The Arabidopsis Transcription Factor *LUH/MUM1* Is Required for Extrusion of Seed Coat Mucilage[1][W][OA]

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During differentiation, the Arabidopsis (*Arabidopsis thaliana*) seed coat epidermal cells secrete mucilage composed primarily of rhamnogalacturonan I that is extruded from the seed coat upon imbibition. The mucilage of the *mucilage modified1* (*mum1*) mutant contains rhamnogalacturonan I 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 nucleus-localized transcriptional activator. *LUH/MUM1* is expressed in all the tissues examined, including the seed coat. Quantitative reverse transcription-polymerase chain reaction data suggest 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 the 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.

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 (*Arabidopsis thaliana*), 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, 2001, 2004; Penfield et al., 2001; Usadel et al., 2004; Macquet et al., 2007a). RG I is a backbone of alternating (1→2)-α-L-Rha and (1→4)-α-D-GalUA to which are attached various polysaccharide side chains (Ridley et al., 2001; Willats et al., 2001). Arabidopsis mucilage RG I is relatively unbranched, with Gal and Ara identified as the principal side chain sugars. The pectin homogalacturonan (HG) has also been identified as a component of Arabidopsis mucilage (Willats et al., 2001; Dean et al., 2007; Macquet et al., 2007a, 2007b). 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 hydrolyze ester linkages (Western et al., 2000; Dean et al., 2007; Macquet et al., 2007a).

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 defense-related signaling (Cosgrove, 1993, 1997; Ridley et al., 2001).

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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 whose products 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 (Western et al., 2001; Gonzalez et al., 2009; Li et al., 2009). 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 Rha synthase that is required to convert UDP-D-Glc to UDP-L-Rha, 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, *MUM1* and *MUM2* (Western et al., 2001), *β-XYLOSIDASE1* (*BXL1*; Arsovski et al., 2009), and *SUBTILISIN-LIKE SERINE PROTEASE1.7* (*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 trim 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 nucleus-localized transcription factor, *LEUNIG_HOMOLOG* (*LUH*; Sitaraman et al., 2008), which is expressed in the seed coat and other tissues. Quantitative reverse transcription (qRT)-PCR and molecular complementation studies show that *MUM1* is required for the normal expression of *MUM2*, *BXL1*, and *SBT1.7*.

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 Fig. S1). To examine the cell structure during development, seeds of wild-type and *mum1* plants were observed using light microscopy at the developmental stages of 4, 7, and 10 DPA. At these stages, the structure of the seed coat epidermal cells of wild-type and *mum1-1* plants were found to be indistinguishable (Supplemental Fig. S2). 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 and 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 0.05 M EDTA, EGTA, or cyclohexane diamine tetraacetic acid (CDTA), and alkali, such as 1 M Na_2CO_3 or 0.5 M 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. S3). Only the wild-type seeds released significant amounts of mucilage in water. NaOH at 0.2 M caused rupture of the primary cell wall in both mutants, with release of mucilage from *mum1* seeds and lesser amounts from *mum2* seeds (Fig. 2; Supplemental Fig. S3). NaOH at 2 M is able to remove most of the inner adherent layer of mucilage in the wild type and some of the adherent layer in the mutants (Fig. 2; Supplemental Fig. S3). The *mum2* mutant seed appears to retain the most mucilage (Supplemental Fig. S3). Thus, the ability to extract mucilage from *mum1* and *mum2* mutant seed is impaired relative to the wild type.

