

Isolation and Characterization of Mutants Defective in Seed Coat Mucilage Secretory Cell Development in Arabidopsis¹

Tamara L. Western², Joanne Burn³, Wei Ling Tan, Debra J. Skinner⁴, Luke Martin-McCaffrey, Barbara A. Moffatt, and George W. Haughn*

Department of Botany, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z4 (T.L.W., J.B., W.L.T., D.J.S., G.W.H.); and Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1 (L.M.-M., B.A.M.)

In Arabidopsis, fertilization induces the epidermal cells of the outer ovule integument to differentiate into a specialized seed coat cell type producing extracellular pectinaceous mucilage and a volcano-shaped secondary cell wall. Differentiation involves a regulated series of cytological events including growth, cytoplasmic rearrangement, mucilage synthesis, and secondary cell wall production. We have tested the potential of Arabidopsis seed coat epidermal cells as a model system for the genetic analysis of these processes. A screen for mutants defective in seed mucilage identified five novel genes (*MUCILAGE-MODIFIED [MUM]1–5*). The seed coat development of these mutants, and that of three previously identified ones (*TRANSPARENT TESTA GLABRA1*, *GLABRA2*, and *APETALA2*) were characterized. Our results show that the genes identified define several events in seed coat differentiation. Although *APETALA2* is needed for differentiation of both outer layers of the seed coat, *TRANSPARENT TESTA GLABRA1*, *GLABRA2*, and *MUM4* are required for complete mucilage synthesis and cytoplasmic rearrangement. *MUM3* and *MUM5* may be involved in the regulation of mucilage composition, whereas *MUM1* and *MUM2* appear to play novel roles in post-synthesis cell wall modifications necessary for mucilage extrusion.

Fertilization of the angiosperm ovule not only results in the development of the embryo and endosperm, but also initiates differentiation of the ovule integuments to form the seed coat. The seed coat consists of multiple specialized cell layers that play important roles in embryo protection and the regulation of germination. One specialization is known as myxospermy, a property of epidermal cells whereby they produce large quantities of pectic polysaccharide (mucilage; Frey-Wyssling, 1976; Grubert, 1981; Boesewinkel and Bouman, 1995). Myxospermy is commonly found in species of the Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae, where mucilage forms a gel-like capsule surrounding the seed

upon imbibition. Proposed roles for mucilage include facilitating seed hydration and/or dispersal. Mucilages are also found in the root cap and transmitting tract (Frey-Wyssling, 1976; Esau, 1977), where they foster root tip and pollen tube growth, respectively.

Mucilages are largely composed of pectins, a heterogeneous group of complex, acidic polysaccharides that also comprise the majority of the plant cell wall matrix. Dicotyledonous pectins largely consist of poly-GalUA (PGA) and rhamnogalacturonan I (RG I; Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997). PGA is composed of an unbranched chain of α 1,4-linked GalUA residues, whereas RG I is a highly branched polysaccharide with a backbone of alternating α 1,4-linked GalUA and α 1,2-linked rhamnose (Rha), with sugar side chains attached to the Rha residues (Brett and Waldron, 1990). The degree of gelling of pectins is largely dependent on ionic bonding between PGA molecules and free divalent calcium. Thus, cell wall fluidity is affected by the degree of methyl esterification of PGA carboxyl groups and the frequency of interruptions of homogalacturonan chains with RG I (Brett and Waldron, 1990; Carpita and Gibeaut, 1993). Studies of PGA and RG I production have shown that they are manufactured in the Golgi apparatus, then transported to the extracellular matrix via secretory vesicles (Brett and Waldron, 1990; Zhang and Staehelin, 1992; Driouich et al., 1993; Staehelin and Moore, 1995; Dupree and Sherrier, 1998). Although some pectin biosynthetic enzymes have been identified biochem-

¹ This work was supported by the Natural Sciences and Engineering Research Council of Canada (research grants to G.W.H. and B.A.M.), by the Killam Foundation (Predoctoral Fellowship to T.L.W.), by the University of British Columbia (University Research Fellowship to W.L.T.), and by the Zimbabwe-Canada General Training Facility Scholarship (to D.J.S.).

² Present address: Waksman Institute, Rutgers University, 190 Frelinghuysen Road, Piscataway, NJ 08854.

³ Present address: Plant Cell Biology, Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra, ACT 2601, Australia.

⁴ Present address: Section of Molecular and Cellular Biology, University of California, 1 Shields Avenue, Davis, CA 95616.

* Corresponding author; e-mail haughn@interchange.ubc.ca; fax 604-822-6089.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010410.

ically (e.g. Rodgers and Bolwell, 1992; Piro et al., 1993; Doong and Mohnen, 1998; Edwards et al., 1999; Perrin et al., 1999; Seitz et al., 2000), little is known about the regulation of complex polysaccharide biosynthesis and secretion.

The genetic model species *Arabidopsis*, a member of the Brassicaceae, is myxospermous. In addition, its epidermal cells are marked by a central volcano-shaped secondary cell wall known as the columella (Vaughn and Whitehouse, 1971; Koornneef, 1981). Differentiation of the outer integument epidermal cells to form the seed mucilage cells involves a highly regulated series of events, including growth, morphogenesis, mucilage biosynthesis and secretion, and secondary cell wall production (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). Although the presence of mucilage in *Arabidopsis* seeds is dispensable under laboratory conditions, only a few genes affecting seed coat morphology have been identified: *TRANSPARENT TESTA GLABRA1* (*TTG1*), *GLABRA2* (*GL2*), *APETALA2* (*AP2*), *ABERRANT TESTA SHAPE*, and *ABSCISIC ACID DEFICIENT1*. In each case, the seed coat defect has been noted as a pleiotropic effect of a mutation in the gene and the seed specific cellular defects have not been investi-

gated in detail. *ttg1* and *gl2* mutants were both originally identified as trichome mutants, which lack leaf hairs (Koornneef, 1981; Rerie et al., 1994). Further study of each revealed epidermal cell defects throughout the plant in the form of extra root hairs and lack of seed coat mucilage and columellae (Koornneef, 1981; Bowman and Koornneef, 1994; Galway et al., 1994; Rerie et al., 1994; Di Cristina et al., 1996; Masucci et al., 1996). *AP2* is involved in the regulation of both flower and ovule development (Bowman et al., 1989, 1991; Kunst et al., 1989; Modrusan et al., 1994; Western and Haughn, 1999). Observation of *ap2* seeds showed altered seed shape and the absence of mucilage and columellae (Bowman and Koornneef, 1994; Jofuku et al., 1994). Mutants defective in *ABERRANT TESTA SHAPE*, a gene involved in ovule integument development, have heart-shaped seeds that have a reduced amount of mucilage (Léon-Kloosterziel et al., 1994). Finally, *abscisic acid deficient1* mutants, as part of their syndrome resulting from lack of abscisic acid, produce a reduced amount of mucilage (Karssen et al., 1983).

As a first step in analyzing the complex process of *Arabidopsis* seed epidermal cell differentiation, we and others have made a detailed study of wild-type

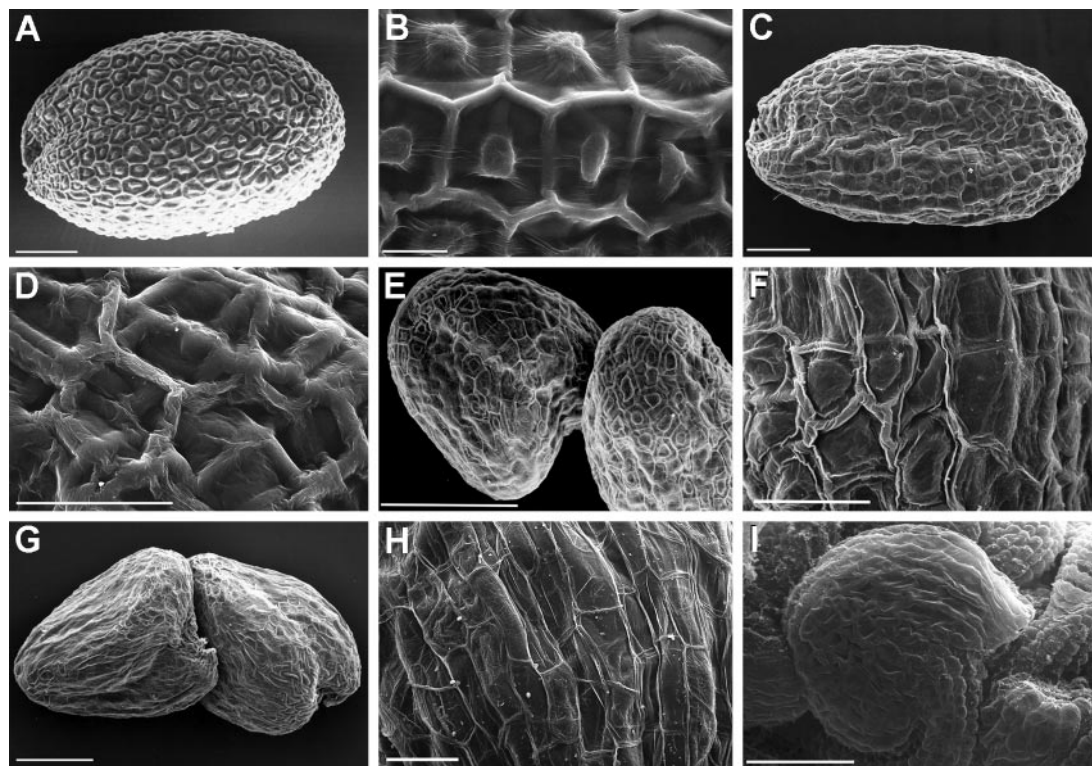


Figure 1. Scanning electron micrographs showing whole seed and seed coat details of wild-type and mutant seeds. A, Wild-type seed. Note hexagonal epidermal cells with thickened radial cell walls and volcano-shaped columellae in the center of each cell. B, Detail of wild-type seed coat. C, *mum4-1* seed. The columellae are absent. D, Detail of *mum4-1* seed coat. E, *ap2-1* seeds. Seeds are heart shaped and columellae are absent or reduced. F, Detail of *ap2-1* seed coat. G, *ap2-6* seeds. Seeds are heart shaped and epidermal cells are thin walled, rectangular, and lack columellae. H, Detail of *ap2-6* seed coat. I, Mature wild-type ovule. Note similarity in shape to *ap2-1* and *ap2-6* seeds. Scale bars: A and C, 100 μm ; B, 20 μm ; D, F, H, and I, 40 μm ; E and G, 200 μm .

development and mucilage composition (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). In this paper, we report the use of a novel screen for the isolation of mutants specifically defective in the mucilage-containing seed coat epidermal cells. Our characterization of the seed coat defects in five such mutants (*mucilage-modified [mum] 1–5*), as well as the previously identified mutants *ap2*, *ttg1*, and *gl2*, indicate that the products of these mutated genes act at multiple steps in seed coat epidermal cell differentiation, ranging from the regulation of outer integument differentiation to mucilage biosynthesis and post-deposition cell wall modification.

