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MYB61 Is Required for Mucilage Deposition and Extrusion in the Arabidopsis Seed Coat

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We have undertaken a systematic reverse genetic approach to understand *R2R3-MYB* gene function in Arabidopsis. Here, we report the functional characterization of *MYB61* based on the phenotype of three independent insertion alleles. Wide-ranging phenotype screens indicated that *MYB61* mutants were deficient in seed mucilage extrusion upon imbibition. This phenotype was expressed in the sporophytic tissues of the seed. Deposition and extrusion of the principal component of the mucilage, a relatively unbranched rhamnogalacturonan, were reduced in the *MYB61* mutant seed coats. Additional defects in the maturation of the testa epidermal cells suggested a potential deficiency in extracellular secretion in *myb61* lines. Consistent with a proposed role in testa development, reverse transcription–polymerase chain reaction analysis showed the highest *MYB61* expression in siliques, which was localized to the seed coat by a β-glucuronidase (*GUS*) reporter gene fusion. Lower levels of *GUS* expression were detected in developing vascular tissue. Parallel analysis of the *ttg1-1* mutant phenotype indicated that this mutant showed more severe developmental defects than *myb61* and suggested that MYB61 may function in a genetic pathway distinct from that of TTG1. The transient nature of seed epidermal characteristics in the *ttg1-1* mutant suggested that TTG1 was required for maintenance rather than initiation of testa epidermal differentiation. Germination and seedling establishment were compromised in the *myb61* and *ttg1-1* mutants under conditions of reduced water potential, suggesting a function for Arabidopsis seed mucilage during germination in dry conditions.

INTRODUCTION

In angiosperms, the mature embryo is surrounded by an outer seed coat that participates in many important processes during seed development and germination. These include the transport of nutrients from the funiculus to the developing embryo, the protection of the embryo, and the control of the length of the dormancy period (Debeaujon et al., 2000a). The seed coat has economic significance as the source of cotton fibers, and during processing of some seed-based products such as coffee and cocoa, the seed coat must be removed. Angiosperm seed coat morphology is extremely diverse, reflecting multiple adaptations to seed dispersal and germination in different environments (Fahn, 1990).

In Arabidopsis, the seed coat differentiates from two ovule integuments (Léon-Kloosterziel et al., 1994). The inner integument forms the tegmen, the site of synthesis of tannins, the characteristic brown pigment of Arabidopsis seeds. The outer integument develops into the testa, the outer epidermis of which differentiates in a complex process into mucilage-containing cells with thickened radial cell walls and central elevations known as columellae (Koornneef, 1981; Western et al., 2000; Windsor et al., 2000). These are reinforced by the deposition of a secondary cell wall. In this respect the development of the seed coat epidermis resembles that of tracheary elements.

The most abundant monosaccharide constituents of Arabidopsis seed mucilage are rhamnose and galacturonic acid, suggesting that the principal polysaccharide is rhamnogalacturonan I (RG I; Goto, 1985; Western et al., 2000). In mature seeds, mucilage is present in a dehydrated form within each epidermal cell; upon contact with water, the mucilage expands, rupturing the primary cell wall and extruding from the seed coat. In this hydrated state, the mucilage envelops the whole seed and forms a pectin hydrogel (Zwieniecki et al., 2001). Seed mucilage has been hypothesized to play a role in germination as an oxygen barrier and in seed dispersal (Gutterman and Shemtov, 1996). However, the function of seed mucilage in Arabidopsis remains unclear.

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To date, three pleiotropic loci have been implicated in the development of the testa epidermis in Arabidopsis. Plants lacking a functional *APETALA2* (*AP2*) gene are deficient in the development of columellae and the biosynthesis of mucilage, and strong *AP2* mutant alleles such as *ap2-1* lead to weakened and misshapen seed coats. This suggests that AP2 regulates multiple aspects of seed coat development (Jofuku et al., 1994), including early steps of seed coat specification. In addition to their well-characterized roles in trichome and root atrichoblast specification, the *TRANPARENT TESTA GLABRA1* (*TTG1*) and *GLABRA2* (*GL2*) genes are essential for columella development in the testa epidermis and for mucilage extrusion during imbibition (Koornneef, 1981; Koornneef et al., 1982). *TTG1* encodes a WD40 repeat protein, and *GL2* encodes a homeodomain transcription factor (Rerie et al., 1994; Walker et al., 1999). These interact genetically with the tissue-specific MYB transcription factors *GLABRA1* (*GL1*) and *WEREWOLF* (*WER*) in the initiation of trichome and root atrichoblast development (Oppenheimer et al., 1991; Lee and Schiefelbein, 1999). The *ttg1-1* phenotype can be complemented by overexpression of the maize basic helix-loop-helix (bHLH) transcription factor *R* (Lloyd et al., 1992), suggesting that TTG1 functions to regulate the activity of Arabidopsis bHLH proteins. One of these has recently been found to be encoded by the *GL3* locus (Payne et al., 2000). Although for some time it has been known that *TTG1* plays a role in the development of the seed coat epidermis, the effects of the *ttg1-1* allele on seed coat development have not been described in detail.

MYB-related proteins comprise a large transcription factor family in higher plants of >100 members (Martin and Paz-Ares, 1997; Arabidopsis Genome Initiative, 2000; Riechmann and Ratcliffe, 2000). They are characterized by the number of conserved helix-loop-helix repeats in the MYB domain, and the largest group in plants containing two repeats is known as *R2R3-MYB* genes. *R2R3-MYB* genes are specific to plants and appear to regulate a wide variety of cellular functions in Arabidopsis. *ASYMMETRIC LEAVES1* downregulates meristem identity genes in lateral organ primordia (Byrne et al., 2000), and the *GL1* and *WER* genes regulate the initiation of trichome and root atrichoblast, respectively (Oppenheimer et al., 1991; Lee and Schiefelbein, 1999). In addition to their function in the control of organ and cell morphology, MYB-related transcription factors also regulate metabolism. The *ATR1* gene controls tryptophan biosynthesis (Bender and Fink, 1998), whereas several *MYB* genes have been shown to regulate phenylpropanoid metabolism (Grotewold et al., 1994; Moyano et al., 1996; Jin et al., 2000) and anthocyanin biosynthesis (Paz-Ares et al., 1987; Cone et al., 1993; Borevitz et al., 2000).

We have undertaken a systematic approach to understanding of *MYB* gene function in Arabidopsis. More than 70 members of the *R2R3-MYB* gene family were characterized and their expression examined in the major tissues and under a variety of conditions (Kranz et al., 1998). Transposon and T-DNA knockouts were obtained for many genes, and these were subsequently screened for phenotypes (Meissner et al., 1999). Here, we report the functional characterization of *AtMYB61*, based on the phenotype of transposon insertion mutants, and propose a role for this gene during the development of the testa epidermis. Using mutants deficient in the accumulation of seed coat polysaccharides, we also demonstrate a function for mucilage during seed germination in reduced water potential conditions. The large quantity of mucilage deposited during the development of the testa provides a significant opportunity to study polysaccharide biosynthesis and secretion in plants and the role of transcription factors in these processes.

RESULTS

Isolation of *MYB61* Insertion Mutants

The isolation of two transposon alleles of *MYB61* (At1 g09450) by use of polymerase chain reaction (PCR)–based screens from the Wisman collection has been described previously (Meissner et al., 1999). In addition, a stable allele was isolated from a hybridization screen of the Sainsbury Laboratory *Arabidopsis thaliana* (SLAT) collection using the Inverse Display of Insertion (IDI) filters (Tissier et al., 1999). This *d*/*Spm* insertion is located downstream of the conserved MYB helix-turn-helix repeats disrupting the codon for the isoleucine residue at position 129. Transposons in the SLAT collection are single copy and stable as the result of selection against the transposase source in *trans*, so this allele was chosen for further characterization and was designated *myb61-1*. This allele functions as a knockout at the RNA level (see Figure 4C). The two additional alleles from the Wisman collection were designated *myb61-2* and *myb61-3*. The relative positions of the three insertions in the *MYB61* gene are shown in Figure 1A.