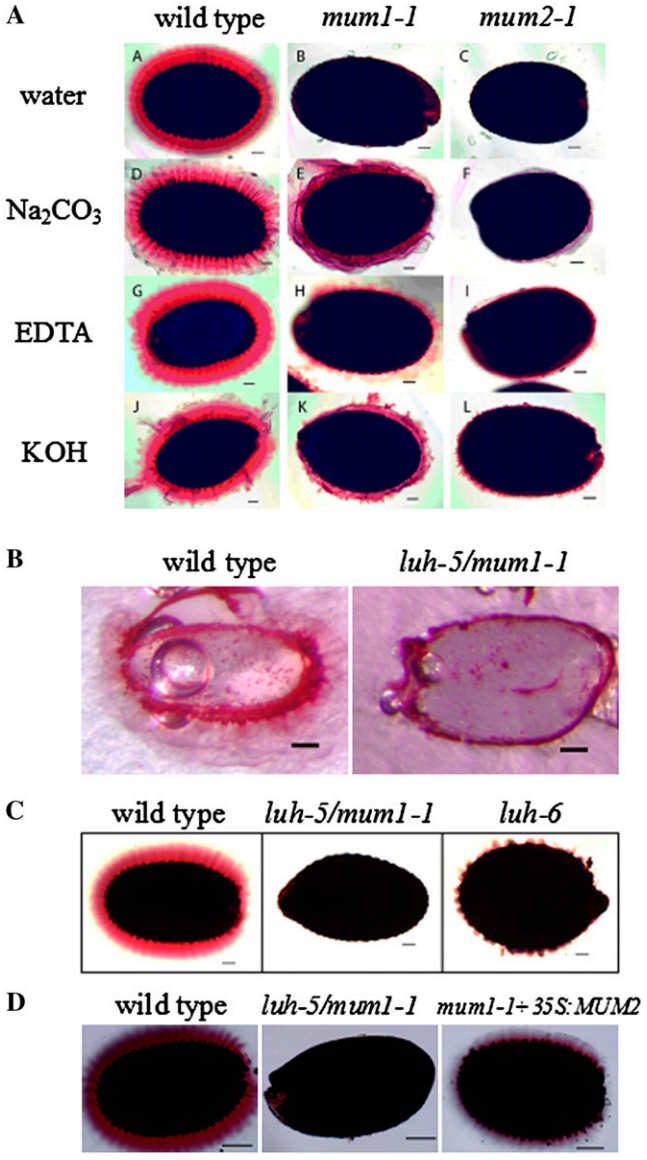

Figure 1. Seed coat mucilage phenotypes of wild-type, mutant, and transgenic plants. A, The *mum1* mucilage lacks the capability to extrude following exposure to water. All the seeds were stained with ruthenium red after treatment with the indicated solutions. *mum1-1* and *mum2-1* could not release 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. Bars = 50 μm. B, The *mum1* mutant mucilage lacks the capability of expansion following hydration. Mucilage expanded from sections of wild-type seed stained with ruthenium red (left), while mucilage in sections of *mum1* seed did not expand (right). Bars = 50 μm. C, Mucilage phenotype of *luh-6*. The *luh-6* seeds extruded much less mucilage than the wild type but more than *luh-5/mum1-1* after seeds were shaken in water and stained with ruthenium red. Bars = 50 μm. 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. Bars = 100 μm.

The Extractable Seed Coat Mucilage Compositions of *mum1* and *mum2* Are Similar and Distinct from the Wild Type

Because of the differences in mucilage extraction profiles, we performed monosaccharide and linkage analyses to determine the compositions. After sequential extraction of the mucilage fractions in water and 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-dideuterio-sugar residues. Monosaccharide analysis showed that for water extracts, the 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 with vanishingly small amounts of Rha (Table I). As described above, addition of 0.2 M NaOH caused rupture of the outer seed coat wall, leading to the release of large amounts of material containing primarily Rha and GalA from the mutants as well as additional mucilage from the wild type (Table I). The tightly attached gel layer extracted by 2 M NaOH was similar in monosaccharide distribution between the wild type and the 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 the wild type and mutant was primarily 2-Rha and 4-GalA, representing a relatively unbranched RG I backbone (Table II). The presence of large

