MUCILAGE-MODIFIED4 Encodes a Putative Pectin Biosynthetic Enzyme Developmentally Regulated by *APETALA2*, *TRANSPARENT TESTA GLABRA1*, and *GLABRA2* in the Arabidopsis Seed Coat[1]

Tamara L. Western[2], Diana S. Young, Gillian H. Dean, Wei Ling Tan[3], A. Lacey Samuels, and George W. Haughn*

Botany Department, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z4

The Arabidopsis seed coat epidermis undergoes a complex process of differentiation that includes the biosynthesis and secretion of large quantities of pectinaceous mucilage, cytoplasmic rearrangement, and secondary cell wall biosynthesis. Mutations in *MUM4* (*MUCILAGE-MODIFIED4*) lead to a decrease in seed coat mucilage and incomplete cytoplasmic rearrangement. We show that *MUM4* encodes a putative NDP-L-rhamnose synthase, an enzyme required for the synthesis of the pectin rhamnogalacturonan I, the major component of Arabidopsis mucilage. This result suggests that the synthesis of monosaccharide substrates is a limiting factor in the biosynthesis of pectinaceous seed coat mucilage. In addition, the reduced cytoplasmic rearrangement observed in the absence of a key enzyme in pectin biosynthesis in *mum4* mutants establishes a causal link between mucilage production and cellular morphogenesis. The cellular phenotype seen in *mum4* mutants is similar to that of several transcription factors (*AP2* [*APETALA2*], *TTG1* [*TRANSPARENT TESTA GLABRA1*], *TTG2* *MYB61*, and *GL2* [*GLABRA2*]). Expression studies suggest that *MUM4* is developmentally regulated in the seed coat by *AP2*, *TTG1*, and *GL2*, whereas *TTG2* and *MYB61* appear to be regulating mucilage production through alternate pathway(s). Our results provide a framework for the regulation of mucilage production and secretory cell differentiation.

The mature seed coat, in addition to forming a protective layer around the embryo, can play roles in such processes as seed dispersal and germination. One adaptation believed to act in these processes is the production of mucilage in the epidermal cells of the seed coat. This specialization, known as myxospermy, is found in many families, including the Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae (Grubert, 1981; Boesewinkel and Bouman, 1995). Seed imbibition in these plants leads to the formation of a gel capsule around the seed that is thought to aid hydration and/or dispersal. Mucilage consists of pectins, a heterogeneous group of acidic polysaccharides that form a gel-like matrix. In the primary cell wall, pectins form the matrix in which cellulose microfibrils and hemicelluloses are embedded. Key pectins include homo-GalUA (HGA) and rhamnogalacturonan I (RGI). HGA consists of an unbranched chain of α-1,4-linked GalUA residues whose carboxyl groups may be modified through methyl-esterification. RGI has a backbone of alternating α-1,2-linked Rha and α-1,4-linked GalUA residues, with sugar side chains attached on varying numbers of Rha residues. Both HGA and RGI are believed to be synthesized in the Golgi apparatus through the activities of glycosyltransferases using nucleotide sugars imported from the cytoplasm (for review, see Ridley et al., 2001). Chemical analysis of Arabidopsis mucilage has shown that it is composed largely of α-1,2 linked Rha and α-1,4 linked GalUA, suggesting that it is comprised primarily of RGI (Penfield et al., 2001). Immunofluorescence studies of mucilage have demonstrated the presence of methyl-esterified HGA (Willats et al., 2001); however, the authors did not test for the presence of RGI.

The production of mucilage in the epidermal cells of the Arabidopsis seed coat is part of a coordinated developmental process that begins with cell growth, followed by biosynthesis and polar secretion of large quantities of pectin, formation of a cytoplasmic column through cytoplasmic constriction and vacuolar contraction, and, finally, the synthesis of a secondary cell wall (columella; Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). Seed coat mucilage is dispensable under laboratory conditions in Arabidopsis and *mum* (*mucilage-modified*) mutants affecting

[1] This work was supported by the Natural Science and Engineering Research Council of Canada (Discovery Grants to G.W.H. and A.L.S. and a Cell Wall Multi-Network Grant to G.W.H.).

[2] Present address: Biology Department, McGill University, 1205 Docteur Penfield Avenue, Montreal, QC, Canada H3A 1B1.

[3] Present address: Ottawa Regional Cancer Centre, Centre for Cancer Therapeutics, Third Floor, 503 Smyth Road, Ottawa, ON, Canada K1H 1C4.

* Corresponding author; email haughn@interchange.ubc.ca; fax 604–822–6089.

Article, publication date, and citation information can be found at http://www.plantphysiol.org/cgi/doi/10.1104/pp.103.035519.

mucilage production can be identified by a simple screening method (Western et al., 2001). Mutations in one of these genes, *MUM4*, yield a phenotype where mucilage is not released from hydrated mature seeds.

Several genes encoding putative transcription factors have been implicated in seed coat epidermal development because mutations in these genes result in seeds that fail to release mucilage upon hydration. Mutants in *AP2* (*APETALA2*), in addition to their defects in floral morphogenesis, lack differentiation past the growth phase of mucilage secretory cells (Jofuku et al., 1994; Western et al., 2001). *TTG1* (*TRANSPARENT TESTA GLABRA1*), *TTG2*, and *GL2* (*GLABRA2*), which were originally identified through their role in trichome specification, have defects in both mucilage and columella production in the seed coat (Koornneef, 1981; Penfield et al., 2001; Western et al., 2001; Johnson et al., 2002). It has been shown at the genetic and molecular levels in trichomes and root hairs that TTG1 interacts with a basic helix-loop-helix (bHLH) protein and a tissue-specific MYB protein to activate both *GL2* and *TTG2* (Payne et al., 2000; Johnson et al., 2002; Schiefelbein, 2003). Recently, mutations in *MYB61* have been found to specifically affect both mucilage and columella production in the Arabidopsis seed coat (Penfield et al., 2001).

The objective of this study was to characterize the role of *MUM4* in the development of the Arabidopsis seed coat. Using positional cloning of *MUM4*, we demonstrate that *MUM4* encodes a putative NDP-L-Rha synthase. Mutations in this gene lead to reduced mucilage in the seed coat and an altered columella. Expression studies show that *MUM4* is developmentally regulated during seed coat differentiation such that its transcript levels are increased at the time of mucilage production. Furthermore, *MUM4* appears to be a downstream target of a cascade of transcription factors that includes AP2, TTG1, and GL2. These results demonstrate the importance of mucilage production for the morphology of seed coat epidermal cells and suggest a regulatory framework for the control of seed coat epidermal differentiation.

RESULTS

mum4 Mutant Phenotype

Two alleles of *mum4* (*mum4-1* and *mum4-2*) were identified in a screen for mutants that lacked extruded mucilage as visualized by staining with Ruthenium red (compare Fig. 1, A with D; Western et al., 2001). A T-DNA insertion allele, *mum4-3*, was also identified. Because all alleles of this mutant had similar phenotypes, only that of *mum4-1* is described.

Viewed with scanning electron microscopy, mature *mum4* seed coats are marked not only by the absence of mucilage extrusion upon hydration but also by the absence of volcano-shaped columellae (compare Fig. 1, B with E). Light microscopy of sections of mature seeds revealed that both columellae and mucilage are present in *mum4* but reduced in comparison with wild type (compare Fig. 1, C with F). No other phenotypes were observed.

