Molecular Genetic Analysis of Seed Coat Mucilage Mutants of *Arabidopsis thaliana*

by

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ABSTRACT

During differentiation, the *Arabidopsis* seed coat epidermal cells produce copious amounts of mucilage that is extruded from the seed coat upon imbibition. Mucilage is composed primarily of pectin, a polysaccharide that is a main component of the cell wall. For this reason, the *Arabidopsis* seed coat is a good system for studying the biosynthesis, secretion and modification of pectin. Mutants with mucilage defects can be used to identify genes involved in the production of pectin. *Mucilage-Modified* mutants, including *mum1*, *mum2* and *mum4*, were identified using screens of EMS mutagenized plants.

Both *mum1* and *mum2* lack the ability to release the mucilage when mature seeds are imbibed. *MUM2* encodes a β-galactosidase that modifies the mucilage structure in the apoplast. I have cloned the *MUM1* gene and shown it to encode a putative transcription factor LEUNIG_HOMOLOG (LUH). Cellular localization and transcriptional assay results suggest that LUH/MUM1 is a nuclear-localized, transcriptional activator. *LUH/MUM1* is expressed in all the tissues examined including the seed coat. qRT PCR data suggest that *LUH/MUM1* is expressed throughout seed coat development, reaching peak expression late in differentiation. *MUM2* expression in the *luh/mum1* mutant was reduced dramatically, relative to that of wild type. Over-expression of *MUM2* could partially rescue the *mum1* phenotype. These data suggest that LUH/MUM1 is a positive regulator of *MUM2*. qRT PCR data revealed a similar expression level of *LUH/MUM1* in wild type compared to plants homozygous for mutations in several genes encoding regulators of seed coat mucilage, namely *APETALA2*, TRANSPARENT TESTA

GLABRA1 (*TTG1*), *TTG2* and *GLABRA2*. Thus the *LUH/MUM1-MUM2* regulatory pathway appears to be independent of other transcription factors known to regulate aspects of seed coat mucilage biology.

Mutations in the *MUM4* gene result in seeds that release little mucilage. A *mum4* mutant was mutagenized and resulting M_2 progeny screened for modifier mutants. Ten enhancers (*mum4* enhancer (*men*)) and ten suppressors (*mum4* suppressors (*msu*)) mutants were isolated and partially characterized genetically and phenotypically. Further studies are needed to characterize these mutants.

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LIST OF ABBREVIATIONS

4-MU	4-methylumbelliferone
ABA1	*ABSCISIC ACID1*
ABRC	Arabidopsis Biological Resource Center
AG	*AGAMOUS*
AP	*APETALA*
ARAD1	*ARABINAN DEFICIENT1*
ATS	*ABERRANT TESTA SHAPE*
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAPS	Cleaved Amplified Polymorphic Sequence
CAT	Chloramphenicol acetyltrasferase
CCRC	Complex Carbohydrate Research Center (Athens, GA)
cDNA	Complementary DNA
CIA	Chloroform:Isoamyl alcohol=24:1
Col	Columbia ecotype
DMSO	dimethyl sulfoxide
DNA	Deoxyribounucleic acid
DPA	Day Post Anthesis
EDTA	ethylene diamine tetraacetic acid, dissodium salt dihydrate
EGL3	*ENHANCER OF GLABRA3*
EIMS	electron impact mass spectrometry
EMS	ethylmethane sulfonate
FIL	*FILAMENTOUS FLOWER*
GA	gibberellic acid
GA3OX4	*GIBBERELLINS 3-OXIDASE4*
GalA	alpha-(1,4)-linked D-galactopyranosyluronic acid
GATL	GAUT-like
GAUT	*GALACTURONOSYLTRANSFERASE*
GD	Gal4 binding domain
GFP	Green fluorescent protein
GL	*GALABRA*

GLC	gas-liquid chromatography
HD-zip	homeodomain-leucine zipper
HG	homogalacturonan
KAN4	*KANADI4*
Ler	Landsberg *erecta* ecotype
LUG	*LEUNIG*
LUH	*LEUNIG HOMOLOG*
MEN	*MUM4 ENHANCER*
MMg	0.4 mM mannitol, 15 mM MgCl2, and 4 mM Mes, pH 5.7
MOR	*MICROTUBLE ORGANIZING*
MS	mass spectrometer
MS medium	Murashige and Skoog medium
MSU	*MUM4 SUPPRESSOR*
MT	methyltransferase
MUG	4-methylumbelliferyl-β-D-glucuronide
MUM	*MUCILAGE-MODIFIED*
NCBI	National Center for Biotechnology Information
NDP-sugars	Nucleoside diphosphate sugars
OSU1	*OVERSENSITIVE TO SUGAR1*
PA	proanthocyanidin
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGM1	*PHOSPHOGLUCOMUTASE1*
PME	pectin methylesterase
Q	Glutamine
qRT-PCR	quantitative reverse transcriptase PCR
QUA	*QUASIMODO*
REB	25 mM Tris-HCl pH 8.0, 25 mM EDTA, 75 mM NaCl, 1% SDS
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
RGXT	*RHAMNOGALACTURONAN XYLOSYLTRANSFERASE*
RHM	*RHAMNOSE BIOSYNTHESIS*
RSW3	*RADIAL SWELLING3*

RT-PCR	Reverse Transcriptional PCR
SBT1.7	*SUBTILISIN-LIKE SERINE PROTEASE1.7; SUBTILASE1.7*
SEM	scanning electron microscopy
SEU	*SEUSS*
SSDP	single-stranded DNA-binding protein
SSLP	simple sequence length polymorphism
TAIR	The Arabidopsis Information Resource
T-DNA	transfer DNA of *Agrobacterium tumefaciens*
TEM	Transmission electron microscopy
TFA	trifluoroacetic acid
TGN	trans-Golgi network
TSD2	*TUMOROUS SHOOT DEVELOPMENT2*
TT	*TRANSPARENT TESTA*
TTG	*TRANSPARENT TESTA GLABRA*
UAS_{Gal4}	upstream activation sequence of Gal4 promoter
UDP	uridine diphosphate
W5	154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes, pH 5.7
WD40	Tryptophan -Aspartic acid dipeptide
WI	0.5 M mannitol, 20 mM KCl, and 4 mM Mes, pH 5.7
YAB	*YABBY*

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1 General Introduction

1.1 Pectin in plant cell walls

The wall is an important structure of plant cells. It determines the cell shape and size, which in turn affect plant morphology. It also has many biological functions including cell communication, counterbalance for osmotic pressure, and protection against pathogen invasion. In addition, the cell wall represents a large pool of terrestrial biomass and renewable energy since most carbon from photosynthesis is incorporated into cell wall polymers (Reiter, 2002). Cell wall polymers are used for biofuels (Pauly and Keegstra, 2010).

The conventional model of the primary cell wall states that walls consist of three polysaccharide components, cellulose, hemicellulose and pectin, as well as structural and enzymatic proteins. The cell wall can be compared to reinforced concrete, in which cellulose and hemicellulose form the steel rod network embedded in a pectinaceous concrete (Emons and Mulder, 2000). The cellulose-hemicellulose network is considered as the major tension-bearing structure (Jarvis and McCann, 2000) while pectin, also found in the middle lamella, is responsible for porosity control, cell adhesion and defense signalling (Carpita and Gibeaut, 1993; Mohnen, 2008).

1.1.1 Pectin structure

There are three common domains in pectin: homogalacturonan (HG), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). HG is a linear unbranched array of 1,4-linked α-D-galactosyluronic acid (GalA) typically in stretches of approximately 100 GalA residues (Figure 1-1; Zhan et al., 1998; Yapo et al., 2007). A high proportion of GalA residues of HG are typically methyl esterified at the C-6 carboxyl group (O'Neill et al., 2001). The backbone of RG I is a linear chain of 1, 2- α - L-rhamnose-1, 4- α-D-galacturonic acid units. Unlike HG, RG I is branched. Polymeric 1,4- β –linked D-galactosyl and 1,5-α-linked L-arabinosyl residues form many of the side chains (Mohnen, 1999). Despite the name, RG II is structurally different from RG I. In fact, RG II shares the same backbone with HG but it has branches. The GalA residues are substituted with two structurally different oligoglycosyl side chains (side chains A and B Figure 1-1) and two structurally different disaccharide side chains (side chains C and D; Figure 1-1; Vidal et al., 2000; Glushka et al., 2003). RG II seems to be much less variable than RG I in structural diversity or modulation of its fine structure (Willats et al., 2001b)

All three domains are believed to be linked covalently to form the matrix (Ridley et al., 2001; Willats et al., 2001c). Ishii and Matsunaga (2001) treated saponified pectin with *endo*- and *exo*-polygalacturonase. The dialysis of the mixture provided evidence that RG II is covalently linked to HG. Mass spectrometry analysis of the segments of HG and RG I with controlled acid hydrolysis indicates covalent linkage between the two. It is not

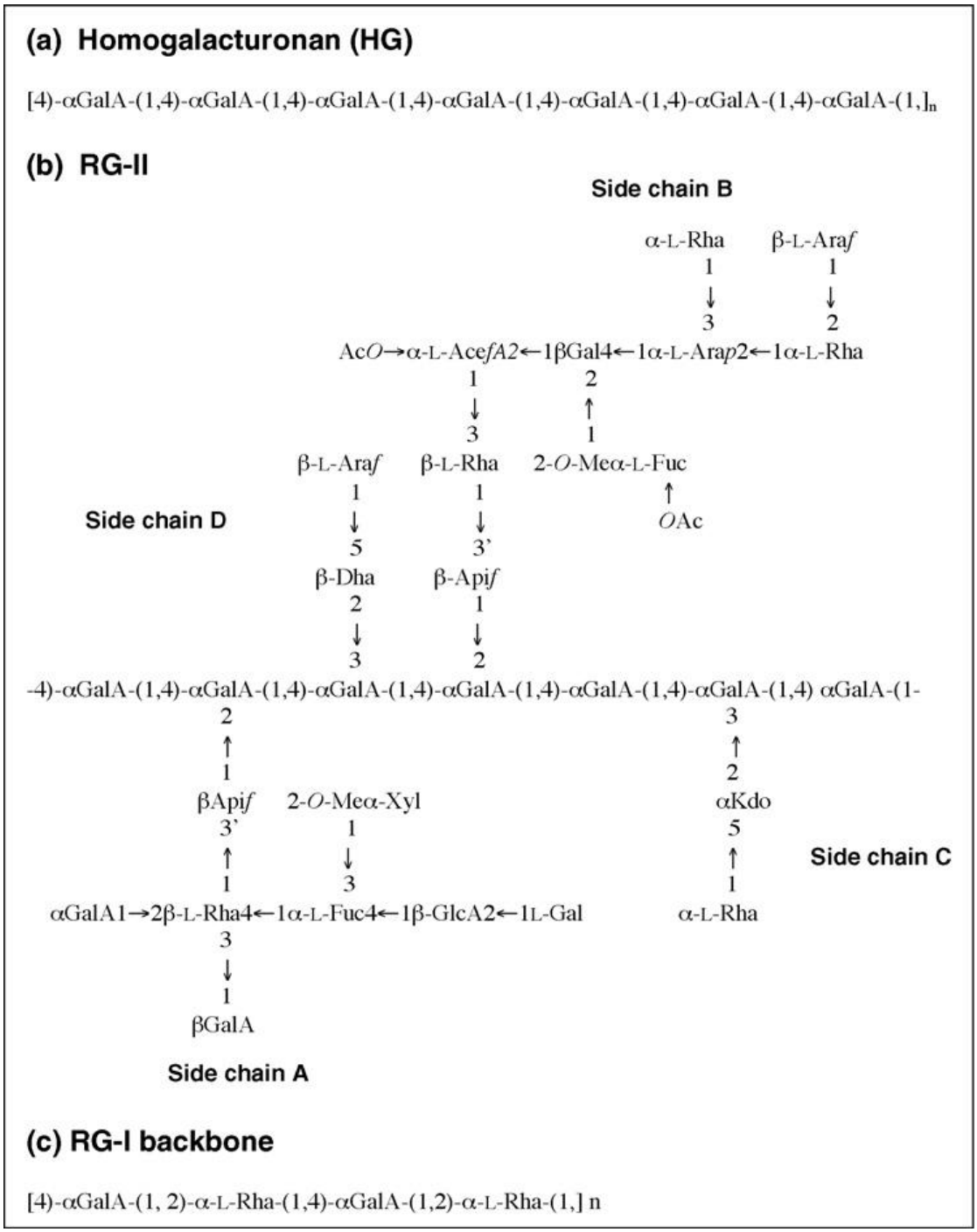

Figure 1-1 Representative structures of common domains of pectin

The structures of HG, RGII and RGI backbone are given. HG is unbranched. RGII has the same side chains (side chains A-D) across species. RGI has varible side chains often comprised of arabinose and galactose (Mohnen, 2008).

clear whether RG I and RG II are covalently linked (Willats et al., 2001c; Coenen et al., 2007).

It is now generally accepted that RG II can dimerize via 1:2 borate-diol diesters (Kobayashi et al., 1996). Dimerization involves a single borate diester cross link and the ester is formed between the apiosyl residue in side chain A of each monomer subunit (O'Neill et al., 1996; Ishii et al., 1999; O'Neill et al., 2001). The antiparallel HG chains can be cross-linked by Ca^{2+}. The hydroxyl group (-OH) of the unesterified C-6 carboxyl group of GalA residues can form alkoxide (-O), two of which can cross-link with one Ca^{2+} to form part of a 'junction zone' (Rees, 1977; Jarvis, 1984). At low concentrations two chains are thought to form a stable junction with maximum strength from about 14 GalA residues up to the length of about 200 GalA residues (Carpita and Gibeaut, 1993; Kobayashi et al., 1999). If sufficient Ca^{2+} exists, more HG chains can be cross-linked to form larger aggregates (Morris et al., 1982). Once the carboxyl group is methyl esterified, the Ca^{2+}-mediated linkage cannot be formed. Thus the methyl ester at C-6 can affect the gelling properties of pectin.

Pectin is believed to be crosslinked to hemicellulosic xyloglucans and xylans as well (Nakamura et al., 2002; Duan et al., 2004; Popper and Fry, 2008). The side chains of pectin can bind to cellulose in vitro (Zykwinska et al., 2005).

1.1.2 Pectin biosynthesis and secretion

Information concerning pectin biosynthesis and secretion is still fragmentary. Autoradiography of isotope-labelled glucose demonstrated that pectin originated in the Golgi apparatus, and was transported in vesicles to the plasma membrane (Northcote and Pickett-Heaps, 1966). Moore et al. (1991) found that HG and RG I epitopes could be identified in both the cis and medial Golgi using immunogold labelling. So it is presumed that the biosynthesis of these pectins occurs in cis Golgi (Moore et al., 1991; Lynch and Staehelin, 1992), and continues into the medial Golgi (Moore et al., 1991). Pectin is believed to be transported as a highly methyl-esterified polymer to the apoplast via vesicles from the Golgi (Liners et al., 1994; Dupree and Sherrier, 1998). New evidence of seed coat mucilage pectin secretion will be discussed in section 1.2.2.

Nucleoside diphosphate sugars (NDP-sugars) are thought to be the precursors of all carbohydrates in the cell wall (Goubet and Mohnen, 1999). All the NDP-sugars are derived from some central NDP-sugars, such as UDP-Glc and GDP-Man. 39 enzymes in 13 groups in *Arabidopsis* have been predicted or proven to be required for NDP-sugar interconversion (Caffall and Mohnen, 2009), including RHAMNOSE BIOSYNTHESIS1-3 (RHM1-3) hypothesized to convert UDP-D-Glc to UDP-L-Rha (Reiter and Vanzin, 2001). RHM2 is also known as MUM4, which will be discussed in section 1.3.6.

The biosynthesis of pectin is thought to be complex, requiring at least 67 distinct biosynthetic glycosyl-, methyl-, or acetyl-transferase enzymes, assuming distinct enzymes are required for each different glucosyl linkage present among pectins (Mohnen,

2008). Much more research is required to understand this process since very few of these enzymes have been characterized.

Glycosyl residues are transferred to growing polysaccharide chains through the action of various glycosyltransferases (Mohnen, 2008). Before any gene encoding a glycosyltransferase was identified, research on the biosynthesis of cell wall polysaccharides focused on the purification and characterization of the enzymatic properties of active glycosyltransferases (Gibeaut and Carpita, 1994). The first pectin biosynthetic enzyme identified both enzymatically and through cloning was the *Arabidopsis* protein GALACTURONOSYLTRANSFERASE1 (GalAT; GAUT1), with HG α-1,4-GalAT activity (Sterling et al., 2006). Another characterized member of 15 GAUT1-related family members is QUASIMODO1 (QUA1)/GAUT8 whose deficiency results in dwarfed plants, reduced cell adhesion and 25% reduction of GalA in leaves. These data suggest that *QUA1* encodes a homogalacturonan α-1-4-D-galacturonosyltransferase activity that is reduced or absent in *qua1-1* stems (Bouton et al., 2002; Orfila et al., 2005). At low humidity, the *parvus* mutant is semi-sterile dwarf with reduced anther dehiscence. PARVUS encodes a putative GAUT-like (GATL) enzyme, which may be responsible for the reduced branching of RG I and an increase in wall pectin (Lao et al., 2003; Brown et al., 2007).

Most enzymes for biosynthesis of RG I have not been identified. *ARABINAN DEFICIENT1* (*ARAD1*) encodes a putative arabinosyltransferase that is believed to form α-1,5-arabinan side chains on RG I (Harholt et al., 2006).

RHAMNOGALACTURONAN XYLOSYLTRANSFERASE1 (RGXT1) or RGXT2 transfers xylose from UDP-α-D-Xylose onto fucose forming α-1,3-linkage, which is found in the side chain A of RG II (Egelund et al., 2006). Though no wall structural alteration was detected in *rgxt1* or *rgxt2* mutants, the RG II from either mutant can be used as an acceptor for D-[^{14}C]-Xylose using either RGXT1 or RGXT2, while the counterpart of wild type cannot (Egelund et al., 2006). NpGUT1 encodes a putative glucuronyltransferase involved in RG II biosynthesis (Iwai et al., 2002).

In addition to glycosyltransferases, methyltransferases are of upmost importance in pectin biosynthesis. Pectin methyltransferases (MT) add methyl groups to the C-6 carboxyl group, or acetyl groups at O-2 or O-3 of GalA residues (Caffall and Mohnen, 2009). The Golgi-localized protein QUASIMODO2 (QUA2)/ TUMOROUS SHOOT DEVELOPMENT2 (TSD2)/ OVERSENSITIVE TO SUGAR1 (OSU1) has a putative MT domain, though the catalytic activity is undetermined. The corresponding mutant shows dwarf, reduced cell adhesion and 50% of HG of wild type (Krupkova et al., 2007; Mouille et al., 2007; Gao et al., 2008). However the degree of methylesterification of HG in the mutant is unaltered. It is noteworthy to observe that *QUA2* is co-transcribed with *QUA1*/*GAUT8* (Mouille et al., 2007).

In contrast, pectin methylesterase (PME) can catalyze the demethylesterification of HG to generate acidic pectin which can cross-link with Ca^{2+}. Given the fact that 70-80% GalA residues of HG are methyl esterified at the C-6 carboxyl group (O'Neill et al., 1990), this kind of enzyme is very important for the formation of pectic gels.

1.1.3 Functions of pectin

Pectin is a multifunctional complex. It helps maintain the mechanical stability of the cell wall, is involved in cell growth and development, and provides a source of oligosaccharide signals.

Given that antiparallel HG chains can be cross-linked by Ca^{2+}, and RG II can dimerize with the help of borate, pectin is believed to play important roles in maintaining the rigidity of the cell wall. Further, since pectin occupies most of the middle lamella, it is thought to be responsible for the adhesion of cells. Several lines of evidence support the connection between cell-cell adhesion and pectin crosslinking. First, a T-DNA insertional mutant from *Nicotiana plumbaginifolia*, *nolac-H18*, has non-organogenic callus with loosely attached cells. The gene, cloned using T-DNA as a probe, was named *NpGUT1*, mentioned above. Examination of RG II from the mutant cells showed that it had less glucuronic acid which would be expected to dramatically reduce the formation of borate cross-linking. It was suggested that the loss of adhesion is the direct result of decrease in borate cross-linking (Iwai et al., 2002). Second, the mutant *quasimodo*, has a reduction in cell adhesion that is correlated with a 25% reduction in GalA (Bouton et al., 2002). Since *QUA* encodes a putative glycosyltransferase it is possible that the cell adhesion in the mutant is a consequence of a decrease in pectin biosynthesis.

Pectin composition can change between cell types and in a given tissue, organ or cell type over time. For example, following the development of bean pods, Stolle-Smits et al. (1999) found that in young pods, exponentially growing cell walls contained large amounts of neutral-, pectic polymers (rhamnogalacturonan); in the elongating pods, more

galatose-rich pectic polymers were deposited into the cell wall, the level of branched rhamnogalacturonan remained constant, and the level of linear homogalacturonan steadily increased; in the mature pods, galactose-rich pectin was degraded, while the accumulation of soluble homogalacturonan continued; in the senescent pods, there was an increase in the amount of ionically complex pectin, mainly at the expense of freely soluble pectin. However, whether such changes in pectin composition are required for cell differentiation is unclear.

Rhamnogalacturonan lyase from *Aspergillus aculeatus* can cleave the RG I backbone at specific sites. Transgenic potato tubers expressing the *Aspergillus* rhamnogalacturonan lyase gene have morphological alterations, including radial swelling of the periderm cells and the development of intercellular spaces in the cortex. Sugar composition analysis and immunocytochemical studies showed a large reduction of galactan and arabinan side chains of RG I. But the corresponding epitopes were mostly found in the expanded middle lamella at cell corners of tubers. So, RG I might be important in anchoring galactans and arabinans at particular regions in cell walls and in normal development of the periderm (Oomen et al., 2002).

Considering its many roles in cell biology, development and physiology, understanding the biosynthesis and secretion of pectin is an important goal. The genetic analysis of mutants defective in cell wall pectin is an effective and, under-utilized approach to studying pectin biosynthesis. However, most such mutants are expected to be lethal making their isolation difficult.

1.2 Seed coat in *Arabidopsis*

Seed coats differentiate from cells of the ovule integuments and play important roles in dormancy, germination and longevity of seeds (Debeaujon et al., 2000). The seed coat contains several cell layers that are highly specialized. One distinctive feature found in the seed coat epidermis is myxospermy, referring to the release of mucilage upon exposure to aqueous solutions. This property is found in a variety of species, including members of Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae (Young and Evans, 1973; Frey-Wyssling, 1976; Grubert, 1981; Van Caeseele et al., 1981; Van Caeseele et al., 1987; Boesewinkel and Bouman, 1995).

Mucilage is primarily composed of pectin (Goto 1985; Western et al., 2000) and is believed to play roles in seed germination. When the seeds of mutants, such as *myb61*, *sbt1.7* and *mum4 men* discussed below, are placed on a filter paper moistened with polyethylene glycol (PEG) solutions to limit the water access to the seeds, the mutant seeds show lower, or slower germination, compared to wild type (Penfield et al., 2001; Rautengarten et al., 2008; Arsovski et al., 2009a).

Arabidopsis thaliana, a member of Brassicaceae, is myxospermous. After maturity, dry seeds of *Arabidopsis* can release mucilage, containing two distinct layers, upon hydration. The outer layer of the mucilage is relatively diffuse, is weakly stained by ruthenium red and can be easily removed from the seed by vigorous shaking (Western et al., 2000; Macquet et al., 2007a). The inner layer, however, is compact, strongly stained by ruthenium red and is much more difficult to remove from the seed (Western et al., 2000; Macquet et al., 2007a).

The *Arabidopsis* seed coats can be used to study pectin biosynthesis and modification. The dry mature seeds release large quantities of a specific pectin that is dispensable under laboratory conditions. Therefore seed coat pectin is amenable to both chemical and molecular genetic analyses, and provides an opportunity to identify genes involved in pectin biology.

1.2.1 Development of the seed coat

During germination, the pollen tube releases two sperm nuclei into the embryo sac to accomplish the double fertilization in angiosperms. One sperm fuses with the egg initiating embryogenesis, while the other sperm fuses with two polar nuclei to form the endosperm which supplies nutrients for the growing embryo. This process initiates the differentiation of seed coat from the maternal ovule integuments that occurs over a period of 2–3 weeks (Haughn and Chaudhury, 2005).

At the beginning stage of seed coat development (0 days post-anthesis, DPA), the four to five layers of cells in the ovule integuments (outer and inner) are relatively undifferentiated (Beeckman et al., 2000; Debeaujon et al., 2003). The outer integument (oi) includes two cell layers, an inner (oil) and an outer (oi2; epidermis) layer while the inner integument (ii) consists of two to three cell layers (iil, iil` and ii2, labelled from inner to outer; Figure 1-2; Schneitz et al., 1995; Beeckman et al., 2000).

