The *Arabidopsis* MUM2 Gene Encodes a β-Galactosidase Required for the Production of Seed Coat Mucilage with Correct Hydration Properties

Gillian H. Dean, a Huanquan Zheng, a,1 Jagdish Tewari, b,2 Jun Huang, a Diana S. Young, a Yeen Ting Hwang, a Tamara L. Western, a,1 Nicholas C. Carpita, c Maureen C. McCann, b Shawn D. Mansfield, d and George W. Haughn a,3

a Department of Botany, University of British Columbia, Vancouver, Canada V6T 1Z4
b Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907
c Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907
d Department of Wood Science, University of British Columbia, Vancouver, Canada V6T 1Z4

Seed coat development in *Arabidopsis thaliana* involves a complex pathway where cells of the outer integument differentiate into a highly specialized cell type after fertilization. One aspect of this developmental process involves the secretion of a large amount of pectinaceous mucilage into the apoplast. When the mature seed coat is exposed to water, this mucilage expands to break the primary cell wall and encapsulate the seed. The mucilage-modified2 (mum2) mutant is characterized by a failure to extrude mucilage on hydration, although mucilage is produced as normal during development. The defect in *mum2* appears to reside in the mucilage itself, as mucilage fails to expand even when the barrier of the primary cell wall is removed. We have cloned the *MUM2* gene and expressed recombinant MUM2 protein, which has β-galactosidase activity. Biochemical analysis of the *mum2* mucilage reveals alterations in pectins that are consistent with a defect in β-galactosidase activity, and we have demonstrated that MUM2 is localized to the cell wall. We propose that MUM2 is involved in modifying mucilage to allow it to expand upon hydration, establishing a link between the galactosyl side-chain structure of pectin and its physical properties.

**INTRODUCTION**

The secretion of large amounts of mucilage in a specialized cell type makes the seed coat an excellent model system for studying aspects of polysaccharide biosynthesis, secretion, and modification. The development of the *Arabidopsis thaliana* seed coat has been studied in detail (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). After fertilization, the cells of the outer ovule integument secrete a large amount of pectinaceous mucilage into the apoplast. This secretion is targeted to the junction of the radial and outer tangential cell walls, resulting in a mucilage-filled apoplastic domain surrounding a volcano-shaped cytoplasm. After mucilage secretion, the secondary cell wall is deposited and eventually fills the entire space occupied by the cytoplasmic column. Therefore, mature seed coat cells of *Arabidopsis* consist of a volcano-shaped secondary cell wall known as the columella surrounded by a donut-shaped ring of mucilage.

When mature seeds are hydrated, the outer radial wall breaks and the mucilage is extruded to form a capsule around the seed. Although it has been proposed that seed mucilage may be important for germination under conditions of water stress in *Arabidopsis* (Penfield et al., 2001), mucilage production and seed coat differentiation are dispensable under laboratory conditions. This feature, as well as the availability of a simple screen that uses Ruthenium Red to stain acidic pectins (Hanke and Northcote, 1975) found in extruded mucilage, allows for relatively straightforward identification of mutants with defects in mucilage biosynthesis (Western et al., 2001).

*Arabidopsis* mucilage is composed primarily of pectin (Western et al., 2000, 2004; Penfield et al., 2001; Usadel et al., 2004; Macquet et al., 2007a, 2007b). Pectins are a group of complex and structurally diverse polysaccharides, with two key pectins being homogalacturonan (HG) and rhamnogalacturonan I (RG-I; O’Neill and York, 2003). HG is structurally related to xylogalacturonan and rhamnogalacturonan II (RG-II); all have a backbone of (1 → 4)-α-D-galacturonic acid residues, with HG being unbranched, xylogalacturonan being substituted with xylose residues, and RG-II having complex but structurally defined, nonvariable side chains. By contrast, RG-I has a backbone consisting of alternating (1 → 4)-α-D-galacturonic acid and (1 → 2)-α-L-rhamnose with variable side chains containing arabinose, fucose, galactose, and glucuronic acid.

Pectin has been ascribed important roles in ion transport, molecular sieving, and cell adhesion because of its physical properties (Knox, 2002) and is also a potential source of
oligosaccharides that can act as signaling molecules (Ridley et al., 2001). The structure of pectin determines these properties, and pectin structure is often modified in particular cell types (Knox, 2002) as well as during growth and developmental processes such as fruit ripening (Rose et al., 2003).

Arabidopsis seed mucilage is composed largely of relatively unbranched RG-I (Western et al., 2000, 2004; Penfield et al., 2001; Usadel et al., 2004; Macquet et al., 2007a, 2007b). Immunocytochemical studies also indicate that HG is present in mucilage (Willats et al., 2001a; Western et al., 2004; Macquet et al., 2007a, 2007b), and there is evidence that 4-linked glycans form a constituent of mucilage (Windsor et al., 2000; Willats et al., 2001a; Macquet et al., 2007a, 2007b; G.H. Dean and G.W. Haughn, unpublished data). Smaller amounts of sugar linkages associated with arabinoxylans and type II arabinogalactans are also detected (Penfield et al., 2001).

A number of mutants have been identified in which development of the seed coat is perturbed (Haughn and Chaudhury, 2005; Western, 2006). One subset of these mutants (mucilage-modified, mum1, mum2, and patchy) appear to undergo normal epidermal cell differentiation and mucilage secretion, but mature seeds do not release mucilage on hydration (Western et al., 2001; T.L. Western, T.M. Popma, and G.W. Haughn, unpublished data). This defect in mucilage extrusion may reflect changes in the primary cell wall that prevent it from breaking or alterations in the ability of mucilage to hydrate and expand on contact with water.

Here, we present a phenotypic analysis of the cytological and biochemical defects in the mum2 mutant. We show by positional cloning that the MUM2 gene encodes the Family 35 β-galactosidase AtBGAL6, which is localized in the apoplast and results in modified pectin side chains to enable increased hydration properties of mucilage.

RESULTS

mum2 Develops Normally but Does Not Release Mucilage on Hydration

When mature wild-type Arabidopsis seeds are exposed to water, mucilage is extruded from the epidermal cells to encapsulate the seed (Figure 1A). Screening a mutagenized population of M3 lines using Ruthenium Red as a stain identified a large number of mutants with altered seed coat mucilage (Western et al., 2001). Complementation tests identified nine mutant alleles of MUM2 from four independent mutagenized populations. As all of the alleles have a similar phenotype, only the mum2-1 allele will be described in detail.

The mum2 mutant is characterized by a complete absence of extruded mucilage in hydrated seeds when examined by Ruthenium Red staining (cf. Figures 1A with 1B; Western et al., 2001). Scanning electron microscopy of mature, dry seeds revealed that mum2 and the wild type are indistinguishable, indicating that the failure of mum2 mucilage to be released on hydration is not associated with an obvious morphological defect (Figures 1C and 1D).

Developing seeds from mum2 and wild-type plants were sectioned, stained with Toluidine Blue, and examined by light microscopy (Figures 1E to 1N). Between 4 and 13 d post-anthesis (DPA), mum2 seeds were indistinguishable from the wild type, with mucilage deposition into the apoplast and columella formation appearing to proceed as normal (cf. Figures 1E to 1H with 1J to 1M; for a detailed description of seed coat epidermal differentiation, see Western et al., 2000). However, unlike the wild type, when 16-DPA mum2 seeds are hydrated during the fixation process, the primary cell wall fails to break and no mucilage is extruded (cf. Figures 1I to 1N). Note that slight variations in staining intensity between different sections is within the bounds of normal Toluidine Blue staining and does not reflect differences in morphology or structure of cell components such as mucilage.

In mutants such as mum4/rhm2 where less mucilage is produced (Usadel et al., 2004; Western et al., 2004) the morphology of epidermal cells tends to be altered. In the case of mum2, the normal appearance of the epidermal cells indicates that the lack of mucilage release is not due to a decrease in mucilage amount but is more likely due to a lack of mucilage release on hydration.

mum2 Seeds Can Release Mucilage on Hydration with Na2CO3

The release of mucilage during seed hydration is thought to involve the expansion of hydrophilic mucilage in the apoplast as it absorbs water, resulting in the breakage of the radial primary cell wall (Western et al., 2000). We investigated a number of chemical reagents and extractants for their ability to release mucilage from mature mum2 seeds.

The chelating agents EDTA, EGTA, and CDTA (0.05 M concentration), which extract Ca2+-cross-linked pectins, were able to extract a small amount of mucilage from mum2 (Figure 1Q; see Supplemental Figure 1 online), whereas 0.5 M ammonium oxalate did not (see Supplemental Figure 1 online). Weak alkali, such as Na2CO3, has been reported to extract pectins by cleavage of cross-linking ester linkages (Selvendran and Ryden, 1990; Fry, 2000; McCartney and Knox, 2002). Treatment of mum2 seeds with 1 M Na2CO3 caused the primary cell wall to break and mucilage to be extruded (cf. Figures 1R and 1B). Although it is apparent that the primary cell wall has broken (columelae can be visualized as dark staining points on the seed surface), mum2 mucilage does not expand as much and does not stain as darkly with Ruthenium Red as wild-type mucilage (cf. Figures 1P and 1R).