MYB61 Is Required for Seed Mucilage Extrusion during Imbibition

Lines homozygous for *MYB61* insertions were subjected to systematic screens to uncover phenotypes associated with the loss of gene function. Plants carrying transposon insertions in *MYB61* appeared phenotypically indistinguishable from the wild type, except for a lack of mucilage extrusion from the seeds during imbibition. Upon contact with water, Arabidopsis seeds release mucilage from the seed coat epidermis that swells to form a gelatinous coating over the seed. This was visualized by staining with ruthenium red, which stains negatively charged biopolymers such as pectin and DNA (Hanke and Northcote, 1975; Koornneef, 1981). Inspection of imbibed *myb61-1*, *myb61-2*, and *myb61-3*

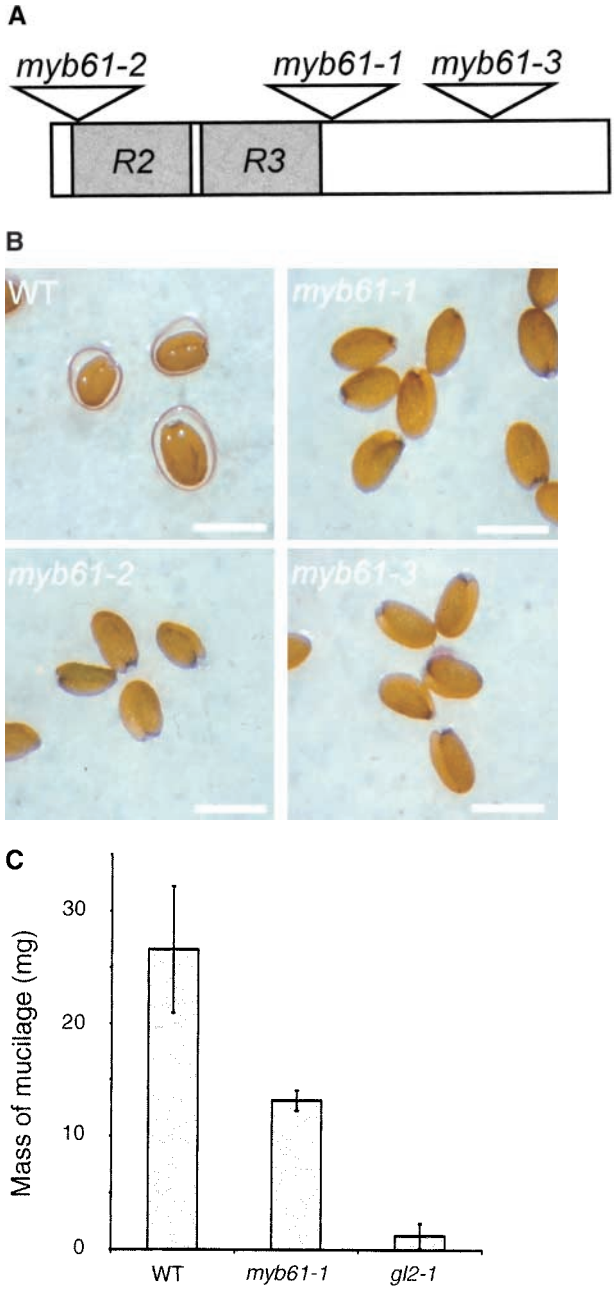

Figure 1. Three Disruptions of the *MYB61* Gene Cause a Seed Mucilage Deficiency.

(A) Cartoon of the *MYB61* gene showing the relative positions of the three insertions.

(B) Imbibed wild-type, *myb61-1*, *myb61-2*, and *myb61-3* seeds stained with 0.2% ruthenium red. The extruded mucilage in wild-type seeds stains as a red halo. Bar = 0.50 mm.

(C) Mass (mg) of freeze-dried aqueous soluble polysaccharide extracted from equal masses of ground dry seeds of the wild type and *myb61-1*, with *gl2-1* as a negative control. Error bars indicate ±SD. WT, wild type.

seeds using ruthenium red indicated that they were deficient in mucilage extrusion upon hydration (Figure 1B). Aqueous extracts of ground seeds showed significantly reduced levels of soluble polysaccharides in *myb61-1* seeds compared with those of the wild type (Figure 1C). To investigate the inheritance of this phenotype, we crossed *myb61-1* plants with the wild type and then observed the segregation of the insertion and the phenotype. The mucilage extrusion phenotype segregated as a single recessive locus, and inheritance patterns showed that the phenotype was expressed in the sporophytic tissues of the seed. This is consistent with the inheritance of previously characterized seed coat mutants and the sporophytic origin of seed coat tissues (Léon-Kloosterziel et al., 1994). Furthermore, the mucilage phenotype faithfully co-segregated with the *myb61-1* insertion (data not shown).

To further analyze the defect present in the *myb61-1* testa epidermis, we examined seeds by scanning electron microscopy (SEM) before and after imbibition. Wild-type seeds, when viewed using SEM, exhibit a reticulate appearance as a result of the presence of the thickened radial cell walls and a raised columella in the center of each epidermal cell (Figure 2A). Examination of seeds containing *MYB61* disruptions revealed that the seeds were the correct size and shape and that the radial cell walls had developed normally, but that the columellae were reduced in stature when compared with those of the wild type (Figure 2B). This contrasts with the phenotype seen in *ttg1-1* and *gl2-1* seeds, which lack a columella at the center of each cell (Léon-Kloosterziel et al., 1994; Figure 2C). Hence, the *myb61-1* seed coat phenotype is distinct from previously characterized seed coat mutants. Observation of *myb61-1 ttg1-1* double mutant seed coats revealed a more severe phenotype than did either the *myb61-1* or *ttg1-1* mutant alone (Figure 2D).

To investigate changes in seed coat appearance caused by imbibition, wild-type and *myb61-1* seeds were imbibed on wet filter paper and air-dried before observation by SEM. After imbibition, deep troughs were visible around the columellae of wild-type epidermal cells (Figure 2E). These corresponded to the areas in which mucilage was deposited (Western et al., 2000) and from which it had extruded during the imbibition process. The *myb61-1* seed coat was unchanged by the imbibition process (Figure 2F), indicating that no mucilage had been extruded, in agreement with observations using ruthenium red stain (Figure 1B).

Reduced Quantities of Mucilage Are Deposited during the Development of the Seed Coat Epidermis in the *myb61-1* Mutants

Mucilage accumulates during the complex developmental process of the testa epidermis. This process begins with a highly vacuolated epidermal cell and is characterized by

