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Name: George Haughn

Address: Botany Department, University of British Columbia, 6270 University Blvd.,

Vancouver, BC, V6T 1Z4, Canada

Telephone: +1 604 822 9089
Fax: +1 604 822 6089
Email: haughn@interchange.ubc.ca
The Arabidopsis Transcription Factor LUH/MUM1 Is Required for Extrusion of Seed Coat Mucilage

Jun Huang1, Danisha DeBowles2, Elahe Esfandiari1, Gillian Dean1, Nicholas C. Carpita2,3, George W. Haughn1,*

1Botany Department, University of British Columbia, 6270 University Blvd., Vancouver, BC, V6T 1Z4, Canada
2Department of Botany & Plant Pathology, Purdue University, 915 West State Street, West Lafayette, Indiana 47906, U.S.A.
3Bindley Bioscience Center, Purdue University, 1203 West State Street, West Lafayette, IN 47907, U.S.A.

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*For correspondence (phone +1 604 822 9089; fax +1 604 822 6089; e-mail george.haughn@ubc.ca)
Abstract

During differentiation, the Arabidopsis seed coat epidermal cells secrete mucilage composed primarily of Rhamnogalacturonan I (RGI) that is extruded from the seed coat upon imbibition. The mucilage of the *mucilage modified1* (*mum1*) mutant contains RGI that is more highly branched, and lacks the ability to be extruded when exposed to water. Our cloning of the *MUM1* gene shows that it encodes 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 suggests that *LUH/MUM1* is expressed throughout seed coat development, reaching peak expression late in differentiation. *LUH1/MUM1* expression in plants homozygous for mutations in several genes encoding regulators of seed coat mucilage was unchanged. Thus *LUH/MUM1* expression appears to be independent of other transcription factors known to regulate aspects of seed coat mucilage biology. The expression in the *luh/mum1* mutant of three genes encoding enzymes needed for mucilage extrusion, *MUM2*, *SUBSILIN PROTEASE1.7* and *β-XYLOSIDASE1*, was reduced relative to that of wild type. Overexpression of *MUM2* could partially rescue the *mum1* phenotype. These data suggest that LUH/MUM1 is a positive regulator of all three genes.
INTRODUCTION

After fertilization, cells of the ovule integuments differentiate as seed coats resulting in several layers of specialized cell types that aid in dormancy, germination, defense and dispersal. In Arabidopsis, differentiation of the seed coat epidermis involves growth, secretion of pectinaceous mucilage to the apoplast, and formation of a volcano-shaped secondary cell wall termed the columella (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). Upon hydration of the mature seed, these epidermal cells extrude this mucilage, which forms a capsule around the seed. Although mucilage has been shown to aid in germination during conditions of water limitation in the laboratory (Penfield et al., 2001; Rautengarten et al., 2008; Arsovski et al., 2009;), the functions of mucilage in the wild are still not clear.

The major component of Arabidopsis mucilage is the pectin rhamnogalacturonan I (RG I) (Western et al., 2000; Western et al., 2001; Penfield et al., 2001; Western et al., 2004; Usadel et al., 2004; Macquet et al., 2007a). RG I is a backbone of alternating (1→2)-α-L-rhamnose and (1→4)-α-D-galacturonic acid to which are attached various polysaccharide side chains (Willats et al., 2001; Ridley et al., 2001). Arabidopsis mucilage RG I is relatively unbranched with Gal and Ara identified as the principle side chain sugars. The pectin homogalacturonan (HG) has also been identified as a component of Arabidopsis mucilage (Willats et al., 2001; Macquet et al., 2007a,b, Dean et al., 2007). HG plays important roles in pectin cross-linking through both Ca\(^{2+}\) bridges and ester linkages. Accordingly, the cohesiveness of pectin can be disrupted by both heavy metal chelators and chemicals that hydrolize ester linkages (Western et al., 2000; Macquet et al., 2007a; Dean et al., 2007).

Pectins, one of the three major polysaccharide groups of the cell wall, maintain the mechanical properties of the wall by forming the matrix in which the network of cellulose and cross-linking glycans is embedded (Carpita and Gibeaut, 1993; Cosgrove, 1997).
They are also found in the middle lamella, where they function in cell to cell adhesion. In addition, pectin is believed to play important roles in cell expansion, control of wall porosity, and plant defence-related signalling (Cosgrove, 1993; Cosgrove, 1997; Ridley et al., 2001).

Seed coat mucilage represents a readily accessible source of pectins from a single cell type that is not required for plant growth and development under laboratory conditions (Western et al., 2000). For these reasons, the seed coat is a valuable model system for gene discovery related to pectin biology. Screens for altered seed coat mucilage phenotypes have identified genes, the products of which are needed for mucilage synthesis or secretion. Included among these are genes encoding transcription factors. For example APETALA2 (AP2) is required for differentiation of the epidermis and palisade layers of seed coat cells (Jofuku et al., 1994; Western et al., 2001). In contrast, TRANSPARENT TESTA GLABRA1 (TTG1), ENHANCER OF GLABRA3 (EGL3), TRANSPARENT TESTA8 (TT8) and MYB5/TT2 form a WD40-bHLH-MYB complex that appears to impact primarily mucilage synthesis (Li et al., 2009; Gonzalez et al., 2009, Western et al., 2001). The complex does so, at least in part by activating TTG2 and GLABRA2 (GL2; Walker et al., 1999; Western et al., 2001). TTG2, a WRKY transcription factor and GL2, a homeodomain protein, are both required for normal levels of mucilage biosynthesis. At least one of the roles of GL2 is to activate the transcription of MUCILAGE MODIFIED4 (MUM4; also known as RHAMNOSE BIOSYNTHESIS2 (RHM2); Usadel et al., 2004; Western et al., 2004), a gene encoding a rhamnose synthase that is required to convert UDP-D-glucose to UDP-L-rhamnose, a substrate for synthesis of the RG I backbone (Usadel et al., 2004; Western et al., 2004; Oka et al., 2007).

