AtBXL1 Encodes a Bifunctional β-d-Xylosidase/α-L-Arabinofuranosidase Required for Pectic Arabinan Modification in Arabidopsis Mucilage Secretory Cells1[C][W][OA]

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Following pollination, the epidermal cells of the Arabidopsis (Arabidopsis thaliana) ovule undergo a complex differentiation process that includes the synthesis and polar secretion of pectinaceous mucilage followed by the production of a secondary cell wall. Wetting of mature seeds leads to the rapid bursting of these mucilage secretory cells to release a hydrophilic gel that surrounds the seed and is believed to aid in seed hydration and germination. A novel mutant is identified where mucilage release is both patchy and slow and whose seeds display delayed germination. While developmental analysis of mutant seeds reveals no change in mucilage secretory cell morphology, changes in monosaccharide quantities are detected, suggesting the mucilage release defect results from altered mucilage composition. Plasmid rescue and cloning of the mutant locus revealed a T-DNA insertion in AtBXL1, which encodes a putative bifunctional β-d-xylosidase/α-L-arabinofuranosidase that has been implicated as a β-d-xylosidase acting during vascular development. Chemical and immunological analyses of mucilage extracted from bxl1 mutant seeds and antibody staining of developing seed coats reveal an increase in (1→5)-linked arabinans, suggesting that BXL1 is acting as an α-L-arabinofuranosidase in the seed coat. This implication is supported by the ability to rescue mucilage release through treatment of bxl1 seeds with exogenous α-L-arabinofuranosidases. Together, these results suggest that trimming of rhamnogalacturonan I arabinan side chains is required for correct mucilage release and reveal a new role for BXL1 as an α-L-arabinofuranosidase acting in seed coat development.

Differentiation of ovule integuments after pollination establishes a number of specialized cell types, including, in some species, the creation of a mucilaginous seed coat epidermis (myxospermy) (Esau, 1977; Fahn, 1982). Upon seed hydration, the epidermal cells of myxospermous seeds burst to release a hydrophilic, polysaccharide gel that has been suggested to play multiple roles, including promotion of seed hydration and prevention of desiccation during germination (Fahn, 1982). Mucilages are primarily composed of pectins, a complex, heterogeneous set of acidic polysaccharides that also compose the matrix of dicot primary cell walls (Fahn, 1979; Grubert, 1981; McCann and Roberts, 1991; Carpita and Gibeaut, 1993). The pectin rhamnogalacturonan I (RG I) is predominant in a number of seed mucilages (Naran et al., 2008) and is comprised of a backbone of alternating (1→2)-α-L-Rha and (1→4)-β-d-GalA. This backbone can be substituted with at least three different types of side chains on the Rha residues: arabinans, galactans, and type I arabinogalactans. Arabinans consist of (1→5)-α-L-Ara with occasional (1→3)- and (1→2)-α-L-Ara branch points, galactans are unbranched chains of (1→4)-β-d-Gal, and type I arabinogalactans are (1→4)-β-d-galactans that can be decorated with terminal Ara residues (Ridley et al., 2001; Willats et al., 2001a; Mohnen, 2008).

The Arabidopsis (Arabidopsis thaliana) seed coat mucilage secretory cells (MSCs) undergo a complex differentiation process, including mucilage synthesis and secondary cell wall production, that makes them an excellent model for understanding the developmental regulation of cell wall polysaccharide synthesis (Haughn and Chaudhury, 2005; Western, 2006). Following pollination, these cells undergo a phase of...
growth mediated by vacuolar expansion, which is succeeded by the biosynthesis and secretion of large quantities of pectinaceous mucilage to the upper tangential corners of the cell. This targeted secretion leads to the establishment of a volcano-shaped cytoplasmic stream of TTG1 that is specifically up-regulated by the epidermal cell differentiation factors APETALA2 (AP2), GLABRA2 (GL2), MYB5, and MYB61, all of which encode transcription factors and play roles in multiple developmental processes beyond seed coat differentiation (Koornneef, 1981; Jofuku et al., 1994; Rerie et al., 1994; Penfield et al., 2001; Johnson et al., 2002; Zhang et al., 2003; Gonzales et al., 2009; Li et al., 2009). A screen for mutants affected in mucilage extrusion led to the identification of the MUCILAGE-MODIFIED genes (MUM1 to MUM5; Western et al., 2001). mum4 mutants make a reduced amount of mucilage, mum3 and mum5 appear to be affected in mucilage composition, while mum1 and mum2 mutants are defective in mucilage release upon seed hydration. MUM4 encodes a UDP-1-Rha synthase (RHAMNOSE SYNTHASE2 [RH2]), an enzyme required for the synthesis of RG I, the primary pectin found in Arabidopsis seed mucilage (Usadel et al., 2004; Western et al., 2004; Oka et al., 2007). Analysis of MUM4/RH2 transcripts in mutants of the MSC-related transcription factors revealed that MUM4 is specifically up-regulated by GL2, which works downstream of TTG1 and AP2 (Western et al., 2004). In contrast, MUM2 encodes a β-galactosidase that modifies mucilage RG I side chains to allow correct hydration properties (Dean et al., 2007; Macquet et al., 2007b). Recently, a subtilisin-like Ser protease, AtSBT1.7, also was found to affect mucilage release, possibly through a role in the regulation of cell wall modification enzymes acting in MSCs (Rautengarten et al., 2008).

In this article, we describe a novel MSC mutant named *patchy* that demonstrates a slow and stochastic mucilage release. Our results reveal that *patchy* mutants are defective in the bifunctional β-D-xylosidase/α-L-arabinofuranosidase BXL1 and have an increased proportion of (1→5)-α-L-arabinan in both their extracted mucilage and seed coat cell walls. These data suggest that BXL1 acts as an α-L-arabinofuranosidase in differentiating MSCs, and such modification of the pectin structure is required for primary cell wall disruption and mucilage release.

### RESULTS

#### patchy Mutants Exhibit Patchy and Delayed Mucilage Release

To identify genes required for the synthesis and extrusion of seed coat mucilage, pools of T-DNA insertion lines (Feldmann, 1991) were screened for the presence of mucilage when hydrated by staining with the pectin dye ruthenium red. With this treatment, wild-type seeds are surrounded by a thick, pink-staining capsule of mucilage (Fig. 1A). One novel mutant identified with this screen demonstrated a patchy mucilage release phenotype, in that mucilage release occurred only from random patches of seed coat epidermal cells, rather than from all cells as in wild-type seeds (Fig. 1, A versus B). Backcrosses revealed this phenotype is due to a mutation at a single locus (223 wild type: 84 mutant; $\chi^2 = 0.9131; P > 0.1$, degrees of freedom [df] = 1), which we named *PATCHY* to reflect the mucilage release phenotype.

