PECTIN METHYLESTERASE INHIBITOR6 Promotes Arabidopsis Mucilage Release by Limiting Methylesterification of Homogalacturonan in Seed Coat Epidermal Cells

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Imbibed seeds of the Arabidopsis thaliana accession Djaryl are affected in mucilage release from seed coat epidermal cells. The impaired locus was identified as a pectin methylesterase inhibitor gene, PECTIN METHYLESTERASE INHIBITOR6 (PMEI6), specifically expressed in seed coat epidermal cells at the time when mucilage polysaccharides are accumulated. This spatio-temporal regulation appears to be modulated by GLABRA2 and LEUNIG HOMOLOG/MUCILAGE MODIFIED1, as expression of PME16 is reduced in mutants of these transcription regulators. In pmei6, mucilage release was delayed and outer cell walls of epidermal cells did not fragment. Pectin methylesterases (PMEs) demethylate homogalacturonan (HG), and the majority of HG found in wild-type mucilage was in fact derived from outer cell wall fragments. This correlated with the absence of methylsterified HG labeling in pmei6, whereas transgenic plants expressing the PME16 coding sequence under the control of the 35S promoter had increased labeling of cell wall fragments. Activity tests on seeds from pmei6 and 35S: PUMEI6 transgenic plants showed that PME16 inhibits endogenous PME activities, in agreement with reduced overall methylesterification of mucilage fractions and demucilaged seeds. Another regulator of PME activity in seed coat epidermal cells, the subtilisin-like Ser protease SBT1.7, acts on different PMEs, as a pmei6 sbt1.7 mutant showed an additive phenotype.

INTRODUCTION

During seed coat formation, the developing seeds of myxospermous species, such as Arabidopsis thaliana, accumulate polysaccharides in the apoplast of epidermal cells. When mature seeds are imbibed, the rehydrated polysaccharides expand and rupture the outer cell wall, and the released polysaccharides then encapsulate the seed as viscous mucilage. The functional role of this mucilage remains unclear, as Arabidopsis seeds without mucilage are viable and germinate under laboratory conditions. Certain reduced mucilage mutants have, however, been found to have delayed germination or increased sensitivity to low water potential compared with wild-type seeds (Penfield et al., 2001; Arsovski et al., 2009a). Diverse physiological roles have been proposed; for example, the adhesive properties of mucilage could glue seeds to animals for dispersion, or to soil particles, thereby impeding predation by ants or retaining seeds in a favorable environment (Young and Evans, 1973; García-Fayos et al., 2010; Engelbrecht and García-Fayos, 2012). Recent studies have shown that in the desert shrub Artemisia sphaerocephala, seed mucilage can promote seedling emergence and the growth of soil microflora and maintain seed viability by enabling the repair of damaged seed DNA (Yang et al., 2011; 2012a; 2012b).

The accumulation of mucilage polysaccharides in the apoplast of seed coat epidermal cells is polarized and requires a high degree of temporal organization (Young et al., 2008). The deposition of mucilage polysaccharides begins at the junction of the outer tangential and radial cell walls and results in the formation of a cytoplasmic column in the center of the epidermis cell. In parallel, an increase in Golgi stacks and the accumulation of starch granules is also observed. Following mucilage synthesis, a secondary cell wall is synthesized and fills the cytoplasmic column, forming the columella and leading to programmed cell death (Western, 2012). At the end of differentiation, the polygonal epidermal cells have central volcano-shaped columella that are connected to reinforced radial cell walls and surrounded by dehydrated polysaccharides under a primary cell wall.

The composition and structure of Arabidopsis seed mucilage is one of the best characterized and it has been shown to be composed of two layers, termed water-soluble (outer layer) and adherent (inner layer) (Western et al., 2000; Macquet et al., 2007a). Both layers are composed mostly of the pectin rhamnogalacturonan I (RG I), a repeat of the disaccharide 4)-α-D-GalA-(1→2)-α-L-Rha-(1→) (Goto, 1985; Western et al., 2000, 2004; Penfield et al., 2001; Usadel et al., 2004; Macquet et al., 2007a). In contrast with the...
water-soluble layer, the adherent layer is tightly attached to the seed and the RG I contains a small number of arabinoid and galactan ramifications (Dean et al., 2007; Macquet et al., 2007a, 2007b; Arsovski et al., 2009b; Huang et al., 2011; Walker et al., 2011). The pectin homogalacturonan (HG), a repeat of galacturonic acid, is also present as a minor mucilage component (Willats et al., 2001; Macquet et al., 2007a). In the adherent mucilage, its degree of methyl esterification (DM) varies, being higher in the outer compared with the inner region of the adherent layer (Macquet et al., 2007a). The adherent mucilage also contains cellulose, which is required for mucilage structuration and adherence (Harpaz-Saad et al., 2011; Mendu et al., 2011; Sullivan et al., 2011). Seed mucilage has become a model system for the study of polysaccharides as its constituents also form part of more complex plant cell walls.

As seed mucilage is nonessential in laboratory conditions, a number of mutants affected in mucilage production have been identified. The defective genes have been characterized and encode transcription regulators or polysaccharide metabolism enzymes. Enzymes implicated in the synthesis of mucilage pectin have been highlighted from the reduced mucilage phenotype of mutants. MUCILAGE MODIFIED4 (MUM4)/RHAMNOSYL TRANSFERASE11 (GAUT11) and GALACTURONOSYLTRANSFERASE-LIKE5 (GATL5) are potentially involved in the synthesis of pectin present in mucilage (Caffall et al., 2009; Kong et al., 2011; Western, 2012). As gat5 mutants present a reduction in both rhamnose and galacturonic acid, while gaut11 only seems to be affected in galacturonic acid content, this suggests different roles in RG I or HG synthesis, respectively (Western, 2012). Recently, mutants defective in the cellulose synthase catalytic subunit CELLULOSY SYNTHASE5 (CES5/MUM3), the Leu-rich receptor kinase FEI2, and the glycoprophathidylinositol-anchored fasciclin-like arabinogalactan protein SALT OVERLY SENSITIVE5 (SOS5) were found to affect the production of cellulose present in mucilage (Caffall et al., 2009; Kong et al., 2011; Western, 2012). As gat5 mutants present a reduction in both rhamnose and galacturonic acid, while gaut11 only seems to be affected in galacturonic acid content, this suggests different roles in RG I or HG synthesis, respectively (Western, 2012).

Most of the transcription regulators identified (APETALA2, ENHANCER OF GLABRA3, GLABRA2 [GL2], MYB5, MYB61, TRANSPARENT TESTA GLABRA2) regulate seed coat differentiation and are required for normal epidermal cell morphology and mucilage production (reviewed in Western 2012). By contrast, mutation of the transcriptional corepressor LEUNIG HOMOLOG1 (LUH1)/MUM1 only affects mucilage extrusion (Bui et al., 2011; Huang et al., 2011; Walker et al., 2011). Three downstream targets of LUH1/MUM1 are enzymes that affect polysaccharide maturation; MUM2 is a β-D-galactosidase and BXL1 a bifunctional β-D-xylosidase/β-D-arabinofuranosidase that trim galactan or arabinan side chains, respectively, from RG I, and the subtilisin-like Ser protease SBT1.7 is implicated in the modulation of HG methyl esterification (Dean et al., 2007; Macquet et al., 2007b; Rautengarten et al., 2008; Arsovski et al., 2009b). These three enzymes appear to alter both mucilage and cell wall mechanical properties important for mucilage liberation (Rautengarten et al., 2008; Arsovski et al., 2009b; Walker et al., 2011). Notably, HG is synthesized and secreted in a highly methyl-esterified state, and the DM is determined after secretion by the relative activity of pectin methyl esterases (PME) and their inhibition by proteinaceous PME inhibitors (PMEIs). It has been hypothesized that SBT1.7 could regulate the DM of HG through either the degradation of PME or activation of PMEI by limited proteolysis (Rautengarten et al., 2008).