Other seed coat mucilage mutants identified in genetic screens produce normal amounts of mucilage that fail to properly extrude when mature seeds are exposed to water. Four genes, mucilagemodified1 and 2 (mum1, mum2; Western et al., 2001), β-xylosidase1 (bxl1, Arsovski et al., 2009), and subtilisin-like serineprotease1.7 (sbt1.7, Rautengarten
et al., 2008) were identified by such a mutant phenotype. Current evidence suggests that MUM2, BXL1 and SBT1.7 are required for modifying the structure of pectin. *MUM2* encodes a cell wall β-galactosidase (Western et al., 2001; Dean et al., 2007; Macquet et al., 2007b) and *BXL1*, encodes a putative bifunctional β-D-xylosidase/α-L-arabinofuranosidase (Arsovski et al., 2009) that trims β-xylan and α-arabinan side-groups from the RG I, respectively. *SBT1.7* encodes a subtilisin-like protease that indirectly affects the pectin methylation status of mucilage and/or the primary cell wall (Rautengarten et al., 2008).

In this study, we have cloned and characterized the *MUM1* gene. The phenotype of the *mum1* mutant closely resembles that of *mum2*. Positional cloning identified the *MUM1* gene as encoding a nuclear-localized transcription factor *LEUNIG_HOMOLOG* (*LUH*; Sitaraman et al., 2008), which is expressed in the seed coat and other tissues. qRT-PCR and molecular complementation studies show that *MUM1* is required for normal expression of *MUM2*.

**RESULTS**

*Mature mum1 seeds require chelators or alkali to release mucilage*

Like the *mum2* mutant (Western et al., 2001; Dean et al., 2007; Macquet et al., 2007b), mature seeds of *mum1-1* do not extrude mucilage when hydrated with water (Fig. 1A). Scanning electron microscopy detected no obvious difference in cell surface features between wild-type and *mum1-1* seeds (Supplemental Figure 1). To examine the cell structure during development, seeds of wild-type and *mum1* were observed using light microscopy at the developmental stages of 4, 7 and 10 days post anthesis (DPA). At these stages, the structure of the seed coat epidermal cells of wild-type and *mum1-1* were found to be indistinguishable (Supplemental Figure 2). These data indicated that the *mum1-1* seed coat mucilage phenotype does not result from an obvious cytological developmental defect.
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* mature seeds were embedded in paraffin wax without fixation, then sectioned and exposed to a solution of ruthenium red (Dean et al., 2007). The thickness of the sections (20µm) ensured that the mucilage of the seed coat epidermal cells was directly exposed to water without the primary cell wall as a barrier to mucilage expansion. The mucilage from wild-type seeds expanded but that from the *mum1* sections did not (Fig. 1B). These results suggest that the *mum1* mucilage, like that of *mum2* (Dean et al., 2007; Macquet et al., 2007a), has reduced capability to expand.

We tested the ability of Ca$^{2+}$ and Mg$^{2+}$ chelators and alkali to allow extrusion of *mum1*-1 mucilage. Chelators, such as 0.05M EDTA, EGTA or CDTA, and alkali, such as 1M Na$_2$CO$_3$ or 0.5M KOH, each resulted in the release of some *mum1*-1 mucilage (Fig. 1A), and *mum1*-1 seeds released more mucilage than *mum2*-1 regardless of treatment. We also determined the amount of mucilage extracted from intact seeds treated sequentially with water, 0.2 M NaOH, and 2 M NaOH, (Fig. 2). Seeds were stained with ruthenium red following extraction to monitor mucilage release (Supplemental Fig. 3). Only the wild-type released significant amounts of mucilage in water. 0.2 M NaOH caused rupture of the cell primary cell wall in both mutants, with release of mucilage from *mum1* seeds and lesser amounts from *mum2* seeds (Fig. 2; Supplemental Fig. 3). 2 M NaOH is able to remove most of the inner adherent layer of mucilage in wild-type, and some of the adherent layer in the mutants (Fig. 2; Supplemental Fig. 3). The *mum2* mutant seed appears to retain the most mucilage (Supplemental Fig. 3). Thus, the ability to extract mucilage from *mum1* and *mum2* mutant seed is impaired relative to wild type.

The extractable seed coat mucilage compositions of *mum1* and *mum2* are similar, and distinct from wild type

Because of the differences in mucilage extraction profiles, we performed monosaccharide and linkage analyses 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 1). As described above, addition of 0.2 M NaOH caused rupture of the outer seed coat wall, leading to release of large amounts of material containing primarily Rha and GalA from the mutants as well as additional mucilage from wild type (Table 1). The tightly attached gel layer 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.

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 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 2). In contrast, the 0.2 M NaOH causes substantial amount of polysaccharide to be released from the seed coats from both of the 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 mutants 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 mum2 extracts (Table 2).
Cloning of MUM1
The MUM1 gene was identified using positional cloning. The region containing MUM1 was mapped to between 13.834 mb and 13.937 mb on chromosome II. There were 28 open reading frames located in this interval. We obtained 29 available SALK insertion lines for the 28 loci and screened for seed coat mucilage phenotypes. The seeds of SALK_107245 showed a mum1 phenotype suggesting that the corresponding gene, At2g32700, represented MUM1. When At2g32700 from mum1 was sequenced, a C-to-T mutation was identified that changes the Glu97 to a stop codon (Fig. 3).