RESULTS

Wild-Type Seed Coat Development and Mucilage Production

The epidermal layer of the wild-type seed coat of *Arabidopsis* is marked both by cell morphology and the presence of mucilage. Figure 1, A and B, shows that the epidermal cells are hexagonal, with thickened radial cell walls and a central, volcano-shaped structure known as the columella. When *Arabidopsis* seeds are hydrated, there is immediate extrusion of pectinaceous mucilage from the epidermal cells. Staining with Ruthenium red (Western et al., 2000) and studies with several pectin-specific antibodies (Willats et al., 2001) revealed that there are both an outer, diffuse layer, and an inner, dense capsule of mucilage directly surrounding the seed as shown in Figure 2A.

The late differentiation of the integuments of the wild-type ovule into the seed coat of *Arabidopsis* has been divided into five dynamic stages (Western et al., 2000). The first stage, passing from Figure 3, A to B, is a period of cell growth driven by vacuolar expansion to give cells with a large central vacuole surrounded by cytoplasm. Stage 2 is marked by the accumulation of large starch granules (amyloplasts) and later by the drawing of the cytoplasm away from the edges of the cell. Stage 3 has further rearrangements such that the cytoplasm becomes a discrete column the center of the cell. In addition, the cells manufacture and secrete large quantities of pectic mucilage between the plasma membrane and primary cell wall on the outer tangential face of the cell as shown in Figure 3, C and D, and Figure 4A. In stage 4, mucilage production has ceased and a new, secondary cell wall is produced that replaces the column of cytoplasm, resulting in the columella (Figs. 3E and 4C). Desiccation of the seed in Stage 5 leaves a central columella surrounded by shrunken mucilage, with the entire cell overlaid by the primary cell wall (Fig. 3K). Upon hydration of the seed, the hydrophilic mucilage swells rapidly, leading to the rupture of the primary cell wall at the thin radial cell walls and release of the gel-like mucilage to surround the seed (Fig. 3F).

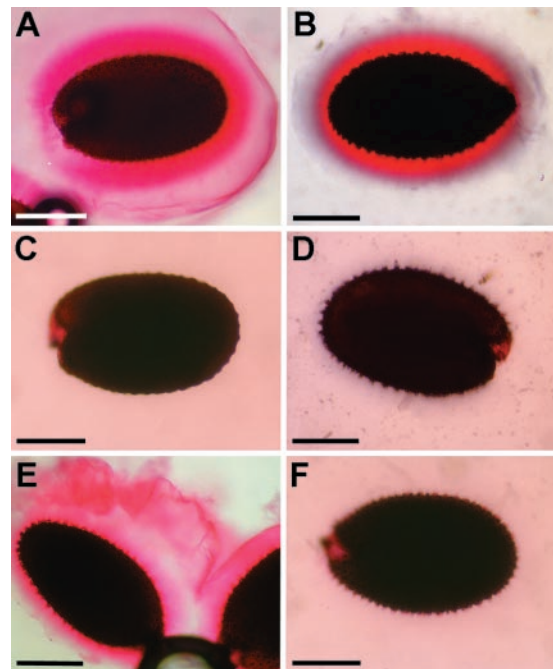


Figure 2. Ruthenium red staining of wild-type and mutant seeds. A, Wild-type seed placed directly into stain without agitation. Two layers of mucilage are present, an outer, cloudy layer, and an inner, intensely staining layer. B, Wild-type seed first shaken in water, then stained; the outer layer of mucilage is not stained. Note sharp outline to the columellae. C, *mum2-1* seed stained after shaking in water. No capsule of mucilage is apparent and the columellae are less defined than in B. D, *mum5-1* seed stained after shaking in water. Columellae are sharply outlined and a thin layer of palely staining mucilage is apparent directly around the seed. E, *mum5-1* seed placed directly in stain without shaking. Both inner and outer layers of mucilage are present, as in A. F, Wild-type seed stained after first shaking in presence of EDTA. Only a thin layer of palely staining mucilage is visible, as in D. Scale bars = 200 μ m.

Identification and Categorization of Mucilage-Modified Mutants

All mutants known to have aberrant seed coat epidermal cells are also defective in other cell types. We sought to isolate mutants affected only in seed coat epidermal cells to identify genes involved specifically in the differentiation of this cell type. To screen for such mutants, we took advantage of the high visibility of both mucilage and seed coat of imbibed seeds stained with Ruthenium red. Wild-type *Arabidopsis* seeds shaken in water, then stained with Ruthenium red are surrounded by a pink capsule of mucilage alone (Fig. 2B). In a preliminary screen of approximately 1,000 ethylmethane sulfonate-mutagenized M3 lines, 12 mutants were isolated that either lacked or had a reduced pink mucilage capsule. Complementation tests among these mutants and with the known seed coat mutants led to the identification of five novel complementation groups that have been named *mucilage-modified (mum) 1* through 5, with only *mum2* and *mum4* having more than one independent allele. Reciprocal crosses to

