinofuranosidases, also has been suggested in fruit ripening of Japanese pear (Tateishi, Mori et al. 2005),

storage of apples (Pena and Carpita, 2004), pedicel abscission in poinsettia (Lee, Derbyshire et al. 2008) and in the growth of suspension-cultured microcalli (Leboeuf, Thoiron et al. 2004). These latter modifications have been correlated with loss of cell adhesion, while modulation of arabinan levels and branching during development may be associated with changes to cell wall elasticity as arabinans have been suggested to act as cell wall plasticizers and/or to form direct linkages between pectins and cellulose (Jones, Milne et al. 2003; Jones, Milne et al. 2005; Zykwinska, Ralet et al. 2005; Zykwinska, Thibault et al. 2007; Moore, Farrant et al. 2008).

The direct effects of a reduction in arabinan side chains has been observed both through the identification of Ara deficient mutants in *Nicotiana plumbaginifolia* (*nolac-H14*) and Arabidopsis (*arad1*), and through ectopic expression of the family 51 α-L-arabinofuranosidase ARAF1 in Arabidopsis and a fungal endo-α-1,5-arabinanase in potato tubers (Iwai, Ishii et al. 2001; Skjot, Pauly et al. 2002; Harholdt, Jensen et al. 2006; Chavez Montes, Ranocha et al. 2008). While no obvious phenotypic effects were observed in Arabidopsis *arad1* mutants, *nolac-H14* mutants were identified through their reduced cell-cell adhesion, ARAF1 overexpression plants had delayed flowering time and altered stem architecture, and tissue from fungal arabinanase-expressing potatoes demonstrate altered wall stiffness (Iwai, Ishii et al. 2001; Ulvskov, Wium et al. 2005; Harholdt, Jensen et al. 2006; Chavez Montes, Ranocha et al. 2008). Our current results complement and extend these data by demonstrating that the loss of arabinan modification in a specific cell type can lead to observable consequences on cell and plant developmental behaviour: namely, the lack of cell

wall breakage to facilitate mucilage release and consequent delayed seed hydration and germination.

Figure 2.1. Ruthenium red staining and seed coat structure of *bxl1-1* versus wild-type Wassileskija (WS) seeds.

(A-B) Wild-type (A) and *bxl1-1* (B) seeds shaken in water then stained with ruthenium red. Note patchy mucilage staining around *bxl1-1* seeds. (C-D) Wild-type (C) and *bxl1-1* (D) seeds stained with ruthenium red after shaking in 0.05 M EDTA. (E-L) Cross sections of developing seed coat epidermal cells stained with toluidine blue. (E-H) Wild-type. (E) 4 DPA with central vacuole filling most of cell. (F) 7 DPA, purple-staining mucilage is accumulating. (G) 10 DPA, dark purple-staining mucilage found in upper tangential regions of the cell, above the blue-staining secondary cell wall forming around the cytoplasm. (H) 13 DPA, secondary cell wall (blue) has filled in the central region of the cell. (I-L) *bxl1-1* mutant sections, note their similarity at each stage to wild type. (I) 4 DPA. (J) 7 DPA. (K) 10 DPA. (L) 13 DPA. The scale bars in panels (A-D) are 200 μm; (E-L) are 50 μm.

Figure2.1

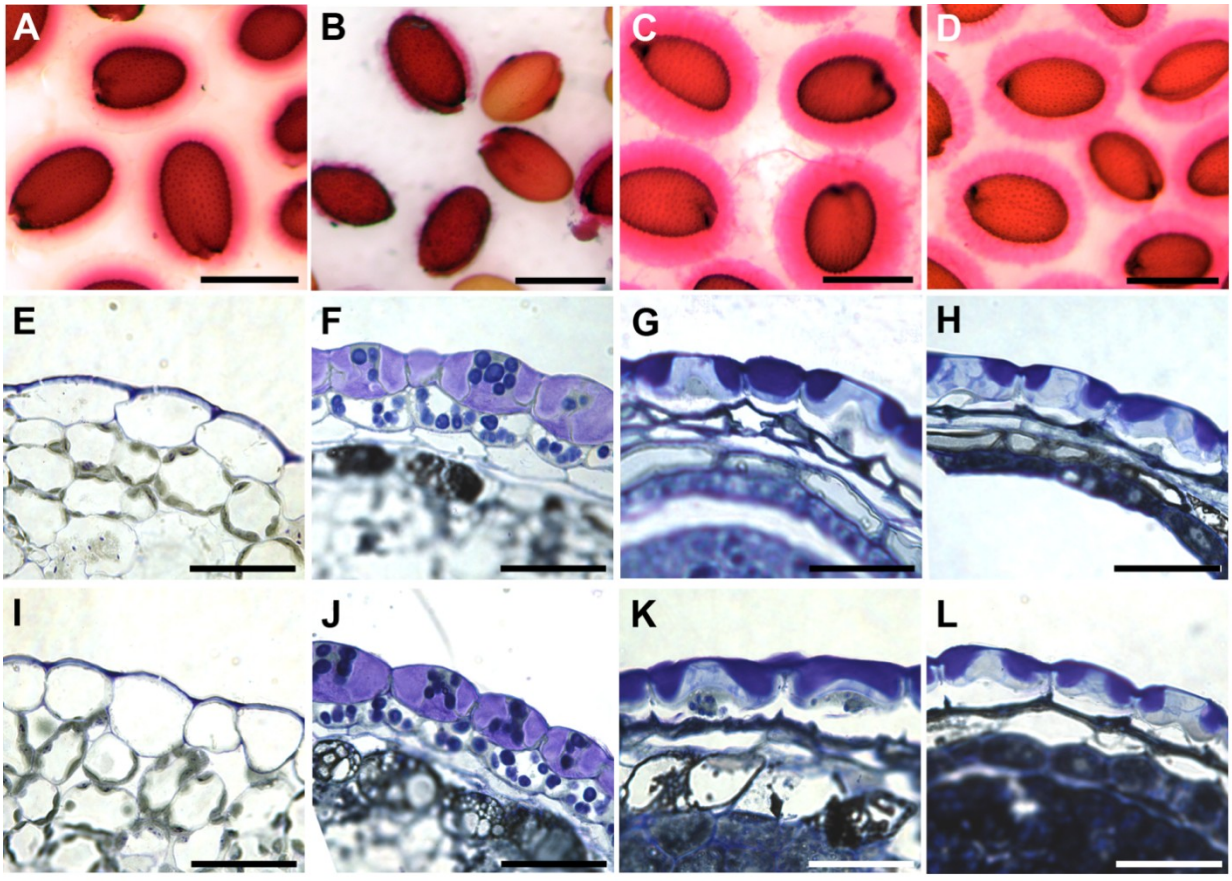


Table 2.1 Quantification of mucilage release of three independent lines of *bxl1-1*.

Seeds were shaken in water for 90 min followed by 60 min shaking in 0.01% ruthenium red, except where noted. The proportion of an individual seed's circumference surrounded by mucilage was then quantified as 100% (seed completely surrounded by mucilage), 75% (3/4 of seed circumference surrounded), 50%, 25% or no mucilage visible. Results are the percent seeds per sample with a particular mucilage category.

Table 2.1

Line	**Percent of seed surrounded by mucilage capsule of specified size**					
	100%	**75%**	**50%**	**25%**	**No mucilage**	**Total seeds**
WS #1	88.0	2.2	3.3	3.8	2.7	184
WS #2	95.7	0	2.9	1.4	0	70
bxl1-1 Line #1	2.7	6.1	43.5	38.8	8.8	147
bxl1-1 Line #2	3.8	12.8	16.0	40.4	26.9	156
bxl1-1 Line #3	17.3	21.0	29.6	21.6	10.5	162
bxl1-1 Line #2[a]	3.6	0	7.2	15.7	73.5	83

[a]Seeds placed directly in ruthenium red with no pre-treatment or shaking and monitored for 90 minutes (sample used for time-lapse in Fig. 2)

Figure 2.2**.** Time-lapse of mucilage release for *bxl1-1* versus wild-type WS seeds.

Seeds were placed in ruthenium red solution and photographed over 90 min.

(A) Wild-type seed releases mucilage within 20 s, then mucilage stains pink over time.

(B) *bxl1-1* seed shows no mucilage release until 83 min, at which time mucilage is observed only for a patch of cells. Scale bars represent 100 μm.

Figure 2.2

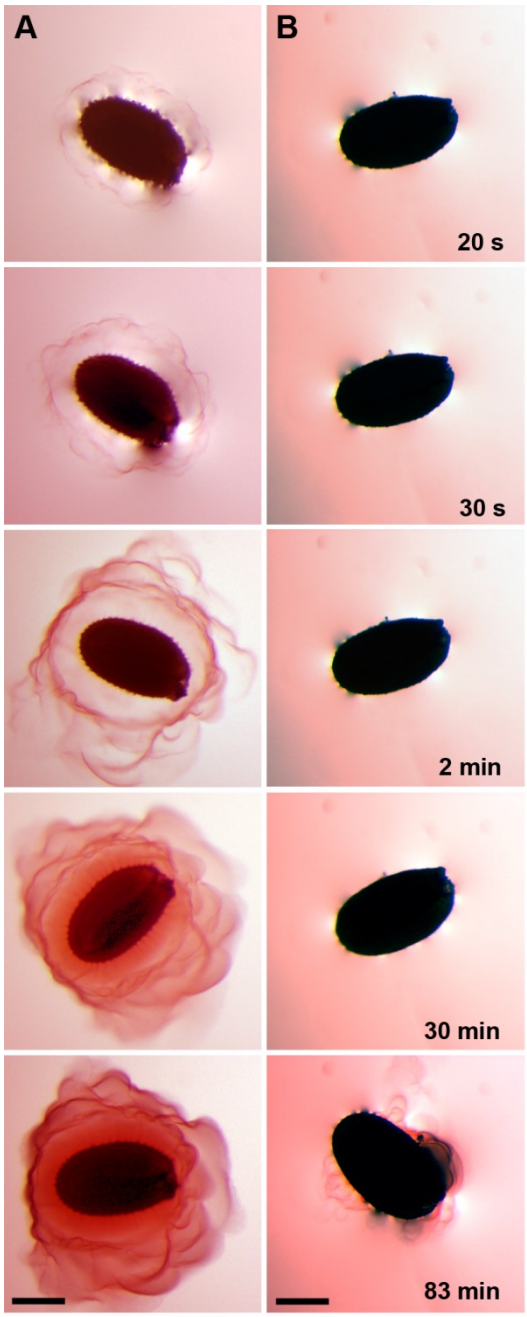


Table 2.2. Monosaccharide quantitation of *bxl1-1* versus wild-type seeds.

Soluble polysaccharides from intact seeds were isolated by shaking in ammonium oxalate (soluble mucilage), followed by grinding of the same seeds and further ammonium oxalate extraction (seed minus soluble mucilage). Samples were then ethanol precipitated and directly hydrolyzed with trifluoroacetic acid followed by derivatization to alditol acetates. Results are given as average μg sugar per 100 mg seed and SE calculated from three independent samples.

Table 2.2

	Soluble mucilage		Seed minus soluble mucilage	
Sugar	**WS**	***bxl1-1***	**WS**	***bxl1-1***
Rha	528 ± 23.6	486 ± 6.8	1090 ± 85.7	937 ± 11.2
Fuc	5.1 ± 0.1	4.0 ± 0.1[a]	74.9 ± 2.4	72.8 ± 4.8
Ara	11.8 ± 0.7	17.5 ± 0.6[a]	1610 ± 87.2	1720 ± 8.5
Xyl	64.4 ± 2.7	53.7 ± 0.6[b]	578 ± 35.4	619 ± 4.7
Man	10.7 ± 0.6	10.4 ± 0.0	141 ± 9.9	137 ± 2.2
Gal	24.7 ± 1.9	25.1 ± 0.3	1030 ± 60.0	985 ± 20.2
Glc	15.3 ± 1.7	13.8 ± 0.6	1540 ± 129	1370 ± 12.9
Total	660 ± 31.2	611 ± 8.6	6050 ± 408	5840 ± 50.3

[a]Significantly different from WS, P-value of <0.005.

[b]Significantly different from WS, P-value of <0.05.

Figure 2.3**.** Germination of *bxl1-1* versus WS wild-type seeds.

Germination of seeds placed on minimal medium agar plates and cold-treated for 72 hrs. Seeds were either plated directly as dry seeds (no trtmt) or pre-hydrated by shaking for 90 min in water (H2O) or 0.05 M EDTA as indicated, followed by suspension in 0.01% agarose and plating on minimal medium. Samples were done in triplicate with 50-60 seeds per sample. Error bars represent SE.

Figure 2.3

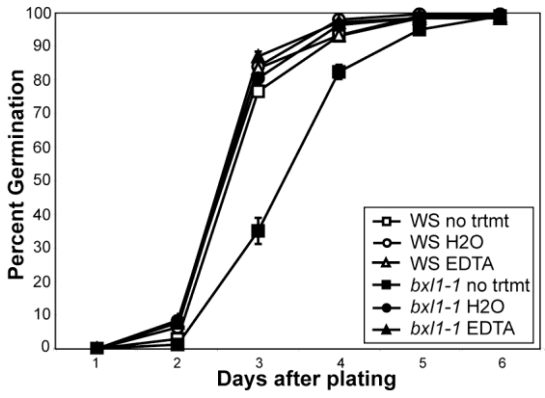

Percent Germination
Days after plating
WS no trtmt
WS H2O
WS EDTA
bxl1-1 no trtmt
bxl1-1 H2O
bxl1-1 EDTA

Figure 2.4**.** Structure and transcription of *AtBXL1*.

(A) Cartoon of the intron-exon structure of *AtBXL1* showing the location of the different T-DNA insertions in *bxl1-1, bxl1-2* and *bxl1-3*. Exons are drawn as boxes with grey shading indicating UTRs. T-DNA insertions are indicated by triangles. Locations of the primers used for the RT-PCR results shown in (B and C) are indicated with arrows.

(B) RT-PCR (30 cycles) using *AtBXL1* primers p15/16 on RNA isolated from Columbia wild-type leaves, stems, seedlings, roots, inflorescence tips, intact siliques at 4, 7, 10 DPA, plus leaf tissue from wild-type WS and *bxl1-1* plants. The loading control is cytosolic glyceraldehyde-3-phosphate dehydrogenase *(GAPC)*.

(C) Real time PCR using *AtBXL1* primers p3/4 on inflorescences (inflo), intact 4 DPA siliques (sd + sil) and separated seeds and siliques at 7 and 10 DPA. Duplicate samples were performed using *GAPC* primers and used to normalize the *AtBXL1* results. Error bars represent SE, n = 3.

(D) Analysis of the α-L-arabinofuranosidase activity in *bxl1-1* mutant versus wild-type siliques using cation-exchange chromatography. Protein extracts were analyzed by SP-Sepharose chromatography and collected as 1 ml fractions. Each fraction was assayed for α-L-arabinofuranosidase activity and normalized against β-galactosidase activity from the same fraction.

Figure 2.4

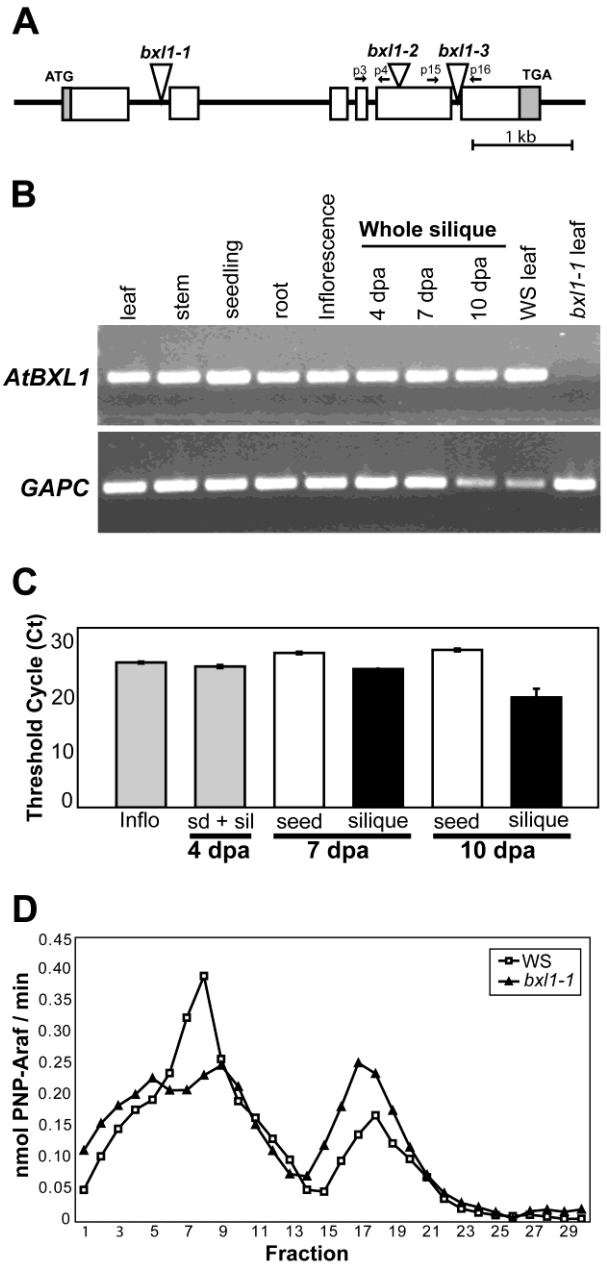

A
bxl1-1
bxl1-2
bxl1-3
ATG
TGA
p3
p4
p15
p16
1 kb
B
leaf
stem
seedling
root
Inflorescence
Whole silique
4 dpa
7 dpa
10 dpa
WS leaf
bxl1-1 leaf
AtBXL1
GAPC
C
Threshold Cycle (Ct)
30
20
10
0
Inflo
sd + sil
seed
silique
seed
silique
4 dpa
7 dpa
10 dpa
D
nmol PNP-Araf / min
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
Fraction
WS
bxl1-1

Table 2.3. Monosaccharide composition of mucilage extracted from *bxl1-1* versus wild-type seeds.

Intact seeds were extracted sequentially with 0.2% ammonium oxalate, 0.2 N and 2 N NaOH, followed by carbodiimide reduction, trifluoroacetic acid hydrolysis and alditol acetate derivatization. Results are given as average mole percentage and SE calculated from five independent samples.

Table 2.3

Sugar	Ammonium oxalate		0.2 N NaOH		2 N NaOH	
	WS	***bxl1-1***	**WS**	***bxl1-1***	**WS**	***bxl1-1***
Rha	49.5 ± 0.4	49.7 ± 0.6	43.6 ± 2.4	44.2 ± 2.1	36.6 ± 1.8	37.1 ± 3.1
Fuc	tr[a]	tr	tr	tr	tr	tr
Ara	0.7 ± 0.1	1.3 ± 0.1[b]	2.6 ± 0.8	4.2 ± 0.7	9.3 ± 2.3	9.1 ± 1.0
Xyl	3.7 ± 0.2	3.3 ± 0.3	6.7 ± 2.0	5.5 ± 1.1	8.2 ± 2.4	9.4 ± 1.0
Man	1.0 ± 0.1	1.2 ± 0.1	2.2 ± 0.6	1.8 ± 0.3	3.2 ± 1.1	2.4 ± 0.9
Gal	1.8 ± 0.1	1.9 ± 0.1	2.9 ± 0.9	2.3 ± 0.3	5.1 ± 0.8	4.2 ± 0.7
Glc	2.3 ± 0.5	2.1 ± 0.3	4.8 ± 1.1	3.9 ± 1.1	9.4 ± 2.3	10.1 ± 6.3
GalA	40.9 ± 0.2	40.4 ± 0.5	37.2 ± 1.2	38.1 ± 1.2	28.2 ± 3.1	27.7 ± 3.3

[a]tr = trace

[b]Significantly different from WS, P-value of 0.002

Table 2.4**.** Linkage analysis of extracted *bxl1-1* versus wild type mucilage.

Intact seeds were extracted sequentially with 0.2% ammonium oxalate, 0.2 N and 2 N NaOH, followed by carbodiimide reduction. Linkage was then determined through per-*O*-methylation, trifluoroacetic acid hydrolysis and alditol acetate derivatization. Results are given as the mean molar percentage ± variance of two samples, where 0 = less than 0.05.

Table 2.4

Linkage	Ammonium oxalate		0.2 N NaOH		2 N NaOH	
	WS	***bxl1-1***	**WS**	***bxl1-1***	**WS**	***bxl1-1***
Fucose						
t*-Fuc*p	tr[a]	tr	tr	tr	tr	tr
Rhamnose						
t*-Rha*p	0.4 ± 0	0.2 ± 0	0.5 ± 0.3	0.3 ± 0	0.3 ± 0.2	2.0 ± 5.5
2-Rha*p*	48.9 ± 0.6	48.6 ± 0.5	42.9 ± 0	42.5 ± 1.4	35.6 ± 3.7	24.5 ± 5.5
2,3-Rha*p*	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.5	0.4 ± 0.3	nd
2,4-Rha*p*	1.0 ± 0.2	1.0 ± 0.1	1.4 ± 0	1.8 ± 0.5	0.7 ± 0.9	nd
Arabinose						
t*-Ara*f	0.1 ± 0	0.2 ± 0	1.2 ± 1.8	1.0 ± 0.6	1.8 ± 4.3	1.3 ± 1.8
2-Ara*f*	0.2 ± 0	0.3 ± 0	0.2 ± 0.1	0.1 ± 0	0.2 ± 0	0.2 ± 0.1
3-Ara*f*	tr	0.1 ± 0	1.3 ± 0	0.7 ± 0	0.2 ± 0.1	0.4 ± 0.3
5-Ara*f*	0.2 ± 0	0.9 ± 0	1.5 ± 0.1	2.4 ± 0.2	0.6 ± 0.7	3.0 ± 0.8
2,5-Ara*f*	tr	0.2 ± 0.1	1.0 ± 2.2	0.5 ± 0.5	0.7 ± 0.9	0.9 ± 1.8
3,5-Ara*f*	nd	0.1 ± 0	0.4 ± 0	0.5 ± 0	nd	nd
Xylose						
t*-Xyl*p	0.3 ± 0	0.2 ± 0	0.5 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0

2-Xyl*p*	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.6 ± 0	0.4 ± 0
4-Xyl*p*	1.9 ± 0	1.6 ± 0	1.4 ± 0	1.6 ± 0	5.2 ± 0.7	3.7 ± 0.2
2,4-Xyl*p*	0.7 ± 0	0.6 ± 0	0.6 ± 0	0.5 ± 0	0.4 ± 0.3	1.2 ± 0.1
3,4-Xyl*p*	0.1 ± 0	0.1 ± 0	0.2 ± 0.1	tr	0.2 ± 0	tr
Mannose						
t*-Man*p	nd	nd	0.1 ± 0	0.1 ± 0	0.2 ± 0.1	0.2 ± 0
4-Man*p*	0.4 ± 0	0.5 ± 0	0.9 ± 0	1.0 ± 0	6.4 ± 0.5	4.6 ± 0
4,6-Man*p*	0.5 ± 0	0.4 ± 0	0.5 ± 0	0.4 ± 0	0.3 ± 0.2	0.6 ± 0
Galactose						
t*-Gal*p	1.1 ± 0	0.9 ± 0	1.0 ± 0.3	0.6 ± 0	0.8 ± 1.4	0.9 ± 0
2-Gal*p*	nd	nd	0.3 ± 0.2	0.3 ± 0.2	nd	nd
3-Gal*p*	0.2 ± 0	0.3 ± 0	0.3 ± 0.1	0.1 ± 0	0.7 ± 1.0	1.6 ± 0.3
4-Gal*p*	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	4.1 ± 28.6	0.4 ± 0
6-Gal*p*	0.1 ± 0	0.1 ± 0	0.8 ± 0	0.5 ± 0	0.9 ± 1.8	nd
3,4-Gal*p*	0.1 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0	0.8 ± 1.4
3,6-Gal*p*	0.2 ± 0	0.5 ± 0	0.4 ± 0.4	1.8 ± 0	1.2 ± 2.7	2.8 ± 0.3
Glucose						
t*-Glc*p	0.1 ± 0	0.1 ± 0	2.5 ± 2.1	3.2 ± 2.6	3.2 ± 1.0	15.5 ± 63.0
3-Glc*p*	nd	nd	nd	0.1 ± 0	0.1 ± 0	nd
4-Glc*p*	0.4 ± 0.3	0.8 ± 0	3.3 ± 0.8	3.4 ± 0.8	11.4 ± 0.2	14.8 ± 30.8
6-Glc*p*	nd	nd	0.2 ± 0.1	0.2 ± 0.0	0.6 ± 0.7	nd

2,4-Glc*p*	0.2 ± 0.1	0.1 ± 0	nd	nd	nd	nd
3,4-Glc*p*	tr	tr	0.4 ± 0	0.4 ± 0.1	0.5 ± 0.5	1.7 ± 1.4
4,6-Glc*p*	0.2 ± 0	0.1 ± 0	0.4 ± 0	0.5 ± 0	1.3 ± 0	2.1 ± 1.5
Galacturonic acid						
t*-GalUA*p	0.6 ± 0.1	0.5 ± 0	0.5 ± 0	0.5 ± 0	0.4 ± 0.2	0.8 ± 0.2
4-GalUA*p*	39.3 ± 0.3	39.2 ± 0.2	32.4 ± 1.1	31.8 ± 0.9	19.7 ± 1.3	13.5 ± 0
3,4-GalUA*p*	1.6 ± 0	1.6 ± 0.1	2.0 ± 0.9	1.8 ± 0.7	0.4 ± 0.4	0.4 ± 0.4
Glucuronic acid						
t*-GlcUA*p	0.1 ± 0	0.2 ± 0	0.3 ± 0	0.5 ± 0	0.7 ± 0	0.9 ± 0

[a]tr = trace, nd = not detected

Figure 2.5. Molecular complementation with PTYg and phenotypic rescue of *bxl1-1* mutants with exogenous enzymes.