As shown in Figure 3, we identified five alleles of At2g32700 from available T-DNA insertional (SALK_107245C and SALK_097509; Alonso et al., 2003) and TILLING mutant lines (luh_172H3, luh_147A6 and CS90546; Seattle TILLING Project, http://tilling.fhcrc.org). Each allele had a phenotype similar to mum1-1 (Fig. 1C; data not shown). Sequence analysis of At2g32700 suggested that the gene is a transcription factor related in sequence 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 F1 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 Glu73 to Lys is a weak allele that results in the release of some mucilage when treated with water (Fig. 1C).

We performed molecular complementation of mum1 to confirm that the mutation in At2g32700 was responsible for the mum1 phenotype. A fragment of genomic DNA containing the wild-type MUM1 gene, including 2.6 kb of 5' sequences, a 4.6 kb ORF and 0.6 kb of 3' sequences was cloned into the binary transformation vector pART27 and transformed into mum1 plants via Agrobacterium-mediated transformation. The mum1 mutant plants transformed with the MUM1 gene extruded mucilage like wildtype while those transformed with the empty vector showed no such rescue (Supplemental Figure 4).

MUM1/LUH is a putative transcription factor with an activator function
The open reading frame of LUH encodes a 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 C terminus contains several WD40 repeats commonly involved in protein-protein interactions (http://smart.embl-heidelberg.de/; Fig. 3).

The LUG gene product is located in the nucleus (Conner and Lui, 2000). To determine if LUH is also nuclear localized, expression of a p35S::GFP-LUH fusion protein in mesophyll protoplasts was performed. Subcellular location of GFP was observed using fluorescence microscopy, placing LUH in the nucleus (Fig. 4).

To determine if LUH also acts as a transcriptional repressor, the gene was fused in frame to the Gal4 DNA binding domain (GD-LUH). GD-LUH and the reporter UAS_{Gal4}-GUS (upstream activating sequence of Gal4 fused with GUS) were co-transfected into leaf mesophyll protoplasts. Because GD binds to UAS_{Gal4}, LUH can control the expression of GUS. 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 (Fig. 5). However, relative to the VP16 positive control, activation by LUH was modest. Given that the high sequence similarity between LUH and LUG, it was surprising to find that one represses while the other activates. For this reason a GD-LUG chimeric gene was also 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 transcriptional activator under the conditions of this assay (Fig. 5).

LUH expression pattern

RT-PCR analysis showed that the LUH transcript was detected in all tissues examined, including siliques, rosette leaves, cauline leaves, roots (6 days after germination), stems and open flowers (Fig. 6A). qRT-PCR analysis showed LUH transcripts to be expressed during all stages of seed coat development, with the highest expression at 10 DPA (Fig. 6B).
LUH regulates *MUM2*

*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* (Dean et al., 2007; Western et al., 2001) suggests that LUH could be a positive regulator of *MUM2*. For this reason, *MUM2* expression was examined by qRT-PCR in seed coats of both wild-type and *luh*-5 at 7 DPA when *MUM2* expression is at its peak (Dean et al., 2007). The *MUM2* transcript levels are markedly decreased in *luh*-5 seed coats, indicating that LUH/MUM1 is required for normal *MUM2* expression in this tissue (Fig. 7). When a p35S::MUM2-GFP chimeric gene (Dean et al., 2007) was introduced into the *luh*-5 mutant, eight of 38 transgenic lines transformed with p35S::MUM2-GFP partially rescued the mucilage phenotype (Fig. 1D). In contrast, none of the 22 plants transformed with the vector alone produced seeds that extruded mucilage. This frequency of complementation of the *mum1* mutant is similar to that achieved by transforming the *mum2* mutant with the same p35S::MUM2-GFP construct. However, unlike the *mum1* transformants, several of the *mum2* complemented lines displayed relatively normal levels of mucilage (Dean et al., 2007).

The observation that p35S::MUM2-GFP could not completely complement the *luh/mum1* mutation could be explained by the fact that LUH/MUM1 controls genes influencing mucilage extrusion other than *MUM2*. Two such genes that have been identified are *BXL1* and *SBT1.7*. Consequently, qRT-PCR was used to determine if *LUH/MUM1* was required for normal levels of transcript for these two genes. Indeed the results (Fig. 7) indicate that transcript levels of both genes are significantly lower in the seed coat of the *mum1* mutant relative to wild type. In contrast *GL2* transcript levels were unaffected by the absence of LUH/MUM1 activity (Fig. 7).

**LUH functions independently from other mucilage-related transcription factors**
A number of transcription factors required for normal levels of seed coat mucilage have been identified and a regulatory pathway of mucilage biosynthesis has been proposed (Western et al., 2004; Gonzalez et al., 2009; Li et al., 2009). To decide if LUH is regulated by any of these transcription factors, the LUH expression level was determined in seed coats of wildtype and the ap2-1, ttg1-1, ttg2-1 and gl2-1 mutants. The data indicate that there is little difference in LUH expression between wild type and any of the mutants (Fig. 6C). These results suggest that LUH is not regulated by any of the transcription factors tested and a modified regulatory pathway for mucilage biosynthesis is proposed (Fig. 8).

Discussion

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). Thus, we have identified a novel role for LUH in producing a water-soluble seed coat mucilage with the correct properties for hydration. The expression of LUH in the seed coat/endosperm (Fig. 6) is consistent with such a role.

Mature dry seeds of luh/mum1 release little or no mucilage upon hydration and compared to the wild type, less mucilage can be extracted from intact mutant seed with water and NaOH. Failure to extrude/extract 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 makes it more cohesive and therefore unable to expand upon hydration. We believe that the former hypothesis is unlikely since the available evidence suggests that luh/mum1 makes
relatively normal amounts mucilage. The surface features and the cytological structure of the epidermal cells as well as the monosaccharide content of ground whole seed of luh/mum1 seeds are indistinguishable from those of wild type and distinct from those of mutants that synthesize low amounts of mucilage (Supplemental Figures 1 and 2; Penfield et al., 2001; Western et al., 2001; Western et al., 2004; Usadel et al., 2004).