To quantify more precisely the differences in extruded mucilage between wild-type and *patchy* mutants, the amount of mucilage release was determined. In analyzed samples of seeds, 88.0% to 95.7% of wild-type seeds were completely surrounded by mucilage, while only 2.7% to 17.3% of *patchy* seeds had complete mucilage envelopes (Table I). Furthermore, when a particular line was stained in ruthenium red without agitation and prehydration in water, it was found that the degree of mucilage release dropped sharply, suggesting that mechanical agitation can aid release for these mutants (Table I). Timing of mucilage release was determined by filming seed hydration in the presence of ruthenium red dye (Fig. 2). With wild-type seeds, bulging of cells is first seen within a few seconds, following which a diffuse cloudy layer forms around the seed that intensifies in staining over time. A second, denser layer of mucilage close to the seed becomes stained later (Fig. 2A; Western et al., 2000). Wild-type seeds consistently released their mucilage in under 1 min ($n = 8$ seeds, all of which released), while those *patchy* seeds that released any mucilage could take from 3.5 to 95 min, with an average release time of 26.3 min ($SE = 5.6$ min; $n = 22$ seeds that released out of 83 total seeds; Fig. 2B).
**patchy** Mutants Appear to Undergo Normal Seed Coat Development But Have an Altered Mucilage Composition

Seeds making a severely reduced quantity of mucilage (e.g. _mum4/rhm2_ mutants) demonstrate morphological changes at the cellular level as well as being defective in mucilage release (Western et al., 2001, 2004). Specifically, when observed with scanning electron microscopy, the columella found in the center of the MSCs is reduced in prominence in comparison to wild-type columellae (Supplemental Fig. S1, A versus B). This change in columella shape is accompanied by reduced intensity of mucilage staining with toluidine blue and smaller mucilage pockets. Sectioning and toluidine blue staining of developing _patchy_ mutant seeds, however, shows similar mucilage staining, mucilage pocket size, and columella shape compared with the wild type (Fig. 1, E–L). This resemblance to the wild type is also observed when scanning electron microscopy is used to visualize the cell surface details (Supplemental Fig. S1, C versus D).

A threshold quantity of mucilage could be present in _patchy_ mutants, making them look morphologically normal, but interfering with mucilage release in the stochastic manner observed. Alternately, mucilage hydration properties could be affected as in _mum2_ mutants (Dean et al., 2007; Macquet et al., 2007b). Mucilage release can be induced in some reduced mucilage mutants by treatment with the heavy metal chelators, such as EDTA. Chelator treatment is believed to reduce binding of pectin chains through calcium bridges, allowing more extensive hydration and swelling of mucilage and/or weakening of the primary cell wall. When _patchy_ seeds are treated with EDTA, and their mucilage levels compared to wild-type seeds, no gross difference was detected between the thickness of wild-type and _patchy_ mucilage enveloping the seeds (compare Fig. 1, A and B with C and D).

To confirm this result, the mild chelator ammonium oxalate was used for extraction of mucilage, and the crude extracts were directly hydrolyzed and derivatized to alditol acetates (Table II). A slight but non-significant drop was observed both in the quantity of Rha and in total sugars. This was reflected in a small but significant decrease in both Fuc and Xyl. In addition, the amount of Ara increased approximately 1.5-fold (P value < 0.005) in _patchy_ mucilage. Following mucilage extraction, the remaining seeds were also analyzed, but no significant differences in monosaccharide levels were observed (Table II). These results suggest that there is a change in mucilage composition that may be responsible for the slow and _patchy_ mucilage release observed in the _patchy_ mutants.

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**Figure 1.** Ruthenium red staining and seed coat structure of _bxl1-1_ versus wild-type Ws seeds. A and B, Wild-type (A) and _bxl1-1_ (B) seeds shaken in water and then stained with ruthenium red. Note patchy mucilage staining around _bxl1-1_ seeds. C and D, Wild-type (C) and _bxl1-1_ (D) seeds stained with ruthenium red after shaking in 0.05 M EDTA. E to L, Cross sections of developing seed coat epidermal cells stained with toluidine blue. E to H, Wild type. E, 4 DPA with central vacuole filling most of cell. F, 7 DPA, purple-staining mucilage is accumulating. G, 10 DPA, dark purple-staining mucilage found in upper tangential regions of the cell, above the blue-staining secondary cell wall forming around the cytoplasm. H, 13 DPA, secondary cell wall (blue) has filled in the central region of the cell. I to L, _bxl1-1_ mutant sections, note their similarity at each stage to the wild type. I, 4 DPA; J, 7 DPA; K, 10 DPA; L, 13 DPA. Bars = 200 μm in A to D and 50 μm in E to L.
**patchy Seeds Show Delayed Germination in the Absence of Prehydration**

General observation of growing **patchy** mutant plants revealed no significant vegetative differences compared to wild-type plants. A time course of seed germination revealed a 1- to 2-d delay of germination such that there is a roughly 40% reduction in germination in **patchy** mutants versus wild-type seeds at 3 d after plating, which becomes only 10% after 4 d (Fig. 3). However, prehydration of **patchy** seeds by shaking for 90 min in either water or EDTA restores germination to wild-type rates (Fig. 3). These data suggest that the changes in the mucilage of **patchy** mutants affect germination, presumably due to the reduced ability for **patchy** seeds to attract or hold water around the seed.

**patchy** Mutants Have Mutations in the Gene Encoding the Bifunctional β-α-L-Arabinofuranosidase BXL1

The **patchy** mutant was isolated from a pool of T-DNA insertional mutants (CS2497; Feldmann, 1991). Kanamycin resistance encoded by the T-DNA was found to segregate with the mutant phenotype. In the F2 of a backcross to Wassilewskija (Ws), kanamycin-resistant segregated with a 3:1 ratio (161 resistant:62 sensitive; $\chi^2 = 0.9188; P > 0.1, df = 1$). Of the kanamycin-resistant plants, one-third had a **patchy** mucilage phenotype (115 wild type:48 **patchy**; $\chi^2 = 1.1203; P > 0.1, df = 1$), and 15 of these **patchy** mutants chosen at random gave rise to 100% kanamycin-resistant progeny. Southern-blot analysis with multiple T-DNA probes confirmed that the **patchy** mutant resulted from an insertion in an intron (Fig. 4A), while pGREEN0229-transformed plants retained the **patchy** mucilage phenotype (11/11) (Fig. 5, A and B; Supplemental Table S1). These results are consistent with the hypothesis that an insertion in *AtBXL1* is responsible for the seed coat phenotype.

Since the **patchy** mutant (renamed **bxl1-1**) has a T-DNA insertion in an intron (Fig. 4A), we examined the transcript level of *AtBXL1* in **bxl1-1** mutants using reverse transcription (RT)-PCR. In leaves, where *AtBXL1* is highly expressed, a strong band is found in wild-type Ws leaves, while a barely detectable band is seen for **bxl1-1** mutants at a saturating cycle number (Fig. 4B). Real-time PCR on 7 DPA seeds suggests that there is an approximately 1,000-fold decrease in transcript amount in **bxl1-1** mutants (Ws threshold cycle $[C_t] = 21.8 \pm 0.1$ se, $n = 3$; **bxl1-1** $C_t = 32.1 \pm 0.2$ se, $n = 3$). Two further T-DNA insertion lines in At5g49360 were obtained from the Salk Sequence Indexed Insertion collection (Alonso et al., 2003), which we named **bxl1-2** (Salk_012090) and **bxl1-3** (Salk_054483). **bxl1-2** has an insertion in exon 5, while the insertion in **bxl1-3** is in intron 5 (Fig. 4A). Both **bxl1-2** and **bxl1-3** have similar **patchy** mucilage release and germination phenotypes to **bxl1-1** and do not complement **bxl1-1** in genetic crosses, confirming they are insertions in the same gene (Supplemental Table S1; data not shown).

**Table 1. Quantification of mucilage release of three independent lines of bxl1-1**

Seeds were shaken in water for 90 min followed by 60 min of shaking in 0.01% ruthenium red, except where noted. The proportion of an individual seed’s circumference surrounded by mucilage was then quantified as 100% (seed completely surrounded by mucilage), 75% (3/4 of seed circumference surrounded), 50%, 25%, or no mucilage visible. Results are the percentage of seeds per sample with a particular mucilage category.

| Line            | 100% | 75% | 50% | 25% | No Mucilage | Total No. of Seeds |
|-----------------|------|-----|-----|-----|-------------|--------------------|
| Ws #1           | 88.0 | 2.2 | 3.3 | 3.8 | 2.7         | 184                |
| Ws #2           | 95.7 | 0   | 2.9 | 1.4 | 0           | 70                 |
| **bxl1-1** line #1 | 2.7  | 6.1 | 43.5| 38.8| 8.8         | 147                |
| **bxl1-1** line #2 | 3.8  | 12.8| 16.0| 40.4| 26.9        | 156                |
| **bxl1-1** line #3 | 17.3 | 21.0| 29.6| 21.6| 10.5        | 162                |
| **bxl1-1** line #2* | 3.6  | 0   | 7.2 | 15.7| 73.5        | 83                 |

*Seeds placed directly in ruthenium red with no pretreatment or shaking and monitored for 90 min (sample used for time lapse in Fig. 2).
lies upstream of the predicted catalytic Glu found in glycosyl hydrolase family three (GH3) enzymes (Minic et al., 2004), this insertion would be expected to render the protein nonfunctional. Because bxl1-1 approaches a transcriptional null, all further analyses were performed on this allele.