To more fully characterize the *mum4* seed coat epidermal cell defects, their development was studied with light microscopy (Fig. 1, G–L). Under our growth conditions, seed maturation to dry seed takes approximately 18 d and has been staged by DPA. In terms of embryo growth, 4 DPA is early to mid globular stage, 7 DPA is mid-torpedo stage, whereas 10 DPA is late upturned-U stage (this study; Western et al., 2000). The early stages of *mum4* epidermal cell differentiation are similar to that of wild type: The cells grow, and mucilage starts to accumulate, accompanied by the initiation of intracellular cytoplasmic rearrangement to form a column in the center of the cell (compare wild type, Fig. 1, G and H, with *mum4*, Fig. 1, J and K). Differences, however, were obvious at 10 DPA. Although wild-type seed coat cells have dark-pink staining mucilage in the extracellular space and a thin secondary cell wall surrounding a central column of cytoplasm (Fig. 1I), the extracellular space containing mucilage in *mum4* seed coat epidermal cells is smaller with lighter staining mucilage and the secondary cell wall has formed around a partially formed cytoplasmic column atop a large vacuole (Fig. 1L). Thus, the seed coat appears to have less mucilage, and the flattened columella appears to be the result of reduced cytoplasmic and vacuolar constriction.

The possibility of a change in mucilage quantity or composition in *mum4* versus wild-type seed coats was addressed by quantifying monosaccharides using gas chromatography. Because *mum4* seed coats release mucilage in the presence of ammonium oxalate, mucilage could be extracted from intact *mum4* seeds for direct comparison with wild-type mucilage. Trimethylsilane (TMS) derivatives were used because this method allows detection of GalUA and neutral sugars simultaneously. The results showed that, for equal seed masses, less sugar was extracted from *mum4* mucilage, suggesting that less mucilage is synthesized compared with wild-type seeds (Fig. 2). In addition, the decrease in monosaccharides in *mum4* mucilage was limited primarily to two sugars, GalUA and a single neutral sugar (identified as either Rha or Fuc; Fig. 2; $H_0\ \mu_1 = \mu_2$, GalUA, $T = 7.897$, $P < 0.01$; Rha, $T = 6.960$, $P = 0.01$). Because the identity of the neutral sugar could not be completely resolved using TMS derivatives, alditol acetate derivatization, which gives a single derivative for each monosaccharide, was used to confirm that it is Rha (Fuc found only at trace levels in wild-type mucilage; data not shown). These results are consistent with the suggestion that Arabidopsis mucilage is comprised primarily of relatively unbranched RGI (Penfield et al., 2001).

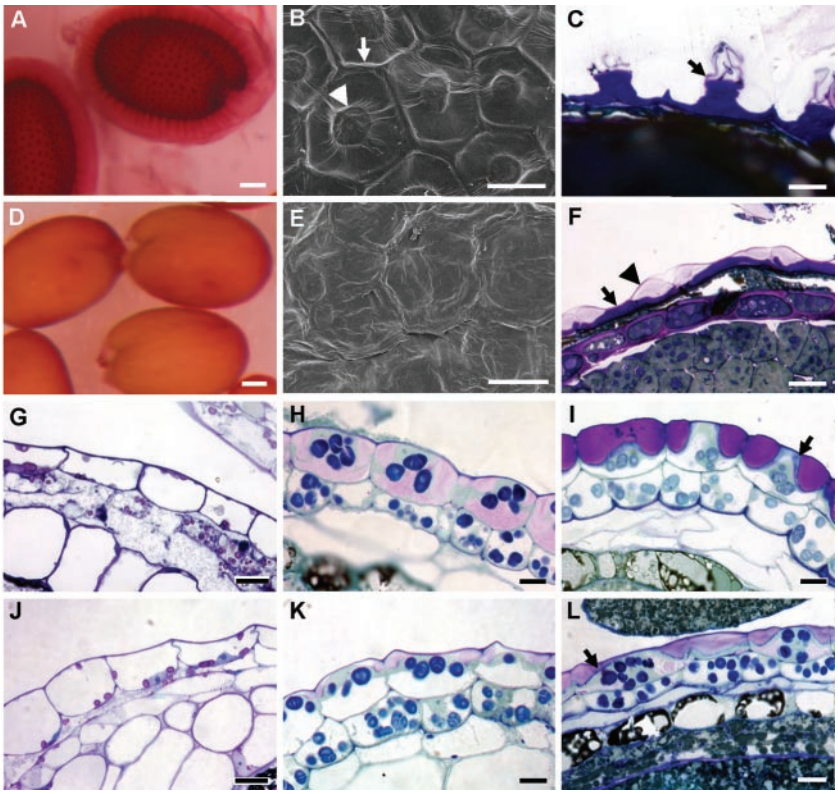

Figure 1. Structure and development of wild-type (Columbia-2 [Col-2]) and *mum4-1* seed coats. A, Whole-mount wild-type seed stained with Ruthenium red. Note red staining capsule of mucilage surrounding seeds. B, Scanning electron micrograph of wild-type seed coat. Note thickened radial cell walls (arrow) and central columella (arrowhead) in each cell. C, Cross section of mature wild-type seed coat epidermis. The cells have burst to release mucilage, leaving cell wall fragments attached to tip of columellae (arrow). D, Whole-mount *mum4-1* seeds stained with Ruthenium red. No mucilage is visible around seeds. E, Scanning electron micrograph of *mum4-1* seed coat. Columellae are not visible. F, Cross section of mature *mum4-1* epidermal cells. Flattened columellae (arrow) are apparent and intact primary cell walls (arrowhead) surround faint pink staining mucilage. G to I, Cross sections of developing wild-type epidermal cells. G, 4 DPA. H, 7 DPA, note accumulation of pink-staining mucilage in cells. I, 10 DPA, the center of each cell contains cytoplasm that is surrounded by a developing secondary cell wall staining blue (arrow), whereas both are surrounded by intensely stained mucilage (dark pink). J to L, Developing *mum4-1* epidermal cells. J, 4 DPA. K, 7 DPA, pink staining mucilage is only apparent in top portion of cells. L, 10 DPA, the cytoplasm fills most of the cell in a dome shape, with a thin, blue secondary cell wall (arrow) forming around it. Pink staining mucilage is found only in the upper corners of the cells. C and F to L, Toluidine blue-stained sections of tissue fixed under aqueous conditions. Scale bars: A and D = 100 μm, B and E = 20 μm, and C and F to L = 10 μm.

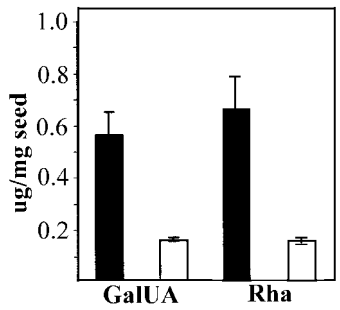

Figure 2. GalUA and Rha levels of wild-type (Col-2) and *mum4-1* mucilage. Comparison of GalUA and Rha levels detected in mucilage isolated from intact *mum4-1* seeds (white bars) versus wild-type mucilage (black bars, Col-2) using TMS derivatization and gas chromatography. The bars represent micrograms of sugar per milligram of seed as determined by comparison with an internal standard. Analyses were done in triplicate. Error bars = SD.