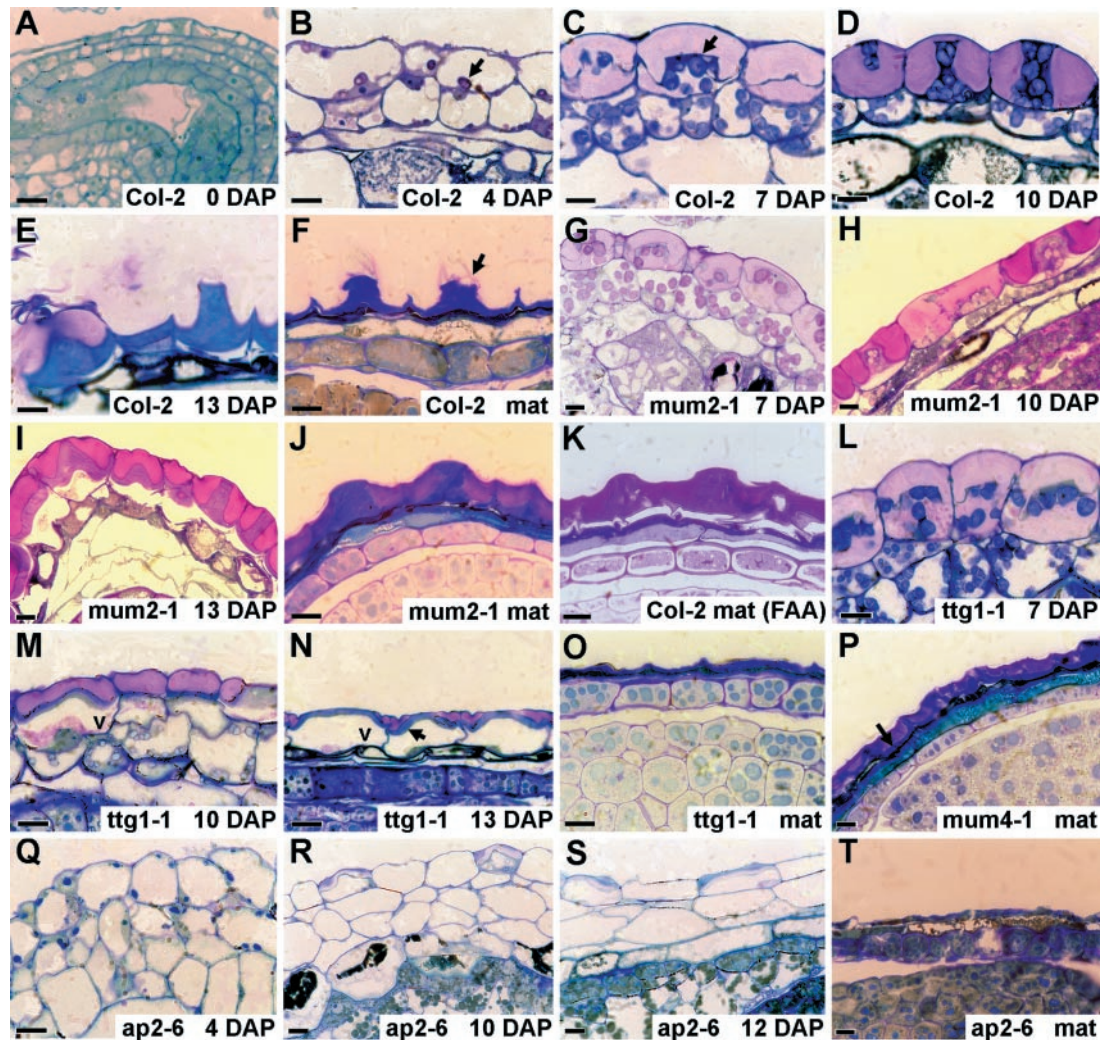


Figure 3. Structure and development of wild-type and mutant seed coats. Plastic sections of tissue fixed in 3% (v/v) aqueous glutaraldehyde (unless otherwise stated) and stained with Toluidine blue. A, Mature wild-type ovule (0 d after pollination [DAP]). B, Wild-type seed coat at 4 DAP. Amyloplasts are visible (arrow) in the outermost two cell layers. C, Wild-type seed coat at 7 DAP. The amyloplasts are larger (arrow) and are found in the center of the epidermal cells, surrounded by pale pink-staining mucilage. D, Wild-type seed coat at 10 DAP. In the epidermal cells, the pink stain is more intense and amyloplasts occupy a vertical column in the center of the cell. E, Wild-type seed coat at 13 DAP. A blue-purple staining column now occupies the center of the cells. The outer cell wall has ruptured on the cell to the right, whereas it is still partially intact on the cell on the left, enclosing pink-staining mucilage. F, Mature wild-type seed coat. The outer cell walls have ruptured, leaving some remnants (arrow) attached to the columellae, and no pink-staining mucilage is apparent. G, *mum2-1* seed coat at 7 DAP. H, *mum2-1* seed coat at 10 DAP. Note partial rupture of center cell, whereas the others are intact. I, *mum2-1* seed coat at 13 DAP. Columellae are fully formed and all cells are intact. J, *mum2-1* mature seed coat. All cells remain intact. K, Wild-type seed coat after fixation in 4% (v/v) formaldehyde in 50% (v/v) ethanol. The cells remain intact, with mucilage retained around the columellae. L, *ttg1-1* seed coat at 7 DAP. M, *ttg1-1* seed coat at 10 DAP. A large vacuole is retained, with intensely staining mucilage apparent above. N, *ttg1-1* seed coat at 13 DAP. A large vacuole occupies the bottom two-thirds of the cell, topped by a flattened layer of cell wall (arrow) that is surrounded by pink-staining mucilage. O, Mature *ttg1-1* seed coat. No mucilage or volcano-shaped columellae are apparent in the epidermal cell layer. P, Mature *mum4-1* seed coat. Flattened columellae (arrow) surrounded by mucilage are visible. Q, *ap2-6* seed coat at 4 DAP. R, *ap2-6* seed coat at 10 DAP. No amyloplasts are visible in either the epidermal or subepidermal (palisade) cell layers. Most epidermal cells are empty while a few have faint staining similar to 10 DAP *ttg1-1* (M). S, *ap2-6* seed coat at 12 DAP. T, Mature *ap2-6* seed coat. No discernable epidermal or palisade cell layers. v, Vacuole. Scale bars = 10 μm .

wild-type plants revealed in each case that the seed phenotype was the result of a recessive mutation to a single locus and that the phenotype was only apparent in the seed of a homozygous mutant mother plant

(Table I). None of the mutants had any other obvious phenotypic abnormalities that cosegregated with the seed coat mucilage defects with the exception of the *mum2* mutants. A percentage of siliques of many

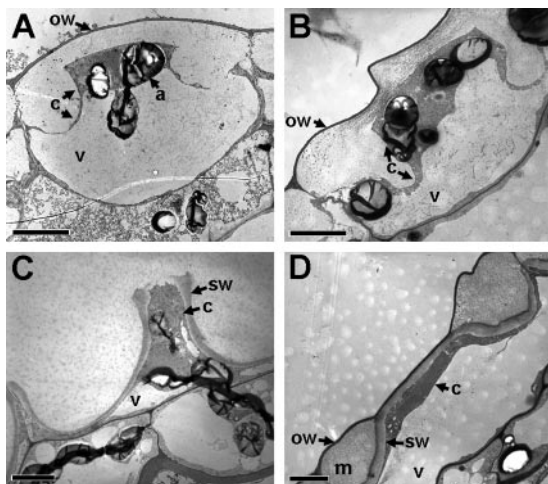


Figure 4. Transmission electron micrographs of developing epidermal cells of wild-type and *ttg1-1* seeds. A, Wild-type seed at 7 DAP. The cytoplasm is drawn into the center of the cell in a sharply defined column above the vacuole. Note large amyloplasts. B, *ttg1-1* seed at 7 DAP. The cytoplasm is in the center of the cell over the vacuole. Some strands of mucilage are apparent between the cytoplasm and the outer tangential cell wall. C, Wild-type seed at 10 DAP. The cytoplasm is found in a narrower column over a much-reduced vacuole and both are surrounded by secondary cell wall. The outer tangential cell wall has ruptured and is no longer visible. D, *ttg1-1* seed at 10 DAP. Similar to 7 DAP (B), the cytoplasm is found over a large vacuole. Some secondary cell wall is apparent over the cytoplasm in a low dome, bounded on both sides by fibrillar mucilage. The outer tangential cell wall is intact. a, Amyloplast; c, cytoplasm; m, mucilage; ow, outer tangential cell wall; sw, secondary cell wall; v, vacuole. Scale bars = 5 μm .

plants homozygous for either of the two independent *mum2* alleles were short suggesting variation in fertility.

Light and scanning electron microscopy (SEM) were used to categorize *ttg1*, *gl2*, *ap2*, and the *mum* mutants into distinct groups based on mature seed phenotypes. Ruthenium red staining after shaking in water divided the mutants into two categories depending on their mucilage capsule phenotype: no mucilage capsule (*mum1-1*, accession no. CS3903; *mum2-1*, accession no. CS3904; *mum2-2*, accession no. CS3905; *mum4-1*, accession no. CS3907; *ttg1-1*; *gl2-1*; *ap2-1*; and *ap2-6*; Fig. 2C), and a very reduced mucilage capsule just seen around the seed periphery (*mum3-1*, accession no. CS3906; *mum4-2*, accession no. CS3908; and *mum5-1*, accession no. CS3909; Fig. 2D). SEM of dry seed determined that the seed coat epidermal cells of *mum2-1*, *mum2-2*, *mum3-1*, and *mum5-1* have wild-type cell surface features, *mum1-1* cells have slightly irregular columellae (data not shown), and *mum4-1*, *mum4-2*, *gl2-1*, and *ttg1-1* have absent or reduced columellae (Fig. 1, C and D). *ap2-1* seeds are heart shaped and have epidermal cells that are often rectangular rather than hexagonal and lack or have reduced columellae (Fig. 1, E and F); *ap2-6* seeds were much more severe than *ap2-1* (Fig. 1, G and H; compare with Fig. 1, E and F, respectively).

Thus, *ttg1*, *gl2*, *ap2*, and *mum1* through 5 can be divided into four groups based on their seed coat defects: (a) no mucilage capsule (*mum1* and *mum2*), (b) no mucilage capsule and reduced columellae (*mum4*, *gl2*, and *ttg1*), (c) reduced mucilage capsule (*mum3* and *mum5*), and (d) no mucilage and aberrant seed coat (*ap2*). In the following sections, we describe further characterization of these four categories to determine the origin of their seed coat defects.

Mutants Affecting Mucilage Extrusion

When mature wild-type seeds are fixed in an aqueous solution of 3% (v/v) glutaraldehyde, the epidermal cells rupture, releasing the mucilage and leaving protruding columellae (Fig. 3F). Fixation in an ethanol-based solution avoids extrusion (Fig. 3K): Cells remain intact, retaining mucilage between the outer primary cell wall and the secondary wall of the columella. When *mum1-1* and *mum2-1* seeds (Group 1) were fixed in 3% (v/v) glutaraldehyde, sectioned, and stained with Toluidine blue, the mature seed coat epidermal cells resembled those of wild-type seeds fixed in 50% (v/v) ethanol. The outer cell wall was intact and pink-staining material was found between the outer cell wall and the columellae (Fig. 3J; compare with formaldehyde-acetic acid-alcohol-fixed wild type in K). These results indicate that these mutants are defective in mucilage extrusion.

To determine whether these changes could be due to an alteration in mucilage composition and/or amount compared with wild type, the carbohydrate content of the mutant seeds was investigated using coupled gas chromatography (GC)-mass spectrometry (MS). To use a single assay to reveal both the basic neutral sugar profile and give a gross estimate of the relative uronic acid levels, trimethylsilane derivatives were used. We have shown previously that the mucilage extruded from wild-type (Columbia [Col]-2) *Arabidopsis* seeds gives a consistent monosaccharide profile (Western et al., 2000; see Fig. 5C). However, mucilage is retained within the epidermal cells of *mum1-1* and *mum2-1* seeds. Thus, as a control for these mutants, we repeated the sugar analysis, this time by grinding wild-type (Col-2) seeds and going through extraction and hydrolysis in the presence of crushed seeds, rather than just hydrolyzing and derivatizing the ammonium oxalate-soluble sugars found in the supernatant after shaking intact seeds. Once again, a consistent sugar profile was obtained (Fig. 5A), though considerably more complex than that from mucilage alone (compare Fig. 5, A with C) because the whole seed extraction also includes sugars from cell walls and other compartments of cells of the seed coat, endosperm, and embryo. Similar to wild type, derivitization of ground *mum1-1* and *mum2-1* seeds yielded reproducible sugar profiles (Fig. 5A). A comparison of the monosaccharide profile obtained from ground *mum2-1* seeds with that of

Table 1. Results of reciprocal backcrosses to wild-type plants and segregation analysis

| Mutant | F ₁ Seed Phenotype ^{a,b} | | F ₂ Seed Phenotype | | F ₃ Seed | |
|---------------|--|------|-------------------------------|------|---------------------|-------------------------|
| | × WT ^c | WT × | × WT | WT × | WT:mutant | Chi square ^d |
| <i>mum1-1</i> | muc ⁻ | + | + | + | 188:50 | 2.0224, <i>P</i> > 0.1 |
| <i>mum2-1</i> | muc ⁻ | + | + | + | 98:33 | 0.0025, <i>P</i> > 0.5 |
| <i>mum3-1</i> | red.muc. | + | + | + | 57:20 | 0.0390, <i>P</i> > 0.5 |
| <i>mum4-1</i> | muc ⁻ | + | + | + | 147:37 | 2.3480, <i>P</i> > 0.1 |
| <i>mum5-1</i> | red.muc. | + | + | + | 142:41 | 0.6576, <i>P</i> > 0.1 |

^a F₁ seed, Seed from cross; F₂ seed, seed of F₁ plants; F₃ seed, seed of F₂ plants. ^b Ruthenium red staining result; +, mucilage; muc⁻, no mucilage; red.muc., reduced mucilage. ^c All crosses written female parent × male parent; WT, wild type. ^d Null hypothesis of 3:1 WT:mutant; degrees of freedom = 1; cutoff at *P* = 0.05.

wild-type seeds revealed that there is no significant difference in either sugar composition or amount (Fig. 5A). In a similar manner, *mum1-1* seeds had a similar composition, but an increased amount of most sugars compared with wild-type seeds. The increased level of sugars in *mum1-1* seeds is not correlated with a gross increase in seed size as viewed externally (data not shown).