Strong alkali, such as KOH, extracts cross-linking glycos from cellulosic microfibrils (Fry, 2000). However, mum2 mucilage was not released by treatment with 2 M KOH or 0.5, 1, or 2 M NaOH (data not shown). Although strong alkali is expected to de-esterify pectins in the same way as Na2CO3, it also swells cellulosic microfibrils by disruption of hydrogen bonds that may entrap the mucilage. Finally, 5.5 M guanidine thiocyanate, an aqueous chaotropic agent that solubilizes mannans and glucomannans (Fry, 2000), did not release mum2 mucilage (data not shown).

mum2 Mucilage Cannot Expand Even When Exposed Directly to Water

The failure of mum2 mutants to release mucilage on hydration with water may be caused by an alteration in the mucilage that
Figure 1. Cytological Analysis of the mum2 Mutant.

(A) Mature wild-type seed stained with Ruthenium Red. A layer of stained mucilage is visible around the seed, and the columellae are clearly visible in the center of each cell, indicating that the outer tangential cell wall has ruptured.

(B) Mature mum2 seed stained in Ruthenium Red. No extruded mucilage is visible, and the outer tangential cell walls have not ruptured.

(C) Scanning electron microscopy of mature wild-type seed. Hexagonal epidermal cells with thickened radial walls and a central columella are visible.

(D) Scanning electron microscopy of mature mum2 seed. The mutant seeds are indistinguishable from the wild type.

(E) Wild-type seed coat epidermal cell at 4 DPA. Amyloplasts are visible; much of the cell volume is occupied by the vacuole.

(F) Wild-type seed coat epidermal cell at 7 DPA. Mucilage secretion into the apoplast has begun, and the cytoplasmic column is visible.

(G) Wild-type seed coat epidermal cell at 10 DPA. Mucilage secretion is complete, and columella formation has begun.

(H) Wild-type seed coat epidermal cell at 13 DPA. Columella formation is complete.

(I) Wild-type seed coat epidermal cell at 16 DPA. The epidermal cells of the seed coat have ruptured during the fixation process, and mucilage has been released. The columella and the radial cell walls are visible.

(J) to (N) The development of the mum2 seed coat epidermal cells at 4 (J), 7 (K), 10 (L), and 13 DPA (M) is indistinguishable from the wild type. However, at 16 DPA (N), the mum2 seed coat does not rupture and the mucilage remains within the primary cell wall (cf. [I] with [N]).

(O) and (P) Mature wild-type seed treated with EDTA (O) or Na$_2$CO$_3$ (P) and stained with Ruthenium Red. Mucilage has been released, and seeds have a similar appearance to those stained directly with Ruthenium Red (cf. with [A]).

(Q) Mature mum2 seed treated with EDTA and stained with Ruthenium Red. Small amounts of mucilage are released from some cells.

(R) Mature mum2 seed treated with Na$_2$CO$_3$ and stained with Ruthenium Red. A thin layer of poorly stained mucilage has been released. The columellae are visible, indicating that the outer tangential cell wall has ruptured (cf. [R] with [B]).

(S) and (T) Sections (20 μm) of mature, unfixed, paraffin-embedded wild-type seed hydrated with Ruthenium Red (S) or Na$_2$CO$_3$ and then Ruthenium Red (T). The mucilage is extruded from the seed coat similar to intact seeds.

(U) Section (20 μm) of mature, unfixed, paraffin-embedded mum2 seed hydrated with Ruthenium Red. The mucilage does not expand, despite being in direct contact with the aqueous dye solution.

(V) Section (20 μm) of mature, unfixed, paraffin-embedded mum2 seed hydrated with Na$_2$CO$_3$ and then Ruthenium Red. The outer tangential cell wall has ruptured, and a thin layer of mucilage has been released, similar to intact seeds treated with Na$_2$CO$_3$.

Bars = 100 μm in (A) to (D) and (O) to (V) and 10 μm in (E) to (N).
affects its ability to hydrate and/or changes in the seed epidermal primary cell wall that prevent it from breaking. To determine whether the \textit{mum2} mucilage itself can hydrate, unfixed mature seeds of both wild-type and \textit{mum2} mutants were embedded in paraffin wax and 20-μm sections prepared. Since seed coat epidermal cells are typically larger than 20 μm, few epidermal cells are intact in such sections, thereby eliminating the need for the mucilage to break the seed coat to expand. The sections were mounted on microscope slides and hydrated in the presence of Ruthenium Red. Wild-type sections prepared in this way showed hydration and expansion of the mucilage as in intact seeds (Figure 1S). By contrast, the \textit{mum2} mucilage did not expand despite being in direct contact with the water with no apparent barrier to hydration (Figure 1U). We also hydrated wild-type and \textit{mum2} sections with 1 M Na₂CO₃ prior to Ruthenium Red staining. In the wild type, expansion of mucilage occurs as in sections hydrated with Ruthenium Red directly (Figure 1T). In \textit{mum2}, only a small amount of mucilage expansion was observed (Figure 1V), consistent with the hypothesis that the hydration of \textit{mum2} mucilage itself is impaire. Although this experiment does not rule out the possibility that alterations in the primary cell wall also prevent breaking during hydration, these data indicate that the \textit{mum2} mucilage itself is altered in its ability to hydrate and expand.

Cloning of the MUM2 Gene

We identified the \textit{MUM2} gene using positional cloning. The \textit{MUM2} gene was localized on chromosome V between markers on BACs MBK5 and MHJ24. Of the 48 loci in this region, several were identified as putative carbohydrate active enzymes and selected as \textit{MUM2} candidates. SALK insertion lines were obtained for these loci, and plants homozygous for an insertion in exon 3 of At5g63800 (SALK_011436) showed a \textit{mum2} phenotype. The T-DNA insertion in this SALK line segregates with the \textit{mum2} phenotype, and crosses to SALK_011436 failed to complement the \textit{mum2} phenotype (Figure 2B). None of 18 independent transformants carrying empty pCAMBIA1200 rescued the \textit{mum2} phenotype (Figure 2C).

\textbf{MUM2 Encodes a Putative β-Galactosidase}

The Carbohydrate Active Enzymes database (http://www.cazy.org; Coutinho and Henrissat, 2006) includes MUM2 as a member of glycosyl hydrolase Family 35 β-galactosidases (Ahn et al., 2007). Members of this family are found in eukaryotes and prokaryotes, and there are 18 members in \textit{Arabidopsis}. The predicted MUM2 protein has 718 amino acids, giving a predicted size of 80 kD (UniProt Version 35 identifier Q9FFN4). Identification of conserved domains using the Pfam database (Version 22.0; http://www.sanger.ac.uk/Software/Pfam; Finn et al., 2006) indicates that the MUM2 protein contains a glycosyl hydrolase catalytic domain near the N terminus as well as several PfamB domains (automatically annotated conserved domains with no assigned function) near the C terminus. Prediction of domains using the InterPro resource (version 16.0; http://www.ebi.ac.uk/interpro; Mulder et al., 2005), which searches a number of subdatabases including Pfam, Prints, SUPERFAMILY, PANTHR, and Prosite simultaneously, also reveals putative galactose binding-like domains (SUPERFAMILY version 1.69, domain ID IPR008979) that overlap with the PfamB domains predicted using the Pfam database. The predicted MUM2 protein structure

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Transcript Analysis in \textit{mum2} Alleles and Genomic Complementation of \textit{mum2} with At5g63800. (A) RT-PCR analyses on 7-DPA silique cDNA from, \textit{mum2}-2, \textit{mum2}-5, and \textit{mum2}-10 using MUM2-specific primers. Cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) was used as a loading control. The larger product in \textit{mum2}-1 caused by inefficient splicing is indicated by an arrow. (B) and (C) Ruthenium red–stained seeds. The \textit{mum2} plants transformed with pMUM2g show complementation of the \textit{mum2} phenotype as indicated by a restoration of wild-type mucilage extrusion (B). The \textit{mum2} plants transformed with empty pCAMBIA1200 retain a \textit{mum2} phenotype (C). Bars = 100 μm.}
\end{figure}
and the positions of the lesions in mum2-1, mum2-2, mum2-5, and mum2-10 are shown in Figure 3.

An N-terminal signal peptide for secretion of the MUM2 protein was predicted using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP; Bendtsen et al., 2004). The NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc) was used to identify Asn residues at positions 291 and 468 as likely sites of N-glycosylation (Figure 3). A possible third N-glycosylation site was also identified by NetNGlyc at position 538, but this was predicted to be unlikely to be glycosylated in vivo.