Immediately after the fertilization, the cells of the iil layer became vacuolated. In these cells the pigment, proanthocyanidin (PA), a flavonoid compound (Dixon et al.,

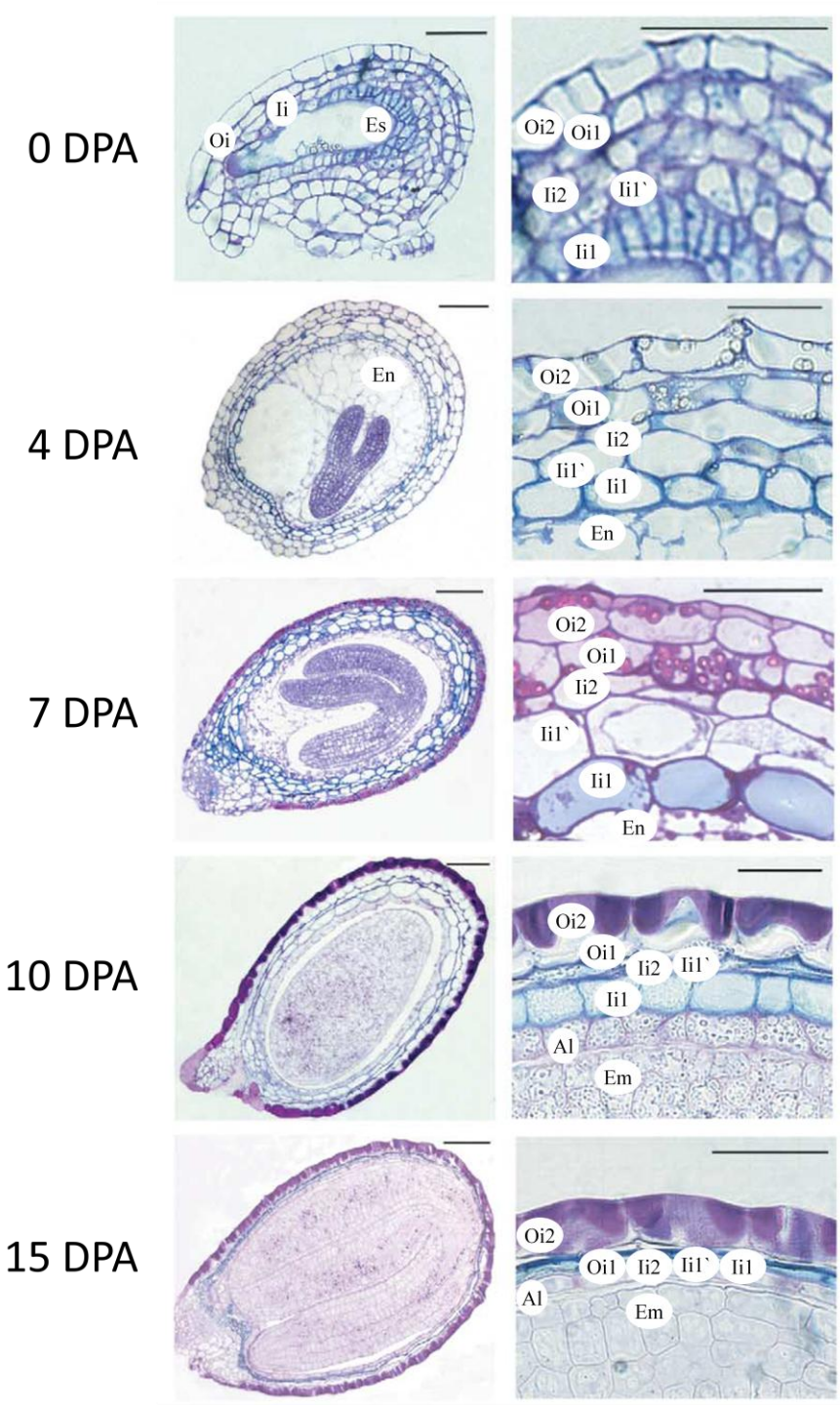

Figure 1-2 The development of ovule integuments

The right column shows the development of the seed coat from ovule integuments, with the seed development as the reference (left column). Al, endosperm aleurone; Em, embryo; En, endosperm; Es, embryo sac; Ii, inner integument; Oi, outer integument (adapted from Haughn and Chaudhury, 2005).

2005) accumulates in the vacuoles eventually forming condensed tannins, and is then deposited in the cytoplasm as well (Beeckman et al., 2000). The cells in this layer eventually go through programmed cell death after which the tannins are released into surrounding layers (Beeckman et al., 2000). Oxidation of the tannins results in the brown colour characteristic of *Arabidopsis* seeds. The cells in the iil` and ii2 layers first expand after the fertilization, with an extensive vacuolisation in cells. The bent-cotyledon embryo stage is the turning point for the cells in these two layers since the cells undergo programmed cell death (Nakaune, et al. 2005) and are ultimately crushed into a thin layer by maturity (Beeckman et al., 2000).

The appearance of starch grains characterizes the early growth of the oi1 and oi2 layers (Western et al., 2000; Windsor et al., 2000; Beeckman et al., 2000). In the oi1 layer, a secondary wall is produced on the inner tangential side of the cell resulting in a palisade-like appearance (Beeckman et al., 2000).

The oi2 (epidermal) layer is the one that produces mucilage and its differentiation has been studied intensely. During the first 4 DPA, the cell size increases roughly 4-fold, with the expansion of the vacuole that occupies the centre of the cell, pushing the cytoplasm to the outer margins (Western et al., 2000; Windsor et al., 2000; Young et al., 2008). Globular prominent amyloplasts are observed in cells beginning at 3 DPA, with both the size and number increasing until 7 DPA. At 6 to 7 DPA, pink-staining acidic polysaccharide is seen in growing pockets at the junction of the radial and outer tangential cell walls indicating the beginning of mucilage synthesis. From 7 through 9 DPA, the cytoplasm and amyloplasts form a column at the centre of the cells defined by the growing mucilage pockets (Western et al., 2000; Windsor et al., 2000; Young et al.,

2008). By 10 DPA, a secondary cell wall called the columella starts to be formed along the outer surface of the cytoplasmic column while the amyloplasts become smaller and darker. As the walls of columellae are thickened, the cytoplasmic column is displaced toward the bottom of the cell until the cytoplasm is replaced entirely by the growing secondary cell wall. Thus, by maturity, the volcano-shape columella is surrounded by a donut-shaped space, which is filled with mucilage (Western et al., 2000; Windsor et al., 2000). Given the correlation between the appearance and disappearance of amyloplasts and the biosynthesis of mucilage and columellae, the starch grains are considered to be the source of much of the carbohydrate for pectin and secondary cell wall, although normal amounts of mucilage are produced in the starchless mutant *phosphoglucomutase1* (*pgm1*) when grown in continuous light many fewer seeds (2 or 3 compared to 50-60 in the wild type) were produced per silique (Caspar et al., 1985; Caspar and Pickard, 1989; Western et al., 2000).

1.2.2 Seed coat mucilage secretion

Similar to cell wall pectin, mucilage pectin is produced in the Golgi stacks and secreted during seed coat development (Young et al., 2008). Accordingly, at 7 DPA, at the height of mucilage synthesis, the Golgi has swollen cis cisternae and more extensive trans-Golgi network (TGN) than Golgi at 4 DPA (Young et al., 2008). In addition, the number of Golgi stacks increases dramatically during this period (Young et al., 2008). An anti-mucilage antibody (CCRC-M36) was used to investigate the appearance and localization of mucilage in developing seed coats (Young et al., 2008; Pattathil et al.

2010). Mucilage was detected in the Golgi Bodies and vesicles and mucilage pockets during (7 DPA) but not before (4 DPA) or after (9 DPA) mucilage deposition (Young et al., 2008).

Although the secretion of mucilage is targeted to a specific region of the cell, the Golgi apparatus is evenly distributed in the cytoplasm (Young et al., 2008). How such targeted secretion occurs is unclear. Cortical microtubules are found lining the mucilage secretion domain of the plasma membrane in the seed coat epidermal cells (McFarlane et al., 2008). By contrast, actin microfilaments are distributed evenly throughout the cytoplasm (McFarlane et al., 2008). To test the hypothesis that microtubules guide mucilage vesicle transportation to the mucilage pocket, mucilage deposition was examined in *microtubule organization1-1* (*mor1-1*), which has disordered cortical microtubules when grown at high temperatures (Whittington et al., 2001; Kawamura et al., 2006). However seed coat development in *mor1-1* was found to be normal suggesting that mucilage secretion is not dependent on microtubule organization (McFarlane et al., 2008).

1.2.3 Seed coat mucilage composition

To investigate the nature of the *Arabidopsis* seed coat mucilage, various approaches have been applied, including chemical analysis, cytological staining, and antibody labelling.

Monosaccharide and linkage analysis of *Arabidopsis* mucilage identified the major sugars in mucilage as 2-Rha and 4-GalA, suggesting that RG I is a major component of mucilage (Goto, 1985; Western et al., 2000, 2001, 2004; Penfield et al., 2001; Usadel et al., 2004; Dean et al., 2007; Macquet et al., 2007a; Arsovski et al., 2009b). In addition, the chemical analysis was consistent with the presence of cellulose, xyloglucan and HG, but at a relatively low level compared to RG I.

Cytological analyses have also identified RG I, HG, XG and cellulose as being present in mucilage. Ruthenium red, which can stain acidic polysaccharides (Luft, 1971; Fletcher and Floodgate, 1973; Frey-Wyssling, 1976), and widely used as an indicator of pectin, stains mucilage (Western et al., 2000; Willats et al., 2001a; Macquet et al., 2007a). The anti-mucilage antibody CCRC-M36 is believed to bind strongly to unbranched backbone of RG I from *Arabidopsis*, tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*), and soybean (*Glycine max*) and mustard (*Sinapis alba*; Young et al., 2008; Pattathil et al. 2010). Cellulose in the mucilage was suggested by the staining by calcoflour white, which can detect β-1, 4-glucans, and demonstrated through the binding of a cellulose binding domain (Willats et al., 2001a; Blake et al., 2006; Macquet et al., 2007a; Young et al., 2008). LM5 and LM6, which recognize (1, 4)-β-galactan and (1, 5)-α-arabinan of the RG I side chains, also bind mucilage (Jones et al., 1997; Willats et al., 1998; Willats et al., 2000). HG was detected in mucilage by anti-HG monoclonal antibodies JIM5 and JIM7 and PAM1 (Willats et al., 2000; Willats et al., 2001a; Macquet et al., 2007a; Young et al., 2008). JIM7 recognizes the outer layer of seed coat mucilage while JIM5 recognizes the inner layer. Thus the data indicate that the outer region of mucilage consists of highly esterified HG, and the inner region more de-esterified HG.

Finally, XG has also been identified as a component using antibody binding (Macquet et al., 2007a; Young et al., 2008). Therefore, the major component of mucilage is RG I, with much lower quantities of cellulose, XG and HG. However, the relative arrangements of different components are still unclear.

1.3 Genetic analysis of seed coat mucilage production

Because the *Arabidopsis thaliana* seed coat epidermal cells are a non-essential cell type that produces copious amounts of easily accessible pectin at a specific time in development, this cell type can be useful as a model to study the processes of biosynthesis, secretion and modification of pectin. Discovering genes involved in mucilage production by identifying mutants defective in seed coat mucilage can help us obtain more insights into the processes of pectin production. Several mutants have already been identified and studied (Table 1-1).

1.3.1 Seed coat development mutants

Among the seed coat mucilage mutants, *aberrant testa shape* (*ats*) and *apetala2* (*ap2*) affect the integument development/ differentiation. Neither can produce normal seed coat cells.

Table 1-1 Seed coat mucilage mutants

Mutant	Mutant phenotype	Gene product
aberrant testa shape (*ats*)	reduced mucilage	KANADI TF
abscisic acid1 (*aba1*)	reduced mucilage release	Zeaxanthin epoxidase
apetala2 (*ap2*)	no mucilage or columella	AP2 TF
enhancer of glabra3 (*egl3*)	reduced mucilage and flattened columella (*egl3 tt8* double mutant)	bHLH TF
galacturonosyltransferase11 (*gaut11*)	reduced mucilage and flattened columella	Galacturonosyltransferase
gibberellin 3-oxidase4 (*GA3ox4*)	reduced mucilage release and abnormal columella	Gibberellin 3-oxidase4
glabra2 (*gl2*)	reduced mucilage and flattened columella	Homeobox TF
mucilage-modified1 (mum1)	no mucilage on hydration with normal development	WD40 TF
mum2	no mucilage on hydration with normal development	Glycosyl hydrolase
mum3	altered mucilage staining	Unknown
mum4	reduced mucilage and flattened columella	NDP-L-rhamnose synthase
mum5	altered mucilage staining	Unknown
myb23	reduced mucilage and flattened columella (constitutive chemeric repressor)	MYB TF
myb5	reduced mucilage and flattened columella	MYB TF
myb61	reduced mucilage and flattened columella	MYB TF
patchy	patchy release of mucilage	Glycosyl hydrolase
radial swelling3 (*rsw3*)	reduced mucilage and flattened columella	Glucosidase II
subtilisin-like serine protease1.7 (subtilase1.7; sbt1.7)	no mucilage on hydration with normal development	Subtilase
transparent testa2 (*tt2*)	reduced mucilage and flattened columella (*myb5 tt2* double mutant)	MYB TF
tt8	reduced mucilage and flattened columella (*egl3 tt8* double mutant)	bHLH TF
transparent testa glabra1 (*ttg1*)	reduced mucilage and flattened columella	WD40 TF
ttg2	reduced mucilage and flattened columella	WRKY TF

ats is a heart-shaped seed mutant that produces much less mucilage than wild type upon hydration. Unlike the wild type ovule, which has two integuments with five layers of cells, *ats* mutant has only one integument with three cell layers. The endothelium of the *ats* mutant develops normally but the epidermal cells fail to produce a normal amount of mucilage. The single integument in *ats* demonstrates normal pigment and less mucilage (Leon-Kloosterziel et al., 1994). *ATS* encodes KANADI4 (KAN4), a member of the KAN family of putative transcription factors. *In situ* hybridization data revealed that *ATS* expression is confined to the abaxial side of the inner integument and the adaxial side of the outer integument at the time of integument primordium initiation. These data suggest that ATS is required for the separation of the two integuments (McAbee et al., 2006). However, the role of ATS in mucilage production is still unclear.

The *ap2* mutant was first isolated as a floral organ identity mutant, but has defects in a number of different processes, including seed coat differentiation. The seeds of *ap2* are heart-shaped and the seed coat epidermis has flattened columellae (Bowman et al., 1989; Kunst et al., 1989; Jofuku et al., 1994). AP2 is a member of a DNA-binding transcription factor family defined by AP2 domain (Okamuro et al., 1997). Electron and light microscopy were used to examine the differences in *ap2* ovule integument development compared to wild type. The cells in outer integument develop normally until 4 DPA. However the cells stop differentiating, and retain a vacuolated state. No discernable epidermal or palisade cell layers develop in mature *ap2* seeds (Western et al., 2001). Therefore, AP2 functions as a regulator of outer integument differentiation.

1.3.2 Mucilage biosynthesis mutants

As discussed previously, the biosynthesis of mucilage likely involves many enzymes. Loss-of-function mutations can help identify genes encoding these enzymes. One example of such a mutant is *mum4*, which has severe mucilage reduction, and the mutated gene encodes a rhamnose synthase. Since this mutant is directly involved in one of my projects, it will be discussed in detail below. Another enzyme-encoding gene related to seed coat mucilage is *GALACTURONOSYLTRANSFERASE11* (*GAUT11*) which was identified in a reverse-genetics study of the GAUT family (Caffall et al., 2009). The reduced mucilage release observed in this mutant and the monosaccharide composition of the mucilage are consistent with a reduction of HG (Caffall et al., 2009). The *radial swelling3* mutant (*rsw3*) shows reduced mucilage and flattened columela typical of mutants deficient in mucilage synthesis. *RSW3* encodes the α-subunit of a putative glucosidase II with homology to enzymes that process N-glycans during ER quality control (Burn et al., 2002). The cellulose in roots of *rsw3* is also reduced (Peng et al., 2000). The exact function of RSW3 is unclear but it could be related to glycosylation defects of cell wall biosynthetic enzymes (Burn et al., 2002).

1.3.3 Mutants affecting the regulation of mucilage biosynthesis

A number of mucilage-defective mutants identified transcription factors involved in the regulation of mucilage biosynthesis. Three such transcription factors are TRANSPARENT TESTA GLABRA1 (TTG1), GLABRA2 (GL2) and TTG2. None of the three mutants, *ttg1*, *gl2* and *ttg2*, release mucilage in water, and their columellae are

flattened (Koornneef, 1981; Rerie et al., 1994; Western et al., 2001; Johnson et al., 2002). Chemical analysis determined that the monosaccharide composition of mucilage is unchanged but the quantity is decreased in all three mutants compared to wild type. Further, the differentiation of epidermal cells is altered. The large vacuole does not contract completely and the secondary cell wall is deposited on top of the vacuole. When the vacuole collapses on desiccation, the columella flattens (Western et al., 2001). *TTG1* encodes a WD40 repeat protein (Walker et al., 1999) and *GL2* a homeodomain-leucine zipper (HD-zip) transcription factor (Rerie et al., 1994). *TTG2* encodes a WRKY transcription factor expressed specifically in the innermost layer of the inner integument and later in multiple layers of the seed coat (Johnson et al., 2002).

The mutants *ttg1*, *gl2* and *ttg2* also have defects in trichome and root hair development and seed colour (Koornneef, 1981; Rerie et al., 1994; Johnson et al., 2002). Trichomes are absent on the leaves of mutants *ttg1* and *gl2*, while *ttg2* has reduced amount of trichome branching (Koornneef, 1981; Rerie et al., 1994; Johnson et al., 2002). Unlike in wild type root epidermis where the hair-bearing cells and hairless cells are arranged alternately, all cells are hair-bearing ones in *ttg1* and *gl2*, which leads to more root hairs in both mutants (Galway et al., 1994; Masucci and Schiefelbein, 1996; Di Cristina et al., 1996; Masucci et al., 1996). The root hair phenotype of *ttg1* is not found in *ttg2* (Johnson et al., 2002). The seeds of *ttg1* and *ttg2* are yellow in appearance resulting from a reduction of tannins in the pigmented cell layer (Koornneef, 1981; Walker et al., 1999; Johnson et al., 2002).

The pleiotropic phenotypes of *ttg1*, *gl2* and *ttg2* suggest that the epidermal cells of seed coat, trichome and root hair share a common mechanism to determine cell

specification. TTG1, a WD40 repeat protein, forms a complex with a MYB protein and a bHLH protein (Payne et al., 2000) to promote epidermal cell fate in a variety of tissues. A specific fate is determined by the specific MYB and bHLH proteins in the complex (Payne et al., 2000; Zhang et al., 2003; Schiefelbein, 2003; Larkin et al., 2003; Schellmann et al., 2007; Larkin et al., 1994; Zhao et al., 2008; Kang et al., 2009). TTG2 and GL2 are downstream targets of the complex (Szymanski et al., 1998; Johnson et al., 2002; Western et al., 2004; Ishida et al., 2007).

One cell type promoted by the WD40-bHLH-MYB complex is the seed coat mucilage epidermal cells (Zhang et al., 2003; Western et al., 2004). EGL3 and TT8 are bHLH proteins that interact with TTG1 and act redundantly to specify the seed coat epidermis (Zhang et al., 2003). Similarly MYB5 and TT2 act redundantly as the MYB component. The data that *myb5* has pleiotropic phenotypes and both *MYB5* and *TTG1* up-regulate the same genes suggest that MYB5 is likely part of the WD40-bHLH-MYB complex (Li et al., 2009; Gonzalez et al., 2009).

Both *TRANSPARENT TESTA8* (*TT8*) and *ENHANCER OF GLABRA3* (*EGL3*) encode a basic helix-loop-helix domain protein (Nesi et al., 2000; Zhang et al., 2003). The *tt8* mutant was isolated as the yellowish seeds in contrast to the wild type brown ones (Nesi et al., 2000). *Egl3* was identified as a mutant with completely hairless plants (Zhang et al., 2003) when screening for the enhancer of *glabra3* (*gl3*), which has reduced trichomes (Payne et al., 2000). Only the double mutant *egl3 tt8* has no mucilage extrusion in water and collapsed columellae (Zhang et al., 2003). These data suggest that EGL3 and TT8 have redundant roles in mucilage production. Much less mucilage and flattened columellae are found in the seed epidermal cells in *myb5*, compared to wild type (Li et al.,

2009; Gonzalez et al., 2009). *MYB5* and *TT2* are redundant in mucilage production since the double mutant has a more severe mucilage phenotype than *myb5*, although *tt2* seed coat has wild type development and mucilage (Gonzalez et al., 2009).

MYB61 is a member of an independent pathway from the TTG1-dependent pathway (Penfield et al., 2001; Western et al., 2004). The *myb61* mutant lacks mucilage extrusion and volcano-shaped columellae, resulting from the reduction of mucilage deposition (Penfield et al., 2001). MYB61 differs from other transcription factors in its control of *MUM4* (Western et al., 2004). All transcription factors in the TTG1 pathway except TTG2 are required for normal levels of *MUM4* expression while MYB61 is not. The role of MYB61 in the mucilage production is still unclear. Another study demonstrated that *MYB61* is required for the control of stomatal aperture and suggested a role in balancing carbon supply with demand (Liang et al., 2005). Perhaps its role in mucilage deposition is connected to carbon supply.

Combining all the information mentioned above, a model of the regulatory pathways of seed coat mucilage biosynthesis have been proposed (Figure 1-3; Western et al., 2004; Li et al., 2009; Gonzalez et al., 2009).

MYB23 is a transcription factor that cannot yet be incorporated into the mucilage biosynthesis regulatory pathway, although there is some evidence for its involvement in mucilage production. A chimeric *MYB23* repressor leads to phenotypes that are similar to the loss-of-function alleles of *TTG1*. These phenotypes include the defects in trichome development, hairy shorter roots, elongation of leaves and of stems, and absence of columellae and seed coat mucilage on hydration (Matsui et al., 2005). More studies showed that *MYB23* participates in cell fate determination in roots and trichomes (Kirik

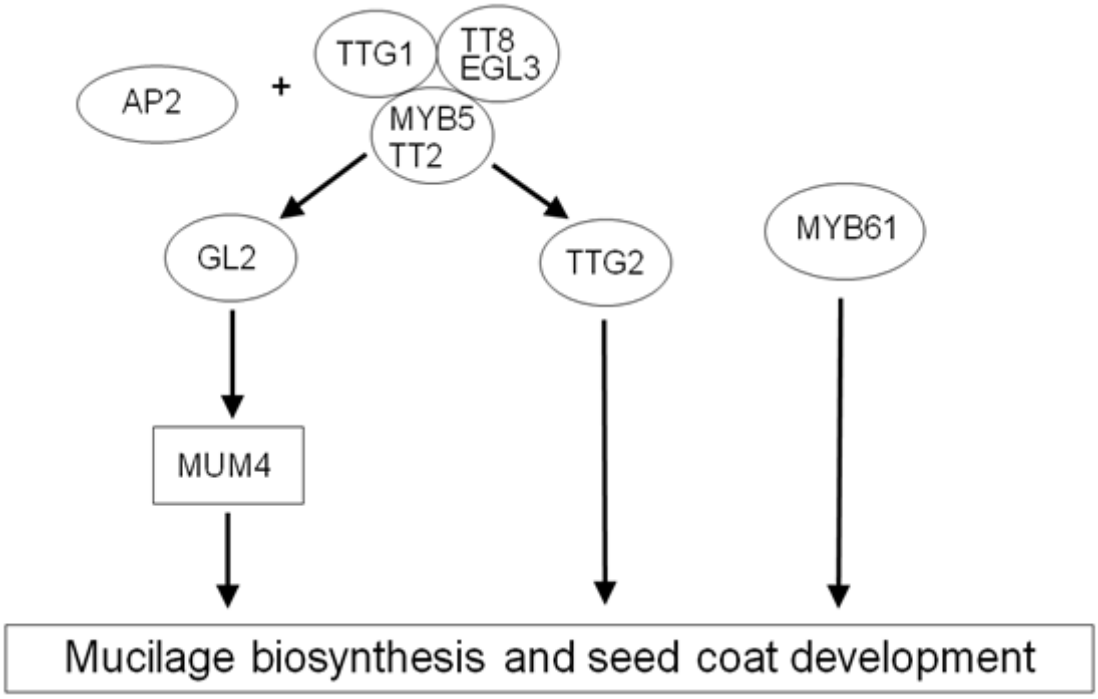

Figure 1-3 Regulatory pathways of seed coat mucilage production

TTG1 forms a complex with TT8/EGL3 and MYB5/TT2. Together with AP2, the complex regulates GL2 and TTG2. GL2 controls mucilage production at least in part through MUM4, a rhamnose synthase. MYB61 is independent from other regulatory components (adapted from Western et al., 2004; Li et al., 2009; Gonzalez et al., 2009).

et al., 2001; Kirik et al., 2005; Kang et al., 2009). *MYB23* and *MYB5* show redundancy in trichome development, however, *MYB23* does not seem to share a similar role as *MYB5* in mucilage production since *myb23* releases a large amount of mucilage and *myb5 myb23* double mutant is similar to a *myb5* single mutant (Li et al., 2009).

1.3.4 Hormone-associated mucilage mutants

Two mutants with seed coat mucilage phenotypes can be rescued by hormones, *abscisic acid1* (*aba1*) and *gibberellin-3 oxidase4* (*GA3ox4*; Karssen et al., 1983; Kim et al., 2005). The *aba1* mutant defective in the ABA biosynthetic enzyme zeaxanthin epoxidase shows reduction of mucilage extrusion which can be recovered by exogenous ABA (Karssen et al., 1983). The only obvious difference in *GA3ox4* seed coat development from wild type is the delayed disappearance of starch granules, which may contribute to the formation of columellae. The defective mucilage release phenotype in the *GA3ox4* mutant can be rescued by the application of gibberellic acid (GA; Kim et al., 2005). These data suggest that ABA and GA simulate the production of mucilage, though the mechanism remains unclear.

1.3.5 Mucilage adhesion mutants

Another group of seed coat mutants are *mucilage-modified1* (*mum1*) through *mum5* which were isolated in a screen for seed coat defects (Western et al., 2001). In *mum3* and *mum5,* mucilage is present when hydrating seeds in ruthenium red without shaking. However, both layers disappear after agitation. The adherent inner layer of

mucilage becomes diffuse and water-soluble, like the outer layer. As yet *MUM3* and *MUM5* remain to be identified (Western et al., 2001; Macquet et al., 2007a).

The mutants *mum1*, *mum2*, *patchy* and *subtilisin-like serine protease1.7* (*subtilase1.7*; *sbt1.7*) are defective in the mucilage release. Since they are the focus of my thesis research, they will be discussed below in detail.

1.3.6 The *mum4* mutant

The mutant *mum4* was found in a screen for *Arabidopsis* mutants defective in mucilage extrusion. When *mum4* is immersed in water, no mucilage comes out. However, the seed coat releases a little mucilage after treatment with EDTA. Light microscopy of *mum4* seeds suggests that the amount of mucilage is lower than that of wild type. In addition, *mum4* has columellae that are flatter than those of wild type (Western et al., 2001; Western et al., 2004).

The *MUM4* gene was identified by map-based cloning. It encodes a protein of 667 amino acids. BLAST of the nucleotide sequence of *MUM4* indicated that the N-terminal domain was similar to dTDP-D-glucose-4, 6-dehydratases and the C-terminal domain was similar to bacterial 4-reductases from the reductase/epimerase/dehydrogenase protein superfamily responsible for rhamnose biosynthesis in bacteria (Western et al., 2004; Usadel et al., 2004). The *MUM4* gene is one of three members of a small gene family: *RHM1, MUM4/RHM2* and *RHM3* (Usadel et al., 2004; Western et al., 2004). *MUM4* gene is expressed ubiquitously in *Arabidopsis* (Usadel et al., 2004; Western et al., 2004)

but it is up-regulated in the silique during mucilage synthesis. Characterization of *MUM4* expression showed that *MUM4* is transcribed less in the mutants *ap2, ttg1* and *gl2* than in the wild type but this was not the case in the mutants *ttg2* and *myb61*. Thus *AP2, TTG1* and *GL2* are necessary for the up-regulation of *MUM4* expression, but not *TTG2* and *MYB61* (Western et al., 2004; Figure 1-3).