**AtBXL1 Is Transcribed throughout the Plant, Including in Differentiating Seed**

RT-PCR was used for an initial determination of the transcription of AtBXL1 throughout various tissues as well as in siliques before, during, and after the time of seed mucilage production (4, 7, and 10 DPA, respectively). AtBXL1 transcripts were found in each of these tissues (Fig. 4B). Promoter-GUS experiments have shown that AtBXL1 is expressed in the vasculature of the siliques (Goujon et al., 2003); thus, we compared the transcription of AtBXL1 in developing seeds versus siliques using real-time PCR (Fig. 4C). AtBXL1 was found to be strongly expressed in both seeds and siliques at 7 and 10 DPA, with higher transcript levels (lower Ct) for siliques versus seeds. Separation of seeds from siliques was not done for 4 DPA due to the difficulty of removing seeds at this stage.

**bxl1 Mutants Have an Increase in the Proportion of Ara and Arabinan-Type Ara Linkages in Their Seed Coat Mucilage and Have Decreased α-L-Arabinofuranosidase Activity in Their Siliques**

Since AtBXL1 encodes a putative β-D-xylosidase/α-L-arabinofuranosidase, the mucilage composition was investigated more closely. Mucilage was sequentially extracted from seeds using the mild chelator ammonium oxalate, 0.2 and 2N sodium hydroxide, to create fractions consisting of loosely attached pectins, more strongly linked pectins, and strongly linked pectins and cross-linking glycans (hemicelluloses), respectively. These extracts were extensively dialyzed to remove monosaccharide and chemical contaminants and analyzed for monosaccharide composition through carbodiimide activation and reduction with sodium borodeuteride followed by hydrolysis and derivatization to alditol acetates to allow for detection of both neutral sugars and uronic acids (Kim and Carpita, 1992; Carpita and McCann, 1996). For all three extracts, the primary sugars were Rha and GalA, reflecting isolation of mucilage that is largely composed of RG I (Table III; Penfield et al., 2001; Western et al., 2004; Naran et al., 2008). A decrease in the proportion of these two sugars, however, was seen in fractions from the harsher extractions, indicating the extraction of increased levels of RG I containing arabinan and galactan side chains as well as cellulose and other complex polysaccharides observed in the inner adherent layer of mucilage (Macquet et al., 2007a). Comparison between the extracts for the wild type and bxl1-1 revealed increases in Ara in both ammonium oxalate and 0.2 N sodium hydroxide frac-

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Figure 2. Time lapse of mucilage release for bxl1-1 versus wild-type Ws seeds. Seeds were placed in ruthenium red solution and photographed over 90 min. A. Wild-type seed releases mucilage within 20 s and then mucilage stains pink over time. B. bxl1-1 seed shows no mucilage release until 83 min, at which time mucilage is observed only for a patch of cells. Bars = 100 μm. [See online article for color version of this figure.]
tions, with concurrent decreases in these same extracts for Xyl. The statistical significance of the ammonium oxalate Ara increase (P value = 0.002) is consistent with our earlier ammonium oxalate extracts without carboxyl reduction. Slight changes were also observed for Glc in 0.2N sodium hydroxide (decrease), and Xyl, Glc (increase), Man, and Gal (decrease) in 2N sodium hydroxide extracts.

To determine if more subtle changes in the chemical structure of the mucilage occur in bxl1-1 extracts, analysis of the sugar linkages present was performed through per-O-methylation (Carpita and Shea, 1989; Gibeaut and Carpita, 1991) for all three extractions (Table IV). Similar to the monosaccharide analysis, few changes are observed between the wild type and bxl1-1 across the extracts. The only difference that is consistently seen across all three extractions is an increase in the ratio of (1\(\rightarrow\)5)-Ara (5-Ara\(p\)) accompanied by an increase in branch-point Rha residues (2,4-Rha\(p\)) and a decrease in 2,3-Rha\(p\). The level of (1\(\rightarrow\)4)-Man is also slightly decreased in this extraction. A similar slight change to branching of type II arabinogalactans is suggested in the 0.2N sodium hydroxide extraction where there is a decrease in (1\(\rightarrow\)6)-Gal and increase in branch-point Gal (3,6-Galp).

To confirm that the changes in Ara and arabinan levels found in extracted bxl1-1 mucilage resulted from reduced \(\alpha\)-L-arabinofuranosidase activity in bxl1-1 mutants, enzyme assays were undertaken. Protein was extracted from developing wild-type and bxl1-1 seedlings. Fourier transform infrared spectroscopy and monosaccharide analysis were performed. While the Fourier transform infrared results suggested the possibility of a slight change in pectin esterification (data not shown), no statistically significant differences were seen in the monosaccharide composition (Supplemental Table S2), consistent with the results of Goujon et al. (2003) for stems of antisense \(BXL1\) plants.

![Figure 3. Germination of bxl1-1 versus Ws (WS) wild-type seeds.](image)

Germination of seeds placed on minimal medium agar plates and cold treated for 72 h. Seeds were either plated directly as dry seeds (no trtmt) or prehydrated by shaking for 90 min in water (H2O) or 0.05 mM EDTA as indicated, followed by suspension in 0.01% agarose and plating on minimal medium. Samples were done in triplicate with 50 to 60 seeds per sample. Error bars represent se.

### Table II. Monosaccharide quantitation of bxl1-1 versus wild-type seeds

Soluble polysaccharides from intact seeds were isolated by shaking in ammonium oxalate (soluble mucilage), followed by grinding of the same seeds and further ammonium oxalate extraction (seed minus soluble mucilage). Samples were then ethanol precipitated and directly hydrolyzed with trifluoroacetic acid followed by derivatization to alditol acetates. Results are given as average mg sugar per 100 mg seed and se calculated from three independent samples.

| Sugar   | Soluble Mucilage Ws | bxl1-1 | Seed Minus Soluble Mucilage Ws | bxl1-1 |
|---------|---------------------|--------|-------------------------------|--------|
| Rha     | 528 ± 23.6          | 486 ± 6.8 | 1,090 ± 85.7                  | 937 ± 11.2 |
| Fuc     | 5.1 ± 0.1           | 4.0 ± 0.1\(^a\) | 74.9 ± 2.4                  | 72.8 ± 4.8 |
| Ara     | 11.6 ± 0.7          | 17.5 ± 0.6\(^b\) | 1,610 ± 87.2                 | 1,720 ± 8.5 |
| Xyl     | 64.4 ± 2.7          | 53.7 ± 0.6\(^b\) | 578 ± 35.4                  | 619 ± 4.7 |
| Man     | 10.7 ± 0.6          | 10.4 ± 0.0               | 141 ± 9.9                   | 137 ± 2.2 |
| Gal     | 24.7 ± 1.9          | 25.1 ± 0.3               | 1,030 ± 60.0                | 985 ± 20.2 |
| Glc     | 15.3 ± 1.7          | 13.8 ± 0.6               | 1,540 ± 129                 | 1,370 ± 12.9 |
| Total   | 660 ± 31.2          | 611 ± 8.6                | 6,050 ± 408                 | 5,840 ± 50.3 |

\(^a\)Significantly different from Ws, P value of <0.005. \(^b\)Significantly different from Ws, P value of <0.05.
Liques, and fractions were collected after separation on a cation exchange column, following which α-L-arabinofuranosidase activity was assayed using p-nitrophenyl (PNP)-α-L-Araf. Two peaks of α-L-arabinofuranosidase activity were detected, one of which was strongly reduced in the bxl1-1 mutant, confirming that bxl1-1 mutants have decreased α-L-arabinofuranosidase activity (Fig. 4D). An increase in the second peak was also observed in the bxl1-1 mutant, suggesting possible compensation by one of the other two α-L-arabinofuranosidases identified in Arabidopsis siliques (Minic et al., 2006).