Arabidopsis Mucilage Is Largely Composed of RGI

To further investigate the effect of *mum4* mutations on mucilage production, immunofluorescence with antipectin antibodies was used. Whole-mount immunofluorescence performed on wild-type Arabidopsis seeds using the polyclonal antipectin antibody α-poly-GalUA (PGA)/RGI gave significant labeling of the mucilage without pretreatment (dry seeds added directly to fixative; Fig. 3A). This polyclonal antibody, although raised to RGI, has some cross-reactivity to HGA (Lynch and Staehelin, 1992). However, JIM5 and JIM7 monoclonal antibodies specific for HGA with moderate or higher states of methylation, respectively, failed to cross-react with the mucilage under the same conditions (Fig. 3C; data not shown). Slight (JIM5; data not shown) or moderate (JIM7; Fig. 3D) amount of labeling of extruded mucilage could be observed only after a pretreatment of

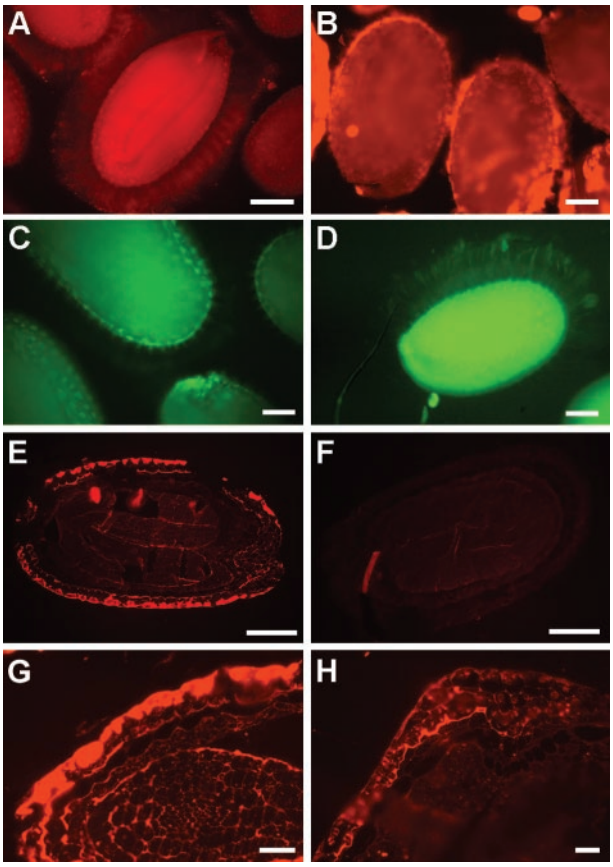

Figure 3. Immunofluorescence of wild-type (Col-2) and *mum4-1* mucilage with antipectin antibodies. A, Immersion immunofluorescence of whole wild-type seeds, labeled with α-PGA/RGI primary and anti-rabbit Alexa 594 secondary antibody. Note autofluorescence of seed itself and stain of mucilage capsule surrounding the seed. B, Immersion immunofluorescence of whole *mum4-1* seed, labeled with α-PGA/RGI after shaking in ammonium oxalate. The stained mucilage capsule is thin compared with wild type. C and D, Immersion immunofluorescence of whole wild-type seeds, labeled with JIM7 as primary antibody, followed by anti-rat fluorescein isothiocyanate secondary antibody. C, Seed treated as in A with no shaking pretreatment. No stain of mucilage capsule. D, Seed pretreated by imbibition of extensive shaking in water. Stain of mucilage capsule is apparent. E to H, Immunofluorescence of sections of 9-DPA seeds incubated with α-PGA/RGI or serum control. E, Wild-type seed incubated with α-PGA/RGI antibody, epidermal cells are labeled on seed surface. F, Wild-type seed incubated with nonimmune normal rabbit serum control, no staining of seed epidermis, same photographic conditions as E. G, Direct comparison of wild-type seed and H *mum4-1* seed show reduced α-PGA/RGI label in *mum4* mutant. Scale bars: A to F = 100 μm and G and H = 50 μm.

imbibition with extensive shaking before fixation. These results suggest that most of the binding found against wild-type mucilage can be attributed to RGI, rather than HGA. When *mum4* seeds were pretreated by shaking in ammonium oxalate, α-PGA/RGI labeling of a thin ring around the seed was observed (Fig. 3B), suggesting less mucilage is produced in *mum4* seeds compared with wild type.

To examine the mucilage in situ, samples were cryo-fixed/freeze substituted, resin embedded, sectioned, and then labeled by indirect immunofluorescence. Labeling was seen on thick sections of developing wild-type seed coats stained with α-PGA/RGI (9 DPA; Fig. 3E), with fluorescence in the epidermal cells on either side of a central, unstained space. Control experiments including omitting the primary antibody or substituting nonimmune, normal rabbit serum for the primary antibody, abolished the fluorescent signal (Fig. 3F). The similarity in staining pattern with α-PGA/RGI in sections compared with the pink staining substance in toluidine blue-stained sections (e.g. Fig. 1I) confirms the identity of the pink staining substance as pectinaceous mucilage. When thick sections of *mum4* seeds were treated with α-PGA/RGI, only a small amount of labeling was observed on the outer edges of the epidermal cells (Fig. 3H) compared with wild-type seeds (Fig. 3G), once again suggesting a reduced amount of mucilage in *mum4* seeds.

Molecular Cloning of *MUM4*

To identify why *mum4* seeds had reduced mucilage, we cloned *MUM4* by map-based cloning. *MUM4* was mapped to a 144-kb region spanning the bacterial artificial chromosomes F12M16 and T3F20. To identify the exact locus, the *mum4-3* T-DNA insertion allele was probed with sequences derived from these bacterial artificial chromosomes, and an insertion was identified in a region of T3F20 containing four predicted genes. PCR performed using gene-specific primers in combination with a T-DNA right border primer led to the identification of a T-DNA insertion in the predicted gene *At1g53500*.

Next, we isolated a putative full-length cDNA by reverse transcriptase (RT)-PCR using a series of primers upstream from the predicted translation start site. Sequencing of a putative full-length *At1g53500* transcript revealed that the gene consists of three exons and two introns (including one in the 5′-untranslated region [UTR]) and encodes a transcript of 2,477 bp (Fig. 4A).

To confirm that *At1g53500* is *MUM4*, we sequenced this locus in *mum4-1*, *mum4-2*, and *mum4-3*. All three alleles contain mutations in the second exon. *mum4-1* and *mum4-2* contain point mutations leading to G to A transitions at nucleotides 595 and 886, respectively (Fig. 4A). These missense mutations should result in a conserved Asp residue being replaced with an Asn in *mum4-1* and a Gly being replaced with an Arg in *mum4-2* (Fig. 5). *mum4-3* contains a T-DNA insertion at nucleotide 1,637 (Fig. 4A). The similar phenotype between the missense alleles and that of the *mum4-3* insertional allele that lacks detectable transcript (data not shown) suggests that these changes have a significant effect on protein activity. A database search of the Salk Institute sequence-indexed Arabidopsis T-DNA insertion lines identified two new alleles of *mum4*, *mum4-4*, and *mum4-5* (Salk_085051 and

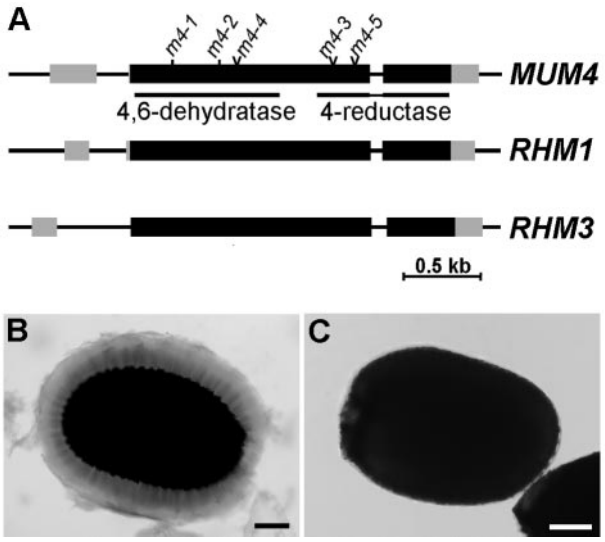

Figure 4. Structure of the *MUM4*, *RHM1*, and *RHM3* genes and complementation of *mum4-1* with *MUM4* coding region. A, Exon-intron structures are based on comparison of the genomic and cDNA sequences. The coding region is shown as black boxes, whereas the gray boxes represent 5′- and 3′-UTRs. The location of the two domains with similarity to bacterial nucleotide sugar interconversion enzymes are represented as thin bars underneath the *MUM4* coding region. |, Location of the two EMS alleles (*mum4-1* and *mum4-2*); ∨, Location of the three T-DNA insertion alleles (*mum4-3*, *mum4-4*, and *mum4-5*). B, Complementation of *mum4-1* with pMUM4g. Mucilage capsule is observed around seed stained with Ruthenium red. C, *mum4-1* seed transformed with empty vector pGREEN0229. No mucilage release is observed. Scale bars = 100 μm in B and C.