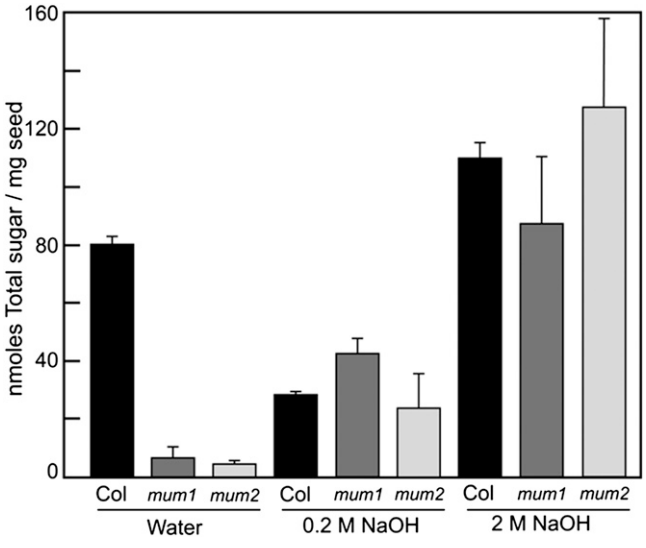

Figure 2. Total sugar released per mg of seeds of wild-type, *mum1-1*, and *mum2-1* plants in water and alkali extracts. Mature dry seeds were treated sequentially with water, 0.2 M NaOH, and 2.0 M NaOH to extract mucilage. These extracts after dialysis were carboxyl reduced with $NaBD_4$ to determine quantities and proportions of uronic acids and neutral sugars by gas chromatography-mass spectrometry. Confirmation of uronic acid content was made by colorimetric assay. The error bars represent SD values from three biological replicates.

Table I. *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*

Values are means ± variance of two independent extractions. trc, Trace amounts less than 0.05%.

Extract	Water			0.2 M NaOH			2.0 M NaOH		
	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

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 II). 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 the wild type. Notably, the degree of branching of the RG I, as determined by the ratio of 2,4-Rha to 2-Rha, was substantially higher in both *mum* mutants compared with the 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 the gel layers in both *mum* mutants also display increased RG I branching, with increases in *t*-Gal residues accounting for much of the increase in branching; increases in *t*-Ara over wild-type amounts were found only in *mum2* extracts (Table II).

Cloning of *MUM1*

The *MUM1* gene was identified using positional cloning. The region containing *MUM1* was mapped to between 13.834 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 Glu-97 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 (luh_172H3, luh_147A6, and CS90546; Seattle TILLING Project [http://tilling.fhcrc.org]) mutant lines. 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 *LUH* with mutant alleles *luh-1*, *luh-2*, *luh-3* (luh_172H3, luh_147A6, and SALK_107245C, respectively; Sitaraman et al., 2008), and *luh-4* (SALK_097509; Stahle et al., 2009). Furthermore, 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 Glu-73 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 coding region, and 0.6 kb of 3′ sequences, was cloned into the binary transformation vector pART27 and transformed into *mum1* plants via *Agrobacterium tumefaciens*-mediated transformation. The *mum1* mutant plants transformed with the *MUM1* gene extruded mucilage like the wild type, while those transformed with the empty vector showed no such rescue (Supplemental Fig. S4).

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 Liu, 2000). To determine if LUH is also nucleus 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).

Table II. *Comparison of linkage distribution in mucilage and other polymers extracted from seeds sequentially with water, 0.2 M NaOH, and 2.0 M NaOH*

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

Sugar and Linkage	Water			0.2 M NaOH			2.0 M NaOH		
	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*	Col	*mum1*	*mum2*
Fuc									
t-Fuc	tr	tr	tr	tr	tr	tr	tr	tr	tr
Rha									
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
Ara									
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
Xyl									
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
Man									
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
Gal									
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
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
Glc									
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
GalUA									
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

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 cotransfected 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 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 act as transcriptional activators 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 d 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).

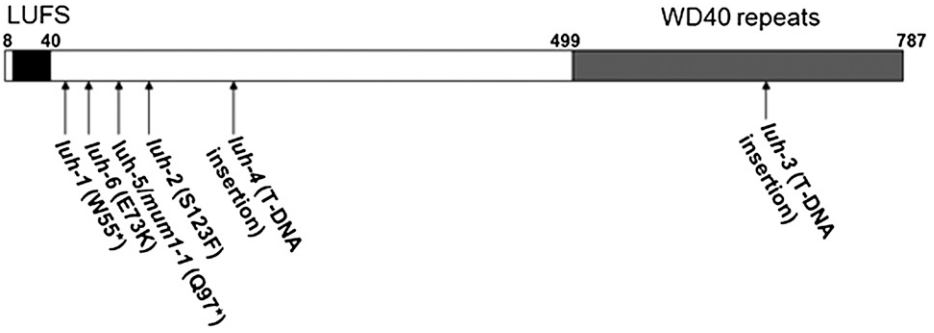

Figure 3. LUH/MUM1 protein structure. The predicted MUM1 protein is 787 amino acids in length. The numbers represent the amino acid positions. The LUFS domain (black bar) is located at the N terminus, and WD40 repeats (gray bar) are located at the C terminus (http://smart.embl-heidelberg.de/). The arrows indicate the positions of the mutations of various alleles.