The *mum4* mutant is a good tool for screening for mutations in new genes that are involved in the biosynthesis and secretion of pectin and/or the differentiation of secondary cell walls. Because the *mum4* mutant seed coats make only small amounts of mucilage, it is easier to detect small changes in the amount (either increases or decreases) of mucilage relative to wild type. For this reason, mutations that affect the amount of pectin can be more easily detected in a *mum4* genetic background. Secondary mutations that result in more mucilage or more pronounced columellae are termed *mum4 suppressors* (*msu*). Secondary mutations that result in less mucilage or less pronounced columellae are termed *mum4 enhancers* (*men*). Such modifier screening can identify genes involved in pectin or cell wall biosynthesis that may not have been recognizable by previous screening methods.

Eight independent *men* mutants were identified, including two alleles of the previously known genes, *MUM2* and *MYB61* (Arsovski et al., 2009b). The other six double mutants identified new loci: *men1-1 mum4-1*, *men4-1 mum4-1*, and *men5-11 mum4-1* deposit less mucilage than *mum4-1*, while *men2-1 mum4-1* and *men6-1 mum4-1* fail to release mucilage even after the treatment of EDTA (Arsovski et al., 2009b). No single mutant shows a seed coat mucilage phenotype, except *men4*, which has less

mucilage in EDTA than wild type, and larger columellae than *mum4* (Arsovski et al., 2009b).

1.4 Mutants effecting pectin modification

Seed coat mucilage mutants that fail to extrude mucilage can result from the reduction of mucilage production, like the ones discussed above. Another class of mucilage mutants do not have a dramatic change in the amount of mucilage, but still fail to extrude mucilage. These mutants may have alterations in the structure of mucilage, the primary cell wall, or both, that limit the capability of mucilage to expand in water. *mum2*, *sbt1.7*, *patchy* and *mum1* are in this class. The *MUM2*, *SBT1.7* and *PATCHY* genes have been cloned and characterized.

1.4.1 The pectin modification mutants

Except for the lack of mucilage extrusion, differentiation of the *mum2* mutant seed coat appears to be cytologically normal, with similar amounts of mucilage deposited into the mucilage pocket (Western et al., 2001; Dean et al., 2007; Macquet et al., 2007b). When seeds are sectioned such that the cell walls are not completely intact, the mucilage still fails to expand, suggesting that the failure to extrude is due, at least in part, to changes to the mucilage itself (Dean et al., 2007; Macquet et al., 2007b). The *MUM2* gene encodes a β-galactosidase secreted to the apoplast (Dean et al., 2007; Macquet et al., 2007b). β-galactosidase activity is absent in the *mum2* mutant and recombinant MUM2

protein shows β-galactosidase activity (Dean et al., 2007; Macquet et al., 2007b). *MUM2* is expressed in many tissues of the plant besides the seed coat. Chemical analysis of the *mum2* mucilage is consistent with the absence of β-galactosidase activity. The *mum2* mucilage has more RG I galactan and arabinan side chains than that of wild type (Dean et al., 2007; Macquet et al., 2007b). The highly branched mucilage resulting from the absence of MUM2 may be responsible for the alteration in the hydration and expansion properties of mucilage in *mum2*.

The *patchy* mutant has only patches of mucilage released from seed coat epidermal cells (Arsovski et al., 2009a). Like in *mum2*, *patchy* seed coat development is normal. Increases in a neutral side chain α-1-5 linked arabinans in mucilage, and in primary cell walls, were detected by chemical analysis and immunofluorescence, respectively (Arsovski et al., 2009a). An exogenous α-arabinofuranosidase helps the wild type mucilage release in *patchy* (Arsovski et al., 2009a). *PATCHY* was found to encode a bi-functional β-xylosidase/α-arabinofuranosidase previously named BXL1 (Goujon et al., 2003), expressed in all the tissues examined (Arsovski et al., 2009a). PATCHY/BXL1 is predicted to be targeted to the extracellular matrix, and to trim arabinan side chains for weakening of primary cell walls and/or normal mucilage release.

SBT1.7 encodes a subtilisin-like serine protease (subtilase) that is expressed ubiquitously, but peaks during seed development (Rautengarten et al., 2008). The seeds of the *sbt1.7* mutant do not release mucilage in water but in EDTA, the mucilage is partially released (Rautengarten et al., 2008). However, no structural or developmental changes were detected during the seed coat development (Rautengarten et al., 2008). The mucilage composition of analysis suggests the RG I level is unchanged (Rautengarten et

al., 2008). Coincidentally, pectin methylesterase (PME) activity was found to increase in *sbt1.7* late in seed coat development (12-14 DPA) when the SBT1.7 protein accumulates in the seed coat (Rautengarten et al., 2008). Low pectin methylesterification in *sbt1.7*, which might lead to higher cross linkage of pectin by Ca^{2+}, could explain the mucilage expansion defect and the cell wall change. Thus SBT1.7 may activate a PME possibly through cleavage of a repressor domain (Rautengarten et al., 2008).

The functions of *MUM2*, *SBT1.7* and *PATCHY* genes suggest that the modification of mucilage/cell wall structure after mucilage biosynthesis is necessary for mucilage extrusion and illustrates the usefulness of seed coat cells as a model system to study cell wall dynamics.

1.4.2 The *mum1* mutant

mum1 has a mucilage extrusion phenotype closely resembling that of the *mum2* mutant (Western et al., 2001). The seed coat development of *mum1* is indistinguishable from that of wild type (Western et al., 2001). Monosaccharide analysis of *mum1* whole seed revealed a similar monosaccharide composition to that of wild type (Western et al., 2001). Methylation analysis shows an increase in *mum1* and *mum2*, compared to wild type (Western et al., 2001). This change was hypothesized to be responsible for the retention of the *mum1* mucilage (Western et al., 2001).

Given the similar phenotypes of *mum1* and *mum2*, it would be interesting to investigate the role of *MUM1* in mucilage modification, and its relationship with *MUM2*.

1.5 The thesis objectives

Given the essential roles of pectin in cell wall architecture, it is important to understand pectin biosynthesis and its regulation, secretion and modification. Therefore the objectives of my thesis are as follows:

PART A: To explore the *mum1* phenotype. Previous studies revealed that *mum1* is defective in releasing mucilage on hydration. Two possible reasons for this phenotype, strengthening of the primary cell wall or modification of mucilage, can be tested. Further, the similar phenotypes of *mum1* and *mum2* raise the question as to whether the functions of the two genes are related.

PART B: To identify the *MUM1* gene. The identity of the *MUM1* gene should provide clues concerning the biochemical role of the gene. In addition, the expression pattern of *MUM1* will suggest when and where the gene product is required in the course of the seed coat development.

PART C: To characterize the activity of MUM1 and its role in pectin biosynthesis. Once *MUM1* is identified, its relationship with other known mucilage-related genes can be determined. The connection between MUM1 and other genes will help to establish how the function of MUM1 fits with other proteins involved in mucilage biosynthesis.

PART D: To identify *mum4* modifier mutants as a means to discover new genes involved in the biosynthesis and/or the secretion of seed coat mucilage, and the biosynthesis of the secondary cell wall. The new genes may provide more information about the biosynthesis and secretion process of mucilage and therefore insight into the mechanism of the biosynthesis of the cell wall.

2 Methods

2.1 Plant material and growth conditions

The *Arabidopsis* ecotypes Columbia-2 (Col-2) and Landsberg *erecta* (Ler) were used as wild type controls. *mum1/luh-5* and *mum2-1* were isolated from an ethyl methanesulfonate (EMS) –mutagenized M3 population of wild type Col-2 *Arabidopsis thaliana* plants (Western et al., 2001). *luh-1* (CS91893), *luh-3* (SALK_107245), *luh-4* (SALK_097509) and *luh-6* (CS90546) were ordered from the *Arabidopsis* Biological Resource Center (ABRC), Ohio State University through TAIR (http://www.arabidopsis.org). *ap2-1*, *gl2-1*, *ttg1-1* and *tt2-1* (Ler ecotype) were obtained from ABRC by Western et al. (2004). The construct 35S:MUM2-GFP was obtained from an earlier study (Dean et al., 2007).

Seeds were placed on AT minimal medium (Haughn and Somerville, 1986) in Petri dishes at 4℃ for 2 days, before being moved to growth chambers at 20℃ under continuous light (90 to 120 μmol $m^{-2}s^{-1}$ photosynthetically active radiation (PAR)). The 7-to-10-day-old seedlings were transferred to prepared soil mix (Sunshine Mix 5; Sun Gro Horticulture), watered once with liquid AT medium, and grown under the same conditions as above.

To isolate different developmental stages of siliques, newly open flowers were defined as 0 day post anthesis (DPA). Open flowers were marked with different colors of nontoxic, water-soluble paint to allow specific developmental stages to be harvested. The

seed coats were collected and used experimentally when they reached the appropriate age (Western et al., 2001; Dean et al., 2007).

The *luh-5/mum1* plants were transformed by the *Agrobacterium tumefaciens*–mediated floral dip method (Clough and Bent, 1998). The transgenic plants were checked for the *mum1* background using the CAPS primers At2g32700 CAPS1/2 (5'-TGAATTACGTAACTGACCAGTGG-3'/ 5'-AGGCTGCTTCATGCGTTCC-3'). The DNA fragments were cut using Pst I which produces 2 bands in the wild type background (87 + 152 bp), but only one band (239bp) in the *mum1* background.

2.2 Microscopy

Seed mucilage was stained by shaking whole seeds in 0.01% (w/v) ruthenium red (Sigma-Aldrich) for 2 h. The seeds were observed using a Leica WILD M8 dissecting microscope. To observe the effects of Ca^{2+} chelators (such as EDTA) and alkali, seeds were shaken in corresponding solutions (for example Na2CO3, see Figure 3-1) for 2 h before being stained with ruthenium red as described above (Dean et al., 2007).

For resin embedding and sectioning, developing seeds were punctured with a needle to allow penetration of the fixative and resin before being fixed with 3% (v/v) glutaraldehyde (Canemco, Montreal, Canada) in 0.5 M sodium phosphate buffer, pH 7. Samples were post-fixed for 1 to 2 h in 1% (v/v) osmium tetraoxide in 0.5 M phosphate buffer, dehydrated using an ethanol solution series and transferred to a solution of

propylene oxide, and then solutions of Spurrs resin (Canemco, Montreal, Canada) in increasing increments for infiltration. Samples were embedded in resin for polymerization at 60℃ in an oven. Seeds were sectioned (0.2 to 0.5 µm) with glass knives on a microtome (Reichert-Jung, Vienna). Sections were mounted on glass slides, and then stained with 1% (w/v) toluidine blue O in a 1% (w/v) sodium borate solution, pH 11 (Western et al., 2000) and examined under a Zeiss AxioScop light microscope (Carl Zeiss, Oberkochen, Germany).

To determine if mucilage would expand from sectioned, hydrated cells, mature dry seeds were added to molten paraplast (Sigma-Aldrich, Saint Louis, USA) at 60 °C. After incubation for 2 h, the paraplast was solidified at room temperature overnight. 20 µm sections were produced on a HM 325 microtome (Microm, Boise, USA), then mounted on slides and hydrated with 0.01% (w/v) ruthenium red and examined as described above (Dean et al., 2007).

Seeds to be examined by SEM were mounted on stubs, coated with gold-palladium in a SEM Prep2 sputter coater (Omicron NanoTechnology, Taunusstein , Germany), and imaged using a Hitachi S4700 scanning electron microscope (Hitachi High-Technologies Schaumburg, IL, USA).

Digital images were cropped and labelled in the softwares ImageJ (National Institutes of Health, DC, USA) and Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

2.3 Positional cloning of *MUM1*

The mapping population of 420 F_2 plants was derived from F_1 plants made by crossing *mum1* (Col-2 background) and wild type Ler. DNA samples of individual F_2 plants were stored by crushing young leaves on FTA classic card (Whatman). Small discs containing samples were punched from FTA cards for PCR reactions (Zhang et al., 2007). Sequence information was obtained from the *Arabidopsis* Genome Initiative (The *Arabidopsis* Genome Initiative, 2000) and Cereon (Jander et al., 2002) at the TAIR website (http://www.arabidopsis.org) to generate simple sequence length polymorphism (SSLP) markers for map-based cloning. Primer sequences are listed in Table 2-1.

The sequences of the *luh-5/mum1-1* mutant allele and Col-2 wild type were determined using 6 sequencing primers for At2g32700 (Table 2-2). Gene sequence was amplified using genomic DNA samples on the FTA classic cards as a template, then sequenced.

Genomic sequences were amplified by primers At2g32700 TF/TR (5'-ATTGCGGCCGCCCGGTTTTGCTTCTTCTTTTTC-3'/ 5'-TTAGCGGCCGCGTTGAAAGAGAGGCAGAGTCATTC-3') with the Not I enzyme site included in both primers in order to conduct transgenic complementation of *luh-5/mum1*. Both the fragment and the vector pART27 were digested with Not I before ligation. The *luh-5/mum1* plants were transformed by the *Agrobacterium tumefaciens*–mediated floral dip method (Clough and Bent, 1998).

Table 2-1 The SSLP maker primers used for map-based cloning of *MUM1*

Primer name	Chromosome	AGI position	F primer	R primer	Ler fragment	Col fragment
NGA361	II	13.2 mb	5'-ACATATCAATATATTAAAGTAGC-3'	5'-AAAGAGATGAGAATTTGGAC-3'	120 bp	114 bp
MASC06579	II	13.51 mb	5'-TTGCAAGGGAAGCTTTGTCT-3'	5'-TTGCTCCAAAATCACGTCAG-3'	98 bp	94 bp
2m13834	II	13.834 mb	5'-GGTTTATTGAGAGTGGAGC-3'	5'-GAATCCTAATCAGAAGAAGAG-3'	82 bp	74 bp
2m13873	II	13.873 mb	5'-TGCAGTGATCAGTTTATAAGG-3'	5'-CTTGCGTCAAATACTAGTTC-3'	103 bp	95 bp
2m13937	II	13.937 mb	5'-TATGTGTGAGGCCAAGAACC-3'	5'-CCACCTCATGCATGTGTTAT-3'	246 bp	279 bp
2m13995	II	13.995 mb	5'-CTCGGCGAACTTCTCCTTC-3'	5'-GTCCAACGTTTCCAATATAAG-3'	96 bp	104 bp
2m1404	II	14.04 mb	5'-TCACCGGTTCAAGATCAGG-3'	5'-CACGCCGGAATTCTACAGG-3'	116 bp	125 bp
2m14315	II	14.315 mb	5'-CTTTCTCACACCAATGCATCC-3'	5'-GTGTTTCATCTTCCAATTTGAG-3'	96 bp	89 bp
MASC06557	II	14.44 mb	5'-GGTCACCTAACTTACCATGG-3'	5'-CTTCAATCACATGATCCTAG-3'	172 bp	180 bp
F3G5II-16	II	15.6 mb	5'-GCTCCTTTATAATGCAAGAATG-3'	5'-CAGTCTCCAACTGTTCTATGTG-3'	125 bp	138 bp

Table 2-2 Sequencing primers of At2g32700

Primer name	Sequence
At2g32700 seq1	5'-TGTTTGGGCTTTTATTCAGG-3'
At2g32700 seq2	5'-ATCAGCAACAAACCATCATGG-3'
At2g32700 seq3	5'-TCTTTCCGTTGCTTGTTGG-3'
At2g32700 seq4	5'-TGTCATGGCCCTAAAACAGC-3'
At2g32700 P1	5'-TCAACAGATTCCACTGCATCC-3'
At2g32700 P2	5'-AAAAGTCCTGAGCTCTCTGC-3'

Sequenced sequences were compared to the databases at TAIR (http://www.arabidopsis.org) and NCBI (http://www.ncbi.nlm.nih.gov/) using BLAST. Sequence alignments were generated using Bioedit software (Hall, 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Gene Runner (version 3.05; Hastings Software Inc., http://www.generunner.net/).

2.4 Extraction of seed mucilage

To an equal amount of seeds (125 mg) 5 mL of water were added, and the suspension gently swirled every 15 min for 1 h. The water extract was removed from the settled seeds; the seeds were rinsed with 2 mL of water and gentle swirling, and the water was combined with the extract. Five mL of 0.2 M and 2.0 M NaOH containing 3 mg ml^{-1} $NaBH_4$ were added sequentially to the settled seeds, with each extraction step repeated as for water. The NaOH extracts were chilled and neutralized with glacial acetic acid, and aliquots saved for carbohydrate analyses. The majority of the extracts was dialyzed against running deionized water for 36 h, and then with several changes of nanopure water for 8 h.

2.5 Monosaccharide and linkage analyses

This part of work was done in the Carpita Lab in Purdue University by Danisha DeBowles. The uronosyl residues in the neutralized and dialyzed mucilage extracts were carboxyl-reduced with $NaBD_4$ after activation with a water-soluble carbodiimide, as described by Kim et al. (1992) and modified by Carpita and McCann (1996). Uronosyl-reduced wall material (1 to 2 mg) was hydrolyzed in 1 mL of 2 M trifluoroacetic acid (TFA) at 120°C for 90 min, and the supernatant was then evaporated in a stream of nitrogen.

The monosaccharides were reduced with $NaBH_4$ and alditol acetates were prepared as described previously (Gibeaut and Carpita, 1991). Derivatives were separated by gas-liquid chromatography (GLC) on a 0.25-mm x 30-m column of SP-2330 (Supelco, Bellefonte, PA). Temperature was held at 80 ℃ during injection, then ramped quickly to 170°C at 25°C min^{-1}, and then to 240°C at 5°C min^{-1} with a 10-min hold at the upper temperature. Helium flow was 1 mL min^{-1} with splitless injection. The electron impact mass spectrometry (EIMS) was performed with a Hewlett-Packard MSD at 70 eV and a source temperature of 250°C. The proportion of 6,6-dideuteriogalactosyl was calculated using pairs of diagnostic fragments *m/z* 187/189, 217/219 and 289/291 according to the equation described in Kim and Carpita (1992) that accounts for spillover of ^{13}C.

For linkage analysis polysaccharides were per-*O*-methylated with Li^+ methylsulfinylmethanide, prepared by addition of *n*-butyllithium to dry dimethyl sulfoxide (DMSO) and methyl iodide according to Gibeaut and Carpita (1991). The per-

O-methylated polymers were recovered after addition of water to the mixture and partitioning into chloroform. The chloroform extracts were washed five times with a three-fold excess of water each, and the chloroform was evaporated in a stream of nitrogen gas. The partly methylated polymers were hydrolyzed in 2 M TFA for 90 min at 120 ℃, the TFA was evaporated in a stream of nitrogen gas, and the sugars were reduced with $NaBD_4$ and acetylated. The partly methylated alditol acetates were separated on the same column as the alditol acetates; after a hold at 80 ℃ for 1 min during injection and rapid ramping, the derivatives were separated in a temperature program of 160 ℃ to 210 ℃ at 2 ℃ per min, then to 240 ℃ at 5 ℃ per min, with a hold of 5 min at the upper temperature. All derivative structures were confirmed by electron-impact mass spectrometry (Carpita and Shea 1989).

2.6 RNA isolation, RT-PCR and qRT PCR

RNA was isolated from plant tissues except siliques using TRIzol reagent (Invitrogen, ON, Canada; Simms et al., 1993). The extraction procedure was adapted (Downing et al., 1992; Western et al., 2004) to extract RNA from siliques because of their high content of polysaccharides. Siliques at specific stages were collected and ground in liquid nitrogen. 1 ml REB (25 mM Tris-Hcl pH 8.0, 25 mM EDTA, 75 mM NaCl, 1% SDS) was added to the dry powder and the RNA extracted with one volume of a decreasing solution series of phenol: CIA (Chloroform:Isoamyl alcohol=24:1) solutions, and finally with CIA. RNA was precipitated with 2 M LiCl on ice. RNA samples were

transcribed with SuperScript II reverse transcriptase (Invitrogen, ON, Canada) according to manufacturer`s instructions. For isolation of RNA specifically from seed coats/endosperm, seed coats of the appropriate stage were separated from the embryo using two pairs of fine forceps in distilled water under dissecting microscope. The seed coat tissue included the single layer of endosperm at later stages. The isolated seed coats were quickly frozen on dry ice and ground in liquid nitrogen. RNAqueous-Micro kit (Ambion) was used to extract RNA. First strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, ON, Canada) according to manufacturer`s instructions.

Gene specific primers (Table 2-3) were used to amplify cDNA fragments spanning an intron. *GAPC* was used as the loading control of RNA samples. *GAPC*, *LUH/MUM1* and *MUM2* were amplified under nonsaturating conditions. SYBR Green Supermix reagent (Bio-Rad, ON, Canada) was used to monitor the real time PCR reactions. qRT-PCR was performed using the MJ Mini Opticon real-time PCR system (Bio-Rad, ON, Canada). Actin2 was used as the internal control. Data were analyzed using Gene Expression Macro software (version 1.1; Bio-Rad, ON, Canada). Reactions were performed in triplicate.

2.7 Protoplast isolation

Arabidopsis mesophyll protoplasts were isolated following the method developed by Wang et al. (Kovtun et al., 2000; Wang et al., 2005). Wild type Col-2 plants were

Table 2-3 Primers for RT-PCR and qRT PCR

Primer name	Sequences
GAPCp1	5'-TCAGACTCGAGAAAGCTGCTAC-3'
GAPCp2	5'-GATCAAGTCGACCACACGG-3'
MUM1RT3F	5'-TGATAGCAACAATGATATTCGC-3'
MUM1RT3R/At2g32700 P2	5'-AAAAGTCCTGAGCTCTCTGC-3'
MUM2/At5g63800 F5	5'-GCAAACGATTCTCTCCTTGG-3'
MUM2/At5g63800 R5	5'-CCATGTAAGCTCCAGAGTCC-3'
MUM1realtime F	5'-CATCCACGAGCTTAGCAACA-3'
MUM1realtime R	5'-GGCCTGCTACCGTCATACAT-3'
MUM2p1	5'-GTTACAACGCCGGTTCAAGT-3'
MUM2p2	5'-ACGTGGACAACATGTCCTGA-3'
Actin2-RT-FW	5'-CCA GAA GGA TGC ATA TGT TGG TGA-3'
Actin2-RT-RW	5'-GAG GAG CCT CGG TAA GAA GA-3'

germinated and grown under the conditions described above. Approximately 1 g of leaves from 4-6 week old plants were collected and cut into 0.5-1 mm strips. The strips were digested in 25 ml of enzyme solution (1% cellulase R10 (SERVA Electrophoresis, Heidelberg, Germany), 0.25% macerozyme R10 (SERVA Electrophoresis, Heidelberg, Germany), 0.4 M mannitol, 80 mM $CaCl_2$, and 20 mM Mes(2(N-morpholino) ethanesulfonic acid), pH 5.7). Vacuum infiltration for 20 min was used to improve digestion. The digestion was conducted in darkness with slow shaking (40 rpm) for 3 hours. Protoplasts were filtered through a 200-mm nylon mesh (Spectrum Laboratories), washed by pre-chilled W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes, pH 5.7), and incubated on ice for 30 min. The protoplasts were pelleted and resuspended in pre-chilled MMg solution (0.4 mM mannitol, 15 mM $MgCl_2$, and 4 mM Mes, pH 5.7) and used for transfection.

2.8 Subcellular localization of MUM1

LUH/MUM1 cDNA was amplified by the primers MUM1 ACT4 F/R (5'-ACGCGTCGACATTAATATGGCTCAGAGTAATTGGGAAGCTGA-3'/ 5'-ACGCGTCGACATCGATCTACTTCCAAATCTTTACGGATTTGT-3'). The fragment was digested with Sal I and introduced into the intermediate vector pBluescript2 SK+ to produce *pBS-LUH*. The *LUH/MUM1* cDNA was excised from pBS-LUH with Cla I and Ase I and ligated into the destination vector (pUC19 containing a GFP sequence driven by the 35S promoter (Dr. Wang, personal communication) digested with Cla I and Nde I

(Ase I and Nde I make compatible ends) to produce the *GFP-LUH* gene. The same plasmid with no insert was used to produce free GFP (unfused to another polypeptide).

Plasmid DNA was prepared by Endofree Plasmid Maxi Kits (Qiagen, ON, Canada). 10 μg of plasmid DNA was used for transfection of mesophyll protoplasts using the polyethylene glycol (PEG) method (Kovtun et al., 2000; Wang et al., 2005). An equal volume of 40% PEG 3350 (Sigma Aldrich) was added to 200 μl of protoplasts (2 X 10^4 protoplasts) together with the plasmid DNA. The PEG solution was removed after incubation for 20 min at room temperature. Protoplasts were resuspended in 1 ml WI solution (0.5 M mannitol, 20 mM KCl, and 4 mM Mes, pH 5.7). After incubation at room temperature for 18-20 hours in darkness, the GFP signals were observed under a Leica MZ6 microscope equipped with a digital camera. The images were manipulated using ImageJ (National Institutes of Health, USA) and Adobe Photoshop (Adobe Systems, CA, USA).

2.9 Transcriptional activity

LUH/MUM1 cDNA was obtained from the subclone *pBS-LUH* described above by digesting with enzymes Cla I and Ase I, and then ligated to the destination vector pUC19 containing the Gal4 binding domain (GD) driven by the 35S promoter (Wang et al., 2005; Wang et al., 2007). LUG was amplified using the primers *LUG* act F/R (5'-TACTATTAATATGTCTCAGACCAACTGGGAAG-3'/ 5'-TTGAGAGCTCTCACTTCCACAGTTTCACTAGCTT-3'), and then linked to the same

destination vector as an Ase I-Sac I fragment. GD, *Gal4-GUS*, *LexA-Gal4-GUS*, *LexA DD (LD)-VP16*, and Chloramphenicol acetyltransferase (*CAT*) plasmids were obtained from Dr. Wang (Tiwari et al., 2003). The plasmid DNA was prepared by Endofree Plasmid Maxi Kits (Qiagen, ON, Canada), and transfected into mesophyll protoplasts by the PEG method as described above. Since different amounts of plasmid DNA were used in transfection assays (1x vs 2x) *CAT* plasmid was used to adjust DNA amounts such that all transfections had the same quantity of DNA. After incubation at room temperature for approximately 20h, protoplasts were lysed by Cell Culture Lysis Reagent (Promega, WI, USA; E153A). 100 µl of 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) was used as the substrate for β-glucuronidase (GUS) reaction to produce 4-methylumbelliferone (4-MU). After incubation for 60 min at 37 ℃, 100 ml of 0.2 M Na_2CO_3 was added to stop the reaction. Fluorescence of 4-MU at 455 nm with excitation at 365 nm was measured from a Fluoroskan Finstruments Microplate Reader (MTX Lab Systems Inc., VA, USA; Jefferson et al., 1987; Fujii and Uchimiya, 1991).

