

Differentiation of Mucilage Secretory Cells of the Arabidopsis Seed Coat¹

Tamara L. Western², Debra J. Skinner³, and George W. Haughn*

Department of Botany, University of British Columbia, 6270 University Boulevard,
Vancouver, British Columbia, Canada V6T 1Z4

In some plant species, including *Arabidopsis*, fertilization induces the epidermal cells of the outer ovule integument to differentiate into a specialized seed coat cell type with a unique morphology and containing large quantities of polysaccharide mucilage (pectin). Such seed coat mucilage cells are necessary for neither viability nor germination under normal laboratory conditions. Thus, the *Arabidopsis* seed coat offers a unique system with which to use genetics to identify genes controlling cell morphogenesis and complex polysaccharide biosynthesis and secretion. As a first step in the application of this system, we have used microscopy to investigate the structure and differentiation of *Arabidopsis* seed coat mucilage cells, including cell morphogenesis and the synthesis, secretion, and extrusion of mucilage. During seed coat development in *Arabidopsis*, the epidermal cells of the outer ovule integument grow and differentiate into cells that produce large quantities of mucilage between the primary cell wall and plasma membrane. Concurrent with mucilage production, the cytoplasm is shaped into a column in the center of the cell. Following mucilage secretion the cytoplasmic column is surrounded by a secondary cell wall to form a structure known as the columella. Thus, differentiation of the seed coat mucilage cells involves a highly regulated series of events including growth, morphogenesis, mucilage biosynthesis and secretion, and secondary cell wall synthesis.

The angiosperm seed coat consists of several layers of specialized tissues that provide protection to the embryo and assist in germination and dispersal. Tissues of the seed coat are derived from cells of the ovule integuments that differentiate in response to fertilization. In some species of plants, including members of the Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae, the epidermal cells of the seed coat contain a large quantity of a pectinaceous, complex polysaccharide (mucilage), a property known as myxospermy (Frey-Wyssling, 1976; Grubert, 1981; Van Caeseele et al., 1981, 1987; Boesewinkel and Bouman, 1995). When dry myxospermous seeds are placed in an aqueous

environment, the mucilage is released (extruded) and completely envelops the seed. Although the role of mucilage is unknown, it is thought to aid in the dispersal and/or protection of the emerging seedling during imbibition and germination. In addition to the seed coat, mucilages are commonly found in the transmitting tract of the pistil and surrounding the root cap (Frey-Wyssling, 1976; Esau, 1977), where they have roles in fertilization and root growth through the soil, respectively.

The major component of mucilage is pectin. Pectins are largely acidic polysaccharides that form gels in the extracellular matrix and are present in all cell walls as well as mucilage. The two most common pectins found in dicotyledonous plants are polygalacturonic acid (PGA) and rhamnogalacturonan I (RG I) (Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997). PGA is an unbranched chain of α -1,4-linked GalUA residues, while RG I is a highly substituted, branched polysaccharide with a backbone of alternating α -1,4-linked GalUA and α -1,2-linked rhamnose (Brett and Waldron, 1990). The fluidity of the extracellular matrix is largely dependent on the degree of bonding between PGA molecules, which is determined by the number of free carboxyl groups and interruptions of homogalacturonan chains with RG I (Bolwell, 1988; Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Reiter, 1998). Complex polysaccharides are synthesized from UDP-sugars by biosynthetic enzymes in the Golgi apparatus (Northcote, 1986; Bolwell, 1988; Brett and Waldron, 1990; Rodgers and Bolwell, 1992; Zhang and Staehelin, 1992; Carpita and Gibeaut, 1993; Driouich et al., 1993; Piro et al., 1993; Staehelin and Moore, 1995; Doong and Mohnen, 1998; Dupree and Sherrier, 1998; Reiter, 1998). Carbohydrate molecules are carried to the plasma membrane in secretory vesicles, and are secreted via exocytosis to form part of the extracellular matrix (Staehelin and Moore, 1995; Dupree and Sherrier, 1998). Little is known about the regulation of complex polysaccharide biosynthesis or secretion.