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 (Figure 1B; Dean et al., 2007; Macquet et al., 2007). This hypothesis is consistent with the chemical analysis of luh/mum1 mucilage that also indicates changes in mucilage structure. Both mum2 and luh-5/mum1-1 mutants have higher mole percentages of the RGI side chain monosaccharides galactose and arabinose relative to the backbone sugars rhamnose and galacturonic acid suggesting the presence of more and/or larger RGI side chains. The wild-type Arabidopsis seed coat epidermal cells synthesizes two forms of the mucilage, one that is loosely adherent and expands greatly upon hydration and one that forms a tight adherent gel attached to the seed coat (Naran et al., 2008). Interestingly the adherent mucilage from wild type appears to have a similar branched composition as the mutants (extracted with 2 M NaOH, Table 1). Taken together, these data suggest that the action of LUH/MUM1 and MUM2 is required to remove mucilage RGI side-chains from the mucilage in the apoplast, converting much of mucilage to an expandable form. Failure to do so, as occurs in the mutants, impacts the ability of mucilage to transition to the water-soluble form that swells upon hydration, rupturing the epidermal wall.

LUH/MUM1 encodes a putative transcription factor

The sequence of LUH/MUM1 has homology to WD40 transcription factors and is closely related to LUG, a known transcriptional repressor (Sridhar, 2004; Sitaraman et al., 2008). Based on the conserved domains of WD40 and LUFS, both LUH/MUM1 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 AGAMOUS (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 factors SEUSS (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 DNA binding domains, suggesting that to function, LUG interacts 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 (Fig. 4). Second, a transcriptional activity assay suggests that LUH works as a transcriptional activator (Fig. 5). 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, p35S::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).
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 RGI side chains from seed mucilage to allow mucilage extrusion (Dean et al., 2007; Macquet et al., 2007b). Data provided in this manuscript strongly support the hypothesis that LUH/MUM1 functions, at least in part, to activate MUM2 expression. The luh/mum1 mutant phenotype is 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/mum1 mutant relative to wild type (Fig 7). Third, p35S::MUM2 can partially rescue the mucilage defect of the luh/mum1 mutant (Fig. 1D). 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/mum1 mutant phenotype appears weaker than that of mum2 even for lines homozygous for putative null alleles (e.g. luh-5; Figs. 1, 2). This could be explained by the fact that MUM2 transcript can still be detected even in a strong luh/mum1 mutant (Fig. 7) and therefore some MUM2 activity likely remains in a luh/mum1 mutant background. These data suggest that, in addition to LUH/MUM1, other positive regulators of MUM2 exist.

In addition to MUM2, LUH/MUM1 also positively regulates BXL1 and SBT1.7, each of which encodes an enzyme needed for normal mucilage extrusion. However, whereas MUM2 expression is reduced over 90% in a luh/mum1 mutant, expression of BXL1 and SBT1.7 are reduced only 40-70% (Fig. 7). These data are consistent with the fact that p35S::MUM2-GFP did not completely rescue the luh/mum1 seed mucilage phenotype. Since all three genes encode enzymes that modify mucilage structure, one role of MUM1 may be to activate such genes in the seed coat epidermis and other similar targets of MUM1 may await identification.

We have shown that LUH/MUM1 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 that LUG (and possibly LUH/MUM1) 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/MUM1 await a more complete understanding of the other proteins with which it must interact to influence transcription.

The role of LUH/MUM1 is independent of other transcription factors controlling seed mucilage biology

In addition to LUH/MUM1, several transcription factors influencing seed mucilage have been identified (reviewed in Arsovski et al., 2010). 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.

Our data reveal a new regulatory pathway required for mucilage modification (Error! Reference source not found.). The significant decrease of transcript levels of MUM2, BXL1 and SBT1.7 in the luh/mum1 mutant compared to that in wild type indicates that LUH/MUM1 positively regulates MUM2, BXL1 and SBT1.7. However the similar transcript levels of LUH/MUM1 in both wild type and corresponding mutants reveal that LUH/MUM1 is not regulated by AP2, the MYB5/TT2-EGL3/TT8-TTG1 complex, GL2 or TTG2 (Fig. 6C). Whether MUM2/BXL1/SBT1.7 is also regulated by AP2 and/or the MYB5/TT2-EGL3/TT8-TTG1 complex remains to be determined (the dash-lined arrow in Fig. 8).

MATERIALS AND METHODS
Plant Material and Growth Conditions

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

Seeds were placed on AT minimal medium (Haughn and Somerville, 1986) in Petri dishes at 4°C for 2 days, before being moved to growth chambers at 20°C 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, open flowers were defined as 0 day post anthesis (DPA) and 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 plasmid p35S::MUM2-GFP was obtained from an earlier study (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').
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.

Plant transformation was done by the Agrobacterium–mediated floral dip method (Clough and Bent, 1998).

**Extraction of seed mucilage**

To an equal amount of seeds (125 mg) was added 5 mL of water, 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 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 NaBH₄ 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 were dialyzed against running deionized water for 36 h, and then with nanopure water several changes for 8 h.

**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 Ca²⁺chelators and alkali, seeds were shaken in corresponding solutions 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) in 0.5 M sodium phosphate, pH 7. Samples were post-fixed for 1 to 2 h in 1% (v/v) osmium tetraoxide in 0.5 M phosphate buffer, and dehydrated by using an ethanol series. Samples were transferred to a solution of propylene oxide and
then solutions of Spurrs resin (Canemco) in increasing increments for infiltration. Samples were embedded in polymerized resin at 60°C 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).

To determine if mucilage would expand from sectioned, hydrated cells, mature dry seeds were added to molten paraplast (Sigma-Aldrich) 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), then mounted on slides and hydrated with 0.01% (w/v) ruthenium red and then 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 (Nanotech). The images were taken using a Hitachi S4700 scanning electron microscope (Hitachi High-Technologies Canada).