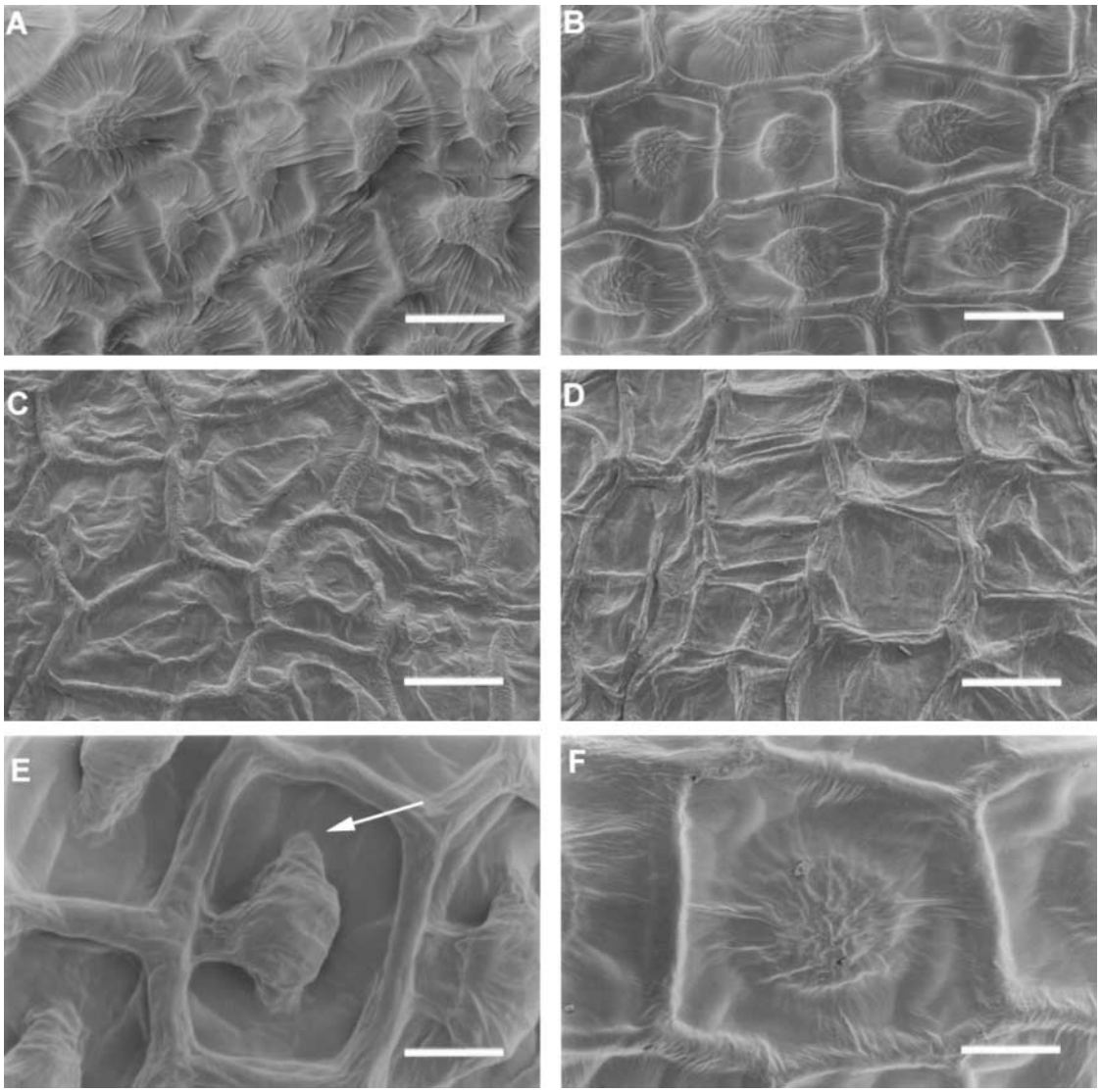

Figure 2. SEM Analysis of Wild-Type and *myb61-1* Seeds.

(A) Wild-type seed coat epidermis.
(B) *myb61-1* seed coat epidermis.
(C) *ttg1-1* seed coat epidermis.
(D) *myb61-1 ttg1-1* double mutant seed coat epidermis.
(E) Wild-type seed coat epidermal cell post-imbibition. The arrow indicates the trough remaining around the central columella from which the mucilage has extruded.
(F) *myb61-1* seed coat epidermal cell post-imbibition. No mucilage extrusion has occurred.
Bars in **(A)** to **(D)** = 20 μm; bars in **(E)** and **(F)** = 10 μm.

the formation of a central cytoplasmic column containing amyloplasts (Beekman et al., 2000; Western et al., 2000; Windsor et al., 2000), which begins to form at approximately the torpedo stage of embryo development, first against the outer tangential cell wall (Figure 3A). The deposition of mucilage occurs around the central column in the space between the primary cell wall and the plasma membrane (Western et al., 2000; Windsor et al., 2000; Figure 3E). After mucilage deposition is completed, the amyloplasts are degraded and a secondary cell wall is deposited, first around the plasma membrane, and then throughout the area occupied by the cytoplasm to form the columella.

Although seed mucilage was not extruded during imbibition of *myb61-1* seeds, mucilage deposition was observed in the course of testa development. The central cytoplasmic column appeared to form normally, but dense mucilage (revealed by light pink staining) was secreted only against the outer cell wall, whereas much of the space between the plasma membrane and the cell wall was composed of diffuse mucilage that stained faintly with toluidine blue (Figure 3F). As the cells neared maturity, a reduced volume of the cell became filled with deposited mucilage when compared with that of the wild type (cf. Figure 3Q, wild type, with Figure 3R, *myb61-1*). Secondary thickenings were deposited to form the columella in *myb61-1*, but a partial slump occurred at this stage, leading to the lower flatter structures observed under SEM (cf. Figure 2C with Figures 3N and 3R). At this stage of development, as a result of the absorption of water from the fixative (Figure 3U), it was usual for mucilage to be extruded from wild-type epidermal cells. This occurrence was rarely observed in the *myb61-1* mutants, which was consistent with previous examinations using ruthenium red stain and SEM. Thus, although no mucilage was extruded from *myb61-1* seeds during imbibition, reduced levels of mucilage were deposited during seed coat development.

Given the known genetic interactions between MYB-related transcription factors and the *TTG1* locus, we characterized the *ttg1-1* and *ttg1-1 myb61-1* double mutant phenotype in the seed coat epidermis. The *myb61-1 ttg1-1* double mutants were isolated using PCR to detect plants showing the *ttg1-1* phenotype that were also homozygous for the *myb61-1* insertion (see Methods). To minimize differences arising from the use of the two ecotypes (the *myb61-1* allele is in the Columbia background, whereas *ttg1-1* is in the Landsberg *erecta* ecotype), developing seeds from both the *ttg1-1* and *ttg1-1 myb61-1* plants without the *erecta* mutation were analyzed. Columellae began to form in *ttg1-1* testa epidermal cells, and mucilage began to be deposited into the space between the plasma membrane and the outer cell wall (Figure 3C). However, the cytoplasmic column was short lived and began to lose organization before disappearing completely to leave a highly vacuolated cell (Figures 3G and 3K). The *ttg1-1* mucilage was more diffuse than in either the wild-type or the *myb61-1* mutants; after the loss of the columellae, only a small amount had been deposited, and this was compressed by the vacuole against the outer cell wall (Figure 3K). After this stage, secondary thickening began; because the organized cytoplasmic column had disappeared, no columella formed, but thickening was observed (Figures 3O and 3S). This gave the flattened seed surface morphology characteristic of the *ttg1-1* mutant observed by SEM (Figure 2D), and no mucilage was extruded from the epidermal cells. Analyses of the *ttg1-1 myb61-1* double mutant (Figures 3D to 3T) suggested an additive phenotype with less mucilage than that of the *ttg1-1* single mutant and a more transient cytoplasmic column. Furthermore, secondary thickening was less well organized (Figure 3T) in the double mutant.

Analysis of Mucilage from Wild-Type *myb61-1* Seeds

One explanation for the failure of mucilage extrusion in the *myb61-1* mutants may be that changes in the composition or structure of the polysaccharide components might reduce the hydrophilic potential of the mucilage. To test this hypothesis, we analyzed soluble polysaccharides from the seeds of the wild type and the *myb61-1* mutants. Because mucilage was not extruded from *myb61-1* mutant seeds upon imbibition, soluble polysaccharides were extracted from ground seeds of mutant and wild-type lines and subjected to gas chromatography–mass spectrometry analysis. The results were normalized to the total amount of freeze-dried material extracted from each sample (Table 1). The most abundant monosaccharides in wild-type samples were rhamnose and galacturonic acid. This is consistent with previous analyses showing rhamnose as the principal monosaccharide of seed mucilage (Goto, 1985; Western et al., 2000) and suggested that seed mucilage comprised a large fraction of the polysaccharides extracted from whole seeds. In addition, there were significant amounts of other monosaccharides, including glucose, arabinose, and mannose, in the soluble extracts. Soluble polysaccharide extracts from *myb61-1* mutants contained approximately half the material obtained from the wild type (Figure 1C), in agreement with microscopic observations that indicated that the *myb61-1* mutant testa epidermal cells contained lower quantities of mucilage (Figure 3R). The largest reductions were seen in the rhamnose and galacturonic acid components, suggesting a reduction in the relative abundance of pectins, the major component of mucilage, in *myb61-1* seeds. Levels of all sugars were reduced with the exception of arabinose, suggesting either that the arabinose content of seed mucilage is not affected by *MYB61* or that the arabinose in the samples may come from elsewhere in the seed.