(A-B) Molecular complementation results demonstrating restoration of mucilage release in *bxl1-1* PTYg transformants (B) and patchy phenotype of *bxl1-1* pGREEN 0229-transformed control lines (A).

(C-F) Treatment of *bxl1-1* with exogenous enzymes. (C) Enzyme buffer control, note patchy mucilage release. (D,E) Treatment with α-L-arabinofuranosidases AN1571 (D) and AN7908 (E), note almost wild-type mucilage levels in (D) and significantly increased mucilage release in (E) compared to (C) and (F). (F) Treatment with β-D-xylosidase AN2359, note patchy mucilage release. Scale bars represent 200 μm.

Figure 2.5

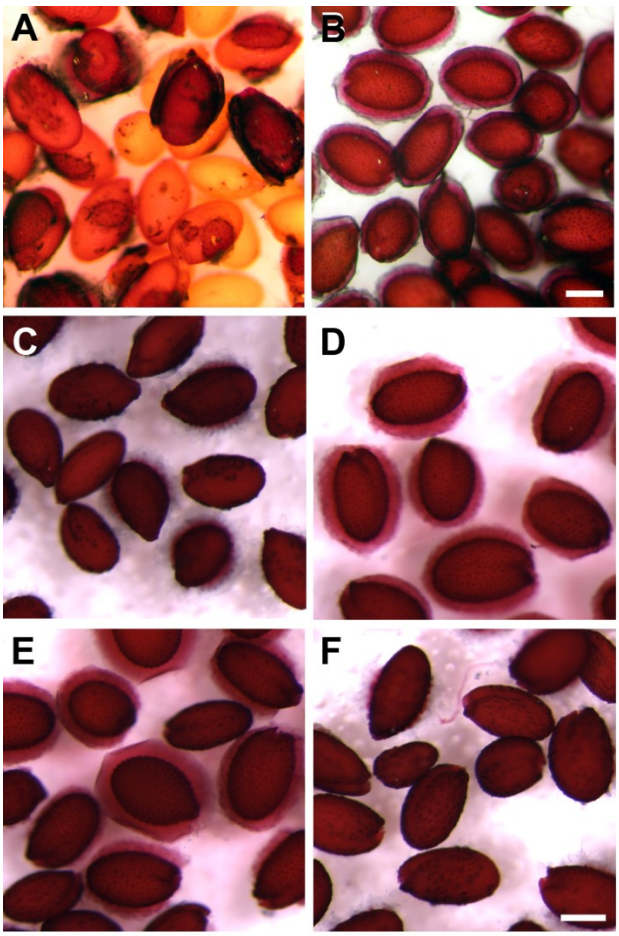


Figure 2.6. Immunofluorescence of developing WS versus *bxl1-1* seeds coats.

(A-B) Confocal sections of 7 DPA WS and *bxl1-1* seed coats stained with anti-arabinan antibody LM6. Arrows indicate lower corners of the mucilage cells, note intense staining around whole cell in *bxl1-1* mutants.

(C-D) Confocal sections of 7 DPA WS and *bxl1-1* seed coats stained with unbranched RG I antibody CCRC-M36.

(E) Control image of WS seed coat stained without primary antibody. Photo contrast was enhanced to visualize cells.

All photographs were taken with the same microscope settings, save (B), where the gain had to be reduced due to saturation of the image due to high intensity of fluorescence, making the image in (B) an under-representation of the degree of labelling. Scale bar represents 50 μm.

Figure 2.6

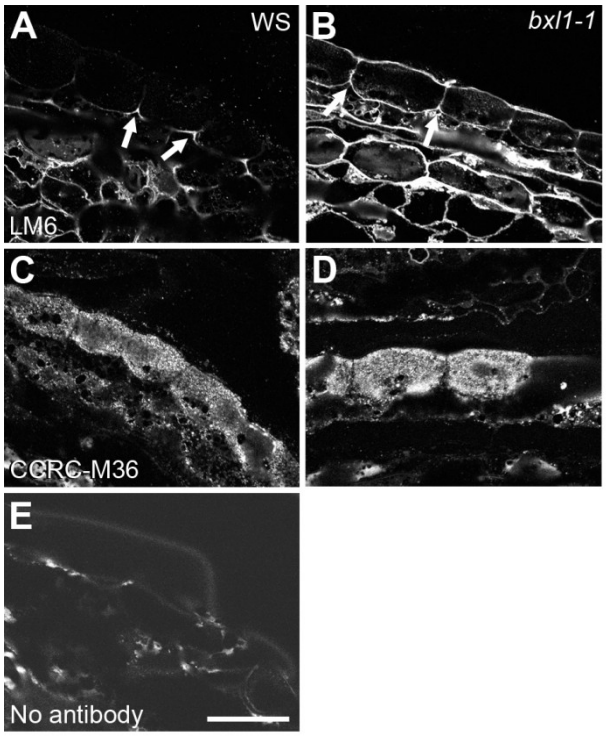
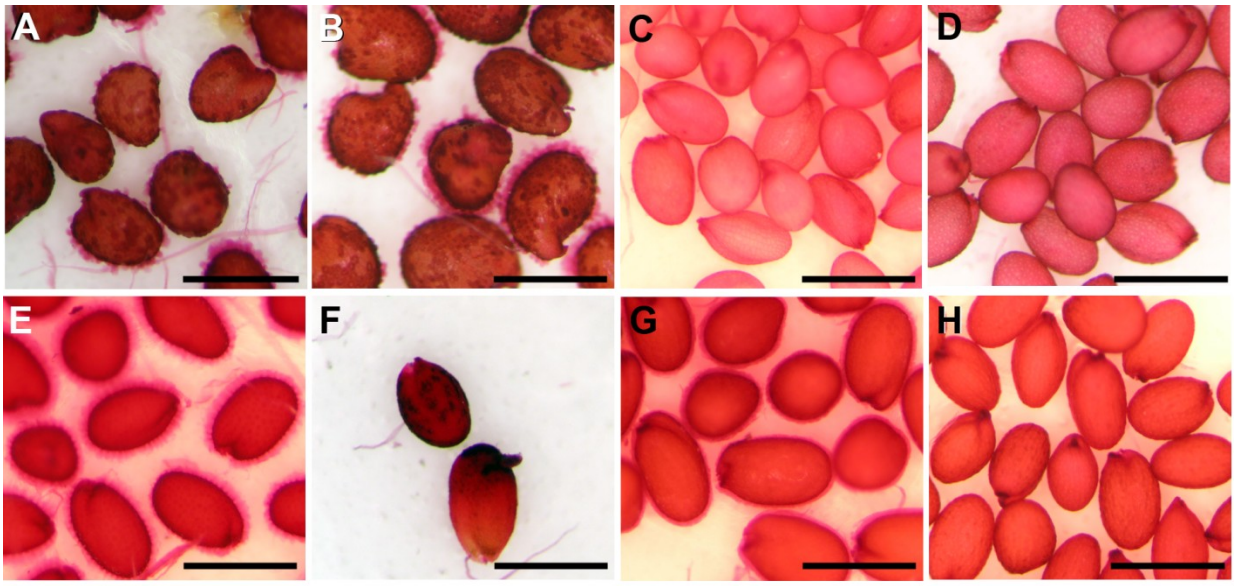


Figure 2.7**.** Phenotype of *bxl1-1* double mutants with *ap2-1, ttg1-1, myb61-1* and *mum4-1*.

Ruthenium red staining of single mutants and double mutants with *bxl1-1*, all shaken in 0.05 M EDTA to promote release of their small amount of mucilage prior to staining, except *myb61-1* and *bxl1-1 myb61-1* (E,F), which were shaken in water since *myb61-1* releases mucilage in water. (A,B) *ap2-1* and *bxl1-1 ap2-1*, respectively. (C,D) *ttg1-1* and *bxl1-1 ttg1-1*, respectively. (E,F) *myb61-1* and *bxl1-1 myb61-1*, respectively; note mucilage release in *myb61-1* but not in double mutant. (G,H) *mum4-1* and *bxl1-1 mum4-1*, respectively; note thin layer of mucilage surrounding *mum4-1* seeds that is not seen in the double mutant. Scale bars represent 500 μm.

Figure 2.7



SUPPLEMENTAL MATERIAL

Supplemental Figure S2.1. Scanning electron microscopy of *bxl1-1* versus *mum4-1* and wild-type seed coat epidermal cells.

(A) Wild-type Col-2 seed, note cells are hexagonal shape with central raised columella. (B) *mum4-1* seed lacking obvious columella. (C) Wild-type WS seed. (D) *bxl1-1* seed. Scale bars represent 50 μm.

Figure S2.1

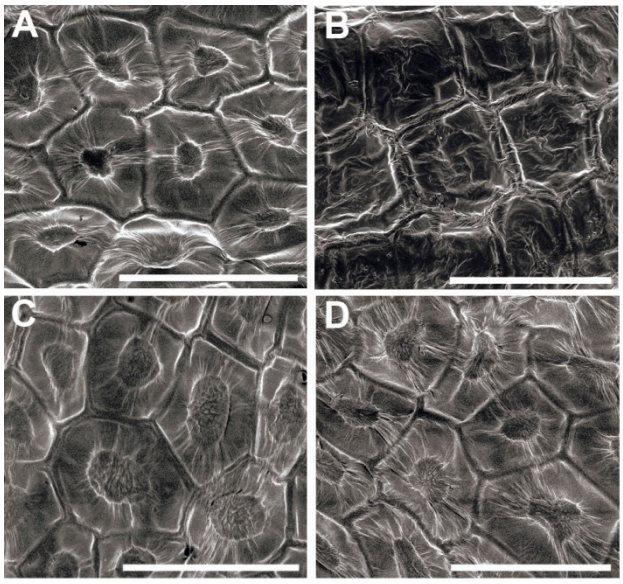


Figure S2.2. Immunoblot of extracted mucilage and whole seed immunofluorescence of WS versus *bxl1-1* seeds.

(A) Immunoblot of EDTA-extracted mucilage from WS and *bxl1-1* seeds hybridized with unbranched RG I antibody CCRC-M36 and anti-arabinan antibody LM6. Concentrated mucilage from 75 mg of seed was diluted as noted in the figure prior to spotting on the membrane.

(B-E) Confocal images of whole seed immunofluorescence (green) with the seed coat outer cell walls counterstained with propidium iodide (magenta), shown as Z-stack projections. (B-C) Hybridization with CCRC-M36, note thick capsule of stained mucilage. (D-E) Hybridization with LM6. (D) Faint staining close to seed at tops of columellae in WS (arrowhead), with one bit of stained intact cell wall (arrow). (E) *bxl1-1* LM6 staining is more intense, with obvious pieces of intact cell wall (arrows). (F-G) Single confocal slice through controls lacking primary antibodies with contrast enhanced to visualize seed outlines. Scale bar represents 100 μm.

Figure S2.2

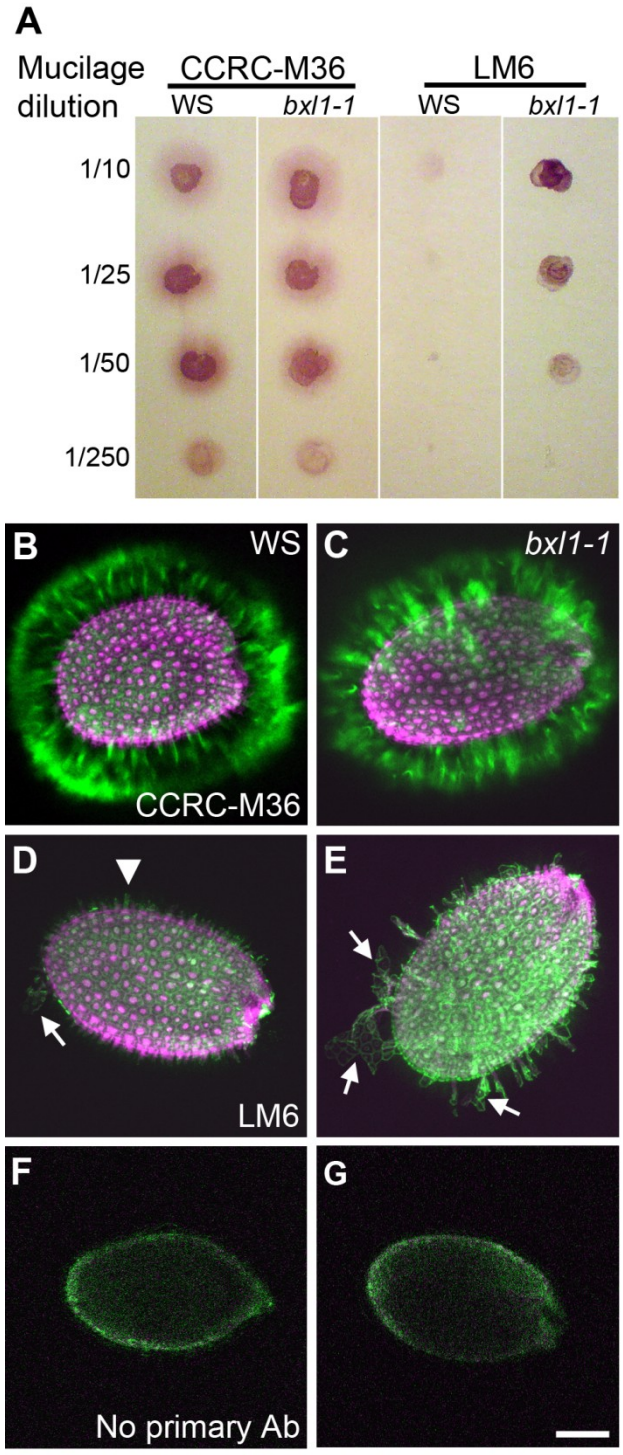

A
Mucilage dilution
CCRC-M36
LM6
WS
bxl1-1
WS
bxl1-1
1/10
1/25
1/50
1/250
B
WS
CCRC-M36
C
bxl1-1
D
LM6
E
F
No primary Ab
G

Figure S2.3. Water absorption of *bxl1-1* versus wild-type mucilage extracts.

Time course of water absorption (μg water/mg freeze-dried mucilage) of ammonium oxalate, 0.2 N and 2 N sodium hydroxide extracts of *bxl1-1* versus wild-type mucilage measured every 5 min for 30 min, then every 15 min up to 120 min. Error bars represent SE, n = 3.

Figure S2.3

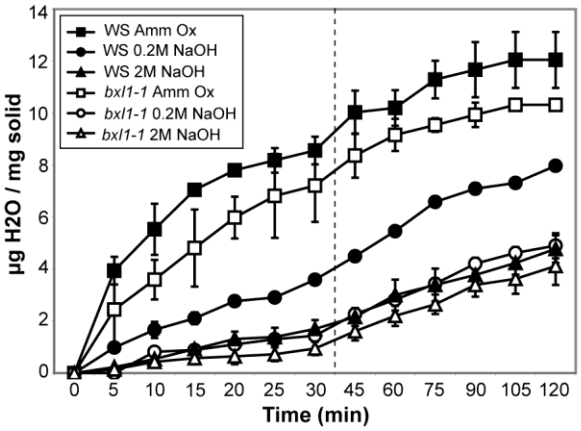

WS Amm Ox
WS 0.2M NaOH
WS 2M NaOH
bxl1-1 Amm Ox
bxl1-1 0.2M NaOH
bxl1-1 2M NaOH
µg H2O / mg solid
Time (min)
0
2
4
6
8
10
12
14
0 5 10 15 20 25 30 45 60 75 90 105 120

Figure S2.4**.** Expression of *AtBXL2* throughout Arabidopsis tissues and during seed and silique development.

Qualitative (35 cycles) RT-PCR using *AtBXL2* primers on RNA isolated from Columbia wild-type leaves, stems, seedlings, roots, inflorescence tips, intact siliques at 4, 7, 10 DPA, plus leaf tissue from wild-type WS and *bxl1-1* plants. The loading control is cytosolic glyceraldehyde-3-phosphate dehydrogenase *(GAPC)*.

Figure S2.4

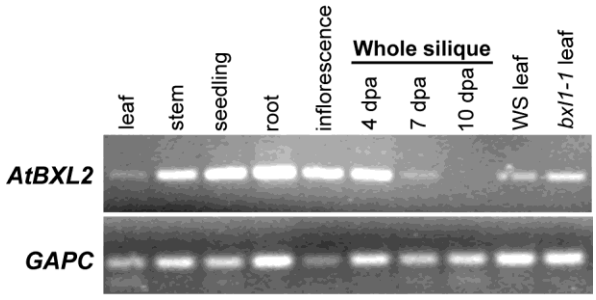

leaf
stem
seedling
root
inflorescence
Whole silique
4 dpa
7 dpa
10 dpa
WS leaf
bxl1-1 leaf
AtBXL2
GAPC

Table S2.1 Quantification of mucilage release of complemented lines of *bxl1-1,* plus *bxl1-2* and *bxl1-3*.

Seeds were shaken in water for 90 min followed by 60 min shaking in 0.01% ruthenium red. The proportion of an individual seed's circumference surrounded by mucilage was then quantified as 100% (seed completely surrounded by mucilage), 75% (3/4 of seed circumference surrounded), 50%, 25% or no mucilage visible. Results are the percent seeds per sample with a particular mucilage category. Vector-only transformed *bxl1-1* seeds (pGR0229) are controls for the PTYg *bxl1-1* complementation lines (to compare to wild type WS see Table 2.1). Col-0 is the wild-type background for *bxl1-2* and *bxl1-3*. Complemented lines were stained in one experiment, while Col-0 wild type and allele samples were stained in separate experiment.

Table S2.1

Line	**Percent of seed surrounded by mucilage capsule of specified size**					
	100%	**75%**	**50%**	**25%**	**No mucilage**	**Total # seeds**
PTYg in *bxl1-1* #1	93.0	2.1	2.1	2.8	0	142
PTYg in *bxl1-1* #2	100	0	0	0	0	122
PTYg in *bxl1-1* #3	91.4	6.6	1.3	0.7	0	151
pGR0229 in *bxl1-1* #1	4.4	15.8	36.8	35.1	7.9	114
pGR0229 in *bxl1-1* #2	7.5	12.8	31.6	34.6	13.5	133
Col-0	79.2	3.8	5.7	5.7	5.7	53
bxl1-2	9.5	29.7	40.5	14.9	5.4	74
bxl1-3	6.5	5.2	33.8	50.6	3.9	77

Table S2.2 Monosaccharide quantitation of *bxl1-1* versus wild type seedlings.

Samples were alcohol insoluble residue prepared from seedlings 12 days after germination that were hydrolyzed with trifluoroacetic acid and derivatized to alditol acetates. Results are average μg sugar plus standard error per 100 mg fresh tissue calculated from three independent samples

Table S2.2

Sugar	WS	*bxl1-1*
Rha	9.3 ± 0.4	8.4 ± 0.8
Fuc	2.6 ± 0.1	2.3 ± 0.1
Ara	18.9 ± 1.1	21.6 ± 1.6
Xyl	18.0 ± 0.6	18.4 ± 1.6
Man	3.3 ± 0.2	2.7 ± 0.3
Gal	14.6 ± 1.1	12.9 ± 1.1
Glc	5.7 ± 0.3	4.9 ± 0.4
Total	72.4 ± 3.8	71.2 ± 4.0

Table S2.3 Primers used for RT-PCR, real-time PCR and genotyping of double mutants.

Table S2.3

Gene	Primer Pair	Sequence
AtBXL1	At5g49360 p3/p4	ACTAGCACTCCGGAAGAAGC
		CAATCCTTTCTTCACTGCACCT
	At5g49360 p8/p11	ACGCCATTCCATTATCAAG
		GGTCCAGGCGCTAAGTTCGG
	At5g49360 p15/p16	TGGTGGACCAATCGATGTAA
		ATTACCGGATGCTCTCATGG
GAPC	GAPC p1/p2	TCAGACTCGAGAAAGCTGCTAC
		GATCAAGTCGACCACACGG
	RT GAPC p5/p6	GACAGATTTGGAATTGTTGAGG
		GGCCCATCAACAGTCTTCTG
AtBXL2	BXL2 p1/p2	AGGAAACTCCCGGTGAAGAT
		ATACGGAACGGTACGTCGAA
MYB61	MYB61 p1/p2	TTTGCAGAGATGTGGGAAGA
		GCCATTGTCGAAGAAATTTGA
	DSPM11	GGTGCAGCAAAACCCACACTTTTACTTC
MUM4	At1g53500 p1/p8	TTGCAGATTTCAAGGATGGA
		CATGGTTTCCTACAGCAGCA

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Link between Chapter 2 and 3

Chapter 2 described the characterization of the *patchy (pty)/beta-xylosidase1(bxl1)* mutant and analyzed the role of the bi-functional β-xylosidase/α-arabinofuranosidase encoded by the *PTY/BXL1* locus. It was concluded that the action of this hydrolyzing enzyme was necessary in order to trim arabinan side chains from the pectin RG I in the mucilage and/or the primary cell wall in order for the mucilage to be released normally. The identification of this gene and its role in mucilage release illustrates the alteration of pectin composition and its effect on the physical properties of mucilage and/or the cell wall. To identify genes acting in cell wall production and modification beyond *AtBXL1* and other characterized genes acting in MSC differentiation, further genetic screens are necessary. **Chapter 3** describes an enhancer/suppressor screen using the *mum4* reduced mucilage mutant. The screen identified six novel mutants named *mum enhancer (men) 1-6*, representing two distinct groups, those that produced a further reduced amount of mucilage compared to *mum4* (*men1, 3, 4, 5*), and those that produced similar amounts of mucilage to *mum4* but failed to release it (*men2, 6*). These groups of mutants represent genes that may be involved in the synthesis or secretion of pectin, or the modification of the cell wall and/or mucilage required for mucilage release, respectively. The work was published in the Journal of Experimental Botany. The full citation for the published report is Arsovski AA, Villota MM, Rowland O, Subramaniam R, Western TL (2009) *MUM ENHANCERS* are important for seed coat mucilage production and mucilage secretory cell differentiation in *Arabidopsis thaliana*. Journal of Experimental Botany 60(9): 2601-2612

Chapter 3

MUM ENHANCERS are important for seed coat mucilage production and mucilage secretory cell differentiation in *Arabidopsis thaliana*

ABSTRACT

Pollination triggers not only embryo development, but also the differentiation of the ovule integuments to form a specialized seed coat. The mucilage secretory cells of the *Arabidopsis thaliana* seed coat undergo a complex differentiation process in which cell growth is followed by the synthesis and secretion of pectinaceous mucilage. A number of genes have been identified affecting mucilage secretory cell differentiation, including *MUCILAGE-MODIFIED4 (MUM4). mum4* mutants produce a reduced amount of mucilage and cloning of *MUM4* revealed that it encodes an UDP-L-rhamnose synthase that is developmentally upregulated to provide rhamnose for mucilage pectin synthesis. To identify additional genes acting in mucilage synthesis and secretion, a screen for enhancers of the *mum4* phenotype was performed. Eight *mum enhancers (men)* have been identified, two of which result from defects in known mucilage secretory cell genes (*MUM2* and *MYB61*). Our results show that, in a *mum4* background, mutations in *MEN1, MEN4* and *MEN5* lead to further reductions in mucilage compared to *mum4* single mutants, suggesting that they are involved in mucilage synthesis or secretion. Conversely, mutations in *MEN2* and *MEN6* appear to affect mucilage release rather than quantity. With the exception of *men4*, whose single mutant exhibits reduced mucilage, none of these genes have a single mutant phenotype, suggesting that they would not have been identified outside of the compromised *mum4* background.