Developmental differences from wild type were also investigated for *mum2-1* using Toluidine blue stained sections, SEM, and transmission electron microscopy (TEM). The timing and production of mucilage and columellae appears to be identical to wild type, with the exception that mucilage is retained within the cells upon maturity (Fig. 3, G–J; compare with Fig. 3, C–F). In developing wild-type seeds exposed to aqueous conditions (i.e. fixation), mucilage release can occur starting at the beginning of Stage 4, once most of the mucilage has been deposited and the new secondary cell wall of the columella is initiated (data not shown; Fig. 3E). Some mucilage release was also observed in *mum2-1* seeds at the beginning of Stage 4 (Fig. 3H), but by the end of this stage, the ability to release mucilage had been lost (Fig. 3, I and J). These results suggest that cell wall or mucilage modification is occurring in the *mum2-1* and possibly *mum1-1* seeds during Stage 4 that prevents subsequent mucilage release. Because a common modification of pectins is demethylation of the PGA carboxyl groups after secretion (Brett and Waldron, 1990; Carpita and Gibeaut, 1993), we performed methylation analysis on ammonium oxalate extracts of intact, wild-type *mum1-1* and *mum2-1* seeds. Our results revealed a significant, 6% to 8% increase in methylation for the mutants compared with wild-type seeds (average values of 25.6% and 23.9% for *mum1-1* and *mum2-1*, respectively, versus 18.3% for Col-2 wild type; measurements were done in triplicate; *mum1-1* versus Col-2, $H_0: \mu_1 = \mu_2$, $T = -33.792$, $P \ll 0.01$; *mum2-1* versus Col-2, $H_0: \mu_1 = \mu_2$, $T = -17.431$, $P \ll 0.01$), which is consistent with an altered methylation state of the mucilage and/or primary cell wall pectins. Because the degree of pectin methylation could affect both cross-linking and hydrophilicity, it is conceivable that an increase in the proportion of methyl esterified PGA molecules could

be responsible for the retention of mucilage within the primary cell wall of the seed coat epidermis of these mutants. However, we cannot exclude the possibility that the observed difference in methylation could be simply a consequence of differential extraction of wild-type seeds bearing extruded mucilage versus *mum1-1* and *mum2-1* seeds where the mucilage is contained within intact epidermal cell walls.

Mutants Having Reduced Mucilage and Flattened Columellae

Unlike wild-type seeds, seeds of *gl2-1*, *ttg1-1*, and strong *mum4* (*mum4-1*) mutant plants do not extrude any mucilage upon hydration and appear to have a reduced amount of mucilage compared with wild-type cells viewed under conditions where mucilage is retained (Fig. 3, O and P; compare with Fig. 3K). In addition, the columellae of the seed epidermal cells are reduced in size (compare Fig. 1, C and D, with Fig. 1, A and B; compare Fig. 3, O and P, with Fig. 3, F and K). With the exception of *ttg1* mutants, which are known to lack anthocyanins in the pigmented layer (Koornneef, 1981), the other seed coat layers (palisade and pigmented layers) appear normal in these mutants (Fig. 3, O and P).

The possibility of a reduced amount or altered chemical composition of mucilage in these mutants was explored using GC-MS. Once again, due to mucilage retention in the epidermal cells, ground seeds were used for analysis and reproducible profiles were obtained for *Ler* wild type, *ttg1-1*, *gl2-1*, *mum4-1*, and *ap2-1* (see Fig. 5B for *Ler*, *ttg1-1*, and *ap2-1*; *gl2* and *mum4-1* data not shown). When *ttg1-1*, *gl2-1* and *mum4-1* seeds were compared with wild type, it was found that all the monosaccharides were present, but the peak for GalUA was significantly reduced (*ttg1-1* versus *Ler*, $H_0: \mu_1 = \mu_2$, $T = 11.758$, $P < 0.01$; *ap2-1* versus *Ler*, $H_0: \mu_1 = \mu_2$, $T = 12.616$, $P < 0.01$). The peak for Rha and Fuc (*ttg1-1* versus *Ler*, $H_0: \mu_1 = \mu_2$, $T = 8.769$, $P < 0.01$; *ap2-1* versus *Ler*, $H_0: \mu_1 = \mu_2$, $T = 11.143$, $P < 0.01$), and the Gal peak were also smaller (*ttg1-1* versus *Ler*, $H_0: \mu_1 = \mu_2$, $T = 8.319$, $P < 0.01$; *ap2-1* versus *Ler*, $H_0: \mu_1 = \mu_2$, $T = 8.642$, $P \ll 0.01$). Thus, all three mutants have a decrease in the monosaccharides (GalUA and Rha)

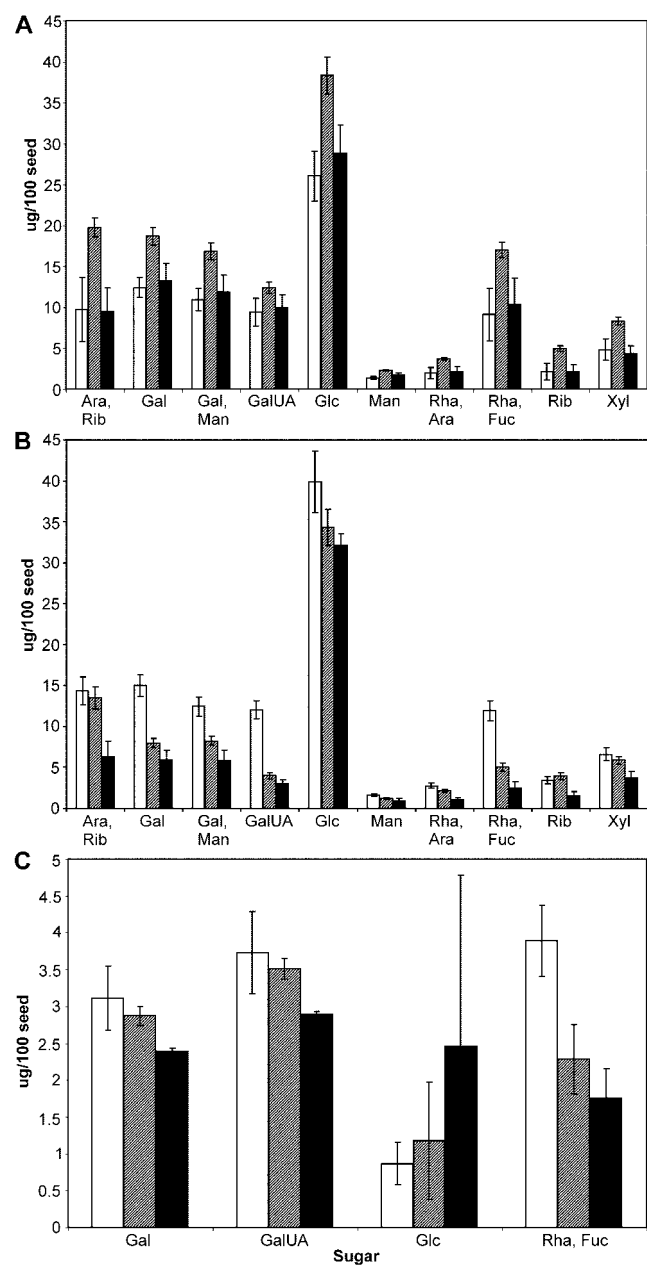


Figure 5. Monosaccharide composition of wild-type and mutant seeds and mucilage. The bars represent the average quantity of monosaccharide found by GC over multiple experiments. The data were standardized and converted to micrograms per 100 seed through comparison to an internal standard. The monosaccharide assignments were determined through coupled GC-MS. The occurrence of a monosaccharide in more than one bar is due to the sensitivity of GC analysis, where each anomer of each sugar is detected separately (Chaplin, 1986). Where anomers of a sugar were found in GC peaks representing a single monosaccharide, they were added to give a single bar. If an anomer of a sugar could not be resolved from that of a different monosaccharide, they were included as a separate, mixed bar. A, Comparison of the monosaccharide composition observed using GC of whole, ground seeds of wild-type Col (white bars), *mum3-1* (striped bars), and *mum5-1* (black bars) seeds. Analyses were done in triplicate for all genotypes except Col mucilage, which was done five times and Col whole, ground seeds, which was done four times. Error bars represent SD.

that are major components of pectin. Because similar decreases were seen in *ap2-1* seeds where little or no mucilage appears to be synthesized (see below), these results suggest that less mucilage may be made in *ttg1-1*, *gl2-1*, and *mum4-1* seed coats.

The origin of the flattened columellae was studied in *gl2-1*, *ttg1-1*, and *mum4-1* by following the differentiation of the seed coat epidermis using both Toluidine blue-stained sections (Fig. 3, L–O) and TEM (Fig. 4). Similar results were seen for all three mutants; thus, only *ttg1-1* will be described in detail. During the first 4 d after pollination, *ttg1-1* development was similar to wild type, with the enlargement and vacuolization of the epidermal cells together with the accumulation of amyloplasts (data not shown). This was followed by rearrangement of the cytoplasm and accumulation of pink-staining mucilage (Fig. 3L). Differences between wild type and *ttg1-1* development became evident just prior to Stage 4, where new cell wall is laid down around the cytoplasm. In wild-type seeds, the cytoplasm is first pulled into the center of the cell over a large basal vacuole, followed by narrowing of the column and reduction of the vacuole; the secondary cell wall is then laid down, forming a tall, volcano-shaped structure (Figs. 3, C–E, and 4, A and C). In *ttg1-1*, *gl2-1*, and *mum4-1* seeds, however, only the first stage of the cytoplasmic rearrangement occurs and the secondary cell wall is laid down in a peaked dome over a large vacuole (Figs. 3, L–N, and 4, B and D). Desiccation leads to the crushing of the vacuole and a flattened columella (Fig. 3O). These data suggest that in all three mutants, there is a defect in the later stages of cytoplasmic rearrangement and/or constriction.