The major rhamnose-containing pectin in cotyledonous plants, RG I, consists of a backbone of alternating (1→2)-linked rhamnose and (1→4)-linked galacturonic acid residues, with side branches consisting of arabinan and arabinogalactan moieties attached to varying numbers of the rhamnose residues (Carpita and Gibeaut, 1993). To investigate the structure of seed mucilage RG I in the wild type and the *myb61-1* mutant, linkage analysis was performed on soluble polysaccharides extracted from wild-type and *myb61-1* seeds (Table 2; see Methods). Wild-type mucilage contained (1→2)-rhamnose and (1→4)-galacturonic acid, consistent with the presence of the rhamnose and galacturonic acid in the pectin RG I. This was not significantly esterified. Only small amounts of rhamnose branch point residues were detected, suggesting that the frequency of arabinan and arabinogalactan side branching in seed mucilage was low. No detectable changes in the linkage profile of seed polysaccharides in *myb61-1* were found, but various sugar components of *myb61-1* polysaccharides were reduced, consistent with the analyses in Table 1. These findings suggest that reduced mucilage secretion in *myb61-1*

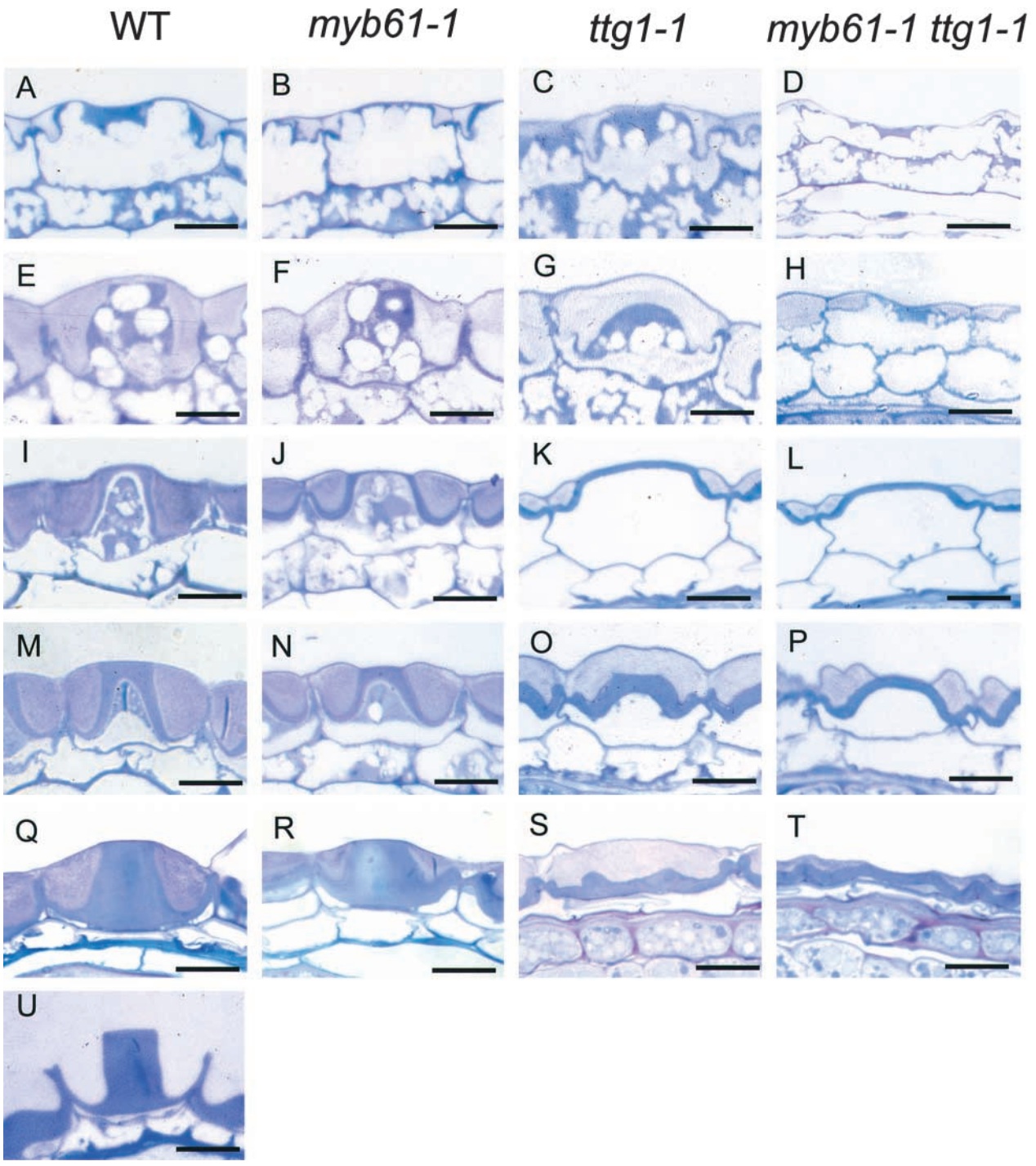

Figure 3. The Effect of the *myb61-1* and *ttg1-1* Mutations on Seed Coat Development.

(A) to **(D)** In stage 1 seeds (torpedo stage embryo), the columella begins to form against the outer tangential cell wall accumulating amyloplasts, and mucilage begins to be secreted.

(E) to **(H)** At stage 2 (initiation of cotyledon formation), a cytoplasmic column extends to the base of the testa epidermal cells in wild-type and *myb61-1* seeds, whereas *ttg1-1* testa cells (**[G]** and **[H])** have begun to lose the cytoplasmic column.

(I) to **(L)** By stage 3 (mid-cotyledon development), mucilage deposition is complete and secondary thickening of the cytoplasmic column has

seeds is not associated with changes in polysaccharide structure.

MYB61 Gene Is Expressed in the Developing Seed Coat and Is Sucrose Induced

Reverse transcription (RT)–PCR was used to assay *MYB61* transcript abundance in several tissues. Relatively high *MYB61* expression was detected in developing siliques, with reduced expression in both stem and root tissue (Figure 4A). This is in agreement with previous observations using reverse RNA gel blot analysis that found the most abundant *MYB61* expression in siliques, with lower levels in stem and young leaf tissue (Kranz et al., 1998). It is likely that the expression in roots detected by RT-PCR was beyond the threshold detectable by the reverse RNA gel blot approach. Because *MYB61* was implicated in the accumulation of polysaccharides, the potential for regulation of the *MYB61* gene by carbohydrates was investigated in seedlings. RT-PCR analysis revealed that *MYB61* expression was increased in seedlings grown on medium containing 100 mM sucrose (Figure 4B). In addition, transcripts from the *myb61-1* allele were not detectable in silique RNA, demonstrating that this allele leads to a likely loss of function (Figure 4C).

To localize the expression of *MYB61* during silique development, the β-glucuronidase (*GUS*) reporter gene (Jefferson et al., 1987) was fused to the *MYB61* promoter. Using PCR, a 2.2-kb fragment corresponding to the *MYB61* promoter was amplified, fused to the *GUS* reporter gene, and transformed into Arabidopsis using the pGREEN II vector (Hellens et al., 2000). Four independent transformants expressed the *GUS* transgene in a similar pattern, although at slightly different levels. The main sites of GUS activity were in developing seeds and the vascular system (Figure 4D). *GUS* expression directed by the *MYB61* promoter in siliques was primarily restricted to the seed coat (Figure 4D, 1 to 4), although low levels of expression were detected in the funiculi and the silique vascular tissue (data not shown). Expression was observed initially after fertilization of the ovule and was present in all cell layers of the developing seed coat, from the globular stage of development until the cotyledon stage. During the cotyledon stage of embryo development, *GUS* expression disappeared from all but the testa epidermal cell layer, where it persisted until the final stages of embryo maturation (Figure 4D, 4). In addition to being observed in the seed coat, *MYB61:GUS* expression was observed in the developing vascular tissue in both the root and inflorescence stem (Figure 4D, 5 to 7), where expression was relatively high in developing tracheary elements (Figure 4D, 5). *GUS* expression was also detected in the root cap and lateral root primordia (Figure 4D, 8 and 9). The patterns of expression of the *MYB61:GUS* transgene were consistent with those observed for *MYB61* using RT-PCR as shown in Figure 4A. No phenotypes associated with *myb61* disruptions were observed in root or vascular development.

Table 1. Cell Wall Polysaccharides from Extracted *myb61-1* versus Wild-Type Seed Soluble Polysaccharide[a]

Sugar	Wild Type[b]	*myb61-1*[b]
Rha	2.96 ± 0.11	1.10 ± 0.01
Ara	0.99 ± 0.04	0.98 ± 0.06
Xyl	0.27 ± 0.02	0.14 ± 0.04
Man	0.94 ± 0.03	0.49 ± 0.01
Gal	0.68 ± 0.06	0.45 ± 0.02
Glu	1.37 ± 0.07	0.57 ± 0.03
Fuc	tr	tr
GalA	1.72 ± 0.03	0.68 ± 0.03

[a] Data are normalized to the total mass of seed extracted. The mole percentages of sugars were calculated from total ion counts between *m/z* 50 and *m/z* 350 on the basis of response factors from authentic sugar standards.