LUH Regulates *MUM2*

MUM2 encodes a β-galactosidase that 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) 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* plants 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 the 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 the wild type and the *ap2-1*, *ttg1-1*, *ttg2-1*, and *gl2-1* mutants. The data indicate that there is little difference in *LUH* expression between the 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 func-

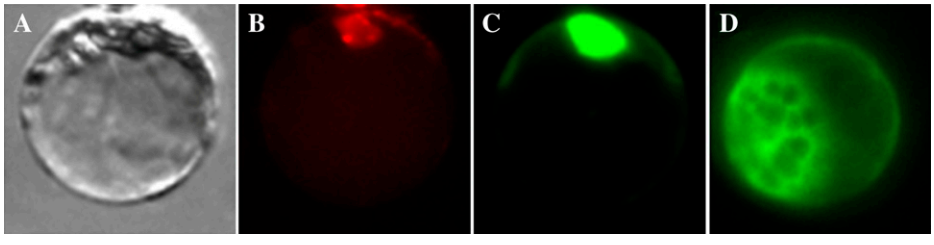

Figure 4. Subcellular localization of LUH. A, Arabidopsis mesophyll protoplast observed by Nomarski optics. B, Localization of the protoplast nucleus using 4′,6-diamidino-2-phenylindole staining. C, Localization of GFP-LUH to the protoplast nucleus. D, Localization of free GFP in a protoplast.

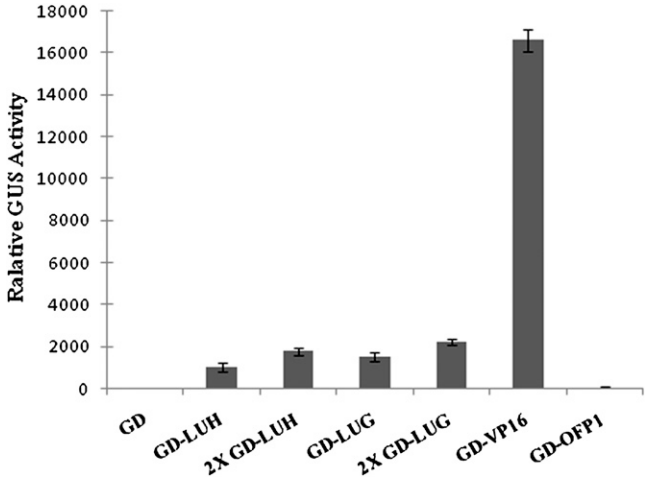

Figure 5. LUH transcriptional activation assay. The vectors *GD*, *GD-LUH*, *GD-LUG*, *GD-OFP1*, and *GD-VP16* were individually transfected into mesophyll protoplasts together with the reporter UAS_{Gal4}-GUS, and the GUS activity was measured. GD was used as a negative control. OFP1 is a known repressor and VP16 a known activator. 2X indicates that the corresponding amount of DNA used in the assay was doubled. Error bars indicate SD.

tion (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 with 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 of 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* are indistinguishable from those of the wild type and distinct from those of mutants that synthesize low amounts of mucilage (Supplemental Figs. S1 and S2; Penfield et al., 2001; Western et al., 2001, 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 those of the primary cell wall (Fig. 1B; Dean et al., 2007; Macquet et al., 2007b). This hypothesis is consistent with the chemical analysis of *luh/mum1* mucilage, which also indicates changes in mucilage structure. Both *mum2* and *luh-5/mum1-1* mutants have higher mole percentages of the RG I side chain monosaccharides Gal and Ara relative to the backbone sugars Rha and GalUA, suggesting the presence of more and/or larger RG I side chains. The wild-type Arabidopsis seed coat epidermal cells synthesize 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 the wild type appears to have a similar branched composition as the mutants (extracted with 2 M NaOH; Table I). Taken together, these data suggest that the action of LUH/MUM1 and MUM2 is required to remove mucilage RG I side chains from the mucilage in the apoplast, converting much of the mucilage to an expandable