**bxl1 Mutants Have an Increase in, and Altered Distribution of, RG I Arabinan Side Chains in Their Seed Coat Cell Walls**

To confirm changes existed in the levels of (1→5)-linked arabinans in bxl1-1 versus wild-type mucilage, immunoblots with extracted mucilage were performed using the arabinan-specific antibody LM6 (Willats et al., 1998, 2001b; Supplemental Fig. S2). The CCRC-M36 antibody specific to unbranched RG I and raised to Arabidopsis mucilage was used in parallel as a control (Young et al., 2008). Strong binding of LM6 was observed for EDTA extracts of bxl1-1 mucilage, while staining was only faintly visible for wild-type samples (Supplemental Fig. S2A). Control immunoblots performed with CCRC-M36 showed roughly equal staining in the wild type and bxl1-1, suggesting a significant increase in (1→5)-linked arabinans in bxl1-1 mucilage (Supplemental Fig. S2A).

Staining of developing seed coats with LM6 and CCRC-M36 was also performed. In wild-type seed coats, only faint staining was detected with LM6. The only significant staining of the MSCs was at the cell junctions on the lower face of the cells (Fig. 6A). By contrast, bxl1-1 seed coats had very intense staining of all cell walls, including all cell walls of the MSCs (Fig. 6B), suggesting both a general increase in arabinans in bxl1-1 seed coats and a specific increase in arabinans in the radial and outer cell walls of the MSCs. Similar to the immunoblot results, no significant differences were detected with CCRC-M36 staining (Fig. 6, C and D). Using whole seed immunofluorescence of mature seeds, the increase in cell wall LM6 staining was reflected in both the intensity of LM6 stain surrounding bxl1-1 mutant seeds and the presence of clearly identifiable small sections of intact, hexagonal primary cell walls (Supplemental Fig. S2, D and E). These results suggest that the increase in LM6 epitopes within the wall may alter the mode of primary cell wall breakage during mucilage release in bxl1 mutants.
The bxl1 Mucilage Release Defect Can Be Rescued by Treatment with Exogenous \( \alpha \)-L-Arabinofuranosidases

To test the potential role for an \( \alpha \)-L-arabinofuranosidase in mucilage release, seeds were treated with recombinant enzymes obtained from an established collection of fungal polysaccharide degrading enzymes expressed in a secreted, affinity-tagged form in \textit{Pichia pastoris} (Bauer et al., 2006). \( bxl1-1 \) seeds were treated with three affinity-purified recombinant arabinofuranosidases (AN1571, AN7908, and AN80401; Bauer et al., 2006). Treatment with all three arabinofuranosidases led to rescue of the patchy mucilage release phenotype (Fig. 5, D and E), while treatment with enzyme buffer and a \( \beta \)-D-xylosidase (AN2359; Bauer et al., 2006) had no effect on mucilage release (Fig. 5, C and F). These rescue results suggest that removal of arabinans can promote mucilage release through modification of MSC walls and/or mucilage of \( bxl1-1 \) mutants.

DISCUSSION

Hydration of Arabidopsis seeds leads to the breakage of the outer primary cell wall of the MSCs and the release of pectinaceous mucilage to surround the seed (Western et al., 2000; Windsor et al., 2000). Our data reveal that the \( \beta \)-D-xylosidase/\( \alpha \)-L-arabinofuranosidase BXL1 may play a role in mucilage release through the degradation of (1\( \rightarrow \)5)-linked arabinans in the mucilage and/or primary cell wall. These results suggest a requirement for the trimming of RG I side chains to allow proper swelling of the mucilage and/or weakening of the primary cell wall to enable mu-
cilage release. The requirement for developmentally coordinated changes to arabinan and RG I side chain numbers and branching is an emerging theme in the regulation of cell wall properties during plant growth and reproduction (Willats et al., 1999; Fulton and Cobbett, 2003; Lee et al., 2003, 2008; Leboeuf et al., 2003; Minic et al., 2006; Xiong et al., 2007; Chávez Montes et al., 2008).

**Mucilage Release Requires Modification of RG I Side Chains**

*bxl1* mutant slow and patchy mucilage release is correlated with a change in mucilage composition. These compositional changes appear to result in altered mucilage hydration properties, as water absorption tests of extracted mucilage suggest the speed of hydration and absorption capacity of *bxl1* mucilage are lower than those of wild-type mucilage (Supplemental Fig. S3). Chemical analysis and immunoblot results for *bxl1* mucilage revealed an increase in Ara and (1→5)-linked arabinans over wild-type mucilage (Tables III and IV; Supplemental Fig. S2), suggesting that chemical modifications to the mucilage in vivo may be required for sufficient mucilage swelling and release. The retention of a similar number of branchpoint 2,3- and 2,4-Rha residues between *bxl1* and wild-type mucilage suggest that trimming of (1→5)-arabinans is occurring rather than the complete removal of the arabinan side chains from RG I polymers.

While chemical changes are observed in the extracted mucilage, a role for BXL1 in weakening of the outer primary cell wall to allow mucilage release is also supported. Mucilage release can be rescued through external treatment of *bxl1* seeds with exogenous α-1,5-arabinofuranosidases (Fig. 5, D and E), suggesting that more or larger arabinans are found in the primary cell wall in *bxl1* seeds. This is consistent with the intense staining of the cell walls of developing *bxl1* seeds hybridized with the arabinan-specific antibody LM6 (Fig. 6B; Willats et al., 1998). Failure to trim arabinans in the MSC primary cell wall may lead to wall stiffening in *bxl1* mutants, resulting in increased difficulty of cell wall breakage and the patchy mucilage release.

The exact contribution of increased cell wall strength versus reduced or slowed mucilage swelling to the altered cell wall breakage pattern seen in *bxl1* mutants is unclear. In wild-type MSCs, rapid pectin swelling is proposed to lead to rupture at the thin, radial cell walls and upward folding of the outer cell wall remnants still attached at the columella (Western et al., 2000; Windsor et al., 2000). In *bxl1* mutants, either slow-building pressure due to altered mucilage and/or prolonged pressure buildup resulting from a stronger primary cell wall could be expected to lead to outward bulging of the cell wall rather than immediate rupture. This continued bulging could eventually result in lifting of the whole surface cell wall upwards, breaking not only at the edges of the cell, but also severing connections to the columella, resulting in the hexagonal cell wall fragments observed in *bxl1* mutants (Supplemental Fig. S2).