Digital images were cropped and labelled in the software ImageJ (National Institutes of Health) and Adobe Photoshop (Adobe Systems).

**Positional Cloning of MUM1**

A mapping population of 420 F2 plants was made by crossing mum1 (Col-2 background) and wild-type Ler. DNA samples 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 Supplemental Table 1.
The sequences of the *luh-5/mum1-1* mutant allele and Col-2 wild-type were determined using 6 sequencing primers for At2g32700 (Supplemental Table 2).

Genomic sequences were amplified by primers At2g32700 TF/TR (5'-ATTGCGGCGCCCGGTTTTGCTTCTTCTTTTTC-3'/ 5'-TTAGCGGCGGCCTTGAAGAGAGGCAGAGTCATTC-3') with the *NotI* enzyme site at both primers in order to conduct transgenic complementation of *luh-5/mum1*. Both the fragment and the vector pART27 were digested with *NotI* before ligation. The *luh-5/mum1* plants were transformed by the *Agrobacterium tumefaciens*–mediated floral dip method (Clough and Bent, 1998).

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.).

**Monosaccharide and linkage analyses**

Neutralized samples of each extract were assayed for total sugar (Dubois et al., 1956) and uronic acid (Filisetti-Cozzi and Carpita, 1991). The uronosyl residues in the neutralized and dialyzed mucilage extracts were carboxyl-reduced with NaBD₄ 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 NaBH₄ 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,
Temperature was held at 80°C 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 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 \(^{13}\text{C}\).

For linkage analysis polysaccharides were per-\(O\)-methylated with \(\text{Li}^+\text{methylsulfinylmethanide}, \) 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°C, the TFA was evaporated in a stream of nitrogen gas, and the sugars were reduced with \(\text{NaBD}_4\) and acetylated. The partly methylated alditol acetates were separated on the same column as the alditol acetates; after a hold at 80°C for 1 min during injection and rapid ramping, the derivatives were separated in a temperature program of 160°C to 210°C at 2°C per min, then to 240°C at 5°C 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).

**RNA Isolation, RT-PCR and qRT PCR**

RNA was isolated from plant tissues except siliques using TRIzol reagent (Invitrogen; Simms et al., 1993). The procedure was adapted (Downing et al., 1992; Western et al.,
2004) to extract RNA from siliques because of its high content of polysaccharides. Siliques at 7 DPA 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 series of phenol: CIA (24:1 Chloroform: isoamyl alcohol) solutions, and finally with CIA. RNA was precipitated with 2 M LiCl on ice. RNA samples were transcribed with SuperScript II reverse transcriptase (Invitrogen). For isolation of RNA specifically from seed coats/endosperm, seed coats of the appropriate stage were separated from the embryo in distilled water under dissecting microscope. The seed coat tissue included the single layer of endosperm at later stages. The tissues 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).

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

**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 germinated and grown under the conditions described above. Approximately 1 g. of leaves from 4-6 week old plants were collected and cut as 0.5-1 mm strips. The strips
were digested in 25 ml of enzyme solution containing 1% cellulase R10 [SERVA Electrophoresis, Heidelberg, Germany], 0.25% macerozyme R10 [SERVA Electrophoresis], 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 with a 200-μm nylon mesh (Spectrum Laboratories), washed in chilled 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. For transfection, the protoplasts were pelleted and resuspended in ice-cold 0.4 M mannitol, 15 mM MgCl$_2$, and 4 mM Mes, pH 5.7.

**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 *SalI* and introduced into the intermediate vector pBluescript2 SK+ to produce *pBS-LUH*. The **LUH/MUM1** cDNA was excised from pBS-LUH with *ClaI* and *AseI* and ligated into the destination vector (pUC19 containing a GFP sequence driven by the 35S promoter (Dr. Wang, personal communication) digested with *ClaI* and *NdeI* (*AseI* and *NdeI* make compatible ends) to produce the **GFP-LUH** gene.

Plasmid DNA was prepared by Endofree Plasmid Maxi Kits (Qiagen). Ten micrograms of plasmid DNA was used for transfection 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 × 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 of 0.5 M mannitol, 20 mM KCl, and 4 mM Mes, pH 5.7). After incubation at ambient 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) and Adobe Photoshop (Adobe Systems).

**Transcriptional Activity**

*LUH/MUM1* cDNA was obtained from the subclone *pBS-LUH* described above by digesting with enzymes *ClaI* and *AseI*, 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 *AseI-SacI* fragment. GD, *Gal4-GUS*, *LexA-Gal4-GUS*, *LexADD(LD)-VP16*, and Chloramphenicol acetyltransferase plasmids were obtained from Dr. Wang (Tiwari et al., 2003). The plasmid DNA was prepared by Endofree Plasmid Maxi Kits (Qiagen), and transfected 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 approximate 20h., protoplasts were lysed by Cell Culture Lysis Reagent (Promega, E153A). One hundred microliters of 1 mM4-methylumbelliferyl-β-D-glucuronide was used as the substrate of β-glucuronidase reaction to produce 4-methylumbelliferone (4-MU). After incubation for 60 min. at 37°C, 100 ml of 0.2 M Na₂CO₃ 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.; Jefferson et al., 1987; Fujii and Uchimiya, 1991).

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Figure 1. Seed Coat Mucilage Phenotypes of Wild Type, Mutants and Transgenic Plants

(A) The *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. *Mum-1* released more mucilage than *mum2-1* under the same treatment. Scale bar 50µm.

(B) The *mum1* mutant mucilage lacks the capability of expansion following hydration. Mucilage expanded from sections of wild-type seed stained with ruthenium red (a) while mucilage in sections of *mum1* seed did not expand. (b). Scale bar 50µm.