2.10 Screening for *mum4-1* modifiers

The *Arabidopsis mum4-1* seeds were mutagenized with ethylmethane sulfonate (EMS) by Dr. Tamara Western. M_2 seeds from approximately 100 M_1 plants were pooled. M_3 seeds from individual M_2 plants were harvested and examined for seed coat phenotypes separately. Dry seeds were treated with 0.5 M EDTA solution for 1 hour,

before staining with aqueous 0.01% (w/v) ruthenium red (Sigma-Aldrich) for 2 hours and examined under a dissecting microscope.

Candidate *mum4-1* modifiers were examined in the next generation (M_4) in order to check if the phenotypes were heritable. Putative suppressors of *mum4-1* were checked for the possibility of wild type contamination of seed stocks by determining if they were homozygous for the *mum4-1* allele. Genomic DNA of putative suppressor mutants was used as a template for PCR amplification of *MUM4* (primers MUM4 p1/p8), the resulting products digested with of Mse I and fragment sizes determined by gel electrophoresis. The wild type produced 3 bands (230+139+97bp) and *mum4-1* 4 bands (165+139+97+65bp).

During genetic analysis of the modifier mutants, Pearson's chi-square test and Student`s T-test were applied to determine if the phenotype segregations match the expected Mendelian ratios.

3 *mum1* Phenotypic Analysis and Cloning of the *MUM1* Gene

3.1 Introduction

As introduced in chapter 1, *Arabidopsis* seed coats are useful as a system to study aspects of cell wall biosynthesis, for the following reasons: 1. seed coats release mucilage upon imbibition; 2. the major component of mucilage is pectin; 3. mucilage is easily observed under the microscope after staining; 4. the seed coat mucilage is dispensable under the laboratory conditions. Thus the seed coat system can be used to identify mutants defective in pectin biosynthesis, and, subsequently, the corresponding genes which provide insight into the process of pectin production. In an effort to identify seed coat mucilage mutants, M_3 seed from a population of 1000 ethylmethane sulfonate (EMS)-mutagenized lines of *Arabidopsis* were checked for a mucilage phenotype. Twelve mutants with altered seed coat mucilage, representing five loci, were identified. The mutants were named *mucilage-modified* (*mum1*) through *mum5* (Western et al., 2001).

The *mum1* and *mum2* mutants were characterized by the inability to release mucilage on hydration (Western et al., 2001). Since the phenotypes could result from changes in the composition and/or the amount of mucilage, *mum1* and *mum2* were checked for mucilage polysaccharide content. Whole seeds were ground for the comparison between wild type and the mutants, because neither *mum1* nor *mum2* extrude

mucilage in water. Both *mum1* and *mum2* showed a similar profile of the monosaccharide to that of wild type seeds, although *mum1* had a slightly increased content of most sugars, compared to wild type seeds. The significance of this increase is unclear.

The *mum2* mutant has been further characterized and the *MUM2* gene cloned. *MUM*2 encodes a β-galactosidase (Macquet et al., 2007b; Dean et al., 2007). The seed coat development of *mum2-1* was identical to that of wild type. Carbohydrate linkage analysis of Na_2CO_3-extracted mucilage indicated that the mucilage RG I of *mum2-1* has more side chain branches than wild type, and mucilage monosaccharide analysis revealed a relative increase in RG I side chain sugars galactose and arabinose to backbone sugars rhamnose and galacturonic acid. Thus MUM2 is proposed to remove side-chain galactose sugars and this impacts, in some way, the ability of mucilage to swell in water.

The primary focus of my thesis is the characterization of the *mum1* mutant. In this chapter, I describe the phenotype analysis, the positional cloning of *MUM1* and the characterization of the *MUM1* expression pattern and subcellular localization. Since the positional cloning data indicated that *MUM1* encodes a putative transcription factor that is a homologue of a known transcriptional repressor, the transcriptional activity of MUM1 was also tested.

3.2 Results

3.2.1 The seed coat mucilage phenotype of *mum1*

When dry mature seeds of wild type are put into water, a thick layer of mucilage extrudes from the epidermal cells. The mucilage can be stained with ruthenium red, and observed easily under a light microscope. However, no visible mucilage was extruded by *mum1-1* seeds under these conditions (Figure 3-1). This phenotype is similar to that of *mum2*. *mum2* can release some mucilage when treated with chelators or alkali (Dean et al., 2007). Chelators help to release mucilage by extracting the Ca^{2+} that cross-links the unesterified GalA, the monosaccharide comprising homogalacturonan (HG). Alkali can hydrolyze ester-linked GalA. HG is one of three major components of pectin. It is partially methylesterified at the C-6 position of GalA (Fry, 2000). We tested the ability of both chelators and alkali to allow for the extrusion of mucilage from *mum1-1* seeds. Similar to their effect on unextruded *mum2-1* mucilage (Dean et al., 2007), EDTA, EGTA and CDTA at 0.05M, the weak alkali Na_2CO_3 (1M), and strong alkali KOH (0.5M) resulted in the release of *mum1* mucilage (Figure 3-1), except that *mum1-1* seeds released more mucilage than *mum2-1* when treated with the same chemical. These data suggest that, as for the *mum2* mutant, the *mum1* mutant synthesizes mucilage but the mucilage does not extrude.

Some mucilage mutants like *mum4/rhm2* have morphological defects such as a flattened columella (Western et al., 2001; Western et al., 2004; Usadel et al., 2004) that can be observed using scanning electron microscopy (SEM). For this reason, I compared

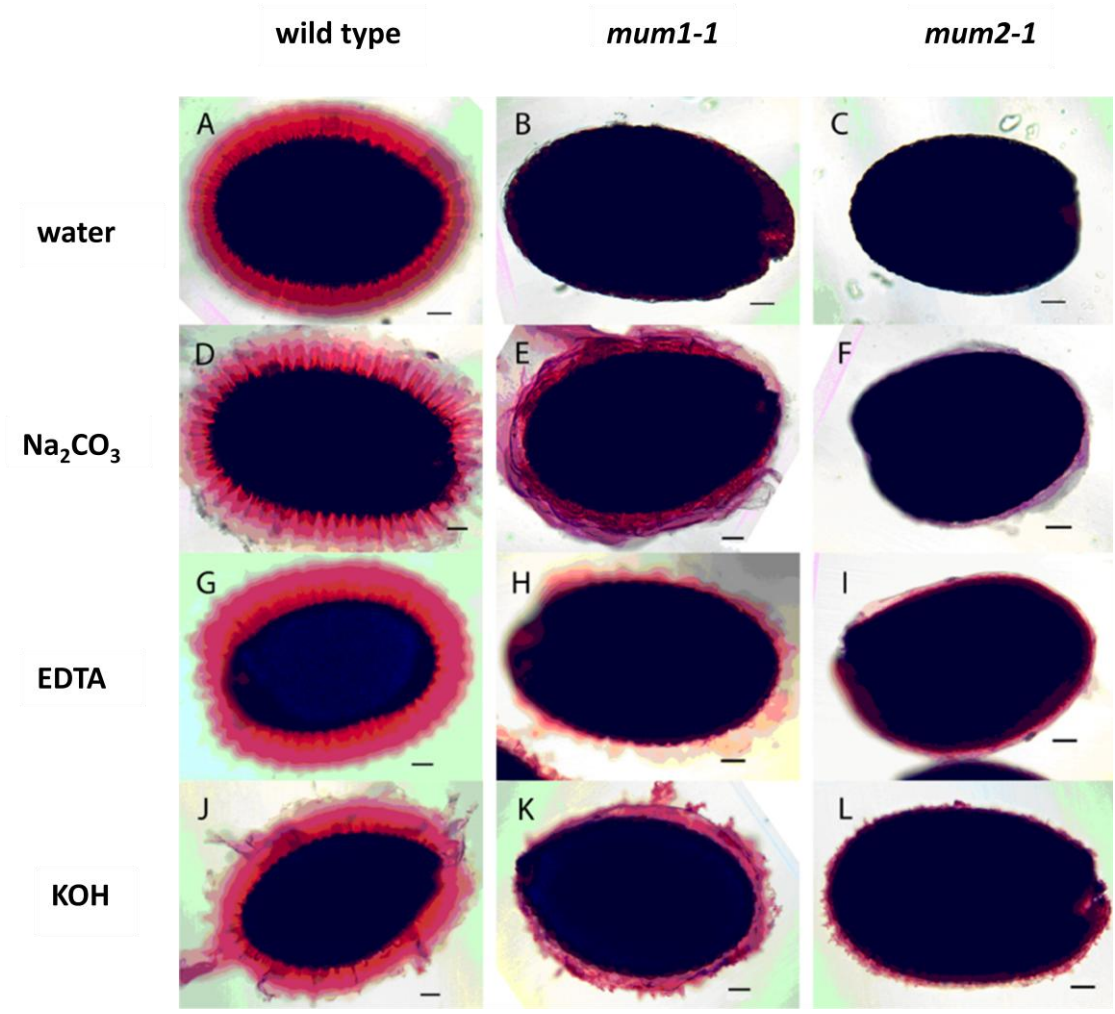

Figure 3-1 Seed coat mucilage phenotypes of wild type, *mum1-1* and *mum2-1*

mum1 mucilage lacks the capability to extrude following exposure to water. All the seeds were stained with ruthenium red after treatment with the indicated solutions. *mum1-1* and *mum2-1* could not release any mucilage when treated with water, unlike wild type seeds which could form a capsule of mucilage surrounding the seeds. Both mutants released a small amount of mucilage in other chemical solutions. *mum1-1* released more mucilage than *mum2-1* under the same treatment. Scale bars=50 μm.

the cell surface features between wild type and *mum1* seeds using SEM, but no differences were observed (Figure 3-2).

The *mum1* seed coat mucilage phenotype might have cytological developmental defects that would provide clues concerning the timing and nature of MUM1 function. To examine the cell structure during development, seeds of wild type and *mum1-1* at 4, 7 and 10 DPA were observed using light microscopy. When examined in this way, the development of wild type and *mum1* were found to be indistinguishable (Figure 3-3).

In order to investigate if the failure of *mum1* seed mucilage to extrude is due to an inability of the mucilage to expand when exposed to water, wild type and *mum1-1* mature seeds were embedded in paraffin wax without fixation, and then sectioned (Macquet et al., 2007b; Dean et al., 2007). The thickness of the sections (20 μm) ensured that most of the seed coat epidermal cells were not intact, exposing the mucilage without the primary cell wall as a barrier. The sections, mounted on slides, were exposed to an aqueous solution containing the stain ruthenium red. The mucilage extruded from wild type but not from the *mum1* sections (Figure 3-4). These results suggest that the *mum1* mucilage, like that of *mum2* (Dean et al., 2007; Macquet et al., 2007b) has lost the capability to expand, although we cannot exclude the possibility that, in addition, the phenotype results from stronger tangential primary cell walls.

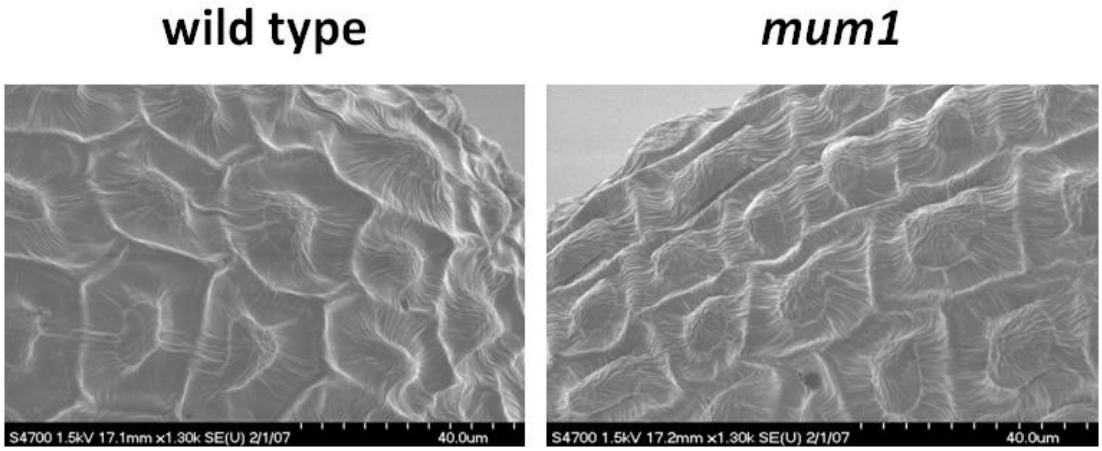

Figure 3-2 Scanning electron microscopy of wild type and *mum1* seeds

Mature dry seeds of wild type and *mum1* show similar epidermal-cell surface features under the scanning electron microscope.

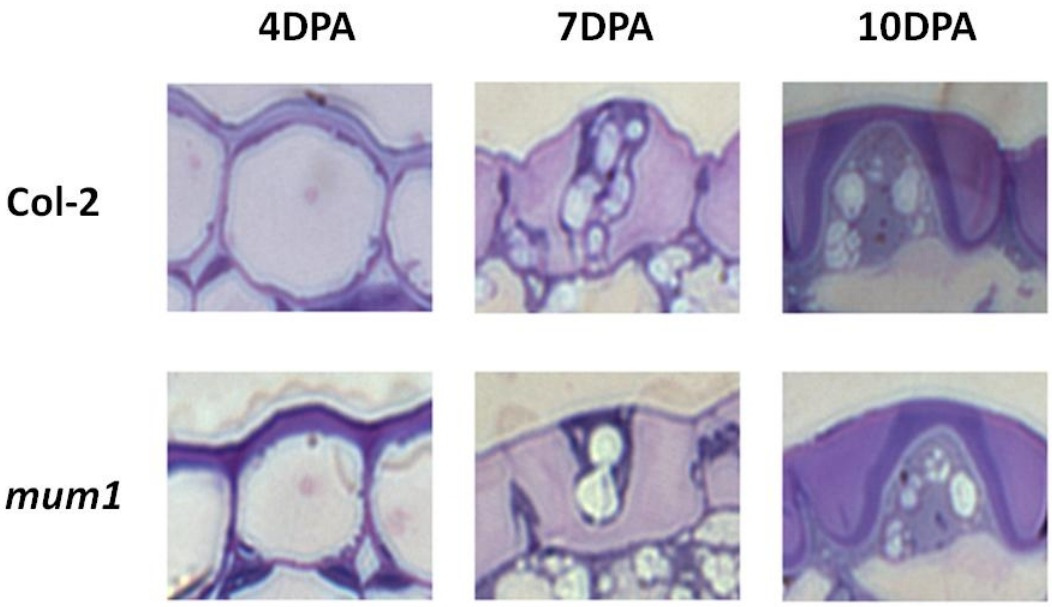

Figure 3-3 Seed coat development of wild type and *mum1*

Developing seeds at 4, 7 and 10 DPA were fixed, sectioned and stained with toluidine blue. The seed epidermal cells at these stages were examined using light microscopy. The cytological characteristics of developing *mum1* seed coat epidermal cells are similar to that of wild type.

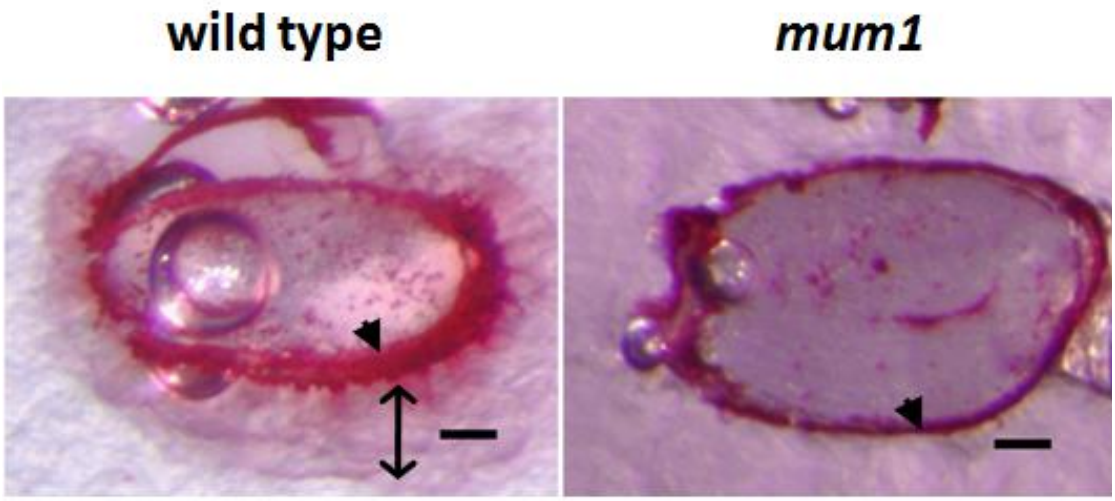

Figure 3-4 Test for seed coat mucilage expansion

mum1 mutant mucilage lacks the capability of expanding following hydration. Mucilage expands (indicated by double-headed arrow) from sections of wild type seed stained with ruthenium red while mucilage in sections of *mum1* seed stays in the mucilage pockets (indicated by arrowhead). Scale bars = 50 μm.

3.2.2 Chemical analysis of *mum1* mucilage

The composition and structural analysis of mucilage reveals differences between *mum1* mucilage and wild type. Given the similar mucilage extrusion phenotypes of *mum1* and *mum2*, *mum2* mucilage was also included for comparison. This part of work was done in the Carpita Lab at Purdue University by Danisha DeBowles. The amount of mucilage released sequentially in water, 0.2 M NaOH, and 2 M NaOH was determined as uronic acid equivalents (Figure 3-5). Only the wild type released significant amounts of mucilage in water. However, 0.2 M NaOH caused rupture of the epidermal wall to release large amounts of mucilage from *mum1* seeds and lesser amounts from *mum2* seeds. The 0.2 M NaOH is not strong enough to remove the gel layer tightly held by wild type and mutant seeds, but 2 M NaOH strips this layer from wild type and mutant equally (Figure 3-5). Because of the differences in mucilage extraction profiles, monosaccharide and linkage analyses were performed to determine the compositions. After extraction of the mucilage fractions in water, 0.2 and 2 M sodium hydroxide, neutralized and dialyzed preparations were reduced with sodium borodeuteride to label former uronic acids as their *6,6-di-deuterio*-sugar residues. Monosaccharide analysis showed that for water extracts, wild type released a high proportion of Rha and GalA, indicative of RG I, whereas the small amounts of material from *mum1* and *mum2* were mostly HG, as judged by high proportions of GalA compared to vanishingly small amounts of Rha (Table 3-1). Addition of 0.2 M NaOH caused disruption of the outer seed coat wall and release of large amounts of material containing primarily Rha and GalA from the mutants as well as additional mucilage from wild type (Figure 3-5, Table 3-1). The tightly held gel layer

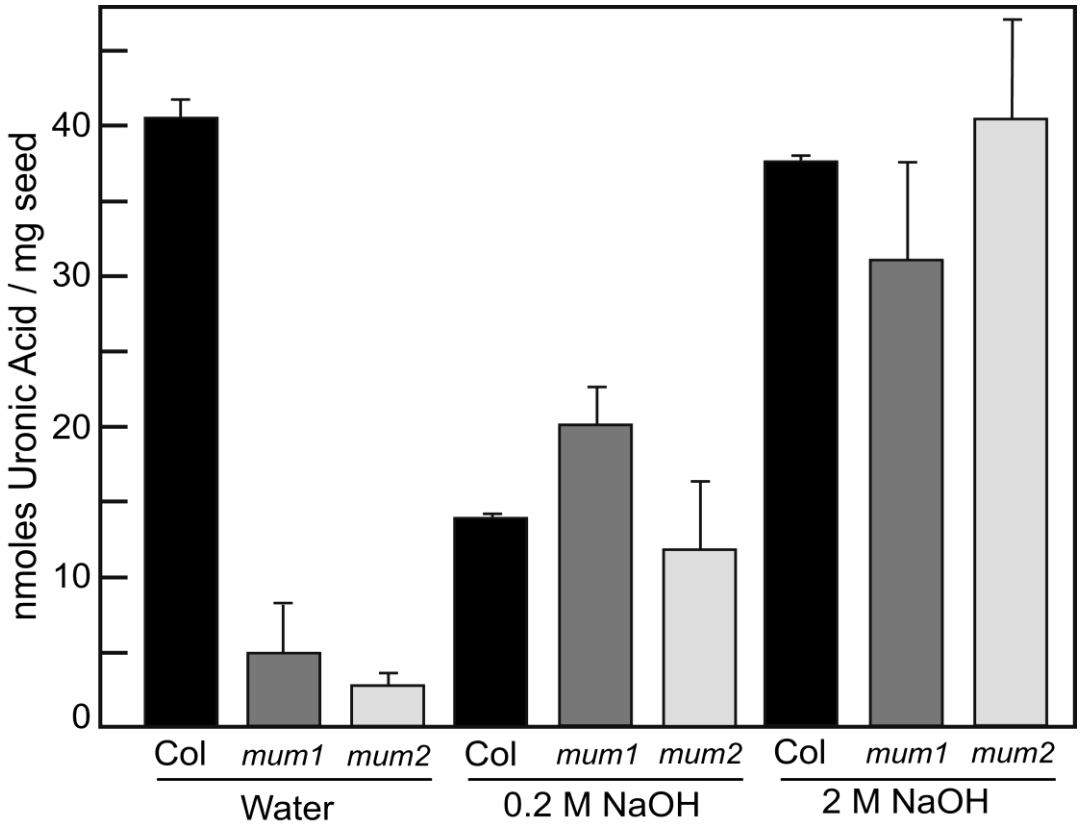

Figure 3-5 The relative uronic acid/fraction of wild type, *mum1-1* and *mum2-1*

Mature dry seeds were treated with water, 0.2 M NaOH, and then 2.0 M NaOH sequentially, in order to extract mucilage. These extracts after dialysis were carboxyl reduced with NaBD4 to reveal uronic acids as their respective neutral sugars. The error bars represent the SD from 3 biological replicates.

Table 3-1 Monosaccharide distribution in mole percentage of carboxyl-reduced mucilage polysaccharides from seeds successively extracted with water, 0.2 M NaOH, and 2.0 M NaOH

Extract	Water			0.2 M NaOH			2.0 M NaOH		
Genotype	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*
Rha	40.9 ±3.3	3.9 ±0.3	5.8 ±4.0	37.7 ±5.1	31.9 ±4.2	27.6 ±1.7	33.9 ±0.7	29.6 ±5.5	27.2 ±1.6
Fuc	trc	trc	trc	trc	trc	trc	trc	trc	trc
Ara	0.4 ±0.0	2.3 ±0.4	2.2 ±0.2	0.8 ±0.0	2.3 ±0.8	4.1 ±1.6	4.7 ±1.3	2.8 ±1.2	6.7 ±0.2
Xyl	2.4 ±0.0	3.2 ±0.3	3.8 ±0.6	3.1 ±0.0	3.2 ±0.7	2.0 ±1.1	6.8 ±0.5	6.8 ±2.0	8.1 ±1.3
Man	0.6 ±0.0	3.8 ±0.8	4.6 ±0.2	1.3 ±0.1	1.8 ±0.8	2.0 ±0.7	5.1 ±0.0	6.0 ±1.9	6.4 ±2.2
Gal	3.9 ±0.1	7.5 ±0.8	6.7 ±0.6	5.0 ±0.4	9.3 ±1.7	12.1 ±0.2	7.3 ±0.7	10.3 ±0.4	10.4 ±0.4
Glc	1.2 ±0.0	11.4 ±1.0	17.3 ±5.7	2.3 ±0.3	4.5 ±2.6	3.7 ±0.6	7.9 ±0.1	9.0 ±3.2	9.6 ±3.5
GalA	50.8 ±3.0	68.1 ±3.4	59.8 ±2.8	49.9 ±4.3	47.2 ±1.1	48.7 ±3.3	34.5 ±1.9	35.6 ±2.3	31.7 ±4.8

Values are mean ±variance of two independent extractions; tr = trace amounts less than 0.05%

Table 3-2 Comparison of linkage distribution in mucilage and other polymers extracted from seeds sequentially with water, 0.2 M NaOH, and 2.0 M NaOH (to be continued)

Extraction	Water			0.2 M NaOH			2.0 M NaOH		
Sugar	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*
Fucose:									
t-Fuc	tr	tr	tr	tr	tr	tr	tr	tr	tr
Rhamnose:									
t-Rha	0.1	tr	tr	tr	0.2	0.2	0.5	tr	0.5
2-Rha	38.7	2.8	5.2	35.7	25.6	22.3	31.0	23.2	21.4
2,3-Rha	0.6	0.3	0.2	0.8	0.5	0.4	0.8	0.4	0.5
2,4-Rha	1.5	0.8	0.4	1.2	5.6	4.7	1.6	6.0	4.8
Arabinose:									
t-Ara*f*	0.1	1.0	0.1	0.7	1.5	2.8	2.7	1.9	3.7
2-Ara*f*	n.d.	tr	tr	n.d.	tr	tr	0.7	0.2	0.8
3-Ara*f*	n.d.	tr	tr	n.d.	tr	tr	tr	tr	tr
5-Ara*f*	0.3	1.2	2.0	0.1	0.7	1.0	1.3	0.7	1.9
2,5-Ara*f*	n.d.	tr	tr	n.d.	tr	0.2	tr	tr	0.1
3,5-Ara*f*	n.d.	tr	tr	n.d.	tr	tr	tr	tr	0.2

Values are mean of two samples, with variance less than 5% for all samples. Values are scaled to monosaccharide analysis in Table I; n.d. = not detected, and tr = trace amounts less than 0.05%.