Mutants Affecting Mucilage Composition

Mutants at the *MUM3* and *MUM5* loci were identified through their apparently reduced amount of seed mucilage. When stained with Ruthenium red after shaking in water, *mum3-1* and *mum5-1* seeds were surrounded by a barely visible layer of unstained mucilage (compare Fig. 2, D with B). However, when these mutants were placed directly in Ruthenium red without agitation, both the outer, diffuse and inner, dense layers of mucilage were apparent (Fig. 2E). To determine if the defect of *mum3-1* and *mum5-1* was due to loss of mucilage with agitation, a dilute solution of India ink was used. India ink is a colloidal liquid whose molecules are too large to penetrate the mucilage gel and thus can serve

the monosaccharide composition observed using GC for extruded mucilage of wild-type Col (white bars), *mum3-1* (striped bars), and *mum5-1* (black bars) seeds. Analyses were done in triplicate for all genotypes except Col mucilage, which was done five times and Col whole, ground seeds, which was done four times. Error bars represent SD.

as a negative stain. *mum3-1* and *mum5-1* seeds first shaken in water, then placed in India ink had a mucilage capsule comparable with wild-type seeds (data not shown), demonstrating that their defect results from altered staining properties with respect to Ruthenium red following agitation.

It is interesting that a phenocopy of *mum3-1* and *mum5-1* mutant seeds resulted from wild-type seeds shaken in the presence of EDTA or EGTA prior to staining with Ruthenium red (compare Fig. 2, F with D). Ruthenium red dye stains molecules with two negative charges 0.42 nm apart (Sterling, 1970). Both EDTA and EGTA are heavy metal chelators, with EGTA being relatively specific for Ca^{2+} . The removal of Ca^{2+} ions could lead to disruption of the ionic cross-linking of pectin (PGA) carboxyl groups, allowing for separation of PGA molecules under agitation and thus loss of Ruthenium red staining. By a similar argument, the defect in *mum3-1* and *mum5-1* could be due to increasing methyl esterification and consequent neutralization of the PGA carboxyl groups. Methylation analysis of ammonium oxalate soluble sugars extracted from intact seeds, however, showed a slight decrease in methylation in these mutants (average values of 15.8% and 14.1% for *mum3-1* and *mum5-1*, respectively, versus 18.3% for Columbia; measurements were done in triplicate with no measurement more than 2.2% from the mean). In an alternate manner, the ability to phenocopy with chelators may reflect a more basic change in mucilage composition. To investigate this possibility, the sugar composition of *mum3-1* and *mum5-1* mucilage was analyzed by GC-MS of ammonium oxalate-soluble sugars obtained from intact seeds. The results (Fig. 5C) showed that for both there is a consistent, significant decrease in the peak containing both Rha and Fuc (*mum3-1* versus Col-2, $H_0: \mu_1 = \mu_2$, $T = 4.188$, $P < 0.01$; *mum5-1* versus Col-2, $H_0: \mu_1 = \mu_2$, $T = 5.823$, $P < 0.01$), suggesting that *mum3-1* and *mum5-1* mutants have defects in mucilage sugar composition, which may affect its basic structure and thus its branching and cross-linking properties.

Mutants Affecting Differentiation of Seed Coat Epidermal and Palisade Layers

ap2 mutant seeds are altered in overall shape and in the shape of the epidermal cells (Bowman and Koornneef, 1994; Jofuku et al., 1994). We used electron and light microscopy to investigate more thoroughly the seed defects associated with the strong *ap2-6* mutant allele. *ap2-6* mutant seeds have a characteristic heart shape more reminiscent of ovules than the oval shape typical of wild-type seeds (compare Fig. 1, G with A and I). The epidermal cells of *ap2-6* seeds lack columellae and are thin walled and rectangular, unlike the hexagonal-shaped cells with thickened radial cell walls found in the wild-type seed coat epidermis (compare Fig. 1, H with B). When

the mature *ap2-6* seeds of the abnormal type were sectioned, of the three layers of the seed coat, only the pigmented layer was apparent (Fig. 3T). The characteristic epidermal and subepidermal palisade cell types were absent, replaced by several layers of crushed cells surrounding the pigmented layer. The development of the aberrant seeds produced by *ap2-6* flowers was followed using light microscopy to determine the timing and nature of the seed coat defects. The aberrant seed coats (Fig. 3, Q–T) appear to develop normally up to 4 d after pollination, with the epidermal and subepidermal palisade cells enlarging, becoming vacuolated, and accumulating amyloplasts (compare Fig. 3, B with Q). However, most *ap2-6* seed coat cells did not differentiate further, failing to undergo cell morphogenesis, produce mucilage or synthesize secondary cell wall (compare Fig. 3, C–E with R and S). At later stages, when large amounts of mucilage and the columellae are the dominant features of wild-type cells, the majority of the *ap2-6* cells remained highly vacuolated (compare Fig. 3, R and S with D and E). A few exceptional cells, however, either appeared to have staining of mucilage and/or columella similar to *ttg1-1* and *gl2-1* seeds, but very faint, or were filled with osmophilic granules normally only seen in the pigmented layer (Fig. 3S). No palisade layer cells were found to develop normally in the aberrant seeds. Thus, it appears that in abnormal *ap2-6* seeds, cell types derived from the outer integument fail to differentiate into the epidermal and palisade layers.

In some seed batches, the seed coat phenotype of *ap2-6* varied in penetrance such that seeds with either mutant or wild-type appearance were produced. Aberrant and normal seeds were generally found in different siliques. Although normal or mutant-bearing siliques both arise from flowers with an *ap2* phenotype, there was a correlation between the severity of morphological defects of the *ap2-6* flower and defects in the seed coat of the seeds derived from it. The “wild-type” *ap2-6* seeds appeared normal in all aspects of seed and seed coat shape and development (data not shown).

The seed coat phenotypes of plant lines homozygous for several other *ap2* mutant alleles were examined by SEM and light microscopy. Seed and seed coat epidermal cell shape defects similar to *ap2-6* have been observed in two other strong alleles of *ap2* (*ap2-7* and *ap2-2*; data not shown). Seeds from plants homozygous for the weak allele *ap2-1* had phenotypes similar to but less severe than *ap2-6* but did not display variation in penetrance. *ap2-1* mutant seeds, like those of *ap2-6*, are heart shaped (Fig. 1E). The epidermal cells, however, were variable in shape ranging from hexagonal cells with almost normal columellae to rectangular, thin-walled cells lacking columellae (Fig. 1F). Sectioning of a developmental series of *ap2-1* seeds showed results consistent with similar but less severe effects on epidermal cell de-

velopment compared with *ap2-6* seeds (data not shown).

DISCUSSION

The development of the epidermal layer of the seed coat from the outer integument of the ovule in *Arabidopsis* is a complex process, involving cell growth, cytoplasmic rearrangement, biosynthesis and secretion of pectinaceous mucilage, and production of a secondary cell wall (Beeckman et al., 2000; Western et al., 2000; Windsor et al., 2000). We have shown here that mutants specifically defective in the seed coat epidermis can be isolated and should be useful in dissecting many of these processes. The loss of the seed coat epidermal cells and those of the palisade layer in the *ap2* mutants had no obvious effect on viability and germination, demonstrating that these cell types are completely dispensable under laboratory conditions. Therefore, the seed coat epidermal cells represent an excellent model system for the use of genetics to study carbohydrate synthesis and secretion, secondary cell wall biosynthesis, and cell morphogenesis.

MUM2 and *MUM1* May Regulate Mucilage Modification

Two of the novel mutants identified (*mum1* and *mum2*) had an unexpected phenotype: Normal mucilage was synthesized and deposited, but not released upon seed wetting. In a previous study of wild-type seeds, we suggested that mucilage release is due to the rapid expansion of dried mucilage upon hydration, leading to the rupture of the primary cell wall (Western et al., 2000). According to this hypothesis, the lack of release in *mum1* and *mum2* seeds may result from either insufficient mucilage expansion due to a change in mucilage amount or composition, or to strengthening of the primary cell wall. The combination of lack of appreciable change in monosaccharide amount or composition in *mum2* seeds and developmental analysis revealing early but not late release of mucilage suggests that for *mum2* seeds, at least, the defect lies in post-deposition modification of the mucilage and/or primary cell wall.

A hint as to the type of defect preventing extrusion in the mutants comes from methylation analyses suggesting an increase in overall methylation of *mum1* and *mum2* seed pectin. The degree of methylation affects the number of free carboxyl groups available in the pectin, which in turn affects both pectin hydrophilicity and its ability to form Ca^{2+} bridges between PGA molecules (Bolwell, 1988; Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Reiter, 1998). An increase in methyl-esterified PGA may reduce the affinity of the mutant's mucilage for water, thereby lessening either the speed or the extent of hydration and decreasing the ability of the hydrated

mucilage to break the primary cell wall. This idea is supported by the observation that some mucilage release can be obtained by treating *mum1* and *mum2* seeds with the Ca^{2+} -specific chelator EGTA (T.L. Western, W.L. Tan, and G.W. Haughn, unpublished data). Treatment with EGTA might remove Ca^{2+} as a competitor with water for the limited number of free carboxyl groups within the mutant's mucilage, thereby partially suppressing the extrusion defect. However, our data do not allow us to determine if the increased methylation is limited to the mucilage or affects pectin of the primary cell wall as well. Thus, we cannot eliminate the possibility that an increase in pectin methylation in the mutant in some way strengthens the primary cell wall, thereby preventing extrusion.

Studies of pectin biosynthesis in root caps have shown that the degree of PGA methyl-esterification differs between cell types and the degree of methyl esterification is likely controlled by the secretion of pectin methyl esterases into the cell wall (Moore et al., 1991; Zhang and Staehelin, 1992; Sherrier and VandenBosch, 1994; Staehelin and Moore, 1995). Assuming an analogous situation in the epidermal cells of *Arabidopsis* seeds, it is possible that *MUM1* and *MUM2* encode either pectin methyl esterases or positive regulators of such an enzyme in the seed coat.