Detailed analysis of the catalytic mechanism has been performed for the glycosyl hydrolase Family 35 β-galactosidases from Xanthomonas manihotis and Bacillus circulans, and the catalytic nucleophile of these enzymes has been identified (Blanchard et al., 2001). Alignment of the predicted MUM2 amino acid sequence with the amino acid sequences of these bacterial enzymes using ClustalW (Chenna et al., 2003) predicts the MUM2 catalytic nucleophile to be Glu-259. The position of the catalytic proton donor has also been predicted for a human Family 35 β-galactosidase (Henrißat et al., 1995). Alignment of the predicted MUM2 protein sequence with this protein sequence using ClustalW predicts that Glu-188 is the MUM2 catalytic protein donor. The positions of these predicted catalytically active residues are shown in Figure 3.

**Recombinant MUM2 Protein Is a β-Galactosidase**

To determine the function of the MUM2 protein, we used the methylotrophic yeast *Pichia pastoris* to express MUM2 under the control of the methanol-inducible AOX1 promoter. To monitor expression of MUM2 protein, we engineered a C-terminal His tag into the MUM2 protein. The parent vector and the parent vector containing the MUM2 coding sequence were introduced into the MUM2 protein. The parent vector and the parent vector containing the MUM2 protein, cell pellet extracts from yeast expressing MUM2 contained a His-tagged protein of ~110 kD (80 kD MUM2 plus the 30 kD α-Factor secretion signal) that was not detected in yeast transformed with the empty vector (see Supplemental Figure 2 online). MUM2 protein was not detected in the growth medium. Maximal expression of MUM2 was seen 26 h after induction.

We tested the ability of cell pellet crude extracts to hydrolyze several β-nitrophenyl-glycosides (PNP-glycosides; Table 1). Extracts containing recombinant MUM2, but not the control extracts, showed significant release of PNP with PNP-β-D-galactopyranoside (PNP-β-D-Gal) as substrate. We determined the activity of MUM2 for PNP-β-D-Gal to be 103 nmoles PNP h⁻¹ mg⁻¹ total *Pichia* protein using six independent replicates (see Supplemental Figure 2 online). Due to high nascent activity of *Pichia* extracts against PNP-β-D-glucopyranoside (PNP-β-D-Glc), the His-tagged recombinant protein was purified by batch elution from Ni⁺⁺ resins and was shown to possess activity against PNP-β-D-Gal at 29% of activity against the galactoside. Trace activities were also detected against PNP-α-arabinopyranoside and PNP-α-L-arabinofuranoside, but activities against several other PNP-glycosides were not detectable (Table 1).

**The Na₂CO₃-Extractable Fraction of mum2 Mucilage Shows Altered Monosaccharide and Linkage Composition**

We used high-performance anion exchange chromatography (HPAEC) to quantify the monosaccharide composition of Na₂CO₃-extracted wild type and mum2 mucilage after hydrolysis with sulphuric acid. These data indicate that the total monosaccharide obtained from extracted mum2 mucilage was significantly less than that obtained from the wild type (Table 2). The amounts of the RG-I backbone sugars rhamnose (Rha) and galacturonic acid (GalA) are decreased significantly in mum2-extracted mucilage, while galactose (Gal) and arabinose ( Ara), which are common side-chain sugars in RG-I, remain constant. In addition, mum2-extracted mucilage has decreased xylose (Xyl), glucose (Glc), and mannose (Man), while trace amounts of fucose (Fuc) remain constant. To examine the relative amount of each sugar in mum2 and wild-type mucilage, the HPAEC data were also expressed as m mole percentage (see Supplemental Table 1 online), highlighting the relative increase of Ara, Gal, and Glc compared with Rha and GalA in mum2-extracted mucilage.
Previous studies have inferred that mucilage accounts for ~60% of the Rha in whole seeds (Penfield et al., 2001; Western et al., 2001). The large differences in monosaccharide composition seen in extracted mucilage were not reflected by similar differences in composition of alcohol-insoluble residue (AIR) prepared from whole mature wild-type and mum2 seeds without an extraction step (Table 2; Western et al., 2001). These data are also presented as mole percentage of each monosaccharide (Table 3). Significant differences in composition of alcohol-insoluble residue (AIR) were identified, including (1→5)-a-arabinan in 2,5- and 3,5-Ara branch point residues and type II arabinogalactan (AG-II) with (1→3)- and (1→6)-linked b-galactan chains connected in (1→3,1→6)-branch points. Very low levels of (1→4)-linked b-galactan are present, and no type I arabinogalactan (AG-I) was detected. The mole percentage of branch-point residues (2,4-Rhap) of the mum2 RG-I correspond in part to the increases in branched (1→5)-a-arabinan as well as the nonreducing t-Galp residues. In addition, mum2 mucilage appears to contain increased AG-II, which may be attached to the RG-I backbone and also forms the carbohydrate moieties of arabinogalactan proteins (AGPs).

Other linkages detected indicate the presence of small amounts of other noncellulosic polysaccharides, including the t-GlcAp, 2-Xylp, and 2,4-Xylp of glucuronoxylans and the 4-GlcAp and 4-Manp of (gluco)mannans. The small amounts of nonreducing t-Xylp and 2-Xylp compared with the presence of 4-GlcAp and 4,6-GlcAp indicates that xyloglucan constitutes very little of the mucilage. The glucosyl residues may be from cellulose linkages by gas-liquid chromatography–mass spectrometry. Data are presented as mole percentage (Table 3). Consistent with the HPAEC results, mum2 mucilage exhibited a decrease in Rha and concomitant increases in Ara, Gal, and Glc when compared with the wild type. The mole percentage GalA is unchanged in the mum2 mucilage.

Consistent with previous studies (Penfield et al., 2001), the predominant linkages in the mucilage of both the wild type and mum2 are 2-linked Rha (2-Rhap) and 4-linked GalA (4-GalAp), indicating that the major polysaccharide in mucilage is RG-I (Table 3). However, in mum2, the amount of 2-Rhap is decreased by almost 50%, indicating that less RG-I backbone is extracted. In the wild type, RG-I is largely unbranched, whereas in mum2, an increase in branch point Rha residues (2,4-Rhap) were detected. Branching at the O-3 position (2,3-Rhap) is observed in both genotypes. The wild-type mucilage RG-I gave an unbranched: branched ratio of nearly 31:1, whereas mum2 mucilage gave ratios less than 4:1. The percentage of 4-GalAp is similar between the wild type and mum2. Some of this (1→4)-a-linked GalA will form part of the RG-I backbone along with 2-Rhap and 2,4-Rhap, while the remainder of 4-GalAp likely represents HG.

Table 1. Activities against PNP-Glycosides Relative to PNP-β-D-Gal

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative Activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNP-β-D-Gal</td>
<td>1.00</td>
</tr>
<tr>
<td>PNP-α-β-D-Glc</td>
<td>UD</td>
</tr>
<tr>
<td>PNP-α-β-D-galactopyranoside</td>
<td>ND</td>
</tr>
<tr>
<td>PNP-α-β-D-glucopyranoside</td>
<td>ND</td>
</tr>
<tr>
<td>PNP-α-β-D-mannopyranoside</td>
<td>ND</td>
</tr>
<tr>
<td>PNP-α-β-D-arabinoxyranoside</td>
<td>ND</td>
</tr>
<tr>
<td>PNP-α-β-D-xylopyranoside</td>
<td>ND</td>
</tr>
<tr>
<td>PNP-α-β-D-rhamnopyranoside</td>
<td>ND</td>
</tr>
<tr>
<td>PNP-α-β-D-rhamnofuranoside</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Base activity against PNP-β-D-Gal was 103 nmoles PNP h⁻¹ mg⁻¹ total Pichia protein. ND, not detectable (<0.01); UD, undetermined due to high endogenous activity in Pichia.

### Table 2. Monosaccharide Composition of Mucilage Determined by HPAEC

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Wild-Type Mucilage</th>
<th>mum2 Mucilage</th>
<th>Wild-Type Whole Seed</th>
<th>mum2 Whole Seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fuc</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>13.65 ± 0.85</td>
<td>11.05 ± 0.76</td>
</tr>
<tr>
<td>Ara</td>
<td>0.78 ± 0.084</td>
<td>0.75 ± 0.1</td>
<td>364.25 ± 29.46</td>
<td>303.77 ± 17.85</td>
</tr>
<tr>
<td>Rha</td>
<td>23.30 ± 1.25</td>
<td>4.66 ± 0.52</td>
<td>351.26 ± 41.02</td>
<td>302.72 ± 25.62</td>
</tr>
<tr>
<td>Gal</td>
<td>1.09 ± 0.05</td>
<td>1.10 ± 0.12</td>
<td>252.01 ± 23.57</td>
<td>236.73 ± 19.37</td>
</tr>
<tr>
<td>Glc</td>
<td>3.82 ± 0.09</td>
<td>2.34 ± 0.15</td>
<td>213.73 ± 5.18</td>
<td>201.40 ± 7.74</td>
</tr>
<tr>
<td>Xyl</td>
<td>2.00 ± 0.08</td>
<td>0.60 ± 0.02</td>
<td>160.06 ± 6.56</td>
<td>127.54 ± 4.5</td>
</tr>
<tr>
<td>Man</td>
<td>0.69 ± 0.04</td>
<td>0.21 ± 0.03</td>
<td>45.38 ± 4.12</td>
<td>46.66 ± 1.49</td>
</tr>
<tr>
<td>GalA</td>
<td>33.66 ± 2.25</td>
<td>9.03 ± 0.52</td>
<td>476.02 ± 49.62</td>
<td>374.79 ± 47.51</td>
</tr>
<tr>
<td>Total</td>
<td>65.43 ± 3.71</td>
<td>18.73 ± 1.27</td>
<td>1876.37 ± 149.29</td>
<td>1604.65 ± 122.39</td>
</tr>
</tbody>
</table>