038898, respectively), both of which also have insertions in the second exon (Fig. 4A). Based on the Ruthenium red assay, both of these mutants have a phenotype (data not shown) similar to *mum4-1* (Fig. 1D). The wild-type *At1g53500* sequence plus 2.1 kb upstream and 0.4 kb downstream sequence was cloned to give the plasmid pMUM4g. Transformation of *mum4-1* plants with pMUM4g versus an empty vector control showed that the wild-type *At1g53500* genomic clone could rescue the mucilage phenotype (Fig. 4, B and C). Together, these results demonstrate that *At1g53500* is *MUM4*.

MUM4 encodes a protein of 667 amino acids (Fig. 5). Comparison with GenBank sequences suggested that MUM4 contains two domains: an N-terminal domain with similarity to bacterial dTDP-D-Glc-4,6-dehydratases (44% identical, 61% similar to RfbB [COG1088] consensus) and a C-terminal domain with some similarity to bacterial 4-reductases (20% identical, 34% similar to RfbD [COG1091] consensus) (Tatusov et al., 2000; Figs. 4A and 5). Both domains contain the conserved N-terminal NAD^+ binding (GxxGxxG) and active site catalytic couple (YxxxK) motifs found in members of the reductase/epimerase/dehydrogenase protein superfamily (Graninger et al., 1999; Allard et al., 2001). Both of the missense alleles, *mum4-1* and *mum4-2*, change codons of conserved amino acids (Fig. 5) in the N-terminal, putative NDP-D-Glc 4,6-dehydratase domain.

A comparison with other Arabidopsis genes predicted from genome annotation revealed that *MUM4* is a member of a small gene family of putative nucleotide sugar interconversion factors that was initially described in an in silico analysis by Reiter and Vanzin (2001). The gene family consists of three genes, *RHM1* (*At1g78570*), *RHM2* (*MUM4*) and *RHM3* (*At3g14790*), which are 83% to 89% identical at the amino acid level (Fig. 5). This suggests that there are at least three genes with a similar function.

Expression Pattern of *MUM4*

The expression pattern for *MUM4* mRNA in different tissues was determined qualitatively using RT-

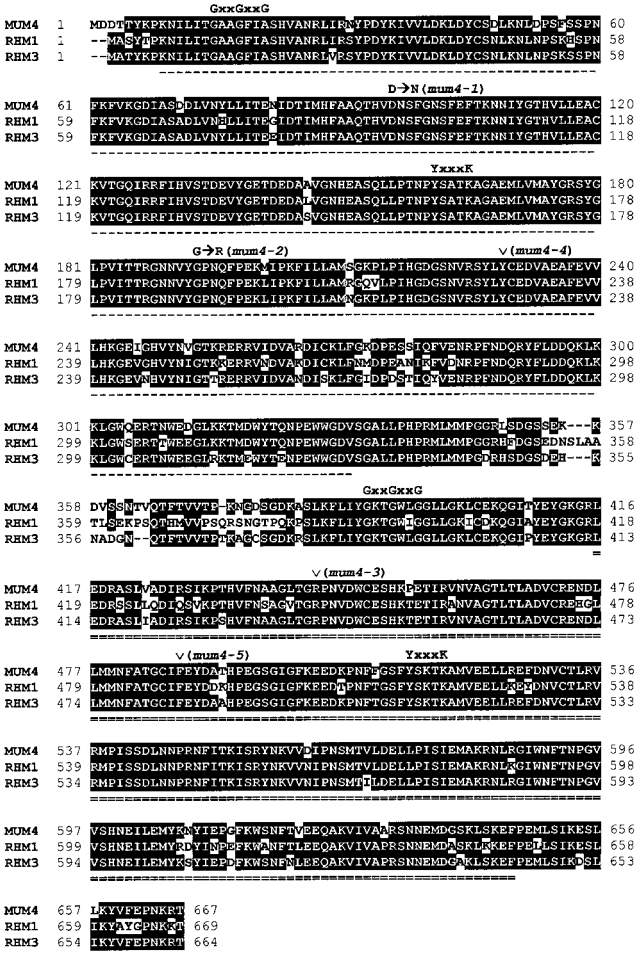

Figure 5. Alignment of the amino acid sequence of the MUM4 protein with those of RHM1 and RHM3. The putative 4,6-dehydratase and 4-reductase domains within MUM4 are marked with single and double underlines, respectively, whereas the highly conserved $NAD(P)^+$ cofactor-binding (GxxGxxG) and active site catalytic couple (YxxxK) motifs are indicated above the sequence alignment. The amino acid substitutions (*mum4-1* and *mum4-2*) and sites of T-DNA insertion (*mum4-3*, *mum4-4*, and *mum4-5*) are also marked.

PCR at a saturating cycle number. As suggested by expressed sequence tag data, *MUM4* transcripts are apparent in all tissues tested: stems, roots, leaves, seedlings, inflorescence tips, and siliques (Fig. 6A). A similar analysis was done for *RHM1* and *RHM3*. As with *MUM4*, both *RHM1* and *RHM3* transcripts were detected in all tissues (Fig. 6A). *RHM3*, however, unlike *MUM4* and *RHM1*, appeared to have only low expression in some tissues (leaves and inflorescences) because bands for these tissues were not saturated in several trials, even at the high cycle number used for qualitative RT-PCR.

Because the only obvious phenotype in *mum4* plants is the defect in the seed coat epidermis, we looked at the expression of *MUM4* during seed coat differentiation (Fig. 6B). RNA was extracted from siliques at three stages of development: (a) before mucilage synthesis (4 DPA; see Fig. 1G), (b) mid-mucilage synthesis (7 DPA; Fig. 1H), and (c) after the completion of mucilage synthesis (10 DPA; Fig. 1I), and subjected to northern analysis. This experiment showed that although *MUM4* is expressed throughout seed coat differentiation, transcript levels are highest at the time of mucilage synthesis (7 DPA; Fig. 6B). To test if the higher levels of *MUM4* detected at 7 DPA may be largely restricted to the seed coat, *ap2-7* mutants were used as a control in gel blots (Fig. 6B). *ap2* was chosen because the outer two layers of the seed coat of *ap2* mutant seeds fail to differentiate, whereas all other cell types of the seed (embryo, endosperm, and seed coat endothelium) appear normal (Western et al., 2001). The low level of *MUM4* transcript seen in 7-DPA *ap2-7* siliques suggests that a large proportion of the *MUM4* transcript at 7 DPA in wild-type siliques may be specific to the outer layers of the seed coat.