[b] Values are the mean ±SD of three (wild type) or four (*myb61-1*) samples; mg/100 mg seed.

Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glu, glucose; Fuc, fucose; GalA, galacturonic acid; tr, trace.

Seed Mucilage Is Required for Efficient Germination and Seedling Establishment in Low–Water Potential Conditions

The hygroscopic properties of mucilage suggest that it may function to enhance water uptake during germination.

Figure 3. (continued).

begun in wild-type and *myb61-1* cells. In the *ttg1-1* and *ttg1-1 myb61-1* mutants, the vacuole expands and the mucilage is pressed to the edge of the cell, and the central cytoplasmic columns have disappeared.

(M) to **(P)** At stage 4 (mature embryo), secondary thickening proceeds in all mutants.

(Q) to **(T)** By stage 5 (maturity seed), secondary thickening is complete and wild-type cells are engorged with mucilage **(Q)**, *myb61-1* columellae have slumped **(R)**, whereas only remnants of the columellae are visible in *ttg1-1* and *ttg1-1 myb61-1* (**[S]** and **[T]**).

(U) Imbibed wild-type seed from which the mucilage has extruded.

WT, wild type. Bars = 10 μm.

Table 2. Comparison of Cell Wall Polysaccharides Extracted from *myb61-1* and Wild-Type Seed Coats[a]

Sugar and Linkage	Wild Type	*myb61-1*
Fucose		
t-Fuc	tr	tr
Rhamnose		
2-Rha	32.0	24.4
2,3-Rha	0.5	tr
2,4-Rha	0.9	0.5
Arabinose		
t-Ara	5.0	6.9
2-Ara	0.6	0.9
3-Ara	1.2	4.9
5-Ara	0.9	2.9
2,5-Ara	3.3	6.0
3,5-Ara	0.2	0.6
Xylose		
t-Xyl	0.2	tr
2-Xyl	tr	tr
4-Xyl	2.3	2.6
2,4-Xyl	0.6	0.5
Mannose		
t-Man	2.1	0.5
4-Man	7.3	9.7
4,6-Man	1.2	0.9
Galactose		
t-Gal	0.8	1.1
3-Gal	0.9	0.2
4-Gal	tr	tr
6-Gal	1.4	1.9
3,4-Gal	ND	ND
3,6-Gal	4.6	7.1
Glucose		
t-Glc	2.7	0.5
4-Glc	11.0	10.7
4,6-Glc	1.8	1.7
Galacturonic acid		
4-GalA	19.4	15.5
4-GalA (methyl ester)	tr	tr

[a] The mole percent values are the average of two independent trials; variance was <5% for all linkage groups.
tr, trace; ND, not determined.

Therefore the efficiency of germination and seedling establishment of the wild type and seed mucilage mutants were tested in conditions of reduced water potential. Seeds were germinated on filter paper moistened with water or aqueous solutions of polyethylene glycol (PEG) 8000 at varying concentrations. Germination and seedling establishment were scored by the appearance of green seedlings after 5 days (Figure 5A). Germination and establishment of wild-type seedlings were only slightly affected by PEG concentrations up to 15%, beyond which the frequency of green seedlings steadily decreased. In contrast, germination and seedling establishment of the *ttg1-1* and *gl2-1* mutants were almost completely inhibited by 10% PEG. The *myb61-1* mutants displayed levels of germination and seedling establishment intermediate between those of the wild type and *ttg1-1* and *gl2-1* seeds. This is consistent with observations showing that the *myb61* phenotype is intermediate between those of the wild type and *ttg1-1* in terms of mucilage deposition, and it suggests that seed mucilage can enhance seedling establishment when water is a limiting factor. Because mucilage was not detectably extruded upon imbibition in *myb61* and *ttg1-1* seeds, these data also suggest that the reduced level of unextruded mucilage in *myb61* seeds still contributed to increasing seedling establishment in conditions of lowered water potential. *myb61-1* plants were fertilized with wild-type pollen to create phenotypically wild-type embryos inside *myb61-1* seed coats. These germinated with an efficiency comparable to that of *myb61-1* seeds on 17% PEG, demonstrating that seed coat characteristics are responsible for the germination phenotypes observed (Figure 5B). No significant difference in germination on PEG was noted between *ttg1-1* and *myb61-1 ttg1-1* double mutant seeds (data not shown).

DISCUSSION

A screen for transposon-mediated disruptions in Arabidopsis led to the isolation of three insertions in the coding regions of the *MYB61* gene (At1 g09450) that are likely to be loss-of-function alleles (Meissner et al., 1999). Systematic phenotype screens revealed a deficiency in mucilage extrusion from the testa epidermis during imbibition in each of the three *myb61* alleles, and this phenotype segregated with the *myb61-1* insertion. No other visible phenotypes were observed. Expression analysis using RT-PCR and *GUS* reporter gene fusions with the *MYB61* promoter showed that the *MYB61* gene is most abundantly expressed in the seed coat. Together these observations demonstrate that the seed mucilage deficiency is a consequence of loss of *MYB61* function. In view of the known genetic interactions between MYB transcription factors and the *TTG1* locus (Oppenheimer et al., 1991; Larkin et al., 1999; Lee and Schiefelbein, 1999), we also undertook a detailed characterization of the *ttg1-1* mutant phenotype in the seed coat. The *myb61* gene disruptions were found to have a phenotype distinct from that of the *ttg1-1* mutant, whereas the effect of combining the *ttg1-1* and *myb61-1* mutations was additive. These results show that *MYB61* performs a novel role in the development of the testa epidermis by regulating processes involved in seed mucilage deposition and extrusion.

Role of *MYB61* in Seed Coat Development

The absence of mucilage extrusion from the seed coat was the only observed phenotype in *myb61* knockout lines in routine screens. This phenotype has been observed in plants with lesions at three known loci (*TTG1*, *GL2*, and

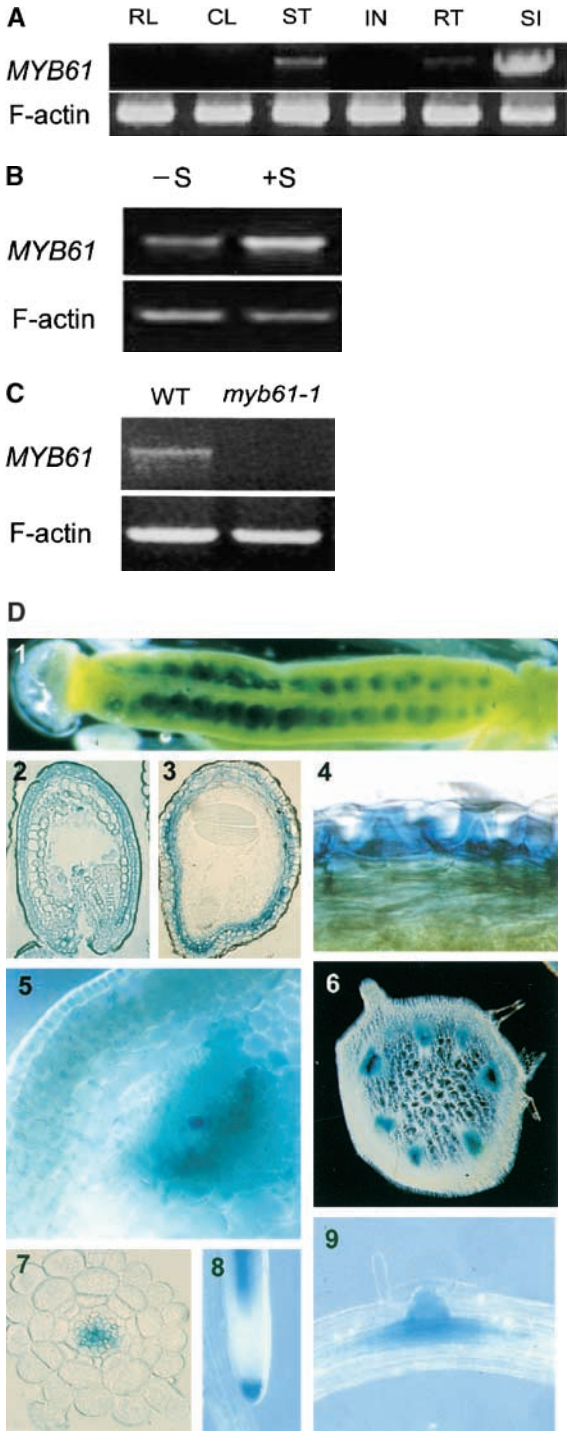

Figure 4. Expression of the *MYB61* Gene.