PMEIs were identified first by purification from kiwi fruit (Actinidia chinensis) as small acidic proteins with four conserved Cys residues and an N-terminal signal sequence for extracellular targeting whose cleavage is required for inhibitor function (Camardella et al., 2000; Giovane et al., 2004). Subsequently, PMEI structure and interactions in a 1:1 complex with PME were described for Arabidopsis PMEI (Hothorn et al., 2004). These studies showed that mature PMEIs have two major structural features, an N-terminal α-helix hairpin anchor that interacts with the PME C terminus and a four-helix bundle that docks into the PME active site, thereby blocking access to the substrate. It has been suggested that PMEI inhibition is not generalized against all PMEs but that different PME isoforms will have preferential PMEI partners (Wolf et al., 2009). In Arabidopsis, the PECTIN METHYLESTERASE INHIBITOR (PMEI) gene family is large, with 69 members, and although five of these have been shown to act as inhibitors, either through assays with recombinant protein or the modification of PME activity in transgenic plants, to date, no PMEI has been directly linked to a precise physiological role or associated with a loss-of-function effect (Wolf et al., 2003, 2012; Raiola et al., 2004; Lionetti et al., 2007; Peaucelle et al., 2008; Pelletier et al., 2010).

The Arabidopsis accession Shahdara was previously shown to be a natural mutant from central Asia affected in MUM2 (Macquet et al., 2007b). Analysis of further accessions from this region has identified Djarly as a novel mutant affected in mucilage release (S. Saez-Aguayo A. Macquet, O. Loudet, and H.M. North, unpublished results). The gene affected in Djarly has been identified through map-based cloning as encoding a PMEI (PMEI6). Genetic, cytological, and biochemical approaches have been used to demonstrate that the expression of PMEI6 in epidermal cells of the seed coat is necessary for the inhibition of PME activity on methyl-esterified HG in mucilage and the outer primary cell wall of these cells.

RESULTS

The Djarly Accession Is Affected in PMEI6

Seeds of new accessions obtained from central Asia (http://www.inra.fr/vast/collections.htm) were imbibed in the pectin dye ruthenium red, and the Djarly accession, from Kyrgyzstan, was found not to release mucilage (cf. Figures 1B and 1A). The gene affecting mucilage release in Djarly was identified using map-based cloning; the affected locus was localized between 19.520 and 19.585 Mb on chromosome 2. Homozygous mutants were obtained for 18 insertion lines in 15 of the 22 annotated genes (see Supplemental Table 1 online) and their seeds analyzed for mucilage release. Only one mutant had seeds with a phenotype equivalent to Djarly, and this had an insertion in the gene At2g47670, predicted to encode a PMEI that we named PMEI6 (Figures 1C and 1G). Two additional insertion mutant alleles, pmei6-2 and pmei6-3, were obtained, and neither released mucilage when they were imbibed with ruthenium red (Figures 1D, 1E,
and 1G). To test for allelism, pmei6-1 was crossed to Djarly, pmei6-2, and pmei6-3, and in all cases, F1 and F2 seed coats did not release mucilage (Figure 1F; data not shown); the Djarly mutant allele was termed pmei6-4. Furthermore, seeds from the backcrossed pmei6-1 mutant still exhibited the phenotype. The release of mucilage from F1 seed coats of F2 seeds used for mapping or from the backcross indicated that pmei6-1 and pmei6-4 mutant alleles were recessive and maternally inherited.

To identify the nature of the mutant allele in the Djarly accession, the At2g47670 gene was sequenced and three nucleotide polymorphisms were identified compared with Columbia-0 (Col-0). The first substituted Ser-110 for Arg, the second at Leu-159 was a synonymous mutation, and the third caused a frameshift mutation due to a 1-bp insertion 581 bp after the ATG codon that changed amino acids 194 to 205 followed by the introduction of a stop codon (Figure 1G). PMEIs inhibit PMEs through the formation of a reversible 1:1 complex (D’Avino et al., 2003; Hothorn et al., 2004). Structural studies have shown that PMEIs have two distinct functional domains: an α-helical hairpin structure that stabilizes the PME-PMEI complex and a C-terminal bundle domain that interacts directly with the PME active site, thereby blocking substrate access (Hothorn et al., 2004). Modeling of the effects of the Djarly mutations on PMEI tertiary structure indicated that the bundle domain would be modified, in particular the α7 helix predicted to interact with the PME binding cleft, which would explain the lack of PMEI6 function (see Supplemental Figure 1 online) (Di Matteo et al., 2005).

Molecular complementation of the pmei6-1 mutant phenotype was also performed; the mutant was transformed with a construct containing the PMEI6 coding sequence (CDS) downstream of the cauliflower mosaic virus 35S promoter, and 16 independent transformants were obtained. When seeds were sown on agarose plates, mucilage release was restored in at least 60% of seeds from all 16 transformants compared with <22% in the pmei6-1 mutant (see Supplemental Figure 2A online).

The expression of PMEI6 was analyzed in the pmei6-1 mutant by RT-PCR on RNA extracts from developing seeds using PMEI6-specific primers situated either side of the pmei6-1 insertion at the 5′ and 3′ extremities of PMEI6 (Figure 1G). Amplification of the control gene EF1α4 was similar in the wild type and pmei6-1 (Figure 1H). By contrast, no amplification was obtained using primers on either side of the pmei6-1 T-DNA insertion site and encompassing the entire CDS, indicating that no full-length transcript was recreated by splicing out the T-DNA insertion and that the pmei6-1 mutant is a knockout. Reduced fragment amplification was observed in the mutant for the PMEI6 5′ region, compared with the wild type, and only a faint fragment was observed for the PMEI6 3′ region that could result from a low level of transcript reinitiation from within the T-DNA insertion (Figure 1H).

**Mucilage Release Is Delayed in pmei6**

Unlike the wild type, pmei6 mutant seeds did not release mucilage within the first few minutes of imbibition in ruthenium red. Nevertheless, it was noted that mucilage was released from pmei6 seeds when imbibition time was increased, indicating that mucilage release was delayed (Figures 2A to 2D). In addition to this delay, the outer primary cell wall did not break up and leave fragments attached to the top of columella; instead, it was observed attached to the hilum, as a large sheet of cell wall (Figure 2D). To quantify this delay in mucilage release, soluble extracts were measured for GaLA contents after imbibition of seeds for different lengths of time in water (Figure 2E). The mucilage release rate for mutant seeds was less than half that of the wild type: $V_{max} [\text{wild type}] = 0.17 \text{ mg}^{-1}\cdot\text{min}^{-1}$ compared with $V_{max}[\text{pmei6-1}] = 0.07 \text{ mg}^{-1}\cdot\text{min}^{-1}$ and $V_{max}[\text{pmei6-2}] = 0.06 \text{ mg}^{-1}\cdot\text{min}^{-1}$. Furthermore, the total amount of GaLA released from pmei6-1 and pmei6-2 seeds never reached wild-type levels, even after 24 h of imbibition (Figure 2E). Ruthenium red staining intensity and the

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**Figure 1. PMEI6 Is Required for the Correct Release of Seed Mucilage.**

(A) to (F) Seeds imbibed directly in the pectin stain ruthenium red. Wild-type seeds (A) release mucilage in contrast with the Djarly accession (pmei6-4) (B) and three pmei6 mutant alleles [C] to [E], that do not release mucilage. Genetic complementation is not observed in the F1 seed coat of F2 seeds from a cross between pmei6-1 and pmei6-4 (F). WT, the wild type. Bars = 500 μm. (G) Schematic representation of the structure of PMEI6 as annotated by The Arabidopsis Information Resource (http://www.Arabidopsis.org/). The sites and orientation of insertion lines in pmei6-1, pmei6-2, and pmei6-3 are indicated (LB, left border) and the position and nature of pmei6-4 mutations are shown. Numbers indicate the position of different features in bp. Colored bars denote the positions of the primers used in (H). (H) Effect of the pmei6-1 mutation on PMEI6 expression. RT-PCR analysis was performed with PMEI6-specific primers. Purple, green, and orange labels indicate primers amplifying the CDS (PMEI6 CDS) and the region 5′ (PMEI6 5′) or 3′ (PMEI6 3′) of the DNA insertion, respectively. A control amplification was performed with primers for EF1α4.
thickness of the adherent mucilage appeared the same in the wild type and *pmei6-1* after imbibition in either water or the cation chelator EDTA (Figures 2C and 2D; see Supplemental Figure 3 online). To confirm these data and to determine in which silique tissues *PMEI6* is expressed, quantitative RT-PCR (qRT-PCR) was performed on dissected seeds, silique envelopes, and whole siliques at developmental stages when mucilage polysaccharides are produced and deposited (8, 10, 12, and 14 d after pollination [DAP]). *PMEI6* expression was highest in developing seeds at 8 DAP, corresponding to seeds with torpedo stage embryos (see Supplemental Figure 4A online). A diminution of transcript accumulation was observed at later stages, in agreement with in silico transcriptome data.