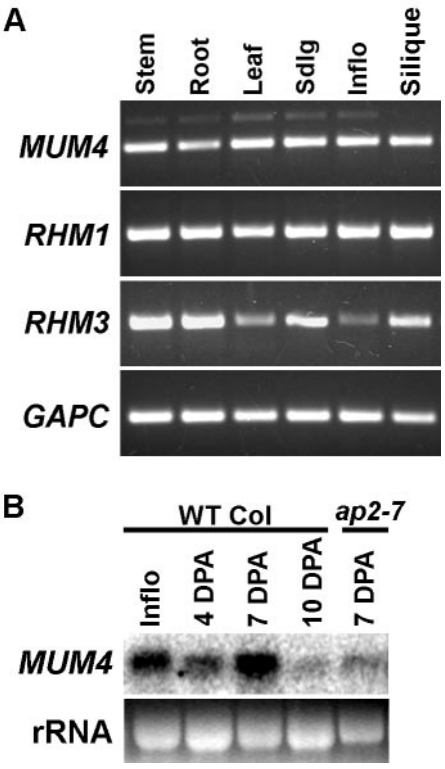

Figure 6. *MUM4*, *RHM1*, and *RHM3* expression in Arabidopsis plants. A, Saturating, qualitative RT-PCR of RNA isolated from wild-type (Col-2) stem, root, leaf, seedling, inflorescence, and siliques using gene-specific primers for *MUM4*, *RHM1*, and *RHM3*. The loading control is *GAPC*, which encodes cytosolic glyceraldehyde-3-phosphate dehydrogenase. B, Changes in *MUM4* expression during silique development. RNA gel-blot analysis using *MUM4* probe on total RNA from wild-type (Col-2) inflorescence, 4, 7, and 10 DPA wild-type siliques, and 7 DPA *ap2-7* siliques. Ethidium bromide-stained rRNA is shown as loading control.

Maximal Accumulation of *MUM4* Transcript Requires Upstream Transcription Factors

Mutations in *AP2*, *TTG1*, *TTG2*, *GL2*, and *MYB61*, which encode putative transcription factors, lead to seeds with reduced mucilage in the seed coat. Furthermore, developmental analyses have shown that the cellular phenotypes for *ttg1*, *gl2*, and *myb61* mucilage secretory cells are almost identical to those of *mum4* mutants, whereas the *ap2* mutant cellular phenotype is more severe (Penfield et al., 2001; Western et al., 2001). Therefore, we tested whether the accumulation of *MUM4* transcript was altered in mutants for each of these genes. RNA was extracted from 7 DPA siliques of wild type and mutants and subjected to northern hybridization (Fig. 7A). To control for ecotype differences between mutants isolated in Col-2 (*ap2-7* and *myb61-1*) versus L*er* (*ap2-1*, *gl2-1*, *ttg1-1*, and *ttg2-1*) backgrounds, both wild types were examined. Interestingly, *MUM4* appeared to be more highly expressed in the L*er* background, possibly because of background mutations affecting morphological differences in the seeds and/or siliques between the two ecotypes. As mentioned above, the level of *MUM4* transcript was reduced in *ap2-7* mutant siliques compared with wild-type Col-2 siliques, a result that was confirmed with *ap2-1* in the L*er* background. Our analysis also indicated that the levels of *MUM4* RNA at 7 DPA are lower for *ttg1-1* and *gl2-1* siliques (Fig. 7A), suggesting that *AP2*, *TTG1*, and *GL2* each are required for maximum levels of *MUM4* expression at the time of mucilage production. Conversely, there was a slight increase in *MUM4* transcript levels in *myb61-1* and *ttg2-1* siliques compared with wild type (Fig. 7A). Thus, the requirement of *MYB61* and *TTG2* for mucilage biosynthesis is for some aspect other than the regulation of *MUM4* transcription.

The Expression of Putative Regulatory Transcription Factors during Mucilage Secretory Cell Differentiation

The *ap2* mutant phenotype differs from that of *ttg1*, *ttg2*, *gl2*, and *myb61* in that the differentiation of the

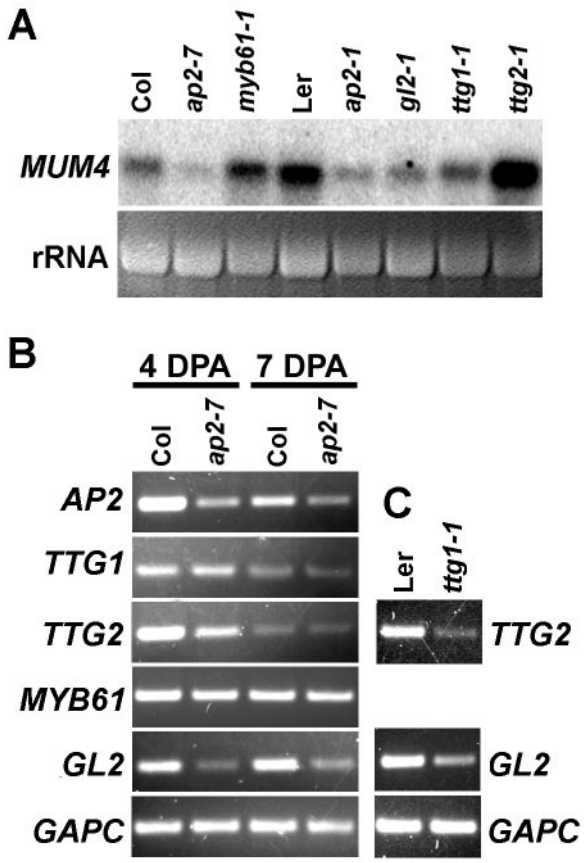

Figure 7. Expression of *MUM4* and genes encoding putative regulatory transcription factors during silique development. A, RNA gel-blot analysis using *MUM4* probe on total RNA from 7-DPA wild-type, *ap2-7*, *myb61-1* (Col-2), wild-type, *ap2-1*, *gl2-1*, *ttg1-1*, and *ttg2-1* (Landsberg *erecta* [L*er*]) siliques. Ethidium bromide stained rRNA is shown as loading control. B, Semiquantitative RT-PCR amplification of RNA isolated from wild-type and *ap2-7* (Col-2) siliques at 4 and 7 DPA using gene-specific primers for *AP2*, *TTG1*, *TTG2*, *MYB61*, and *GL2*. The loading control is *GAPC*, which encodes cytosolic glyceraldehyde-3-phosphate dehydrogenase. C, Semiquantitative RT-PCR amplification of RNA isolated from wild-type and *ttg1-1* (L*er*) 7-DPA siliques using gene-specific primers for *TTG2* and *GL2*. The loading control is *GAPC*, which encodes cytosolic glyceraldehyde-3-phosphate dehydrogenase.

mucilage secretory cells is halted before mucilage and columella production in *ap2* whereas the others resemble *mum4*, having low levels of mucilage and flattened columellae. Thus, phenotypically, *AP2* appears to be working upstream of the other genes. To determine whether AP2 acts by controlling the transcription of the other genes, semiquantitative RT-PCR was used to determine the levels of *TTG1*, *TTG2*, *GL2*, and *MYB61* in *ap2-7* mutants versus wild type at 4 and 7 DPA. The results showed a significant decrease in transcript levels for *GL2* at both 4 and 7 DPA (Fig. 7B) and *TTG2* at 4 DPA (*TTG2* levels at 7 DPA too low to differentiate between wild type and *ap2*; Fig. 7B). This result suggests that *AP2* is required for maximal levels of both *GL2* and *TTG2* expression during seed coat differentiation.