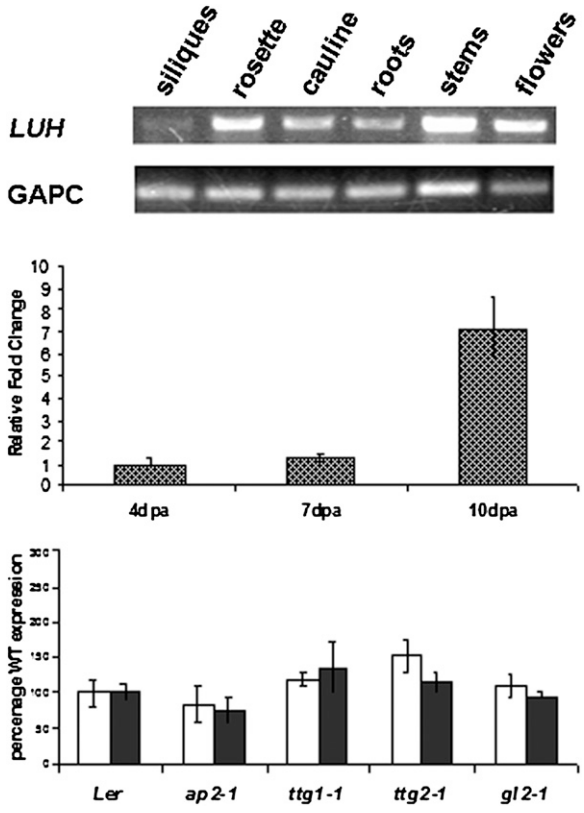

Figure 6. LUH expression analyses. A, Presence of *LUH* transcripts in different tissues. RT-PCR data revealed that *LUH* was expressed in all the tissues examined. *GAPC* was used as the internal control. B, The temporal expression pattern of *LUH* in seed coats. The amount of *LUH* transcripts in three 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 (WT) and mutant plants. The level of *LUH* transcript in 10-DPA seed coats (stage of maximum expression of *LUH*; see B) of wild-type and various mutant plants was determined using qRT-PCR. The data are presented as a percentage of the wild type. The white and black bars represent results of two independent experiments. The error bars indicate SD, derived from three technical replicates.

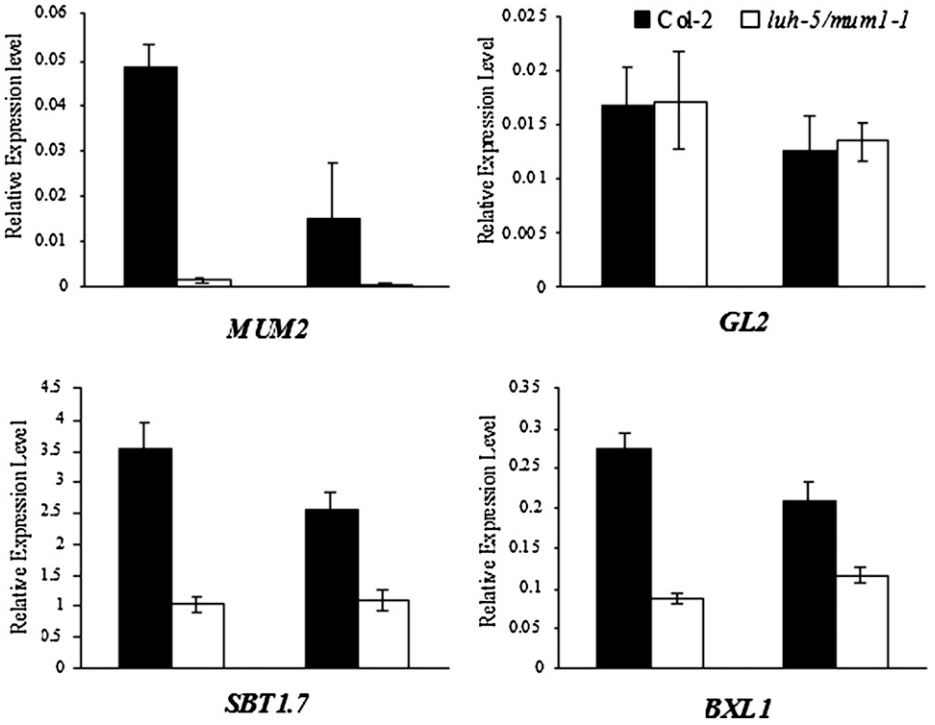

Figure 7. Comparison of *MUM2*, *BXL1*, *SBT1.7*, and *GL2* seed coat expression in wild-type and *luh-5/mum1-1* plants. qRT-PCR analysis was used to determine the *MUM2*, *BXL1*, *SBT1.7*, and *GL2* transcript levels in 7-DPA seed coats of both wild-type (black bars) and *luh-5/mum1-1* (white bars) plants. 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.