TTG1, *GL2*, and *MUM4* Regulate Cytoplasmic Rearrangement and Amount of Mucilage Produced

Previous, external studies of *gl2* and *ttg1* seeds showed that these mutants fail to extrude mucilage and lack columellae (Koornneef, 1981; Bowman and Koornneef, 1994; Rerie et al., 1994). Our more detailed characterization of *gl2*, *ttg1*, and *mum4* phenotypes has shown that both mucilage and columellae are present in the seed coat epidermis, but are reduced compared with that of wild type. The early stages of differentiation involving growth and early cytoplasmic rearrangement appear to occur correctly. The first obvious defect in *gl2*, *ttg1*, and *mum4* seeds is the failure to completely retract the vacuole and form a normal cytoplasmic column, suggesting that the role of the gene products is in cell morphogenesis. This hypothesis is consistent with the roles of the *TTG1* and *GL2* in other tissues (trichomes and root hairs; Koornneef, 1981; Galway et al., 1994; Rerie et al., 1994; Di Cristina et al., 1996; Masucci et al., 1996). However, in the seed coat epidermal cells, unlike the trichomes and root hairs, the changes occur within the existing cell boundaries, rather than involving cell outgrowth.

Because the *gl2*, *ttg1*, and *mum4* phenotypes all include changes in both the amount of seed coat mucilage deposited in the extracellular space and the cytoplasmic rearrangements during seed coat epidermal cell differentiation, these two processes are likely to be related. This functional relationship between

the late events of cellular morphogenesis and mucilage deposition, events that occur simultaneously during differentiation, could be explained in several ways. First, both processes probably rely on the cytoskeleton. Recent studies have demonstrated the important role of the cytoskeleton in trichome morphogenesis (Oppenheimer et al., 1997; Mathur et al., 1999; Szymanski et al., 1999; Mathur and Chua, 2000). In a similar manner, movement of the cytoplasm in the seed coat epidermal cells from the edges of the cell to a precise column in the center could be an active process that involves the cytoskeleton. Mucilage deposition requires polar secretion, which is likely to be dependent on directional trafficking of vesicles via the cytoskeleton (Hyde, 1970; Van Caeseele et al., 1981; Staehelin et al., 1990; Lynch and Staehelin, 1992, 1995; Western et al., 2000). Thus, the *gl2*, *ttg1*, and *mum4* mutant phenotypes could be due to defects in the cytoskeletal rearrangements required for cell differentiation. Second, the complete compression of the cytoplasm and expulsion of water from the vacuole of differentiating seed coat epidermal cells may be dependent on pressure created by the accumulation of mucilage between the plasma membrane and the outer tangential cell wall. According to this hypothesis, the improper restriction of the cytoplasm and vacuole in the mutant seed coats would be a secondary effect of the reduced amount of mucilage produced in these mutants. Third, mucilage secretion may be dependent on cell morphogenesis to provide an optimal amount of membrane surface relative to the amount of extracellular space. If so, the lower mucilage deposition in the mutants could be a secondary effect of a defect in cell morphogenesis. Identification of a seed coat mutant defective in either cell morphogenesis or mucilage deposition but not both would help distinguish between these possibilities.

Both *GL2* and *TTG1* have been cloned. *GL2* encodes a putative homeodomain transcription factor (Rerie et al., 1994; Di Cristina et al., 1996), whereas *TTG1* encodes a protein of unknown function with a putative WD40 protein-protein interaction domain (Walker et al., 1999). In addition to their roles in the seed coat epidermis, *GL2* and *TTG1* are required for normal development of both trichomes and root hairs (Koornneef, 1981; Galway et al., 1994; Rerie et al., 1994; Di Cristina et al., 1996; Masucci et al., 1996). In a converse manner, *MUM4* appears to be seed specific, making it a candidate downstream target or specificity factor for these genes.

***MUM3* and *MUM5* Affect Mucilage Biosynthesis**

Two mutants that affect the composition of mucilage, *mum3* and *mum5*, have been identified. This altered composition resulted in aberrant staining qualities with Ruthenium red only after mechanical agitation of seeds in water prior to staining. Staining

with Ruthenium red is dependent upon the positioning of two negative charges 0.42 nm apart (Sterling, 1970). The loss of staining in these mutants, therefore, may be due to a reduced number of pectin cross-linkages that allows for separation of negatively charged PGA molecules in pectin following mechanical agitation in water. This hypothesis is supported by the ability to phenocopy the *mum3* and *mum5* mutants by treating wild-type seeds with the Ca^{2+} chelator, EGTA, a treatment that disrupts the Ca^{2+} bridges between PGA molecules.

The molecular structure of Arabidopsis mucilage is unknown, but similar to other mucilages, it is likely to consist of a network containing not only PGA and RG I, but other complex polysaccharides, including hemicelluloses (Lynch and Staehelin, 1992, 1995), and possibly even structural cell wall proteins. This network is held together through Ca^{2+} bridges between PGA molecules and bonding among complex polysaccharides and between proteins (Bolwell, 1988; Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Reiter, 1998). Therefore, weakening of the network in *mum3* and *mum5* seeds could be due to anything from neutralization of PGA by increased methyl esterification to reduced bonding between complex polysaccharides or proteins. The former possibility is unlikely due to the slight decrease in methylation state in these mutants. In a converse manner, the alteration of relative monosaccharide levels in *mum3* and *mum5* mucilage would seem to reflect a change in the polysaccharide composition itself. This could be due to a change in the number or types of polysaccharides present, which could in turn lead to decreased cross-linkages between molecules within the gel. Under this assumption, *MUM3* and *MUM5* could encode biosynthetic enzymes or regulators of complex polysaccharide production.

Very few biosynthetic and regulatory genes have been identified for complex polysaccharide biosynthesis in plants. These include the recently identified *MUR* genes of Arabidopsis, which were identified through altered leaf sugar composition (Reiter et al., 1993, 1997; Zablackis et al., 1996; Bonin et al., 1997; Burget and Reiter, 1999). Because several of the *mur* mutants have lower Fuc and/or Rha levels (*mur1*, *mur2*, *mur8*, and *mur11*), we tested them with our Ruthenium red staining assay to see if any had a phenotype similar to *mum3* and *mum5*. Under these conditions, all the *mur* mutants tested appeared wild type (T.L. Western, W.L. Tan, and G.W. Haughn, unpublished data), suggesting that *MUM3* and *MUM5* represent novel loci affecting complex polysaccharide biosynthesis.

***AP2* Is a Regulator of Outer Integument Differentiation during Seed Coat Development**

The *AP2* gene encodes a putative transcription factor expressed throughout the developing plant and

required for many aspects of plant development including floral organ identity, floral meristem identity, ovule development, and seed morphology (Bowman et al., 1989, 1991, 1993; Kunst et al., 1989; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993; Jofuku et al., 1994; Modrusan et al., 1994; Western and Haughn, 1999). Here, we have characterized *ap2* defects of the seed coat that include altered seed and epidermal cell shape, lack of mucilage and columellae in the epidermal layer, and the absence of a lignified secondary cell wall in the subepidermal palisade layer. All of these defects, including the abnormal seed shape, probably result directly from the failure, following fertilization, of *ap2* cells of the outer integument to differentiate after initial expansion of the integument cells (early Stage 2). Thus, it appears that AP2 acts as a regulator of differentiation of the outer integument cells into seed coat-specific cell types.

The *AP2* gene is required for ovule morphogenesis (Modrusan et al., 1994; Western and Haughn, 1999). A small percentage of *ap2* mutant ovules develop as carpel-like structures with the remaining ovules developing normally. Because the seed coat is derived from the ovule integuments, it is possible that the failure in seed coat differentiation is a consequence of earlier defects in ovule development. In an alternate manner, *AP2* may regulate ovule morphogenesis and seed coat differentiation independently of one another. Given the variability of the ovule defects and the specificity of the seed coat defects, we tend to favor the latter hypothesis.

The *MUM* Genes Define Important Steps in Seed Coat Differentiation

Arabidopsis mucilage cell differentiation has been divided into five stages (Western et al., 2000). Based on their phenotypes, the mutants described in this paper further delineate the processes involved in seed coat differentiation (Fig. 6). Fertilization triggers cell growth (Stage 1), followed by amyloplast accumulation and initial cytoplasmic rearrangement (Stage 2). *AP2* appears to be a developmental regulator required for epidermal cell differentiation to move beyond early Stage 2. Stage 3 (mucilage biosynthesis and cytoplasmic rearrangement) is initiated properly but not completed in *ttg1*, *gl2*, and *mum4* seeds. Thus, *TTG1*, *GL2*, and *MUM4* are needed to maintain intracellular differentiation during the later stages of Stage 3, with *MUM4* acting in a seed-specific manner. *MUM3* and *MUM5* act at a fundamentally different level: They affect the polysaccharide structure of the mucilage being synthesized, either as regulators or biosynthetic enzymes. Finally, *MUM2*, and possibly *MUM1*, play an unexpected role during Stage 4: regulation of a post-secretion chemical modification of either mucilage or primary cell wall necessary for mucilage extrusion upon seed hydration.