(C) Mucilage phenotype of *luh-6*. The *luh-6* seeds extrude much less mucilage than wild type but more than *luh-5/mum1-1* after seeds were shaken in water and stained with Ruthenium Red seeds. Scale bar 50µm.

(D) The seed coat mucilage phenotype of the transgenic plant *luh-5/mum1-1, p35S::MUM2*. Seeds from the transgenic plant *luh-5/mum1-1, p35S::MUM2* showed partial rescue of the *luh-5/mum1-1* mucilage phenotype. Scale bar 100µm.

Figure 2. Total sugar released per mg of seeds of wild type, *mum1-1* and *mum2-1* in water and alkali extracts.

Mature dry seeds were treated sequentially with water, 0.2 M NaOH, and 2.0 M NaOH, to extract mucilage. These extracts after dialysis were carboxyl reduced with NaBD4 to determine quantities and proportions of uronic acids and neutral sugars by GC-MS. Confirmation of uronic acid content was made by colorimetric assay. The error bars represent the SD from 3 biological replicates.
Figure 3. LUH/MUM1 Protein Structure

The predicted MUM1 protein is 787 amino acids in length. The numbers 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 4. Subcellular Localization of LUH

(A) Arabidopsis mesophyll protoplast observed by Nomarski optics.

(B) Localization of the protoplast nucleus using DAPI staining.

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

(D) Localization of free GFP in a protoplast.

Figure 5. LUH Transcriptional Activation Assay

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 UASGal4-GUS and the GUS activity was measured. GD was used as a negative control. OFP1 is a known repressor and VP16 a known activator. 2X indicates that the corresponding amount of DNA used in the assay was doubled. Error bars indicate SD.

Figure 6. LUH Expression Analyses

(A) Presence of LUH transcripts in different tissues. RT-PCR data revealed that LUH was expressed in all the tissues examined. GAPC was used as the internal control.

(B) The temporal expression pattern of LUH in seed coats. The amount of LUH transcripts in three stages (4, 7 and 10 DPA) of seed coat development was examined by
qRT-PCR. The data are presented as relative change where the \textit{LUH} expression level at 4 DPA is arbitrarily set at 1.0. \textit{LUH} was expressed at all stages but most highly at 10 DPA. The error bars indicate SD.

(C) Comparison of \textit{LUH} expression in wild type and mutants. The level of \textit{LUH} transcript in 10 DPA seed coats (stage of maximum expression of \textit{LUH}, see B) of wild type and various mutants was determined using qRT-PCR. The data are presented as a percentage of wild type. The white and the black bars represent results of two independent experiments. The error bars indicate SD, derived from three technical replicates.

**Figure 7. Comparison of MUM2, BXL1, SBT1.7 and GL2 Seed Coat Expression in Wild Type and luh-5/mum1-1.**

qRT-PCR analysis was used to determine the \textit{MUM2, BXL1, SBT1.7} and \textit{GL2} transcript levels in 7 DPA seed coats of both wild type (black bars) and \textit{luh-5/mum1-1} (white bars). For each of the four genes results for two independent experiments are shown. Data are presented as relative expression. The error bars indicate SD for technical replicates within each experiment.

**Figure 8. A Proposed Regulatory Pathway For Seed Coat Mucilage Biosynthesis**

\textit{TTG1, EGL3/TT8} and \textit{MYB5/TT2} form a complex, which regulates \textit{GL2} and \textit{TTG2}. \textit{LUH/MUM1} is independent of the other transcription factors and can activate \textit{MUM2}. Since over-expression of \textit{MUM2} can only partially rescue the \textit{mum1} phenotype, \textit{LUH/MUM1} may be needed to activate other elements (the question mark) for the normal mucilage production. Whether \textit{MUM2/BXL1/SBT1.7} is also regulated by AP2 and/or the \textit{MYB5/TT2-EGL3/TT8-TTG1} complex remains to be determined (the dash-lined arrow).
Table 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 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.

| 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-Araf     | 0.1   | 1.0        | 0.1        | 0.7 | 1.5   | 2.8  | 2.7 | 1.9   | 3.7  |
| 2-Araf     | n.d.  | tr         | tr         | n.d.| tr    | tr   | 0.7 | 0.2   | 0.8  |
| 3-Araf     | n.d.  | tr         | tr         | n.d.| tr    | tr   | tr  | tr    | tr   |
|      | 5-Araf | 2,5-Araf | 3,5-Araf | Xylose: | Mannose: | Galactose: |
|------|--------|----------|----------|---------|----------|-----------|
|      |        |          |          | t-Xyl   | t-Man    | t-Gal     |
| 0.3  | 1.2    | 2.0      | 0.1      | 0.4     | tr       | 2.8       |
| tr   | tr     | n.d.     | tr       | 0.2     | tr       | 1.8       |
| 0.7  | 1.0    | 1.3      | 0.1      | 0.7     | 0.2      | 3.2       |
| 1.9  | 0.1    | 0.2      | 0.2      | 1.0     | 0.5      | 8.4       |
| n.d. | tr     | tr       | n.d.     | 0.2     | tr       | 8.6       |
| tr   | tr     | tr       | tr       | 0.2     | tr       | 6.8       |
| tr   | tr     | tr       | tr       | 0.2     | tr       | 9.7       |
|      |        |          |          | 0.2     | 0.2      | 9.4       |