**Mucilage Release Requires the Activity of Multiple Genes**

In addition to *AtBXL1*, several other genes have been demonstrated to play roles in mucilage release: *MUM1*, *MUM2*, and *AtSBT1.7* (Western et al., 2001; Dean et al., 2007; Rautengarten et al., 2008). While *MUM1* is yet to be cloned, the others appear to be involved, directly or indirectly, in structural modifications of the mucilage and/or outer cell wall that appear to be necessary for proper mucilage hydration and release. Similar to *AtBXL1*, *MUM2* encodes a glycosyl hydrolase, specifically a β-galactosidase, that is believed to be involved in the degradation of RG I side chains during MSC differentiation to allow the proper swelling and release of mucilage (Dean et al., 2007; Macquet et al., 2007b). *mum2* mucilage shows abnormally high levels of arabinans as well as terminal

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**Table III. Monosaccharide composition of mucilage extracted from *bxl1*-1 versus wild-type seeds**

Intact seeds were extracted sequentially with 0.2% ammonium oxalate, 0.2 N and 2 N NaOH, followed by carbodiimide reduction, trifluoroacetic acid hydrolysis, and alditol acetate derivatization. Results are given as average mole percentage and SE calculated from five independent samples.

| Sugar | Ammonium Oxalate | 0.2 N NaOH | 2 N NaOH |
|-------|------------------|------------|----------|
|       | Ws               | *bxl1*-1   | Ws       | *bxl1*-1   | Ws       | *bxl1*-1   |
| Rha   | 49.5 ± 0.4       | 49.7 ± 0.6 | 43.6 ± 2.4 | 44.2 ± 2.1 | 36.6 ± 1.8 | 37.1 ± 3.1 |
| Fuc   | tr²              | tr         | tr       | tr         | tr       | tr       |
| Ara   | 0.7 ± 0.1        | 1.3 ± 0.1  | 2.6 ± 0.8 | 4.2 ± 0.7  | 9.3 ± 2.3 | 9.1 ± 1.0 |
| Xyl   | 3.7 ± 0.2        | 3.3 ± 0.3  | 6.7 ± 2.0 | 5.5 ± 1.1  | 8.2 ± 2.4 | 9.4 ± 1.0 |
| Man   | 1.0 ± 0.1        | 1.2 ± 0.1  | 2.2 ± 0.6 | 1.8 ± 0.3  | 3.2 ± 1.1 | 2.4 ± 0.9 |
| Gal   | 1.8 ± 0.1        | 1.9 ± 0.1  | 2.9 ± 0.9 | 2.3 ± 0.3  | 5.1 ± 0.8 | 4.2 ± 0.7 |
| Glc   | 2.3 ± 0.5        | 2.1 ± 0.3  | 4.8 ± 1.1 | 3.9 ± 1.1  | 9.4 ± 2.3 | 10.1 ± 6.3 |
| GalA  | 40.9 ± 0.2       | 40.4 ± 0.5 | 37.2 ± 1.2 | 38.1 ± 1.2 | 28.2 ± 3.1 | 27.7 ± 3.3 |

²tr, Trace. ³Significantly different from Ws, *P* value of 0.002.

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Gal residues and type 2 arabinogalactans (Dean et al., 2007; Macquet et al., 2007b), suggesting that MUM2 β-galactosidase activity may be required for activity of BXL1, possibly to allow access of BXL1 to its substrate. A requirement for the concerted activity of two or more glycosyl hydrolases for proper degradation of polysaccharides has been suggested previously for many enzymes, including other bifunctional β-D-xylosidases/α-L-arabinofuranosidases (Minic and Jouanin, 2006; Xiong et al., 2007; Minic, 2008).

Unlike the glycosyl hydrolases produced by AtBXL1 and MUM2, the subtilisin-like Ser protease encoded by AtSBT1.7 appears to work indirectly on primary cell wall and/or mucilage structure. sbt1.7 mutants lack mucilage release when hydrated with water; however, treatment with EDTA leads to mu-

### Table IV. Linkage analysis of extracted bxl1-1 versus wild-type mucilage

| Linkage | Ammonium Oxalate | 0.2 N NaOH | 2 N NaOH |
|---------|------------------|------------|----------|
|         | Ws | bxl1-1 | Ws | bxl1-1 | Ws | bxl1-1 |
| Fuc     |     |       |     |       |     |       |
| Rha     |     |       |     |       |     |       |
| t-Fucp  | tr  | tr    | tr  | tr    | tr  | tr    |
| t-Rhap  | 0.4 ± 0.2 | 0.2 ± 0.2 | 0.5 ± 0.3 | 0.3 ± 0.2 | 0.3 ± 0.2 | 2.0 ± 0.55 |
| 2-Rhap  | 48.9 ± 0.6 | 48.6 ± 0.5 | 42.9 ± 0.0 | 42.5 ± 1.4 | 35.6 ± 3.7 | 24.5 ± 5.5 |
| 2,3-Rhap | 0.4 ± 0.1 | 0.5 ± 0.2 | 0.4 ± 0.1 | 0.5 ± 0.5 | 0.4 ± 0.3 | nd |
| 2,4-Rhap | 1.0 ± 0.2 | 1.0 ± 0.1 | 1.4 ± 0.0 | 1.8 ± 0.5 | 0.7 ± 0.9 | nd |
| Ara     |     |       |     |       |     |       |
| t-Araf  | 0.1 ± 0.0 | 0.2 ± 0.0 | 1.2 ± 1.8 | 1.0 ± 0.6 | 1.8 ± 4.3 | 1.3 ± 1.8 |
| 2-Araf  | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.1 |
| 3-Araf  | tr     | 0.1 ± 0.0 | 1.3 ± 0.0 | 0.7 ± 0.0 | 0.2 ± 0.1 | 0.4 ± 0.3 |
| 5-Araf  | 0.2 ± 0.0 | 0.9 ± 0.0 | 1.5 ± 0.1 | 2.4 ± 0.2 | 0.6 ± 0.7 | 3.0 ± 0.8 |
| 2,5-Araf | tr  | 0.2 ± 0.1 | 1.0 ± 2.2 | 0.5 ± 0.5 | 0.7 ± 0.9 | 0.9 ± 1.8 |
| 3,5-Araf | nd   | 0.1 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.0 | nd   | nd   |
| Xyl     |     |       |     |       |     |       |
| t-Xylp  | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.5 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 2-Xylp  | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.6 ± 0.0 | 0.4 ± 0.0 |
| 4-Xylp  | 1.9 ± 0.0 | 1.0 ± 0.0 | 1.4 ± 0.0 | 1.6 ± 0.0 | 5.2 ± 0.7 | 3.7 ± 0.2 |
| 2,4-Xylp | 0.7 ± 0.0 | 0.6 ± 0.0 | 0.6 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.3 | 1.2 ± 0.1 |
| 3,4-Xylp | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.1 | tr     | 0.2 ± 0.0 | tr     |
| Man     |     |       |     |       |     |       |
| t-Manp  | nd   | nd    | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.0 |
| 4-Manp  | 0.4 ± 0.0 | 0.5 ± 0.0 | 0.9 ± 0.0 | 1.0 ± 0.0 | 6.4 ± 0.5 | 4.6 ± 0.6 |
| 4,6-Manp | 0.5 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.0 | 0.3 ± 0.2 | 0.6 ± 0.0 |
| Gal     |     |       |     |       |     |       |
| t-Galp  | 1.1 ± 0.0 | 0.9 ± 0.0 | 1.0 ± 0.3 | 0.6 ± 0.0 | 0.8 ± 1.4 | 0.9 ± 0.0 |
| 2-Galp  | nd    | nd    | 0.3 ± 0.2 | 0.3 ± 0.2 | nd   | nd   |
| 3-Galp  | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.1 ± 0.0 | 0.7 ± 1.0 | 1.6 ± 0.3 |
| 4-Galp  | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 4.1 ± 28.6 | 0.4 ± 0.0 |
| 6-Galp  | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.8 ± 0.0 | 0.5 ± 0.0 | 0.9 ± 1.8 | nd   |
| 3,4-Galp | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.8 ± 1.4 |
| 3,6-Galp | 0.2 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.4 | 1.8 ± 0.0 | 1.2 ± 2.7 | 2.8 ± 0.3 |
| G1c     |     |       |     |       |     |       |
| t-Glcp  | 0.1 ± 0.0 | 0.1 ± 0.0 | 2.5 ± 2.1 | 3.2 ± 2.6 | 3.2 ± 1.0 | 15.5 ± 63.0 |
| 3-Glcp  | nd    | nd    | nd    | 0.1 ± 0.0 | 0.1 ± 0.0 | nd   |
| 4-Glcp  | 0.4 ± 0.3 | 0.8 ± 0.8 | 3.3 ± 0.8 | 3.4 ± 0.8 | 11.4 ± 0.2 | 14.8 ± 30.8 |
| 6-Glcp  | nd    | nd    | 0.2 ± 0.1 | 0.2 ± 0.0 | 0.6 ± 0.7 | nd   |
| 2,4-Glcp | 0.2 ± 0.1 | 0.1 ± 0.1 | nd    | nd    | nd    | nd   |
| 3,4-Glcp | tr   | tr    | 0.4 ± 0.0 | 0.4 ± 0.1 | 0.5 ± 0.5 | 1.7 ± 1.4 |
| 4,6-Glcp | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.0 | 1.3 ± 0.0 | 2.1 ± 1.5 |
| GalA    |     |       |     |       |     |       |
| t-GalAp | 0.6 ± 0.1 | 0.5 ± 0.0 | 0.5 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.2 | 0.8 ± 0.2 |
| 4-GalAp | 39.3 ± 0.3 | 39.2 ± 0.2 | 32.4 ± 1.1 | 31.8 ± 0.9 | 19.7 ± 1.3 | 13.5 ± 0.0 |
| 3,4-GalAp | 1.6 ± 0.0 | 1.6 ± 0.1 | 2.0 ± 0.9 | 1.8 ± 0.7 | 0.4 ± 0.4 | 0.4 ± 0.4 |
| G1cA    |     |       |     |       |     |       |
| t-GlCP  | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 | 0.5 ± 0.0 | 0.7 ± 0.0 | 0.9 ± 0.0 |