To obtain more detailed information about the spatial expression of *PMEI6* within the seed, β-glucuronidase (*GUS*) reporter gene expression was observed in the *pmei6-3* mutant. This mutant was generated using an enhancer trap Ds element that contains the *GUS* reporter gene (Sundaresan et al., 1995). In *pmei6*-3, the *GUS* gene is correctly orientated for control by the endogenous *PMEI6* promoter. In addition, wild-type transformants containing a translational fusion with GFP were examined and in situ hybridization performed using a *PMEI6*-specific probe. The *PMEI6*-GFP (for green fluorescent protein) produced was not functional, since the phenotypes of the *pmei6-1* mutant were not restored when transformed with the same construct. For reporter gene analyses, cell walls were counterstained with propidium iodide to facilitate visualization. Analyses were performed on the same four seed developmental stages as for qRT-PCR. Expression of *PMEI6* transcripts and protein was observed to be specific to the epidermal cells of the seed coat. At 10 DAP, expression delimited the cytoplasm found at the base of the epidermal cells and in a column containing accumulated starch granules (see Supplemental Figures 4B, 4D, 4F, 4H, 4J, and 4L online). As seed coat differentiation progresses, secondary cell wall material accumulated to form the columella and reinforce radial cell walls. The column of cytoplasm was thus reduced at 14 DAP, and *PMEI6* expression was observed in this restricted cytoplasm (see Supplemental Figures 4C, 4E, 4G, 4I, 4K, and 4M online).

**Seed Coat Structure and Function Are Unaffected by *PMEI6* Mutation**

The structure of mutant seed coat epidermal cells was examined in more detail by scanning electron microscopy on mature dry seeds. The epidermal cells of both wild-type and *pmei6-1* mutant seeds were equivalent in size and morphology with a characteristic polygonal form and a central columella (see Supplemental Figures 5A to 5D online). No difference in the outer and radial cell walls was observed that could explain the delay in mucilage release and the nonfragmentation of the outer cell wall in *pmei6*. Developing seed coat epidermal cells from the *pmei6-1* mutant were also compared with the wild type in seed sections staged from 8 to 14 DAP (see Supplemental Figures 5E to 5L online). Cell structure and the timing of differentiation looked the same; in particular, outer and radial cell walls and mucilage accumulation appeared unchanged.

**Expression of *PMEI6* in Seeds Is Specific to the Epidermal Cells of the Seed Coat**

In accordance with the delayed mucilage release observed for *pmei6* seeds, available in silico expression data indicated highest expression levels in whole siliques or seeds and in particular the seed coat (Le et al., 2010; http://seedgenenetwork.net/arabidopsis); expression observed in other tissues, such as stems and flowers buds, was weak in comparison (Schmid et al., 2005).
PMEI6 Mutation Modifies HG Methylesterification in Adherent Mucilage

The adherent mucilage layer of wild-type Col-0 has previously been shown to be composed of two domains that both contain HG as a minor component. The DM of this HG is variable, being moderate in the inner domain and high in the outer domain (Macquet et al., 2007a; Figures 3A and 3B). To determine whether PMEI6 influences this DM, whole-mount immunolabeling was performed on mature dry seeds of wild-type, pmei6-1, and PMEI6 overexpressor lines (PMEI6ox) (wild-type plants transformed with the same 35S-PMEI6 construct used to complement the pmei6-1 mutant that show higher steady state levels of PMEI6 transcripts in developing seeds; see Supplemental Figure 6A online). Seeds were costained for β-glycans present in the epidermal cell columella and cell walls, as well as cellulose in the mucilage, using the fluorescent probe Calcofluor (Willats et al., 2001; Macquet et al., 2007a). In the pmei6-1 mutant, neither moderately nor highly methyl-esterified HGs were detectable with JIM5 and JIM7 antibodies, respectively (Figures 3C and 3D). By contrast, labeling with both JIM antibodies was increased in PMEI6ox (Figures 3E and 3F) compared with the wild type (Figures 3A and 3B). Similar increases in labeling were also observed for pmei6-1 seeds complemented with the same construct (see Supplemental Figures 2E to 2H online). Furthermore, Calcofluor labeling in the pmei6-1 mutant showed naked columella, with no fragments from outer cell wall breakage attached to their tops (Figures 3C and 3D).

PMEI6 Inhibits PME Activity in Seeds

To determine if PMEI6 acts as an inhibitor of PME activity found in seeds, assays were performed using total protein extracts from mature dry seeds of the wild type, pmei6-1, and PMEI6ox; PME activities were normalized to the average wild-type activity (Figure 4A). Activity levels were higher in pmei6-1 mutant seeds, with a 14% increase in global PME activity. In addition, PME activity was decreased in seeds of overexpressor lines by 40% compared with the wild type. Similarly, activity levels were reduced in three pmei6-1 transmants complemented with the same 3SS-PMEI6 construct (see Supplemental Figure 2B online). PME activity was also determined for proteins released with soluble mucilage from imbibed seeds, which correspond to proteins present in the apoplastic mucilage pocket of seed coat epidermal cells (Figure 4B). The diameters of activity halos and their staining intensity showed the same tendencies as for whole seeds, with higher and lower PME activities in pmei6-1 and PMEI6ox-21, respectively, compared with the wild type.

Mucilage Composition and Partitioning Are Modified in the pmei6 Mutants

Water-soluble mucilage, adherent mucilage, and demucilaged seeds were obtained from the wild type, pmei6-1, and pmei6-2 mutants using a sequential extraction procedure (see Supplemental Figure 7 online) and analyzed for their sugar and methanol contents. As the major component of Arabidopsis mucilage is RG I, the majority of sugars present in the soluble and adherent mucilage from the wild type were Rha and GalA in a molar ratio close to 1, in agreement with previous studies (Table 1) (Macquet et al., 2007a, 2007b; Huang et al., 2011; Sullivan et al., 2011; Walker et al., 2011). In pmei6-1 and pmei6-2 soluble mucilage, a reduction in total sugars of up to 50% was observed, which was mainly due to a reduction in Rha and GalA (Table 1). By contrast, adherent mucilage total sugars increased by over 25% in pmei6-1 and pmei6-2, again due to modified Rha and GalA contents (Table 1). The pmei6 mutants thus showed a redistribution of water-soluble to adherent mucilage; in the wild type, water-soluble mucilage represented ~63% of total mucilage, whereas in pmei6 mutants, this was reduced to <50% (Table 1). Furthermore, an overall reduction in mucilage total sugars was measured in mutant extracts, as previously observed in mucilage release assays, and this was mainly due to a reduction in GalA (Table 1; Figure 2E). By contrast, GalA contents increased slightly in demucilaged seed extracts (Table 1).
The occurrence of methylesterified HG domains in soluble mucilage was assessed by MS analysis of pectin lyase digests. Pectin lyase degrades highly methylesterified HG via a β-elimination mechanism, producing unsaturated methylesterified GalA oligomers that can be detected and identified by MS (Ralet et al., 2012). The MS spectrum of the pectin lyase digest of wild-type soluble mucilage was characterized by the presence of peaks attributed to unsaturated methylesterified GalA oligomers with a degree of polymerization of 3 to 7. The ions corresponding to dp6 and dp7 oligomers were visible in a zoom of the wild-type mass spectrum region corresponding to a mass-to-charge ratio (m/z) 1100 to 1400 (Figure 5A). Unsaturated GalA oligomers of dp6 with 4 to 6 methyl groups (m/z 1135, 1149, and 1163) and unsaturated GalA oligomers of dp7 with 5 or 6 methyl groups (m/z 1325 and 1339) were observed. In addition, single sodiation (+22) of the carboxylic functions of predominant ions was detected at m/z 1157, 1171, 1347, and 1361, and potassium adducts (m/z 1165 and 1179) and single sodiation of potassium adducts (m/z 1187 and 1377) were observed. By contrast, none of these peaks could be detected in the mass spectrum of the pectin lyase digest of pmei6-1 soluble mucilage (Figure 5B).