TTG1 has been shown to be required for maximal expression of both *TTG2* and *GL2* in leaves (Johnson et al., 2002; Schiefelbein, 2003). To determine if the same is true in seed coats, the expression of both *GL2* and *TTG2* were tested in *ttg1-1* compared with wild-type *Ler* 7 DPA siliques using semiquantitative RT-PCR. The transcript levels for both *GL2* and *TTG2* were reduced in *ttg1-1* mutants (Fig. 7C), suggesting that *TTG1* also acts upstream of *GL2* and *TTG2* in the seed coat.

DISCUSSION

The development of the mucilage secretory cells of the Arabidopsis seed coat is a complex process involving the biosynthesis and secretion of a large quantity of pectinaceous mucilage, intracellular cytoplasmic rearrangement, and secondary cell wall production. We have shown that the putative NDP-L-Rha synthase encoded by *MUM4* is required for wild-type levels of mucilage biosynthesis and columella formation. Expression of *MUM4* is upregulated during seed coat differentiation and is regulated by several transcription factors required for seed coat epidermal cell differentiation. These data validate the utility of the mucilage secretory cells as a model system for polysaccharide biosynthesis, establish a regulatory framework for seed coat epidermal differentiation, and provide a causal link between pectin biosynthesis and cell morphogenesis.

MUM4 Encodes a Putative NDP-L-Rha Synthase

Our data support the hypothesis that *MUM4* encodes an enzyme involved in RGI biosynthesis. First, analysis of *mum4* mucilage using antibodies and gas chromatography have demonstrated a significant reduction in RGI and its composite backbone monosaccharides Rha and GalUA in comparison with wild-type seeds. Second, cloning of *MUM4* revealed a putative protein containing similarity to bacterial nucleotide sugar interconversion enzymes, suggesting that it, too, is required for the production of activated sugars. The N-terminal portion of MUM4 is most similar to bacterial dTDP-D-Glc 4,6-dehydratases, the first of three enzymes required for the conversion of dTDP-D-Glc to dTDP-L-Rha (Tonetti et al., 1998). These data favor a role for MUM4 in the synthesis of NDP-L-Rha, a key step in the production of RGI. A reverse genetics approach taken by another group has come to a similar conclusion regarding the putative enzymatic function of MUM4 (also designated *RHM2* [*RHAMNOSE BIOSYNTHESIS* 2]; B. Usadel et al., 2004). However, another role in RGI biosynthesis cannot be ruled out without evidence of enzymatic activity in the conversion of NDP-D-Glc to NDP-L-Rha.

Gram-negative bacteria such as *Escherichia coli* encode three separate enzymes (4,6-dehydratase, 3,5-epimerase, and 4-reductase) to convert dTDP-D-Glc

to dTDP-L-Rha (Tonetti et al., 1998). The putative MUM4 protein not only contains an N-terminal domain with similarity to the bacterial dTDP-D-Glc 4,6-dehydratases but also a C-terminal domain with some similarity to 4-reductases (this study; Reiter and Vanzin, 2001). There is a precedent in Arabidopsis for a bifunctional 3,5 epimerase, 4-reductase in the synthesis of GDP-L-Fuc from GDP-D-Man (GER1, GER2) (Bonin and Reiter, 2000). Therefore, based on sequence analysis, it is attractive to postulate a single, multifunctional protein acting in the conversion of NDP-D-Glc to NDP-L-Rha in Arabidopsis (Reiter and Vanzin, 2001).

MUM4 is a member of a small gene family consisting of three genes (*RHM1*, *MUM4/RHM2*, and *RHM3*; Reiter and Vanzin, 2001) that exhibit high identity at both nucleotide and amino acid levels. The ubiquitous expression of *RHM1* and *RHM3* can be postulated to provide a mechanism for the formation of the normal primary cell wall in *mum4* mutants and the residual mucilage present in *mum4* seed coats. Redundancy in genes coding for putative NDP-L-Rha synthases is unsurprising because of the importance of the Rha-containing pectins RGI and RGII in primary cell walls. In fact, the presence of multiple genes coding enzymes involved in nucleotide sugar interconversions is common in plants (Reiter and Vanzin, 2001). The roles of MUM4 and its paralogs may not be completely redundant, however, because the three genes do not have identical patterns/levels of expression throughout all plant tissues (Fig. 6A; Arabidopsis expressed sequence tag database at The Institute for Genomic Research [http://www.tigr.org]; T.L. Western and G.W. Haughn, unpublished data).

Developmental Regulation of *MUM4* during Seed Coat Secretory Cell Differentiation by AP2, TTG1, and GL2

MUM4 transcript increases in differentiating siliques at the time of mucilage production. Two lines of evidence suggest that this up-regulation occurs in the seed coat epidermis to support mucilage biosynthesis. First, the only obvious phenotypic defect in *mum4* plants occurs in the seed coat epidermis. Second, *MUM4* expression is severely attenuated in siliques of *ap2* mutants that fail to differentiate the outer two layers of the seed coat. Such a specific up-regulation of a putative NDP-L-Rha synthase may be required to provide extra Rha for the production of the large quantity of RGI required for mucilage synthesis. If so, the amount of this enzyme must be the limiting factor in Rha biosynthesis and the amount of Rha a limiting factor in RGI biosynthesis.

Our data indicate that *MUM4* transcription is decreased in *ttg1* and *gl2* mutants but is essentially wild type in *ttg2* and *myb61* mutants (Fig. 7A). This result suggests a regulatory framework for mucilage secretory cell differentiation in which there are at least two pathways controlling mucilage biosynthesis in the seed coat. In one of these pathways, it appears that TTG1 and GL2 control the transcription of *MUM4* (Fig. 8). In other epidermal systems, such as trichomes and root hairs, TTG1 has been shown to interact with a tissue-specific MYB protein through the bHLH protein GLABRA3 (Payne et al., 2000; Schiefelbein, 2003) to activate *GL2*. Therefore, we favor a model in which a seed coat-specific complex of TTG1-bHLH-MYB causes activation of *GL2* and the subsequent up-regulation of *MUM4* during mucilage production (Fig. 8). Recent results suggest that EGL3 (ENHANCER OF GLABRA3) and/or TT8 (TRANSPARENT TESTA8) are the bHLH proteins acting with TTG1 in the seed coat (Zhang et al., 2003). A possible MYB component of this trimeric complex, *MYB23*, is expressed in developing seeds and groups to the same subfamily of MYBs as the bHLH-TTG1 interacting proteins GLABROUS1 and WEREWOLF (Kirik et al., 2001).