Table 3-2 Comparison of linkage distribution in mucilage and other polymers extracted from seeds sequentially with water, 0.2 M NaOH, and 2.0 M NaOH (continuing)

Extraction	Water			0.2 M NaOH			2.0 M NaOH		
Sugar	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*
Xylose:									
t-Xyl	0.3	tr	0.2	0.4	0.4	0.2	1.0	0.5	0.6
2-Xyl	0.1	0.2	0.3	0.3	0.3	0.2	0.6	0.5	0.7
4-Xyl	0.7	1.2	1.7	1.5	1.5	0.8	3.1	3.4	3.7
2,4-Xyl	1.1	1.5	1.4	0.8	0.9	0.7	1.9	2.1	2.7
3,4-Xyl	0.2	0.3	0.2	0.1	0.1	0.1	0.2	0.3	0.4
Mannose:									
t-Man	tr	tr	tr	tr	tr	tr	tr	tr	tr
4-Man	0.5	3.4	3.7	1.1	1.5	1.7	1.3	1.3	2.1
4,6-Man	0.1	0.4	0.9	0.2	0.3	0.3	3.8	4.7	4.3
Galactose:									
t-Gal	tr	2.8	1.8	3.2	8.4	8.6	6.8	9.7	9.4
3-Gal	tr	tr	tr	tr	tr	0.5	tr	tr	tr
4-Gal	3.7	4.7	4.9	1.5	0.1	0.3	tr	0.5	0.4

Values are mean of two samples, with variance less than 5% for all samples. Values are scaled to monosaccharide analysis in Table I; n.d. = not detected, and tr = trace amounts less than 0.05%.

Table 3-2 Comparison of linkage distribution in mucilage and other polymers extracted from seeds sequentially with water, 0.2 M NaOH, and 2.0 M NaOH (continuing)

Extraction	Water			0.2 M NaOH			2.0 M NaOH		
Sugar	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*
6-Gal	n.d.	tr	tr	tr	tr	0.5	tr	tr	tr
3,4-Gal	0.2	tr	tr	0.3	0.8	0.6	0.3	0.1	0.6
3,6-Gal	tr	tr	tr	tr	0.1	1.6	0.2	tr	tr
Glucose:									
t-Glc	0.1	0.8	2.2	0.2	0.3	0.1	0.7	0.9	0.9
4-Glc	0.7	9.8	10.8	1.5	2.3	2.5	5.3	5.3	5.7
4,6-Glc	0.4	0.8	4.3	0.6	1.9	1.1	1.9	2.8	3.0
Galacturonic Acid:									
t-GalA	tr	0.2	1.7	1.2	2.9	2.8	2.7	0.1	0.3
4-GalA	46.7	67.1	55.4	46.4	40.4	41.8	29.3	31.4	26.3
3,4-GalA	4.1	0.8	2.7	2.3	3.9	4.1	2.5	4.1	5.1

Values are mean of two samples, with variance less than 5% for all samples. Values are scaled to monosaccharide analysis in Table I; n.d. = not detected, and tr = trace amounts less than 0.05%.

extracted by 2 M NaOH was similar in monosaccharide distribution between wild type and mutant. The gel layer is rich in Rha and GalA but also contains other sugars in greater abundance such as Xyl, Ara, Gal, and Glc.

Carbohydrate linkage analyses confirmed that the mucilage released in wild type and mutant was primarily 2-Rha and 4-GalA, representing a relatively unbranched RG I backbone (Table 3-2). The presence of large amounts of primarily 4-GalA in the water extracts of *mum1* and *mum2* seeds confirms that a small amount of HG was the principal material present (Table 3-2). In contrast, the 0.2 M NaOH causes substantial uronic acid-rich material to be released from the seed coats from both *mum* mutants, and linkage analysis shows most of the carbohydrate to be RG I. Additional amounts of mucilage are also released from wild type. Notably, the degree of branching of the RG I, as determined by the ratio of 2,4-Rha : 2-Rha, was substantially higher in both *mum* mutants compared to wild type; *t*-Ara and *t*-Gal residues in both *mum*s were higher, accounting for the differences in Rha branch point residues. The 2 M NaOH extracts of both *mum* mutants of the gel layers also display increased RG I branching; whereas increases in *t*-Gal residues account for much of the increases in branching, increases in *t*-Ara over wild type amounts were found only in *mum*2 extracts (Table 3-2).

3.2.3 Positional cloning of *MUM1*

The *mum1* mutant was isolated from an EMS-mutagenized seed population (Western et al., 2001). A backcross to wild type indicated that *mum1* is a recessive

mutant (Western et al., 2001). Map-based cloning was used to identify the *MUM1* gene. I generated a *mum1* mapping population by crossing *mum1* (*Col-2* ecotype) and *Landsberg erecta* (*Ler*). Since the seed coat is maternal tissue, the phenotype of F_2 plants (F_2 lines) was scored by examining the F_3 progeny of individual plants.

Thirty-nine F_3 lines showing the *mum1* phenotype were used to determine the chromosome on which *MUM1* is located. Seven simple sequence length polymorphism (SSLP) markers spread over all the 5 chromosomes were tested for linkage with *MUM1*. The marker NGA168 showed high linkage with *MUM1* indicating that the *MUM1* gene is located on Chromosome II. More SSLP markers from chromosome II, including T30L20II-10, F6K5II-13, NGA361 and F3G5II-16 were used to confirm the linkage. Fine structure mapping was then done with 420 F_3 lines having either wild type or *mum1* phenotypes using 15 SSLP markers (Figure 3-6). The region containing *MUM1* was positioned between the molecular markers 13834 and 13937. There were 28 open reading frames located in this 103Kb interval. 29 available SALK insertion lines for the 28 genes were obtained and checked for seed coat mucilage phenotypes. Only the seeds of SALK_107245 showed a *mum1* phenotype suggesting that the gene mutated in this line, At2g32700, is *MUM1*. When At2g32700 from *mum1* and wild type are sequenced, a C-to-T transversion was identified. This nucleotide change is predicted to be a nonsense mutation that changes the 97th codon encoding a Glutamine to a Stop.

We identified five alleles of At2g32700 from available T-DNA insertional (SALK_107245C and SALK_097509) and TILLING mutant lines (luh_172H3, luh_147A6 and CS90546; Figure 3-7; Seattle TILLING Project, http://tilling.fhcrc.org; Alonso et al., 2003). Each allele had a phenotype similar to *mum1-1* (Figure 3-8; data not

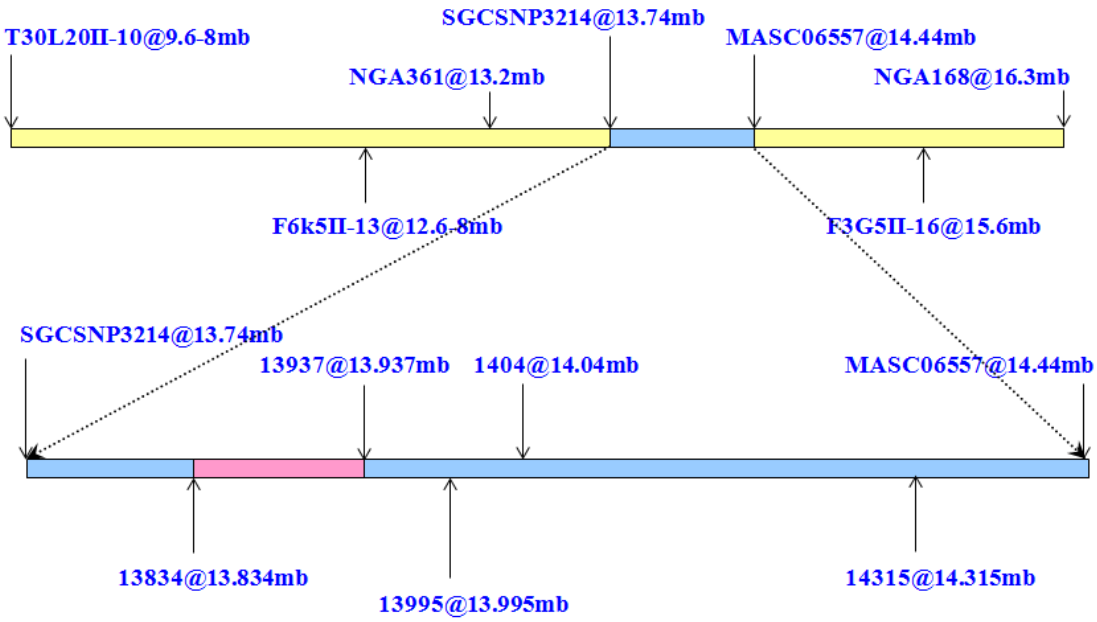

Figure 3-6 The positional cloning of *MUM1*

The genetic map containing the *MUM1* gene on the Chromosome II of *Arabidopsis* is represented schematically. The positions of SSLP markers on the chromosome II are indicated with arrows as well as numerically by the number following the @ symbol. The bottom map is an expanded view of the blue region in the top map. The pink region is the smallest interval obtained for the *MUM1* locus.

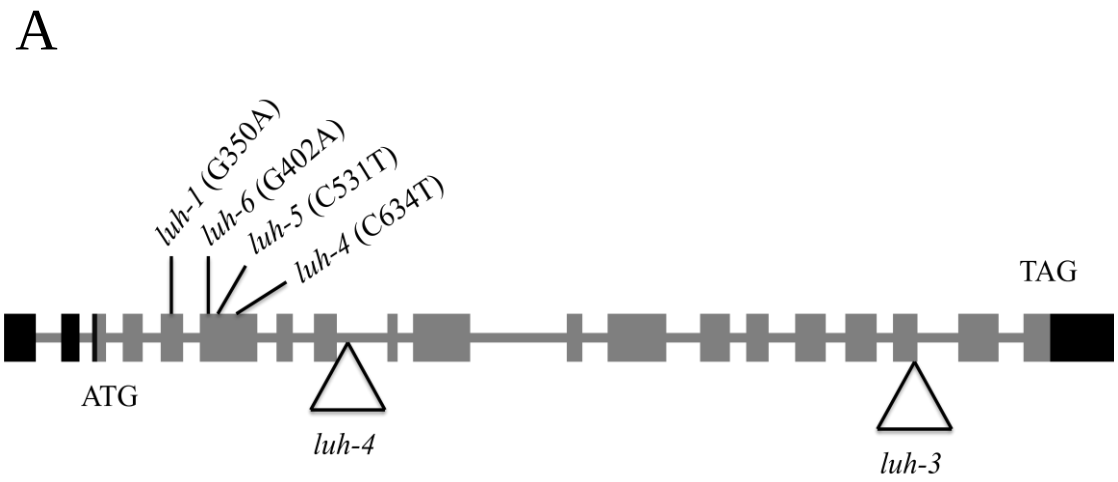

Figure 3-7 LUH/MUM1 gene and protein structure (to be continued)

B

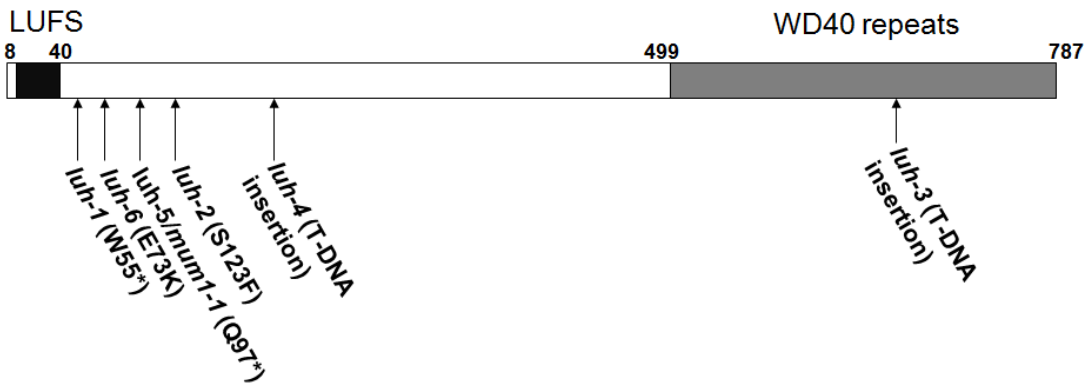

Figure 3-7 LUH/MUM1 gene and protein structure (continuing)

A. The exons of the *LUH/MUM1* gene are represented as bars, linked by lines representing introns. The coding regions are labelled in grey, from ATG to TAG. The untranslated regions (UTR) are labelled in black. The positions of the mutations of various alleles are shown.

B. The predicted LUH/MUM1 protein is 787 amino acids in length. The numbers above the bar represent the amino acid position. The LUFS domain (black bar) is located at the N-terminus, and WD40 repeats (grey bar) at the C terminus (http://smart.embl-heidelberg.de/). The arrows indicate the positions of the mutations of various alleles.

Figure 3-8 The phenotype of the weak allele of *luh-5*/*mum1-1*

Mucilage phenotype of *luh-6*. *luh-6* seeds extrude much less mucilage than wild type after shaking in water and staining in ruthenium red but more than *luh-5/ mum1-1* seeds.Scale bars = 50 μm.

shown). Sequence analysis of At2g32700 suggested that the gene encodes a transcription factor related to *LEUNIG* (*LUG*) named *LEUNIG_HOMOLOG* (*LUH*) with mutant alleles *luh-1, luh-2, luh-3* (luh_172H3, luh_147A6, SALK_107245C respectively; Sitaraman et al., 2008) and *luh-4* (SALK_097509; Stahle et al., 2009). Further, crosses between *mum1-1* and the known *luh* alleles *luh-1* and *luh-4* produced F_2 progeny that failed to extrude mucilage confirming that all three mutants represent *luh* alleles. We designate the two new alleles as *luh-5* (*mum1*), and *luh-6* (CS90546). The latter, a missense allele causing a change of Glu_{73} to Lys is a weak allele that results in the release of some mucilage when treated with water (Figure 3-8).

To confirm that the mutation in At2g32700 was responsible for the *mum1* phenotype, molecular complementation of *mum1* was done. Wild type *MUM1* genomic sequence, including 2.6 kb of 5` sequence, a 4.6 kb ORF and 0.6 kb of 3` sequence was cloned into the binary transformation vector pART27 and transformed into *mum1* plants via Agrobacterium transformation. The *mum1* mutant plants transformed with the *MUM1* gene had a wild type phenotype while those transformed with the empty vector showed no such rescue (Figure 3-9).

The open reading frame of *LUH* encodes a predicted protein of 787 amino acids. The N terminus of the predicted protein is defined as the LUFS domain, since this domain is found to be conserved in LUG, LUH, yeast Flo8, and human SSDP (for single-stranded DNA-binding protein). The function of the LUFS domain is still unclear, although in LUG, it is crucial for interaction with the cofactor SEUSS (SEU; Sridhar et al., 2004). The LUFS domain contains a LisH (Lissencephaly homology) domain that

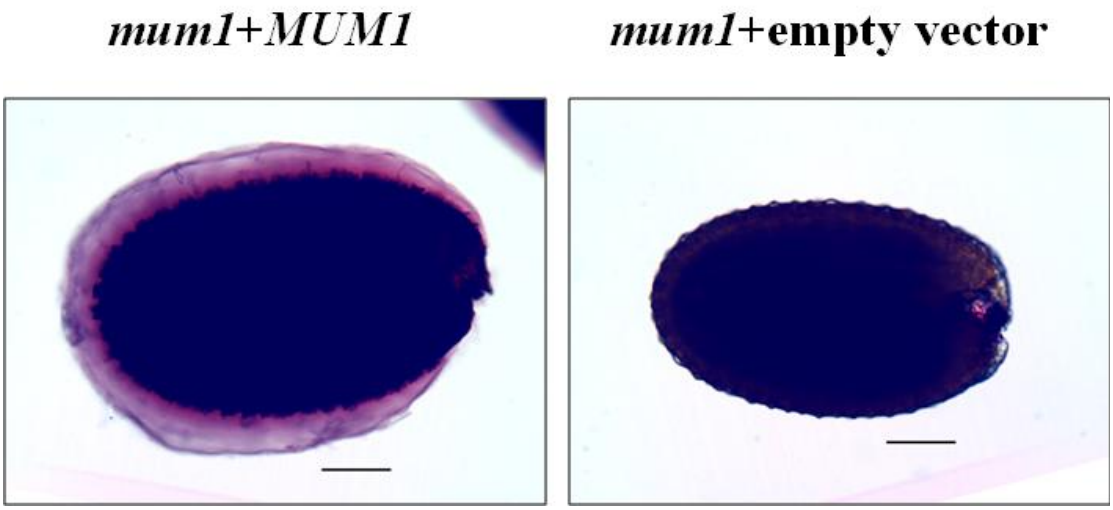

Figure 3-9 Complementation test of *MUM1*

The *MUM1* genomic sequence including sequences both upstream and downstream of *MUM1* was cloned into the vector pART27. Both this construct and the empty plasmid were transformed into *mum1* via Agrobacteriam. Only the plants with MUM1 showed a mucilage phenotype different from *mum1*. Scale bars=100 μm.

retains protein-binding function to result in protein dimerisation and tetramerisation (Cerna and Wilson, 2005). The C terminus contains several WD40 repeats. The WD40 repeat is usually 40 amino acids long, with a Tryptophan -Aspartic acid (W-D) dipeptide at the end. This motif is believed to be involved in protein-protein interactions. (http://smart.embl-heidelberg.de/; Figure 3-10). There are Q (Glutamine)-rich regions between the LUFS and WD40 domains (Sitaraman et al., 2008).

3.2.4 *MUM1* expression pattern

To understand the relationship between *LUH* and the *mum1* phenotype, it is important to investigate when and where the *LUH* gene is expressed in the course of the seed development. I used reverse transcriptional (RT) PCR to determine the qualitative pattern of *MUM1* expression among the major plant organs: stem, root, leaf, and silique and quantitative reverse transcription PCR (qRT-PCR) to measure the transcript level quantitatively.

RT-PCR data showed that *LUH* transcript was present in all tissues examined (siliques at 7 DPA, rosette leaves, cauline leaves, roots (6 days after germination), stems and open flowers; Figure 3-11A).

In order to investigate more accurately the *LUH* expression in the seed coat, qRT-PCR analysis was used. RNA was isolated from the seed coats at 4, 7 and 10 DPA. Because it is difficult to remove the endosperm from the seed coat at later stages of the

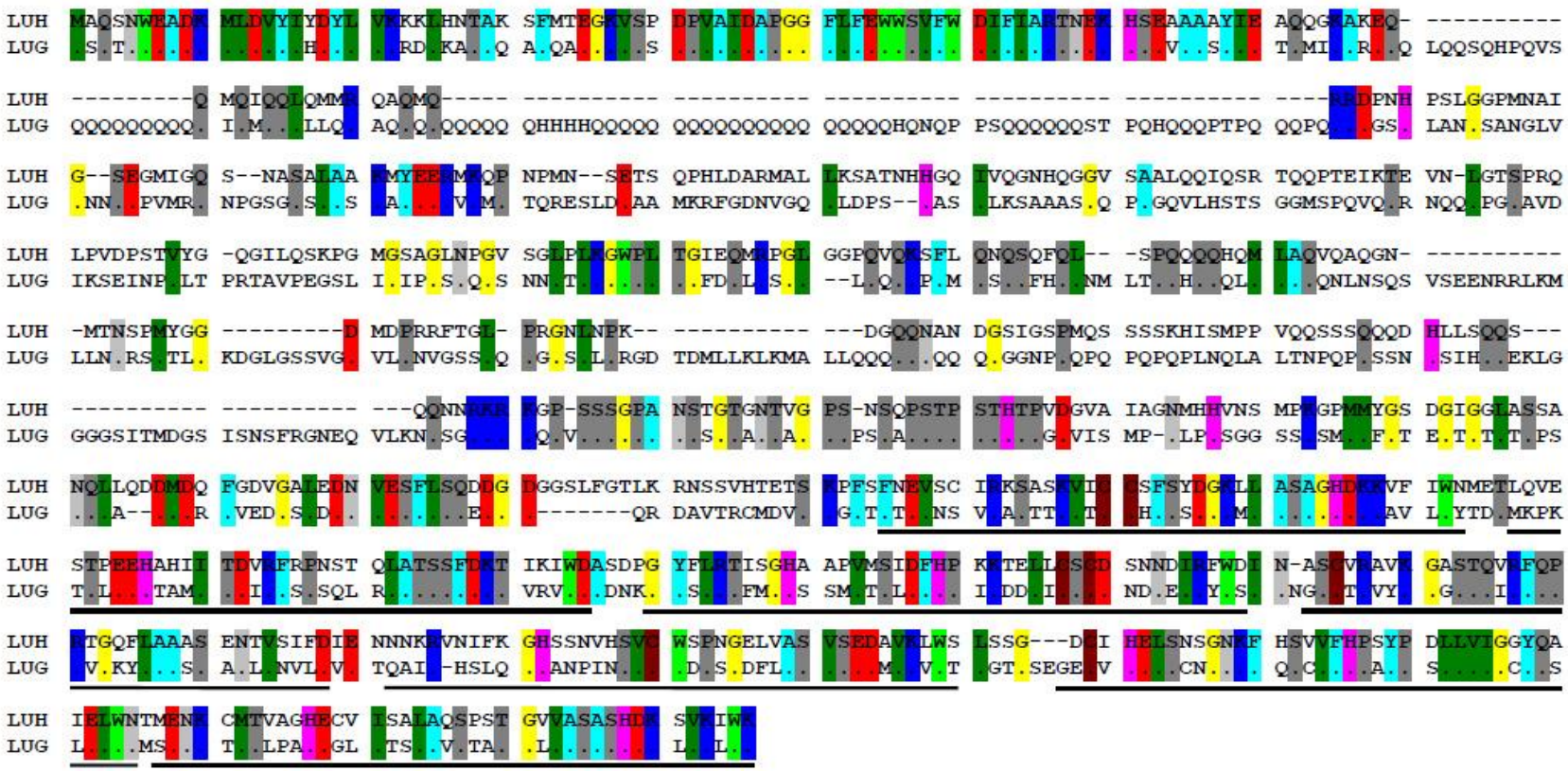

Figure 3-10 The alignment of LUH and LUG proteins

The predicted protein sequences of LUH and LUG were aligned in the BioEdit software. Identical residues are in colour and represented as dots in the LUG sequence. The dashes represent the gaps. The WD40 repeats at the C terminus are underlined.

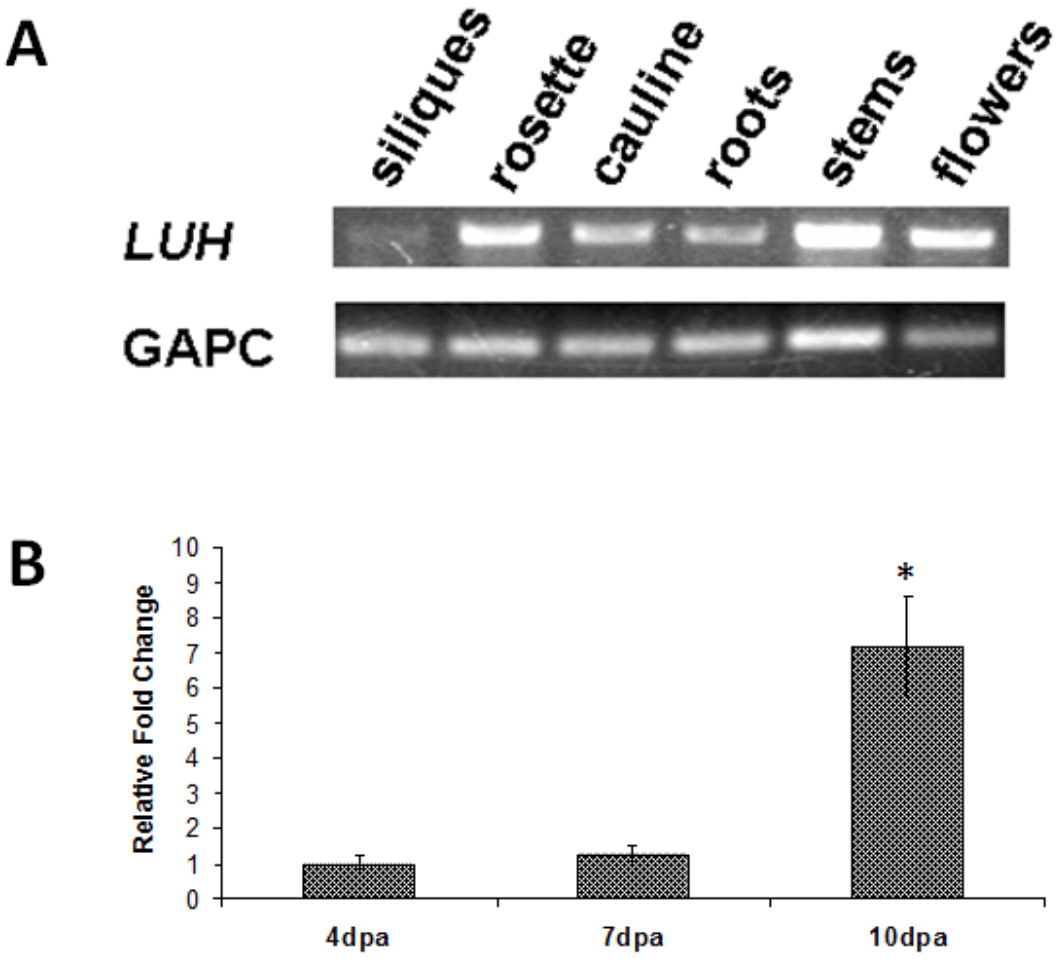

Figure 3-11 LUH expression analyses

(A) LUH expression in the indicated tissues was determined by RT-PCR. *LUH* was expressed in all the tissues examined. *GAPC* was used as the loading control.

(B) The amount of *LUH* transcripts in three stages (4, 7 and 10 DPA) of seed coat development were examined by qRT-PCR. The data are presented as the relative change, where the *LUH* expression level at 4DPA was set as 1.0. *LUH* was expressed at all stages but most highly at 10 DPA. The error bars indicate SD derived from three independent biological replicates. The star indicates a significant difference from the expression level of 4 DPA assessed by Student`s t test ($T=7.4475$, $P<0.01$).

seed development, the gene expression data in seed coat includes expression in the endosperm. The results obtained from 3 biological replicates show that *LUH* is expressed during all stages of the seed coat development examined with the highest expression at 10 DPA (Figure 3-11B).

3.2.5 Subcellular localization of MUM1

The *LUG* gene product is located in the nucleus where it acts as a transcriptional repressor limiting the expression of *AGAMOUS* (*AG*), the C class floral homeotic gene, to the outer whorls of a flower (Liu and Meyerowitz, 1995).