Note: tr = trace, n.d. = not detected.
|            | 3-Gal | 4-Gal | 6-Gal | 3,4-Gal | 3,6-Gal | Glucose: | Galacturonic Acid: |
|------------|-------|-------|-------|---------|---------|----------|-------------------|
| 3-Gal      | tr    | tr    | tr    | tr      | tr      | t-Glc    | t-GalA            |
|            |       | 3.7   | 4.7   | 4.9     | 1.5     | 0.1      | 46.7             |
| 4-Gal      |       |       |       |         |         | 4-Glc    | 39.1             |
|            |       |       |       |         |         | 3.7      | 67.1             |
| 6-Gal      | n.d.  | tr    | tr    | tr      | tr      | 4,6-Glc  | 4,6-GalA         |
|            |       |       |       |         |         | 0.4      | 0.4              |
| 3,4-Gal    | 0.2   | tr    | tr    | 0.3     | 0.8     | 4,6-Glc  | 4,6-GalA         |
|            |       |       |       |         |         | 0.2      | 0.4              |
| 3,6-Gal    | tr    | tr    | tr    | tr      | tr      | 3,4-Gal  | 3,4-GalA         |
|            |       |       |       |         |         | 0.1      | 0.1              |
| Glucose:   |       |       |       |         |         |          |                   |
| t-Glc      | 0.1   | 0.8   | 2.2   | 0.2     | 0.3     | 0.7      | 46.7             |
| 4-Glc      | 0.7   | 9.8   | 10.8  | 1.5     | 2.3     | 5.3      | 39.1             |
| 4,6-Glc    | 0.4   | 0.8   | 4.3   | 0.6     | 1.9     | 1.1      | 4,6-GalA         |
| Galacturonic Acid: |       |       |       |         |         |          |                   |
| t-GalA     | tr    | 0.2   | 1.7   | 1.2     | 2.9     | 2.7      | 29.3             |
| 4-GalA     | 46.7  | 67.1  | 55.4  | 46.4    | 40.4    | 41.8     | 31.4             |
| 3,4-GalA   | 4.1   | 0.8   | 2.7   | 2.3     | 3.9     | 4.1      | 26.3             |

Values are mean of two samples, with variance less than 5% for all samples. Values are scaled to monosaccharide analysis in Table 1; n.d. = not detected, and tr = trace amounts less than 0.05%.
Supplemental Figure 1. 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. Scale bar 10μm.

Supplemental Figure 2. 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. Scale bar 10μm.

Supplemental Figure 3. Seeds following sequential extraction of mucilage.

Seeds were extracted sequentially first with water, followed by 0.2 M NaOH and 2.0 M NaOH. Samples of seeds were taken following each extraction and stained with ruthenium red.

Supplemental Figure 4. Complementation Test of MUM1

The MUM1 genomic gene including sequences both upstream and downstream was cloned into the vector pART27. Both this construct and the empty plasmid were transformed into mum1. Only the plants with the transgenic MUM1 showed a wild-type mucilage phenotype. Scale bars=100 μm.
### Supplemental Table 1 The 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'-ACATATCAATATATTTAAAGTAGC-3'                | 5'-AAAGAGATGAGAATTTGGAC-3'                    | 120 bp       | 114 bp       |
| MASC06579        | II         | 13.51 mb     | 5'-TTGCAAGGGGAAGCTTTGTCT-3'                   | 5'-TTGCTCCAAAATCAGTCAG-3'                     | 98 bp        | 94 bp        |
| 2m13834          | II         | 13.834 mb    | 5'-GGTTTATTGAGAGTGGAGC-3'                     | 5'-GAATCCTAATCAGAAGAGAG-3'                    | 82 bp        | 74 bp        |
| 2m13873          | II         | 13.873 mb    | 5'-TGCAGTGATCAGTTTATAAGG-3'                   | 5'-CTTGCCTGAATAACTAGTTC-3'                    | 103 bp       | 95 bp        |
| 2m13937          | II         | 13.937 mb    | 5'-TATGTGTGAGGGCAAGAACCC-3'                   | 5'-CCACCTCATGATGTGTATATT-3'                   | 246 bp       | 279 bp       |
| 2m13995          | II         | 13.995 mb    | 5'-CTCGGCAGAATTCTCTCTCT-3'                    | 5'-GTCCAACGTTTCAATATAAG-3'                    | 96 bp        | 104 bp       |
| 2m1404           | II         | 14.04 mb     | 5'-TCACCGGTTCAAGATCAGG-3'                     | 5'-CAGCCGGAATTCTACAGG-3'                      | 116 bp       | 125 bp       |
| 2m14315          | II         | 14.315 mb    | 5'-CTTCTCTCAGCAATGCATCC-3'                    | 5'-GTGTTTCATCTTCAATTTAG-3'                    | 96 bp        | 89 bp        |
| MASC06557        | II         | 14.44 mb     | 5'-GGTCACCTAACCCTACCCTAG-3'                   | 5'-CTTCAATCACATGATCTCTAG-3'                   | 172 bp       | 180 bp       |
| F3G5II-16        | II         | 15.6 mb      | 5'-GCTCCTTTATAATGCAAGATG-3'                   | 5'-CAGTCTCAACTTGTCTATGTG-3'                   | 125 bp       | 138 bp       |
# Supplemental Table 2 sequencing primers of At2g32700

| Primer name   | Sequence                                      |
|---------------|-----------------------------------------------|
| At2g32700 seq1 | 5'-TGTTTGGGCTTTTATTCAGG-3'                    |
| At2g32700 seq2 | 5'-ATCAGCAACAAACCATCATGG-3'                   |
| At2g32700 seq3 | 5'-TCTTTCCGTTGCTTGGG-3'                       |
| At2g32700 seq4 | 5'-TGTGTCCTGGCCCTAAAACAGC-3'                  |
| At2g32700 P1   | 5'-TCAACAGATCCACTGCATCC-3'                    |
| At2g32700 P2   | 5'-AAAAGTCCTGAGCTCTGCTGC-3'                   |
### Supplemental Table 3 Primers for RT-PCR and qRT PCR