Values are the mean ± SE of four samples (mucilage) or three samples (whole seed) and are expressed as nmoles sugar normalized to milligrams of seed used for extraction (mucilage) or milligrams of AIR hydrolyzed (whole seed).
Table 3. Linkage Analysis of Wild-Type and mum2 Mucilage

<table>
<thead>
<tr>
<th>Sugar and Linkage</th>
<th>Wild Type</th>
<th>mum2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose t-Fuc</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>Rhamnose t-Rha</td>
<td>0.2 ± 0</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td>2-Rha</td>
<td>49.2 ± 0.4</td>
<td>25.4 ± 1.9</td>
</tr>
<tr>
<td>2,3-Rha</td>
<td>0.4 ± 0</td>
<td>0.3 ± 0</td>
</tr>
<tr>
<td>2,4-Rha</td>
<td>1.2 ± 0</td>
<td>6.3 ± 0.5</td>
</tr>
<tr>
<td>Arabinose t-Araf</td>
<td>0.1 ± 0</td>
<td>0.3 ± 0</td>
</tr>
<tr>
<td>2-Araf</td>
<td>tr</td>
<td>0.2 ± 0</td>
</tr>
<tr>
<td>5-Araf</td>
<td>0.8 ± 0</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>2,5-Araf</td>
<td>tr</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>3,5-Araf</td>
<td>tr</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td>Xylose t-Xylp</td>
<td>0.6 ± 0</td>
<td>0.4 ± 0</td>
</tr>
<tr>
<td>4-Xylp</td>
<td>2.2 ± 0.2</td>
<td>3.4 ± 0</td>
</tr>
<tr>
<td>2,4-Xylp</td>
<td>0.9 ± 0</td>
<td>0.7 ± 0</td>
</tr>
<tr>
<td>3,4-Xylp</td>
<td>tr</td>
<td>0.2 ± 0</td>
</tr>
<tr>
<td>Mannose t-Manp</td>
<td>ND</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>4-Manp</td>
<td>1.3 ± 0.3</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>Galactose t-Galp</td>
<td>1.2 ± 0.1</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>3-Galp</td>
<td>0.4 ± 0</td>
<td>0.5 ± 0</td>
</tr>
<tr>
<td>4-Galp</td>
<td>0.1 ± 0</td>
<td>0.3 ± 0</td>
</tr>
<tr>
<td>6-Galp</td>
<td>ND</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>3,6-Galp</td>
<td>0.2 ± 0</td>
<td>0.8 ± 0</td>
</tr>
<tr>
<td>Glucose t-GlcP</td>
<td>tr</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>4-GlcP</td>
<td>3.1 ± 1.0</td>
<td>4.5 ± 0.9</td>
</tr>
<tr>
<td>2,4-GlcP</td>
<td>0.8 ± 0.3</td>
<td>0.5 ± 0</td>
</tr>
<tr>
<td>3,4-GlcP</td>
<td>0.3 ± 0</td>
<td>0.5 ± 0</td>
</tr>
<tr>
<td>4,6-GlcP</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Galacturonic Acid t-GalAp</td>
<td>0.3 ± 0</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>4-GalAp</td>
<td>31.6 ± 1.2</td>
<td>32.9 ± 2.2</td>
</tr>
<tr>
<td>3,4-GalAp</td>
<td>1.5 ± 0.1</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>Glucuronic acid t-GlcAp</td>
<td>0.3 ± 0</td>
<td>0.1 ± 0</td>
</tr>
</tbody>
</table>

Values are the mole percentage and variance of two samples (0 = <0.05). tr, trace; ND, not detected.

(4-GlcP) or from amylose and amyllopectin, which are readily extracted by dilute alkali from underlying cell layers.

The increase in the relative proportion of t-Gal residues in mum2 mucilage and in the degree of branching of RG-I are consistent with a defect in the β-galactosidase activity of the MUM2 protein.

Fourier Transform Infrared Spectroscopy Reveals Altered Polysaccharide Structures in the Na2CO3-Extractable Fraction of mum2 Mucilage

Samples of Na2CO3-extracted mum2 and wild-type mucilage were desalted by dialysis and freeze-dried before analysis by Fourier transform infrared (FTIR) spectroscopy. For comparison, wild-type mucilage samples were also prepared in water and freeze-dried.

Pectins give rise to peaks in the infrared spectrum at 1144, 1100, 1047, 1017, 953, 896, 857, and 835 cm−1, and RG-I gives rise to peaks at 1150, 1122, 1070, 1043, 989, 951, 902, 846, and 823 cm−1 (Kacuráková et al., 2000). Samples extracted from wild-type seeds using water and Na2CO3 (see Supplemental Figure 3 online) have peaks characteristic of RG-I (1126, 1068, 1041, 987, and 821 cm−1). The peaks at 1612 and 1423 cm−1 are carboxylate ion stretches from galacturonic acid (Kacuráková et al., 2000). Consistent with our monosaccharide analysis, this indicates that extraction with Na2CO3 allows the extraction of mainly pectic components from wild-type seed mucilage.

Samples of Na2CO3-extracted mucilage from mum2 showed RG-I and acetylation peaks as for wild-type Na2CO3-extracted mucilage (peaks at 1145, 1068, 1037, 987, and 821 cm−1, and 1731 and 1238 cm−1, respectively). The carboxylate stretches at 1596 and 1415 cm−1 are shifted from 1612 and 1423 cm−1 and are also increased in intensity. There are also additional absorbances at 1095, 1014, 956, 898, and 860 cm−1 that can be assigned to pectin (see Supplemental Figure 3 online) (Kacuráková et al., 2000).

To highlight the differences between Na2CO3-extracted mucilage from the wild type and mum2, a difference spectrum representing proportional differences between the genotypes was generated by digitally subtracting an average wild-type spectrum from an average mum2 spectrum (see Supplemental Figure 3 online). The negative peaks at 1153, 1122, 1068, 1041, and 822 cm−1 indicate a higher proportion of RG-I in wild-type mucilage. The positive peaks at 1577 and 1404 cm−1 indicate increased proportions of carboxylate ions in mum2 mucilage. HPAEC (see Supplemental Table 1 online) and linkage analysis (Table 3) indicate that the mole percentage of Rha is decreased in mum2 mucilage but that the 4-linked GalA content remains at wild-type levels, indicating that HG may constitute a higher proportion of Na2CO3-extracted mum2 mucilage. In addition, the shift in wave number from the expected GalA absorbances of 1600 and 1420 cm−1 may indicate that GalA is complexed with other cell wall moieties. Positive peaks at 964, 887, and 860 cm−1 also indicate altered proportions of pectins in mum2 mucilage.

MUM2 Expression Is Not Limited to the Developing Seed Coat

RT-PCR analysis using RNA extracted from developing wild-type siliques at 4, 7, 10, and 13 DPA (Figure 4A) indicated that MUM2 transcript is present in siliques throughout development. We also detected MUM2 transcript in roots (6 d after germination), rosette leaves, cauline leaves, stems, and open flowers (Figure 4A), suggesting that MUM2 has biological functions additional to those in seed coat development.

The outer layer of epidermal cells in the ap2-7 mutant does not differentiate (Western et al., 2001), making ap2-7 a useful control to distinguish between transcripts that are expressed in the outer epidermal cells during differentiation and transcripts that are expressed in other tissues of the developing silique. MUM2 transcript is present in 7-DPA ap2-7 siliques (Figure 4A), suggesting that MUM2 is expressed in other tissues of the developing seed and silique and is not restricted to outer epidermal cells at this stage in development. As MUM2 is expressed in ap2
siliques, it appears that MUM2 is not under control of AP2 in these tissues; however, we cannot rule out the possibility that AP2 regulates MUM2 expression specifically in the outer layer of the seed coat.

To examine the expression of MUM2 more precisely, we next performed real-time PCR using RNA derived specifically from seed coats isolated from wild-type plants at 4, 7, and 10 DPA (Figure 4B). Consistent with a role in mucilage modification, these data indicate that MUM2 is upregulated at 7 DPA when mucilage production in seed coats is at its peak.