INTRODUCTION

Pollination in flowering plants leads not only to the initiation of embryogenesis and endosperm development, but also to differentiation of the ovule integuments to form the seed coat. The seed coat layers are derived from maternal tissue and can undergo a number of specializations that aid embryo nutrition, seed dispersal, germination and seed longevity (Esau 1977; Fahn 1982; Boesewinkel and Bouman 1984). One such specialization is the production of a hydrophilic polysaccharide slime, known as mucilage, in the seed coat epidermis. This trait, known as myxospermy, is found in a number of species, including the Brassicaceae, Solanaceae, Linaceae and Plantaginaceae. Seed coat mucilage has been suggested to play a number of roles, including promotion of seed hydration and germination, prevention of gas exchange, and attachment to soil substrates and animal vectors (Esau, 1977; Fahn, 1982; Grubert, 1981).

The seed coat mucilage secretory cells of the model genetic plant, *Arabidopsis thaliana* (Arabidopsis), undergo a complex differentiation process, including separable stages of pectinaceous and cellulosic cell wall production, making them an excellent model in which to study the regulation of cell wall biosynthesis in a developmental context (Haughn and Chaudhury 2005; Western 2006). Epidermal cells of the seed coat first grow by vacuolar expansion. This growth phase is followed by the biosynthesis of a large quantity of pectinaceous mucilage, which is secreted to the apical tangential regions of the cell, forming a donut-shaped mucilage pocket between the plasma membrane and primary cell wall (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). Mucilage

production is accompanied by an increase in number of Golgi stacks, consistent with the synthesis of pectins in the Golgi apparatus (Western, Skinner et al. 2000; Young, McFarlane et al. 2008). Concurrent with mucilage synthesis and accumulation, the vacuole contracts towards the bottom of the cell and the cytoplasm is constricted to a volcano shape in the centre of the cell. Pectin biosynthesis and secretion is succeeded by the production of a cellulosic secondary cell wall that fills in the remaining cytoplasm to form a volcano-shaped columella in the center of the cell (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). Programmed cell death is followed by seed desiccation and the shrinking of the mucilage around the columella to reveal hexagonal-shaped cells with thickened radial cell walls surrounding the columella. Seed wetting leads to almost instantaneous hydration of the hydrophilic mucilage, followed by rupture of the primary cell wall and release of mucilage to form a gel capsule surrounding the seed (Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). Arabidopsis seed mucilage is primarily composed of an unbranched form of the pectin rhamnogalacturonan I (RG I), with smaller quantities of pectic side chains (arabinans, galactans), homogalacturonan, hemicellulose and cellulose (Penfield, Meissner et al. 2001; Willats, McCartney et al. 2001; Western, Young et al. 2004; Macquet, Ralet et al. 2007).

Mutations in several genes have been identified to have pleiotropic effects on Arabidopsis mucilage secretory cell differentiation. These include the developmental regulator, *APETALA2 (AP2)*, the epidermal cell differentiation factors *TRANSPARENT TESTA GLABRA1 (TTG1), TTG2, GLABRA2 (GL2),*

TRANSPARENT TESTA2 (TT2), TT8, ENHANCER OF GLABRA3 (EGL3), MYB5, and the transcription factor *MYB61* (Koornneef 1981; Bowman and Koornneef 1993; Jofuku, den Boer et al. 1994; Penfield, Meissner et al. 2001; Johnson, Kolevski et al. 2002; Zhang, Gonzalez et al. 2003; Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009). Loss of function mutants of each of these regulators result in a reduced amount of mucilage and flattened columellae. The most severe are *ap2* mutants, which completely lack mucilage and columellae. Reduced mucilage has also been observed in mutants for the KANADI family transcription factor *ABERRANT TESTA SHAPE (ATS)*, the putative glucosidase II, *RADIAL SWELLING3*, *MICROTUBULE ORGANIZATION1*, the abscisic acid biosynthetic gene *ABSCISIC ACID1,* and the gibberellin biosynthetic gene *GIBBERELLIN-3 OXIDASE4* (Karssen, Brinkhorst-van der Swan et al. 1983; Leon-Kloosterziel, Keijzer et al. 1994; Burn, Hurley et al. 2002; Kim, Nakajima et al. 2005; Messmer McAbee, Hall et al. 2006; McFarlane, Young et al. 2008). A screen for mucilage-specific genes led to the identification of *MUCILAGE-MODIFIED1—5 (MUM1—5)* (Western, Burn et al. 2001). *mum4* mutants have reduced mucilage and flattened columellae, while *mum3* and *mum5* mutants have mucilage of altered composition. By contrast, *mum1, mum2* and the recently identified *subtilase1.7 (Atsbt1-7)* mutants have defects in mucilage release (Western, Burn et al. 2001; Rautengarten, Usadel et al. 2008). Both *MUM2* and *MUM4* have been cloned. *MUM2* encodes a β-galactosidase, which, along with a putative pectin methylesterase target of *SBT1.7*, appears to be required to modify pectin structure in the mucilage and/or primary cell wall to facilitate mucilage release (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007;

Rautengarten, Usadel et al. 2008). Conversely, *MUM4* encodes an UDP-L-rhamnose synthase (also known as RHM2) required for the production of the primary mucilage pectin RG I (Usadel, Kuchinsky et al. 2004; Western, Young et al. 2004; Oka, Nemoto et al. 2007). Expression of *MUM4* is specifically upregulated at the time of mucilage synthesis. AP2 and a TTG1-EGL3-TT8-MYB5-TT2 transcription factor complex activate GL2, which in turn regulates *MUM4* gene expression (Johnson, Kolevski et al. 2002; Western, Young et al. 2004; Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009). Alternate pathways of mucilage production appear to be regulated by TTG2, also downstream of AP2 and the TTG1-EGL3-TT8-MYB5-TT2 complex, and MYB61, which may be acting indirectly on mucilage production through a role in sugar allocation (Johnson, Kolevski et al. 2002; Zhang, Gonzalez et al. 2003; Newman, Perazza et al. 2004; Western, Young et al. 2004). Thus, while many regulatory genes, and even some cell wall modification genes, have been identified for roles in mucilage secretory cell differentiation, only one biosynthetic gene has yet been identified.

An enhancer mutant screen of the reduced mucilage mutant *mum4* was performed to identify additional downstream genes in the mucilage production pathway. In addition to isolating new alleles of *mum2* and *myb61*, six new mucilage secretory cell differentiation genes, *MUM ENHANCER1—6 (MEN1—6)* were identified and characterized. *men1—6* mutants demonstrate varying degrees of enhancement of the *mum4* phenotype, with three having significant loss of mucilage, suggesting direct roles in mucilage biosynthesis or secretion.

MATERIALS AND METHODS

Plant lines, mutagenesis and growth conditions

Lines of *Arabidopsis thaliana* used were *mum4-1* (Col-2 ecotype)(Western, Young et al. 2004) and *ttg1-1* (L*er* ecotype; Arabidopsis Biological Resource Center, Columbus, OH). Seeds were planted on AT minimal medium plates (Haughn and Somerville, 1986) or directly on soil (Sunshine Mix #5, SunGro Horticulture), stratified for 3-4 days at 4°C and then transferred to growth chambers at 22°C under continuous light (90-120 μE m^{-2} s^{-1} photosynthetically active radiation), unless otherwise specified. Flower staging for days post anthesis (DPA) was performed as in (Western, Burn et al. 2001).

For mutagenesis, 0.33 g *mum4-1* seeds (~15,000) were treated for 12 h with 0.25% (v/v) ethyl methanesulfonate. After rinsing, mutagenized seeds were planted in 80 batches of ~150 plants (M1) and bulk-harvested. For screening, seeds from individual M2 plants were isolated and stained with ruthenium red after pre-treatment with EDTA as described below. The nine mutants described were isolated from screening approximately 5,000 M3 lines from 10 batches (~1,500 parental lines). *men2 mum4* and *men3 mum4* were isolated from a common parental batch, as were *men6-2 mum4* and *myb61-6 mum4*; the rest were single isolates from separate parental batches. Prior to study, all double mutant lines were backcrossed at least twice to *mum4-1*.

In accordance to journal policy on distribution of novel materials, the *men* mutants will be made available by the authors upon request.

Sequencing of *mum2-13* and *myb61-6*

The coding regions of *MUM2* and *MYB61* were PCR amplified from the *mum2-13 mum4-1* and *myb61-6 mum4-1* double mutants, respectively, using the overlapping primer sets presented in Table S1. Sequencing was performed at the McGill-Genome Quebec Innovation Centre sequencing facility and alignments were performed against the wild-type sequences using DNAMAN (Lynnon Corporation).

Microscopy

For ruthenium red staining, seeds were either placed directly in 0.01% (w/v) ruthenium red without shaking, shaken directly in ruthenium red for 90 min, or pre-hydrated with shaking in 0.05 M EDTA for 90 min followed by ruthenium red stain, as indicated. For the seeds stained with shaking, samples were rinsed in dH_2O prior to visualization. Seeds were observed on a Leica MZ-16F stereomicroscope and imaged with a Micropublisher 3.3 camera (Qimaging) operated via Openlab 5 (Perkin Elmer).

Developing seeds were prepared for brightfield microscopy, sectioned and stained with toluidine blue O as described in (Western, Burn et al. 2001). Samples were examined using a Leica DM 6000B compound microscope and images captured with a Qimaging Retiga CCD camera operated through Openlab. Scanning electron microscopy of dry seeds was performed as described in (Western, Burn et al. 2001).

To test for mucilage release after extraction with ammonium oxalate, intact seeds were incubated in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 h at 30 ºC. Seeds were then either shaken in 0.01% (w/v) ruthenium red for 90 min,

mounted on a depression slide and observed with a Leica DM 6000B compound microscope, or air-dried before mounting on stubs and observed by scanning electron microscopy.

Seed coat permeability was determined using tetrazolium salts as described by (Debeaujon, Leon-Kloosterziel et al. 2000). In short, seeds were incubated in 1% (w/v) tetrazolium red for 2 d in the dark at 37°C and the percentage of red seeds calculated as a measure of permeability.

Chemical analysis

To quantify neutral sugars in crude mucilage extracts, 50 mg of intact seeds were incubated in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 h at 30 °C. 1 μmole of myo-inositol was added to the supernatant and samples were precipitated with 5 volumes ethanol, directly hydrolyzed with 2M trifluroacetic acid and derivatized to alditol acetates. Derivatization to alditol acetates and gas chromatography were performed as in (Gibeaut and Carpita 1991), but with an HP-23 glass capillary column (30 m x 0.25 mm i.d.; Agilent Technologies). Seeds used for chemical analyses were collected from mutant and control plants cultivated together.

Germination time-course

Two 70 mm diameter -Whatman #1 filter papers (Whatman) were placed in the lid of a 100 mm plastic Petri dish. To these were added 2 ml of water and 40-80 seeds of each mutant line. The plates were sealed with parafilm and stratified in the dark at 4°C for 72 h. Seeds were incubated at 22°C under 16 h light: 8 h dark, following which they were counted every day for six days and germination was scored by the

presence of open green cotyledons. The plates were counted again after nine days to confirm all lines reached approximately 100% germination. Seeds used for germination analyses were collected from mutant and control plants cultivated together and stored as distinct seed sets for six or eight months, depending on the set. Seeds were stored in microfuge tubes with holes in the lids at room temperature under ambient humidity and light conditions. Each time-course was done in triplicate and the whole was performed twice using two independent sets of seeds with similar results.

RESULTS

Identification of *mum enhancers*

When Arabidopsis seeds are hydrated, the seed coat mucilage swells rapidly, leading to the bursting of the primary cell wall and release of mucilage to surround the seed in a gel-like capsule (Fig. 1A) (Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). *mum4* mutants make a significantly reduced amount of mucilage (Western, Burn et al. 2001; Western, Young et al. 2004) that remains within the cells when seeds are hydrated. Addition of a heavy metal chelator such as EDTA or EGTA, however, leads to the release of *mum4* mucilage. This is likely due to the withdrawl of Ca^{2+} ions from the cell wall pectins, leading to weakening of the cell wall and/or permitting increased swelling of the mucilage present. *mum4* seeds shaken in EDTA prior to ruthenium red staining reveal a thin layer of stained

mucilage around the seeds, consistent with their reduced mucilage production (Fig.3.1A) (Western, Young et al. 2004). By contrast, mutants for *TTG1*, which acts upstream of both the *GL2* and *TTG2* pathways of mucilage production, make very little mucilage and show no obvious mucilage release when EDTA-treated (Fig.3.1A). The moderate level of mucilage release found for *mum4* mutants, as well as the ability to differentiate *mum4* mutants from mutants with further reduced mucilage, allowed us to perform a genetic screen for phenotypic enhancers of *mum4*. *mum4-1* seeds were mutagenized with ethyl methanesulfonate and seeds from individual M2 plants (M3 lines) were collected and screened for reduced levels of mucilage compared with *mum4-1* as observed with ruthenium red staining after EDTA pretreatment. Over 5,000 M3 lines derived from ten parental M1 batches (1,000-1,500 M1 parents) were screened, leading to the identification of nine *mum4-1* enhancers (named *mum enhancers (men)*) that have no visible mucilage release when treated with EDTA (Figs. 3.1A and 3.2A).

Backcrosses to *mum4-1* plants revealed in each case that the seed phenotype was the result of a recessive mutation to a single locus (Table 3.1). Complementation tests were also performed between the nine *men mum4-1* lines. Only one pair of mutants did not complement each other, revealing the identification of eight mutant loci. To determine if any of the *men mum4-1* lines represented known mucilage mutant loci beyond *MUM4*, several assays were performed. First, no changes in seed shape were observed as in *ap2* and *ats* mutants (Leon-Kloosterziel, Keijzer et al. 1994). Second, their identity as new alleles of *GL2, TTG1* and *TTG2* was tested through an examination of seed coat colour and trichome presence (Koornneef

1981; Rerie, Feldmann et al. 1994; Johnson, Kolevski et al. 2002). All *men mum4-1* lines had trichomes, and none had obviously yellow seeds, suggesting that they are different genes. Third, to eliminate *tt* mutants that were not obviously yellow, tetrazolium red staining was used to detect the increased permeability to solutes found for most *tt* mutants, including *ttg1, tt2* and *tt8* that are known to affect mucilage production (Debeaujon and Koornneef 2000). One line that appeared to be wild-type seed colour (named *men3-1 mum4-1)* showed significant staining with tetrazolium red (data not shown). However, closer examination of *men3-1 mum4-1* seeds revealed that they were slightly paler than wild-type seeds. The seed colour phenotype was found to segregate away from the mucilage phenotype, suggesting a background mutation in a *tt* or related gene that is unlikely to significantly affect mucilage release (data not shown). Fourth, complementation tests were performed with *myb61*, another reduced mucilage mutant (Penfield, Meissner et al. 2001). One line was found not to complement *myb61-1* and sequencing confirmed that it is a new allele of *MYB61,* which we have named *myb61-6* (G to A transition leading to the conversion of Trp at position 252 to a stop codon). Fifth, the remaining *men mum4-1* lines were backcrossed to wild-type Columbia-2 (Col-2) plants to determine if there was a mutant phenotype in the absence of *mum4-1*, as all mucilage mutants identified to date other than *egl3*, *tt2* and *tt8* have detectable mucilage release phenotypes in a wild-type background. Only two mutant lines had detectable single mutant phenotypes where no mucilage was released when shaken in ruthenium red stain without EDTA pretreatment (Fig.3.1; data not shown). Since they were already shown not to be allelic to known reduced mucilage mutants, both of these lines were

backcrossed to the mucilage release mutants *mum1-1, mum2-1* and *patchy* (Chapter 2) (Western, Burn et al. 2001). One line was found to complement all three mutants, while the other only complemented *mum1-1* and *patchy*. While sequencing did not reveal an obvious mutation in the coding sequence of *MUM2* for the non-complementing line, we cannot rule out regulatory region or intron-related mutations. This line did, however, map to the *MUM2* region, which, in combination with multiple complementation tests using both the double and isolated single mutants, and similar results between the single mutant and *mum2* in all further assays (data not shown), suggest that this is, indeed, a new allele of *MUM2* that we have named *mum2-13*. The other line represents a different gene that we have named *MEN4*. EDTA pretreatment of *men4* single mutants revealed the release of a reduced amount of mucilage compared to wild-type seeds (compare Fig.3.1A with 3.2A).

Phenotypic characterization of *men* seed coats

Wild-type epidermal seed coat cells, when observed by scanning electron microscopy (SEM), are shown to be roughly hexagonal in shape with thickened radial cell walls and a narrow, volcano-shaped columella in the centre of each cell (Fig.3.1B) (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). A characteristic of reduced mucilage synthesis mutants, such as *mum4* and *ttg1*, is a flattened columella that is subtly visible or missing when observed with SEM (Fig.3.1B) (Koornneef 1981; Western, Burn et al. 2001; Western, Young et al. 2004). To determine if an exacerbated phenotype was apparent in the *mum4* enhancer lines,

dry seeds of each double mutant plus the *men4-1* single mutant were subjected to SEM. In each case for the double mutants, no significant enhancement of the *mum4* phenotype was obvious (Figs.3.1B and 3.2B). *men4-1* epidermal seed coat cells, however, have apparent columellae, but they are much broader and less prominent than those of wild-type seeds (Fig.3.2B).

A more detailed view of the presence of mucilage and the shape of the columella can be gained through the use of sectioning and staining of seed coats with toluidine blue. Toluidine blue is a polychromatic dye that stains various cell components different colours. For example, acidic polysaccharides such as pectins stain pink-purple, and cell walls purple-blue (O'Brien, Feder et al. 1964). The timing of mucilage and columella production has been extensively studied, demonstrating that both are generally complete by 13 days post anthesis (DPA) (Western, Skinner et al. 2000). Wild-type mucilage secretory cells at 13 DPA tend to release their mucilage upon wetting in aqueous fixative, leaving only the tall, volcano-shaped columellae and empty spaces where the mucilage accumulated prior to hydration (Fig3.1C) (Western, Skinner et al. 2000). *mum4-1* mucilage secretory cells, by contrast, remain intact, with small amount of pink-purple staining mucilage found in the apical cell corners above a dome-shaped secondary cell wall (columella), all found above a large vacuole (Fig.3.1C)(Western, Young et al. 2004). *ttg1-1* mucilage cells have a more severe phenotype than that of *mum4-1*, with less mucilage and a very thin secondary cell wall (Fig.3.1C) (Penfield, Meissner et al. 2001; Western, Burn et al. 2001). All of the *mum4* enhancer lines resemble *mum4-1* or *ttg1-1* to varying degrees (Figs.3.1C and 3.2C). *mum2-13 mum4-1* and*, men2-1 mum4-1* resemble *mum4-1*, while *men1-1 mum4-1,*

men4-1 mum4-1 and *men5-1 mum4-1* are similar to *ttg1-1*. *myb61-6 mum4-1, men3-1 mum4-1* (with background *tt* mutation) and *men6-1 mum4-1* all appear to have an intermediate phenotype.

Quantitative analysis of *men* mucilage

To quantify the amount of mucilage produced by the different *mum4* enhancers, ammonium oxalate soluble mucilage was extracted from intact seed samples, hydrolyzed, derivatized to alditol acetates and subjected to gas chromatography. Alditol acetate derivatization allows for the production of a complete neutral sugar profile of cell wall material (fucose, rhamnose, arabinose, xylose, mannose, galactose and glucose) (Chaplin 1986). However, the soluble cell wall material from these mutants could be derived from the cell wall and/or the mucilage. Arabidopsis mucilage is primarily comprised of unbranched RG I, a pectin whose backbone is composed of alternating residues of rhamnose and galacturonic acid (Penfield, Meissner et al. 2001; Western, Young et al. 2004). Comparison between ground wild-type seeds and those of an *ap2* mutant, which makes little or no mucilage, suggests that approximately 80% of the rhamnose of Arabidopsis seeds is found in the mucilage (Western, Burn et al. 2001). Thus, to focus more specifically on changes to mucilage levels, only rhamnose was considered for comparison between mutants (Fig.3.4). Wild-type Col-2 and L*er* extracted mucilage contained 339.6 ± 9.7 and 332.9 ± 6.3 μg of rhamnose per 50 mg seed, respectively (SE, n = 3). By contrast, *mum4-1* seeds have approximately one-tenth that amount (38.9 ±3.5

μg/50 mg seed) and *ttg1-1* seeds have approximately half as much rhamnose as *mum4-1* mutants (17.5 ± 2.6 μg/50 mg seed) (Fig.3.4).

men2-1 mum4-1 and *men6-1 mum4-1* were found to have approximately the same amount of rhamnose as *mum4-1* single mutants (Fig.3.4). While this correlates with the similar appearance of mucilage and columella between these two double mutants and *mum4-1* observed with toluidine blue-stained sections (Figs.3.1C and 3.2C), it is intriguing that the double mutant seeds pretreated with EDTA do not appear to release mucilage (Fig.3.2A). In order to determine if this resulted from differential release of mucilage with ammonium oxalate at 30ºC versus EDTA at room temperature, ammonium oxalate-treated seeds were stained with ruthenium red. Similar to the EDTA results, *mum4-1* seeds showed substantial mucilage release with ammonium oxalate treatment, while *men2-1 mum4-1* and *men6-1 mum4-1* seeds had only slight puffing of the cell walls (Fig.3.5). No mucilage release or cell wall puffing was seen for *mum2-1* mutants that cannot release mucilage due to mucilage hydration defects (Fig.3.5A) (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007).

myb61-6 mum4-1 has an approximately 30% drop compared with *mum4-1*, confirming the enhanced phenotype seen with toluidine blue-stained sections. This was also the case for *men4-1 mum4-1* and *men5-1 mum4-1*, which are similar to *ttg1-1,* both in terms of a 50% reduction in rhamnose compared with *mum4-1* and in their phenotype in their cross-sections. *men1-1 mum4-1*, which also appears similar to *ttg1-1* in toluidine-blue stained sections, has a further drop in soluble mucilage as reflected as another 40-50% drop compared to *ttg1-1*. The lowest amount of rhamnose observed was for *mum2-13 mum4-1*, reflecting the lack of mucilage release observed in

mum2 single mutants (Fig.3.5) (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). The rhamnose level was also determined for the *men4-1* single mutant and found to be approximately 35% of wild-type rhamnose levels in its extracted mucilage, consistent with the ruthenium red and toluidine blue section results.

Germination of *men* lines

Altered seed germination responses have been correlated with changes in seed coat structure, including mucilage quantity and release (Leon-Kloosterziel, Keijzer et al. 1994; Debeaujon, Leon-Kloosterziel et al. 2000; Penfield, Meissner et al. 2001; Rautengarten, Usadel et al. 2008). To determine the effect of reduced mucilage levels in the *mum4* single mutant, as well as in the *men* lines, a time-course of germination was performed (Fig. 3.6). *mum4-1* germination lagged significantly behind that of wild-type seeds at three days (23% for *mum4-1* versus 67% for Col-2), but reached approximately wild-type levels by four days (Fig.3.6). Similar, or even more severe delays, at three days were detected for the set of *men mum4-1* lines plus *myb61-6 mum4-1* and *mum2-13 mum4-1* double mutants, all of which continued to stay significantly below wild-type germination levels at day four, with the exception of *men4 mum4* (Fig.3.6). All lines reached 95-100% germination within nine days (data not shown). Together these results suggest not only that the reduction of mucilage in *mum4-1* has an effect on the speed of germination, but also that this delay may be enhanced by further defects in both mucilage release and quantity. Conversely, *men4-1*, which has approximately three times more mucilage than *mum4-1* (Fig.3.4), shows

no delay in germination. Interestingly, both *men4-1 mum4-1* and *men5-1 mum4-1* seeds demonstrate precocious germination relative to *mum4-1* at two days. This is reflected in *men4-1*, which germinates faster than wild type at two days. This "early germination" may explain the similar if not faster germination exhibited by *men4-1* versus wild type, and *men4-1 mum4-1* and *men5-1 mum4-1* versus *mum4-1* exhibited at three days. It is possible that in these lines there is a germination phenotype beyond that resulting from reduced mucilage levels.