As a protein with high sequence similarity to LUG, I asked whether LUH might also be localized to the nucleus. To determine the subcellular localization of LUH, a *35S:GFP-LUH* chimeric gene was expressed in mesophyll protoplasts (Sheen, 2002) and the subcellular location of GFP observed using fluorescence miscroscopy. DAPI staining was used to indicate the position of the nucleus. My results (Figure 3-12) confirmed that the subcellular localization of LUH is in the nucleus.

3.2.6 The transcriptional regulatory activity of LUH

To determine whether *LUH* acts as a transcriptional repressor or activator, the gene was fused in frame to the gene encoding Gal4 DNA binding domain (*GD-LUH*). In the test for a transcriptional repressor, LD-VP16 (LexA binding domain-VP16) and

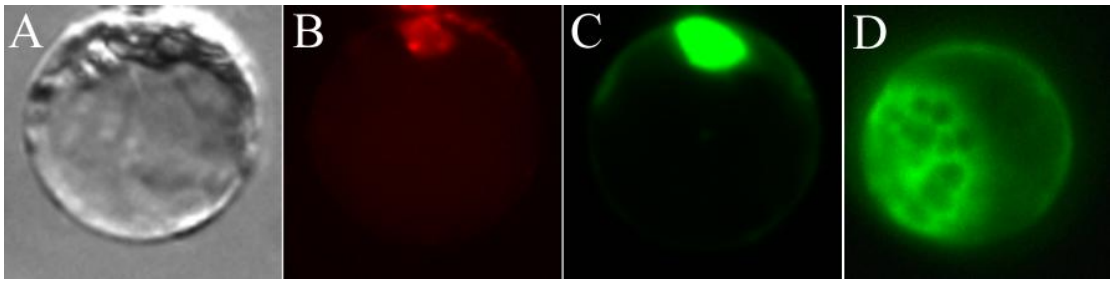

Figure 3-12 Subcellular localization of LUH

(A) Arabidopsis mesophyll protoplast observed by Nomarski optics.

(B) Localization of protoplast nucleus using DAPI staining (false coloured red).

(C) Localization of GFP-LUH to the protoplast nucleus (false coloured green).

(**D**) Localization of free GFP in a protoplast (false coloured green).

reporter LexA-UAS_{Gal4}:GUS (UAS_{Gal4} is an upstream activation sequence of Gal4 promoter) were co-transfected with GD-LUH. Since VP16 is a known activator, it increases the background level of GUS expression. However, GD-LUH failed to decrease GUS expression (Figure 3-13A), suggesting that LUH is not a transcriptional repressor. To test whether LUH is an transcriptional activator, GD-LUH and the reporter UAS_{Gal4}-*GUS* were co-transfected into mesophyll protoplasts (Liu et al., 1994; Sheen, 2002; Wang et al., 2005). Because of the binding of GD to UAS_{Gal4}, the properties of LUH can control the expression of *GUS*. The LUH protein significantly increased the GUS activity above that of the empty vector negative control, suggesting that LUH acts as a transcription activator rather than a repressor (Figure 3-13B). However, relative to the VP16 positive control (Triezenberg et al., 1988), activation by LUH was modest. Given the high sequence similarity between *LUH* and *LUG*, it was surprising to find that *LUG* is reported to be a repressor in flower (Sridhar et al., 2004) while *LUH* seems to be an activator. For this reason a GD-LUG chimeric gene was constructed and the activation assay was repeated using both GD-LUH and GD-LUG. The results of this assay suggest that LUG as well as LUH acts as modest transcriptional activators under the conditions of this assay (Figure 3-13).

3.3 Discussion

The mature dry seeds of *luh-5*/*mum1-1* cannot release mucilage upon hydration, although the seed epidermal features and the cellular features throughout development

A

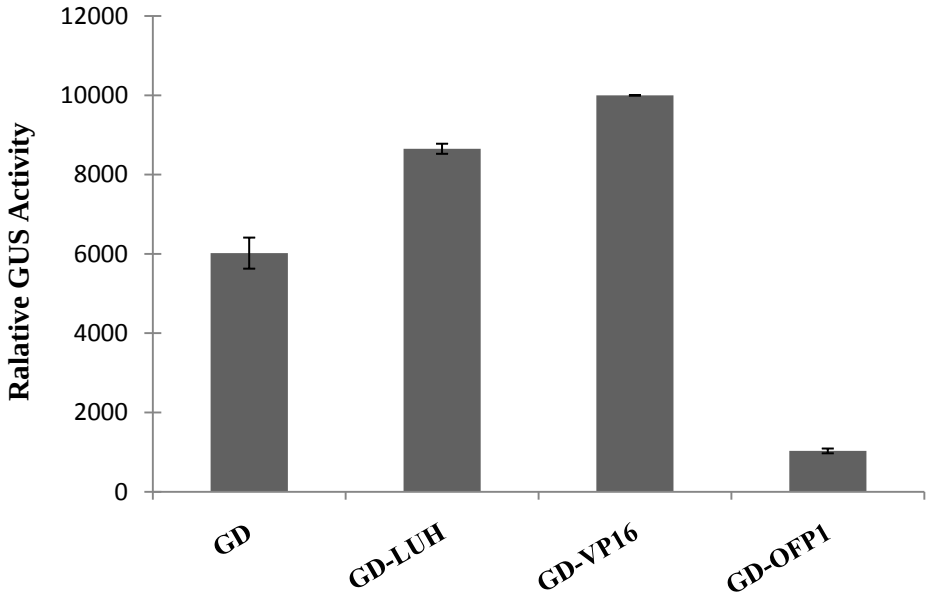

Figure 3-13 LUH transcriptional activation assay (to be continued)

B

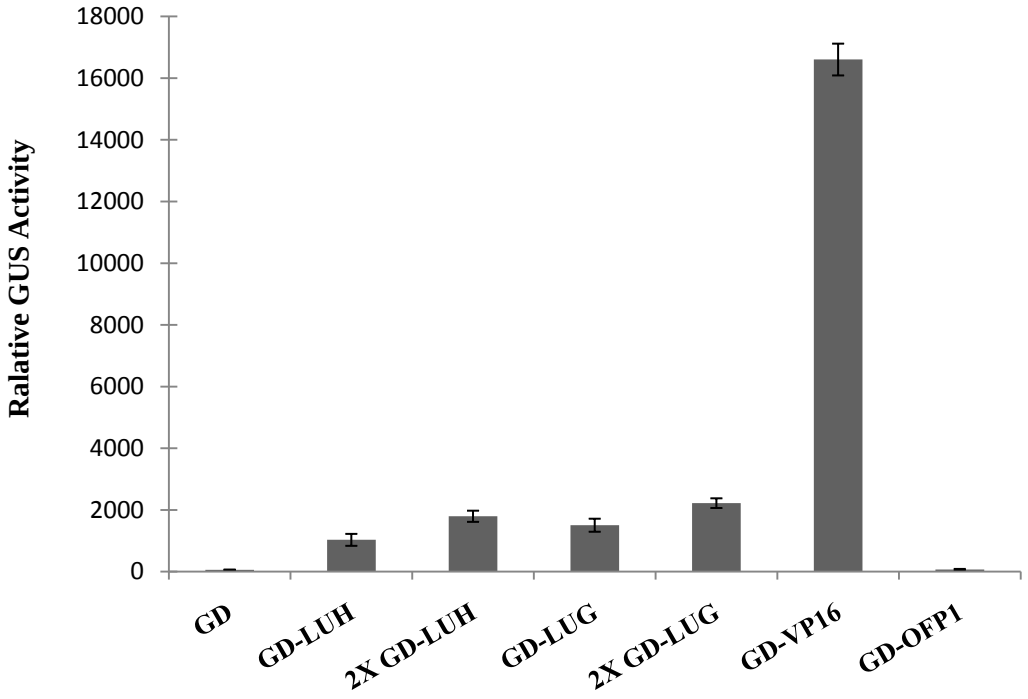

Figure 3-13 LUH transcriptional activation assay (continuing)

A. GD-LUH was tested initially for the transcriptional repressor activity, given that LUG is a repressor. GD was used as a negative control. OFP1 is a known repressor (Wang et al., 2007) and VP16 a known activator (Triezenberg et al., 1988). The relative GUS activity was measured by detecting the GUS reaction product 4-MU, which excites at 365nm.

B. The vectors *GD* (Gal4 DNA Binding Domain), *GD-LUH*, *GD-LUG*, *GD-OFP1* and *GD-VP16* were individually transfected into mesophyll protoplasts together with the reporter UAS_{Gal4}-GUS and the GUS activity was measured. 2X indicates that the corresponding amount of DNA used in the assay was doubled. Error bars indicate SD.

appear indistinguishable from those of wild type. A mucilage expansion assay indicated that the *luh-5*/*mum1-1* mucilage loses the capability to expand in water. The chemical analysis of *luh-5*/*mum1-1* mucilage reveals that more side chains are found in the mutant than wild type. The *LUH* gene was cloned and its product identified as a putative transcription factor, which is localized in the nucleus, and has a transcriptional activator function. Our data suggest that *MUM1* is indirectly involved in mucilage structural modification and that its targets could be genes encoding carbohydrate-active enzymes.

3.3.1 LUH /MUM1 is required for normal mucilage structure

The *MUM1* gene was identified on the basis of a mutation that results in the failure of seed coat mucilage to extrude on hydration of mature seeds. We have cloned *MUM1* and shown that it corresponds to the previously identified *LUH* gene (Conner and Liu, 2000; Sitaraman et al., 2008). Genetic analysis has suggested that LUH is redundant with LUG function in controlling floral morphogenesis, leaf polarity, embryo development and shoot apical meristem function (Sitaraman et al., 2008; Stahle et al., 2009). We have identified a novel role of LUH in producing seed coat mucilage with the correct hydration properties. The expression of *LUH* in the seed coat/endosperm (Figure 3-11) is consistent with such a role.

Mature dry seeds of *luh* release little or no mucilage upon hydration. Failure to extrude mucilage could be due to the inability to synthesize high enough amounts of mucilage, a strengthened primary cell wall that fails to rupture during hydration, or

production of mucilage with modified composition that is unable to expand upon hydration. The *luh/mum1* appears to make normal amounts of mucilage. This is reflected in the epidermal features and the cytological structure of *mum1* seeds that are indistinguishable from those of wild- type throughout development, unlike mutants that synthesize low amounts of mucilage (Penfield et al., 2001; Western et al., 2001b; Western et al., 2004; Usadel et al., 2004). Indeed, monosaccharide analyses of ground whole seed suggest that *luh/mum1* mutant seed is not deficient in mucilage (Western et al., 2001). Therefore it is unlikely that LUH/MUM1 influences the amount of mucilage produced.

Similar to *mum2* mutants, *luh/mum1*seed mucilage fails to expand even when sectioning directly exposes the mucilage to water, suggesting that the lack of mucilage extrusion in the seed is due to changes in the chemical properties of the mucilage rather than that of the primary cell wall (Dean et al., 2007; Macquet et al., 2007b). This hypothesis is consistent with the chemical analysis of *luh/mum1* mucilage that also indicates changes in mucilage structure. The *mum2* and *luh-5/mum1-1* mutants have higher mole percentages of the RG I side chain monosaccharides galactose and arabinose relative to the backbone sugars rhamnose and galacturonic acid, suggesting the presence of more RG I side chains. Taken together, these data suggest that LUH/MUM1, like MUM2, is required for modification of mucilage RG I that impacts the ability of mucilage to swell upon hydration.

3.3.2 *LUH* /*MUM1* encodes a putative transcription factor

The sequence of *LUH* has homology to WD40 transcription factors and is closely related to LUG, a known transcription factor reported to be a repressor in flowers (Sridhar, 2004; Sitaraman et al., 2008). Based on the conserved domains of WD40 and LisH (lissencephaly homology), both LUH and LUG are grouped into a small gene family of 13 members in *Arabidopsis* (Liu and Karmarkar, 2008), although not all these genes are highly related phylogenetically. The best studied of these, *LUG*, was identified on the basis of a mutation that enhanced the phenotype of the floral homeotic mutant *ap2*. Ectopic expression of the class C homeotic gene *AG* in *lug* suggests that *AG* expression is repressed by *LUG* in the whorls of sepals and petals (Liu and Meyerowitz, 1995). Besides flower development (Franks et al., 2002), *LUG* is also involved in gynoecial (Roe et al., 1997; Liu et al., 2000; Chen et al., 2000; Kuusk et al., 2006), leaf (Navarro et al., 2004; Cnops et al., 2004; Stahle et al., 2009) and vascular (Navarro et al., 2004; Franks et al., 2006) development. *LUG* localizes to the nucleus, has transcriptional repressor activity, and interacts both physically and genetically with transcription factor SEU (Sridhar et al., 2004) as well as FILAMENTOUS FLOWER (FIL), YABBY3 (YAB3) and YABBY5 (YAB5; Stahle et al., 2009). For these reasons, LUG is considered to be a transcription factor. Both LUG and SEU lack a DNA binding domain, suggesting that in order to function, LUG must interact with additional transcription factors (Sridhar et al., 2004).

On the basis of deduced amino acid sequence, LUH is structurally similar to LUG with an overall amino acid identity of 44% (Conner and Liu, 2000), suggesting that, like

LUG, LUH acts as a transcription factor. This hypothesis is supported by several additional lines of evidence. First, LUH localizes to the nucleus (Figure 3-12). Second, a transcriptional activity assay suggests that LUH works as a transcriptional activator (Figure 3-13). Third, LUH has been shown to physically interact with the transcription factors SEU, FIL, YAB3 and YAB5 (Sitaraman et al., 2008; Stahle et al., 2009). Finally, *luh* can enhance *lug* phenotypes suggesting that its function is redundant with that of LUG (Sitaraman et al., 2008; Stahle et al., 2009).

Despite the functional similarities between LUH and LUG noted above, significant differences have also been identified. Their single mutant phenotypes are distinct, *35S-LUH* was unable to rescue the *lug* mutant phenotype, and the global expression profiles of *LUG* and *LUH* are significantly different (Sitaraman et al., 2008). These phenotypic differences extend to seed coat mucilage as both *lug* and *seu* mutants have normal seed mucilage extrusion (Huang and Haughn, unpublished results).

3.3.3 The temporal and spatial expression of LUH

LUH is expressed throughout seed coat development with its highest level detected at 10 DPA (Figure 3-11B). These results are in agreement with recent microarray expression data using RNA derived from 3, 7 and 11 DPA seed coats (unpublished data from Haughn Lab and Datla Lab). In addition, the public database *Arabidopsis* eFP browser reveals a similar expression pattern, although in this case the RNA was derived from siliques and seeds (http://bar.utoronto.ca/) rather than specifically

from seed coats. Therefore our data represent the temporal expression pattern of *LUH* that *LUH* is expressed at every stage, and reaches its peak at the late stage of seed coat development.

During seed coat development, mucilage production starts at about 4 DPA, and finishes by 8-9 DPA (Beeckman et al., 2000; Windsor et al., 2000; Western et al., 2000). Since MUM1 is required for correct mucilage biosynthesis/processing, it would be expected that the *LUH* gene is expressed prior to or during mucilage biosynthesis (4-9 DPA) as was observed. Curiously, the highest level of *LUH* expression detected occurred after the end of mucilage biosynthesis marked by the beginning of synthesis of the secondary cell wall (columella). One reasonable explanation could be that LUH also functions in other seed coat or endosperm processes that occur at 10 DPA. *LUH* is expressed in all organs tested suggesting that the gene has functions other than in seed coat development. Indeed, LUH plays a partially redundant role with LUG in flower development. Expression was also observed in tissues where no function is known, such as stems and leaves. Interestingly, although the seed coat mucilage phenotype is the most obvious difference in comparison with wild type plants, the *LUH* expression in siliques is much lower than that in other tissues (Figure 3-11A). The low expression may result from the specific expression of *LUH* in seed coat, and non-expression in other cell types in siliques.

4 The Regulatory Roles of LUH

4.1 Introduction

The previous chapter revealed that *LUH* encodes a transcription factor, with an activator function. One proposed model for LUH is to regulate the expression of some enzyme required for normal mucilage production. Given that *luh* and *mum2* have a similar seed coat mucilage phenotypes (Western et al., 2001), and that *MUM2* encodes a *β*-galactosidase that modifies mucilage structure (Dean et al., 2007; Macquet et al., 2007b), *MUM2* is a good candidate for regulation by LUH either directly or indirectly. Therefore, I decided to test the putative direct relationship between *LUH* and *MUM2*.

As described in the General Introduction, it was shown that a several transcription factors are involved in the mucilage regulatory pathway including AP2, TTG1, TTG2, GL2, MYB5/TT2, and TT8/EGL3 (Figure 1-3). The regulatory pathway controls the expression of *MUM4*, an enzyme essential for the mucilage biosynthesis. Since LUH is a newly identified transcription factor regulating mucilage production, I also attempted to determine the relationship between LUH and the other transcription factors influencing seed mucilage accumulation.

4.2 Results

4.2.1 The regulation of *MUM2* by MUM1

MUM2 encodes a β-galactosidase, which is required for proper mucilage structure and impacts its hydration properties (Dean et al., 2007; Macquet et al., 2007b). The fact that LUH is a transcription factor and *luh* has a phenotype similar to *mum2* (Western et al., 2001; Dean et al., 2007) is consistent with the hypothesis that LUH is a positive regulator of *MUM2*. For this reason, I checked *MUM2* expression by qRT-PCR in seed coats/endosperm of both wild type and *luh-5*, at 7 DPA when *MUM2* has its peak expression (Dean et al., 2007). The dramatic decrease of *MUM2* transcript levels in *luh-5* seed coats, compared to wild type, indicates that LUH is a positive regulator of *MUM2* in this tissue (Figure 4-1).

Since MUM2 is required for mucilage extrusion, the *luh* seed coat phenotype could be due in large part to the decrease in MUM2 activity. This hypothesis was tested by introducing 35S:MUM2-GFP protein fusions into the *luh-5* mutant. Eight of 38 transgenic lines transformed with 35S:MUM2-GFP partially rescued the mucilage phenotype (Figure 4-2). In contrast, none of the 22 plants transformed with the vector alone, produced seeds that extruded mucilage. These results suggest that the *luh-5*/*mum1* phenotype is due in large part to the loss of MUM2 activity.

Among the eight transgenic lines with partial recovery, line 5 and 9 were randomly selected to check for contamination. The *luh-5* mutant has a transition from C to T, which removes a Pst I site. Cleaved Amplified Polymorphic Sequence (CAPS)

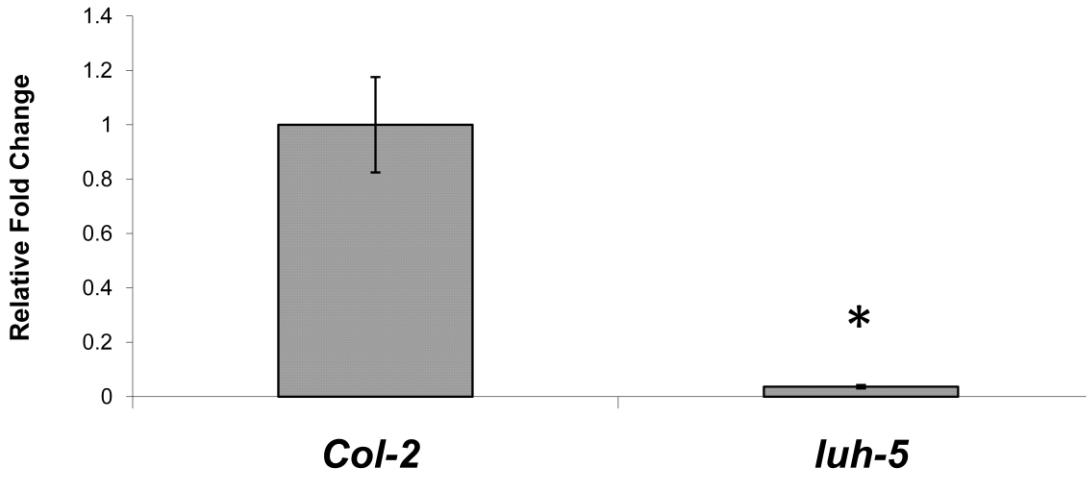

Figure 4-1 Comparison of *MUM2* seed coat expression in wild type and *luh-5*

qRT-PCR analysis was used to determine the *MUM2* transcript levels in 7 dpa seed coats/endosperm of both wild type and *luh-5*. Data are presented as the relative fold change, where the *MUM2* expression in the wild type was set as 1.0. *MUM2* transcript in *luh-5* seed coats is less than 10% of that in the wild type. The error bars indicate SD. The star indicates that the expression in *luh-5* is significantly different from that in Col-2 as assessed by Student`s t test ($T= 9.5045$, $P<0.01$).

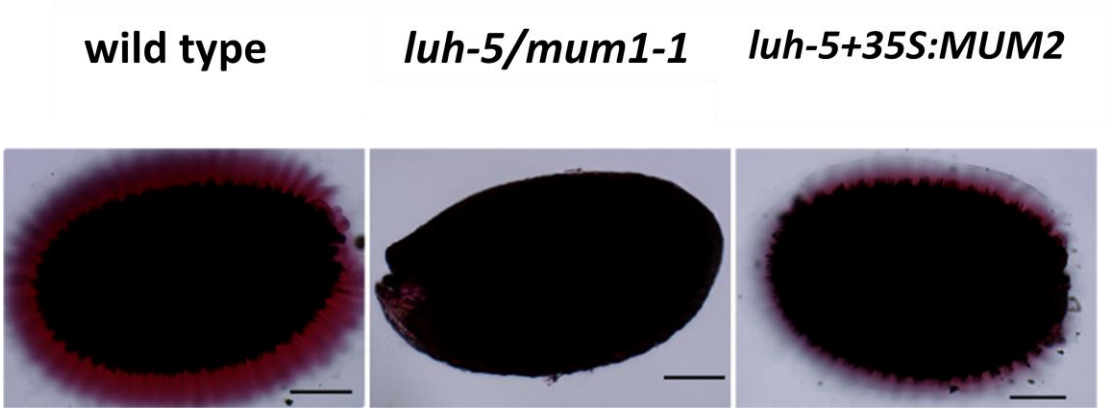

Figure 4-2 The seed coat mucilage phenotype of the transgenic plant *luh-5*+35S:MUM2

Dry mature seeds were harvested and imbibed in water. The seed coat mucilage phenotypes were examined under a light microscope after staining with ruthenium red. Wild type and *luh-5* were used as the positive and negative controls respectively. The seeds from the transgenic plant *luh-5+35S:MUM2* showed partial recovery of the mucilage phenotype. Scale bars = 100 μm.

primers were designed. The PCR products were digested with the enzyme Pst I. The wild type product showed two bands: 87 + 152 bp, and the *luh-5* product showed only one band: 239bp, indicating that the transformants were indeed homozygous for the mutant allele and not contaminants.

4.2.2 LUH functions independently from other mucilage-related transcription factors

Several transcription factors were found to be involved in mucilage production and a regulatory pathway has been proposed (Western et al., 2004; Li et al., 2009; Gonzalez et al., 2009). TTG1, TT8/EGL3 and MYB5/TT2 form a complex, together with AP2, to regulate the expression of GL2 and TTG2 (Figure 1-3) in the outer seed coat. LUH, as a transcription factor, might also be involved in this regulatory pathway although the *luh* phenotype is distinct from that of loss-of-function mutations in the other genes. To determine if *LUH* is regulated by any of these transcription factors, the *LUH* expression levels were identified in seed coats of wild type and mutants *ap2-1*, *ttg1-1*, *tt2-1* and *gl2-1*. The ecotype of Landsberg erecta (Ler) was chosen for the wild type control since all the mutants have the Ler background. The data indicate that the expression level of *LUH* in the seed coat is not reduced significantly in the mutants *ap2-1*, *ttg1-1*, *tt2-1* and *gl2-1* compared to wild type (Figure 4-3). These data suggest that *LUH* is not regulated by any of the transcription factors known to influence mucilage biosynthesis. *LUH* is involved in a distinct regulatory pathway for mucilage biosynthesis that includes *MUM2* (Figure 4-4).

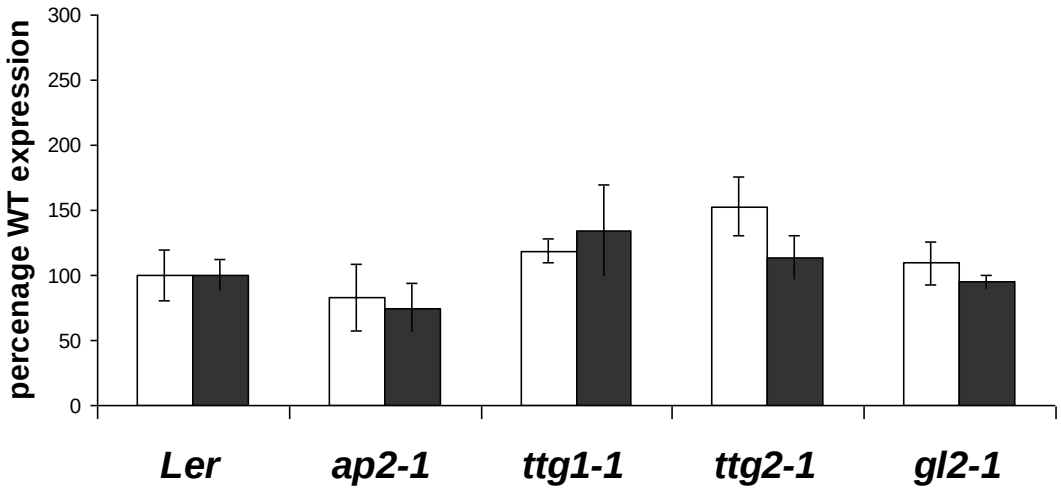

Figure 4-3 The comparison of *LUH* expression in wild type and mutants

The *LUH* transcripts were examined by qRT-PCR at 10 DPA seed coats, when *LUH* reached the highest expression during the seed coat development (Figure 3-11B). The data are presented as the percentage of wild type. The white and the black bars represent two biological replicates. The error bars indicate SD, derived from three technical replicates.