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 et al., 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; Chen et al., 2000; Liu et al., 2000; Kuusk et al., 2006), leaf (Cnops et al., 2004; Navarro 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 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 (J. Huang and G.W. Haughn, unpublished data).

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

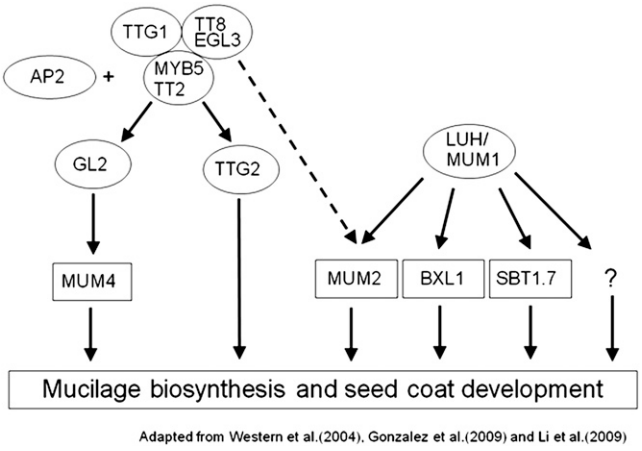

Figure 8. Proposed regulatory pathway for seed coat mucilage biosynthesis. TTG1, EGL3/TT8, and MYB5/TT2 form a complex, which regulates GL2 and TTG2. LUH/MUM1 is independent of the other transcription factors and can activate *MUM2*. Since overexpression of *MUM2* can only partially rescue the *mum1* phenotype, LUH/MUM1 may be needed to activate other elements (the question mark) for 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 (dashed arrow).

in the removal of RG I side chains from seed mucilage to allow mucilage extrusion (Dean et al., 2007; Macquet et al., 2007b). Data provided in this paper 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 the 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 and 2). This could be explained by the fact that *MUM2* transcript can still be detected even in a strong *luh/mum1* mutant (Fig. 7); 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* is reduced only 40% to 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 either as an activator or a repressor depending on the specific coregulator with which it interacts and that the available coregulators 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 (for review, see 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 the synthesis of mucilage. One target of GL2 is the *MUM4* gene encoding a Rha synthase.

Our data reveal a new regulatory pathway required for mucilage modification (Fig. 8). The significant decrease of transcript levels of *MUM2*, *BXL1*, and *SBT1.7* in the *luh/mum1* mutant compared with that in the wild type indicates that LUH/MUM1 positively regulates *MUM2*, *BXL1*, and *SBT1.7*. However, the similar transcript levels of *LUH/MUM1* in both the 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 (dashed arrow in Fig. 8).

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) ecotypes Columbia-2 (Col-2) and Landsberg *erecta* (L*er*) were used as wild-type controls. The *mum1/luh-5* and *mum2-1* mutants were isolated from an ethyl methanesulfonate-mutagenized M3 population of wild-type Col-2 Arabidopsis 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 through The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org), and the *ap2-1*, *gl2-1*, *ttg1-1*, and *tt2-1* (L*er* ecotype) mutants were obtained from the Arabidopsis Biological Resource Center by Western et al. (2004).

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

To isolate different developmental stages of siliques, open flowers were defined as 0 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 cleaved-amplified polymorphic sequence primers At2g32700 CAPS1/2 (5′-TGAATTACGTAACTGACCAGTGG-3′/5′-AGGCTGCTTCATGCGTTCC-3′). The DNA fragments were cut using *Pst*I, which produces two bands in the wild-type background (87 + 152 bp) but only one band (239 bp) 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 was 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 milliliters 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 were saved for carbohydrate analyses. The majority of the extracts were dialyzed against running deionized water for 36 h and then with several changes of nanopure water 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 Ca^{2+} 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 postfixed 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 Spurr's resin (Canemco) in increasing increments for infiltration. Samples were embedded in polymerized resin at 60°C in an oven. Seeds were sectioned (0.2–0.5 μm) with glass knives on a microtome (Reichert-Jung). 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 with 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. Sections (20 μm) were produced on a HM 325 microtome (Microm), 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 scanning electron microscopy were mounted on stubs and 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 labeled with 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 L*er*. DNA samples were stored by crushing young leaves on FTA classic card (Whatman). Small discs containing samples were punched from FTA cards for PCR (Zhang et al., 2007). Sequence information was obtained from the Arabidopsis Genome Initiative (2000) and Cereon (Jander et al., 2002) at the TAIR Web site (http://www.arabidopsis.org) to generate simple sequence length polymorphism markers for map-based cloning. Primer sequences are listed in Supplemental Table S1.