Taken together, the results obtained from MS and biochemical analyses reveal the presence of highly methylesterified HG domains in wild-type mucilage that are absent from pmei6-1 mucilage.

**LUH/MUM1 and GL2 Regulate PMEI6 Expression**

Previous studies implicated LUH/MUM1 as a positive regulator of MUM2, BXL1, and SBT1.7 expression and GL2 in the induction of MUM4 expression (Western et al., 2004; Bui et al., 2011; Huang et al., 2011; Walker et al., 2011). To assess whether PMEI6 is regulated by these transcription factors, transcript abundance was analyzed by qRT-PCR in developing seeds of the wild type, luh/mum1, and gl2. Steady-state PMEI6 transcript levels were markedly reduced in both gl2 and luh/mum1 relative to the wild type (Figure 6A; see Supplemental Figure 6B online). These results indicate that LUH/MUM1 and GL2 are required for normal PMEI6 expression in the seed coat. To confirm this analysis, PME activity assays were performed on protein extracts from mature dry seeds of luh/mum1 and gl2. Figure 6B shows that the PME activities of gl2 and luh/mum1 were both altered compared with the wild type, confirming that these transcription factors influence PME activity. Nevertheless, while activity levels in gl2 were higher than those of the wild type, as observed for the pmei6-1 mutant, in luh/mum1, activity levels decreased.

**PMEI6 and SBT1-7 Regulate PME Activity through Independent Pathways**

A previously identified mutant, sbt1.7, does not release mucilage and has similar phenotypes to those observed for pmei6 mutants: higher PME activity in seeds, reduced methylesterification of mucilage, nonfragmentation of the outer cell wall, and its shedding in large pieces attached to the chalazal end of the seed (Rautengarten et al., 2008). It was hypothesized that the SBT1.7 subtilisin-like Ser protease could activate PMEI by removing the
predicted inhibitory N-terminal signal sequence or inactivate PME by proteolysis. To determine whether SBT1.7 and PMEI6 could act through regulating the same PME activity, double mutants were obtained from a cross between pmei6-1 and sbt1.7-1. On direct imbibition of seeds in ruthenium red, the seeds of pmei6-1 sbt1.7-1 did not release mucilage, as observed for seeds of the parental genotypes (Figures 7A to 7D). Furthermore, when seeds were imbibed for 1 h in either water or EDTA, mucilage was released from all genotypes and staining of adherent mucilage was similar in width and intensity (Figures 7E to 7L). In the same way as for the parent genotypes, the outer cell wall did not fragment in the double mutant and remained attached to the hilum (Figures 7H and 7L). By contrast, while PME activities measured on dry seeds of the parental genotypes exhibited similar increases in PME activity compared with the wild type, activities in pmei6-1 sbt1.7-1 extracts were higher and corresponded to the addition of the increases observed for the parents (Figure 7M), indicating that they act on different PME activities.

DISCUSSION

The Djarly Accession Is a Naturally Occurring Mutant of a PMEI Expressed in Seed Coat Epidermal Cells

A screen exploiting variability between Arabidopsis accessions was used to identify a new accession, Djarly, with altered seed mucilage release (Figure 1B). The locus affecting mucilage release in Djarly was identified through a map-based cloning approach as At2g47670, predicted to encode a PMEI, named here as PMEI6. Mutations identified in PMEI6 in the Djarly accession are likely to modify protein structure such that interaction of PMEI6 with the

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<th>Table 1. Composition of Sequentially Extracted Mucilage and Demucilaged Seeds from the Wild Type, pmei6-1, and pmei6-2</th>
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<tr>
<td>The Wild Type</td>
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<tr>
<td>Water-soluble mucilage polysaccharides</td>
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<td>GalA</td>
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<td>Total sugars (SM)</td>
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<td>SM CH₂OH (µmol·g⁻¹)</td>
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| Adherent mucilage polysaccharides | | | |
| GalA | 6.17 (0.37) | 7.87 (0.72) | 7.85 (0.31) |
| Rha | 3.73 (0.23) | 5.61 (0.22) | 5.45 (0.27) |
| Fuc | – | – | – |
| Ara | 0.45 (0.19) | 0.49 (0.20) | 0.42 (0.12) |
| Xyl | 0.1 (0.1) | 0.07 (0.07) | – |
| Man | 0.29 (0.04) | 0.36 (0.08) | 0.33 (0.01) |
| Gal | 2.28 (0.12) | 2.19 (0.08) | 2.35 (0.05) |
| Glc | – | – | – |
| Total sugars (AM) | 13.01 (0.89) | 16.59 (1.19) | 16.4 (1.18) |
| AM CH₂OH (µmol·g⁻¹) | 0.62 (0.08) | 0.24 (0.02) | 0.32 (0.06) |

| Total mucilage polysaccharides | | | |
| Total mucilage sugars | 34.84 (4.42) | 31.67 (0.76) | 27.03 (2.89) |
| Total mucilage GalA | 17.46 (2.57) | 15.41 (0.17) | 13.31 (1.2) |
| Total mucilage Rha | 12.83 (2.69) | 11.96 (0.37) | 9.9 (0.5) |
| Total mucilage CH₂OH (µmol·g⁻¹) | 4.12 (1.45) | 0.30 (0.09) | 0.45 (0.1) |

| Demucilaged seed polysaccharides | | | |
| GalA | 36.03 (0.32) | 37.99 (0.75) | 39.1 (0.2) |
| Rha | 7.11 (0.51) | 7.65 (0.39) | 8.35 (0.45) |
| Fuc | 2 (0.17) | – | 1.4 (1.4) |
| Ara | 31.72 (0.65) | 30.79 (1.12) | 28.85 (0.25) |
| Xyl | 11.55 (0.45) | 10.92 (0) | 10.9 (0.5) |
| Man | 5.99 (0.4) | 6.12 (0.35) | 7.55 (1.05) |
| Gal | 22.51 (1.38) | 22.01 (0.57) | 22.35 (0.75) |
| Glc | 54.96 (0.2) | 50.4 (1.02) | 51.5 (0.2) |
| Total sugars (DS) | 171.87 (6.49) | 165.87 (6.95) | 170 (6.19) |
| DS CH₂OH (µmol·g⁻¹) | 25.9 (1.44) | 24.07 (2.28) | 20.57 (0.45) |

Values are means of four independently extracted samples from two biological repeats (±SD). AM, adherent mucilage; CH₂OH, methanol; DS, demucilaged seed; SM, water-soluble mucilage; –, not detected. Details of sequential extraction method are given in Supplemental Figure 7 online.
active site of target PMEs would be affected, thereby leaving the site free for pectin binding (see Supplemental Figure 1 online). The role of PMEI6 in mucilage release was confirmed by the observation of the same mucilage defect in three independent allelic mutants and molecular complementation of pmei6-1 phenotypes with the PMEI6 CDS (Figures 1C to 1F; see Supplemental Figure 2 online).