4.3 Discussion

4.3.1 LUH/MUM1 is required for activation of the *MUM2* gene

The *MUM2* gene encodes a β-galactosidase that is secreted into the apoplast and is believed to be involved in the removal of RG I side chains from seed mucilage to allow for mucilage extrusion (Dean et al., 2007; Macquet et al., 2007b). Data provided in this chapter strongly support the hypothesis that LUH/MUM1 functions, at least in part, to activate *MUM2* expression. The *luh* mutant phenotype is strikingly similar to that of *mum2*, as would be expected for an upstream regulator. Second, levels of *MUM2* transcript are drastically reduced in seed coats of the *luh* mutant relative to wild type (Figure 4-1). Third, p35S::MUM2-GFP can partially rescue the mucilage defect of the *luh* mutant (Figure 4-2). Thus formally, LUH/MUM1 can be considered to be a positive regulator of *MUM2* although whether such regulation is direct or not remains to be determined. Interestingly, the *luh* mutant phenotype appears weaker than that of *mum2* even for lines homozygous for putative null alleles (e.g. *luh-5*; Figures 3-1, 5). This could be explained by the fact that *MUM2* transcript can still be detected even in a strong *luh* mutant (Figure 4-1) and therefore some MUM2 activity likely remains in a *luh* mutant background. These data suggest that, in addition to LUH, other positive regulators of *MUM2* exist.

Since all aspects of the *luh* seed coat phenotype can be explained by the loss of MUM2 activity it is possible that the *MUM2* gene is the only target of LUH/MUM1 in the seed coat. However, since p35S::MUM2-GFP did not completely rescue the

luh/mum1 seed mucilage phenotype, the possibility that LUH/MUM1 regulates other genes required for seed mucilage remains.

We have shown that LUH can act as a transcriptional activator, albeit a weak one relative to the strong activator VP16. This is consistent with its role as a positive regulator of *MUM2* but not with its role as a regulator redundant with LUG previously shown to have repressor activity (Sitaraman et al., 2008). Curiously, LUG also acted as a positive regulator in our assays. A possible explanation for this discrepancy is that LUG (and possibly LUH) can function both as an activator or repressor depending on the specific co-regulator with which it interacts, and that the available co-regulators can vary depending on the origin of the cells used in the transcription assay. In any case, strong conclusions concerning the molecular mode of action of LUH await a more complete understanding of the other proteins with which it must interact to influence transcription.

4.3.2 The role of *LUH* is independent of other transcription factors controlling seed mucilage biology

In addition to LUH, several transcription factors influencing seed mucilage have been identified (reviewed in Arsovski et al., 2010; Figure 4-4). Differentiation of seed coat mucilage epidermal cells requires AP2 and the TTG1 protein complex. The TTG1 protein complex, which includes proteins TTG1, EGL3 and/or TT8 and MYB5 and/or TT2, activates at least two genes, *GL2* and *TTG2,* encoding transcription factors required for synthesis of mucilage. One target of GL2 is the *MUM4* gene encoding a rhamnose synthase.

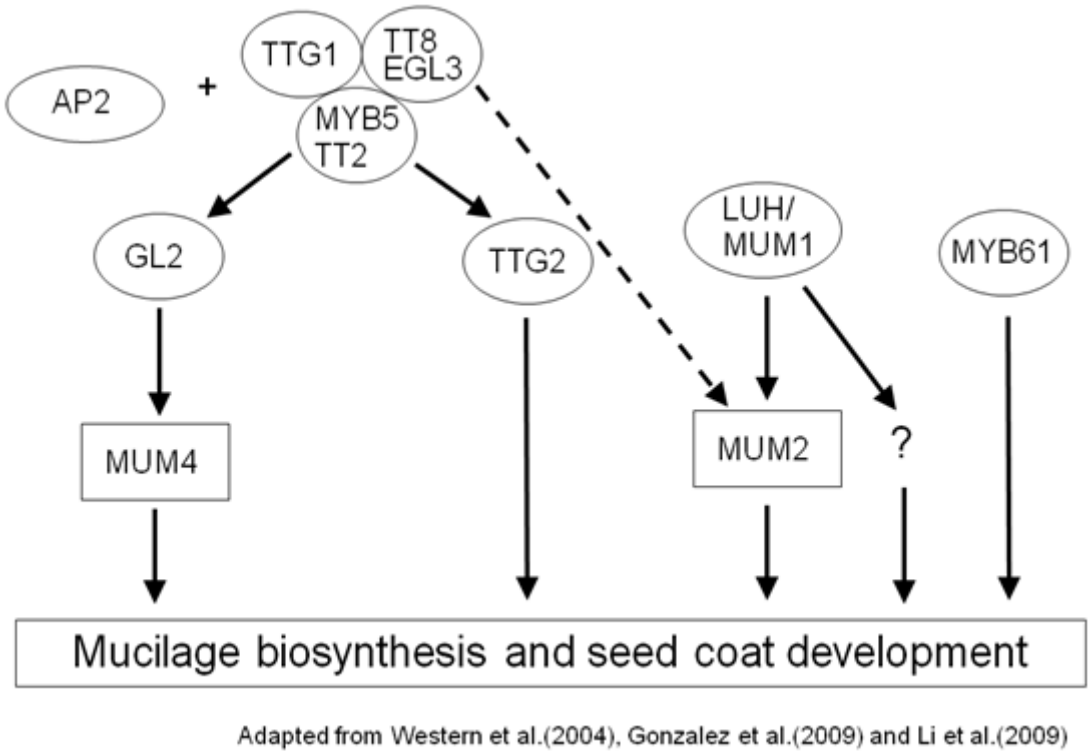

Figure 4-4 Proposed regulatory pathways for seed coat mucilage biosynthesis

TTG1, EGL3/ TT8 and MYB5/TT2 form a complex, which regulates GL2 and TTG2. LUH/MUM1 is independent of the other transcription factors and can activate *MUM2*. Since over-expression of *MUM2* can only partially rescue the *mum1* phenotype, LUH/MUM1 may be needed to activate other genes (the question mark in the figure) for normal mucilage production. The dotted line indicates possible regulation of *MUM2* by TTG1 complex and AP2. MYB61 is independent of other regulatory components.

Our data reveal a new regulatory pathway required for mucilage modification (Figure 4-4). LUH regulates *MUM2* but is not regulated by AP2, the MYB5/TT2-EGL3/TT8-TTG1 complex, GL2 or TTG2 (Figure 4-3). Whether *MUM2* is also regulated by AP2 and/or the MYB5/TT2-EGL3/TT8-TTG1 complex remains to be determined.

5 Genetic Modifiers of *mum4*

5.1 Introduction

As introduced in chapter 1, *mum4* was isolated in a screen for seed coat mucilage defects among approximately 1,000 ethylmethane sulfonate (EMS) mutagenized M_3 lines. The *mum4* seeds cannot release any mucilage in water, unlike the wild type. However a thin layer of mucilage can be released when seeds are treated with the chelator EDTA, consistent with the low production of mucilage in *mum4* (Western, 1998; Western et al., 2004; Usadel et al., 2004). This phenotype provides a background to screen for mutations in new genes which have a small impact on seed mucilage and may not be recognized when screening in a wild type background.

Two possible types of modifier phenotypes can be identified: enhancers (*mum4 enhancers = men*) can release even less mucilage than *mum4*, and suppressors (*mum4 suppressors = msu*) can release more mucilage. Both *MEN* and *MSU* genes can provide insights into mucilage biosynthesis and/or secretion. Six novel *men* mutants have already been isolated using such a screen. These mutants were affected in either mucilage biosynthesis or mucilage release (Arsovski et al., 2009).

I have conducted a similar screen for *mum4* modifiers. As described below, more *men* were isolated, and *msu* mutants were identified for the first time.

5.2 Results

5.2.1 Screen for the modifiers of *mum4-1*

The seeds of *mum4-1* were mutagenized with EMS by Dr. Tamara L. Western while she was a postdoctoral fellow in the Haughn laboratory. Half of the population, in 32 independent batches, remained in the Haughn laboratory to be screened, while Dr. Western screened the remaining half of the population in her own laboratory (Arsovski et al., 2009). Each of the 32 batches represented pooled seeds from approximately 100 M_2 plants.

I have completed the screening of all 32 batches once, using 50-70 seeds from each batch (about 2000 plants in total). M_2 seed was planted and plants harvested individually. The dry seeds of each line were treated with 0.05M EDTA and then stained with ruthenium red. *mum4-1* served as negative control. Col-2 was used as the positive control for suppressors, and *ttg1-1*, which cannot release mucilage even when treated with EDTA, for enhancers. From this screen 28 putative enhancers and 20 putative suppressors were identified. The progeny of the putative mutants were re-screened for the mutant phenotypes. Ten of the putative enhancers and 15 of the putative suppressors were found to be heritable.

Among the putative suppressors, several mutants have a wild type like mucilage phenotype. Because these could be true suppressors or wild type contamination, I checked whether these plants were homozygous for the *mum4-1* allele. The *mum4-1* allele has an extra MseI site compared to wild type (Western et al., 2004). On the basis of

this assay, three of the 15 suppressors were determined to be wild type contaminants. An additional 2 suppressor lines were lost.

Therefore, following screening and re-screening I identitifed 10 *men* and 10 *msu* mutants (Table 5-1).

5.2.2 The enhancers of *mum4-1*

As described earlier, *mum4-1* can only release a small amount of mucilage after treatment with EDTA solution. The *men* mutants in a *mum4-1* background (*men mum4-1*) can release little or no mucilage after being treated with EDTA (Figure 5-1) but otherwise have a *mum4-1*-like phenotype, lacking a volcano-shaped columella.

Twenty-two *mum4-1* modifier mutants from 2000 M_2 plants screened is a high frequency even assuming there are many genes involved in the complex processes of pectin biosynthesis and secretion. One explanation is that some mutants are homozygous for mutations in the same gene, a possibility that increases in likelihood when one considers that some mutants were found in the same seed batch (refer to Table 5-1) and may not have arisen independently. In an attempt to determine allelism, 44 reciprocal crosses were conducted (Table 5-2). However, because some enhancers showed only subtle differences from *mum4-1*, and the phenotypes varied with growth conditions, it was often difficult to distinguish the phenotypes of the crossed F_2 generation. As a result, the data of the backcross and the complementation were unreliable. For these reasons I was unable to determine how many different modifier genes I have identified.

ENHANCERS	G5-13	G5-21	H2-28	H2-8	H3-43	H6-8	H7-61	I3-49	I7-48	J4-1
Nomenclature	*men7 mum4-1*	*men8 mum4-1*	*men9 mum4-1*	*men10 mum4-1*	*men11 mum4-1*	*men12 mum4-1*	*men13 mum4-1*	*men14 mum4-1*	*men15 mum4-1*	*men16 mum4-1*
Heritable	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
water	0	0	0	0	0	0	0	0	0	0
EDTA	0+	0+	0++	0	0+	0+	0+	0+	0	0+

SUPPRESSORS	H4-55	H5-40	H6-49	H8-62	I2-2	I6-33	I6-38	I6-54	J3-47	J7-41
Nomenclature	*msu1 mum4-1*	*msu2 mum4-1*	*msu3 mum4-1*	*msu4 mum4-1*	*msu5 mum4-1*	*msu6 mum4-1*	*msu7 mum4-1*	*msu8 mum4-1*	*msu9 mum4-1*	*msu10 mum4-1*
Heritable	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
avoid contamination	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
water	1	1	1	1	1	1	1	1	1	1
EDTA	1++	1+++	1++++	1+	1++++	1+	1++	1++	1++	1+++

Table 5-1 The modifiers of *mum4-1*

The modifiers of *mum4-1* were identified from an EMS mutagenized population and determined to be heritable if their progeny produced seed with a similar phenotype. The phenotypes of modifiers were checked in either water or EDTA solutions. '0' represents no mucilage. '1' stands for the level of *mum4-1* mucilage quantity and '+' indicates an increase in mucilage amount relative to *mum4-1*. Every batch was named as a letter plus a number. The number after dash represents an individual M_3 line.

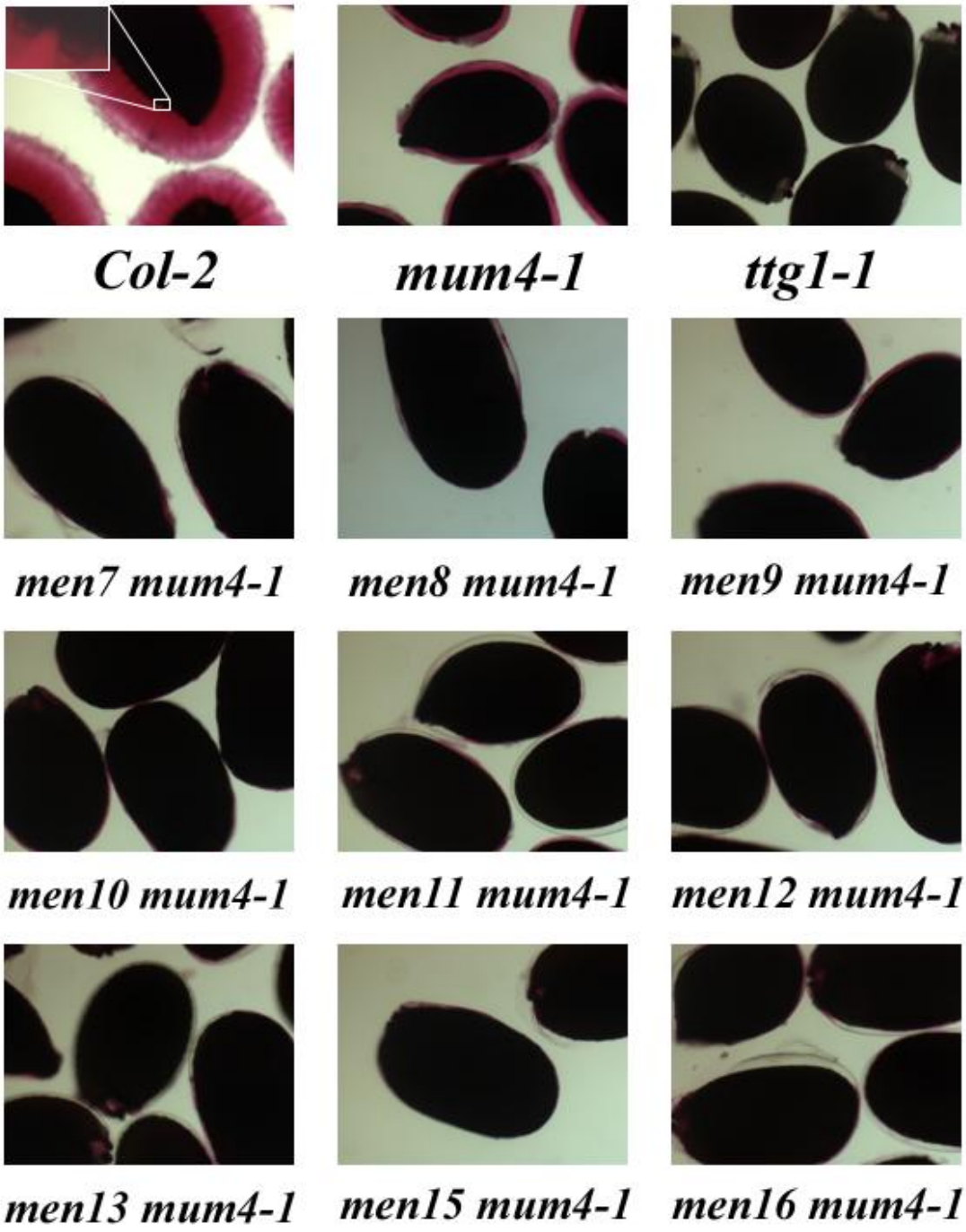

Figure 5-1 The phenotypes of the *mum4-1* enhancer mutants

Mature dry seeds were first shaken in EDTA, before being transferred into ruthenium red solution. The phenotypes were observed under the dissecting microscope. Col-2, *mum4-1* and *ttg1-1* were used as controls for comparison. The inset shows the collumela.

F2	phenotypes		NOTES	*men* alleles?
	mum4	*men*		
men16 mum4-1 X *men14 mum4-1*		✓		14=16
men12 mum4-1 X *men14 mum4-1*		✓		12=14
men14 mum4-1 X *men15 mum4-1*		✓		14=15
men11 mum4-1 X *men14 mum4-1*		✓		11=14
men13 mum4-1 X *men7mum4-1*		✓		7=13
men12 mum4-1 X *men13 mum4-1*		✓		12=13
men7mum4-1 X *men15 mum4-1*		✓		7=15
men7mum4-1 X *men12 mum4-1*		✓		7=12
men12 mum4-1 X *men8 mum4-1*		✓		8=12
men14 mum4-1 X *men7mum4-1*		✓		7=14
men13 mum4-1 X *men8 mum4-1*		✓		8=13
men14 mum4-1 X *men13 mum4-1*		✓		13=14
men14 mum4-1 X *men16 mum4-1*		✓		14=16
men12 mum4-1 X *men10 mum4-1*		✓		12=10
men11 mum4-1 X *men12 mum4-1*		✓		11=12
men14 mum4-1 X *men10 mum4-1*		✓		14=10
men7mum4-1 X *men11 mum4-1*		✓		7=11
men11 mum4-1 X *men10 mum4-1*		✓	some WT, contamination?	11=10

Table 5-2 The F_2 phenotypes of reciprocal crosses between *men mum4-1* lines

(To be continued)

The F_2 progeny of individual F_1 plants from reciprocal crosses between the *men mum4-1* lines were examined for mucilage phenotypes to determine if two lines are allelic to each other. The check marks represent the corresponding phenotypes. '*men* alleles?' infers if two genes are alleles based on the corresponding phenotype. '=' represents alleles, while '<>' not alleles.

F2	phenotypes		NOTES	*men* alleles?
	mum4	*men*		
men15 mum4-1 X *men11 mum4-1*	✓			11<>15
men14 mum4-1 X *men9 mum4-1*	✓			9<>14
men16 mum4-1 X *men12 mum4-1*	✓			12<>16
men16 mum4-1 X *men8 mum4-1*	✓			8<>16
men13 mum4-1 X *men9 mum4-1*	✓			9<>13
men16 mum4-1 X *men11 mum4-1*	✓			11<>16
men13 mum4-1 X *men15 mum4-1*	✓			13<>15
men7mum4-1 X *men8 mum4-1*	✓			7<>8
men14 mum4-1 X *men7mum4-1*	✓			7<>14
men7mum4-1 X *men8 mum4-1*	✓			7<>8
men11 mum4-1 X *men13 mum4-1*	✓			11<>13
men16 mum4-1 X *men7mum4-1*	✓			7<>16
men14 mum4-1 X *men13 mum4-1*	✓			13<>14
men13 mum4-1 X *men8 mum4-1*	✓			8<>13
men16 mum4-1 X *men10 mum4-1*	✓		less than mum4-1	16<>10
men16 mum4-1 X *men14 mum4-1*	✓		less than mum4-1	14<>16
men11 mum4-1 X *men8 mum4-1*	✓		less than mum4-1	8<>11
men16 mum4-1 X *men8 mum4-1*	✓		less than mum4-1	8<>16
men16 mum4-1 X *men7mum4-1*	✓		less than mum4-1	7<>16
men13 mum4-1 X *men7mum4-1*	✓			7<>13
men16 mum4-1 X *men13 mum4-1*	✓			16<>13
men13 mum4-1 X *men10 mum4-1*	✓			13<>10
men14 mum4-1 X *men8 mum4-1*	✓			8<>14
men14 mum4-1 X *men7mum4-1*	✓			7<>14
men16 mum4-1 X *men14 mum4-1*	✓		more than mum4-1, ???	14<>16

Table 5-2 The F_2 phenotypes of reciprocal crosses between men mum4-1 lines (continuing)

Some of the known mucilage mutants have pleiotropic phenotypes. Unlike the mutants *apetala2* (*ap2*) (Jofuku et al., 1994; Western et al., 2001) and *aberrant testa shape* (*ats*) (Leon-Kloosterziel et al., 1994), the seed shapes of *men mum4-1* lines reassembled those of the wild type. Therefore, *men* mutants are unlikely to be homozygous for alleles of *ap2* or *ats*. The mutation in *TTG1* results in a glabrous leaf phenotype, and the seeds show the color of yellow cotyledons because of the transparent testa lacking of purple anthocyanin pigments (Koornneef, 1981). The mutant *ttg2* shows similar trichome phenotype and seed color defects as *ttg1* (Johnson et al., 2002). The mutant *gl2* reveals short spike-like trichomes (Rerie et al., 1994). None of *men* mutants showed abnormalities in seed color or leaf trichomes indicating that the *MEN* genes are unlikely to be *TTG1*, *TTG2* and *GL2*.

Genetic complementation was used to determine if the new mutants carry alleles of any of the genes known to be required for normal seed mucilage including *ATS*, *AP2*, *TTG1*, *TTG2*, *GL2*, *MYB61*, *MUM1* and *MUM2*. Since the known mutants do not have *mum4* in their background, the F_2 seeds of individual F_1 plants from crosses of a known mutant to *mum4* enhancer mutants will show the corresponding known mutant phenotype if *men* is allelic. Otherwise the F_2 seeds will show a wild type phenotype (if *men* is recessive). The results reveal that the *men* mutants are not alleles of any of the mutants tested (Table 5-3).

F2	phenotypes			NOTES
	Col-2	*mum4*	*ttg1*	
men10 mum4-1 X *gl-2*	✓			
men10 mum4-1 X *ttg1-1*	✓			
men7mum4-1 X *ttg2-1*	✓			
mum2-1 X *men7mum4-1*	✓			
myb61 X *men7mum4-1*	✓			
men9 mum4-1 X *mum2-1*	✓			
myb61 X *men11 mum4-1*	✓			
men11 mum4-1 X *ttg2-1*	✓			
men11 mum4-1 X *gl2-2*	✓			
men11 mum4-1 X *mum2-1*	✓			
myb61 X *men12 mum4-1*	✓			
men12 mum4-1 X *ttg2-1*	✓			
men13 mum4-1 X *ttg2-1*	✓			
men13 mum4-1 X *ap2-7*	✓			
myb61 X *men13 mum4-1*	✓			
men13 mum4-1 X *gl2-1*	✓			
men13 mum4-1 X *ttg2-1*	✓			
men14 mum4-1 X *ap2-7*	✓			
men14 mum4-1 X *ttg1-1*	✓			
mum2-1 X *men15 mum4-1*	✓			
mum2-1 X *men16 mum4-1*	✓			
myb61 X *men16 mum4-1*	✓			

Table 5-3 The F_2 phenotypes of modifiers crossed with some known mucilage mutants

Genetic complementation analysis was used to determine if Mum4 modifier mutants and previously characterized mucilage mutants were allelic. F_2 seeds of individual F_1 plants from the indicated crosses were checked for the mucilage phenotypes after being treated with EDTA and ruthenium red.

5.2.3 The suppressors of *mum4-1*

Seeds of the *mum4-1 msu* double mutants can release more mucilage than *mum4-1* when treated with EDTA solutions. However none completely suppress the *mum4* defect such that all double mutants can typically be distinguished from the wild type (Figure 5-2). Like *mum4-1*, the *msu mum4-1* lines do not release mucilage in water (data not shown).

No irregular seed shape or abnormal seed color was found in the *msu mum4-1* lines. Although the trichomes of *msu1 mum4-1* and *msu3 mum4-1* reveal unusual shapes (data not shown) the trichome phenotypes were found to be unlinked to the seed coat mucilage phenotypes (data not shown).

Reciprocal crosses between *msu* mutants were conducted to determine the number of genes involved. Because of difficulty in scoring the phenotypes, the F_2 seeds derived from 46 crosses failed to reveal clear relationships among the *msu mum4-1* lines (Table 5- 4). Therefore allelism among the *msu mum4-1* lines was undetermined.

5.2.4 Genetic segregation analysis of *mum4* modifier lines

Once the mutant phenotypes were confirmed to be heritable, genetic analysis was used to determine how many genes were responsible for the phenotype and if the mutant phenotype of interest was recessive or dominant, crucial information for both complementation tests and positional cloning of the genes of interest.

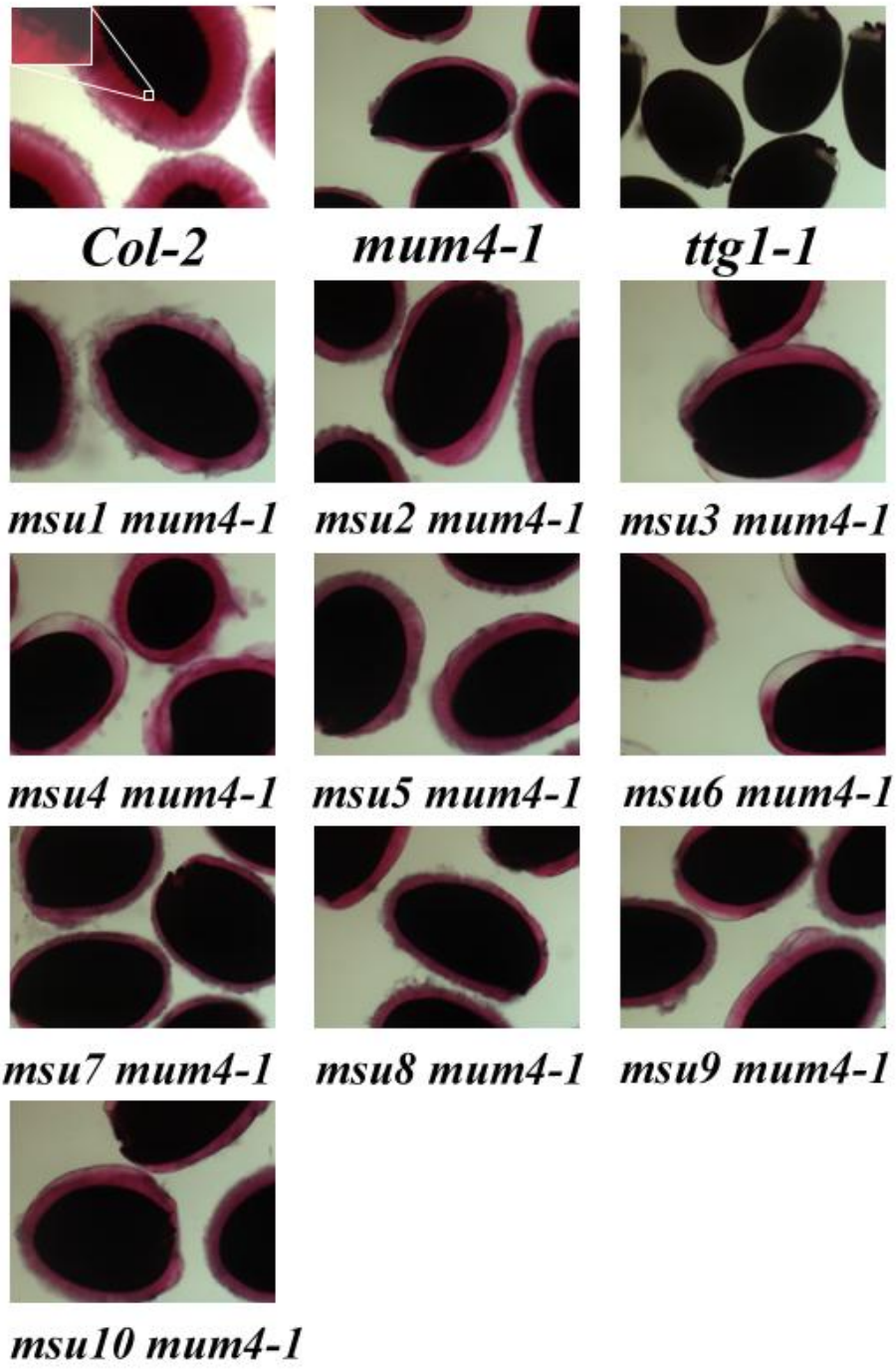

Figure 5-2 The phenotypes of the suppressors of *mum4-1*

Mature dry seeds were treated with EDTA and ruthenium red solutions sequentially and observed with a dissecting microscope. Col-2, *mum4-1* and *ttg1-1* were used as controls to compare the amount of the stained mucilage. The inset indicates the wild type structure of collumela.