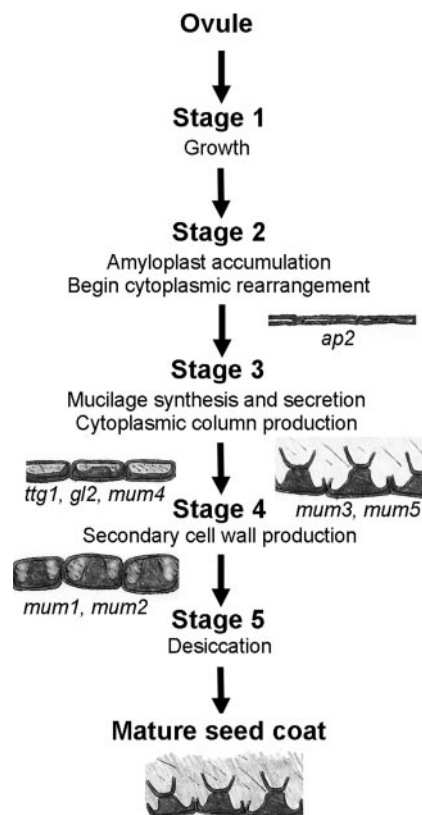


Figure 6. A time course of mucilage secretory cell development showing the timing of *TTG1*, *GL2*, *AP2*, and *MUM1* through 5 action as determined from their phenotypes. Mucilage secretory cell development can be divided into five stages: (1) growth, (2) amyloplast accumulation and commencement of cytoplasmic rearrangement, (3) simultaneous mucilage production/secretion and cytoplasmic column formation, (4) secondary cell wall production to form the columella, and (5) desiccation of the seed coat. The genes discussed in this paper appear to affect seed coat epidermal cell differentiation at three of these stages. *AP2* is necessary to continue differentiation of the cells past the growth phase (transition of Stage 2 to Stage 3). *TTG1* and *GL2* reiterate their roles in cell morphology, being necessary for the completion of Stage 3 mucilage production and cytoplasmic rearrangement, with seed-specific *MUM4* acting alongside or downstream, whereas *MUM3* and *MUM5* apparently affect Stage 3 mucilage biosynthesis with respect to sugar composition. *MUM2* (and possibly *MUM1*) may play a role in post-deposition mucilage or cell wall modifications in late Stage 4.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Lines of Arabidopsis used were *ap2-1* (*Ler* ecotype; seed stock no. CS29; gift from Maarten Koornneef, Laboratory of Genetics, Wageningen University, The Netherlands), *ap2-2* (*Ler*; seed stock no. CS3082; gift from Elliot Meyerowitz, California Institute of Technology, Pasadena), *ap2-6*, *ap2-7* (Col-2 ecotype; seed stock nos. CS6240 and CS6241, respectively; Kunst et al., 1989), *gl2-1*, and *ttg1-1* (*Ler*; Arabidopsis Biological Resource Centre, Ohio State University, Columbus, stock nos. CS65 and CS89, respectively). Seeds were stratified at 4°C for 3 d on Terra-Lite Redi Earth prepared

soil mix (W.R. Grace and Co. Canada Ltd., Ajax, ON) and then transferred to growth chambers at 20°C under continuous light (90–120 $\mu\text{E m}^{-2} \text{s}^{-1}$ photosynthetically active radiation).

Due to the maternal origin of the seed coat, mutant screens were performed on M3 lines. The M3 lines were derived from plants randomly chosen from several independent ethylmethane sulfonate-mutagenized M2 populations of Arabidopsis ecotype Col-2 (M1 population size > 40,000). Seeds were screened by first shaking seeds in water, then placing them in a 0.01% (w/v) aqueous solution of Ruthenium red before inspection under a dissection microscope. Suspected mutants were then rescued through germination on Arabidopsis minimal medium (Haughn and Somerville, 1986), then transferred to soil at the two-true-leaf stage.

Staging of Flower Age

The time of pollination (0 DAP) was defined as the time at which the flowers are just starting to open and the long stamens grow over the gynoecium (Bowman et al., 1994). Each day for 5 d, flowers at this stage were marked with a different color of nontoxic, water-soluble paint. The color of the paint identified the date of pollination and allowed selection of developing siliques at precise ages.

Resin Embedding for Bright-Field and TEM

Developing seeds for embedding in resin were either fixed in the silique or removed from the silique prior to fixation in 3% (v/v) glutaraldehyde (Canemco, Lachine, Quebec, Canada) in 0.5 M sodium phosphate buffer at pH 7 with post-fixation with 1% (v/v) osmium tetroxide or in formaldehyde-acetic acid-alcohol (4% [v/v] paraformaldehyde [Canemco], 15% [v/v] acetic acid, and 50% [v/v] ethanol) without post-fixation. Dehydration, embedding, and sectioning were as described by Western et al. (2000). For brightfield microscopy, 0.2- to 0.5- μm sections were stained with 1% (w/v) Toluidine blue O in 1% (w/v) sodium borate (pH 11) and photographed using a Leitz DRB (Leica, Wetzlar, Germany) light microscope with Kodak Gold Plus or Royal Gold 100 ASA film (Eastman Kodak, Rochester, NY). In preparation for electron microscopy, thin sections (silver to gold) were stained in 1% to 2% (w/v) uranyl acetate for 30 min, followed by 15 min in lead acetate. Specimens were observed and photographed on a Zeiss 10C transmission electron microscope (Carl Zeiss, Oberkochen, Germany), which was operated at an accelerating voltage of 60 or 80 kV. Photographs were digitized and manipulated with Adobe Photoshop (Adobe Photosystems, Mountain View, CA) to prepare figures.

Scanning Electron Microscopy

Samples were dry mounted on stubs, coated with gold or gold-palladium in a SEMPrep2 sputter coater (Nanotech, Manchester, UK), and observed using a Cambridge model 250T scanning electron microscope (Leica, Cambridge, UK) with an accelerating voltage of 20 kV and photographed

using Polaroid Polapan 55PN film. Photographs were digitized and manipulated with Adobe Photoshop to prepare figures.

GC and MS

Extruded mucilage for derivatization (Col-2, *mum3-1* and *mum5-1*) was isolated from samples of 100 intact seeds by incubating in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 h at 30°C (Goto, 1985). The solution was then drawn off from the seeds and 10 μL of internal standard (4.8 mg mL^{-1} myo-inositol) was added prior to addition of 5 volumes of absolute ethanol and drying under nitrogen gas. Derivatization and GC of trimethylsilyl ethers was performed as described previously (Western et al., 2000). The isolation of cell wall components from whole seeds (Col-2, *Ler*, *mum1-1*, *mum2-1*, *mum4-1*, *ttg1-1*, *gl2-1*, and *ap2-1*) was accomplished by grinding 100 seed in 0.2% (w/v) ammonium oxalate prior to shaking. Addition of internal standard and hydrolysis was performed in the presence of the ground seeds, following which samples were drawn off the seed debris and derivatization was performed in the same manner as for mucilage alone (Western et al., 2000), except a hexane extraction (2 volumes hexane to 1 volume sample) was performed after the acetylation step to remove seed oil.

Compounds were identified initially through comparison with the retention times obtained with individual sugar standards, and then confirmed through GC-MS. GC-electron impact MS was performed by the University of British Columbia Mass Spectrometry Centre (Vancouver). Individual sugar standards and a composite standard were made from the following monosaccharides: myo-inositol (used as internal standard), Fuc, Man, Gal, Glc, Ara, Rha, Xyl, GlcUA, and GalUA (Chaplin, 1986).

Assay of Pectin Methylation in Seed Mucilage

Seed (100 mg) was stirred in 1.5 mL of 0.5% (w/v) ammonium oxalate (Sigma, Oakville, Ontario, Canada) at 80°C for 1 h. After centrifugation (13,000g, 20 min) the supernatant was poured into 5 volumes of ethanol and centrifuged again (2,300g, 20 min). The precipitate was dissolved in a minimal amount of water (about 5 mL), dialyzed using tubing with a 10,000- M_r cutoff for 40 h against running water (first 20 h using tap water; last 20 h using distilled water), and lyophilized. Two colorimetric assays for saponifiable methanol and GalUA were used to determine the degree of methyl esterification. The assay for saponifiable methanol was carried out as described by Kim and Carpita (1992). The GalUA assay was that of Filisetti-Cozzi and Carpita (1991) except sodium tetraborate was not added to the H_2SO_4 and 10 μL of carbazole (Sigma) in ethanol (1 mg mL^{-1}) was used for color development in place of m-hydroxydiphenyl. The addition of sulfamate in the assay of uronic acids suppresses nonspecific color production by neutral sugars (Filisetti-Cozzi and Carpita, 1991) without sacrificing sensitivity, when used in combination with carbazole. Both assays were scaled down by a

factor of 10 to preserve plant material. Results were quantified based on standard curves generated using methyl-esterified PGA (22%, 67%, and 89% [w/v] MePGA; Sigma).

ACKNOWLEDGMENTS

We thank Drs. Elaine Humphrey, Lacey Samuels, Mary Berbee, and Mr. Réza Shahidi (University of British Columbia, Vancouver, Canada) for assistance with microscopy; Ms. Yeen Ting Hwang (University of British Columbia) for valuable technical assistance; and Dr. Gunter Eigendorf (University of British Columbia Chemistry Mass Spectrometry Facility) and Dr. Anthony Millar (University of British Columbia) for help with chemical analysis of mucilage. We also thank Drs. Ljerka Kunst, Linda Matsuuchi, Jennifer Klenz, Mr. Mark Pidkowich, Ms. Yeen Ting Hwang, and Mr. Theodore Popma (University of British Columbia) for helpful discussions and comments on the manuscript.

Received May 1, 2001; returned for revision June 12, 2001; accepted July 27, 2001.