MUM2 Protein Is Localized to the Cell Wall via the Golgi

The MUM2 protein has a predicted signal peptide for secretion to the cell wall. We constructed MUM2–green fluorescent protein (GFP) fusions under control of the 35S promoter and used them for transient expression in tobacco (Nicotiana tabacum). The fluorescence of MUM2-GFP was weak but could be detected in the cell wall of tobacco epidermal pavement cells, indicating that the MUM2 protein was secreted (Figure 5A). To confirm cell wall localization, tissue was stained with FM4-64 to visualize the plasma membrane. Localization of GFP external to the plasma membrane confirms that MUM2 is a secreted protein (Figure 5A, inset). Expression of the pVKH18-GFPC parent vector shows a characteristic free GFP localization pattern, with GFP present in the cytoplasm and nucleus (but not the nucleolus) (Figure 5B).

The fluorescence of GFP in the cell wall is usually weak because of the acidic pH in the apoplast (Zheng et al., 2004). We cotransformed tobacco with the MUM2-GFP fusion protein and a dominant-negative Rab1b mutant protein AtRab1b(N121I). AtRab1b is required for protein transport between the endoplasmic reticulum (ER) and the Golgi in plant cells, and the presence of AtRab1b(N121I) causes proteins that are normally secreted via the Golgi to be retained in the ER (Batoko et al., 2000). Tobacco cotransformed in this way showed an accumulation of MUM2-GFP...
GFP in a structure typical of the cortical ER (Figure 5C), as confirmed by examination of tobacco transformed with the ER-localized GFP-HDEL (Figure 5D). These data confirm that MUM2 is secreted, and its secretion to the cell wall is mediated by the Golgi.

The pVKH18-GFPC parent vector and P3SS:MUM2-GFP constructs were introduced into mum2 plants, and stable transformants were recovered. Seed from lines transformed with P3SS:MUM2-GFP showed complementation of the mum2 phenotype, indicating that the fusions were functional (see Supplemental Figure 4 online). Seed from lines transformed with pVKH18-GFPC were not complemented (see Supplemental Figure 4 online). We were unable to visualize the MUM2-GFP fusion protein in complemented plants due to a combination of dim GFP fluorescence in the apoplast caused by acidic pH (Zheng et al., 2004) and strong cell wall autofluorescence in seeds and roots.

To confirm that the MUM2-GFP fusion protein was present in the transgenic tobacco, protein gel blotting was performed using an antibody against GFP. A band of ~110 kD (79 kD corresponding to the predicted size of MUM2 plus 30 kD corresponding to GFP) was detected, indicating that the fusion protein was present and not degraded (see Supplemental Figure 4 online). We also detected full-length fusion protein in seedlings germinated from complemented seeds using protein gel blotting as described above (see Supplemental Figure 4 online).

**DISCUSSION**

The MUM2 locus was identified in a screen for mutants with modified seed coat mucilage. We cloned the MUM2 gene and identified it as a member of glycosyl hydrolase Family 35 with β-galactosidase activity. Functional MUM2-GFP protein fusions have demonstrated that the MUM2 protein is secreted to the apoplast via the ER-Golgi network. Cytological and biochemical analyses reveal that the MUM2 gene does not greatly influence the amount of mucilage produced or the morphology of the seed coat but is required for production of mucilage with the correct structure to permit expansion when hydrated. These biochemical analyses are consistent with the β-galactosidase function of MUM2. Taken together, our data show that MUM2 functions to modify carbohydrates in the apoplast following their biosynthesis and secretion and establishes a direct link between the function of a β-galactosidase and the modification of pectin structures with specific biochemical properties. Concurrent with our investigation into the role of MUM2 in Arabidopsis seed coat development, complementary work conducted in the lab of Helen North corroborates the major results and conclusions of this research (Macquet et al., 2007b).

**MUM2 Is Required for Expansion of Mucilage**

The failure of mucilage to extrude from mum2 seed coats could be due to a decrease in the total amount of mucilage as observed in mum4/rhm2 mutants (Usadel et al., 2004; Western et al., 2004), changes in the primary cell wall that restricts mucilage expansion, or chemical alteration of the mucilage that prevents such expansion.

Our cytological data suggest that mum2 mutants make and secrete an amount of mucilage that is comparable to the wild type. Significant decreases in total mucilage are normally associated with obvious changes in cellular morphology (Penfield et al., 2001; Western et al., 2001, 2004; Usadel et al., 2004), yet no such changes have been observed in mum2 mutants (Figure 1). In addition, monosaccharide analysis of wild-type and mum2 whole-seed AIR (prepared without an extraction step) reveals no major difference in the monosaccharide profiles of these two genotypes (Tables 2 and 3; Western et al., 2001). Given that much of the seed Rha and GalA appears to be derived from the mucilage (Penfield et al., 2001; Western et al., 2001), major changes in the amount of mucilage RG-I should be reflected in the total Rha observed in whole seeds, but no such decrease in Rha from whole ground seeds is apparent. These data indicate that the amount of secreted mucilage is not altered in mum2 mutants.

**MUM2 impacts seed mucilage hydration 4015**

MUM2 is required for production of mucilage that can be extruded on exposure of mature seeds to water (Figures 1A and 1B; Western et al., 2001). Although treatment of mature mum2 seeds with Na2CO3 allows mucilage extrusion via breakage of the primary cell wall, it appears that the mucilage does not expand as much as in the wild type (Figures 1Q and 1R). Hydration of sectioned seeds also indicates that the mucilage is unable to expand even when unrestricted by the primary cell wall (Figures 1S and 1T). Although it is possible that the primary cell wall is also a target for the action of MUM2, these data indicate that MUM2 affects the hydration properties of mucilage directly. Consistent with our data indicating that mum2 mucilage is impaired in its ability to expand, quantification of monosaccharides shows that far less total sugar is obtained from Na2CO3-extractable mucilage mum2 seeds (Table 2).

Based on the data discussed here and in more detail in the following section, we propose that MUM2 is required to modify mucilage structure after secretion to produce mucilage with correct hydration properties.

**MUM2 Modifies the Pectin Component of Mucilage**

The chemical analyses presented here are consistent with previous studies indicating that wild-type mucilage is composed primarily of relatively unbranched RG-I (Penfield et al., 2001; Willats et al., 2001a; Western et al., 2004; Macquet et al., 2007a) and that RG-I side chains contain various neutral sugars, with arabinose and galactose as the major glycosyl residues (Ridley et al., 2001). The differences between mum2 and wild-type mucilage involve these pectic components.

Partial extrusion of mucilage can be induced by treatment with Na2CO3, a chemical that has been used to extract pectin. This suggests that mum2 mutant pectin is structurally different from the wild type. Monosaccharide analysis by HPAEC shows decreased Rha and GalA (the major sugars of the RG-I backbone) and a relative increase in side-chain sugars Gal and Ara. FTIR data also indicate that mum2 mucilage has relatively less RG-I than the wild type and reveals relative increases in pectic components other than RG-I. A decrease in unbranched Rha accompanied by corresponding increases in the number of branch point Rha residues, arabinan side chain, and terminal
Gal residues determined by linkage analysis suggests a decrease in unbranched and an increase in branched RG-I. These data indicate that the pectic fraction and more specifically the RG-I present in Na2CO3-extractable mum2 mucilage are reduced in amount and more highly branched than in the wild type. In addition, there are also increases in AG-II side chains. These may by present as part of RG-I and are also found as the carbohydrate moiety of AGPs (Vincken et al., 2003).

The altered hydration properties of mum2 mucilage may be caused by the increased branching observed in the Na2CO3-extractable RG-I fraction. However, we cannot eliminate the possibility that other more subtle changes in RG-I or another component of the mum2 mucilage, such as an increase in mum2 seed methylation reported earlier (Western et al., 2001), are responsible for the observed changes.

Potential Targets for the MUM2 Enzyme in Mucilage

We have identified MUM2 as a member of glycosyl hydrolase Family 35 and demonstrated that it functions as a β-galactosidase. However, the in vivo target of MUM2 remains to be determined; previous studies have revealed that the substrate specificities of plant β-galactosidases can vary widely, indicating functional diversity in their activity. For example, all three isoforms of β-galactosidase isolated from ripening tomato fruit (Solanum lycopersicum) could hydrolyze PNP-β-D-Gal, but only one was capable of hydrolyzing the β-D-(1→4)-Gal, characteristic of galactan side chains of RG-I (Pressey, 1983). Recently, a radish (Raphanus sativus) β-galactosidase that showed activity toward β-D-(1→3)-Gal and β-D-(1→6)-Gal oligosaccharides and the AG-II moieties of AGPs but showed no activity toward β-D-(1→4)-Gal has been reported (Kotake et al., 2005), and the Arabidopsis BGAL4 protein has been shown to hydrolyze β-D-(1→3), β-D-(1→4), and β-D-(1→6)-linked galactobiosides and galactotriosides (Ann et al., 2007). Furthermore, β-galactosidases isolated from nasturtium (Tropaeolum majus) cotyledons (Edwards et al., 1991) and Copalifera langsfordii (de Alcantara et al., 1999) show activity toward the β-D-Gal-(1→2)α-L-xyllose side chains of xyloglucan but show little or no activity toward pectic β-D-(1→4)-Gal.