The *mum4* enhancer lines also were tested for gross changes in whole plant developmental phenotypes by following the Arabidopsis Gantlet Project protocol (http://thale.biol.wwu.edu/). In short, seeds were plated side-by-side with wild-type and *mum4-1* control seeds, grown 14 days vertically on plates, and then transplanted to soil, with regular observation across all stages (daily while on plates and weekly once in soil). No gross developmental phenotypes were observed for any of the lines.

DISCUSSION

The mucilage secretory cells of the Arabidopsis seed coat are a useful model for the identification and study of genes involved in cell wall production and metabolism. In particular, they have started to allow the dissection of genes involved in the regulation of pectin synthesis and in pectin modification (Penfield, Meissner et al. 2001; Johnson, Kolevski et al. 2002; Zhang, Gonzalez et al. 2003; Western, Young et al. 2004; Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007; Rautengarten, Usadel et al. 2008; Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009). However, to

date, only one gene involved directly in mucilage synthesis has been identified, and no genes have been directly implicated in polar secretion of mucilage (Usadel, Kuchinsky et al. 2004; Western, Young et al. 2004). Here, six new genes involved in mucilage production have been identified as enhancers of the *mum4* reduced mucilage mutant. Three of these genes appear to have further reductions in mucilage production compared with *mum4*, making them promising candidates for roles in mucilage synthesis and/or secretion.

MEN **genes affect mucilage production**

Mutations in six new genes (*MEN1—6)* affecting mucilage secretory cell differentiation were identified, along with new alleles of two known genes: *MUM2* and *MYB61*. The finding of new alleles of these two genes validated the screen in its ability to find genes acting in parallel with *MUM4* for mucilage production (*MYB61*) (Penfield, Meissner et al. 2001), as well as genes acting in pectic mucilage modifications required for mucilage swelling and release (*MUM2*) (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). Further, this screen revealed the utility of such a sensitized screen, as only one of the new genes identified (*men4)* had an obvious single mutant phenotype. Characterization of the *mum4* enhancers revealed two phenotypic categories: reduced mucilage production and lack of mucilage release.

Three of the *men mum4* double mutants identified in this screen (*men1 mum4, men4 mum4* and *men5 mum4*) appear to make reduced amounts of mucilage compared to *mum4* as determined by both their cell structure and soluble rhamnose levels (Figs.

3.1, 3.2 and 3.4). *men1 mum4* double mutants have the most significant reduction of mucilage, while *men4 mum4* and *men5 mum4* have slightly more mucilage. The interpretation of these mutants as being affected in mucilage production is supported by their shared phenotypes with *myb61 mum4* and *ttg1*. TTG1 regulates both the GL2 and TTG2 pathways of mucilage production (Johnson, Kolevski et al. 2002; Zhang, Gonzalez et al. 2003; Western, Young et al. 2004), thus, *ttg1*-like mutants may be expected to be affected in both pathways downstream of TTG1. *myb61* mutants have reduced mucilage resulting from disruption of a TTG1-independent pathway (Penfield, Meissner et al. 2001; Western, Young et al. 2004), so the *myb61 mum4* double mutant serves as a control for disruption of two independent pathways of mucilage production, as *MUM4* acts downstream of TTG1. In addition, the single mutant for *men4* has significantly reduced mucilage compared to wild-type seeds (Figs.3.1, 3.3 and 3.4), confirming a role in mucilage production for one member of this class of *mum4* enhancers.

As mucilage is primarily comprised of the pectin RG I, genes involved in its manufacture and transport, or the regulation of these processes, would be the most obvious candidates for the *men* genes affected in mucilage quantity. While our phenotypic and complementation analyses have ruled out most mucilage regulatory mutants, it is possible that one of the *men* genes could encode a new allele of *EGL3* or a weak allele of *MYB5* without an obvious single mutant phenotype (Zhang, Gonzalez et al. 2003; Gonzalez, Mendenhall et al. 2009; Li, Milliken et al. 2009). Our preliminary mapping data for *MEN1, MEN4* and *MEN5*, however, suggest that this is not the case (AA Arsovski, M Wang, N Martin, J Schafhauser and TL Western,

unpublished results). *MUM4* is a member of a small gene family that encodes three full length, trifunctional UDP-L-rhamnose synthase proteins (RHM1, MUM4/RHM2, RHM3), and a protein that catalyzes only the latter part of the conversion of UDP-D-glucose to UDP-L-rhamnose (UER) (Usadel, Kuchinsky et al. 2004; Watt, Leoff et al. 2004; Western, Young et al. 2004; Oka, Nemoto et al. 2007). All members are expressed throughout the plant, allowing for genetic redundancy in rhamnose synthesis and the production of some mucilage in *mum4* seeds. A mutation in *RHM1, RHM3* or *UER* could result in a further reduction in mucilage production. By contrast, one of the *men* genes could encode a member of the *UDP-D-GLUCOURONATE 4-EPIMERASE (GAE)* family, which are required for the synthesis of UDP-D-galacturonic acid, the other sugar comprising the backbone of RG I, as well as the backbone of homogalacturonan (Willats, McCartney et al. 2001; Lao, Long et al. 2003; Usadel, Schluter et al. 2004). Pectins are synthesized in the Golgi apparatus through the activity of glycosyltransferases (GT) that use nucleotide sugars as substrates. Members of GT family 8 have been implicated in pectin synthesis through both mutant studies and enzyme isolation (Scheller, Doong et al. 1999; Sterling, Quigley et al. 2001; Willats, McCartney et al. 2001; Bouton, Leboeuf et al. 2002; Lao, Long et al. 2003; Shao, Zheng et al. 2004; Mohnen 2008), so it is possible that one of the *men* genes could encode a GT8 protein required either for RG I backbone synthesis or for the synthesis of one of the other pectins found in Arabidopsis mucilage. Indeed, a gene encoding a GT8 family protein that has a mild mucilage production phenotype has been identified as being upregulated in seed coats at the time of mucilage synthesis, similar to *MUM4* (J Schafhauser, A Abdeen

and TL Western, unpublished results). Alternately, a *men* mutant could be affected in a gene required for secretion of mucilage from the Golgi apparatus to the apoplast. These could include vesicle trafficking factors such as small G-proteins, their effectors or activators, or the multisubunit exocytosis complex known as the exocyst (Cole and Fowler 2006; Hala, Cole et al. 2008; Nielsen, Cheung et al. 2008; Rojo and Denecke 2008; Yalovsky, Bloch et al. 2008)

Both *men2 mum4* and *men6 mum4* double mutants did not release mucilage after pretreatment with EDTA, but appear to make a similar amount of mucilage to *mum4* as assessed both by cellular appearance and rhamnose levels in soluble mucilage (Figs.3.2 and 3.4). Specific mucilage release in ammonium oxalate used for mucilage extraction was tested by staining seeds after shaking in ammonium oxalate and revealed only slight puffing of the cell wall for *men2 mum4* and *men6 mum4* (Fig.3.5). It is possible that some primary cell wall breakage occurred in parallel with this puffing, allowing mucilage extraction through fissures in the wall. Conversely, chelator treatment may have extracted pectins from the primary cell wall, allowing further extraction of highly soluble mucilage through the weakened wall. Extraction of only primary cell wall pectins in the case of *men2 mum4* and *men6 mum4* is unlikely since both have higher levels of soluble rhamnose than other non-releasing mutants that have little or no mucilage (*e.g. ttg1)* or that have been shown to affect mucilage hydration (*mum2 mum4)* (Figs.3.2, 3.4 and 3.5) (Western, Skinner et al. 2000; Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007).

The *mum4*-like phenotypes make it difficult to assess the cause of the lack of mucilage release in *men2 mum4* and *men6 mum4*. One possibility is that there is a

decrease in mucilage production that is sufficient to prevent release, but not large enough to be detected though observation of secretory cell structure or rhamnose levels. Alternately, there could be a defect in cell wall or mucilage structure that prevents mucilage release. While the extremely low soluble rhamnose levels of *mum2 mum4* double mutants would seem to argue against this latter hypothesis, *mum2* mutants are characterized by a lack of mucilage swelling that likely results from reduced hydration capacity, explaining the *mum2 mum4* double mutant phenotype (Fig.3.5) (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007). It is possible for the *men2 mum4* and *men6 mum4* double mutants that the mucilage and/or outer cell wall structural changes are such that mucilage solubility is less significantly affected than for mutations in *MUM2*. Candidate genes for *men2 mum4* and *men6 men2*, therefore, may be new cell wall modification factors. While preliminary mapping places *MEN2* away from known mucilage release genes (MM Villota, TL Western, O Rowland and R Subramaniam, unpublished data), it cannot be ruled out that the two *men6* alleles are weak alleles of *MUM1, MUM2* or *SBT1.7* that lack obvious phenotypes in the absence of *mum4*.

Effect of mucilage changes on seed germination

A time-course of germination revealed that *mum4-1* has delayed germination compared with wild-type seeds (Fig.3.6). This is consistent with previous studies that demonstrated that reduced mucilage mutant seeds (*myb61-1, ttg1-1, gl2-1*) had a decreased ability to germinate under conditions of limited water supply (as exerted by

increasing concentrations of polyethylene glycol) compared to wild-type seeds (Penfield, Meissner et al. 2001). A similar reduction in germination in the presence of polyethylene glycol was seen for *Atsbt1.7* mutants, which are defective in mucilage release (Rautengarten, Usadel et al. 2008). Due to its significant hydrophilicity, Arabidopsis mucilage has been proposed to promote seed hydration, and thus germination, through the attraction and retention of water surrounding the seed (Penfield, Meissner et al. 2001). In *mum4-1*, seed hydration, and thus imbibition, could be slowed by either the reduced quantity of mucilage or the lack of release of mucilage to form a hydrated gel around the seed. While pleiotropic effects cannot be ruled out, the enhanced delay seen in both *myb61 mum4* and *mum2 mum4* double mutants, as well as *men mum4* double mutants (Fig.3.6), strongly suggests that both mucilage quantity and release are important for efficient seed hydration and germination, even under moist conditions.

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Figure 3.1 Mucilage release and seed coat structure of controls and *mum4* enhancer lines resulting from mutations in *MUM2* and *MYB61*.

(A) Seeds pretreated in EDTA with shaking and stained with ruthenium red. Note the thick layer of mucilage surrounding wild-type Col-2 seeds and a very thin layer around *mum4-1* seeds. (B) Scanning electron microscopy of dry seeds. Note the prominent hexagonal cell walls and central volcano-shaped columella in the centre of Col-2 cells. (C) Toluidine blue-stained resin sections of 13 DPA seeds. Wild-type mucilage cells have burst open in the aqueous fixative, leaving tall, blue-stained, volcano-shaped columellae with some cell wall material attached to the centre of the columella. *mum4-1* cells do not burst, and contain small pockets of purple-stained mucilage in the upper apical corners, subtended by a blue dome of secondary cell wall. *ttg1-1* cells, similar to *mum4-1*, do not burst, however, the mucilage pockets are smaller and the secondary cell wall is thinner in appearance. Scale bars in (A) = 500 μm, in (B) = 50 μm, and in (C) = 10 μm.

Figure 3.1

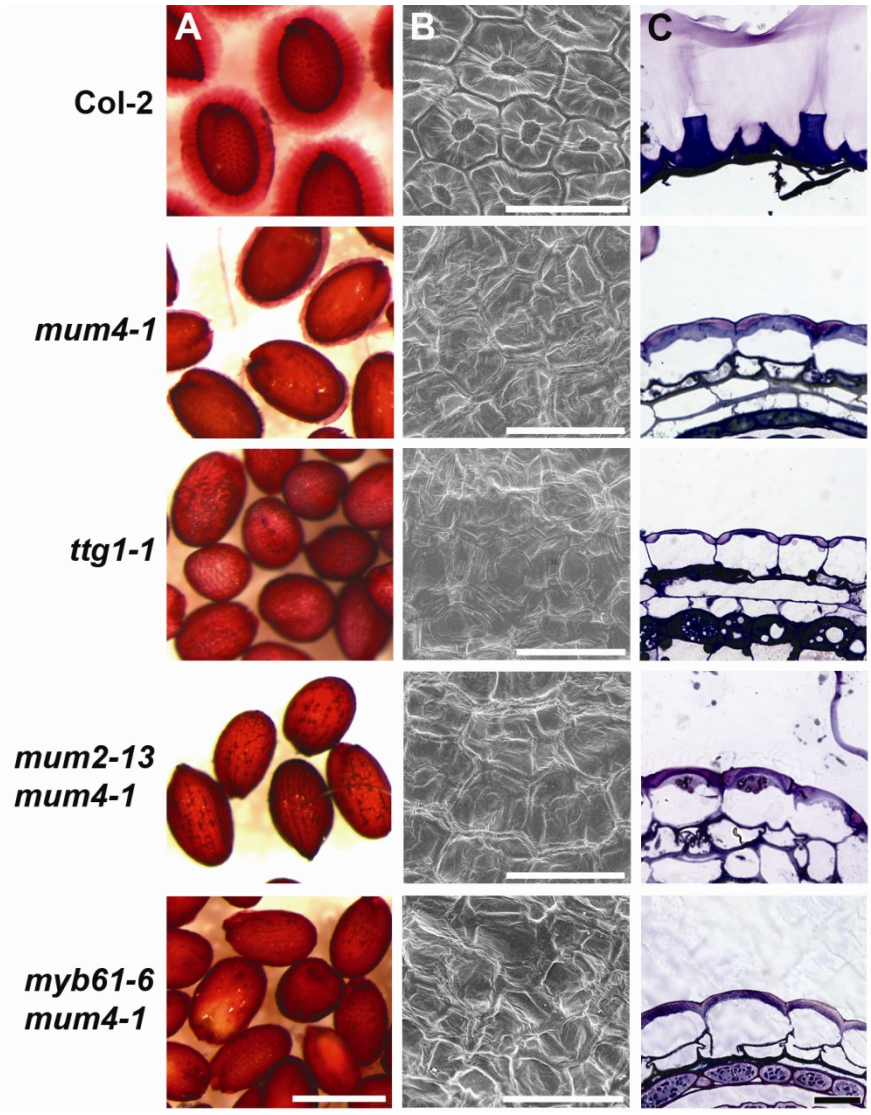


Figure 3.2 Mucilage release and seed coat structure of *mum4* enhancer lines.

(A) Seeds pretreated in EDTA with shaking and stained with ruthenium red. (B) Scanning electron microscopy of dry seeds. (C) Toluidine blue-stained resin sections of 13 DPA seeds. Scale bars in (A) = 500 μm, in (B) = 50μm, and in (C) = 10 μm.

Figure 3.2

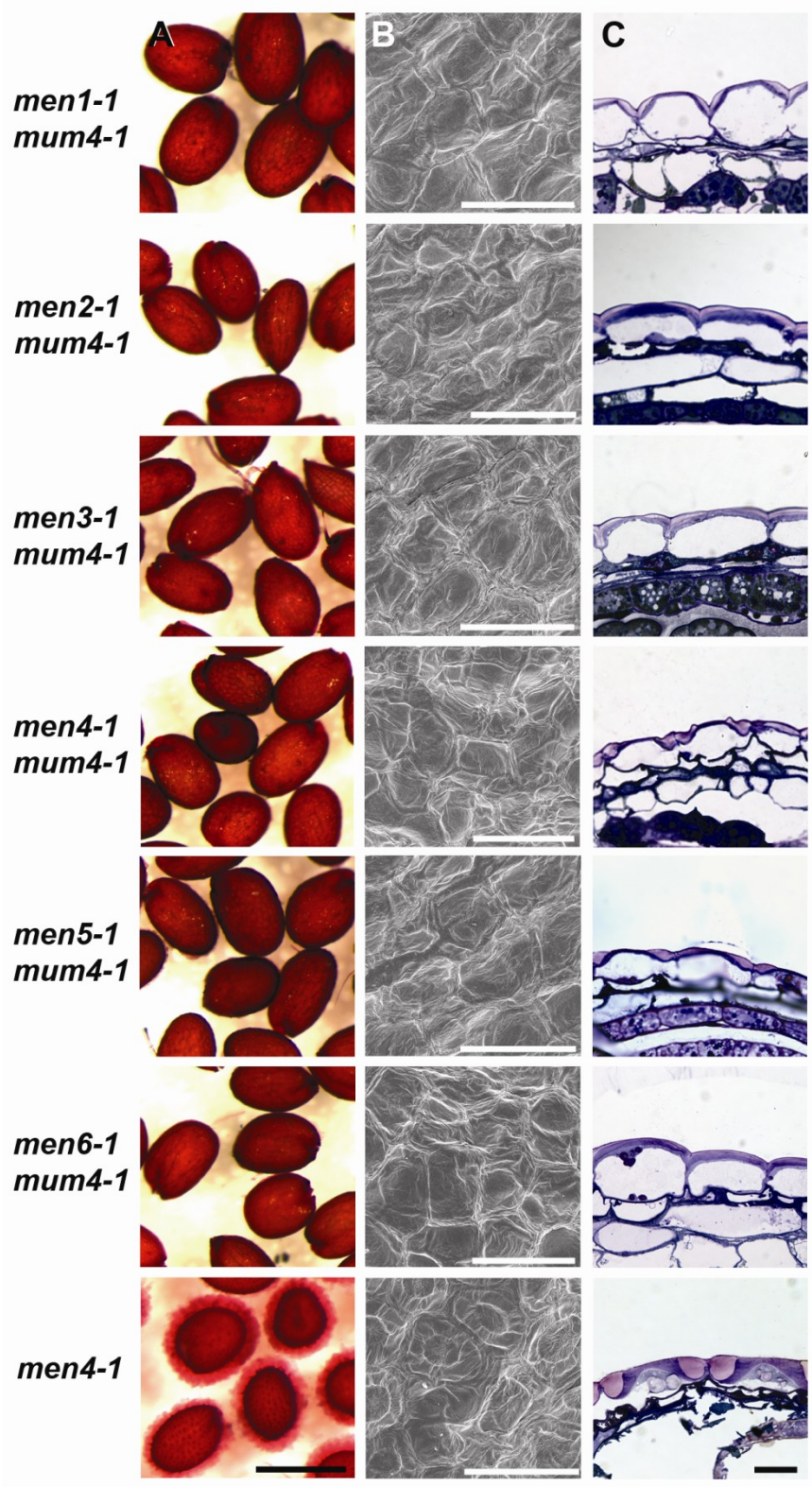

A
B
C
men1-1
mum4-1
men2-1
mum4-1
men3-1
mum4-1
men4-1
mum4-1
men5-1
mum4-1
men6-1
mum4-1
men4-1

Table 3.1 *Segregation analysis and Chi-square test results for mum4 enhancer lines backcrossed to* mum4-1.

Table 3.1

Mutant line	***mum4*: no mucilage**[a]	**Chi square**[b]
men1-1 mum4-1	68 : 17	1.1333, $P > 0.1$
men2-1 mum4-1	60 : 17	0.3506, $P > 0.5$
men3-1 mum4-1	85 : 27	0.0476, $P > 0.5$
men4-1 mum4-1	57 : 16	0.3699, $P > 0.5$
men5-1 mum4-1	60 : 21	0.0370, $P > 0.5$
men6-1 mum4-1	80 : 19	1.7811, $P > 0.1$

[a]F3 seed phenotype (seed of F2 plants), shaken 90 min in EDTA followed by staining in 0.01% ruthenium red.

[b]Null hypothesis of 3:1 *mum4*:no mucilage; degrees of freedom = 1; cutoff at $P = 0.05$.

Figure 3.3 Mucilage release of *men4-1* versus wild-type seeds in ruthenium red with and without shaking.

(A, B) Col-2 wild-type seeds. (A) Seeds put directly into ruthenium red without shaking are surrounded by an outer, diffuse layer of mucilage and an inner, dense layer of mucilage, while those shaken in dye lose the soluble outer layer (B). (C, D) *men4-1* seeds lack mucilage release when treated directly with ruthenium red, with or without agitation. Scale bar = 500 μm.

Figure 3.3

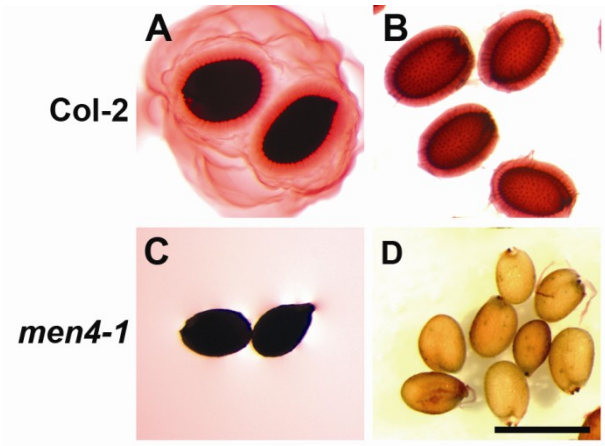


Figure 3.4 Rhamnose levels of soluble mucilage extracted from *mum4* enhancers and controls.

Ammonium oxalate extracts of concurrently-grown seed batches were hydrolyzed with trifluoroacetic acid and derivatized to alditol acetates, followed by gas chromatography. Extractions were done in triplicate, error bars = SE.

Figure 3.4

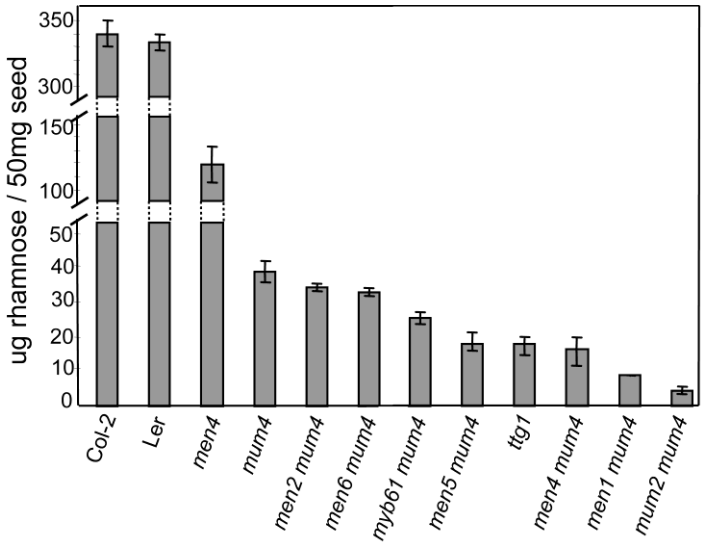

ug rhamnose / 50mg seed
350
300
150
100
50
40
30
20
10
0
Col-2
Ler
men4
mum4
men2 mum4
men6 mum4
myb61 mum4
men5 mum4
ttg1
men4 mum4
men1 mum4
mum2 mum4

Figure 3.5 Seed coat phenotype of *men2 mum4*, *men6 mum4* and control seeds following extraction with ammonium oxalate.

(A) Seeds extracted with ammonium oxalate with shaking at 30°C, then stained with ruthenium red. Note substantial mucilage release for Col-2 seeds. *mum4-1* seeds release less mucilage, with the outer wall appearing to remain largely intact. (B) Scanning electron microscopy of air-dried ammonium oxalate extracted seeds. Only Col-2 seeds show obvious rupture of outer primary cell wall. Scale bars in (A) = 10 μm, and (B) = 25 μm.