The expression of PMEI6 in seeds was analyzed by qRT-PCR, in situ hybridization, and the expression of reporter genes from the PMEI6 promoter. Expression of both transcripts and protein for PMEI6 was specific to the epidermal cells of the seed coat where mucilage polysaccharides accumulate (see Supplemental Figures 4B to 4O online). Maximal expression was observed in seeds with embryos at the linear cotyledon stage (8 to 10 DAP, depending on culture conditions) (see Supplemental Figure 4 online). Therefore, the spatio-temporal expression patterns were in agreement with PMEI6 participating in the release of mucilage polysaccharides on imbibition.

### PMEI6 Modulates the Degree of HG Methylesterification by Regulating PME Activity

A comparison of PME activity in protein extracts from mature dry seeds of a pmei6 mutant and transgenic plants expressing the PMEI6 CDS under the control of the 35S promoter showed that activity levels were modulated with respect to the presence or absence of PMEI6 expression (Figure 4; see Supplemental Figure 2B online). Mucilage release was restored in pmei6 transformants, confirming that the decreased activity observed in transformants was due to increased inhibition of PME activity by PMEI6 expression. In addition, PME activity was detected in protein extracts obtained from imbibed seeds; these extracts correspond to proteins present in the apoplasm of seed coat epidermal cells. As these extracts also had increased activity in pmei6 mutants and reduced activity in PMEI6ox transformants, this confirms the expression of PME6 in epidermal cells of the seed coat and suggests that it is secreted to the apoplasm (Figure 4B).

### Table 2. GalA Distribution between Rhamnogalacturonan and HG in Sequentially Extracted Mucilage and Demucilaged Seeds from the Wild Type, pmei6-1, and pmei6-2

<table>
<thead>
<tr>
<th></th>
<th>The Wild Type</th>
<th>pmei6-1</th>
<th>pmei6-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg·g⁻¹ seeds</td>
<td>mg·g⁻¹ seeds</td>
<td>mg·g⁻¹ seeds</td>
</tr>
<tr>
<td>Total GalA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>11.29 (0.33)</td>
<td>7.54 (0.29)</td>
<td>5.46 (0.89)</td>
</tr>
<tr>
<td>AM</td>
<td>6.17 (0.37)</td>
<td>7.87 (0.72)</td>
<td>7.85 (0.3)</td>
</tr>
<tr>
<td>DS</td>
<td>36.03 (0.32)</td>
<td>37.99 (0.75)</td>
<td>39.09 (0.21)</td>
</tr>
<tr>
<td>Total</td>
<td>53.49 (0.60)</td>
<td>53.4 (0.51)</td>
<td>52.376 (0.99)</td>
</tr>
<tr>
<td>HG GalA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>0.633 (0)</td>
<td>0.078 (0)</td>
<td>0.053 (0)</td>
</tr>
<tr>
<td>AM</td>
<td>0.213 (0)</td>
<td>0.017 (0)</td>
<td>0.022 (0)</td>
</tr>
<tr>
<td>DS</td>
<td>27.48 (0.28)</td>
<td>28.77 (0.32)</td>
<td>29.02 (0.33)</td>
</tr>
<tr>
<td>Total</td>
<td>28.32 (0.28)</td>
<td>28.87 (0.32)</td>
<td>29.10 (0.33)</td>
</tr>
<tr>
<td>RG I GalA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>10.66 (0.56)</td>
<td>7.46 (0.48)</td>
<td>5.41 (1.51)</td>
</tr>
<tr>
<td>AM</td>
<td>5.96 (0.37)</td>
<td>7.85 (0.72)</td>
<td>7.83 (0.30)</td>
</tr>
<tr>
<td>DS</td>
<td>8.56 (0.61)</td>
<td>9.22 (0.42)</td>
<td>10.07 (0.44)</td>
</tr>
<tr>
<td>Total</td>
<td>25.18 (0.32)</td>
<td>24.54 (0.18)</td>
<td>23.28 (0.66)</td>
</tr>
</tbody>
</table>

Values for HG, GalA, soluble mucilage, and adherent mucilage are means of two independently extracted samples from two biological repeats (±sd). Values for total GalA are from Table 1. Other values were derived by calculations based on measured values. AM, adherent mucilage; DS, demucilaged seeds; SM, soluble mucilage. Details of sequential extraction procedure are given in Supplemental Figure 7 online.

Figure 5. Matrix-Assisted Laser-Desorption Ionization Time-of-Flight Mass Spectra (Positive Ion Mode) of Soluble Mucilage Digested with PectinLyase. Hydrolysates from wild-type (WT) (A) and pmei6-1 (B) soluble mucilage. u, unsaturated; numbers in bold indicate degree of polymerization; exponent number indicates the degree of methylesterification. [See online article for color version of this figure.]
PMEI6 and SBT1.7 Regulate Different PME Targets

In the *pmei6* mutant, mucilage release was not completely blocked, as observed for *luh/mum1, mum2*, and *mum4* mutants, but delayed (Figure 2E; Western et al., 2001). When mucilage was released, it was found that this delay was not due to reduced mucilage expansion and only a minor decrease was observed in total mucilage amount (Figure 2D, Table 1; see Supplemental Figure 3B online). Strikingly, on imbibition, the outer cell wall of the seed coat epidermal cells did not rupture into fragments as in the wild type but was observed as a sheet attached to the hilum of *pmei6* seeds, as has been reported for *sbt1.7* mutants imbibed in EDTA (Figures 7F, 7G, 7J, and 7K; Rautengarten et al., 2008). In addition, imbibition of *sbt1.7* seeds for 1 h in water resulted in a similar extrusion of mucilage from most seeds, as observed in *pmei6* (Figure 7G). SBT1.7 was implicated in the regulation of pectin methylesterification, as mutants had higher seed PME activities and the overall degree of methylesterification was reduced in soluble mucilage and seeds after EDTA extraction (Figure 7M; Rautengarten et al., 2008). Although similar increases in PME activity were observed in *pmei6* and *sbt1.7* (Figure 7M; Rautengarten et al., 2008), the *pmei6-1 sbt1.7-1* mutant showed an additive phenotype for PME activity in seed extracts, demonstrating that PMEI6 and SBT1.7 inhibit the activity of different PMEs (Figure 7M). Nevertheless, the similarity of mutant phenotypes implies that these PMEs act, at least in part, on the same targets.

PMEI6 Modulates HG Methylesterification in the Outer Cell Wall of Seed Coat Epidermal Cells

In measurements of sugars in sequential extracts from mature dry seeds, the amounts of GalA derived from HG were reduced in *pmei6* mucilage with a corresponding increase in GalA in HG from demucilaged seeds (Tables 1 and 2). Furthermore, none of the methylesterified HG oligomers detected in wild-type soluble mucilage were present in *pmei6-1* (Figure 5). A possible source for the HG that is present in wild-type seed mucilage, but associated with *pmei6* demucilaged seeds, is the sheet of outer cell wall that remains attached to the hilum. In accordance, when methylesterified HG in adherent mucilage was labeled with JIM5 and JIM7 antibodies, no immunofluorescence was observed in the mucilage of the *pmei6-1* mutant, although labeling of the cell wall fragment attached to the hilum was observed (see Supplemental Figure 2D online). By contrast, increased fluorescence was seen for complemented mutants and PMEI6ox transgenic plants, compared with the wild type, but fluorescence was not present in the region of adherent mucilage to the sides of the columella (Figure 3; see Supplemental Figures 2E to 2H online). In certain complemented mutants, outer cell wall rupture sometimes produced large cell wall fragments, and generally no immunofluorescence was observed in the mucilage beneath these fragments, while the cell wall fragments themselves showed strong labeling (see Supplemental Figure 2H online). This confirms that the majority of the methylesterified HG labeled in mucilage is in fact derived from fragments of the outer cell wall, either through direct labeling of fragments or labeling of HG that has diffused from fragments. The increased labeling observed in transgenic plants expressing the PMEI6 CDS (Figure 3; see Supplemental Figures 2E to 2H online) would therefore be...
due mainly to increased methylesterification of HG in the outer cell wall.