There are a number of substrates in mucilage that MUM2 may act upon, including terminal galactose (t-Gal), β-D-(1→4)-galactans attached to RG-I, AG-I, AG-II, and xyloglucans. However, as only low levels of β-D-(1→4)-galactans are detected in wild-type mucilage and these are not significantly altered in the mutant, and no galactosylated xyloglucans or AG-I have been observed in the wild type or mutant mucilage, the only Gal residues associated with RG-I that are available are t-Gal on RG-I. The linkage analysis indicates increased t-Gal in mum2 mucilage, which may be the target of MUM2 activity. In addition, there are also increased AG-II residues in mum2 mucilage, and it is not clear if these form RG-I side chains or AGP carbohydrate moieties. AGPs are implicated in a number of cellular processes, such as vegetative and reproductive growth and development, cell differentiation, and programmed cell death, but the molecular mechanisms of their actions have not been elucidated (Showalter, 2001). It has been suggested that AGPs may be involved in signaling by direct interaction with cell surface molecules (Schultz et al., 1998) or by providing a source of oligosaccharide signals (Ridley et al., 2001). Recently, it was proposed that AGPs can alter the porosity of pectins in the cell wall (Lamport et al., 2006), which may in turn affect access of modifying enzymes to pectic components. It could be envisaged that MUM2 is required to produce functional AGPs by modifying AG-II side chains.

It is also possible that MUM2 action is required as the first step in a series of modifications that together cause a more dramatic alteration in mucilage structure and properties. Such cascade effects have been documented as a feature of cell wall modification, for example, in xyloglcan breakdown during storage carbohydrate mobilization (Crombie et al., 1998) where β-galactosidase is required to carry out the first step in xyloglcan hydrolysis. The removal of galactosyl residues then allows α-xyllosidase (Fanuzzi et al., 1991), β-glucanase (Crombie et al., 1998), and xyloglucan endotransglycosylase (Fanuzzi et al., 1993) to complete the hydrolysis. A similar cascade for breaking down xyloglucans has recently been identified in the Arabidopsis apoplast (Iglesias et al., 2006). It may also be that modification of Gal in the primary cell wall of epidermal seed coat cells is important to set the stage for other changes, such as acetylation and methylation in the mucilage itself, which causes the changes in properties of mum2 mucilage. A model such as this, where the action of MUM2 does not directly alter the properties of mucilage but is required to process cell wall components to allow access for other cell wall modifying enzymes, may explain why there is a large change in mucilage properties upon loss of MUM2 function and may also explain the increase in arabinan RG-I side chains in mum2 mucilage.

Cell Wall Modification by MUM2 in Other Tissues

Our results and other studies indicate that MUM2 is expressed in all tissues examined (Iglesias et al., 2006; Macquet et al., 2007b); therefore, MUM2 may be required to play roles in wall modification elsewhere in the plant. Indeed, FTIR data available at the Purdue University Cell Wall Genomics website (http://cellwall.genomics.purdue.edu/families/index.html) indicate that mum2-10 seedlings have an altered cell wall structure, with increased methyl esters, increased pectin, and decreased protein. It is necessary to investigate these alterations in more detail to determine the degree of similarity to the seed coat mucilage defects described here. This may provide insights into the functions of MUM2 in different tissues with potentially different substrates and interacting partners.

The Impacts of Pectin Modification on Cell Wall Properties

The structure of pectin is important in determining wall characteristics, such as mechanical properties, wall porosity, cell adhesion, and cell expansion, which in turn impact various developmental processes, such as growth, abscission, and fruit ripening. Pectin modification therefore allows the wall to fulfill different functions during the lifetime of an individual cell or in a specific tissue (Willats et al., 2001b; Knox, 2002).

One developmental process where β-galactosidase in particular has been implicated is fruit ripening. In numerous fruits, increased β-galactosidase activity is correlated with fruit ripening, and in many species, the loss of galactose and arabinose
from the wall is associated with fruit softening and an increase in pectin solubility (for a review, see Brummell, 2006). Although the mechanisms by which β-galactosidases are involved in cell wall modifications are not entirely clear, a functional role for β-galactosidases that alter wall properties during fruit ripening has been demonstrated. For example, the carambola β-galactosidase from ripe fruit is active against various galactans and can depolymerize structurally intact pectin and modify hemicelluloses (Balasubramaniam et al., 2005), and papaya β-galactosidase from ripe fruit has been shown to be capable of hydrolyzing cell wall components in unripe fruit (Lazan et al., 2004). It may be that there are analogies between fruit ripening and mucilage modification in the seed coat.

Establishing a relationship between pectin structure and function can be difficult, and predicting the effects of altering pectin structure is not trivial. The spatial and temporal distribution of epitopes in the wall is likely to be important in determining pectin properties, as are interactions between both pectins themselves and other cell wall components, such as hemicellulose and cellulose (Vinczen et al., 2003). Our data show that the seed coat model system can be used to identify enzymes that are involved in cell wall modification and that impact the physical and biochemical properties of cell walls. Further investigation into the pectin modifications in mucilage and into cell wall modifications in other tissues in mum2 mutants may further improve our understanding of this structure-function relationship. The mechanism by which MUM2, a β-galactosidase whose function is to remove terminal galactose residues from polysaccharides, can alter the hydration properties of mucilage so radically remains a key question. In particular, the identification of the in vivo substrate of MUM2 should provide insights into how modification of cell wall architecture can have a dramatic effect on wall properties.

METHODS

Plant Material and Growth Conditions

*mum2-1* (Western et al., 2001), *mum2-2*, and *mum2-9* were isolated from an ethyl methanesulfonate–mutagenized M3 population of wild-type Col-2 *Arabidopsis thaliana* plants: *mum2-3* and *mum2-4/scm4* are T-DNA insertion lines in theWs-2 background isolated from the Feldman collection (gift from Scott Satter and David Marks, University of Minnesota); mum2-5, mum2-6, and mum2-7 are Fast Neuron lines in a Col-0 background carrying a *gl1* mutation; *mum2-8* is a Fast Neuron line in Col-0; mum2-10 (SALK_011436; Alonso et al., 2003) is a T-DNA insertion line in theCol-0 background obtained from the ABRC. Alleles are available from ABRC through TAIR (www.arabidopsis.org).

Seeds were germinated on AT minimal medium (Haughn and Somerville, 1986) in growth chambers at 20°C under continuous light (90 to 120 μmol m−2 s−1 PAR) and then transferred at 7 to 10 d after germination to prepared soil mix (Sunshine Mix 5; Sun Gro Horticulture), watered with liquid AT medium, and grown under the same conditions as above.

For staging of siliques, open flowers 0 DPA were marked with nontoxic, water-soluble paint to allow the selection of developing siliques at precise ages.

Microscopy

Whole seeds were stained by shaking in 0.01% (w/v) Ruthenium Red (Sigma-Aldrich) for 2 h and then mounted in water and viewed using a dissection microscope. To investigate release of mucilage by Ca2⁺ chelators, alkali, and guanidine thiocyanate, seeds were shaken for 2 h in the chelator, alkali, or guanidine solution at room temperature, rinsed twice with water, and then stained with Ruthenium Red, as described above.

For resin embedding and sectioning, developing seeds were fixed with 3% (v/v) glutaraldehyde (Canemco) in 0.5 M sodium phosphate, pH 7. Postfixation with 1% (v/v) osmium tetroxide, dehydration, embedding, and sectioning were as described by Western et al. (2000). Thick sections (0.2 to 0.5 μm) were stained with 1% (w/v) Toluidine Blue O in 1% (w/v) sodium borate, pH 11, and photographed using a Zeiss AxioScope light microscope (Carl Zeiss).

Mature unfixed seeds were prepared for sectioning by embedding in Paraplast (Sigma-Aldrich). Seeds were added to molten Paraplast medium and then incubated at 60°C for 2 h before hardening overnight at ambient temperature. Sections approximately one cell’s width (20 μm thick) were prepared and hydrated with 0.01% (w/v) Ruthenium Red and then imaged as described above.

Samples were dry-mounted on stubs, coated with gold-palladium in a SEMPrep2 sputter coater (Nanotech), and imaged using a Hitachi S4700 scanning electron microscope (Hitachi High-Technologies Canada).

Digital photographs were manipulated with Adobe Photoshop (Adobe Systems) to prepare figures.

Positional Cloning of MUM2

A mapping population of 2016 F2 plants was generated from a cross between *mum2-1* (Col-2 background) and wild-type Landsberg erecta plants. Simple sequence length polymorphisms were identified using sequence information available from the Arabidopsis Genome Initiative (2000) and Cereon (Jander et al., 2002). Primer sequences are available at TAIR (www.arabidopsis.org).

SALK_011436 was genotyped by PCR using gene-specific primers (AT5g3800 F5, 5'-GCAAGACATCTCGGTGAGGAG-3' and AT5g3800 R5, 5'-CCATGTAAGCTCCAGATGGG-3') and a primer to the left border of the T-DNA insertion (LBb1, 5'-GGTGAGCGCTTGACGCAACT-3').