Figure 3.5

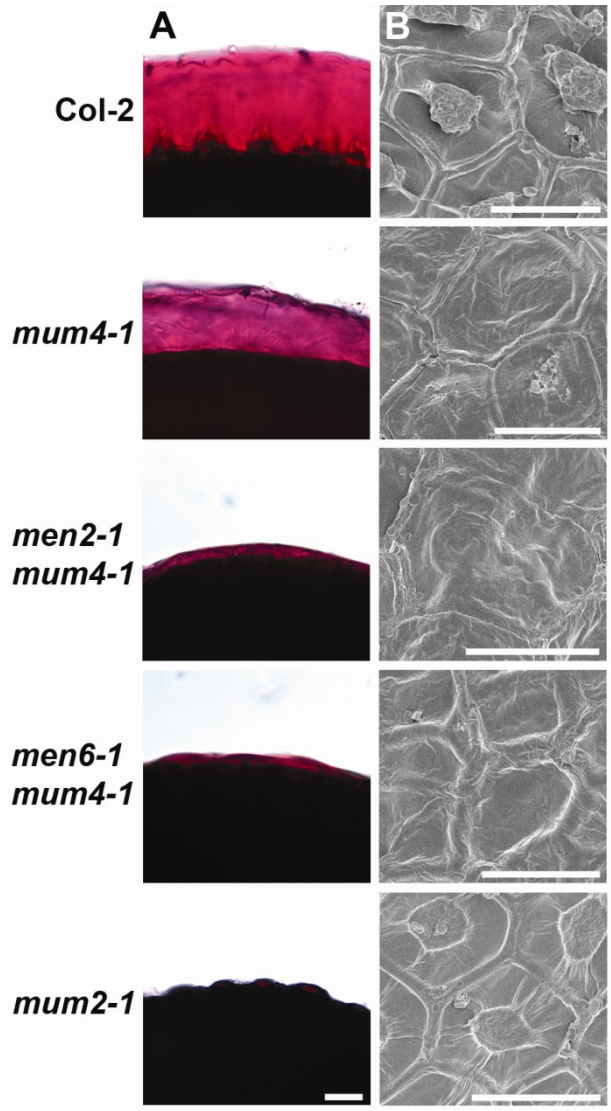
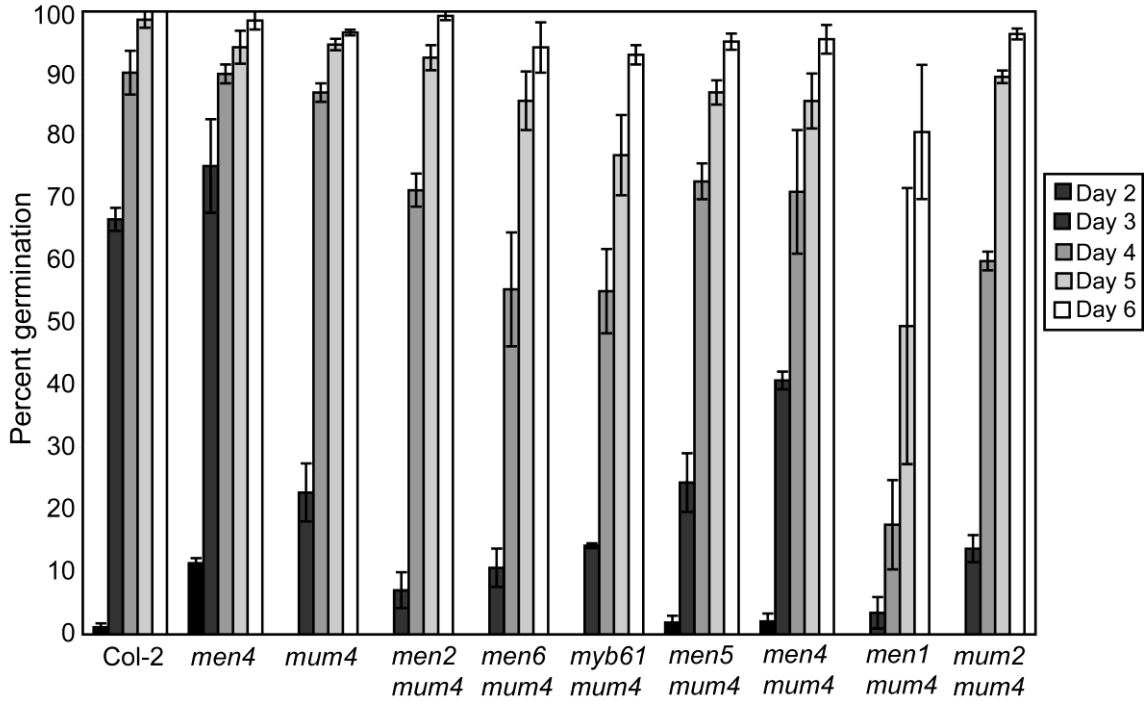


Figure 3.6 Time-course of germination of *mum4* enhancers and controls.

Genotypes are organized in the same order as Fig. 4 for comparison. Seeds were stratified for three days at 4°C, followed by germination at 22°C under 16 h light: 8 h dark. 40-80 seed of each genotype were sowed on filter paper with water, error bars = SE. Similar results were obtained in a separate experiment using seed from an independent set of plants.

Figure 3.6


Percent germination
100
90
80
70
60
50
40
30
20
10
0
Day 2
Day 3
Day 4
Day 5
Day 6
Col-2
men4
mum4
men2 mum4
men6 mum4
myb61 mum4
men5 mum4
men4 mum4
men1 mum4
mum2 mum4

SUPPLEMENTARY MATERIAL

Table S3.1 *Primers used for the sequencing of* mum2-13 *and* myb61-6.

Table S3.1

Primer Name	**Sequence (5' → 3')**
mum2 p327F	AGCTTCTCTTCTCCGGTTCC
mum2 p2302R	ATCACATCGATGCCTCCTTC
mum2 p1874F	CACAGATCCGTTGTCTGTGC
mum2 p3741R	GCGTGAAGCTCCTTCAAATG
mum2 p3740F	GCTGCGATAAAATCCTCTGC
mum2 p5586R	ACCCCCAATTACCCAAAAAC
mum2 p844F	TTTTCGCCAAAAATGAAAAA
mum2 p955R	TGATCCCCGCTAGAATTTTG
mum2 p2694F	ATACCGAACCGACAATGAGC
mum2 p2865R	TTCGGTTTGGTGACATTGAA
mum2 p3808R	CATTGGTCCCAAGGAGAGAA
mum2 p4482F	TTGACGGTCCATTGCACTTA
mum2 p4622R	TGTCTCTGCTTCCATGTCCA
myb61 p59F	CATTCTCTCTGCTCACAACTTTT
myb61 p1715R	TGCAAAAGAAAGAAGAATCTTTGA
myb61 p804F	CACACAAGCCCATCTCTGAA
myb61 p942R	GGCCTTTCGAGGAAGAAGTC

Link between Chapter 3 and 4

Chapter 3 described the use of an enhancer/suppressor screen and its identification of novel mutants that may be involved in the synthesis or secretion of mucilage *(men1, 3, 4, 5)* or the modification of mucilage and/or the cell wall, required for mucilage release *(men2, 6)* . **Chapter 4** focuses on further characterization of the *men4* mutant and its reduced mucilage phenotype. Monosaccharide analysis and immunolabelling suggests *MEN4* may be required for the synthesis of the pectin RG I. Preliminary mapping places the *MEN4* locus on the upper arm of chromosome 5. In addition, *men4* and other mutants are employed to examine whether mucilage synthesis and/or its release is necessary for normal germination rates.

Chapter 4

Characterization of the *men4* mutant, mapping of the *MEN4* locus, and the effects of mucilage on germination

ABSTRACT

Pollination triggers the differentiation of the ovule integuments to form a specialized seed coat. One specialization is the production of pectinaceous mucilage in the epidermal cells. This mucilage is released upon wetting and forms a gel capsule that is believed to aid seed hydration and germination. The mucilage secretory cells of *Arabidopsis thaliana* undergo a complex differentiation process in which cell growth is followed by the synthesis and secretion of a large amount of mucilage. Mucilage secretion to the apoplast in the outer tangential portion of the cell is accompanied by constriction of the vacuole and formation of a volcano-shaped columnella beneath the mucilage pocket. A novel gene that appears to play a role in mucilage synthesis is *MUM ENHANCER 4* (*MEN4*). *men4* mutant mucilage has approximately one-third of the rhamnose found in wild type mucilage. Cross sections of developing *men4* seeds show a wider columnella and reduced mucilage pockets compared with wild type seeds. In addition, staining with pectin-specific antibodies suggests a decrease in the pectin rhamnogalacturonan I. Together these results suggest a role for *MEN4* in mucilage synthesis or secretion to the apoplast. Initial mapping places the *MEN4* locus between 26 and 32cM on chromosome 5. Differing degrees of mucilage reduction between *men4, mum4* and *men4 mum4* double mutants, along with mutants affected in mucilage release, allowed testing of the effects of mucilage levels versus release in seed hydration and germination. Preliminary results indicate that the production of mucilage may be more important than its release in the promotion of germination.

INTRODUCTION

In *Arabidopsis thaliana,* the seed coat epidermal cells undergo a differentiation process that leads to the production of a pectinaceous substance referred to as mucilage (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). Upon wetting of mature seeds, the mucilage is released and forms a gelatinous capsule that envelops the seed (Western, Skinner et al. 2000). Found in many species, this hydrophilic mucilage has been proposed to play a role in seed germination by absorbing water and maintaining the hydration of the seed (Grubert 1981; Boesewinkel and Bouman 1995). Additionally, the adhesive properties of mucilage may play a role in the dispersal of the seed. In Arabidopsis, mucilage is not vital for germination or viability of the seed under laboratory conditions (Western, Skinner et al. 2000; Western, Burn et al. 2001).

The mature, dry seed coat epidermal cells of Arabidopsis have a donut-shaped pocket of mucilage under the apical cell wall that surrounds a central columella (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). The differentiation of these mucilage secretory cells (MSCs) from the ovule integument is triggered by pollination (Beeckman, De Rycke et al. 2000; Western, Skinner et al. 2000; Windsor, Symonds et al. 2000). The initial growth of the MSCs is driven by vacuolar expansion, which is followed by the synthesis and secretion of large quantities of pectinaceous mucilage. The polar secretion of large quantities of mucilage to the apical cell face results in a cytoplasmic column in the centre of the cell. A secondary cell wall is then deposited over the cytoplasmic column to form a cellulosic columella (Western, Skinner et al. 2000). Eventually the cell undergoes

apoptosis and desiccation. Upon wetting, the hydrophilic mucilage rapidly expands, ruptures the primary cell wall and surrounds the seed in a gel-like capsule (Western, Skinner et al. 2000). The presence of two clearly defined stages of cell wall biosynthesis (the synthesis and secretion of pectinaceous mucilage, followed by the deposition of secondary cell wall), as well as the dispensability of mucilage, make the MSCs a convenient model for studying the synthesis, secretion, and modification of cell wall components (Haughn and Chaudhury 2005; Western 2006).

A number of regulatory genes have been identified that play a role in the differentiation and development of the MSCs. In particular, *TRANSPARENT TESTA GLABRA1 (TTG1), TTG2, GLABRA2 (GL2),* and *APETALA2 (AP2)* were found to be important for the regulation of two associated pathways for MSC development and mucilage production (Fig.1.4) (Koornneef 1981; Jofuku, den Boer et al. 1994; Rerie, Feldmann et al. 1994; Western, Burn et al. 2001; Johnson, Kolevski et al. 2002; Western, Young et al. 2004). However, only one biosynthetic gene has been identified to date. *MUM4*, also known as *RHM2*, has been extensively characterized and encodes a putative NDP-L-rhamnose synthase, an enzyme required for the synthesis of rhamnogalacturonan I (RG I), the major pectin component of mucilage (Usadel, Kuchinsky et al. 2004; Western, Young et al. 2004; Oka, Nemoto et al. 2007). The reduced levels of rhamnose, galacturonic acid and RG I in the *mum4* mutant correspond with its reduced mucilage phenotype. Expression studies demonstrated that *MUM4* is regulated by the transcription factors *TTG1, GL2* and *AP2* (Western, Young et al. 2004). In order to identify additional downstream genes in the mucilage production pathway, an enhancer mutant screen of the reduced mucilage mutant *mum4*

was performed (Chapter 3). In addition to isolating new alleles of the known mucilage-related genes *MUM2* and *MYB61*, mutants for six new MSC differentiation genes were identified from the screen that demonstrated varying degrees of enhancement of the *mum4* phenotype. Of these, the *men4* mutant had a single mutant phenotype of significantly reduced mucilage, suggesting a role in the synthesis or secretion of mucilage (Chapter 3).

Here I describe further characterization of the *men4* mutant, as well as initial mapping of the *MEN4* locus. *men4* MSCs appear to develop and synthesize mucilage normally compared with wild type seeds until 13dpa where wider columnella and smaller mucilage pockets indicate a decrease in mucilage deposition. Chemical and immunological analyses suggest this reduction might be due to decreased levels of the pectin RG I. Initial mapping places the *MEN4* locus to a 5cM region on the upper arm of chromosome 5. In addition to pursuing the role and identity of *MEN4*, *men4* was used along with *mum4* and the *men4 mum4* double mutant, and mutants affected in mucilage release (*mum2*, *atsbt1.7-1, atsbt1.7-2*), to study the relative effects of mucilage level versus mucilage release on germination. Preliminary results suggest that mucilage amount has a greater effect on germination rates than mucilage release.

MATERIALS AND METHODS

Plant lines, mutagenesis and growth conditions

Lines of *Arabidopsis thaliana* used were *mum4-1* (Col-2 ecotype) (Western *et al.*, 2004), *atsbt1.7-1* and *atsbt1.7-2* (gift from B. Usadel; Rautengarten, Usadel et al. 2008), *mum2*, *men4* and *men4 mum4* (Arsovski, Villota et al. 2009). Seeds were planted on AT minimal medium plates (Haughn and Somerville, 1986) or directly on soil (Sunshine Mix #5, SunGro Horticulture), stratified for 3-4 days at 4°C and then transferred to growth chambers at 22°C under continuous light (90-120 μE m^{-2} s^{-1} photosynthetically active radiation), unless otherwise specified. Flower staging for days post anthesis (dpa) was performed as in Western *et al.* (2001).

Microscopy

Developing seeds were prepared for brightfield microscopy, sectioned and stained with toluidine blue O as described in Western *et al.* (2001). Samples were examined using a Leica DM 6000B compound microscope and images captured with a Qimaging Retiga CCD camera operated through Openlab.

Seed Size Measurements

100 seeds of each line were observed on a Leica MZ-16F stereomicroscope and imaged with a Micropublisher 3.3 camera (Qimaging) operated via Openlab 5 (Perkin Elmer). Seed size was measured using Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA,

http://rsb.info.nih.gov/ij/, 1997-2009). A paired t-test was used to compare seed sizes to wild type.

Chemical Analysis

To quantify neutral sugars in crude mucilage extracts, 50 mg of intact seeds were incubated in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 h at 30 ºC. 1 μmole of myo-inositol was added to the supernatant and samples were precipitated with 5 volumes ethanol, directly hydrolyzed with 2M trifluroacetic acid and derivatized to alditol acetates. Derivatization to alditol acetates and gas chromatography were performed as in Gibeault and Carpita (1991), but with an HP-23 glass capillary column (30 m x 0.25 mm i.d.; Agilent Technologies). Seeds used for chemical analyses were collected from mutant and control plants cultivated together.

Immunofluorescence

For immunofluorescence on developing seeds, seeds were dissected from 7 and 10 dpa siliques and fixed for 2 hr in 4% (v/v) formaldehyde (freshly prepared from paraformaldehyde) in 50 mM PIPES (pH 7.0). Samples were rinsed, dehydrated through an ethanol series and embedded in LR White resin. Embedded samples were sectioned to 0.5 μm, affixed to slides with poly-L-lysine, and subjected to antibody detection as described in Young et al. (2008), except primary antibodies were used full strength and secondary antibodies were diluted as described below for whole seed samples.

Whole seed immunofluorescence was performed as in Young et al. (2008). Primary antibodies (1:20 [v/v]) were detected with a 1:100 (v/v) dilution of Alexfluor 488

conjugated goat anti-mouse (CCRC-M36), goat anti-rat (LM18, JIM5) secondary antibodies (Molecular Probes, Invitrogen). Seeds were counterstained with 0.2 μg/ml propidium iodide in 50 mM phosphate buffer pH 7.4 to visualize the outer cell wall. Treatments without primary antibody were included to test for non-specific staining, and all seeds were mounted in 1:100 (v/v) India ink in 90% (v/v) glycerol in water to confirm the presence of released mucilage. Immunofluorescence samples were observed with a Zeiss Meta 510 LSM confocal microscope.

Mapping

A mapping population of approximately 700 plants derived from a cross between *men4-1* and wild-type L*er* was used for mapping using simple sequence length polymorphism and cleaved-amplified polymorphic sequence markers generated from sequence information provided by the Arabidopsis Genome Initiative (2000) and Cereon (Lukowitz, Gillmor et al. 2000; Jander, Norris et al. 2002). Molecular markers used included the set defined by Lukowitz, Gillmor et al. 2000, along with the following additional markers:

Nga249 (**forward primer:** 5'-GGATCCCTAACTGTAAAATCCC-3';
reverse primer: 5'-TACCGTCAATTTCATCGCC-3') and
nga106 (**forward primer:** 5'-TGCCCCATTTTGTTCTTCTC-3';
reverse primer: 5'-GTTATGGAGTTTCTAGGGCACG-3'.

Germination time-course

Two 70 mm diameter Whatman #1 filter papers (Whatman) were placed in the lid of a 100 mm plastic Petri dish. To these were added 2 ml of water, 450mM mannitol, or

400mM sorbitol, and 40-80 seeds of each mutant line. Prior to plating, dry seeds were placed in 1.5ml microfuge tubes and stratified in the dark at 4°C for 72 h. The plates were sealed with parafilm and incubated at 22°C under 16 h light: 8 h dark, following which they were counted at 24, 30, 45, 52, 70, 92, and 118hrs and germination was scored by protrusion of the radicle through the testa. The plates were counted again after nine days to determine if all lines reached approximately 100% germination. Seeds used for germination analyses were collected from mutant and control plants cultivated together and stored as a seed set for six months, one seed set was used. Seeds were stored in microfuge tubes with holes in the lids at room temperature under ambient humidity and light conditions. Each time-course was done in triplicate.

Seed swelling

Two 70 mm diameter Whatman #1 filter papers (Whatman) were placed in the lid of a 100 mm plastic Petri dish. To these were added 2 ml of water and 40-50 seeds of each mutant line. Seeds were photographed immediately after plating, and again after 4 hours. The seed area was measured at both time points and was measured using Image J software. The percent change in seed size was calculated using the measured seed areas of both time points.

RESULTS

Phenotypic characterization of the *men4* mutant

As described in the previous chapter, *men4* mutants make a reduced amount of mucilage compared to wild-type seeds. In order to determine if there were any developmental irregularities accounting for the reduced mucilage phenotype in *men4*, seeds at distinct time points during MSC development were embedded, sectioned and stained with the polychromatic dye toluidine blue. These MSC developmental stages correspond to cell growth (4dpa, Fig.4.1A and E), mucilage synthesis and accumulation (7dpa, Fig.4.1B and F), deposition of a secondary cell wall over the central cytoplasm (10dpa, Fig.4.1C and G) and approaching maturity when the remaining cytoplasm is lost from wild type cells (13dpa, Fig. 4.1D and H arrows) (Western, Skinner et al. 2000). Sectioning and toluidine blue staining of *men4* seeds showed initial MSC development similar to that of wild type. Cell growth appears normal and mucilage synthesis is similar to wild type (compare Fig.4.1 A to E and B to F). The deposition of a secondary cell wall also is comparable to that observed in wild type (compare Fig.4.1 C to G). However, at the point where seed coat desiccation commences and cytoplasm is no longer visible in wild type cells, a difference is observed. *men4* MSCs show a decrease both in the mucilage pocket size and in columella height, suggesting a decrease in the amount of synthesized mucilage (compare Fig.4.1 D to H).

Multiple observations of *men4* versus wild type seeds suggested that *men4* seeds were smaller than those of wild type. Seed area measurements from 100 seeds of each line revealed a size decrease in *men4* mutants, whose seeds measured 1300.83 ± 25.41 pixels compared to 1643.07 ± 45.4 for Col-2 seeds (Fig 4.2). Further examination showed a similar decrease in the *men4 mum4* mutant, with an average seed size of 1310.28 ± 17.0 pixels, as well as a smaller but significant decrease in the *mum4* mutant, which had a seed size of 1549.90 ± 19.2 pixels ($p<<0.05$ for all mutants versus wild type seeds using a paired t-test) (Fig.4.2). More plants are being grown in order to confirm these preliminary observations.

Quantitative analysis of *men4* mucilage

To provide a neutral sugar profile of *men4* mucilage versus that of wild type and *mum4* seeds, ammonium oxalate soluble mucilage was extracted from intact seed samples, hydrolyzed, derivatized to alditol acetates and subjected to gas chromatography. As noted in Chapter 3, wild type (Col-2) mucilage contained 339.6 ± 9.7 μg of rhamnose per 50mg of seed, while *mum4-1* seeds are severely reduced at 38.9 ± 3.5 μg/50mg seed (SE, n=3) (Table 4.1, Fig.4.3). The *men4* mutant mucilage was found to have a 65% reduction of rhamnose levels when compared with wild type mucilage (119.12 ± 13.0 μg/50mg seed). The *men4 mum4* double mutant mucilage contained only 15.9 ± 3.4 μg of rhamnose per 50 mg seed, showing a further reduction than the *mum4* or *men4* single mutants. In addition to reduced rhamnose, there was also a decrease in extracted xylose in the *men4* mutant with

11.72 ± 0.11 μg, compared to 25.5 ± 0.02 μg in the wild type. Additionally, there appears to be a 44 percent decrease in galactose between the *men4* mutant and wild type. Similar decreases in xylose and galactose were also observed in *mum4* mucilage. Finally, all mutants show an increase in extracted glucose, *mum4* had 22.24 ± 0.45 μg, *men4* was similar with 23.50 ± 1.32μg, and *men4 mum4* had 35.79 ± 4.28μg, while the wild type Col2 had 15.99 ± 0.88 μg per 50 mg seed. Fucose, arabinose, and mannose did not show significant change between the mutants and wild type with the exception of slight increases in the *men4 mum4* double mutant (Table 4.1, Fig.4.3).

Immunolabelling of whole and sectioned *men4* seeds

In order to confirm if the observed decrease in rhamnose in *men4* mucilage was due to a decrease in the pectin RG I, staining of developing seed coats with the antibody CCRC-M36 was performed. The CCRC-M36 antibody is specific to unbranched RG I and was raised to Arabidopsis mucilage (Young, McFarlane et al. 2008). In wild type seed coats, strong staining was observed throughout the mucilage pocket at 7dpa, and, by 10dpa, the MSCs had burst and released their mucilage in response to the antibody buffer (Fig4.4A and B). The *men4* mutant did not show an observable difference in CCRC-M36 staining at 7dpa, but at 10dpa the cells had not released their mucilage, making direct comparisons of the degree of labelling difficult (Fig.4.4C and D). As expected, the *mum4* mutant showed a reduced amount of staining corresponding to the decrease in mucilage production (Fig4.4E and F). The *men4 mum4* double mutant displayed a further decrease in CCRC-M36

staining confirming the severe reduction in rhamnose observed in the monosaccharide analysis as well as the decreased staining with toluidine blue (Fig.4.4G and H). The decrease in label in the *men4 mum4* double mutant compared to the *mum4* single mutant suggests the *men4* mutation further decreases mucilage production and lends additional support for *MEN4*'s role in RG I and mucilage synthesis or deposition. In order to determine if the *men4* mutant mucilage was affected in other pectins, immunolabelling of developing seeds with an array of anti-pectin antibodies was performed. The JIM5 and LM19 antibodies detect HG epitopes with a low degree of methyl-esterification, and can also bind to un-esterified HG, while JIM7 and LM20 recognize more highly methyl-esterified epitopes of HG. The LM18 antibody also recognizes partly methyl-esterified HG, but can also bind to un-esterified HG (Willats, Limberg et al. 2000; Yves Verhertbruggen, Susan E. Marcus et al. 2009). All of these HG antibodies only stained primary cell walls and did not show significant differences between any of the mutants and wild type (data not shown). Lack of staining of the intact mucilage pocket by several of these antibodies has been observed previously, despite their ability to bind released mucilage, presumably due to reduced epitope availability in the densely-packaged mucilage of intact cells (Young, McFarlane et al. 2008, and T. L. Western personal communication).

Because some of the anti-pectin antibodies failed to stain resin-embedded sectioned material, immunolabelling of whole mature seeds was also performed in order to observe any additional alterations in the mucilage composition of *men4* mutant seeds. When stained with the CCRC-M36 antibody as a control, wild type

seeds showed bright rays of label extending from the MSC and surrounding the seed. Instead of this pattern, the *men4* mutant showed labelling in small 'puffs' that did not uniformly surround the seed (Fig.4.5 compare A to B). Ruthenium red staining after shaking seeds in the antibody buffer confirmed that the antibody buffer promoted mucilage release, and that these 'puffs' correspond to areas of mucilage release similar to what is seen with EDTA pre-treatment (data not shown, Fig.3.2). The *mum4* mutant showed very small amounts of label, while the *men4 mum4* mutant did not show any (Fig.4.5C and D).