Differences observed in the localization of JIM5 and JIM7 labeling indicate that HG observed in the outer region of the adherent mucilage is more highly methylesterified than that in mucilage closer to the seed (Figures 3A and 3B; Macquet et al., 2007a). The increased number of negatively charged carboxyl groups on partially methylesterified HG would allow interactions through Ca\(^{2+}\) cross-links, and this could slow its diffusion compared with highly methylesterified HG. Although most HG-derived GalA would appear to be associated with cell wall fragments in the wild type, a small amount was still detected in the mucilage of pmei6 (Table 2). This is in agreement with the detection of patches of HG epitopes within mucilage polysaccharides accumulated in the apoplast of developing seed sections from the wild type (Walker et al., 2011).

**RG I Partitioning into Adherent and Soluble Layers Is Affected by HG Methylesterification**

Sugar contents determined for water-soluble and adherent mucilage extracts revealed that the major component of seed mucilage, RG I, had a modified distribution from soluble to adherent mucilage in pmei6 mutants (Tables 1 and 2). As PME6 modulates PME activity, this indicates that the DM of the small amount of HG domains present in mucilage plays a significant role in RG I solubility.
The conformation of RG I must also be regulated by HG within mucilage, as imbibition of wild-type seeds in Ca2+ chelators causes increased adherent mucilage expansion compared with imbibition in water (Figures 7E and 7I; Willats et al., 2001; Rautengarten et al., 2008; Arsovski et al., 2009b; Harpaz-Saad et al., 2011). Modifications in mucilage partitioning between the two layers of mucilage has previously been reported for mum2, cesa5, fei2, and sos5, with more adherent mucilage in the former and less in the latter three (Macquet et al., 2007b; Mendu et al., 2011; Harpaz-Saad et al., 2011; Sullivan et al., 2011). A similar redistribution probably occurs in sbt1.7, as the amount of soluble mucilage extracted by EDTA was 50% less than that of the wild type, while mum5 had 50% more soluble mucilage (Macquet et al., 2007a; Rautengarten et al., 2008). It is interesting to note that the three mutants with increased proportions of adherent mucilage are affected in proteins required for pectin modifications; MUM2 trims RG I galactan side chains, while PMEI6 and SBT1.7 modify HG methylesterification (Table 1; Dean et al., 2007; Macquet et al., 2007b; Rautengarten et al., 2008). Nevertheless, defective arabinan side chain trimming in an Arabidopsis bxl1 mutant did not affect partitioning (Arsovski et al., 2009b). This suggests that while the major factor determining mucilage adherence to the seed is cellulose, the amount of pectin associated with the cellulose network is determined by relatively minor modifications of certain pectin properties.

Modulation of Mucilage Release by HG Methylesterification

The question remains as to how reduced methylesterification of HG in mucilage and the outer cell wall delays mucilage release and prevents cell wall fragmentation. In the wild type, cell wall rupture occurs at the junction between the outer and radial cell wall and leaves fragments attached to the top of columella. In the mutant, although outer cell walls detach from radial cell walls, they remain attached to adjoining outer cell walls and detach from the top of columella, implying that links between the former are reinforced and to the latter weakened. While thicker radial cell walls have been observed in luh/mum1 and mum2, defective mucilage release in these mutants has not been associated with this modification (Walker et al., 2011). Furthermore, defects in CESA2, CESA5, and CESA9 also affect radial cell wall thickness, and mucilage release is not affected in the corresponding mutants (Stork et al., 2010; Mendu et al., 2011). In effect, a comparison of pmei6 seed coat epidermal cell morphology and development did not reveal any difference with the wild type (see Supplemental Figure 5 online). Plants overexpressing PMEI6 showed no obvious difference in seed coat epidermal cell size compared with the wild type (Figure 3), in contrast with 3SS: PMEI5-overexpressing lines (Müller et al., 2013). This suggests that the PMEs inhibited by PMEI5 intervene in different seed coat epidermal cell processes to the PME partners of PMEI6. Demethylesterified HG can form Ca2+ cross-links, which reinforce pectin structure and rigidify cell walls. The reduced methylesterification observed in pmei6 would therefore most likely reinforce links between adjoining outer cell walls. The outer cell wall fragments connected to the top of the columella were detached in cesa5, fei2, and sos5 mutants treated with Ca2+ chelators (Harpaz-Saad et al., 2011), indicating that fixation occurs through cellulose and Ca2+ cross-links. It is possible that even if Ca2+ cross-links are more abundant in pmei6, detachment

Figure 8. Model Integrating PMEI6 Regulation and Action on PME Activity in Seed Coat Epidermal Cells.

The transcription regulators TTG1, EGL3/TT8, and MYB5/TT2 form a complex that regulates GL2 and TTG2. GL2 is required for MUM4 and PMEI6 expression. In addition, two independent pathways act via the regulators MYB61 and LUH/MUM1. The latter is required for the expression of MUM2, BXL1, SBT1.7, and PMEI6. PME activity is negatively regulated by both PMEI6 and SBT1.7 through independent PMEs. MUM1 is also predicted to modulate PMEs. Arrows with dotted lines indicate links identified in this study. Boxes denote enzymes and oval transcription regulators.

The conformation of RG I must also be regulated by HG within mucilage, as imbibition of wild-type seeds in Ca2+ chelators causes increased adherent mucilage expansion compared with imbibition in water (Figures 7E and 7I; Willats et al., 2001; Rautengarten et al., 2008; Arsovski et al., 2009b; Harpaz-Saad et al., 2011). Modifications in mucilage partitioning between the two layers of mucilage has previously been reported for mum2, cesa5, fei2, and sos5, with more adherent mucilage in the former and less in the latter three (Macquet et al., 2007b; Mendu et al., 2011; Harpaz-Saad et al., 2011; Sullivan et al., 2011). A similar redistribution probably occurs in sbt1.7, as the amount of soluble mucilage extracted by EDTA was 50% less than that of the wild type, while mum5 had 50% more soluble mucilage (Macquet et al., 2007a; Rautengarten et al., 2008). It is interesting to note that the three mutants with increased proportions of adherent mucilage are affected in proteins required for pectin modifications; MUM2 trims RG I galactan side chains, while PMEI6 and SBT1.7 modify HG methylesterification (Table 1; Dean et al., 2007; Macquet et al., 2007b; Rautengarten et al., 2008). Nevertheless, defective arabinan side chain trimming in an Arabidopsis bxl1 mutant did not affect partitioning (Arsovski et al., 2009b). This suggests that while the major factor determining mucilage adherence to the seed is cellulose, the amount of pectin associated with the cellulose network is determined by relatively minor modifications of certain pectin properties.
of the outer cell walls from the top of the columella occurs because increased resistance to expanding mucilage polysaccharides at the intersection between outer and radial cell walls allows pressure from the swelling mucilage to build up for longer at the junction between the outer cell wall and columella. Muclage expansion could also be reduced by an increased number of Ca\(^{2+}\) cross-links between HGs, and together with decreased amounts of soluble mucilage in mutant seeds (Table 1), this would lessen pressure on cell walls. Therefore, it seems likely that modifications in both mucilage and cell wall properties contribute to a delay in pmei6 mucilage release.

Transcriptional Regulation of HG Methylsterification in Seed Coat Epidermal Cells

The transcription regulators GL2 and LUH/MUM1 appear to regulate PME6 expression, as the transcript steady state levels in gl2 and luh/mum1 were strongly reduced (Figure 6A; see Supplemental Figure 6B online). Furthermore, PME activity was also modified in seed protein extracts from both mutants (Figure 6B). A similar increase in activity was observed in gl2 and pmei6 extracts (Figure 4A; see Supplemental Figure 2B online), in accordance with PME6 expression being regulated by GL2 (Figure 8). LUH/MUM1 has been implicated in a regulatory pathway for pectin modification, with MUM2, BXL1, and SBT1.7 as downstream targets and PME6 can now be integrated into this pathway (Figure 8; Walker et al., 2011). Nevertheless, in the pmei6-1 sbt1.7-1 mutant, PME activity levels were even higher than in pmei6-1 or sbt1.7-1 alone, and the decreased activity observed in luh/mum1 extracts is therefore unexpected. Moreover, increased labeling of methylsterified HG was observed in both mucilage and outer cell walls of the luh/mum1 mutant, which led Walker et al. (2011) to propose that LUH/MUM1 promotes PME expression during seed coat maturation. Therefore, it is possible that LUH/MUM1 also regulates the expression of genes coding the PME targets of PME6 and SBT1.7 and acts as a coordinator of pectin maturation during seed coat epidermal cell development. In effect, transcriptome analyses have found that both PME and PME3 genes are high confidence targets of the BRASSINAZOLE-RESISTANT1 transcription factor (Sun et al., 2010), indicating that in other cell types they share common regulators of expression. Alternatively, reduced PME activity could occur in luh/mum1 by feedback regulation above a threshold DM of HG. A feedback mechanism has recently been described that mediates the level of HG methylsterification in the cell wall and induces PME expression through the brassinosteroid signaling pathway (Wolf et al., 2012).