Molecular complementation of mum2-1 was performed using a PCR fragment amplified from BAC MKB5 (AT5g3800compF, 5'-GTA-CTTCACCGGTGTAAGCAGC-3', and AT5g3800compR, 5'-ATATCTGCGATCTCGTACCCAGG-3'), the fragment was cloned into pCAMBIA1200 (http://www.cambia.org/daisy/cambia/home.html) to give pMUM2g. As the inserts were not sequenced, four independent clones were selected for transformation. *mum2-1* plants were transformed with either pMUM2g or pCAMBIA1200 by the Agrobacterium tumefaciens dipping method (Clough and Bent, 1998) using strain LBA4404 (Hoekema et al., 1983). Hygromycin-resistant plants were identified by growth of T1 plants on AT medium supplemented with 25 μg/mL hygromycin B (Invitrogen Life Technologies).

Heterologous Expression of MUM2 in *Pichia pastoris* and Determination of MUM2 Enzyme Activity

The MUM2 coding sequence without the endogenous signal peptide was cloned into the pPIC9 vector supplied with the Invitrogen *Picha* expression kit by PCR using the primers pPIC9F (5'-ATCTCGAGAAAAAGAGGCTGAGCTCGAGAAGGAATAGTACGAGCGACG-3') and pPIC9R-His (5'-GCTAGCGCCCGCCTCAATGTAGTATGATAGTATGAGGGAATTAGGATTAGCTGACTGCA-3'). *Picha* strain GS115 was transformed with the finished construct and the parent pPIC9 vector by electroporation, and pure clonal isolates were obtained according the kit instructions. Transformed yeast colonies were maintained on MD plates (1.34% yeast nitrogen base without amino acids, 4 × 10⁻² M biotin, 2% dextrose, and 15 g/L agar). For induction, cultures were grown in MD liquid medium before induction was performed in MM liquid medium.
(1.34% yeast nitrogen base without amino acids, 4 × 10⁻⁵% biotin, and 1% methanol). For filter assays, induction was performed on MM plates (MM liquid medium plus 15 g/L agar).

Filter assays were prepared by transferring yeast colonies onto nitrocellulose membranes that were then flash-frozen in liquid nitrogen to permeabilize the yeast cells. Filters were then incubated at 37°C in 50 mM sodium acetate buffer, pH 5.0, containing 2 mM X-Gal or X-Gluc.

Protein samples from supernatants after cell culture were prepared by trichloroacetic acid precipitation. Protein samples from cell pellets for protein gel blots were prepared using yeast protein extraction reagent (Y-PER; Pierce Biotechnology) according to the manufacturer's instructions. Five microliters of the resulting supernatant was separated by SDS-PAGE on a 10% gel (Laemmli, 1970) and transferred to nitrocellulose membrane by semidyed electrobloiling. Penta-His antibody (Qiagen) and whole molecule anti-Mouse IgG-Alkaline Phosphatase conjugate (Sigma-Aldrich) were used as directed by the manufacturer and developed using NBT/BCIP (Roche).

Cell pellets for enzyme assays were stored at −80°C until required. Pellets were thawed on ice and resuspended in 2 mL of 50 mM sodium acetate, pH 5.0, per 100 mL of induced culture. Cells were disrupted using glass beads and a bead beater according to standard protocols. The cell debris was removed, and the supernatant cleared by two successive centrifugations at 14,000 rpm for 10 min at 4°C. Protein in the resulting extract was determined using protein assay reagent (Bio-Rad) according to the manufacturer's instructions, and 50 mM sodium acetate, pH 5.0, was added if necessary to equalize the protein concentrations in extracts from different genotypes. Protein extract (100 μL) and 24 μL of 10 mM PNP-glycoside were incubated at 37°C for 20 h before the reaction was stopped by the addition of 60 μL of 1 M sodium carbonate. PNP release was determined by measuring absorption at 405 nm. Standard curves for PNP were prepared to allow conversion of absorption to μmoles PNP. Controls consisting of boiled extracts from both genotypes were used to correct for background hydrolysis of PNP-glycoside during the assay. Low amounts of activity were seen in extracts containing the empty pPIC9 vector, and these were used to correct for endogenous activity. All samples were prepared in duplicate.

Other than PNP-β-D-glucoside, reactions against all substrates were negligible in the control Pichia extracts containing the empty pPIC9 vector. However, because of a high background activity against the β-D-glucoside in the Pichia extracts, this activity relative to that against PNP-β,β-D-galactoside was determined after purification of the His-tagged recombinant protein on Ni⁺ resin. Four milliliter aliquots of each of the Pichia control and MUM2 extracts in ice-cold 20 mM imidazole[HEPES], pH 7.4, containing 0.5 M NaCl, were added to a 200-μL suspension of the Ni⁺ resin (Ni Sepharose 6 Fast-Flow; Amersham) and incubated on a rocking shaker for 1 h. The resin was washed with three times with 15 mL each of the loading buffer and then three times with 15 mL each of 50 mM imidazole[HEPES], pH 7.4, containing 1.0 M NaCl, and then the proteins eluted with 1.5 mL of 300 mM imidazole[HEPES], pH 7.4, containing 1.0 M NaCl. To change buffers, the eluate was passed through a Sephadex G-25 desalting column (PD-10; GE Healthcare) equilibrated in 50 mM sodium acetate, pH 5.0. The protein fraction devoid of imidazole was collected and used in the assay directly. No protein was detected from the control extracts, but the same elution volumes were collected and used in the assays. No activities against either PNP-β,β-D-galactoside or PNP-β-D-glucoside were detected in the control reactions.

**Extraction of Mucilage with Na₂CO₃**

Approximately 30 mg of seed (exact weight recorded) was mixed with 0.7 mL of 1 M Na₂CO₃, and the seeds were shaken on an orbital shaker for 20 min to facilitate wetting. The supernatant (0.5 mL) was removed and retained as a mucilage fraction, 0.7 mL of water was added to the seeds, and they were shaken for a further 2 h using a vortex mixer on medium speed. Half a milliliter of this supernatant was removed and pooled with the 0.5 mL of supernatant in 1 M Na₂CO₃, giving 1 mL of mucilage in ~0.5 M Na₂CO₃. Samples were desalted by dialysis using 100 D cutoff cellulose ester membrane (Spectra/Por Biotech; Spectrum Labs) for 30 h in 0.05% chlorobutanol in water to prevent microbial growth. Dialyse was changed three to four times during this period.

**Determination of Monosaccharide Composition by HPAEC**

To prepare mucilage samples for HPAEC, ~30 mg of seeds (exact weight recorded) were mixed with 50 μL of 1 M sodium carbonate and left to stand at room temperature for 20 min before 1.2 mL of water and 10 μL of 5 mg/mL D-erythritol was added as an internal standard and the samples vortexed for 2 h on medium speed. Samples were then dried at 60°C under a stream of nitrogen. Sulphuric acid (72%, 20.25 μL) was added to each sample, and they were incubated on ice for 2 h. Water (480 μL) was then added to give a final concentration of 4% sulphuric acid, and the samples were autoclaved for 60 min at 121°C before being filtered through 0.45-μm nylon syringe filters.

To prepare AIR, whole seeds (~15 mg) were ground to a fine powder by mortar and pestle in liquid nitrogen. The powders were suspended in 1 mL of 70% (v/v) ethanol and heated at 60°C for 10 min to inactivate enzymes. Three 30-min washes in 1 mL 70% ethanol with shaking were then performed to remove small soluble sugars. The AIR fractions were collected by centrifugation in a microcentrifuge between washes. The AIR was dried under nitrogen and weighed before 10 μL of 5 mg/mL D-erythritol was added. Two to five milligrams of AIR was hydrolyzed using sulphuric acid as described above.

Neutral sugar standards (fucose, arabinose, rhamnose, galactose, glucose, manannose, and xylose) and acid sugar standards (galacturonic acid), prepared with and without sodium carbonate as required, were also processed in this way.

The identity and concentration of neutral monosaccharide sugars was determined using HPAEC analysis on a DX-600 BioLC chromatograph (Dionex). Separation was achieved on a CarboPac PA1 anion exchange column (Dionex), and detection was achieved via pulsed amperometry across a gold electrode. A 20-μL volume of sample was injected. The column was equilibrated with 250 mM NaOH and eluted with deionized water at a flow rate of 1.0 mL min⁻¹. Detection of carbohydrates was facilitated with a postcolumn addition of 200 mM NaOH at 0.5 mL min⁻¹. The concentration of acid sugars in the filtrate was determined with the identical HPLC system and column, with a different elution gradient: sugars were eluted in 100 mM NaOH with a linear gradient (curve 5) of 0 to 400 mM sodium acetate from 5 to 40 min, followed by a 10 min 300 mM NaOH wash. The column was then equilibrated to 100 mM NaOH prior to the next injection.