The plant species most widely exploited for genetic analyses, *Arabidopsis*, is included among the Brassicaceae species possessing myxospermy. In addition to carrying mucilage, like several other myxospermous species, *Arabidopsis* seed coat epidermal cells have a unique morphology dominated by the presence of an intracellular volcano-shaped structure known as the columella (Vaughan and Whitehouse, 1971; Koornneef, 1981). The composition of the columella and the manner in which it is formed during seed coat

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

³ 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.

development are unclear. Indeed, almost nothing is known about the structure and differentiation of Arabidopsis seed coat epidermal cells.

Given the unique cellular characteristics of the seed coat epidermis and the facility for genetic analysis in Arabidopsis, seed coat mucilage cells of Arabidopsis represent a potentially excellent model system for the studying the regulation of both the synthesis and secretion of complex carbohydrates and cellular morphogenesis. As a first step toward developing this system, we have used microscopy to investigate the structure and differentiation of this cell type in wild-type Arabidopsis. Our data suggest that Arabidopsis seed coat mucilage production occurs via the differentiation of ovular epidermal cells into active secretory cells that synthesize and secrete large quantities of complex polysaccharides from Golgi stacks. The columella, a volcano-shaped structure observed in mature seed coat epidermal cells, develops through active cytoplasmic rearrangement and synthesis of a secondary cell wall. The extrusion of mucilage following exposure of dry seed to an aqueous environment is the result of hydration and expansion of mucilage and the rupture of the radial segment of the primary cell wall. Our analysis establishes the timing of key events during the differentiation of seed coat mucilage cells, information that is a prerequisite for analyzing mutants defective in this process.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Lines of Arabidopsis used were the Columbia-2 (Col-2) ecotype and a homozygous *pgm1* mutant (Arabidopsis Biological Resource Center, Ohio State University, Columbus; stock no. CS210).

Seeds were stratified at 4°C for 3 d on prepared soil mix (Terra-Lite Redi Earth, W.R. Grace and Co., Ajax, Ontario, Canada) 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 [PAR]). Twelve hours of light/12 h of dark (100–150 $\mu\text{E m}^{-2} \text{s}^{-1}$ PAR) was used as indicated.

Staging of Flower Age

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

Clearing and Differential Interference Contrast Optics

Developing seeds were first stained for starch with $\text{I}_2\text{-KI}$ (Caspar et al., 1985), and were then placed in a quick-clearing solution of chloral hydrate, glycerol, and water (Léon-Kloosterziel et al., 1994) before being observed as whole-mount squashes. The samples were photographed

under differential interference contrast optics using a light microscope (DRB, Leica, Wetzlar, Germany) and Gold Plus 100 ASA film (Eastman Kodak, Rochester, NY). Photographs were digitized and manipulated with Photoshop (Adobe, Mountain View, CA) to prepare the figures.

Resin Embedding for Bright-Field and Transmission Electron Microscopy

Developing seeds for embedding in resin were either fixed in the silique or removed from the silique prior to fixation in 3% (w/v) glutaraldehyde (Canemco, Montreal) in 0.5 M sodium phosphate buffer at pH 7.0. After incubation at 4°C overnight, samples were washed with phosphate buffer, post-fixed for 1 to 2 h in 1% (v/v) osmium tetroxide in 0.5 M phosphate buffer, and dehydrated using a series of graded ethanol solutions. Alternatively, samples were fixed in FAA (4% [v/v] paraformaldehyde [Canemco], 15% [v/v] acetic acid, and 50% [v/v] ethanol) and directly dehydrated without post-fixation. All samples were then transferred to a propylene oxide solution and slowly infiltrated with Spurr's epoxy resin (Canemco). For bright-field microscopy, 0.2- to 0.5- μm sections were cut with glass knives on a microtome (Reichert-Jung, Vienna), mounted on glass slides, and stained with 1% (w/v) toluidine blue O in 1% (w/v) sodium borate (pH 11.0). Sections were photographed using a light microscope with Gold Plus or Royal Gold 100 ASA film. In preparation for electron microscopy, thin sections (silver-gold) were cut using a diamond knife on a microtome (Ultracut E, Reichert-Jung, Vienna) and collected onto formvar-coated, carbon-coated, and nickel grids. Sections 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 transmission electron microscope (model 10C, Carl Zeiss, Oberkochen, Germany) operated at an accelerating voltage of 60 or 80 kV. Photographs were digitized and manipulated with Adobe Photoshop to prepare figures.