LITERATURE CITED

- Beeckman T, De Rycke R, Viane R, Inzé D** (2000) Histological study of seed coat development in *Arabidopsis thaliana*. *J Plant Res* **113**: 139–148
- Boesewinkel FD, Bouman F** (1995) The seed: structure and function. In J Kigel, G Galili, eds, *Seed Development and Germination*. Marcel Dekker Inc., New York, pp 1–24
- Bolwell GP** (1988) Synthesis of cell wall components: aspects of control. *Phytochemistry* **27**: 1235–1253
- Bonin CP, Potter I, Vanzin GF, Reiter W-D** (1997) The *MUR1* gene of *Arabidopsis thaliana* encodes an isoform of GDP-d-mannose-4,6-dehydratase, catalyzing the first step in the *de novo* synthesis of GDP-l-fucose. *Proc Natl Acad Sci USA* **94**: 2085–2090
- Bowman JL, Alvarez J, Weigel D, Meyerowitz EM, Smyth DR** (1993) Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development* **119**: 721–743
- Bowman JL, Koornneef M** (1994) Mutations affecting seed morphology. In JL Bowman, ed, *Arabidopsis: An Atlas of Morphology and Development*. Springer-Verlag, New York, pp 398–401
- Bowman JL, Mansfield SG, Koornneef M** (1994) Embryogenesis. In JL Bowman, ed, *Arabidopsis: An Atlas of Morphology and Development*. Springer-Verlag, New York, pp 398–401
- Bowman JL, Smyth DR, Meyerowitz EM** (1989) Genes directing flower development in *Arabidopsis*. *Plant Cell* **1**: 37–52
- Bowman JL, Smyth DR, Meyerowitz EM** (1991) Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development* **112**: 1–20
- Brett C, Waldron K** (1990) Physiology and Biochemistry of Plant Cell Walls. Unwin Hyman, London
- Burget EG, Reiter W-D** (1999) The *mur4* mutant of *Arabidopsis* is partially defective in the *de novo* synthesis of uridine diphospho l-arabinose. *Plant Physiol* **121**: 383–389
- Carpita NC, Gibeaut DM** (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* **3**: 1–30
- Chaplin MF** (1986) Monosaccharides. In MF Chaplin, JF Kennedy, eds, *Carbohydrate Analysis: A Practical Approach*. IRL Press, Washington, DC, pp 1–36
- Cosgrove DJ** (1997) Assembly and enlargement of the primary cell wall in plants. *Annu Rev Cell Dev Biol* **13**: 171–201
- Di Cristina M, Sessa G, Dolan L, Linstead P, Biama S, Ruberti I, Morelli G** (1996) The *Arabidopsis* *Athb-10* (GLABRA2) is an HD-Zip protein required for regulation of root hair development. *Plant J* **10**: 393–402
- Doong RL, Mohnen D** (1998) Solubilization and characterization of a galacturonosyltransferase that synthesizes the pectic polysaccharide homogalacturonan. *Plant J* **13**: 363–374
- Drriouch A, Faye L, Staehelin LA** (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. *Trends Biochem Sci* **18**: 210–241
- Dupre P, Sherrier DJ** (1998) The plant Golgi apparatus. *Biochem Biophys Acta* **1404**: 259–270
- Edwards ME, Dickson CA, Chengappa S, Sidebottom C, Gidley MJ, Reid JSG** (1999) Molecular characterization of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. *Plant J* **19**: 691–697
- Esau K** (1977) *Anatomy of Seed Plants*, Ed 2. Wiley, Toronto
- Filisetti-Cozzi TMCC, Carpita NC** (1991) Measurement of uronic acids without interference from neutral sugars. *Anal Biochem* **197**: 157–162
- Frey-Wyssling A** (1976) The plant cell wall. In *Encyclopedia of Plant Anatomy*, Ed 3. Gebruder Borntraeger, Berlin
- Galway ME, Masucci JD, Lloyd AM, Walbot V, Davis RW, Schiefelbein JW** (1994) The *TTG* gene is required to specify epidermal cell fate and cell patterning in the *Arabidopsis* root. *Dev Biol* **166**: 740–754
- Goto N** (1985) A mucilage polysaccharide secreted from testa of *Arabidopsis thaliana*. *Arab Inf Serv* **22**: 143–145
- Grubert M** (1981) Mucilage or Gum in Seeds and Fruits of Angiosperms: A Review. Minerva Press, Munich
- Haughn G, Somerville C** (1986) Sulfonyleurea-resistant mutants of *Arabidopsis thaliana*. *Mol Gen Genet* **204**: 430–434
- Hyde BB** (1970) Mucilage-producing cells in the seed coat of *Plantago ovata*: developmental fine structure. *Am J Bot* **57**: 1197–1206
- Jofuku KD, den Boer BGW, Van Montagu M, Okamoto JK** (1994) Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. *Plant Cell* **6**: 1211–1225
- Karsen CM, Brinkhorst-van der Swan DLC, Breckland AE, Koornneef M** (1983) Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. *Planta* **157**: 158–165
- Kim J-B, Carpita NC** (1992) Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiol* **98**: 646–653

- Koornneef M** (1981) The complex syndrome of TTG mutants. *Arab Inf Serv* **18**: 45–51
- Kunst L, Klenz JE, Martinez-Zapater J, Haughn GW** (1989) AP2 gene determines the identity of perianth organs in flowers of *Arabidopsis thaliana*. *Plant Cell* **1**: 1195–1208
- Léon-Kloosterziel KM, Keijzer CJ, Koornneef M** (1994) A seed shape mutant of *Arabidopsis* that is affected in integument development. *Plant Cell* **6**: 385–392
- Lynch MA, Staehelin LA** (1992) Domain-specific and cell type-specific localization of two types of cell wall matrix polysaccharides in the clover root tip. *J Cell Biol* **118**: 467–479
- Lynch MA, Staehelin LA** (1995) Immunocytochemical localization of cell wall polysaccharides in the root tip of *Avena sativa*. *Protoplasma* **188**: 115–127
- Masucci JD, Rerie WG, Foreman DR, Zhang M, Galway ME, Marks MD, Schiefelbein JW** (1996) The homeobox gene *GLABRA2* is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. *Development* **122**: 1253–1260
- Mathur J, Chua N-H** (2000) Microtubule stabilization leads to growth reorientation in *Arabidopsis* trichomes. *Plant Cell* **12**: 465–477
- Mathur J, Spielhofer P, Kost B, Chua N-H** (1999) The actin cytoskeleton is required to elaborate and maintain spatial patterning during trichome cell morphogenesis in *Arabidopsis thaliana*. *Development* **126**: 5559–5568
- Modrusan Z, Reiser L, Feldmann KA, Fischer RL, Haughn GW** (1994) Homeotic transformation of ovules into carpel-like structures in *Arabidopsis*. *Plant Cell* **6**: 333–349
- Moore PJ, Swords KMM, Lynch MA, Staehelin LA** (1991) Spatial organization of the assembly pathways of glycoproteins and complex polysaccharides in the Golgi apparatus of plants. *J Cell Biol* **112**: 589–602
- Oppenheimer DG, Pollock MA, Vacik J, Szymanski DB, Ericson B, Feldmann K, Marks MD** (1997) Essential role of a kinesin-like protein in *Arabidopsis* trichome morphogenesis. *Proc Natl Acad Sci USA* **94**: 6261–6266
- Perrin RM, DeRocher AE, Bar-Peled M, Zeng W, Norambuena L, Orellana A, Raikhel NV, Keegstra K** (1999) Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. *Science* **284**: 1976–1979
- Piro G, Zuppa A, Dalessandro G, Northcote DH** (1993) Glucosylmannan synthesis in pea epicotyls: the mannose and glucose transferases. *Planta* **190**: 206–220
- Reiter W-D** (1998) The molecular analysis of cell wall components. *Trends Plant Sci* **3**: 27–32
- Reiter W-D, Chapple C, Somerville CR** (1993) Altered growth and cell walls in a fucose-deficient mutant of *Arabidopsis*. *Science* **261**: 1032–1035
- Reiter W-D, Chapple C, Somerville CR** (1997) Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J* **12**: 335–345
- Rerie WG, Feldmann KA, Marks MD** (1994) The *GLABRA2* gene encodes a homeodomain protein required for normal trichome development in *Arabidopsis*. *Genes Dev* **8**: 1388–1399
- Rodgers MW, Bolwell GP** (1992) Partial purification of Golgi-bound arabinosyltransferase and two isoforms of xylosyltransferase from French bean (*Phaseolus vulgaris* L.). *Biochem J* **288**: 817–822
- Schultz EA, Haughn GW** (1993) Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis*. *Development* **119**: 745–765
- Seitz B, Klos C, Wurm M, Tenhaken R** (2000) Matrix polysaccharide precursors in *Arabidopsis* cell walls are synthesized by alternate pathways with organ-specific expression patterns. *Plant J* **21**: 537–546
- Shannon S, Meeks-Wagner DR** (1993) Gene interactions that regulate inflorescence development in *Arabidopsis*. *Plant Cell* **5**: 639–655
- Sherrier DJ, VandenBosch KA** (1994) Secretion of cell wall polysaccharides in *Vicia* root hairs. *Plant J* **5**: 185–195
- Staehelin LA, Giddings TH Jr, Kiss JZ, Sack FD** (1990) Macromolecular differentiation of Golgi stacks in roots of *Arabidopsis* and *Nicotiana* seedling as visualized in high pressure frozen and freeze-substituted samples. *Protoplasma* **157**: 75–91
- Staehelin LA, Moore I** (1995) The plant Golgi apparatus: structure, function organization and trafficking mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* **46**: 261–288
- Sterling C** (1970) Crystal-structure of ruthenium red and stereochemistry of its pectin stain. *Am J Bot* **57**: 172–175
- Szymanski DB, Marks MD, Wick SM** (1999) Organized F-actin is essential for normal trichome morphogenesis in *Arabidopsis*. *Plant Cell* **11**: 2331–2347
- Van Caesele L, Mills JT, Sumner M, Gillespie R** (1981) Cytology of mucilage production in the seed coat of Candle canola (*Brassica campestris*). *Can J Bot* **59**: 292–300
- Vaughn JG, Whitehouse JM** (1971) Seed structure and the taxonomy of the Cruciferae. *Bot J Linn Soc* **64**: 383–409
- Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC** (1999) The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* **11**: 1337–1349
- Western TL, Haughn GW** (1999) *BELLI* and *AGAMOUS* genes promote ovule identity in *Arabidopsis thaliana*. *Plant J* **18**: 329–336
- Western TL, Skinner DJ, Haughn GW** (2000) Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiol* **122**: 345–355
- Willats WGT, McCartney L, Knox JP** (2001) In-situ analysis of pectic polysaccharides in seed mucilage and at the root surface of *Arabidopsis thaliana*. *Planta* **213**: 37–44
- Windsor JB, Symonds VV, Mendenhall J, Lloyd AL** (2000) *Arabidopsis* seed coat development: morphological differentiation of the outer integument. *Plant J* **22**: 483–493
- Zablackis E, York WS, Pauly M, Hantus S, Reiter W-D, Chapple CCS, Albersheim P, Darvill AG** (1996) Substitution of l-fucose by l-galactose in cell walls of *Arabidopsis mur1*. *Science* **272**: 1808–1810
- Zhang GF, Staehelin LA** (1992) Functional compartmentation of the Golgi apparatus of plant cells: immunocytochemical analysis of high-pressure frozen- and freeze-substituted sycamore maple suspension culture cells. *Plant Physiol* **99**: 1070–1083