Monosaccharides were quantified with the D-erythritol internal standard after correction of response factors with monosaccharide standards of different concentrations to allow area to be converted to molar amounts. Sugars from mucilage were normalized to the mass of seed extracted, and sugars from whole-seed AIR were normalized to the mass of AIR.

**Carbohydrate Linkage Analysis**

Dialyzed mucilage samples extracted with Na₂CO₃ were used for carbohydrate linkage analysis. The uronosyl groups in mucilage polysaccharides were carboxyl reduced with sodium borodeuteride after activation with a water-soluble diimide (Carpita and McCann, 1996). The sample was then divided, with half of it was used for determination of monosaccharide composition and the other half used for linkage analysis (see below). For monosaccharide composition, mucilage was hydrolyzed with 2 M TFA containing 1 μmol of myo-inositol (internal standard) for 90 min at
120°C. The TFA was evaporated under a stream of nitrogen, and the sugars were converted to alditol acetates (Gibeaut and Carpita, 1991). The alditol acetates were separated by gas-liquid chromatography on a 0.25 mm × 30 m vitreous silica capillary column of SP-2330 (Supelco). Temperature was programmed from an initial 80°C hold for 1 min during injection, to 170°C at 25°C/min, and then to 240°C at 5°C/min with a 6-min hold at the upper temperature. The proportion of galacturonic acid was determined as the proportion of 6,6-dideuterogalactose in total galactose by electron-impact mass spectrometry according to the equation described by Kim and Carpita (1992), which accounts for 13C spillover.

For determination of sugar linkages within the mucilage, the second portion of the carboxyl-reduced mucilage samples were per-O-methylated with Li+ methylsulfinylmethanide 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 at least threefold excess of water, and the chloroform was evaporated under a stream of nitrogen. The methylated polymers purified by chloroform partitioning were hydrolyzed in 2 M TFA for 90 min at 120°C. The sugars were then reduced with NaBD₄ and acetylated. The partly methylated alditol acetates were separated by gas-liquid chromatography on a 0.25 mm × 30 m vitreous silica capillary column of SP-2330 (Supelco). Temperature was programmed from an initial 80°C hold for 1 min during injection, to 160°C at 25°C/min, then to 210°C at 2°C/min, and then to 240°C at 5°C/min, with a 6-min hold at the upper temperature. All linkages were deduced by a combination of retention time and electron-impact mass spectrometry of the derivatives (Carpita and Shea, 1989).

**FTIR Spectroscopy**

Na₂CO₃-extracted dialyzed mucilage samples were freeze-dried then dissolved in water (1 mg/mL). Ten microfilters were air-dried in the wells of IR-reflective, gold-plated microscope slides (Thermo-Electron) and supported on the stage of a Nicolet Continuum series microscope accessory to a 670 IR spectrophotometer with a liquid nitrogen-cooled mercury-cadmium telluride detector (Thermo-Electron). An area of sample (up to 125 × 125 μm) was selected for spectral collection in transfectance mode. One hundred and twenty-eight interferograms collected with 8 cm⁻¹ resolution were co-added to improve the signal-to-noise ratio for each sample. For mucilage samples, 30 spectra were collected, and each was area averaged and baseline corrected prior to generating average spectra and digital subtraction spectra as described previously (Chen et al., 1998).

**RT-PCR**

RNA was isolated from plant tissue as described by Western et al. (2004). RNA was treated with DNeasy (Invitrogen) and reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The complete MUM2 cDNA including 5′ and 3′ untranslated regions was amplified with At5g63800 F9 (5′-TCTCAAAATCTCAATCTCAA-3′) and At5g63800 R6 (5′-GAGTTTCTTTACATGTC-3′). For RT-PCR under nonsaturating conditions, gene-specific primers surrounding an intron were designed for MUM2 (At5g63800 F5, 5′-GCAAACGGCTTCTCTCTG-3′, and At5g63800 R5, 5′-CTATGTAAGTCCAGAGTCC-3′) and GAPC (GAPC P1, 5′-TCTAGACTCGAGAAGGCTCTAC-3′, and GAPC P2, 5′-GATCAAGTCCAGCCACACGG-3′; Western et al., 2004). The number of PCR cycles required for amplification in the linear range was determined for GAPC (23 cycles) and MUM2 (25 cycles). Amplification of GAPC was used as a loading control for MUM2 RT-PCR. RT-PCR was performed a minimum of three times using each set of primers to confirm results.

**Real-Time PCR**

Total RNA was extracted from 4-, 7-, and 10-DPA Col-2 seed coats using the RNAqueous kit (Ambion), and cDNA was synthesized using SuperScript III RNA polymerase (Invitrogen). Amplification was performed using the MJ mini opticon real-time PCR system (Bio-Rad) with GAPC as the internal control. The following gene-specific primers were used: GAPCp1, 5′-TCAGACTCGAGAAGGCTCTAC-3′; GAPCp2, 5′-GATCAAGTCCAGCCACACGG-3′; MUM2p1, 5′-ATTCAACGCCGTTCAAGT-3′; MUM2p2, 5′-ACGTGGACACATGTCGTGTA-3′. Each 20-μL reaction contained 10 μL of SYBR Green Supermix reagent (Bio-Rad), 1 μL of cDNA, and 0.2 μM each primer. Reactions were performed in triplicate. PCR conditions were 95°C for 3 min and then 40 cycles of 95°C for 10 s, 54°C for 30 s, and 72°C for 30 s. Data were analyzed using Gene Expression Macro software (version 1.1; Bio-Rad).

**Subcellular Localization of MUM2 Protein**

To generate GFP-fused MUM2, the vector pVKH18-GFP was first constructed using the following steps. The coding sequence of GFP5 in pVKH18-GFP-HDEL (Batoko et al., 2000) was amplified with primers GFP5C5 (5′-CGGATCCGGCTCGAGATGTAAGGAAACGTTTACGTGG-3′) and GFP5C3 (5′-CGGGATCCGGATGCTCTATTGGTAGTTCTCATCATGACATG-3′) and subcloned into pBlueScript KS as a BamHI-BamHII fragment. The subcloned GFP was sequenced and used as a BamHI-SacI fragment to replace secGFP in pVKH18-sGFP (Batoko et al., 2000) to create pVKH18-GFP. The cDNA of the MUM2 gene was then subcloned into pVKH18-GFP to produce the C-terminal MUM2-GFP fusion under the control of the cauliflower mosaic virus 35S promoter. Due to the presence of a putative ER targeting signal peptide in MUM2, only C-terminal GFP fusion was constructed. pVKH18 carrying the MUM2-GFP fusion was named pSS5:MUM2-GFP. The generation of pSS5:GFP-HDEL and pSS5:ArFAB1b(N121) was described by Batoko et al. (2000).

pVKH18-GFP, pSS5:MUM2-GFP, pSS5:ArFAB1b(N121), and pSS5:GFP-HDEL constructs were introduced transiently into tobacco (Nicotiana tabacum) according to the method of Batoko et al. (2000) with the modification that the infiltration buffer consisted of 50 mM MES, pH 5.6, 10 mM MgCl₂, and 150 μM acetylsyringone (Sigma-Aldrich). Transiently transformed tobacco leaf discs were incubated in 8.2 μM FM4-64 dye (Molecular Probes, Invitrogen Life Technologies) for 1 h to allow visualization of the plasma membrane.

Confocal imaging of samples mounted in water was performed according to Zheng et al. (2005) using a Bio-Rad Radiance Plus on an inverted Zeiss Axiovert (Carl Zeiss). ImageJ software (http://rsb.info.nih.gov/ij/) and Adobe Photoshop (Adobe Photosystems) were used for post-acquisition image processing.

The mum2-1 plants were transformed with either pVKH18-GFP or PSS5:GFP-MUM2 as described above. To confirm the presence of full-length MUM2-GFP protein fusion in plants, protein samples were prepared by grinding tissue in 2 × SDS loading buffer, boiling for 10 min, and then centrifuging to remove cell debris. Protein gel blotting was performed as described above using polyclonal anti-GFP (lgG fraction from rabbit; Molecular Probes, Invitrogen Life Technologies) and whole molecule anti-Rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_125775 and Q9FFN4 (At5g63800; MUM2), P16278 (human β-galactosidase), BAA21669 (Bacillus circulans β-galactosidase), and P48982 (Xanthomonas manihotis β-galactosidase).
Supplemental Data

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

Supplemental Figure 1. Extraction of Mucilage from mum2 Seeds Using Chelating Agents.

Supplemental Figure 2. Heterologous MUM2 Expression in Pichia pastoris.

Supplemental Figure 3. FTIR Spectroscopy Analysis of Wild-Type and mum2-Extracted Mucilage Extracted with Water and Na2CO3.

Supplemental Figure 4. Complementation of mum2 with P3SS: MUM2-GFP.

Supplemental Table 1. Monosaccharide Composition of Mucilage Determined by HPAEC Expressed as Mole Percentage of Each Monosaccharide.

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