Scanning Electron Microscopy

Samples were dry-mounted on stubs, coated with gold or gold-palladium in a sputter coater (SEMPrep2, Nanotech, Manchester, UK), observed using a scanning electron microscope (model 250T, 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.

Gas Chromatography and Mass Spectrometry (GC-MS)

Mucilage 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). Ten microliters of internal standard (4.8 mg/mL myo-inositol) was added prior to precipitation with 5 volumes of absolute ethanol. Derivatization of trimethylsilyl ethers was adapted from the method of Chaplin (1986). Samples were hydrolyzed overnight at 70°C in 4:1 1 N methanolic HCl:methyl acetate.

After transfer to Reactivials (Pierce Chemical, Rockford, IL), samples were precipitated with one-quarter volume of 2-methyl-2-propanol and dried under nitrogen gas. Acetylation of amino sugars was performed by a 15-min incubation in 10:1:1 methanol:pyridine:acetic anhydride and samples dried under nitrogen gas. The monosaccharides were trimethylsilylated for 1 h using Tri-Sil reagent (Pierce Chemical), dried under nitrogen gas, then resuspended in hexane. Samples were run on a gas chromatograph (model 5890A, Hewlett-Packard, Mississauga, Ontario, Canada) on a DB-5 fused silica column (30-m \times 0.25-mm i.d., $df = 0.10 \mu\text{m}$) with helium as the carrier gas. The temperature program was 140°C for 2 min, then increasing 8°C/min up to 240°C, followed by 5 min at 240°C. Compounds were initially identified 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 Chemistry Mass Spectrometry Centre (Vancouver).

The isolation of cell wall components from whole seeds was accomplished by grinding 100 seeds in 0.2% (w/v) ammonium oxalate. Derivatization was performed in the same manner as for mucilage alone, except a hexane extraction (2 volumes hexane to 1 volume sample) was performed after the acetylation step to remove seed oil. Individual sugar standards and a composite standard were made from the following monosaccharides: myo-inositol (used as an internal standard), Fuc, Man, Gal, Glc, Ara, rhamnose, Xyl, GlcUA, and GalUA (Chaplin, 1986).

RESULTS

Arabidopsis Mucilage

When an Arabidopsis seed is placed in water, a transparent, gel-like coating of mucilage is extruded and envelops the seed within seconds. When seeds are immersed in an aqueous solution of Ruthenium red, a dye that stains acidic polysaccharides (Frey-Wyssling, 1976), a pink-stained capsule with two distinct layers is observed (Fig. 1A). The outer layer is cloudy and diffuse, extending outwards from the seed surface approximately a seed width (approximately 200 μm), while the inner capsule resembles a bright-pink halo directly around the seed. If seeds are shaken in water before staining, the outer layer is absent (Fig. 1B). Upon closer examination, the inner capsule has dark-staining rays radiating out from the columellae of the cells below (Fig. 1B). The staining of Arabidopsis seed mucilage with Ruthenium red suggests that it is composed largely of pectin. This was confirmed by treating seeds with pectinase, which resulted in the loss of the mucilage capsule but not the rays (data not shown).

To confirm the composition of Col-2 mucilage, and for future comparison with mutants, the monosaccharide composition of Col-2 mucilage was determined using GC-MS (Table I). As expected, a reproducible sugar profile was obtained for wild-type (Col-2) mucilage, which revealed the presence of both rhamnose and GalUA, the major components of the pectins RG I and PGA. Other neutral monosaccharides, including Glc and Fuc, were also found.

While most of the peaks could be identified as consisting of monosaccharides, MS of some late peaks showed little similarity to previously studied molecules. It is possible that these peaks represent disaccharides or other molecules resulting from incomplete hydrolysis of the mucilage.

The Structure of the Mature Seed Coat

To determine the structure of the mucilage cells and the method of extrusion, mature seeds of the Col-2 ecotype were studied before and after wetting using both scanning electron and light microscopy. Scanning electron microscopic analysis of dry Arabidopsis seeds reveals an epidermal layer of hexagonal cells with thickened radial cell walls and a raised structure known as the columella (Fig. 1, C and D). After wetting and air-drying, the seeds appeared to be surrounded by a film, presumably mucilage, that also coated the tips of the columellae (Fig. 1, compare E to D). In addition, the outer tangential wall appeared to be missing, as the depressions around the columellae were more extensive and no obvious cell wall was draped over the thickened radial cell walls. These observations suggest that the release of mucilage is correlated with rupturing of the outer tangential cell wall of the epidermal cells.