In order to determine whether the pectin HG was affected in the *men4* mutant, the HG specific antibodies JIM5, JIM7, LM18, LM19 and LM20 were used to label whole mature seeds. LM19, LM20 and JIM7 did not show any label in the experiment and are not shown. In wild type seeds, JIM5 shows a labelling pattern similar to that of CCRC-M36, consisting of rays extending from the MSC and surrounding the seed, though the "rays" extend less far than for CCRC-M36 (Fig.4.5E). This is also seen with LM18, however, the staining appears confined closer to the seed (Fig.4.5G). The *men4* mutant shows a significant decrease in JIM5 labelling and this label is confined to 'puffs' and what seems to be part of the radial cell walls of the MSC (Fig.4.5F). The labelling pattern of LM18 is likewise altered appearing in random 'puffs' and in sections of cell wall (Fig4.5H). These results suggest that in addition to a decrease in RG I, *men4* mucilage may also be affected in HG.

Initial mapping of the *MEN4* locus

In order to determine the function of *MEN4*, the molecular cause of the observed phenotype must be determined. Mapping using molecular markers identifies the affected gene responsible for a mutant phenotype by measuring genetic linkage between the mutant phenotype and molecular markers whose physical position in the genome are known. Preliminary mapping using 36 *men4 mum4* mutants suggested that the *MEN4* locus is located on the upper arm of chromosome 5 (data not shown). Due to the comparative ease of identifying *men4* single mutants, further mapping was continued using *men4* mutants from the F2 generation of a wild type (L*er* ecotype) X *men4* (Col2 ecotype) cross. 22 established markers across the Arabidopsis genome were analyzed using 14-76 mutants for each marker (Lukowitz, Gillmor et al. 2000). The recombination frequency at markers CTR1 and ciw8 on chromosome 5 were found to be 16.4% and 16.5%, respectively (n=73 and 76 mutant plants, respectively), suggesting strong linkage between the mutation and these two markers (Table 4.2). These recombination frequencies confirmed the preliminary *men4 mum4* mapping and further suggested that the *MEN4* locus may be located on chromosome 5 somewhere between 10 and 42 cM. At other markers across the genome recombination frequencies ranged between 31% and 78% (Table 4.2). Although recombination frequencies in the 30-40% range on these other chromosomes could be supposed to suggest linkage, the low number of plants tested at these loci, the lack of linkage observed with these same markers with the 36 *men4 mum4* mutants in the preliminary mapping (data not shown), and the strong linkage

shown on chromosome 5 with both the *men4* and preliminary *men4 mum4* mapping suggests that linkage to markers nga280, ciw2, ciw4, nga6 and ciw7 was unlikely. In order to further narrow down the location of the *MEN4* locus, two new markers were found located on chromosome 5 between CTR1 and ciw8. The markers nga249 and nga106 are located at 23 cM and 33 cM, respectively. When 70-71 putative *men4* mutants were analyzed for recombination at nga249 and nga106, the recombination frequencies obtained were 8.5% and 7.1%, respectively (Table 4.2, Fig.4.6). These data suggest the *MEN4* locus may be located between 25.9 and 31.5 cM on the upper arm of chromosome 5 (Fig.4.6).

Mutants with reduced mucilage levels show altered germination in water-limiting conditions

In Arabidopsis, changes in germination rates have been correlated with changes in seed coat structure, including mucilage quantity and release (Leon-Kloosterziel, Keijzer et al. 1994; Debeaujon, Leon-Kloosterziel et al. 2000; Penfield, Meissner et al. 2001; Rautengarten, Usadel et al. 2008). A germination time-course revealed a 40% reduction in germination in *bxl1-1* mutants versus wild-type seeds at 3 days after plating, resulting in a brief delay of germination (Chapter 2, Fig. 2.3). Additionally, various levels of germination delays were observed when the *men* lines were subjected to a similar evaluation of germination (Chapter 3, Fig.3.6). Germination delays have also been reported when attempting to germinate Arabidopsis seeds on water–limiting substrates such as mannitol, sorbitol, NaCl and

glycerol (Chen, Hong et al. 2005). Seeds with altered mucilage release, and seeds with a reduction in mucilage production have been shown to experience a further reduction in germination when water is limited (Penfield, Meissner et al. 2001; Rautengarten, Usadel et al. 2008).

In order to directly compare the effects of different levels of mucilage release versus mucilage quantity on germination timing, one set of mutant lines affected in mucilage amount and release were subjected to a germination time-course under water-limiting conditions. Mutant lines that synthesized but failed to release mucilage included the subtilase-like mutants *sbt1.7-1* and *sbt1.7-2*, as well as the β-galactosidase mutant *mum2-1* (Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007; Rautengarten, Usadel et al. 2008). Lines that synthesized a reduced amount of mucilage included a range of mucilage amounts as represented by *men4-1*, *mum4-1* and *men4-1 mum4-1* mutants.

Initially, Col2 wild type seeds were germinated on filter paper and time-lapse photographs taken at 45 minute intervals to accurately time germination events and thus determine significant time points at which to score the water-limiting germination experiments. Upon placement on wetted filter paper, Col2 seeds ruptured the testa on average after 26.4 ± 3.05 hours (SD, n=9), similar to previously reported timing (Chen, Hong et al. 2005). Germination, defined by the protrusion of the radicle through the seed coat, occurred after an average of 31.6 ± 2.6 hours (SD, n=14) (Fig.4.7). Preliminary germination assays using a range of concentrations of several potential water-limiting agents (polyethylene glycol, mannitol, sorbitol and NaCl) were performed with all seven mutants to determine the optimum

concentrations of solutions to affect germination (data not shown). Due to the extreme variability of germination on polyethylene glycol in several trials (data not shown), and the multiple cellular effects of NaCl, mannitol and sorbitol were chosen for further analyses.

When germinating on water, at 70 hours after plating there was little observable difference in germination between most mutant lines and the wild type control (Col2), with all exhibiting 94-100% germination. The exception was *men4,* which exhibited 75 ± 0.8% at this time point (Fig.4.8A). All lines, however, reached 100% percent by 92 hours after plating (Fig.4.8A). When these lines were germinated on plates containing 400mM sorbitol, wild type germination was delayed with 62 ± 6.9% of seeds germinating at 70hrs, and 93 ± 0.9 % by 118hrs after plating (Fig.4.8B). The mucilage release mutants *sbt1.7-1*, *sbt1.7-2* and *mum2* showed a similar pattern to Col2 and nearly all seeds germinated by 118hrs after plating (Fig.4.8B). The reduced mucilage group, however, showed a significant germination lag compared to Col2, and to their own germination on water. At 70hrs after plating *mum4*, *men4* and *men4 mum4* germinated at 35 ± 2.2%, 32 ± 9.3%, and 40 ± 5.5% respectively. This delay persisted at 118hrs after plating with *mum4* germinating at 67.2 ± 1.9%, *men4* at 52.2 ± 17.2%, and *men4 mum4* at 57.8 ± 10.9% (Fig.4.8B). These lines did not reach 100% germination when examined after 9 days (data not shown).

In order to confirm these observations, the same lines were germinated on filter paper containing 450mM mannitol. Col2 germination appeared more delayed at 70hrs compared to germination on sorbitol-containing plates with 49 ± 11.7% of

seeds germinating, and 84 ± 5.9% after 118hrs (Fig.4.8C). The *sbt1.7-1* and *sbt1.7-2* mutants lagged behind Col2 germination at 70hrs, with germination rates of 16 ± 3.6% and 25 ± 4.0% respectively. This delay was overcome by 94 hours, and after 118hrs, *sbt1.7-1* and *sbt1.7-2* had 78 ± 7.5% and 96 ± 4.4% of seeds germinated, respectively. *mum2* mutant seeds appeared to germinate precociously compared to Col2 with 72 ± 2.8% germinating at 70hrs, and 100% at 118hrs. The reduced mucilage group displayed a similar delay in germination as seen on sorbitol-containing filter paper. At 70hrs after plating, *mum4* germinated at 20 ± 4.6%, while *men4* and *men4 mum4* germinated at 15.6 ± 4.7% and 19.6 ± 9.1%, respectively. As in the sorbitol experiment, this lag persisted after 118hrs with *mum4* germinating at 52 ± 2.4%, *men4 mum4* at 57 ± 11.8%. *men4* appeared much more affected than on the sorbitol-containing plates, only germinating at 28 ± 1.9% (Fig.4.8C). Together, these results suggest that the amount of mucilage present may affect the rate of germination; however, wild type germination rates may be achieved without complete mucilage release.

Seeds with reduced mucilage levels show reduced swelling upon hydration

Germination commences with the rapid uptake of water by the seed (Bewley and Black 1994). This uptake includes three phases, starting with a rapid initial uptake of water by the seed. This is followed by a plateau phase and a resumption of water absorption following germination (Schopfer and Plachy 1984; Manz, Muller et al. 2005). In order to determine if a decrease in mucilage production is affecting the

rate of water uptake by the seed, the percent size change of the seed upon hydration was measured in the *men4*, *mum4*, *men4 mum4* mutant seeds used in the water-limiting germination experiments. After 4 hours on moistened filter paper, wild type (Col2) seed size increased 41 ± 1.24%, while *men4* seeds only showed a 26 ± 1.7% increase in seed size. Similarly, the *mum4* mutant seeds also showed less expansion after 4 hours compared to wild type seeds, growing 34 ± 2.7% in size. Accordingly, the *men4 mum4* double mutant, which has less mucilage than either single mutant, exhibited the smallest seed expansion with only 18.4 ± 1.4% (SE, n=45-50) (Fig.4.9). This suggests that mucilage may aid the seed in absorbing water from its surroundings and commence the events leading to germination. Additional biological replications are required to confirm the observed results.

DISCUSSION

The Arabidopsis mucilage secretory cells have been used to identify genes involved in cell wall synthesis and modification, particularly the production, regulation and metabolism of pectic polysaccharides (Zhang, Gonzalez et al. 2003; Western, Young et al. 2004; Dean, Zheng et al. 2007; Macquet, Ralet et al. 2007; Rautengarten, Usadel et al. 2008; Gonzalez, Mendenhall et al. 2009). With respect to mucilage synthesis, only one gene, encoding the rhamnose synthase *MUM4/RHM2,* has been identified to date (Usadel, Kuchinsky et al. 2004; Western, Young et al. 2004). The *men4* mutant was identified using an enhancer/suppressor screen of the *mum4* phenotype and was found to exhibit a single mutant phenotype of reduced

mucilage compared to wild type. Preliminary analysis of the *men4* mucilage suggests the product of the *MEN4* locus may be involved in the synthesis or secretion of mucilage, particularly the RG I component of mucilage. Additionally, the reduction of mucilage in the *men4* mutant may be affecting the seed's ability to germinate in water-limiting conditions by decreasing the seeds ability to absorb water.

MEN4 affects mucilage production

From the *men* mutants identified in the *mum4* enhancer/suppressor screen, *men4* proved particularly interesting for future study due to its single mutant phenotype of reduced mucilage. *men4* seeds sectioned at stages throughout development suggest *men4* MSCs initially develop similarly to wild type, with wider columnella and a reduction in size of the mucilage pocket becoming evident after seed columnella production, when seed coat dessication and compression are initiated (Fig.4.1). This suggests that, similar to what is observed for *mum4* (Western, Young et al. 2004), the defect is in the synthesis or secretion of mucilage, rather than altered developmental timing, such as delayed mucilage production. Immunolabelling using an RG I-specific antibody showed that, while *men4* mutants did not have obviously less RG I staining compared to wild type prior to seed coat compression, *men4 mum4* mutants appeared more affected than either single mutant, supporting a role for *MEN4* in the production of RG I in mucilage (Fig.4.4).

In addition to a significant reduction in rhamnose, the *men4* mutant also showed decreases in xylose and galactose (Fig.4.3 and Table 4.1). These same

decreases were observed in the *mum4* mutant that is specifically affected in UDP-L-rhamnose and RG I synthesis (Fig.4.3 and Table 4.1) (Western, Young et al. 2004). This suggests that the decreases in xylose and galactose may reflect the overall reduction in mucilage content in both mutants, which would in turn result in lower quantities of mucilage monosaccharides. A decreased and altered pattern of partially-esterified HG is observed in *men4* whole seeds using JIM5 and LM18 antibodies (Fig. 4.5). This, like the galactose and xylose levels, could be due to an overall reduction in mucilage produced, such that HG levels are reduced along with RG I, for example, due to co-regulation or crosstalk during their synthesis. Alternatively, it may be an artefact of the altered mucilage extraction in the *men4* mutant in the presence of antibody buffer. A more detailed chemical analysis of the mucilage, particularly the quantification of galacturonic acid, which makes up the HG backbone, may help clarify this result, as would cloning of *MEN4* and determination of its molecular function. Increases in mannose and glucose also are seen for *men4*, *mum4* and *men4 mum4,* likely due to increased primary cell wall extraction in the absence of large amounts of mucilage.

Arabidopsis mucilage is mostly composed of the pectin RG I, therefore genes involved in its transport or manufacture, as well as the regulation of these processes, would be the most likely candidates for genes affected in the *men4* mutant. Phenotypic analysis, genetic complementation analyses, and ongoing mapping results have ruled out *men4* as an allele of previously identified mucilage mutants (Chapter 3; Fig.4.6 and Table 4.2). Molecular mapping has placed the *MEN4* locus somewhere between 26 and 32 cM on chromosome 5. A search of the Arabidopsis genome using

'TAIR gene search' (http://www.arabidopsis.org) between these locations results in 136 loci matches with 177 distinct gene models (Table 4.3). A cursory examination of the resulting genes does not reveal any obvious pectin biosynthetic candidates that would explain the reduced mucilage phenotype. However, the *MEN4* gene might encode a regulatory factor that may, for example, regulate any number of the proposed glycosyltransferases, monosaccharide transporters or nucleotide sugar interconversion enzymes required for the synthesis of pectins (Mohnen 2008). A mutation in any of the many predicted transcription factors identified in this region or an mRNA stability factor such as At5g09610 could therefore result in the reduced manufacture of RG I, or both RG I and HG, producing the mucilage reduction phenotype observed in the *men4* mutant (Table 4.3). Conversely, a mutation in a gene encoding a protein involved in the transport of synthesized pectins, such as the predicted Rab GDP-dissociation factor encoding-At5g09550, may also affect the transport of RG I or RG I and HG to the apoplast and thus produce the decreases in rhamnose observed in the monosaccharide analysis and altered staining with anti-RG I and anti-HG antibodies (Table 4.3).

The effect of reduced mucilage on germination

Previous studies have demonstrated that seeds with a reduced amount of mucilage and impaired mucilage release have a decreased ability to germinate under conditions where water is limited (Penfield, Meissner et al. 2001; Rautengarten, Usadel et al. 2008; Panikashvili, Shi et al. 2009). In Chapter 2, the *bxl1-1* mutant was

observed to have a slight delay in germination compared to wild type seeds. Similarly, *men* enhancers in Chapter 3 showed varying degrees of germination delay compared to wild type. Due to its hydrophilicity, Arabidopsis mucilage has been proposed to attract and retain water surrounding the seed (Penfield, Meissner et al. 2001). This hydration is an essential step in the germination process, which begins with the initial uptake of water and culminates with the protrusion of the radicle through the testa (Schopfer and Plachy 1984; Bewley 1997; Manz, Muller et al. 2005). In a direct comparison between mutant seeds that fail to release mucilage and mutants that produce less mucilage, mutant seeds that produced but did not release their mucilage (*sbt1.7-1, -2, mum2*) were found to behave similarly to wild type seeds (Fig.4.8). Interestingly, the *sbt1.7-1* and *sbt1.7-2* mutants did not appear to be as affected by the limitation of water as previously reported (Rautengarten, Usadel et al. 2008). This may be due to the difference in water-limiting agents used in our experiments (mannitol and sorbitol versus polyethylene glycol), or unaccounted for laboratory-specific germination conditions. Conversely, those mutants with reduced mucilage (*mum4*, *men4*, *men4 mun4*) had significantly decreased germination in the presence of sorbitol and mannitol (Fig.4.8). Together these data suggest that mucilage release has less effect on germination than the amount of mucilage present. Similar results were obtained in the study of the *men mum4* double mutants, where those double mutants that make less mucilage than the *mum4* single mutant were more severely delayed then those mutants that make similar mucilage amounts to *mum4* but fail to release it (Fig.3.6). In addition, *ttg1-1* mutants, which make very little mucilage, had decreased germination under water-limiting conditions compared with

myb61 mutants that have a less significant mucilage reduction, (Penfield, Meissner et al. 2001). The altered germination observed in reduced mucilage mutants may be due to decreased or significantly slowed hydration of the seed. Indeed, a reduction in seed swelling in the *men4*, *mum4* and *men4 mum4* mutants compared to the wild type strongly suggests that mucilage plays a key role in the seeds ability to absorb water (Fig.4.9). By altering this initial uptake, the initiation of critical cellular processes may be delayed, thus slowing the rate of germination.

Figure 4.1 Seed coat development of the *men4* mutant.

Cross sections of developing MSCs stained with toluidine blue. A to D, Wild type. A, 4dpa with central vacuole filling most of the cell. B, at 7dpa purple staining mucilage is accumulating. C, at 10dpa mucilage is staining dark purple and found in the upper tangential regions of the cell, a secondary cell wall is being deposited around the central cytoplasm. D, 13dpa, the secondary cell wall deposition is complete and the columnella is formed, hydration in the aqueous fixative has caused bursting of the MSCs . E to H, *men4* at the same stages. Note the wider, flatter columnella, smaller mucilage pocket, and lack of MSCs bursting visible in H. Arrows indicate cytoplasm in H, or lack thereof in D. Bar=50μm.

Figure 4.1

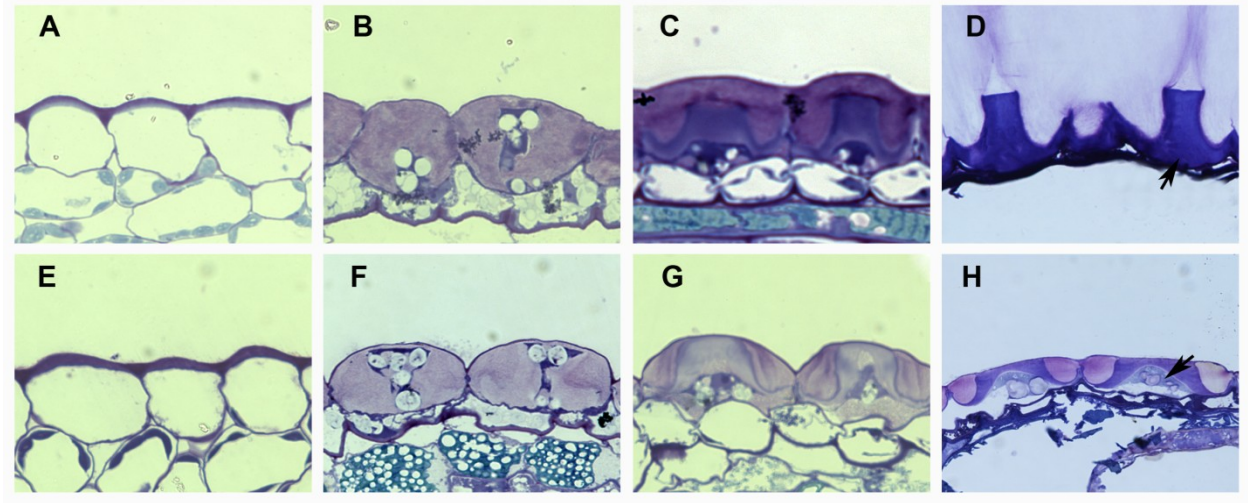


Figure 4.2 Seed size comparison

Seed size comparison for Col-2, *mum4, men4*, and *men4 mum4*. 100 seeds from each line were measured. Seed size is given in pixels. Error bars represent SE.

Figure 4.2

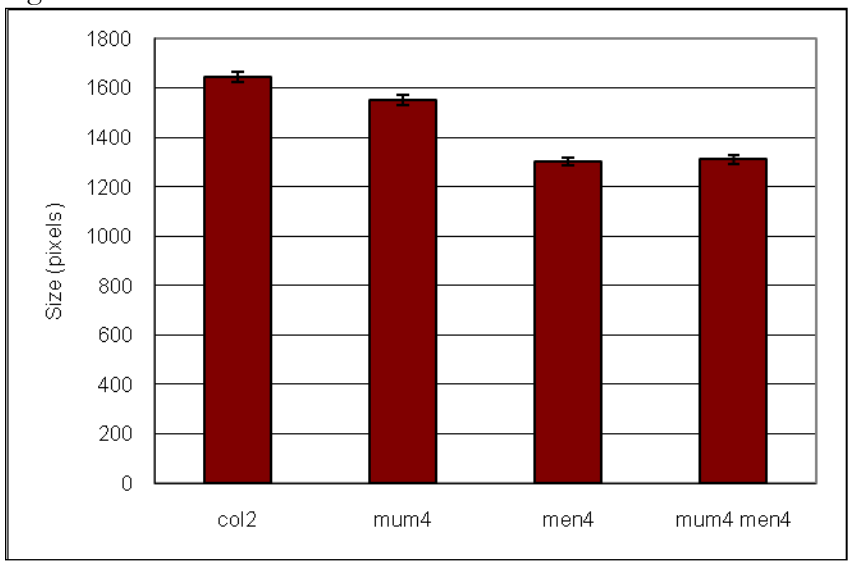

1800
1600
1400
1200
1000
800
600
400
200
0
Size (pixels)
col2
mum4
men4
mum4 men4

Figure 4.3 Monosaccharide quantification of extracted mucilage

Soluble polysaccharides from intact seeds were isolated by shaking in ammonium oxalate (soluble mucilage). Samples were then ethanol precipitated and directly hydrolyzed with trifluoroacetic acid followed by derivatization to alditol acetates. Results are given as average μg sugar per 50 mg seed and SE calculated from three independent samples.

Figure 4.3

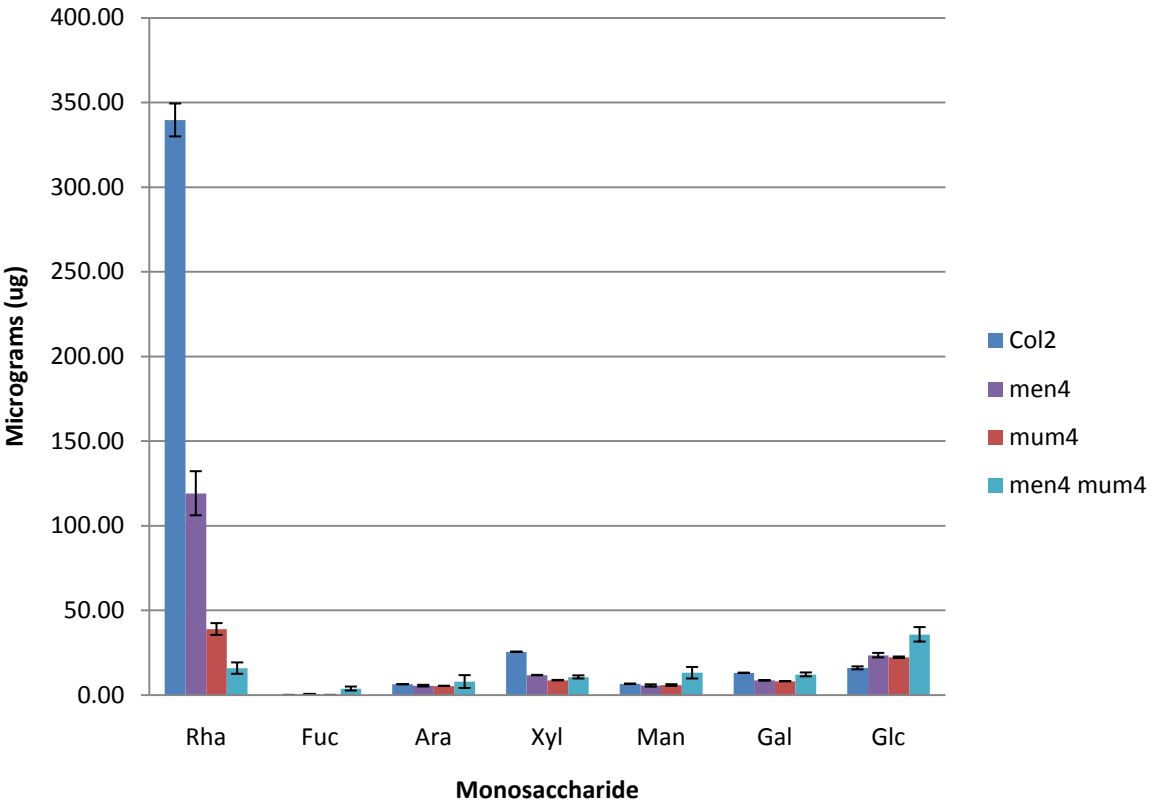

400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Micrograms (ug)
Rha
Fuc
Ara
Xyl
Man
Gal
Glc
Monosaccharide
Col2
men4
mum4
men4 mum4

Table 4.1 Monosaccharide quantification of extracted mucilage

Data from Figure 4.3. Results are given as average μg sugar per 50 mg seed and SE calculated from three independent samples.