The sequences of the *luh-5/mum1-1* mutant allele and Col-2 wild type were determined using six sequencing primers for At2g32700 (Supplemental Table S2).

Genomic sequences were amplified by primers At2g32700 TF/TR (5′-ATTGCGGCCGCCCGGTTTTGCTTCTTCTTTTTC-3′/5′-TTAGCGGCCGCGTTGAAAGAGAGGCAGAGTCATTC-3′) with the *Not*I 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 *Not*I before ligation. The *luh-5/mum1* plants were transformed by the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998).

Sequences were compared with the databases at TAIR (http://www.arabidopsis.org) and the National Center for Biotechnology Information (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).

Monosaccharide and Linkage Analyses

Neutralized samples of each extract were assayed for total sugar (Dubois et al., 1956) and urnoic acid (Filisetti-Cozzi and Carpita, 1991). 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 and Carpita (1992) and modified by Carpita and McCann (1996). Uronosyl-reduced wall material (1–2 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 on a 0.25-mm × 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. Electron-impact mass spectrometry 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, mass-to-charge ratio 187/189, 217/219, and 289/291, according to the equation described by 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 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 3-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 $NaBD_4$ 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 min^{-1}, then to 240°C at 5°C min^{-1}, 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. One milliliter of REB (25 mM Tris-HCl, pH 8.0, 25 mM EDTA, 75 mM NaCl, and 1% SDS) was added to the dry powder, and the RNA was extracted with 1 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 with a 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. The 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 S3) 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 and colleagues (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- to 6-week-old plants were collected and cut as 0.5- to 1-mm strips. The strips were digested in 25 mL of enzyme solution containing 1% cellulase R10 (Serva Electrophoresis), 0.25% macerozyme R10 (Serva Electrophoresis), 0.4 M mannitol, 80 mM $CaCl_2$, and 20 mM MES, 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 h. 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 Glc, 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 *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. S. Wang, personal communication]) digested with *Cla*I and *Nde*I (*Ase*I and *Nde*I make compatible ends) to produce the *GFP-LUH* gene.

Plasmid DNA was prepared with 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 to 20 h in darkness, the GFP signals were observed with 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 *Cla*I and *Ase*I and then ligated to the destination vector pUC19 containing the GD driven by the 35S promoter (Wang et al., 2005, 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*, *LexADD(LD)-VP16*, and chloramphenicol acetyltrasferase plasmids were obtained from Dr. S. Wang (Tiwari et al., 2003). The plasmid DNA was prepared with 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 (1× versus 2×), chloramphenicol acetyltrasferase plasmid was used to adjust DNA amounts such that all transfections had the same quantity of DNA. After incubation at room temperature for approximate 20 h, protoplasts were lysed with Cell Culture Lysis Reagent (Promega; E153A). One hundred microliters of 1 mM 4-methylumbelliferyl-β-D-glucuronide was used as the substrate of GUS reaction to produce 4-methylumbelliferone. After incubation for 60 min at 37°C, 100 mL of 0.2 M Na_2CO_3 was added to stop the reaction. Fluorescence of 4-methylumbelliferone at 455 nm with excitation at 365 nm was measured from a Fluoroskan Finstruments Microplate Reader (MTX Laboratory Systems; Jefferson et al., 1987; Fujii and Uchimiya, 1991).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: LUG (NM_119407, At4g32551), LUH (NM_128829, At2g32700), MUM2 (NM_125775; Q9FFN4, At5g63800), BXL1 (NM_124313, At5g49360), and SBT1.7 (ARA12; NM_126136, At5g67360).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Scanning electron microscopy of wild-type and *mum1* seeds.

Supplemental Figure S2. Seed coat development of the wild type and *mum1*.

Supplemental Figure S3. Seeds following sequential extraction of mucilage.

Supplemental Figure S4. Complementation test of *MUM1*.

Supplemental Table S1. Primers used for map-based cloning of MUM1.

Supplemental Table S2. Sequencing primers of At2g32700.

Supplemental Table S3. Primers for RT-PCR and qRT-PCR.

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