| Primer name               | Sequences                                      | Reference                        |
|---------------------------|------------------------------------------------|---------------------------------|
| MUM1RT3F                  | 5'-TGATAGCAACAATGATATTCGC-3'                   | this study                      |
| MUM1RT3R/At2g32700P2      | 5'-AAAAGTCCTGAGCTCTCTGC-3'                     | this study                      |
| MUM1 realtime F            | 5'-CATCCACGAGCTTAGCAACA-3'                     | this study                      |
| MUM1 realtime R            | 5'-GGCCTGCTACCGTCATACTAC-3'                    | this study                      |
| MUM2 realtime F/MUM2p1     | 5'-GTTACAACGCCCGTCAAGT-3'                      | Dean et al., 2007               |
| MUM2 realtime R/MUM2p2     | 5'-ACGTCGGACACATGTCCTGA-3'                     | Dean et al., 2007               |
| fwd AtSBT1.7 RT            | 5'-CTTCGGGAGACGAGGAGTC-3'                      | Rautengarten et al., 2008       |
| rev AtSBT1.7 RT            | 5'-GACGGCTTCGAGCTCTAC-3'                       | Rautengarten et al., 2008       |
| fwd At5g49360 p3/p4       | 5'-ACTAGCACTCCGGAAGAGC-3'                      | Arsovski et al., 2009           |
| rev At5g49360 p3/p4       | 5'-CAATCTTTCTGCACAGT-3'                        | Arsovski et al., 2009           |
| fwd GL2                   | 5'-AGACACATGGAAGCGCTATTC-3'                    | this study                      |
| rev GL2                   | 5'-TCTCGTGCCTTGCTTGATAG-3'                     | this study                      |
| fwd GAPC                  | 5'-TCAGACTCGAGAAAGCTGCTAC-3'                   | this study                      |
| rev GAPC                  | 5'-GATCAAGTCGACCACACGG-3'                      | this study                      |
| Actin2-RT-FW              | 5'-CAGAAGGATCATATGTGGGTGTA-3'                  | this study                      |
| Actin2-RT-RW              | 5'-GAGGAGCCTCGGTAAGAAGA-3'                     | this study                      |
Figure 1. Seed Coat Mucilage Phenotypes of Wild Type, Mutants and Transgenic Plants

(A) The *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. *Mum-1* released more mucilage than *mum2-1* under the same treatment. Scale bar 50µm.

(B) The *mum1* mutant mucilage lacks the capability of expansion following hydration. Mucilage expanded from sections of wild-type seed stained with ruthenium red (a) while mucilage in sections of *mum1* seed did not expand. (b). Scale bar 50µm.

(C) Mucilage phenotype of *luh-6*. The *luh-6* seeds extrude much less mucilage than wild type but more than *luh-5/mum1-1* after seeds were shaken in water and stained with Ruthenium Red seeds. Scale bar 50µm.

(D) The seed coat mucilage phenotype of the transgenic plant *luh-5/mum1-1*, p35S::MUM2. Seeds from the transgenic plant *luh-5/mum1-1*, p35S::MUM2 showed partial rescue of the *luh-5/mum1-1* mucilage phenotype. Scale bar 100µm.
Figure 2. Total sugar released per mg of seeds of wild type, *mum1-1* and *mum2-1* in water and alkali extracts.

Mature dry seeds were treated sequentially with water, 0.2 M NaOH, and 2.0 M NaOH, to extract mucilage. These extracts after dialysis were carboxyl reduced with NaBD4 to determine quantities and proportions of uronic acids and neutral sugars by GC-MS. Confirmation of uronic acid content was made by colorimetric assay. The error bars represent the SD from 3 biological replicates.
Figure 3. LUH/MUM1 Protein Structure

The predicted MUM1 protein is 787 amino acids in length. The numbers 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 4. Subcellular Localization of LUH

(A) Arabidopsis mesophyll protoplast observed by Nomarski optics.

(B) Localization of the protoplast nucleus using DAPI staining.

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

(D) Localization of free GFP in a protoplast.
Figure 5. LUH Transcriptional Activation Assay

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 UASGal4-GUS and the GUS activity was measured. GD was used as a negative control. OFP1 is a known repressor and VP16 a known activator. 2X indicates that the corresponding amount of DNA used in the assay was doubled. Error bars indicate SD.
Figure 6. LUH Expression Analyses

(A) Presence of LUH transcripts in different tissues. RT-PCR data revealed that LUH was expressed in all the tissues examined. GAPC was used as the internal control.

(B) The temporal expression pattern of LUH in seed coats. The amount of LUH transcripts in three stages (4, 7 and 10 DPA) of seed coat development was examined by qRT-PCR. The data are presented as relative change where the LUH expression level at 4 DPA is arbitrarily set at 1.0. LUH was expressed at all stages but most highly at 10 DPA. The error bars indicate SD.

(C) Comparison of LUH expression in wild type and mutants. The level of LUH transcript in 10 DPA seed coats (stage of maximum expression of LUH, see B) of wild type and various mutants was determined using qRT-PCR. The data are presented as a percentage of wild type. The white and the black bars represent results of two independent experiments. The error bars indicate SD, derived from three technical replicates.
Figure 7. Comparison of MUM2, BXL1, SBT1.7 and GL2 Seed Coat Expression in Wild Type and luh-5/mum1-1.

qRT-PCR analysis was used to determine the MUM2, BXL1, SBT1.7 and GL2 transcript levels in 7 DPA seed coats of both wild type (black bars) and luh-5/mum1-1 (white bars). For each of the four genes results for two independent experiments are shown. Data are presented as relative expression. The error bars indicate SD for technical replicates within each experiment.
Figure 8. A Proposed Regulatory Pathway 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 elements (the question mark) for the normal mucilage production. Whether MUM2/BXL1/SBT1.7 is also regulated by AP2 and/or the MYB5/TT2-EGL3/TT8-TTG1 complex remains to be determined (the dash-lined arrow).