The identification of PME6 as a PMEI that modulates mucilage release by regulating the DM of HG present in the outer cell wall and mucilage of seed coat epidermal cells is an important step toward understanding pectin function. In Arabidopsis, PMEI-related proteins are encoded by a large multigene family with 69 members, with only PME6 associated with a loss-of-function phenotype. Future studies will be required to identify the PME protein target(s) of PME6 and SBT1.7; in silico data and promoter:GUS constructs have shown that several of the 66 members of the PME gene family are expressed in seed tissues and seven PMEs appear to be strongly expressed in the seed coat (Louvet et al., 2006; Le et al., 2010). It will also be important to determine the precise nature of their HG substrates and to elucidate how PME activity is fine-tuned in seed coat epidermal cells. Finally, it will be interesting to ascertain whether the modulation of mucilage release through pectin modifications conveys a physiological advantage to Arabidopsis accessions.

METHODS

Plant Materials and Growth Conditions

The nine accessions corresponding to the Arabidopsis thaliana population Djarly were collected from their natural habitat, and Dja-1 was used in this study (http://www.inra.fr/internet/Produits/vast/collections.htm). The pmei6-1 (SM_3.19557), pmei6-2 (GK-790812), luh-3 (SALK_107245C), gl2-6 (SM_3.16350), and sbt1.7-1 (GK_140B02) (Col-0 accession) mutants were obtained from the Nottingham Arabidopsis Stock Centre (http://Arabidopsis.info) (Tissier et al., 1999; Alonso et al., 2003; Rosso et al., 2003). The enhancer trap line ET6082 (Landsberg erecta accession) pmei6-3 was obtained from the Martienssen lab at the Cold Spring Harbor Laboratory (http://genetrap.cshl.edu). Homozygous lines were identified by PCR using the primers indicated in Supplemental Table 2 online with genomic DNA extracts; an amplicon was only obtained for the insertion border and not with primers flanking the insertion.

Seed production was performed in either a growth chamber (photoperiod of 16 h light at 21°C, 8 h dark at 18°C, 65% relative humidity, and 170 μmol m⁻² s⁻¹) or a glasshouse with a minimum photo-period of 13 h assured by supplementary lighting. Plants were grown in compost (Tref substrates) and watered with Plan-Prod nutritive solution (Fertil). Three successive backcrosses to wild-type Col-0 were performed on the mutant pmei6-1. All phenotypic analyses were performed using these backcrossed seeds. Reciprocal crosses were performed between homozygous pmei6-1 and pmei6-2, pmei6-3, and Djarly. As the seed coat is a maternal tissue, mucilage phenotypes of F1 and F2 seed coats were determined on F2 and F3 seed, respectively. In all comparative analyses, seeds used were from mutant and wild-type plants that had been simultaneously cultivated and harvested. Developing seeds and silique envelopes were obtained by tagging flowers on the day of pollination and dissecting siliques at the specified number of days afterwards. For inclusions, whole silique envelopes were harvested.

Expression Analysis

Total RNA was extracted from developing seeds using the RNeasy plant mini kit (Qiagen) and used as a template (1 μg) for first-strand cDNA synthesis according to the manufacturer’s instructions. For amplification of PCR products from single-stranded cDNA from the wild type and pmei6-1 (Figures 1G and 1H), the following exon primers were used: PME6 CDS, forward primer 5'-GTGCAATTCTCTTTAAAAAGTTGCGATTCCCTCAAATAAAGAACATGACTTC3' and reverse primer 5'-CATAAGGTTACTAAGAATCTCGCCAC-3'; PME6 5', reverse primer 5'-GCAAGGCCTGCCACCCGCT-3'; PME6 3', forward primer 5'-GACGAGAAGCAGTGTACCAT-3'; EF1α4 primers were as described by North et al. (2007). Quantitative real-time PCR reactions were performed as previously described using primers that had been tested for their efficiency rates and sensitivity on dilution series of cDNAs (Plessis et al., 2011). PME6 and seed reference gene At4g12590 (Dekkers et al., 2012) specific primers were as follows: PME6, forward primer 5'-GGCAAGATTGCGATCTGCGCC-3'; PME6, reverse primer 5'-AGCCAGAAGCTGGTCATGCG-3'; PME6, forward primer 5'-GAGTAGAACGAGCAGTCA-3'; EF1α4 primers were as described by Plessis et al. (2011).
In situ hybridization was performed on paraffin-embedded developing seed sections as described by Sullivan et al. (2011). PME6-specific probes were PCR amplified from single-stranded cDNA reverse transcribed from wild-type RNA extracted from developing siliques using forward primer 5'-CTCTTCTTCTCTCC-3' and reverse primer 5'-TTTATAAGGTTTCC-3' and antisense probe and forward primer 5'-TTTATAAGGTTTCC-3' and reverse primer 5'-GCCCACCCCTGGTGTGGT-3' for sense probe.

Cloning and Plant Transformation

DNA fragments containing 2 kb of promoter region and the PME6 CDS or the PME6 CDS alone were amplified from wild-type Col-0 genomic DNA by PCR using the following primers containing attB1 and attB2 recombination sequences: pPM6E-PME6, forward primer 5'-GGGGACAAGTTGTAGCTCTACTCCAAGGGTGCCAGAC-3'; and pPM6E-PME6, reverse primer 5'-GGGGACCTTGTAGCAACAGA-AGCTTGACCAAGCTTCAAAAGCAACTG-3'; PME6 CDS, forward primer 5'-GGGGACAAGTTGTAGCTCTACTCCAAGGGTGCCAGAC-3'; and PME6 CDS, reverse primer 5'-GGGGACCTTGTAGCAACAGA-AGCTTGACCAAGCTTCAAAAGCAACTG-3'. (Note: nucleotides in bold type were added so that PME6 codons would be in phase with GFP codons; underlined nucleotides correspond to the start codon.) The resulting PCR products were then recombined into the pDONR207 vector with BP clonase (Invitrogen) according to manufacturers' instructions and transformed into Escherichia coli strain DH10B. The PME6 gene fragments were sequenced to confirm that no PCR-generated errors were present and then recombined into the binary vector pGW2b or pGW4b, which allow the expression of the PME6 CDS under the control of the cauliflower mosaic virus 35S promoter, or the production of a PME6/GFP translational fusion, respectively (Nakagawa et al., 2007). The resulting binary vectors were transformed into Agrobacterium tumefaciens C58C1mpP90 by electroporation prior to stable transformation of wild-type or pmel6-I plants using the floral dip method of Clough and Bent (1998).

Cytochemical Staining and Immunolabeling Procedures

Mucilage released from mature dry seeds was either stained directly with 500 µg mL−1 ruthenium red or after imbibition in water or 50 mM EDTA, pH 8.0, for 1 h. After EDTA treatment, seeds were rinsed with water prior to staining. Ruthenium red staining was observed with a light microscope (Axioplan 2; Zeiss). Developing seed sections were obtained from staged buds of F2 progeny in a 96-tube format as described by Simon et al. (2008). An approximate genome position was determined based on recombination percentages for 90 F2 progeny genotyped with simple sequence length polymorphism markers. The mapping interval was then reduced using recombinants identified among an additional 6344 F2 progeny. Mucilage release phenotypes for F2 seed coats were determined by ruthenium red staining of F3 seed lots.