*tr, Trace. *nd, Not detected.
BXL1 Acts as a Bifunctional $\beta$-D-Xylosidase/\n$\alpha$-L-Arabinofuranosidase in Vivo and Plays Different
Roles in Different Tissues

$AtBXL1$ was identified by Goujon et al. (2003) as a gene encoding a $\beta$-xylosidase expressed in the vasculature, for which antisense plants with reduced $\beta$-xylosidase activity were found to have various growth defects, including short siliques and curled leaf edges. Our identification of an insertional mutant in $AtBXL1$ with significantly reduced transcript, however, revealed only the patchy release of seed coat mucilage and delayed germination. The lack of a reported mucilage defect for the antisense lines may be due either to the patchy nature of the phenotype or the poor expression of the 35S promoter in MSCs (Young et al., 2008). The difference in whole plant phenotypes between $bxl1$ and the $AtBXL1$ antisense lines may be due to the additional knockdown of $AtBXL2$ in the antisense plants. $AtBXL2$ is 70% identical to $AtBXL1$ at the nucleotide level, and its knockdown could not be ruled out by Goujon et al. (2003) using northern blots. RT-PCR of $AtBXL2$ reveals that it has a lower, but overlapping, transcription pattern in most tissues where $AtBXL1$ is expressed (leaves, stems, seedlings, roots, inflorescences, and 4-DPA siliques; Supplemental Fig. S4), and $BXL2$ has been copurified from stems with $BXL1$ in a proteomic analysis (Minic et al., 2007). Preliminary results suggest that $bxl1$ $bxl2$ double mutants have shortened siliques and curled leaf edges similar to that observed in the antisense lines (data not shown).

While Goujon et al. (2003) suggested that $BXL1$ was functioning as a $\beta$-D-xylosidase in the stems, our chemical, enzymatic, and immunological analyses of $bxl1$-1 mucilage and seed coat suggest that it is working as an $\alpha$-L-arabinofuranosidase in seed MSCs due to the accumulation of both Ara and arabinans in $bxl1$ mutants. The $\alpha$-L-arabinofuranosidase function correlates with the data of Minic et al. (2004), who isolated $BXL1$ enzyme (XYL1) from Arabidopsis stems and demonstrated its activity as a bifunctional $\beta$-D-xylosidase/$\alpha$-L-arabinofuranosidase with a substrate preference for sugar beet (Beta vulgaris) (1→5)-linked arabinan in in vitro enzyme assays. Taking both our data and those of Goujon et al. (2003), it appears that $BXL1$ performs two roles: that of a $\beta$-xylosidase and/or a bifunctional $\beta$-xylosidase/$\alpha$-L-arabinofuranosidase in the remodeling of xylans in vascular development and that of an $\alpha$-L-arabinofuranosidase in the cell wall of MSCs. $BXL1$ belongs to GH3, from which a number of enzymes have been characterized.
to have β-xylosidase (XYL4/[At]BXL4), α-1-L-arabinofuranosidase ([At]BXL3, PpARF2, and [Hv]ARA-I), or bifunctional β-xylosidase/α-1-L-arabinofuranosidase activities (MsXyl1, [Hv]XYL, and RsAraf1; Lee et al., 2003; Minic et al., 2004, 2006; Tateishi et al., 2005; Kotake et al., 2006; Xiong et al., 2007). The bifunctional enzymes tend to have a substrate preference for arabinans in vitro and to be expressed in developing tissues, suggesting roles in the modification of primary cell walls rather than acting on secondary cell wall xylans (Kotake et al., 2006; Xiong et al., 2007). The bifunctionality of these enzymes has been suggested to allow flexibility of cell wall modifications with a limited number of enzymes. The activity of BXL1 as a β-xylosidase in stems (Goujon et al., 2003) and as an α-1-L-arabinofuranosidase in MSCs is the first in vivo demonstration of a bifunctional cell wall enzyme playing different roles in different tissues.

Regulation of RG I and Arabinan Side Chain Structure in Plant Growth and Development

Cell walls are heterogeneous and dynamic structures that vary in composition throughout growth and development (Carpita and Gibeaut, 1993; Somerville et al., 2004; Farrokhi et al., 2006). Arabinan and galactan side chains show developmental, tissue, cell type, and within-cell wall specificity in their localization (Willats et al., 1999, 2001a; McCartney et al., 2000; Orfila et al., 2001; Ridley et al., 2001; McCartney and Knox, 2002; Verheertbruggen et al., 2009). Roles for arabinans and their modification during development have come from the localization of arabinan epitopes to meristematic and proliferating root cells in carrots (Daucus carota) as well as the transcript expression pattern of both GH3 and GH51 α-1-L-arabinofuranosidasases and bifunctional β-D-xylosidase/α-1-L-arabinofuranosidases in developing roots and stems (Willats et al., 1999; Fulton and Cobbett, 2003; Lee et al., 2003; Minic et al., 2006; Xiong et al., 2007; Chávez Montes et al., 2008). Modification of arabinan side chains, particularly debranching or trimming by α-1-L-arabinofuranosidases, also has been suggested in fruit ripening of Japanese pear (Pyrus serotina; Tateishi et al., 2005), storage of apples (Malus domestica; Pena and Carpita, 2004), pedicel abscission in poinsettia (Euphorbia pulcherrima; Lee et al., 2008), and in the growth of suspension-cultured microcalli (Leboeuf et al., 2004). These latter modifications have been correlated with loss of cell adhesion, while modulation of arabinan levels and branching during development may be associated with changes to cell wall elasticity as arabinans have been suggested to act as cell wall plasticizers and/or to form direct linkages between pectins and cellulose (Jones et al., 2003, 2005; Zykwinska et al., 2005, 2007; Moore et al., 2008).