Table 4.1

Line	**Rha**	**Fuc**	**Ara**	**Xyl**	**Man**	**Gal**	**Glc**
Col-2	339.6 ± 9.72	0.00	6.33 ± 0	25.49 ± 0.02	6.57 ± 0.17	13.06 ± 0.13	15.99 ± 0.88
men4	119.12 ± 13.0	0.50 ± 0.13	5.48 ± 0.51	11.72 ± 0.11	5.57 ± 0.67	8.62 ± 0.19	23.50 ± 1.32
mum4	38.93 ± 3.48	0.00	5.35 ± 0	8.73 ± 0.15	5.83 ± 0.51	8.11 ± 0.10	22.24 ± 0.45
men4 mum4	15.85 ± 3.37	3.79 ± 1.16	7.90 ± 3.79	10.63 ± 0.92	13.12 ± 3.40	12.13 ± 1.14	35.79 ± 4.28

Figure 4.4 Immunofluorescence of developing seed coats.

A and B, Confocal sections of 7dpa and 10dpa Col-2 seed coats stained with the anti-RG I antibody CCRC-M36. Note the intense staining in the MSCs in A, and the released mucilage in B. C and D, confocal images of *men4* at 7dpa and 10dpa. Note the lack of mucilage release in D. E and F, *mum4* at 7dpa and 10dpa. Note the significantly reduced staining compared to Col-2 and *men4*, columnella appear flatter. G and H, *men4 mum4* at 7dpa and 10dpa. Mucilage staining and pocket size are severely reduced compared to *mum4*. Bar=50μm

Figure 4.4

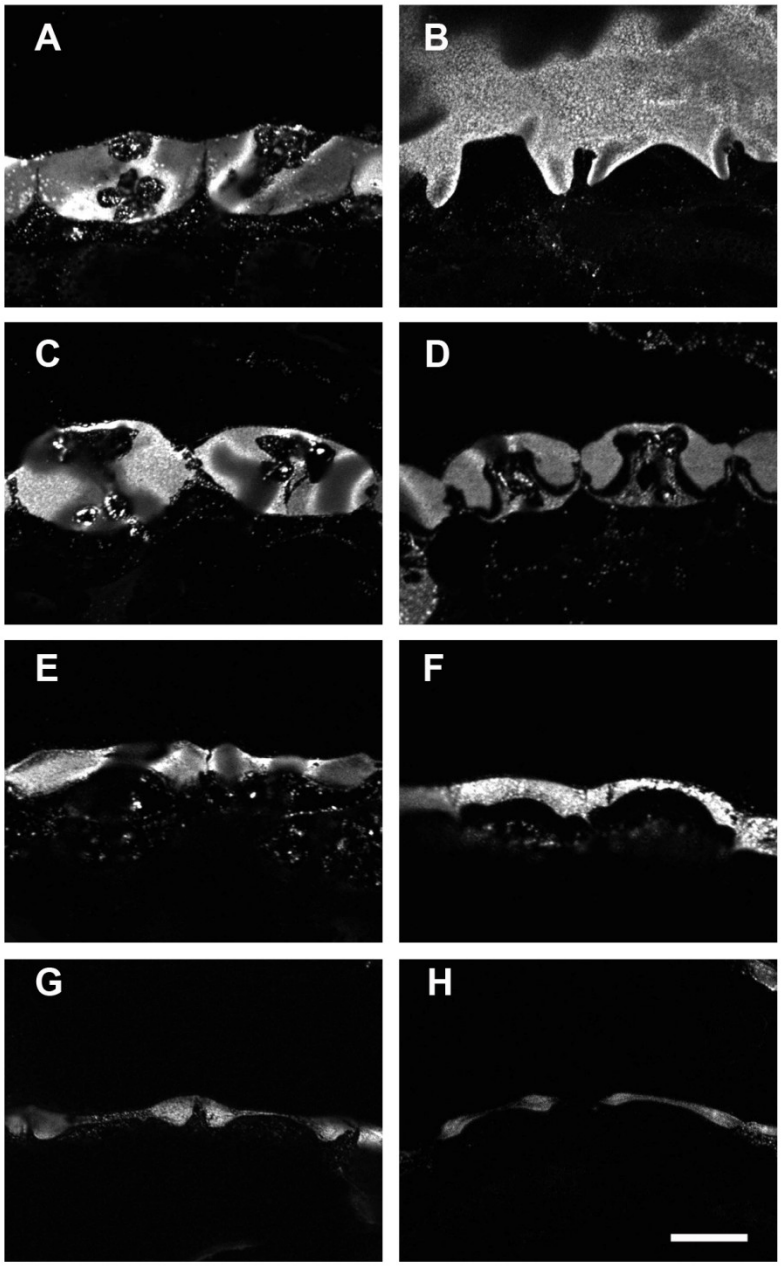


Figure 4.5 Whole seed immunofluorescence

Whole, mature, dry seeds were stained with antibodies (green) specific to RG I and HG with low methyl-esterification. A, Col-2 stained with the anti-RG I antibody CCRC-M36. Note the intense rays of label surrounding the seed. B, *men4* stained with CCRC-M36. Note the decrease in label quantity and distribution. C and D, *mum4* and *men4 mum4* seeds stained with CCRC-M36, note the complete lack of released mucilage. E, Col-2, stained with the anti-HG JIM5. Intense label is close to the seed. F, *men4* with JIM5. Staining appears reduced and limited to sections of the apical cell wall of the MSCs. G, Col-2 with the anti-HG LM18. H, *men4* with LM18, reduction in label quantity and distribution similar to JIM5. All seeds counterstained with propidium iodide to label cell wall (red).

Figure 4.5

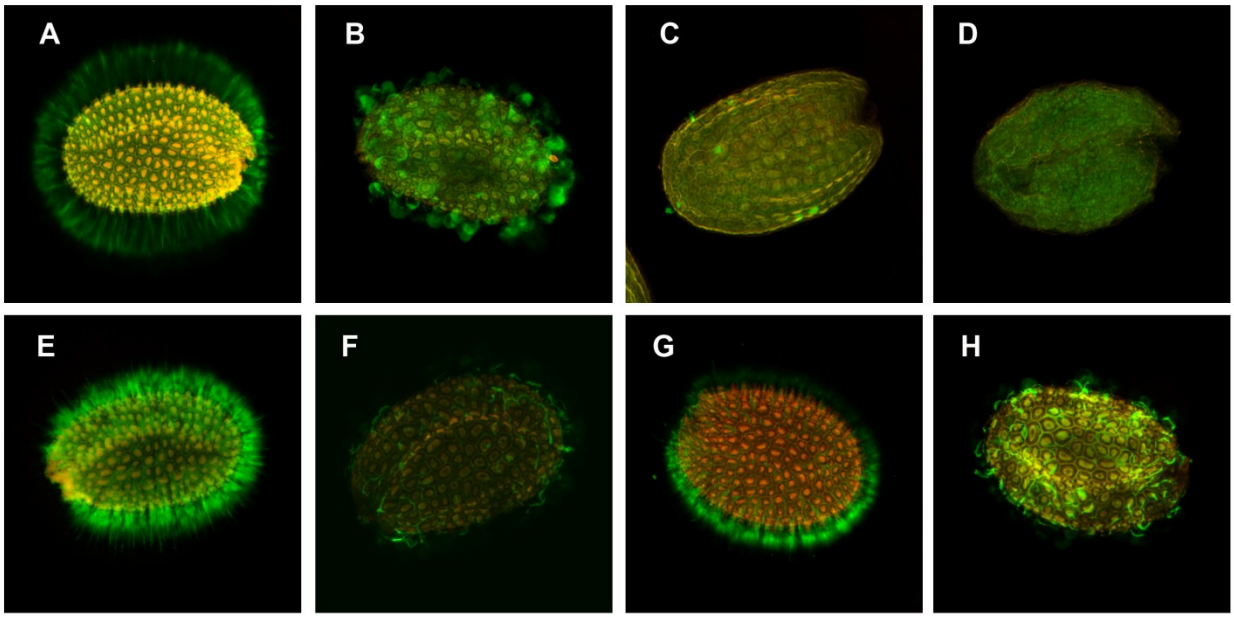


Table 4.2 Molecular markers and recombination frequencies in mapping of the *MEN4* locus

men4 mutants isolated from the F2 of a mapping cross between the *men4* mutant (Col-2 ecotype) and wild type L*er* ecotype plants were assayed for the presence of parental Col-2 (C)or recombinant L*er* (L) alleles using PCR of molecular markers found across the five Arabidopsis chromosomes. The recombination frequency (RF) was calculated as the number of recombinant (L) chromosomes divided by the total number of chromosomes assayed (double the number of mutants assayed since Arabidopsis is diploid). Low recombination frequencies on chromosome 5 indicate linkage (bold).

Table 4.2

	Marker	**location (cM)**	**# mutant individuals**	**L L**	**C L**	**RF (%)**
Chr. 1	F2IM12	10	29	8	19	60.34
	ciw 12	39	63	14	32	47.62
	ciw 1	72	27	4	19	50.00
	nga 280	81	20	2	14	45.00
	nga 111	113	68	14	39	49.26
Chr. 2	ciw 2	11	14	2	7	39.29
	ciw 3	30	33	9	6	36.36
	nga 1126	50	40	13	20	57.50
	nga 168	73	37	8	16	43.24
Chr. 3	nga 162	20	47	11	27	52.13
	ciw 11	43	57	16	23	48.25
	ciw 4	70	38	0	27	35.53
	nga 6	86	34	5	11	30.88
Chr. 4	ciw 5	10	48	17	23	59.38
	ciw 6	47	39	29	3	78.21
	ciw 7	65	44	9	12	34.09
	nga 1107	104	46	17	15	53.26
Chr. 5	**CTR 1**	10	73	4	16	16.44
	nga 249	23	71	3	6	8.45
	nga 106	33	70	4	2	7.14
	ciw 8	42	76	2	21	16.45
	PHYC	72	50	5	14	24.00
	ciw 9	88	48	11	19	42.71
	ciw 10	115	46	15	21	55.43

Figure 4.6 Location of the *MEN4* locus

Graphical representation of chromosome 5 showing presumed *MEN4* location.

Figure 4.6

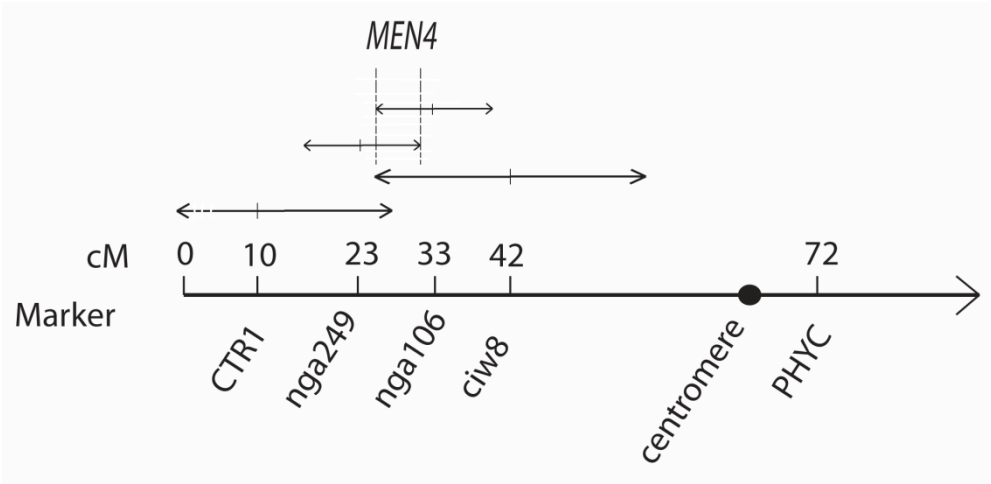
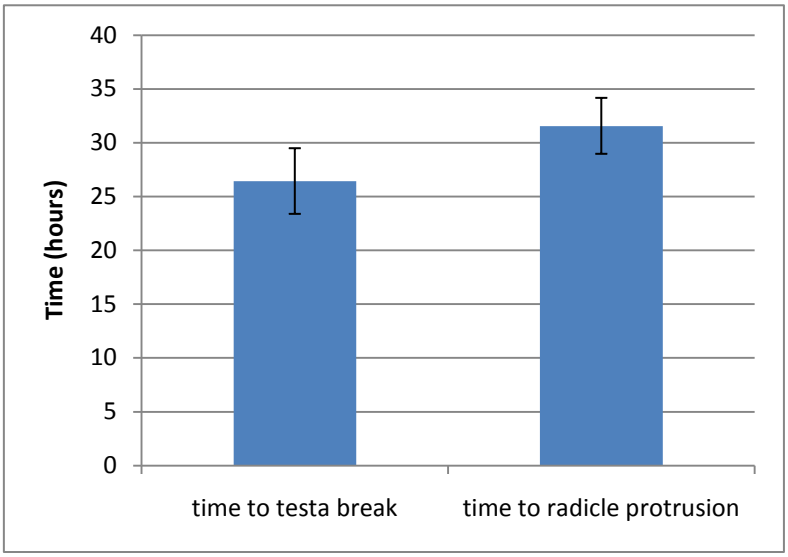

MEN4
cM
0
10
23
33
42
72
Marker
CTR1
nga249
nga106
ciw8
centromere
PHYC

Figure 4.7 Germination timing in wild type seeds

A, Graph showing the time to testa breakage versus time to radicle protrusion. Wild type seeds were placed on wet filter paper and time lapse photographs were taken at 45 minute intervals in order to determine the time of testa rupture and radicle emergence. Testa rupture was scored as the first sign of seed coat breakage. Radicle emergence was scored as any significant protrusion of the embryonic root (radicle) out of the seed coat. B, Seed showing testa breakage (arrowhead). C, Same seed showing radicle protrusion (arrow).

Figure 4.7

A


40
35
30
25
20
15
10
5
0
Time (hours)
time to testa break
time to radicle protrusion

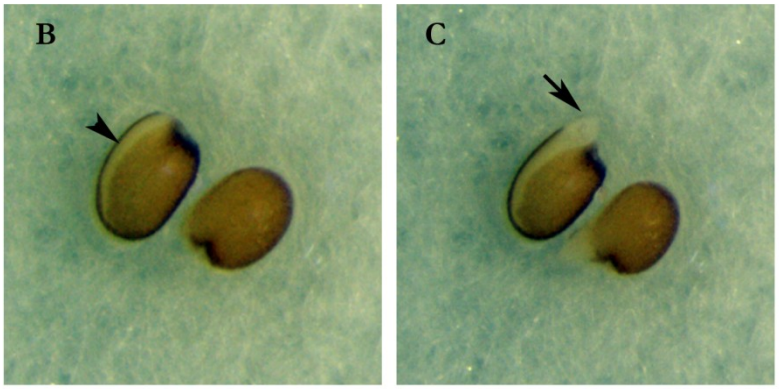
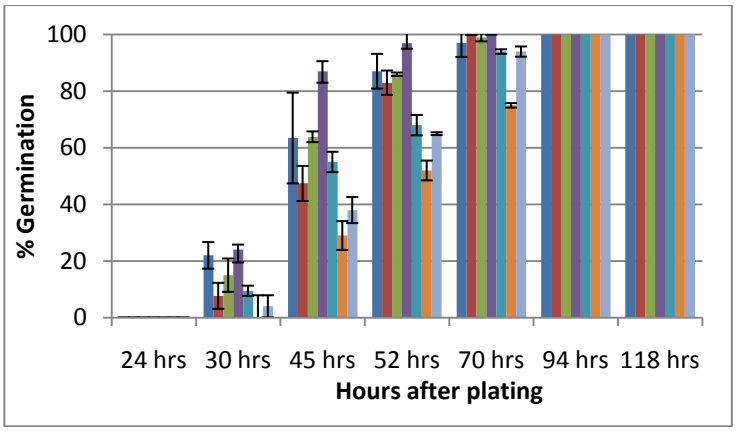

B
C

Figure 4.8 Germination of mucilage mutants with and without water-limiting conditions

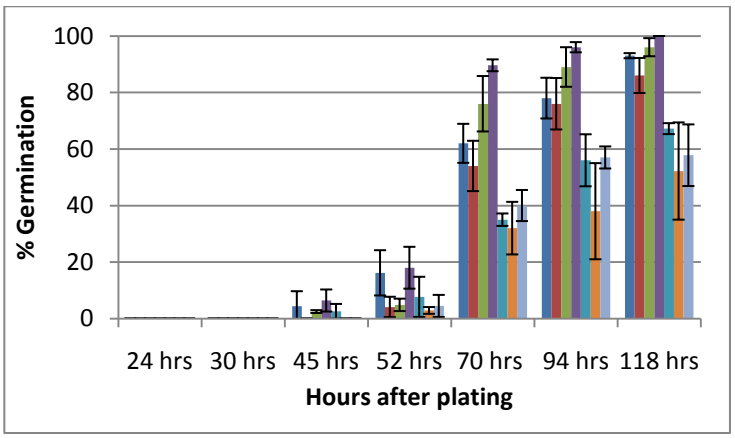
A, Germination of seeds placed on filter paper containing 2ml of water. Dry seeds were cold-treated for 3 days prior to plating. B, germination on 400mM sorbitol. C, germination on 450mM mannitol. Samples were done in triplicate with 40-60 seeds. Germination was checked a final time after 9 days and revealed that all lines except *men4*, *mum4*, and *men4 mum4* reached 100% germination. Error bars represent SE.

Figure 4.8

A



B



C

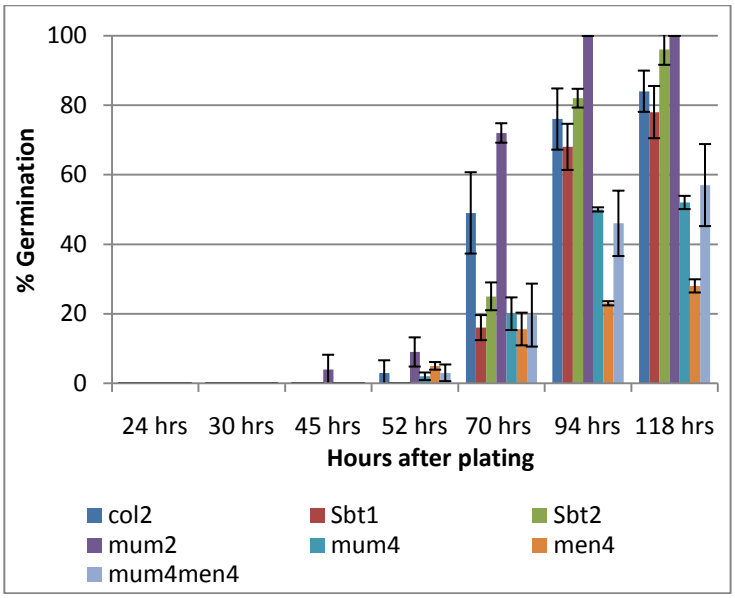


Figure 4.9 Seed swelling upon hydration

Seed swelling for Col-2, *mum4*, *men4*, and *men4 mum4* was determined by placing dry seeds on filter paper containing 2ml water. Photographs were taken of the seeds immediately upon plating and after 4 hours. Percent seed swelling was determined by comparing the area of the seed at the two time points.

Figure 4.9

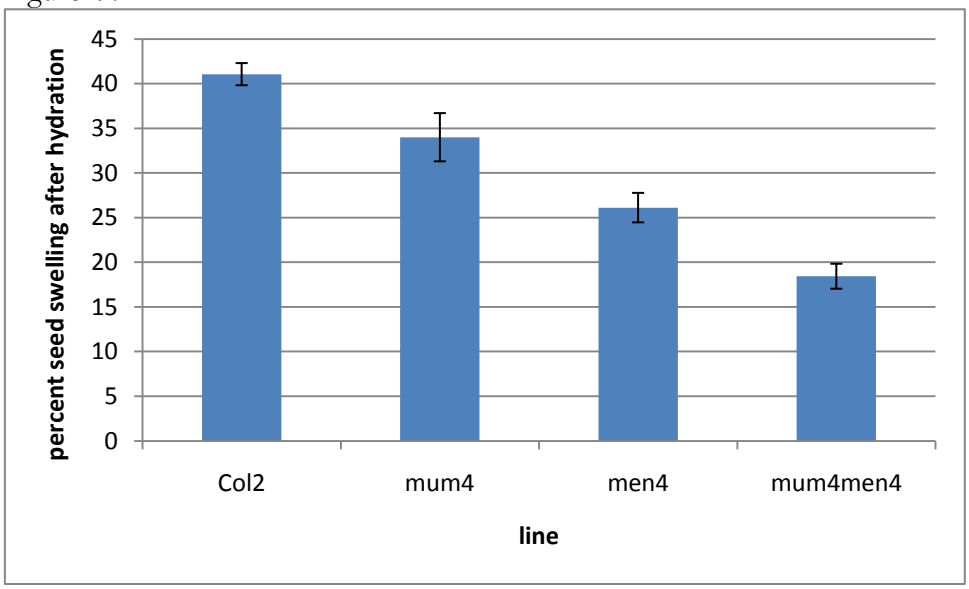

percent seed swelling after hydration
45
40
35
30
25
20
15
10
5
0
Col2
mum4
men4
mum4men4
line

Table 4.3 *MEN4* candidate genes

A listing of the genes located between 26 and 32cM on chromosome 5, the predicted location of the *MEN4* locus

Table 4.3

Locus	Gene Model	Gene Type	Description
AT5G09610	AT5G09610.1	protein_coding	Encodes a member of the Arabidopsis Pumilio (APUM) proteins containing PUF domain. PUF proteins regulate both mRNA stability and translation through sequence-specific binding to the 3' UTR of target mRNA transcripts.
AT5G09620	AT5G09620.1	protein_coding	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein
AT5G09540	AT5G09540.1	protein_coding	DNAJ heat shock N-terminal domain-containing protein
AT5G09710	AT5G09710.1	protein_coding	magnesium transporter CorA-like family protein
AT5G09630	AT5G09630.1	protein_coding	protein binding / zinc ion binding
AT5G09550	AT5G09550.1	protein_coding	RAB GDP-dissociation inhibitor; RAB GDP-dissociation inhibitor activity; regulation of GTPase activity, protein transport;
AT5G09720	AT5G09720.1	protein_coding	metal ion transmembrane transporter; metal ion transmembrane transporter activity;
AT5G09640	AT5G09640.1	protein_coding	encodes a serine carboxypeptidase-like (SCPL) protein.
AT5G09730	AT5G09730.1	protein_coding	encodes a protein similar to a beta-xylosidase located in the extracellular matrix. This is a member of glycosyl hydrolase family 3 and has six other closely related members.
AT5G09560	AT5G09560.1	protein_coding	KH domain-containing protein; RNA binding, nucleic acid binding;
AT5G09650	AT5G09650.1	protein_coding	Encodes a protein with inorganic pyrophosphatase activity.
AT5G09570	AT5G09570.1	protein_coding	FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: chloroplast;
AT5G09740	AT5G09740.1	protein_coding	Encodes an enzyme with histone acetyltransferase activity.
AT5G09660	AT5G09660.1	protein_coding	encodes a microbody NAD-dependent malate dehydrogenase
AT5G09580	AT5G09580.1	protein_coding	unknown protein;
AT5G09750	AT5G09750.1	protein_coding	HECATE 3 (HEC3); FUNCTIONS IN: transcription factor activity, DNA binding;
AT5G09670	AT5G09670.1	protein_coding	loricrin-related; FUNCTIONS IN: molecular_function unknown;
AT5G09590	AT5G09590.1	protein_coding	heat shock protein 70 (Hsc70-5); nuclear
AT5G09760	AT5G09760.1	protein_coding	pectinesterase family protein; FUNCTIONS IN: enzyme inhibitor activity, pectinesterase activity; INVOLVED IN: cell wall modification;
AT5G09680	AT5G09680.1	protein_coding	cytochrome b5 domain-containing protein
AT5G09600	AT5G09600.1	protein_coding	Encodes one of the membrane anchor subunits of the mitochondrial respiratory complex II.
AT5G09770	AT5G09770.1	protein_coding	ribosomal protein L17 family protein
AT5G09690	AT5G09690.1	protein_coding	magnesium transporter CorA-like family protein (MRS2-7)
AT5G09780	AT5G09780.1	protein_coding	transcriptional factor B3 family protein;
AT5G09700	AT5G09700.1	pseudogene	pseudogene of glycosyl hydrolase family 3 protein