Prediction of PME6 Protein Structure

Structural predictions for PME6 were obtained with the ESyPred3D program (Lambert et al., 2002) using the homology modeling approach and the kiwi PME as template (Di Matteo et al., 2005; Protein Data Bank ID code 1X32). Protein Data Bank files were analyzed with Rasmol 2.7.5.2 software (http://rasmol.org/).

Determination of PME Activity

Total protein extracts were obtained by grinding 200 mg of dry seeds in 400 µL of extraction buffer (1 M NaCl, 12.5 mM citric acid, and 50 mM Na2HPO4, pH 6.5). The resulting homogenate was shaken for 1 h at 4°C and then centrifuged at 20,000 g for 15 min, and the supernatant was retained. Protein concentrations were determined according to Bradford (1976), and equal quantities of protein (>8 µg) in the same volume (20 µL) were loaded into 6-mm-diameter wells in gels prepared with 0.1% (w/v) agarose, 12.5 mM citric acid, and 50 mM Na2HPO4, pH 6.5. After incubation overnight at 28°C, plates were stained with 500 µg mL−1 ruthenium red for 45 min and destained with water for >3 h. Measurements of stained areas were performed with ImageJ 1.34S software (Freeware, National Institute of Health).

DNA Sequencing and Genetic Analyses

Genomic DNA was extracted from flower buds of Col-0 and Djarly accessions as described by Doyle and Doyle (1990). PME6 DNA fragments were then amplified by PCR, gel purified using the Wizard SV gel and PCR cleanup system according to the manufacturer's instructions (Promega), and sequenced (GenoScreen). For mapping, crosses were performed between Col-0 and Djarly accessions, and DNA was extracted from flower buds of F2 progeny in a 96-tube format as described by Simon et al. (2008). An approximate genome position was determined based on recombination percentages for 90 F2 progeny genotyped with simple sequence length polymorphism markers. The mapping interval was then reduced using recombinants identified among an additional 6344 F2 progeny. Mucilage release phenotypes for F2 seed coats were determined by ruthenium red staining of F3 seed lots.

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Genomic DNA was extracted from flower buds of Col-0 and Djarly accessions as described by Doyle and Doyle (1990). PME6 DNA fragments were then amplified by PCR, gel purified using the Wizard SV gel and PCR cleanup system according to the manufacturer’s instructions (Promega), and sequenced (GenoScreen). For mapping, crosses were performed between Col-0 and Djarly accessions, and DNA was extracted from flower buds of F2 progeny in a 96-tube format as described by Simon et al. (2008). An approximate genome position was determined based on recombination percentages for 90 F2 progeny genotyped with simple sequence length polymorphism markers. The mapping interval was then reduced using recombinants identified among an additional 6344 F2 progeny. Mucilage release phenotypes for F2 seed coats were determined by ruthenium red staining of F3 seed lots.

Extraction and Analysis of Monosaccharides from Mucilage and Demucilaged Seeds

For determination of monosaccharides from soluble and adherent mucilage and demucilaged seeds, a sequential extraction procedure was used (see Supplemental Figure 7 online). Soluble and adherent mucilage extracts were obtained as described previously (Sullivan et al., 2011). Demucilaged seeds were ground and then washed with 70% ethanol (10 min at 20°C), hexane (1 h at 40°C), and acetone (20 min 20°C) and dried prior to analyses. Material lost during this delipidation process was determined and taken into account in calculations to obtain values for whole seeds as indicated in Tables 1 and 2. Rates of mucilage release were determined using 20 mg of dry seeds that were imbibed by mixing in 1 mL of distilled water for the indicated times (1, 2, 4, 6, 16, and 24 h) at 20°C. After imbibition, seeds were centrifuged (3000g, 5 min) and supernatants removed, filtered, and stabilized by treating for 5 min at 100°C. Extracts were conserved at 4°C prior to acid and neutral sugar analyses.
For the recovery of HG domains, soluble and adherent mucilage fractions were obtained as above from 2 g of seeds. Soluble mucilage was then subjected to digestion with 1 nkat of rhamnogalacturonan hydrolase (Swiss-Prot P000018) provided by Novozymes after adjusting with 100 mM sodium acetate, pH 4.5, to a final volume of 50 mM; samples were incubated for 16 h at 40°C. Enzyme hydrolysates were concentrated, dialyzed to remove all traces of salts, and then dried. Samples were suspended in 2 mL of water and then HG domains precipitated by adding an equal volume of ethanol and incubating for 1 h at 4°C. Precipitated HG was recovered after centrifugation (3000g, 15 min), and pellets were rinsed twice with 3 mL of 48% (v/v) ethanol for 30 min at 4°C. To remove all traces of ethanol, the resulting pellet, corresponding to HG, was re-suspended twice in 1 mL of water and dried.

Uronic acid (as GaA) was determined by the automated m-hydroxybiphenyl and orcinol methods, respectively (Thibault, 1979). Individual neutral sugars were analyzed as their alditol acetate derivatives (Blakeney et al., 1983) by gas–liquid chromatography after hydrolysis with 2 M trifluoroacetic acid at 121°C for 2.5 h for soluble fractions and prehydrolysis (26 N H₂SO₄, 2 h, 100°C) for insoluble fractions (demucilaged seeds).

The degree of methylsterification of mucilage and demucilaged seeds was measured from methanol released by alkaline deesterification of extracts with 0.2 M NaOH for 1 h at 4°C. After neutralization of extracts with 0.2 M HCl, released methanol was measured as described previously (Ralet et al., 2012).

MS Analysis
Soluble mucilage was obtained from 100 mg of seeds by imbibition in 1 mL of 50 mM Na-acetate, pH 5.0, and shaking for 3 h at 20°C. Supernatants with soluble mucilage were recovered by centrifugation (3000g, 5 min) and then filtered and incubated with pectin lyase for 16 h (1 nkat). For MS, all chemical reagents used were HPLC grade. N-N-dimethylaniline/2,5-dihydroxybenzoic acid matrix solution was prepared by dissolving 2% (v/v) methanol into 1 mL of water/ acetonitrile/N,N-dimethylaniline (Fisher Scientific) (49:49:2). One micro-liter of sample was directly mixed with 1 μL of matrix solution on a polished steel target plate. Acquisition was performed on an Autoflex III matrix-assisted laser-desorption ionization tandem time of flight mass spectrometer (Bruker Daltonics) equipped with a Smartbeam laser (355 nm) in positive ion mode with a reflector. Laser power was adapted for each sample. Mass spectra were automatically processed by FlexAnalysis software (Bruker Daltonics).

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: EFT1α, AtSg60390; GLZ, At1g7840; LUH/MUM1, At2g32700; PME6, At2g47670; SBT1.7, At5g67360; and seed reference gene, At4g12590.

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

Supplemental Figure 1. Predicted Three-Dimensional Structure for PME6 Wild-Type and Mutant Proteins.

Supplemental Figure 2. Complementation of pmei6-1 Mucilage Phenotypes by Transformation with a 35S Promoter:PME6 Construct.

Supplemental Figure 3. Ruthenium Red Staining of Adherent Mucilage Released from Seeds Imbied in a Cation Chelator.

Supplemental Figure 4. Expression of PME6 in Developing Seeds.

Supplemental Figure 5. Differentiation of Seed Coat Epidermal Cells in the pmei6-1 Mutant.

Supplemental Figure 6. Expression of PME6 in Overexpressor Lines or GLZ and LUH/MUM1 Mutant Seeds.

Supplemental Figure 7. Schematic Representation of Extraction Procedure Used for Analyses Presented in Tables 1 and 2.

Supplemental Table 1. Genes in the Chromosome 2 Mapping Interval for Which Homozygous T-DNA Insertion Mutants Were Obtained and Examined for Mucilage Release.

Supplemental Table 2. Sequences of Gene-Specific Primers used for Genotyping of Homozygous pmei6 Mutants.

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AUTHOR CONTRIBUTIONS

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