Correct regulation of *MUM4* transcription at the time of mucilage production does not require *TTG2* or *MYB61* (Fig. 7A). However, *TTG1* is required to activate *TTG2* in the seed coat (Fig. 7C) and in other tissues (Johnson et al., 2002). This result places *TTG2* downstream from *TTG1* in a second pathway that controls mucilage biosynthesis in the developing seed. This alternate pathway, along with another that involves *MYB61*, may regulate the expression of other proteins involved in RGI synthesis including an NDP-D-GlcUA 4-epimerase (Feingold, 1982; Reiter and Vanzin, 2001), a nucleotide sugar transporter or an RGI-backbone glycosyltransferase. Alternatively, *MYB61* and *TTG2* may be involved in the transport of newly synthesized RGI to the plasma membrane. Feedback inhibition of mucilage production in the

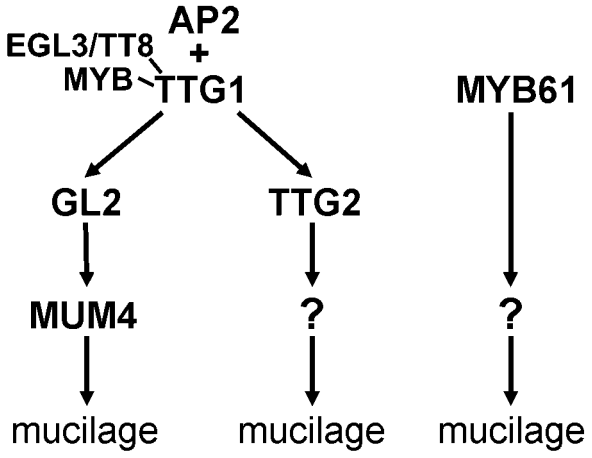

Figure 8. Proposed genetic pathway for the regulation of mucilage production during seed coat secretory cell differentiation. AP2 and a TTG1 complex with a bHLH protein (candidates include EGL3 and/or TT8) and a tissue-specific MYB transcription factor activate *GL2* and *TTG2*. *GL2* acts upstream of *MUM4*. In contrast, both *TTG2* and *MYB61* appear to affect aspects of mucilage production independent from *MUM4*. For details, see text.

absence of secretion has been demonstrated in the root caps of the maize (*Zea mays*) mutant Ageotropic (Millar and Moore, 1990).

AP2 encodes a putative transcription factor that is required for the differentiation of the outer two layers of the seed coat (Western et al., 2001). Not surprisingly, *ap2* seeds lack mucilage and fail to activate *MUM4* transcription beyond its baseline level of expression (Fig. 7A). Although *AP2* is required for maximum *GL2* and *TTG2* transcript levels (Fig. 7B), transcription of *TTG1* and *MYB61* was independent of *AP2* activity (Fig. 7B). Our data are consistent with the hypothesis that AP2 functions in parallel with TTG1 to activate *GL2* and *TTG2* in the seed coat epidermis (Fig. 8).

Columella Production during Seed Coat Secretory Cell Differentiation

The *mum4* phenotype is characterized by both a reduction in the amount of mucilage produced and by a flattened columella in the seed coat epidermis. The flattened appearance of the columella in *mum4* seeds appears to be the result of incomplete cytoplasmic rearrangement and vacuole constriction, leaving a dome of cytoplasm over which the secondary cell wall is laid. Although the defect in mucilage production is readily explained by the putative function of *MUM4* as an NDP-L-Rha synthase, the connection with the columella shape change is less obvious. At least two hypotheses can explain the dependence of cell morphogenesis on mucilage synthesis. The formation of the apoplastic space and cytoplasmic column may be driven by the pressure of accumulating mucilage in the extracytoplasmic space and/or the formation of an osmotic gradient between the cell and the hydrophilic pectin may lead to the loss of turgor and size reduction of the vacuole. Such passive processes may contribute to cell morphogenesis but seem too simple to account for the apparent complexity of the shape of the cytoplasmic column. An alternate but not mutually exclusive explanation is that the synthesis of mucilage may represent or lead to the production of an oligosaccharide signal that stimulates the cell to undergo morphogenesis (Dumville and Fry, 2000; Ridley et al., 2001). Determination of the exact processes involved would be facilitated by the isolation of mutants affected specifically in the shaping of the columella.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Lines of Arabidopsis used were *mum4-1*, *mum4-2* (Col-2 ecotype; Western et al., 2001), *mum4-3* (see below), *mum4-4*, *mum4-5* (Salk_085051 and 038898, respectively, obtained from the Arabidopsis Biological Resource Center [ABRC], Ohio State University, Columbus), *ap2-1* (L*er* ecotype; ABRC), *ap2-7* (Col-2; Kunst et al., 1989), *gl2-1*, *ttg1-1*, *ttg2-1* (L*er*; ABRC), and *myb61-1* (Col-0; gift from Michael Bevan, John Innes Centre, Norwich, UK). Growth conditions were as described by Western et al. (2001).

mum4-3 was isolated by screening T-DNA mutagenized lines (Feldmann and Marks, 1987; ABRC) by staining with a 0.01% (w/v) aqueous solution of Ruthenium red.

Staging of Flower Age

Flowers were staged as by Western et al. (2001).

Immunofluorescence

Developing seeds for bright-field microscopy were prepared as described by Western et al. (2000).

For immunofluorescence, developing seeds were placed in copper hats in a 0.2 M Suc solution and high-pressure frozen using a Balzers HPM 101 (Balzers Instruments, Balzers, Liechtenstein). The samples were freeze substituted over 5 d in 0.25% (v/v) glutaraldehyde/8% (v/v) dimethoxypropane in acetone using a dry ice/acetone bath at −80°C, then gradually warmed to −20°C, 4°C, and then 20°C (3 h at each temperature) before embedding in LR White resin. Sectioning and microscopy were performed as for bright field.

Immersion immunofluorescence was performed according to the procedure of Willats et al. (2001), except dry wild-type seeds were placed directly into the fixative. Alternatively, the seeds were imbibed for 24 h in buffer with gentle shaking before fixation, all subsequent steps also being performed with shaking. Antipectin monoclonal antibodies JIM5, JIM7, and polyclonal α-PGA/RGI at 1:5 and 1:10 (v/v) dilutions were used as primary antibodies. Pre-immune serum controls were rat (JIM5 and JIM7) and rabbit (α-PGA/RGI). Secondary antibodies were 1:100 (v/v) dilutions in 5% (w/v) nonfat dry milk (NFDM) of goat anti-rat IgG conjugated to fluorescein isothiocyanate (JIM5 and JIM7) and anti-rabbit IgG (α-PGA/RGI). Sections were mounted in either anti-fade agent (JIM5 and JIM7; Slowfade Antifade, Molecular Probes, Eugene, OR) or 1:2 (v/v) glycerol:water (α-PGA/RGI) before observation.

For immunofluorescence, 0.5-μm sections were incubated in 50 mM EGTA for 1 h, blocked with 5% (w/v) NFDM for 20 min, then incubated for 1 h with primary antibodies at 1:5 and 1:10 (v/v) dilutions in 5% (w/v) NFDM, and for 1 h in secondary antibodies diluted at 1:100 (v/v). Rinses were performed before and after each incubation with 1× Tris-buffered saline/0.1% (v/v) Tween 20. Primary antibodies, secondary antibodies, pre-immune controls, and mounting were done as described for immersion immunofluorescence.

Scanning Electron Microscopy

Dry-mounted seeds were prepared and observed as by Western et al. (2001).

Gas Chromatography

Mucilage was extracted from samples of approximately 5 mg of intact seeds by shaking in 0.2% (w/v) ammonium oxalate for 2 h at 30°C. No significant difference in mass was observed between Col-2 and *mum4-1* seed (four lots of 100 counted seed weighed in triplicate; Col-2 = 2.0 ± 0.3 mg, *mum4-1* = 2.2 ± 0.1 mg SD). Ten microliters of internal standard (5 mg mL^{-1} myo-inositol) was added before precipitation with 5 volumes of absolute ethanol and drying under nitrogen gas. Derivatization, gas chromatography, and identification of trimethylsilyl ethers were performed as described previously (Western et al., 2000).