F2	phenotypes		NOTES	*msu* Alleles?
	msu	*mum4*		
msu4 mum4-1 X *msu2 mum4-1*		✓		2<>4
msu2 mum4-1 X *msu3 mum4-1*		✓		2<>3
msu4 mum4-1 X *msu1 mum4-1*		✓		1<>4
msu1 mum4-1 X *msu9 mum4-1*		✓		1<>9
msu1 mum4-1 X *msu3 mum4-1*		✓		1<>3
msu4 mum4-1 X *msu10 mum4-1*		✓		4<>10
msu4 mum4-1 X *msu3 mum4-1*		✓		3<>4
msu9 mum4-1 X *msu3 mum4-1*		✓		3<>9
msu4 mum4-1 X *msu3 mum4-1*		✓		3<>4
msu4 mum4-1 X *msu9 mum4-1*		✓		4<>9
msu7 mum4-1 X *msu4 mum4-1*		✓		4<>7
msu9 mum4-1 X *msu7 mum4-1*		✓		7<>9
msu1 mum4-1 X *msu7 mum4-1*		✓		1<>7
msu9 mum4-1 X *msu3 mum4-1*		✓		3<>9
msu4 mum4-1 X *msu2 mum4-1*		✓		2<>4
msu5 mum4-1 X *msu3 mum4-1*		✓		3<>5
msu1 mum4-1 X *msu6 mum4-1*		✓		1<>6
msu6 mum4-1 X *msu2 mum4-1*		✓		2<>6
msu6 mum4-1 X *msu2 mum4-1*		✓		2<>6
msu6 mum4-1 X *msu3 mum4-1*		✓		3<>6
msu6 mum4-1 X *msu3 mum4-1*		✓		3<>6
msu6 mum4-1 X *msu4 mum4-1*		✓		4<>6
msu5 mum4-1 X *msu6 mum4-1*		✓		5<>6
msu5 mum4-1 X *msu6 mum4-1*		✓		5<>6
msu6 mum4-1 X *msu9 mum4-1*		✓		6<>9
msu6 mum4-1 X *msu9 mum4-1*		✓		6<>9
msu6 mum4-1 X *msu8 mum4-1*		✓		6<>8
msu1 mum4-1 X *msu8 mum4-1*		✓		1<>8
msu4 mum4-1 X *msu8 mum4-1*		✓		4<>8
msu4 mum4-1 X *msu8 mum4-1*		✓		4<>8
msu5 mum4-1 X *msu8 mum4-1*		✓		5<>8
msu7 mum4-1 X *msu8 mum4-1*		✓		7<>8
msu9 mum4-1 X *msu8 mum4-1*		✓		8<>9
msu10 mum4-1 X *msu8 mum4-1*		✓		8<>10

Table 5-4 The F_2 phenotypes of reciprocal crosses between *msu mum4-1* lines

(To be continued)

F2	phenotypes		NOTES	*msu* Alleles?
	msu	*mum4*		
msu5 mum4-1 X *msu1 mum4-1*		✓	more than *mum4-1*	1=5
msu1 mum4-1 X *msu5 mum4-1*		✓	more than *mum4-1*	1=5
msu7 mum4-1 X *msu5 mum4-1*		✓	more than *mum4-1*	5=7
msu5 mum4-1 X *msu2 mum4-1*		✓	more than *mum4-1*	5=2
msu5 mum4-1 X *msu2 mum4-1*		✓	more than *mum4-1*	5=2
msu5 mum4-1 X *men7 mum4-1*		✓	more than *mum4-1*	5=7
msu1 mum4-1 X *msu10 mum4-1*		✓	more than *mum4-1*	1=10
msu4 mum4-1 X *msu1 mum4-1*		✓	more than *mum4-1*	4=1
msu9 mum4-1 X *msu2 mum4-1*		✓	more than *mum4-1*	9=2
msu9 mum4-1 X *msu3 mum4-1*		✓	more than *mum4-1*	3=9
msu5 mum4-1 X *msu10 mum4-1*		✓	more than *mum4-1*	5=10
msu4 mum4-1 X *msu5 mum4-1*		✓	more than *mum4-1*	4=5

Table 5-4 The F_2 phenotypes of reciprocal crosses between *msu mum4-1* lines (continuing)

The reciprocal crosses between the *msu mum4-1* lines reveal if two lines are allelic to each other. The F_2 seed phenotypes were checked as described before. The check marks represent the corresponding phenotypes. 'Notes' indicate more details about the phenotypes. '*msu* alleles?' infers if two genes are alleles based on the corresponding phenotype. '=' represents alleles, while '<>' not alleles.

The mutants were crossed with *mum4-1* since the modifiers have the *mum4-1* background, and the phenotype of the F_1 plants was determined by examining mucilage extrusion in their F_2 seeds. Mutants where individual F_1 plants produced seed with a *mum4* phenotype were considered to be recessive whereas those with a modifier phenotype were considered to be dominant. The number of genes underlying each mutant phenotype was determined by the ratio of mutant to wild type F_2 plants (based on F_3 seed phenotype).

Results (Table 5-5) suggested that all mutants were the result of recessive mutations except *msu5* and *msu9* which were dominant. Two enhancers and three suppressors having the most obvious phenotypes were selected to check the F_2 segregation ratios. In the cases of *men10, men15* and *msu3,* the segregation showed *mum4-1*: modifier=3:1 (Table 5-6) suggesting that these phenotypes result from a single recessive mutation. However *msu1* and *msu2* appear to be single dominant mutations, since the segregation ratios are *mum4-1*: modifier=1:3. Noticeably, *msu1* and *msu2* are predicted to be recessive in Table 5-5. The hypothesis that both *msu1* and *msu2* are dominant however has better experimental support since it is based on the analysis of over 70 F_2 lines instead of a few F_1 lines. These opposing results are likely due to the difficulty of scoring phenotypes accurately.

A cross with wild type (Col-2) can be used to determine if the modifier mutation has a phenotype in the absence of *mum4*. All the F_2 seeds showed wild type phenotypes except that *msu9 mum4-1* X Col-2 showed *mum4*-like phenotype (data not shown). Since *MUM4* is recessive (Western et al., 2004; Usadel et al., 2004), the *mum4*-like phenotype may result from the mutation of dominant *MSU9*. Given the reasons mentioned above,

F2	phenotypes			Recessive or Dominant
	msu	*mum4*	*men*	
men7 mum4-1 X *mum4-1*		✓		recessive
men8 mum4-1 X *mum4-1*		✓		recessive
men9 mum4-1 X *mum4-1*		✓		recessive
men10 mum4-1 X *mum4-1*		✓		recessive
mum4-1 X *men11 mum4-1*		✓		recessive
men12 mum4-1 X *mum4-1*		✓		recessive
men13 mum4-1 X *mum4-1*		✓		recessive
men14 mum4-1 X *mum4-1*		✓		recessive
men14 mum4-1 X *mum4-1*		✓		recessive
men15 mum4-1 X *mum4-1*		✓		recessive
mum4-1 X *men15 mum4-1*		✓		recessive
mum4-1 X *men16 mum4-1*		✓		recessive
mum4-1 X *men16 mum4-1*		✓		recessive
mum4-1 X *msu1 mum4-1*		✓		recessive
mum4-1 X *msu2 mum4-1*		✓		recessive
mum4-1 X *msu3 mum4-1*		✓		recessive
mum4-1 X *msu3 mum4-1*		✓		recessive
msu4 mum4-1 X *mum4-1*		✓		recessive
mum4-1 X *msu5 mum4-1*		✓		recessive
mum4-1 X *msu5 mum4-1*	✓			a little more than mum4-1, dominant
mum4-1 X *msu6 mum4-1*		✓		recessive
mum4-1 X *msu7 mum4-1*		✓		recessive
msu8 mum4-1 X *mum4-1*		✓		recessive
mum4-1 X *msu9 mum4-1*	✓			a little more than mum4-1, dominant
mum4-1 X *msu10 mum4-1*		✓		recessive

Table 5-5 The F_2 phenotypes of the modifiers backcrossed with *mum4-1*

The confirmed homozygous modifiers were crossed with *mum4-1* in order to determine the mutation is recessive or dominant. The F_2 seeds were treated with EDTA solution before staining with ruthenium red. Col-2, *mum4-1* and *ttg1-1* were used as controls to score the phenotypes. The check marks represent the corresponding phenotypes.

	Col-2 X (F3)	***mum4-1* X (F3)**	Note
men10 mum4-1			
Expected	Col-2 : *mum4-1* : *men10* : *men10 mum4-1* (9:3:3:1=81:27:27:9)	*mum4-1* : *men10 mum4-1* (3:1=54:18)	single recessive gene, *men10* has its own phenotype.
Observed	82:27:29:6 (Chi square=1.16, p=0.7625)	57:15 (Chi square=0.667, p=0.4142)	
men15 mum4-1			
Expected	Col-2 : *mum4-1* : *men15 mum4-1* (12:3:1=108:27:9)	*mum4-1* : *men15 mum4-1* (3:1=54:18)	single recessive gene.
Observed	121:17:6 (Chi square=6.269, p=0.0435)	53:19 (Chi square=0.074, p=0.7855)	
msu1 mum4-1			
Expected	Col-2 : *mum4-1* : *msu1 mum4-1* (12:1:3=108:9:27)	*mum4-1* : *msu1 mum4-1* (1:3=18:54)	Single, Dominant. *mum4-1* X *msu1 mum4-1* F_2 seeds *msu1 mum4-1* like. Confirmed dominant.
Observed	125:10:9 (Chi square=14.787, p=0.0006)	13:59 (Chi square=1.852, p=0.1736)	
msu2 mum4-1			
Expected	Col-2 : *mum4-1* : *msu2 mum4-1* (15:1:0=135:9:0)	*mum4-1* : *msu2 mum4-1* (1:3=18:54)	Single, Dominant. No *msu2 mum4-1* like phenotypes among the Col-2 X *msu2 mum4-1* F_3 seeds.
Observed	115:27:0 (Chi square=38.963, p=0)	17:55 (Chi square=0.074, p=0.7855)	
msu3 mum4-1			
Expected		*mum4-1* : *msu3 mum4-1* (3:1=54:18)	single recessive gene
Observed	not obtained	55:17 (Chi square=0.074, p=0.7855)	

Table 5-6 The F_3 segregation ratios of the selected modifiers backcrossed with Col-2 or *mum4-1*

Modifiers with the most obvious phenotypes were backcrossed to either Col-2 or *mum4-1* and the F_2 phenotypic segregation ratios determined by examining F_3 seed mucilage derived from individual F_2 plants. The 'expected' value are the predicted results based on the known phenotypes and Mendelian laws.

only 5 modifiers were checked for the F_3 segregation ratios. Among the 5 modifiers, only *men10* appeared to show a mucilage phenotype independent of *mum4* (see section 5.2.5), with a *men10,* Col-2: *mum4-1*: *men10*: *men10 mum4-1* ratio of 9:3:3:1. In the case of *men15*, a Col-2: *mum4-1*: *men15 mum4-1* ratio of 12:3:1 was observed suggesting that *men15* alone has a wild type mucilage phenotype. Similarly, the Col-2: *mum4-1*: *msu1 mum4-1*=12:1:3 ratio observed indicates that the *msu1* phenotype alone is similar to wild type. Finally the observed Col-2: *mum4-1*: *msu2 mum4-1* ratio of 15:1:0 indicates that not only does *msu2* alone have a wild type phenotype but that the suppression of *msu2* over *mum4* is strong enough to make it difficult to distinguish the *msu2 mum4-1* phenotype from wild type as well.

5.2.5 MEN10

The two *men* mutants with the strongest phenotypes were selected for genetic segregation analysis. Only *men10* showed a mucilage phenotype independent of *mum4*. Shaken in EDTA solutions, the mucilage volume of *men10* was between those of wild type and *mum4-1* (Figure 5-3). The ruthenium red staining of *men10* mucilage is also paler than that in wild type suggesting that the monosaccharide composition is altered.

No seed shape, seed color or trichome phenotypes were found to associate with the seed coat mucilage phenotype suggesting that *men10* is not a new allele of *ATS, AP2*, *TTG1, TTG2,* or *GL2*.

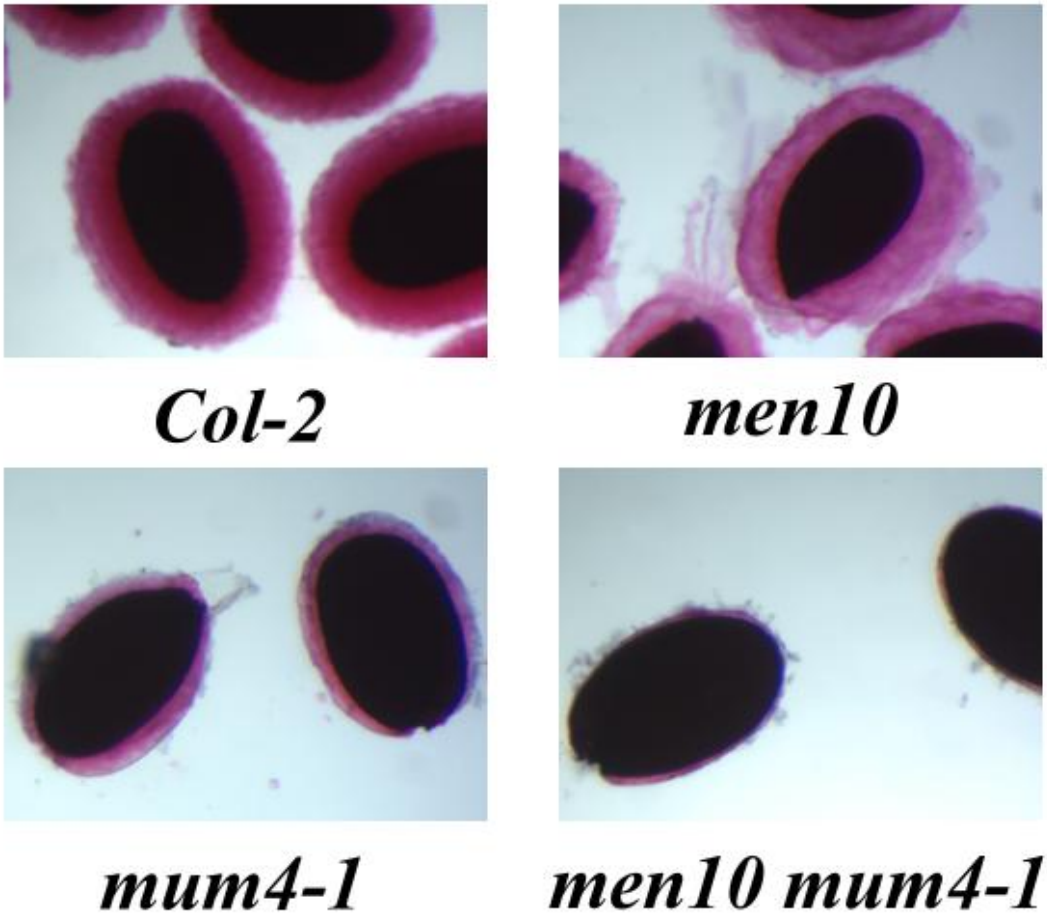

Figure 5-3 The seed coat mucilage phenotype of *men10*

men10 was isolated from the cross between Col-2 and *men10 mum4-1*. The dry seeds were successively treated with EDTA and ruthenium red. The phenotypes were examined and photographed using a dissecting microscope.

In order to test the reliability of the *men10* phenotype independent of the *mum4* background, I made the crosses *men10* X *mum4-1*, *men10* X Col-2 and *men10* X *mum4-1men10*. The F_3 seeds of *men10* X *mum4-1* and *men10* X Col-2 and the F_2 seeds of *men10* X *mum4-1men10* were examined for mucilage defects. In the case of *men10* X *mum4-1*, the expected segregation ratio is WT: *mum4-1*: *men10: mum4-1men10*=9:3:3:1. The F_3 seeds segregate as WT: *mum4-1*: *men10: mum4-1men10*=39:9:12:3. For the cross of *men10* X Col-2 a phenotypic ratio of WT: *men10*=3:1 was expected. The result was WT: *men10*=33:12 (Chi square= 0.067, p= 0.7963). The F_2 seeds of *men10* X *mum4-1men10* showed a *men10* phenotype. The data indicated that the new phenotype resulted from the mutation in a single nuclear gene.

5.3 Discussion

5.3.1 *mum4* is a good genetic background for screening for new genes affecting mucilage

Six mutants, including *mum1* through *mum5* (Western et al., 2004) and *patchy* (Arsovski et al., 2009a), have been isolated by screening for seed coat mucilage phenotypes in the wild type background with the forward genetic approach. All these mutants have obvious differences from wild type. However many genes involved in mucilage production may not result in a phenotype easily distinguishable from wild type. With the benefit of the *mum4* modifier screen, 6 more mutants, *men1* through *men6*, were

identified, along with the new alleles of *mum2* and *myb61* (Arsovski et al., 2009b). Among these mutants, *men1*, *men4* and *men5* in the *mum4* background have a reduction of mucilage deposition compared to *mum4*, while *men2* and *men6* have a defect in mucilage release (Arsovski et al., 2009b). None of these single mutants exhibits defects in mucilage phenotype, except *men4* which has reduced mucilage release (Arsovski et al., 2009b).

In our screening in the *mum4* background, 20 *mum4* modifiers, including 10 *men* and 10 *msu* mutants, were isolated. Of these, only 5 modifiers were selected for genetic segregation analysis given the difficulty of scoring the phenotypes accurately. *men10* is the only one which has a mucilage phenotype independent of *mum4*. Therefore, most of these single mutants could not be found by screening for mucilage mutants in the wild type background. My data demonstrate that the *mum4* modifier screen is a good approach to identify new genes involved in mucilage biosynthesis, secretion and modification.

It is intriguing that no *msu* mutants were identified in the secreening by Arsovski et al. (2009b, personal communication). 10 *MSU* genes found in our screen could play important roles in mucilage biosynthesis, given the nature of MUM4. *MUM4* encodes a rhamnose synthase predicted to convert UDP-D-Glc to UDP-L-Rha (Reiter and Vanzin, 2001). Its family members, *RHM1* and *RHM3*, are expressed redundantly. *MSU* genes may increase the expression of *RHM1* or *RHM3* to compensate for the absence of *RHM2*/*MUM4*. It is also possible that *MSU* genes result in increased extrusion by modifying cell walls or mucilage. Thus *msu* mutants may provide novel insights into cell wall biosynthesis.

Our data indicate that the secondary screening in the *mum4* background is valid to identify new genes affecting mucilage production or modification. No mutants with more mucilage than wild type have been reported. With the help of the *mum4* system, it may be possible to characterize genes which increase mucilage production.

5.3.2 Further characterization of modifier mutants

From the population of 2,000 plants, a total of 20 mutants were eventually identified. This mutant frequency is high considering a frequency of 2 X 10^{-4} mutations/locus (10 mM EMS, 24 hr) in a typical mutagenized population (Koornneef et al., 1982). Thus my results indicate that the mucilage biosynthesis is a complicated process involving many genes. Alternatively some of the mutants may be allelic to each other. Further characterization is needed to determine how many new genes are represented by the new mutants. To characterize the mutants thoroughly, a more reliable method of recognizing the mucilage phenotype is required. For some of the mutants however the phenotype may be too subtle to accurately characterize and clone the genes. Investigations on the seed coat development and the mucilage composition of the mutants may help us catagorize the mutants into phenotypic subgroups. Discovery of the new gene identities and their characterization may provide insights into pectin biosynthesis, secretion and modification.

6 Conclusions and Future Directions

Pectin is an essential component of cell walls. It plays important roles in plant growth regulation and defense signaling. In my thesis, I used the *Arabidopsis* seed coat, which releases pectinaceous mucilage, as a system to study pectin biology. I carried out two projects, namely cloning and characterization of *MUM1*, and screening for *mum4* modifiers.

6.1 Cloning and characterization of *MUM1*

mum1 was isolated as a mutant defective in mucilage release (Western et al., 2001). The development of the seed coat and the surface features of the epidermal cells showed no difference from that of wild type. The *mum1* mucilage lost the capability to expand on hydration. The possibility that the cell walls of *mum1* were stronger than wild type cannot be ruled out. Finally, *mum1* mucilage RG I has more side chains than wild type. All these features resemble the phenotype of *mum2*, which is defective in a β-galactosidase needed to remove pectic side chains for normal mucilage release (Dean et al., 2007) suggesting that MUM1 and MUM2 function in the same pathway.

Map-based cloning revealed that *MUM1* encodes a putative transcription factor LEUNIG_HOMOLOG (LUH) with WD40 repeats at the C terminus of the protein. *LUH*/*MUM1* is expressed in many tissues including the seed coat. *LUH*/*MUM1* is expressed throughout the period of seed coat development (4, 7 and 10 DPA), peaking at

the later stages. The sub-cellular location of LUH/MUM1 is the nucleus. The transcriptional activity analysis indicates that LUH/MUM1 functions as a weak activator.

Since no DNA binding domain is found in the *LUH*/*MUM1* gene, another transcription factor is needed to form a complex with LUH/MUM1 to accomplish the regulatory function such as SEU does for LUG. However, *seu* does not show mucilage defects. A yeast two-hybrid screen can be applied to identify the proteins which interact with LUH/MUM1 in the future. Such an experiment should help to better understand how LUH/MUM1 regulates mucilage modification.

Given the similar phenotypes of *mum1* and *mum2*, and the molecular identities of the proteins encoded by *LUH*/*MUM1* and *MUM2*, I tested the hypothesis that LUH/MUM1 regulates *MUM2*. *MUM2* expression is dramatically decreased in the *luh*/*mum1* background, relative to wild type. Over-expression of *MUM2* can compensate for the loss of LUH/MUM1. These data suggest that LUH/MUM1 positively regulates *MUM2*.

Although I confirmed that LUH/MUM1 regulates *MUM2* positively, we are still not sure if LUH/MUM1 binds to the *MUM2* gene directly. To discover whether *MUM2* is a target of LUH/MUM1 regulation, chromatin immunoprecipitation (CHIP) technology can be applied. Recombinant protein of LUH/MUM1 or its co-regulator, fused with common epitope such as His-tag, FLAG-tag etc., can be recognized by the corresponding common antibody. This recombinant protein-common antibody can be used to identify and purify the antibody to LUH/MUM1 or its co-regulator. Using the antibody of LUH/MUM1 or its co-regulator, we can precipitate the transcription factor protein

complex, still bound to sheared-sonicated chromatin. PCR amplification using MUM2 primers can be used to identify if *MUM2* sequences are represented among the precipitated chromatin.

The similar expression levels of *LUH/MUM1* in both wild type and mutants (Figure 4-3) indicates that LUH/MUM1 – MUM2 pathway is not under the regulation of other transcription factors known to be required for mucilage biosynthesis including AP2, TTG1, TTG2, GL2, MYB5/TT2 and TT8/EGL3 (Western et al., 2004; Li et al., 2009; Gonzalez et al., 2009). It is still possible that LUH/MUM1 is upstream of the TTG1 complex. Though this kind of hypothesis is not supported by the facts that *ttg1* and *luh-5/mum1-1* have distinct mucilage phenotypes, it is still an experiment worth doing. Similarly, the connection of LUH/MUM1 and MYB61 was not tested because of the difference between these two mutants: *myb61* shows decreased mucilage amount, and *luh-5/mum1-1* mucilage structure is changed. However, exploring the relationship of LUH/MUM1 and MYB61 in the future could bring novel information.

As described in the first chapter, two other mutants, *patchy*/*bxl1*and *sbt1.7*, have similar phenotypes to *mum1* and *mum2*. Each of these mutants has seed mucilage incapable of expanding normally in water, although seed coat development is indistinguishable from wild type. *BXL1* was found to encode a bi-functional β-xylosidase/α-arabinofuranosidase (Arsovski et al., 2009a). *SBT1.7* encodes a subtilisin-like serine protease (Rautengarten et al., 2008).. It would be interesting to check if MUM1 regulates either of these genes.

6.2 Screening for *mum4* modifiers

The reduced mucilage release of *mum4* provides us with a good opportunity to identify genes involved in the pectin biosynthesis, secretion and modification that were missed by previous screens. I obtained 10 enhancer mutants with more severe phenotypes, and 10 suppressor mutants with milder phenotypes than *mum4*. The allelism among the mutants could not be determined because of the difficulty with scoring at least some of the phenotypes accurately. Genetic segregation data of five selected mutants with most obvious phenotypes demonstrate that *MEN10*, *MEN15* and *MSU3* are recessive genes, while *MSU1* and *MSU2* appear to be dominant. Mucilage chemical analysis and seed coat development can provide us the hints of defects of new mutants. Positional cloning of the new mutants is needed to identify the mutated genes.

Analysis of mucilage composition and seed coat development can help to categorize the remaining mutants. The complementation tests between mutants within the same category could reduce the misleading information.

The *mum4 msu* mutants extrude more mucilage than *mum4*. Given the role of MUM4 in the essential conversion of UDP-D-Glc to UDP-L-Rha in mucilage, it is reasonable to hypothesize that the mutation of *MSU* compensates for the loss of MUM4. MUM4 is also known as RHM2, a member of RHM family, along with RHM1 and RHM3. Therefore, the mutation of *MSU* may increase the expression of RHM1 and/or RHM3, thus increasing the mucilage release. Alternatively, it is still possible that the mutation of *MSU* modifies the mucilage structure to make it extrude more easily.

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