AT5G09790	AT5G09790.1	protein_coding	Encodes a SET-domain protein. Contains a PCNA-binding domain. ATXR5 accumulates preferentially during the late G1 or S phase, suggesting that it plays a role in cell-cycle regulation or progression
AT5G09800	AT5G09800.1	protein_coding	U-box domain-containing protein
AT5G08440	AT5G08440.1	protein_coding	unknown protein;
AT5G08450	AT5G08450.1	protein_coding	molecular_function unknown;
AT5G08390	AT5G08390.1	protein_coding	unknown protein
AT5G08400	AT5G08400.1	protein_coding	unknown protein
AT5G08410	AT5G08410.1	protein_coding	ferredoxin/thioredoxin reductase subunit A (variable subunit) 2 (FTRA2);
AT5G08420	AT5G08420.1	protein_coding	RNA binding
AT5G08430	AT5G08430.1	protein_coding	SWIB complex BAF60b domain-containing protein / plus-3 domain-containing protein / GYF domain-containing protein
AT5G08610	AT5G08610.1	protein_coding	DEAD box RNA helicase (RH26)
AT5G08530	AT5G08530.1	protein_coding	51 kDa subunit of complex I (CI51); FUNCTIONS IN: 4 iron, 4 sulfur cluster binding, NAD or NADH binding, FMN binding
AT5G08620	AT5G08620.1	protein_coding	Similar in sequence to DEAD-box RNA helicases. Binds RNA. Involved in drought, salt and cold stress responses.
AT5G08540	AT5G08540.1	protein_coding	unknown protein
AT5G08630	AT5G08630.1	protein_coding	DDT domain-containing protein
AT5G08550	AT5G08550.1	protein_coding	Encodes a transcriptional repressor that is homologous to the C-terminal region of mammalian GC binding factor. It regulates endoreduplication through control of CYC2A expression.
AT5G08560	AT5G08560.1	protein_coding	transducin family protein / WD-40 repeat family protein
AT5G08570	AT5G08570.1	protein_coding	pyruvate kinase, putative
AT5G08580	AT5G08580.1	protein_coding	calcium-binding EF hand family protein
AT5G08590	AT5G08590.1	protein_coding	Encodes a member of SNF1-related protein kinases (SnRK2) whose activity is activated by ionic (salt) and non-ionic (mannitol) osmotic stress.
AT5G08600	AT5G08600.1	protein_coding	U3 ribonucleoprotein (Utp) family protein
AT5G09810	AT5G09810.1	protein_coding	Member of Actin gene family.Mutants are defective in germination and root growth.
AT5G09820	AT5G09820.1	protein_coding	plastid-lipid associated protein PAP / fibrillin family protein
AT5G09900	AT5G09900.1	protein_coding	Encodes one of two isoforms for the 26S proteasome regulatory protein (RN) subunit RPN5.
AT5G09830	AT5G09830.1	protein_coding	BolA-like family protein
AT5G09910	AT5G09910.1	protein_coding	ATP binding / GTP binding / transcription factor binding
AT5G09920	AT5G09920.1	protein_coding	Non-catalytic subunit specific to DNA-dependent RNA polymerase II; the ortholog of budding yeast RPB4)
AT5G09840	AT5G09840.1	protein_coding	unknown protein
AT5G09930	AT5G09930.1	protein_coding	member of GCN subfamily
AT5G09850	AT5G09850.1	protein_coding	transcription elongation factor-related
AT5G09860	AT5G09860.1	protein_coding	nuclear matrix protein-related
AT5G09870	AT5G09870.1	protein_coding	Encodes a cellulose synthase isomer, related to CESA6.

AT5G09880	AT5G09880.1	protein_coding	RNA recognition motif (RRM)-containing protein
AT5G09890	AT5G09890.1	protein_coding	protein kinase, putative
AT5G09300	AT5G09300.1	protein_coding	2-oxoisovalerate dehydrogenase, putative / 3-methyl-2-oxobutanoate dehydrogenase, putative / branched-chain alpha-keto acid dehydrogenase E1 alpha subunit, putative
AT5G09310	AT5G09310.1	protein_coding	unknown protein
AT5G09400	AT5G09400.1	protein_coding	potassium transporter
AT5G09320	AT5G09320.1	protein_coding	VPS9B
AT5G09410	AT5G09410.1	protein_coding	calmodulin-binding protein, similar to another ethylene-upregulated calmodulin-binding protein ER1 GI:11612392 from (Nicotiana tabacum)
AT5G09330	AT5G09330.1	protein_coding	Arabidopsis NAC domain containing protein 82 (anac082); FUNCTIONS IN: transcription factor activity
AT5G09500	AT5G09500.1	protein_coding	40S ribosomal protein S15 (RPS15C)
AT5G09420	AT5G09420.1	protein_coding	ARABIDOPSIS THALIANA TRANSLOCON AT THE OUTER MEMBRANE OF CHLOROPLASTS 64-V (ATTOC64-V)
AT5G09340	AT5G09340.1	protein_coding	ubiquitin, putative
AT5G09510	AT5G09510.1	protein_coding	40S ribosomal protein S15 (RPS15D)
AT5G09430	AT5G09430.1	protein_coding	hydrolase
AT5G09350	AT5G09350.1	protein_coding	Encodes a phosphatidylinositol 4-OH kinase, PI-4Kbeta2. Important for polarized root hair growth as the loss of this gene and its close relative PI-4kbeta1, leads to the formation of abnormal root hairs.
AT5G09440	AT5G09440.1	protein_coding	EXORDIUM LIKE 4 (EXL4)
AT5G09280	AT5G09280.1	protein_coding	pectate lyase family protein
AT5G09360	AT5G09360.1	protein_coding	putative laccase, a member of laccase family of genes (17 members in Arabidopsis).
AT5G09450	AT5G09450.1	protein_coding	pentatricopeptide (PPR) repeat-containing protein
AT5G09290	AT5G09290.1	protein_coding	3'(2'),5'-bisphosphate nucleotidase, putative / inositol polyphosphate 1-phosphatase, putative
AT5G09370	AT5G09370.1	protein_coding	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein; FUNCTIONS IN: lipid binding
AT5G09460	AT5G09460.1	protein_coding	transcription factor/ transcription regulator
AT5G09380	AT5G09380.1	protein_coding	DNA-directed RNA polymerase III RPC4 family protein
AT5G09470	AT5G09470.1	protein_coding	Encodes one of the mitochondrial dicarboxylate carriers (DIC): DIC1 (AT2G22500), DIC2 (AT4G24570), DIC3 (AT5G09470).
AT5G09390	AT5G09390.1	protein_coding	CD2-binding protein-related
AT5G09480	AT5G09480.1	protein_coding	hydroxyproline-rich glycoprotein family protein
AT5G09490	AT5G09490.1	protein_coding	40S ribosomal protein S15 (RPS15B)
AT5G08460	AT5G08460.1	protein_coding	GDSL-motif lipase/hydrolase family protein
AT5G08470	AT5G08470.1	protein_coding	an AAA-ATPase that is the probable Arabidopsis orthologue of one of the AAA-ATPases involved in peroxisome biogenesis in yeasts and mammals.
AT5G08480	AT5G08480.1	protein_coding	VQ motif-containing protein
AT5G08490	AT5G08490.1	protein_coding	pentatricopeptide (PPR) repeat-containing protein
AT5G08500	AT5G08500.1	protein_coding	transmembrane CLPTM1 family protein.

AT5G08510	AT5G08510.1	protein_coding	pentatricopeptide (PPR) repeat-containing protein
AT5G08520	AT5G08520.1	protein_coding	myb family transcription factor
AT5G08640	AT5G08640.1	protein_coding	Encodes a flavonol synthase that catalyzes formation of flavonols from dihydroflavonols.
AT5G08650	AT5G08650.1	protein_coding	GTP-binding protein LepA, putative
AT5G09210	AT5G09210.1	protein_coding	DNA binding / transcription factor
AT5G09220	AT5G09220.1	protein_coding	member of AAAP family
AT5G09230	AT5G09230.1	protein_coding	Encodes SRT2, a member of the SIR2 (sirtuin) family HDAC (histone deacetylase) (SRT1/AT5g55760, SRT2/AT5G09230).
AT5G09240	AT5G09240.1	protein_coding	transcriptional coactivator p15 (PC4) family protein
AT5G09250	AT5G09250.1	protein_coding	putative transcriptional co-activator (KIWI) mRNA, complete
AT5G09260	AT5G09260.1	protein_coding	VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 20.2 (VPS20.2)
AT5G09270	AT5G09270.1	protein_coding	unknown protein
AT5G09520	AT5G09520.1	protein_coding	hydroxyproline-rich glycoprotein family protein
AT5G09530	AT5G09530.1	protein_coding	hydroxyproline-rich glycoprotein family protein
AT5G08415	AT5G08415.1	protein_coding	lipoic acid synthase family protein
AT5G08535	AT5G08535.1	protein_coding	D111/G-patch domain-containing protein
AT5G08565	AT5G08565.1	protein_coding	positive transcription elongation factor/ zinc ion binding
AT5G08740	AT5G08740.1	protein_coding	NAD(P)H dehydrogenase C1 (NDC1)
AT5G08750	AT5G08750.1	protein_coding	zinc finger (C3HC4-type RING finger) family protein
AT5G08690	AT5G08690.1	protein_coding	Encodes the mitochondrial ATP synthase beta-subunit
AT5G08670	AT5G08670.1	protein_coding	Encodes the mitochondrial ATP synthase beta-subunit
AT5G08790	AT5G08790.1	protein_coding	induced by wounding, belongs to a large family of putative transcriptional activators with NAC domain.
AT5G08770	AT5G08770.1	protein_coding	unknown protein
AT5G08660	AT5G08660.1	protein_coding	unknown protein
AT5G08680	AT5G08680.1	protein_coding	Encodes the mitochondrial ATP synthase beta-subunit.
AT5G08710	AT5G08710.1	protein_coding	regulator of chromosome condensation (RCC1) family protein / UVB-resistance protein-related
AT5G08720	AT5G08720.1	protein_coding	unknown protein
AT5G08730	AT5G08730.1	protein_coding	IBR domain-containing protein
AT5G08780	AT5G08780.1	protein_coding	histone H1/H5 family protein
AT5G08535	AT5G08535.2	protein_coding	D111/G-patch domain-containing protein
AT5G08480	AT5G08480.2	protein_coding	VQ motif-containing protein
AT5G08450	AT5G08450.2	protein_coding	unknown protein
AT5G09655	AT5G09655.1	pre_trna	pre-tRNA; tRNA-Val (anticodon: AAC)
AT5G09585	AT5G09585.1	small_nuclear_rna	U2.5; snRNA; gi|17664|emb|X06476.1|ATU25 Arabidopsis thaliana U2 RNA gene (U2.5)
AT5G09755	AT5G09755.1	pre_trna	pre-tRNA; tRNA-Glu (anticodon: CTC)
AT5G09690	AT5G09690.2	protein_coding	magnesium transporter CorA-like family protein (MRS2-7)
AT5G09670	AT5G09670.2	protein_coding	loricrin-related
AT5G09270	AT5G09270.2	protein_coding	unknown protein

AT5G09240	AT5G09240.2	protein_coding	transcriptional coactivator p15 (PC4) family protein
AT5G09230	AT5G09230.4	protein_coding	Encodes SRT2, a member of the SIR2 (sirtuin) family HDAC (histone deacetylase) (SRT1/AT5g55760, SRT2/AT5G09230).
AT5G09230	AT5G09230.2	protein_coding	Encodes SRT2, a member of the SIR2 (sirtuin) family HDAC (histone deacetylase) (SRT1/AT5g55760, SRT2/AT5G09230).
AT5G09230	AT5G09230.3	protein_coding	Encodes SRT2, a member of the SIR2 (sirtuin) family HDAC (histone deacetylase) (SRT1/AT5g55760, SRT2/AT5G09230).
AT5G09370	AT5G09370.2	protein_coding	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein;
AT5G09345	AT5G09345.1	pre_trna	pre-tRNA; tRNA-Leu (anticodon: CAA)
AT5G09690	AT5G09690.3	protein_coding	magnesium transporter CorA-like family protein (MRS2-7)
AT5G09900	AT5G09900.2	protein_coding	Encodes one of two isoforms for the 26S proteasome regulatory protein (RN) subunit RPN5. For many functions it acts redundantly with the paralogous gene RPN5b but also appears to exert independent effects.
AT5G09240	AT5G09240.3	protein_coding	transcriptional coactivator p15 (PC4) family protein
AT5G09230	AT5G09230.5	protein_coding	Encodes SRT2, a member of the SIR2 (sirtuin) family HDAC (histone deacetylase) (SRT1/AT5g55760, SRT2/AT5G09230).
AT5G09230	AT5G09230.6	protein_coding	Encodes SRT2, a member of the SIR2 (sirtuin) family HDAC (histone deacetylase) (SRT1/AT5g55760, SRT2/AT5G09230).
AT5G09225	AT5G09225.1	protein_coding	unknown protein
AT5G09300	AT5G09300.2	protein_coding	2-oxoisovalerate dehydrogenase, putative / 3-methyl-2-oxobutanoate dehydrogenase, putative / branched-chain alpha-keto acid dehydrogenase E1 alpha subunit, putative
AT5G09660	AT5G09660.2	protein_coding	encodes a microbody NAD-dependent malate dehydrogenase encodes an peroxisomal NAD-malate dehydrogenase that is involved in fatty acid beta-oxidation through providing NAD to the process of converting fatty acyl CoA to acetyl CoA.
AT5G09680	AT5G09680.2	protein_coding	cytochrome b5 domain-containing protein
AT5G09890	AT5G09890.2	protein_coding	protein kinase, putative
AT5G09820	AT5G09820.2	protein_coding	plastid-lipid associated protein PAP / fibrillin family protein
AT5G09390	AT5G09390.2	protein_coding	CD2-binding protein-related
AT5G08450	AT5G08450.3	protein_coding	unknown protein
AT5G08400	AT5G08400.2	protein_coding	unknown protein
AT5G08505	AT5G08505.1	protein_coding	Encodes a defensin-like (DEFL) family protein.
AT5G08717	AT5G08717.1	miRNA	Encodes a microRNA that targets several HD-ZIPIII family members including PHV, PHB, REV, ATHB-8, and ATHB-15
AT5G08712	AT5G08712.1	miRNA	Encodes a microRNA that targets several HD-ZIPIII family members including PHV, PHB, REV, ATHB-8, and ATHB-15.
AT5G08560	AT5G08560.2	protein_coding	transducin family protein / WD-40 repeat family protein
AT5G08750	AT5G08750.2	protein_coding	zinc finger (C3HC4-type RING finger) family protein
AT5G08750	AT5G08750.3	protein_coding	zinc finger (C3HC4-type RING finger) family protein
AT5G08760	AT5G08760.1	protein_coding	unknown protein
AT5G09230	AT5G09230.7	protein_coding	Encodes SRT2, a member of the SIR2 (sirtuin) family HDAC (histone deacetylase) (SRT1/AT5g55760, SRT2/AT5G09230).

AT5G09330	AT5G09330.2	protein_coding	Arabidopsis NAC domain containing protein 82 (anac082)
AT5G09330	AT5G09330.3	protein_coding	Arabidopsis NAC domain containing protein 82 (anac082)
AT5G09445	AT5G09445.1	protein_coding	unknown protein
AT5G09510	AT5G09510.2	protein_coding	40S ribosomal protein S15 (RPS15D)
AT5G09600	AT5G09600.2	protein_coding	Encodes one of the membrane anchor subunits of the mitochondrial respiratory complex II.
AT5G09660	AT5G09660.3	protein_coding	encodes a microbody NAD-dependent malate dehydrogenase encodes an peroxisomal NAD-malate dehydrogenase that is involved in fatty acid beta-oxidation through providing NAD to the process of converting fatty acyl CoA to acetyl CoA.
AT5G09690	AT5G09690.4	protein_coding	magnesium transporter CorA-like family protein (MRS2-7)
AT5G09740	AT5G09740.2	protein_coding	Encodes an enzyme with histone acetyltransferase activity.
AT5G09790	AT5G09790.2	protein_coding	Encodes a SET-domain protein. Contains a PCNA-binding domain.
AT5G09805	AT5G09805.1	protein_coding	Similar to Inflorescence deficient in abscission (IDA). Involved in floral organ abscission.
AT5G08695	AT5G08695.1	protein_coding	nucleic acid binding / nucleotide binding
AT5G09250	AT5G09250.2	protein_coding	putative transcriptional co-activator (KIWI) mRNA, complete
AT5G09410	AT5G09410.2	protein_coding	calmodulin-binding protein, similar to another ethylene-upregulated calmodulin-binding protein ER1 GI:11612392 from (Nicotiana tabacum)
AT5G09443	AT5G09443.1	other_rna	Potential natural antisense gene, locus overlaps with AT5G09445
AT5G09461	AT5G09461.1	protein_coding	Upstream open reading frames (uORFs) are small open reading frames found in the 5' UTR of a mature mRNA, and can potentially mediate translational regulation of the largest, or major, ORF (mORF)
AT5G09462	AT5G09462.1	protein_coding	Upstream open reading frames (uORFs) are small open reading frames found in the 5' UTR of a mature mRNA, and can potentially mediate translational regulation of the largest, or major, ORF (mORF)
AT5G09463	AT5G09463.1	protein_coding	Upstream open reading frames (uORFs) are small open reading frames found in the 5' UTR of a mature mRNA, and can potentially mediate translational regulation of the largest, or major, ORF (mORF)
AT5G09513	AT5G09513.1	pseudogene	pseudogene of hAT dimerisation domain-containing protein
AT5G09660	AT5G09660.4	protein_coding	encodes a microbody NAD-dependent malate dehydrogenase encodes an peroxisomal NAD-malate dehydrogenase that is involved in fatty acid beta-oxidation through providing NAD to the process of converting fatty acyl CoA to acetyl CoA.
AT5G09672	AT5G09672.1	protein_coding	Upstream open reading frames (uORFs) are small open reading frames found in the 5' UTR of a mature mRNA, and can potentially mediate translational regulation of the largest, or major, ORF (mORF)
AT5G09711	AT5G09711.1	protein_coding	unknown protein; Has 0 Blast hits to 0 proteins in 0 species (source: NCBI BLink).
AT5G09795	AT5G09795.1	pseudogene	pseudogene of F-box family protein
AT5G09900	AT5G09900.3	protein_coding	Encodes one of two isoforms for the 26S proteasome regulatory protein (RN) subunit RPN5.
AT5G09330	AT5G09330.4	protein_coding	Arabidopsis NAC domain containing protein 82 (anac082)

CONCLUSIONS AND FUTURE WORK

The plant cell wall is a complex structure that acts as the extracellular matrix mediating plant interaction with both the biotic and abiotic environments. The cell wall is composed of cellulose microfibrils cross linked by various hemicelluloses, this network is embedded in a more malleable matrix of acidic polysaccharides known as pectins. The cell wall components are under constant dynamic modifications in order to accommodate cell growth, elongation, signalling and division. Understanding this complex harmonization of elements is necessary in order to understand the plant cell wall and its immense ecological and economic applications. This research describes the use of the mucilage secretory cells (MSCs) of the Arabidopsis seed coat to gain insight of both the synthesis and secretion of pectin as well as aspects of the mechanical alteration of the primary cell wall.

Chapter 2 describes the characterization of the *patchy* mutant and its affected gene (*AtBXL1*). Based on the results of my study, it can be concluded that the action of this bi-functional β-xylosidase/α-arabinofuranosidase is required for the modification of RG I in either the mucilage, cell wall, or both in order for the hydrated mucilage to be released. In particular, the α-arabinofuranosidase activity appears necessary in order to hydrolyze 1, 5-linked arabinan chains from RGI and promote mucilage release upon seed imbibition. The discovery and characterization of *AtBXL1* has provided a glimpse into the active and complex cell wall and pectin modifications occurring during MSC development, and is one of the first examples that illustrates the effects of pectin modification on the mechanics of the plant cell wall.

Chapter 3 describes the use of genetic enhancer screen of the previously known *mum4* mutant to identify novel genes involved in mucilage production. The screen identified six mutants named *mum enhancer (men) 1-6,* which may represent novel genes involved in MSC differentiation. Characterization of these *men mum4* double mutants revealed two distinct groups. One group of mutants produced similar amounts of mucilage to *mum4* but failed to release it (*men2, 6*). These mutants may represent mutations in cell wall modifying genes, similar to *AtBXL1*. A second group produced a further reduced amount of mucilage compared to *mum4* (*men1, 3, 4, 5*). These mutants may represent mutations in biosynthetic genes such as *MUM4/RHM2,* or genes involved in the transport and/or deposition of mucilage. Positional cloning of the *MEN* loci is necessary to identify the genes responsible for the various observed phenotypes. Their identification, along with further genetic and phenotypic analyses of the mutants, promise to yield new knowledge about the factors involved in the synthesis, secretion and modification of cell wall components, as well as mucilage production. Further study may identify the *MEN* genes as modifying enzymes that act in concert with enzymes such as AtBXL1 modifying the mucilage and/or the cell wall to allow for mucilage release, or synthetic enzymes part of a cascade involved in synthesis of pectins.

Of the *men* mutants, *men4* displayed a single mutant phenotype of reduced mucilage compared to wild type. Chapter 4 presents further characterization and mapping of the *men4* mutant. Based on the results of my study, the *MEN4* gene has a role in the synthesis or secretion of the pectin RG I, or RG I and HG. Further chemical analysis and cell wall analyses will be required to provide more insight into

the chemical composition of *men4* mucilage and thus aid in determining the role of *MEN4*. Direct examination of the level of methyl esterification as well as the water absorption properties of the *men4* mucilage may also help determine the effect of the mutation and thus aid in the identification of the *MEN4* gene. Molecular mapping places the *MEN4* locus on the upper arm on chromosome 5, away from known mucilage production or pectin-related genes. In order for this location to be narrowed down, a larger mapping population of approximately 2000 plants will be required. These plants are currently being genotyped at the molecular markers nga249 and nga106, following which further markers will be used to determine fine-scale genetic linkage. Ideally this will narrow the location of the mutation to a fewer number of candidate loci, allowing the use of available T-DNA insertion mutants, sequencing and molecular complementation to determine *MEN4*'s identity. Once the *MEN4* gene is identified, a number of analyses will be required to ascertain its function. If *MEN4* encodes a regulator of synthesis or secretion its downstream targets will need to be identified, and their direct effects examined. Conversely, *MEN4* may encode a protein involved in pectin secretion. In this scenario the role of the protein, from pectin packaging at the Golgi membrane to exocytosis at the plasma membrane and delivery into the apoplast, could be elucidated through a combination of cell biological analyses to determine changes in the endomembrane system (using transmission electron microscopy) or dynamics (through the use of fluorescent markers in live cell experiments). The use of a fluorescently-tagged MEN4 fusion protein and/or an antibody to MEN4 additionally could be used to determine the subcellular localization of MEN4.

Finally, Chapter 4 also examined the role of mucilage during seed germination using a series of mucilage mutants affected in either mucilage production or release upon seed hydration. Though the hydrophilicity of the mucilage suggests a role in seed hydration and germination, its specific function has remained somewhat enigmatic. Results from germination tests under water-limiting conditions suggest that the production of mucilage may be more important than its release in the promotion of germination. Though further experimentation is currently being performed in order to confirm these results, a role for mucilage in initial water absorption is an exciting prospect. Additional seed swelling tests using mutants with altered mucilage composition could be done in order to determine if the monosaccharide composition of mucilage has an effect on the mucilage's rate of water absorption. The ability of the mucilage to absorb ionized versus de-ionized water could also provide insight into the physical properties of mucilage. The direct influence of water absorption on transcriptional resumption upon hydration could also be determined using mutants such as *men4* that are altered in seed swelling. These studies may indicate a role for mucilage as yet another factor influencing the highly complex process of germination.

Together these studies provide new information into the development of the Arabidopsis MSCs, as well as pectin synthesis and cell wall modification in general. Additionally, the effect the genes described on pectinaceous mucilage was also shown to have an effect on germination and with that may suggest a possible role of mucilage during germination.

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