For alditol acetates, samples were hydrolyzed in trifluoroacetic acid for 2 h at 100°C, dried under nitrogen gas, then reduced for 90 min at 40°C in 245 μL of 2.6 M ammonia and 700 μL of 2% (w/v) sodium borohydride in dimethylsulfoxide. After addition of 175 μL of acetic acid, the samples were acetylated for 10 min at room temperature with 175 μL of 1-methylimidazole and 2.8 mL of acetic anhydride. Water (5.6 mL) was added, and the alditol acetates were extracted with 1.05 mL of dichloromethane (DCM). The recovered phase was dried and resuspended in 200 μL of DCM, then re-extracted with 1 mL of water. This recovered DCM phase was used for analysis. Samples were run on a gas chromatograph (model 5890A, Hewlett-Packard, Mississauga, ON, Canada) on a HP-23 glass capillary column (30-m × 0.25-mm i.d.) with helium as the carrier gas. The temperature program was 2 min at 180°C, increase 10°C min^{-1} up to

200°C, 5 min at 200°C, increase 10°C min^{-1} to 250°C, and hold at 250°C for 10 min. Monosaccharides were identified through comparison with the retention times obtained with individual sugar standards and a composite standard consisting of Rib, Fuc, Man, Gal, Glc, Ara, Rha, Xyl, GlcUA, and GalUA.

Positional Cloning of MUM4 and Determination of cDNA Sequence

A mapping population of 519 plants derived from a cross between *mum4-1* and wild-type L*er* was used for progressive fine mapping using simple sequence length polymorphism and cleaved-amplified polymorphic sequence markers generated from sequence information provided by the Arabidopsis Genome Initiative (2000) and Cereon (Jander et al., 2002). Genomic DNA was isolated from the T-DNA-tagged *mum4-3* mutant and wild-type Wassilewskija plants, and Southern hybridization was performed using radiolabeled probes derived from F12M16 and T3F20. An insertion was detected in a 9.5-kb *Xho*I fragment from T3F20. Gene-specific primers for the four predicted genes on that fragment were used in PCR with a T-DNA right border primer (KF-RB p1 5′-gttgaagttggcgagttcgt-3′). An insertion was identified in *At1g53500* using a 3′ primer (*At1g53500* p2 5′-tctgaaactgcctaggtggaa-3′).

To confirm that the gene was *MUM4*, *mum4-1* and *mum4-2* were sequenced. Sequencing of the Salk Institute sequence-indexed Arabidopsis T-DNA insertion lines 085051 and 038898 revealed two further alleles, named *mum4-4* and *mum4-5*, respectively. Molecular complementation of *MUM4* was carried out using a 5.2-kb *Sal*I/*Bst*Z17I fragment of T3F20, including *At1g53500* plus 2.1 kb upstream and 0.4 kb downstream sequence, into pGREEN0229 (Hellens et al., 2000). *mum4-1* plants were transformed either with the genomic clone (pMUM4g) or the empty vector (pGREEN0229) using the *Agrobacterium tumefaciens* dipping method (Clough and Bent, 1998). Basta-resistant transformants were isolated by spraying young seedlings with 0.1% (v/v) glufosinate (Final EV150, AgrEvo EH, Paris) in 0.1% (v/v) Silwet L-77, and putative transformants were verified by PCR analysis.

The 5′-UTR of *MUM4* was located using RT-PCR with primers located up to 900 bp upstream from the predicted ATG in combination with an internal primer. RNA was isolated from developing siliques according to the protocol of Downing et al. (1992), with the addition of a sodium acetate wash to remove excess polysaccharides. One-microgram samples were treated with DNaseI (Invitrogen Life Technologies, Carlsbad, CA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. RT-PCR using two sequential primers identified bands approximately 200 bp smaller than predicted from genomic sequence. The longer fragment was isolated and sequenced.

Analyses of Gene Expression

RNA was isolated as described above. For RNA gel blots, 10 μg of total RNA was separated on formaldehyde gels, blotted to Hybond-XL nylon membranes (Amersham Biosciences Corp., Baie d'Urfé, QC, Canada), and hybridized according to the manufacturer's instructions. A ^{32}P-labeled *MUM4* probe corresponding to the degenerate region between the two predicted enzymatic domains was synthesized using PCR (*MUM4*p11 5′-ggaagactttctgatggatctagt-3′/*MUM4*p12 5′-gatcaaaaacttcaacgaagc-3′). 25S rRNA bands from the ethidium bromide-stained RNA gel were used as loading controls.

RNA isolation and RT of DNase-treated RNA was performed as described above. Gene-specific primers surrounding an intron were designed for each gene, with the exception of *TTG1*, which lacks an intron. Primers were as follows: *MUM4*, p1-ttcgtgtaaatgtcgcaggt/p2 5′-gagaagctcgtctagtacggtc-3′; *RHM1*, p1 5′-gagactatccgtgccaatgta-3′/p2 5′-taataactcgtccaacacagtc-3′; *RHM3*, p1 5′-ccgagtcaacgttgctgga-3′/p2 5′-ggtaagagttcatctagtatggtc-3′; *GAPC*, p1 5′-tcagactcgagaaagctgctac-3′/p2 5′-gatcaagtcgaccacacgg-3′; *MYB61*, p3 5′-tggggagacattcttgctg-3′/p4 5′-gatgggcttgtgtgtgtttg-3′; *GL2*, p1 5′-aacggtcactccaaggtcac-3′/p2 5′-agaaacccgcatgtcttgtc-3′; *TTG2*, p1 5′-gccatcttgtcctcttccac-3′/p2 5′-ccttgcgatactcctgcttc-3′; *AP2*, p1 5′-cagggaatcctactactccacaag-3′/p2 5′-atctgattgtgatgatgaggagag-3′; and *TTG1*, p1 5′-catcctccggtcacagaatc-3′/p2 5′-tttcggctctacatcgttcc-3′. Loading for all RT-PCRs was determined by standardizing against *GAPC* test PCRs (at 22–23 cycles) for each RT reaction. Saturating, qualitative RT-PCR for *MUM4*, *RHM1*, and *RHM3* was done with 30 cycles. Semiquantitative RT-PCR (for *AP2*, *TTG1*, *TTG2*, *GL2*, and *MYB61*) was performed a minimum of four times for each set of primers using two to three different cycle numbers (from 25–30 cycles) to confirm results.

Accession Numbers

The GenBank accession numbers for genes mentioned in this article are AY328518 (*MUM4* full-length cDNA), AY042833 (*RHM1*), and AY078958 (*RHM3*). *mum4-3* has been submitted to ABRC under the seed stock number CS6382.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining permissions will be the responsibility of the requestor.

Received October 30, 2003; returned for revision November 12, 2003; accepted November 20, 2003.

ACKNOWLEDGMENTS

We thank Ms. Yeen Ting Hwang (University of British Columbia, Vancouver, BC Canada) and Mr. Adrian Wladichuk (University of British Columbia, Vancouver, BC Canada) for technical assistance, Dr. Michael Bevan (John Innes Centre, Norwich, UK) for supplying *myb61-1* seed, Dr. Mark Smith (University of British Columbia, Vancouver, BC Canada) and Ms. Michelle Fawcett (Carnegie Institution, Stanford University, CA) for help with chemical analysis of mucilage, Drs. Andrew Staehelin (University of Colorado, Boulder) and Paul Knox (University of Leeds, Leeds, UK) for gifts of antibodies, and the Salk Institute Genomic Analysis Laboratory (La Jolla, CA, USA) for providing the sequence-indexed Arabidopsis T-DNA insertion mutants. We also thank Drs. Markus Pauly (Max-Planck Institute for Molecular Plant Physiology, Golm, Germany) and Björn Usadel (Max-Planck Institute for Molecular Plant Physiology, Glom, Germany) for communicating unpublished data and delaying publication of their results on *RHM2*. We thank members of the Ljerka Haughn (University of British Columbia, Vancouver, BC Canada) and George Kunst (University of British Columbia, Vancouver, BC Canada) labs for comments on the manuscript.

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