The direct effects of a reduction in arabinan side chains have been observed both through the identification of Ara-deficient mutants in Nicotiana plumbaginifolia (nolac-H14) and Arabidopsis (arad1) and through ectopic expression of the family 51 α-1-arabinofuranosidase ARAF1 in Arabidopsis and a fungal endo-α-1,5-arabinanase in potato (Solanum tuberosum) tubers (Iwai et al., 2001; Skjøt et al., 2002; Harholt et al., 2006; Chávez Montes et al., 2008). While no obvious phenotypic effects were observed in Arabidopsis arad1 mutants, nolac-H14 mutants were identified through their reduced cell-cell adhesion, ARAF1 overexpression plants had delayed flowering time and altered stem architecture, and tissue from fungal arabinanase-expressing potatoes demonstrate altered wall stiffness (Iwai et al., 2001; Ulvskov et al., 2005; Harholt et al., 2006; Chávez Montes et al., 2008).
2006; Chávez Montes et al., 2008). Our results complement and extend these data by demonstrating that the loss of arabinan modification in a specific cell type can lead to observable consequences on cell and plant developmental behavior: namely, the lack of cell wall breakage to facilitate mucilage release and consequent delayed seed hydration and germination.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Lines of Arabidopsis (Arabidopsis thaliana) used were bxl1-1 (Ws ecotype; CS16299), bxl1-2, bxl1-3 (Salk_012090 [CS16300] and 054483 [CS16301]), Arabidopsis Biological Resource Center (ABRC), Columbus, OH), ap2-1, tgl1-1 (Landsberg erecta ecotype; ABRC), myb61-1 (Col-0; gift from Michael Bevan, John Innes Centre, Norwich, UK), and mum4-1 (Col-2; Western et al., 2004). Plants were grown and flowers staged as described by Western et al. (2001).

**Microscopy**

Developing seeds were staged and prepared for bright-field and scanning electron microscopy as described by Western et al. (2001). Ruthenium red staining was performed with 0.01% (w/v) ruthenium red with prehydration in either water or 0.05% EDTA, as indicated.

**Germination Tests**

Seeds were either plated dry or pretreated by shaking for 90 min in water or 0.05% EDTA and plated in 0.1% (w/v) agarose after rinsing. Seeds were stratified at 4°C for 72 h and incubated at 22°C under 16 h light:8 h dark, following which they were counted every day for 6 d and germination was scored by the presence of open green cotyledons.

**Cloning of AtBXL1 via Plasmid Rescue**

**Plasmid Rescue**

DNA isolated from patchy mutants was digested with Sall, EcoRI, or BglII, ligated, and transformed into Escherichia coli. Plasmid DNA isolated from the resulting colonies was sequenced to identify the genomic region flanking the T-DNA insertion using T-DNA right border and left border primers (Ponce et al., 1998).

**Molecular Complementation**

An 8.1 kb PstI/PheI fragment of BAC K7J8, including At5g49360 plus 2.4 kb upstream and 1.2 kb downstream sequences, was cloned into pGREEN0229 (Hellens et al., 2000) to give the PTyr construct. patchy plants were transformed with PTyrG or the empty vector as in Clough and Bent (1998). Transformants were selected by germinating seeds on plates containing 25 µg/mL glufosinate, and putative transformants were verified by PCR.

**Genetic Complementation**

patchy mutants were crossed to Salk_012090 (bxl1-2) and Salk_054483 (bxl1-3), whose identities as At5g49360 mutants were verified through sequencing of the site of T-DNA insertion and use of RT-PCR to demonstrate reduced transcription and/or transcript truncation.

**Qualitative and Real-Time RT-PCR**

RNA was isolated as described by Western et al. (2004) or with a modified RNeasy plant mini protocol: two silicas were ground in liquid nitrogen, resuspended in 600 µL RLT-PVP40 (540 µL RLT + 60 µL 10% [w/v] polyvinylpyrrolidone) plus 30 µL β-mercaptoethanol per ml buffer and processed according to the manufacturer’s instructions (Qiagen). One-microgram samples of total RNA were treated with DNaseI and transcribed with SuperScript II Reverse Transcriptase using an oligo(dT) primer according to the manufacturer’s instructions (Invitrogen).

PCR for AtBXL1 RT-PCR (Fig. 4B) was performed for 30 cycles using primers At5g49360 p 15/16, tests for truncated transcripts were performed with At5g49360 p 11/8, and AtBXL2 RT-PCR was done with BXL2 p1/p2 (see Supplemental Table S3 for primer sequences). Real-time PCR was performed with an iCycler iQ Real-Time PCR system using the iQ SYBR Green Supermix (Bio-Rad). PCR conditions were 95°C for 10 min, 40 cycles of 30 s at 95°C, and 1 min at 55°C. Transcript levels were normalized against GAPC. Primers used were At5g49360 p5/p4 and RT-GAPCp5/p6.

**Chemical Analyses**

To quantify sugars in crude mucilage extracts, 50 mg of intact seeds were incubated in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 h at 30°C. No significant difference in mass was observed between Ws and bxl1-1 seed (100 counted seed; Ws = 1.5 ± 0.1 mg; bxl1 = 1.6 ± 0.0 mg; n = 3). One µmole of myo-inositol was added to the supernatant, and samples were precipitated with 5 volumes ethanol, directly hydrolyzed with 2 µL trifluoroacetic acid, and derivatized to alditol acetates (see below). For seedling cell walls, seedlings were dark treated for 48 h prior to harvest (150–200 mg of fresh weight), and alcohol-insoluble residues were prepared by grinding tissue in N2 (l) plus 1% SDS (w/v), followed by extensive washing in alternating hot (80°C) water and 50% ethanol (60°C) with vacuum filtration. For determination of monosaccharide ratios including GalA, five independent samples of 250 mg of seeds were extracted sequentially with 0.2% ammonium oxalate, 0.2 and 2 mM sodium hydroxide, for 1 h each with vigorous shaking at 37°C. Both sodium hydroxide extractions contained 3 mg/mL sodium borohydride to prevent end degradation and were neutralized with acetic acid. The supernatants for each extraction were filtered through a glass fiber filter, dialyzed, and freeze dried. Carboxyl reduction was performed as described by Kim and Carpita (1992), as modified by Carpita and McCann (1996). Derivatization to alditol acetates was performed as described by Gibeaut and Carpita (1991). Linkage analysis through per-O-methylation was also performed as described by Gibeaut and Carpita (1991), with inferences on linkage structure as described by Carpita and Shea (1989).

**Protein Isolation and Enzyme Assays**

**Preparation of Protein Extract**

The 7-DPA Ws and bxl1-1 siliques were ground in liquid nitrogen and extracted with 500 µL 25 mM MOPS, pH 7.0, and 0.5 mM Pefabloc and complete EDTA was collected for 10 min at 1,000g. The supernatant was collected and this constituted the soluble fraction. This was repeated three times and the fractions pooled. The remaining cell wall fraction was extracted with 2.5 mL 25 mM MOPS, pH 7.0, and 200 mM CaCl2 during 1 h of vigorous shaking at 4°C. The tube was then centrifuged for 10 min at 1,000g and the supernatant (cell wall fraction) was recovered. The cell wall fraction was salt purified on a PD-10 column (GE Healthcare) according to the manufacturer’s instructions.

**Cation Exchange Chromatography**

The cell wall fraction was equilibrated in 25 mM sodium acetate buffer (pH 5.0) containing 5% glycerol (v/v) and 0.015% Triton X-100 (v/v) and loaded on a HiTrap-FF SP-Sepharose column (GE Healthcare). The proteins were eluted with 500 mM NaCl wash buffer, first alone and then with a 0.0 to 0.5 M NaCl continuous gradient. One-millilitre fractions were collected and 100 to 200 µL assayed from each fraction for α-L-arabinofuranosidase and β-D-galactosidase activity. Arabino- furanosidase activity was equalized to galactosidase activity in each fraction.