(A) RT-PCR showing expression of the *MYB61* cDNA in the main tissue types. RL, rosette leaf; CL, cauline leaf; ST, inflorescence stem; IN, inflorescence; RT, root; SI, silique. F-actin transcript levels were used to normalize samples.

(B) The effect of sucrose on *MYB61* expression in seedlings. −S, MS medium contained no sucrose; +S, MS medium contained 100 mM sucrose.

(C) RT-PCR showing undetectable expression of *MYB61* in siliques of the *myb61-1* mutant. WT, wild type.

(D) Histochemical analysis of *GUS* reporter gene expression from the *AtMYB61* promoter. (1) Expression in developing seeds within a silique; expression in the seed coat in (2) heart stage and (3) cotyledon stage seeds; (4) expression in the seed coat epidermis during late stages of seed development; and expression in vascular tissue of inflorescence stem (5 and 6), primary root (7), root cap (8), and lateral root primordia (9). Developing seed material was stained for 1.5 to 3 hr, and other tissues were stained for up to 10 hr.

AP2; Koornneef, 1981; Koornneef et al., 1982; Jofuku et al., 1994) and was thought to be the result of the absence of mucilage deposition in the testa epidermis. Sections of developing *myb61-1* seed coats revealed the deposition of mucilage, but in reduced amounts when compared with that of the wild type. This observation was supported by biochemical analyses of soluble polysaccharides from ground seeds that showed a 50% reduction in soluble polysaccharides in *myb61-1* seeds. Deposition of mucilage in *myb61-1* seed coats was characterized by areas that stained only faintly with toluidine blue compared with the wild type, and these may represent areas in which the mucilage is diffuse and possibly swollen by absorbed water during the fixation of sections. Such differential mucilage depositions have been observed in other plant species, including Hibiscus and Cinnamon (Bakker and Baas, 1993). Mucilage deposition was also observed in the *ttg1-1* (and *gl2-1*; S. Penfield and M.W. Bevan, unpublished data) seed coats, although only in small quantities.

Mucilage extrusion occurs upon hydration of the mucilage as a result of the rupture of the primary cell wall under hydrostatic pressure (Western et al., 2000). The reduced levels of mucilage probably contribute to reduced pressure on the primary cell wall in the *myb61* and *ttg1-1* mutants and the lack of extrusion at imbibition. The unchanged structure of mucilage components in the *myb61-1* mutant (Table 2) suggests that it is unlikely that the structure of mucilage components is responsible for reduced extrusion by the mucilage. The observation that mucilage does not extrude even from immature wild-type cells suggests that the mucilage or the epidermal cells may be modified during the final stages of testa maturation. One possibility is a partial hydrolysis of the primary wall to ease the extrusion process. This could require the secretion of hydrolytic enzymes and may be defective in both the *myb61* and *ttg1-1* mutants. Hence, they may promote the deposition of both mucilage polysaccharides and cell wall–modifying proteins between the wall and plasma membrane. GUS activity expressed from a *MYB61:GUS* transgene was detected in the developing seed coat, consistent with the data obtained by RT-PCR.

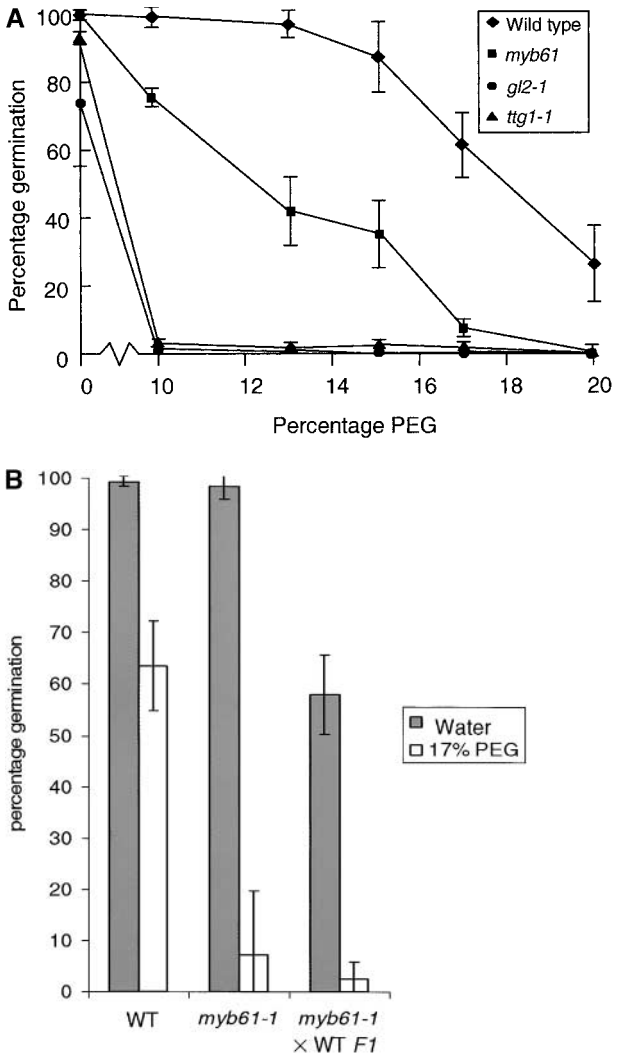

Figure 5. Seedling Establishment of Arabidopsis Seed Mucilage Mutants on Media Containing Polyethylene Glycol.

(A) Comparison of the germination of the wild type and seed mucilage mutants on increasing concentrations of PEG.
(B) Germination of heterozygous (phenotypically wild type [WT]) embryos in *myb61-1* seed coats on 17% PEG.
Data points represent the mean and standard deviation of three independent experiments in both panels.

This expression pattern conforms to the proposed role of *MYB61* in seed coat development and mucilage deposition.

Another feature of the *myb61-1* phenotype was the slump of the columella during secondary thickening, which may be because mucilage is required to stabilize the columella structure as thickening proceeds. *Brassica campestris* mucilage is secreted against the center of the outer tangential cell wall, and no columellae are formed (Van Caeseele et al., 1981), suggesting a correlation between the area in which mucilage is secreted and the final morphology of the seed coat epidermis. There is considerable variation in seed coat morphology throughout the Brassicacae (Vaughan and Whitehouse, 1971), including species such as Capsella, in which the seed coat resembles the *myb61-1* mutant phenotype. It is possible that changes in the expression or activity of MYB transcription factors may play an important role in the evolution of adaptive seed coat traits.

MYB61 Promotes the Accumulation of Linear Rhamnogalacturonans

Both wild-type and *myb61-1* mucilage were rich in the pectin RG I, as demonstrated by the presence of (1→2)-linked rhamnose and (1→4)-linked galacturonic acid residues as the most abundant monosaccharides (Carpita and Gibeaut, 1993). Linkage analysis demonstrated that the frequency of side branching is low in Arabidopsis seed mucilage RG I. This agrees with and extends previous analyses that have shown that rhamnose is the main neutral sugar component of Arabidopsis mucilage (Goto, 1985; Western et al., 2000). The monosaccharide composition of mucilage in *myb61-1* lines showed a reduction of both rhamnose and galacturonic acid in whole-seed soluble polysaccharides, suggesting reduced abundance of RG I in *myb61-1* seeds. Linkage analysis demonstrated that there were no detectable differences in the branching structure of RG I in wild-type and *myb61-1* seeds. Mucilage RG I was not subject to substantial esterification in either wild-type or *myb61-1* seeds, and it is similar to the non-methylesterified pectin in mucilage secreted from clover root cap cells (Lynch and Staehelin, 1992). RG I molecules are also released from in vitro–differentiating *Zinnia elegans* tracheids (Stacey et al., 1995). *MYB61* was also expressed at lower levels in developing vascular tissue and in the root cap (Figure 4D), suggesting a potential link between *MYB61* expression and pectin synthesis, secretion, or deposition. This suggests a more specific role for *MYB61* in regulating RG I pectin levels in the developing testa. However, *MYB61* is also expressed in other cell layers of the seed coat during early seed development. Little is known about the synthesis of polysaccharides in these cells, but it is possible that *MYB61* has either a function in pectin accumulation in these cells or more general functions, including pectin accumulation.