Further study was done using thick plastic sections (0.2–0.5 μm) stained with toluidine blue O (Fig. 1, F and G), a polychromatic dye that stains different cell components a different color (O'Brien et al., 1964). First, the seeds were examined after fixation in 4% (v/v) formaldehyde in 50% (v/v) ethanol (FAA) to prevent mucilage release (Fig. 1F). The epidermal layer has cells with a thin outer tangential cell wall, a thickened inner tangential cell wall, and a very large, volcano-shaped columella in the center of each cell. The columella and the cell walls stained dark purple, suggesting that the columella is made up of cell wall material. The outer cell wall appears to be draped over the columella. Between the columella and the radial cell walls, there was pink-staining acidic polysaccharide: mucilage.

In seeds that were fixed with an aqueous solution of 3% (v/v) glutaraldehyde (Fig. 1G), the inner cell wall thickenings and the columellae are still present, but the polysaccharide is absent and the outer cell wall appears to have ruptured, leaving cell wall remnants attached to the top of the columellae. The underlying two cell layers of the seed coat remain unchanged between the two fixative treatments. The second cell layer, the palisade layer, has thickened inner tangential cell walls that stain dark blue, while the contents of the cells of the innermost layer, the pigmented layer, stain blue-green. Based on the staining properties of toluidine blue (O'Brien et al., 1964), these results are in agreement with the expected presence of lignin and condensed tannins in the second and third layers, respectively.

Outline of Arabidopsis Seed Development

Arabidopsis seed development has been studied by various groups primarily interested in embryo development (Meinke and Sussex, 1979; Mansfield et al., 1991; Mansfield and Briarty, 1991; Bowman and Mansfield, 1994). To corre-