**Enzyme Activity**

The reaction mixture contained 2 mM PNP-α-L-arabinoside or PNP-β-D-galactopyranoside (Sigma-Aldrich), 0.1 mM acetate buffer (pH 5.0), and 100 to 200 µL protein extract in a total volume of 0.5 mL. The reaction was carried out at 37°C for 90 min and stopped by the addition of 0.5 mL of 0.4 M sodium bicarbonate to the assay mixture. Concentration of the resulting PNP was determined spectrophotometrically at 405 nm, and its amount estimated from a calibration curve.
Immunoblotting and Immunofluorescence

For immunoblotting, 75 mg of seed were shaken in 0.05x SDS-TA for 90 min at 37°C. Extracts were concentrated by evaporation and resuspended in 100 μL of PBS, pH 7.4, and 4 μL of concentrated mucilage spotted on nitrocellulose membranes as 2 × 2 μL aliquots. Hybridization was performed as described by Willats et al. (2001b) with the following modifications: membranes were blocked in antibody solution for 1 h, followed by incubation in primary antibody (1:10 [v/v] dilution of CCRC-M36 or LM6) for 90 min. Alkaline phosphatase-conjugated secondary antibodies (anti-mouse and anti-rat, respectively; Invitrogen) were diluted 1:1,000 (v/v) and detected using the BCIP/NBT-Purple liquid substrate (Sigma-Aldrich), with the reaction stopped by rinsing with water. CCRC-M36, an antibody specific for RG I, was obtained from CarboSource (University of Georgia, Athens; http://www.carboxylation.co.uk/). CCRC-M36 was specific to (1→5)-linked arabinans (Willats et al., 1998) and was obtained from PlantProbes (University of Leeds, Leeds; http://www.plantprobes.net).

For immunofluorescence on developing seeds, seeds were dissected from 7- and 9-DPA siliques and fixed for 2 h in 4% (v/v) paraformaldehyde in 50 mM PIPES (pH 7.0). Samples were rinsed, dehydrated through an ethanol series, and embedded in LR White resin. Embedded samples were sectioned to 0.5 μm, affixed to slides with poly-lys and subjected to antibody detection as described by Young et al. (2008), except primary antibodies were used full strength and secondary antibodies were diluted as described below for whole seed samples. Whole seed immunofluorescence was performed as described by Young et al. (2008). Primary antibodies (1:20 [v/v]) were detected with a 1:100 (v/v) dilution of AlexaFluor 488-conjugated goat anti-mouse (CCRC-M36) or goat anti-rat (LM6) secondary antibodies (Molecular Probes, Invitrogen). Seeds were counterstained with 0.2 μg/mL propidium iodide in 50 mM phosphate buffer, pH 7.4, to visualize the outer cell wall. Treatments without primary antibody were included to test for nonspecific staining, and all seeds were mounted in 1:100 (v/v) India ink in 90% (v/v) glycerol in water to confirm the presence of released mucilage. Immunofluorescence samples were observed with a Zeiss Meta 510 LSM confocal microscope.

Seed Treatment with Exogenous Enzymes

*Pichia pastoris* clones for three inducible, secreted, recombinant α-1-arabinofuranosidases (AN1571, AN7908, and AN80401), and one β-1,4-xylanase (AN2359) were obtained from the Fungal Genetic Stock Center (www.fgsc.net). Bauer et al., 2006. Methanol treatment was used to induce secretion of recombinant enzymes into the medium. Cultures were then centrifuged and the enzymes were purified by affinity to their His tag from the supernatant as described by Bauer et al. (2005), with the exception that proteins were affinity isolated in a batch method (rather than in a column) using 1 mL of 50% Ni-NTA His Bind Slurry (EMD Biosciences) and 4 mL Ni-NTA Bind buffer (buffer A; EMD Biosciences). Protein quantitation and activity assays were carried out using PNP-glycosides as described above. For seed treatment, 10 units of goat anti-mouse (CCRC-M36) or goat anti-rat (LM6) secondary antibodies (Molecular Probes, Invitrogen) were used full strength and secondary antibodies were diluted as described below for whole seed samples. Whole seed immunofluorescence was performed as described by Young et al. (2008). Primary antibodies (1:20 [v/v]) were detected with a 1:100 (v/v) dilution of AlexaFluor 488-conjugated goat anti-mouse (CCRC-M36) or goat anti-rat (LM6) secondary antibodies (Molecular Probes, Invitrogen). Seeds were counterstained with 0.2 μg/mL propidium iodide in 50 mM phosphate buffer, pH 7.4, to visualize the outer cell wall. Treatments without primary antibody were included to test for nonspecific staining, and all seeds were mounted in 1:100 (v/v) India ink in 90% (v/v) glycerol in water to confirm the presence of released mucilage. Immunofluorescence samples were observed with a Zeiss Meta 510 LSM confocal microscope.

Water Absorption Measurements

A modified Bowmann capillary apparatus was set up as described by Cui (2001), using 2-3 mm glass tubing connecting a 1-mL serological pipet and a 15-mL sintered glass funnel. Water was added from the pipet end until it reached the sintered glass. Filter paper was placed on top of the sintered glass and allowed to equilibrate. Two to five milligrams of the diazylized, freeze-dried mucilage fractions described above were placed on the saturated filter paper and the level of water in the pipet determined every 5 min for 30 min, followed by every 15 min up to 2 h.

Isolation of Double Mutants

F2 seeds were first screened for visual phenotypes (ap2-1 heart shape seeds and itg1-1 yellow seeds) and/or aberrant of mucilage release in ruthenium red dye (ap2-1, itg1-1, xyb1-1, and mum4-1). Candidate plants were genotyped for the bx1-1 T-DNA insertion using PCR with P154690760p/p11 (Supplemental Table S5) and T-DNA LB primer iPCR-LB (Ponce et al., 1999). Putative bx1-1 myb61-1 and bx1-1 mum4-1 double mutants, which lack non-seed coat phenotypes, had their myb61-1 and mum4-1 genotypes verified using PCR. The myb61-1 dsPm insertion was confirmed using MYB61 p1/p2 and dsPm11 (Supplemental Table S3; Penfield et al., 2001). The mum4-1 point mutation leads to the addition of a new Msel site, which can be detected by digesting the PCR products from A1tg53500 p1/p8 (Supplemental Table S3; Western et al., 2004).

Supplemental Data

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

**Supplemental Figure S1.** Scanning electron microscopy of bx1-1 versus mum4-1 and wild-type seed coat epidermal cells.

**Supplemental Figure S2.** Immunoblot of extracted mucilage and whole seed immunofluorescence of ws versus bx1-1 seeds.

**Supplemental Figure S3.** Water absorption of bx1-1 versus wild-type mucilage extracts.

**Supplemental Figure S4.** Transcription of AI2XL2 throughout Arabidopsis tissues and during seed and silique development.

**Supplemental Table S1.** Quantification of mucilage release of complemented lines of bx1-1, plus bx1-2 and bx1-3.

**Supplemental Table S2.** Monosaccharide quantitation of bx1-1 versus wild-type seedlings.

**Supplemental Table S3.** Primer sequences for RT-PCR, real-time PCR, and genotyping of double mutants.

ACKNOWLEDGMENTS

We thank Drs. Gillian Dean, Hugo Zheng, and Ms. Heather McFarlane for helpful discussions and comments on the manuscript. We are also grateful for the help of Tieeling Zhang, Phoenix Bouchard-Kerr, Amin Osmani, and Kathryn Brown for their assistance in chemical analysis, isolation of double mutants, and RNA preparations and Dr. François Parcy for the modified RNeasy protocol suitable for seed and silique RNA.

Received March 10, 2009; accepted May 14, 2009; published May 20, 2009.

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