Similarities in the Development of Vascular and Testa Tissues

Although there is little vascular development in the Arabidopsis seed coat, the seed coats of many dicotyledonous species exhibit varying degrees of vascularization (Fahn,

1990), suggesting that vascular tissue and the seed coat may share elements of a common developmental pathway. There are several similarities between vascular development and seed coat development: in Arabidopsis, both cell types exhibit pectin secretion, secondary cell wall deposition, primary cell wall modification, and eventual cell death (Fukuda, 1997). It is possible that *MYB61* plays a role in the morphogenesis of cell types that share this set of developmental characteristics. As described above, this role may include promotion of secretory processes.

Role of TTG1 in Seed Coat Epidermal Development

The *TTG1* gene is required for the patterning and initiation of trichomes, root atrichoblasts, and flavonoid biosynthesis as well as mucilage secretion (Koornneef, 1981; Lloyd et al., 1992). Formation of cytoplasmic columns and secretion of mucilage began normally in the *ttg1-1* mutant, suggesting that TTG1 is not required for the initiation of testa epidermal cell development. However, *ttg1-1* cells cease mucilage secretion during early stages of testa development, and the cytoplasmic columns degrade before secondary thickening of the collumella occurs, whereas the vacuole expands to occupy most of the space within the cell. This state is similar to the morphology of the subepidermal palisade cell layer at this stage and suggests a progressive loss of epidermal identity of the mucilage-secreting cells. The mucilage secreted in *ttg1-1* appears to swell and absorb water, filling a large volume of the epidermal cells, but it is not extruded through the primary cell wall. This contrasts with both wild-type and *myb61-1* cells. However, it is clear that when the vacuole expands and the mucilage is compressed, little mucilage is deposited, even compared with the *myb61-1* mutants. The extra swelling may be the result of the presence of more space in the cells as a result of the degradation of the cytoplasmic columns that constrain the mucilage in wild-type and *myb61-1* testa cells. The observation that secondary thickening occurs in the remaining cytoplasm of both *ttg1-1* and *myb61-1* mutants suggests that this process is genetically separable from earlier events in the maturation of the testa epidermis.

These observations are consistent with a model in which *TTG1* activity is not required for the early stages of specification of the seed coat epidermis but is required to maintain the differentiated state of the epidermal cells after the assumption of the mucilage-secreting cell fate. Further support for the hypothesis that *TTG1* activity is not required for the earliest events of epidermal cell differentiation comes from the analysis of an atrichoblast-specific Green Fluorescent Protein marker J2301 in roots (Berger et al., 1998). Its expression is confined to atrichoblasts even in the *ttg1-1* mutant background, demonstrating the occurrence of atrichoblast-specific events in the absence of *TTG1* function (S. Costa and L. Dolan, personal communication). Hence, further factors must be necessary for the initial specification of mucilage cell fate. These may include the *AP2* gene product, strong alleles of which cause a more severe epidermal cell phenotype than those of *ttg1-1* or *gl2-1* (Jofuku et al., 1994). Alternately, it is possible that one or more bHLH transcription factors, known to interact with the TTG1 protein (Payne et al., 2000), may perform this function.

Analysis of *myb61-1 ttg1-1* double mutants showed an additive effect of these genes on columella development and mucilage accumulation at later stages of testa development. The *myb61-1 ttg1-1* double mutants secreted less mucilage than either *myb61-1* or *ttg1-1* alone, and this was accompanied by a more severe columella defect. Thus, *MYB61* may function in a genetic pathway independent of *TTG1*, yet it is also possible that both *MYB61* and *TTG1* act together in a genetic pathway that still retains some function in the *ttg1-1* mutant background. It is also possible that MYB61 function may overlap with that of closely related MYB transcription factors, and consequently the phenotype of the *myb61* mutants may not reflect that of a complete loss-of-function mutant. The possible residual function of the *ttg1-1* allele and the time and place of MYB61 and TTG1 function during testa development will need to be established to distinguish between these possibilities.

Seed Mucilage Facilitates Germination and Seedling Establishment in Dry Conditions

Seed mucilage is a natural example of a hydrogel (Zwieniecki et al., 2001) and is an efficient absorber of water. We therefore tested the hypothesis that mucilage takes up water to increase and stabilize water potential surrounding the seed, thereby promoting efficient germination and seedling establishment. The seed mucilage mutants tested germinated and grew normally under standard laboratory conditions, but as the water potential of the medium was lowered, establishment of *ttg1-1*and *gl2-1* seedlings was strongly reduced compared with that of the wild type, whereas *myb61-1* seeds displayed intermediate levels of seedling establishment. This effect was unrelated to effects of the *ttg1-1* and *gl2-1* mutants on seed dormancy, because they have been reported to exhibit decreased and increased seed dormancy, respectively (Debeaujon et al., 2000a). The stronger phenotype of *ttg1-1* and *gl2-1* compared with *myb61-1* may reflect the smaller quantity of mucilage deposited or, alternately, the ease of mucilage extrusion from the testa epidermal cells. This experiment also demonstrates that the remaining mucilage in *myb61-1* mutants performs a partial function. The definition of plant seed mucilage function during germination suggests that the production of seed mucilage is an adaptation to environments of low or variable water availability, such as those encountered during the evanescent growth of Arabidopsis. Further definition of the roles of *MYB61* in this process may suggest ways in which seed mucilage production could be modified to alter seedling establishment patterns.

Testa as a Model System

The development of the Arabidopsis seed coat epidermis is an excellent model system for establishing links between the regulation of cell differentiation and control of the biosynthesis and exocytosis of polysaccharides. Copious amounts of mucilage are secreted that are easily extractable for biochemical analysis, and facile mutant screens should identify a number of genes required for these processes. In addition, similarities between seed coat and xylem suggest that understanding morphogenesis of the testa epidermis may be relevant to defining mechanisms controlling the development of vascular cells. Future work aims to establish the sets of genes regulated by MYB61. Among these may be genes encoding components of polysaccharide synthesis and secretion. The isolation and characterization of additional testa mutants should improve our understanding of the complex processes underlying polysaccharide secretion and its contribution to plant cell differentiation and architecture.

METHODS

Plant Materials

Arabidopsis thaliana plants carrying the *myb61-1* allele in the Columbia background were obtained from the Sainsbury Laboratory *Arabidopsis thaliana* (SLAT) population using Inverse Display of Insertion filter screens with a digoxygenin-labeled (Roche, Lewes, UK) *MYB61*-specific probe (Meissner et al., 1999; Tissier et al., 1999). The isolation of the *myb61-2* and *myb61-3* alleles has been described by Meissner et al. (1999). The *ttg1-1* allele was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK).

Ruthenium Red Staining of Seed Mucilage

Whole seeds were allowed to imbibe on moist filter paper for between 5 min and 1 hr, before the application of 0.2% w/v aqueous ruthenium red (Sigma) solution. Seeds were photographed with a WILD MZ8 dissecting microscope (Leica UK Ltd., Milton Keynes, UK).

Microscopy and Histology

For scanning electron microscopy (SEM), dry seeds were gold coated using a High Resolution Sputter Coater (Agar, Redding, CA) and imaged in a Philips (FEI UK Ltd., Cambridge, UK) XL30 scanning electron microscope with an accelerating voltage of 3 kV. Imbibed seeds were air dried overnight on filter paper and then treated as dry seeds. For thin sections of seed coats, whole siliques were fixed in 2.5% glutaraldehyde (Sigma Aldrich) in 0.05 M sodium cacodylate buffer, which was vacuum infiltrated before dehydration in a graded ethanol series, and infiltrated with LR White resin (London Resin Company). Polymerization was performed overnight at 60°C. Sections of between 200 and 400 nm in thickness were made with a Reichert Ultramicrotome (Leica UK Ltd., Milton Keynes, UK) and stained with toluidine blue.

AtMYB61 Promoter::*GUS* Fusion

Standard molecular biology techniques were employed as described (Sambrook et al., 1989). The *MYB61* promoter was amplified as a 2.2-kb XhoI-NcoI fragment from genomic DNA by use of primers F61P (5′-CCTCGAGCTACACTTTCTGACCAAGAC-3′) and R61P (5′-CCATGGTTAGTTATTCACAGCTGCAATG-3′) and cloned into the pGEM-T vector (Promega, Southampton, UK) according to the manufacturer's instructions. The β-glucuronidase (*GUS*) gene fused to the nopaline synthase terminator was subcloned from the pRAJ275 vector (Jefferson et al., 1987) as a HindIII-EcoRI fragment into the pGREEN II vector (Hellens et al., 2000) to make plasmid pGREENGUS. The XhoI-NcoI MYB61 promoter was then subcloned upstream of the *GUS* gene in pGREENGUS. This was transformed into Arabidopsis Columbia ecotype by Agrobacterium-mediated floral dip (Clough and Bent, 1998). Histochemical detection of *GUS* expression was performed as described (Jefferson et al., 1987). Seeds and roots were fixed in 2.5% glutaraldehyde (Sigma) in 0.05% sodium cacodylate buffer before dehydration in a graded ethanol series and embedding in Technovit Historesin (Kulzer Heraeus, Wehreim, Germany) according to the manufacturer's protocol. Hand-cut sections were made from GUS-stained stem tissue and photographed on a Microphot (Nikon, Kingston, UK) 600 microscope. Whole-mount tissues were photographed using a WILD MZ8 dissecting microscope (Leica).

Reverse Transcription–Polymerase Chain Reaction

RNA was isolated using TRIZOL reagent (Gibco BRL) from aerial tissues of greenhouse-grown plants and from roots grown for 3 weeks in liquid culture (Kranz et al., 1998), using the manufacturer's protocol. Seedlings were grown for 7 days in tissue culture on Murashige and Skoog (Duchefa, Haarlem, The Netherlands) media with or without 100 mM sucrose (Sigma). First-strand cDNA was synthesized from 5 μg of RNA using M-MLV Reverse Transcriptase (Gibco BRL) according to the manufacturer's instructions. Reverse transcription–polymerase chain reaction (RT-PCR) for the *MYB61* gene used primers F61Bam (5′-GGATCCATGGGGAGACATTCTTTGCTGTTAC-3′) and R61Eco (5′-GAATTCTAAAGGGACTGACCAAAAGAGAC-3′), using 1 μL of the first-strand reaction on a Touchdown thermocycler (Hybaid, UK) under 90°C for 2 min, and then 35 cycles of the following: 90°C for 40 sec, 65°C for 1 min, 72°C for 2 min, and then 72°C for 5 min. The actin control was amplified using primers ACT1 (5′-GCCAAAGCAGTGATCTCTTTGCTC-3′) and ACT2 (5′-GTGTTGGACTCTGGAGATGGTGTG-3′), using the above reaction conditions with either 25 or 35 amplification cycles.

Isolation of *myb61-1 ttg1-1* Double Mutants

ttg1-1 plants were crossed to *myb61-1*, and double mutants were isolated from the resulting F2 population by PCR. Plants showing the *ttg1-1* phenotype were tested using primers F61Bam and R61Eco and the above PCR conditions to detect the wild-type allele, whereas R61Eco and the En8130 primer (Wisman et al., 1998) were used to amplify a product from the *myb61-1* insertion.

Seed Mucilage Extraction

Equal masses of ground dry wild-type and *myb61-1* seeds were initially extracted with 2 M imidazole, pH 7.5; insoluble material was removed by centrifugation, and the supernatant was precipitated with 5 volumes of ethanol. The precipitated material was redissolved in water; insoluble material was removed by centrifugation and precipitated with 5 volumes of ethanol. This was repeated three times before freeze-drying the soluble polysaccharide before measuring yield.

Determination of Sugar and Polymer Composition of the Pectic and Alkali-Soluble Polysaccharides

Uronic acid units in polysaccharides in ammonium oxalate extracts of freeze-dried soluble polysaccharides were activated by the water-soluble diimide, 1-cyclo-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMC), and reduced with $NaBD_4$ to their respective 6,6-dideuterio neutral sugars, as described by Kim and Carpita (1992). One to two mg of each fraction was hydrolyzed with 2 M trifluoroacetic acid (TFA) containing 1 μmol of *myo*-inositol (internal standard) for 90 min at 120°C. The TFA was evaporated under a stream of nitrogen, and the sugars were converted to alditol acetates (Gibeaut and Carpita, 1991). The alditol acetates were separated by gas-liquid chromatography (GLC) on a 0.25-mm × 30-m vitreous silica capillary column of SP-2330 (Supelco, Bellefonte, PA). Temperature was programmed from 170°C to 240°C at 5°C per min with a 6-min hold at the upper temperature. The 6,6-dideuteriogalactosyl units, diagnostic of GalA, were determined by the shift of 2 amu of the secondary fragments from electron-impact mass spectrometry (EIMS) from those of Gal. Diagnostic pairs used were *m/z* 189/191, *m/z* 217/219, *m/z* 259/261, and *m/z* 289/291, and amounts were calculated by the equation described by Kim and Carpita (1992).

Linkage Analyses

Polysaccharides, with uronic acids that had been reduced with $NaBD_4$ to their respective 6,6-dideuterio sugars, were per-*O*-methylated with Li^+ methylsulfinylmethanide and methyl iodide according to Gibeaut and Carpita (1991). The per-*O*-methylated polymers were recovered after addition of water to the mixture and partitioning into chloroform. The chloroform extracts were washed five times with a threefold excess of water, and the chloroform was evaporated. The permethylation step was repeated, and the methylated polymers were purified by chloroform partitioning and were hydrolyzed in 2 M TFA for 90 min at 120°C. The TFA was evaporated in a stream of nitrogen gas, and the sugars were then reduced with $NaBD_4$ and acetylated. The partly methylated alditol acetates were separated on the column as the alditol acetates but in a temperature program of 160°C to 210°C at 2°C per min, then to 240°C at 5°C per min with a hold of 5 min at the upper temperature. GLC-EIMS analysis was used to verify all derivative structures (Carpita and Shea, 1989).

Determination of Degree of Esterification

The esterified uronic acid residues, regardless of the leaving group, were chemically reduced to their respective neutral sugars by addition of sodium borohydride (Maness et al., 1990). We devised a variation of this method whereby the pectic material was dissolved or suspended in 4 mL of 25 mM sodium acetate, pH 4.6 (Carpita and McCann, 1996). The suspension was split into two equal samples, chilled to ice temperature, and while stirring continuously, 1 mL of 2 M imidazole (HCl), pH 7.0, and 300 mg of sodium borohydride or sodium borodeuteride were added, respectively, to the two samples. The mixtures were slowly warmed and incubated at ambient temperature with continuous stirring. The reactions were stopped by addition of glacial acetic acid to destroy the excess borohydride and bring the pH to below 5. The samples were dialyzed overnight against running deionized water. The samples then were returned to 25 mM sodium acetate, pH 4.6, with continuous stirring, and the remaining unesterified uronic acids were activated with CMC. The mixtures were chilled to ice temperature, and 1 mL of 2 M imidazole (HCl), pH 7.0, and 300 mg of sodium borodeuteride or sodium borohydride were added, respectively, to the samples. The samples were incubated, and the reactions stopped as described previously; after extensive dialysis against running deionized water, the materials were freeze-dried. As described above, alditol acetate derivatives were prepared and separated by GLC, and the mole fractions of 6,6-dideuteriogalactose were determined by EIMS. The mole fraction of 6,6-dideuteriogalactose deduced from the primary borodeuteride reduction–secondary borohydride reduction represented the esterified uronic acid, whereas the amount deduced from primary borohydride reduction–secondary borodeuteride reduction represented the amount of free acid; the remainder was nascent galactose.

Germination on Polyethylene Glycol

Two-week-old seeds were placed on 9-cm filter paper discs sealed in an upturned Petri dish and moistened with 1 mL of water or polyethylene glycol (PEG) 8000 solution from 10 to 20% concentration (Sigma). Seeds were stratified for 3 days at 4°C. Seedling establishment was scored as the appearance of green seedlings after 5 days. Each treatment was performed in triplicate with ~50 seeds per genotype per treatment.

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