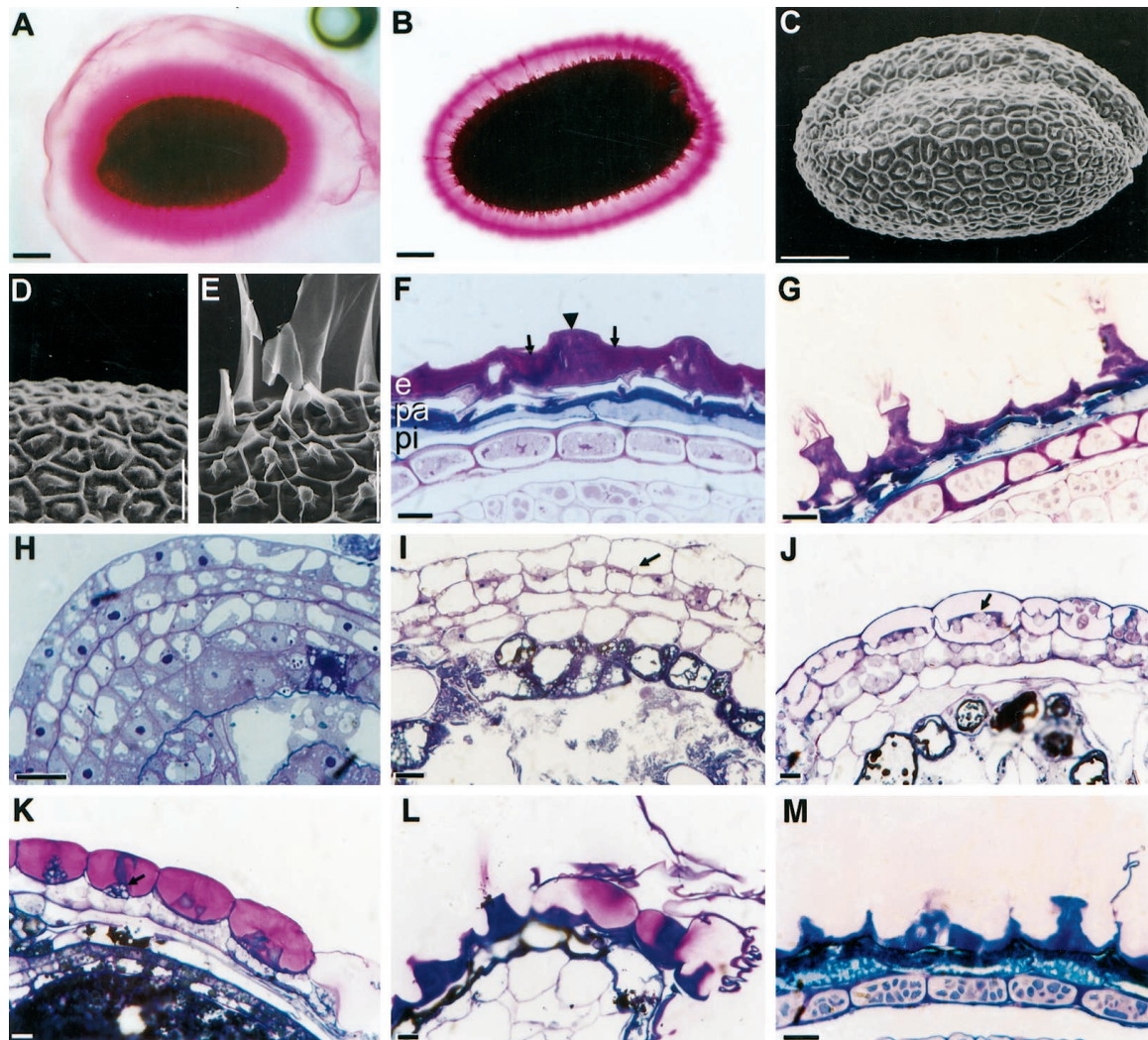


Figure 1. Structure and development of the wild-type seed coat. A and B, Stained with Ruthenium red; C to E, scanning electron micrograph; F to M, plastic sections of tissue fixed in aqueous 3% (v/v) glutaraldehyde and stained with toluidine blue. A, Seed stained without agitation. Two layers of mucilage are present, an outer cloudy layer and a darkly staining inner capsule. B, Seed stained after first shaking in water; only the inner capsule is present. Note the "rays" radiating from each columella. C, Scanning electron micrograph of dry seed. Note the hexagonal epidermal cells with thickened radial cell walls and columellae in the center. D, Scanning electron micrograph of epidermal cells from a dry seed. E, Scanning electron micrograph of seed that has been wetted and air-dried. Note the mucilage on tips of columellae and deep shadows around the columellae where the outer cell wall has been torn away. F, The seed coat when fixed in 4% (v/v) formaldehyde and 50% (v/v) ethanol. Mucilage (arrows) is retained in the epidermal layer (e) surrounding the columella (arrowhead). Note that the thick bottom cell wall of the palisade layer (pa) stains dark blue and the contents of pigmented layer (pi) cells are pale blue-green, both suggesting the presence of polyphenols. The other visible cell layers subtending the seed coat are embryonic in origin, including the aleurone layer immediately adjacent to the pigmented layer of the seed coat. G, Seed coat fixed under aqueous conditions. The outer cell wall of the epidermal cells has burst and mucilage has been released to surround the seed. Note cell wall material attached to the columella. H, Mature ovule (0 DAP). I, Seed at 4 DAP. Note small globular inclusions (arrow) in cells. J, Seed at 7 DAP. The globular inclusions (arrow) are larger and found in center of the epidermal cells, which also have faint pink staining. K, Seed at 10 DAP. Intense pink staining polysaccharide is in epidermal cells; globular inclusions are small and are found at the bottom of cell (arrow). Purple-staining cell wall material can be seen in the center of some cells. Thickening of the inner tangential cell wall is also apparent in the palisade (subepidermal cell layer). L, Seed at 13 DAP. The outer cell wall has burst, releasing mucilage from the epidermal cells. In the cells that are still partially intact, dark pink mucilage is present. The columellae consist completely of cell wall material. M, Seed at 18 DAP. The outer cell wall of the epidermal cells has burst and mucilage has been released to surround the seed. Directly below the base of the columellae is the dark blue, thickened cell walls of the palisade layer. The contents of the pigmented layer cells stains pale blue. Scale bars: A to C = 100 μm ; D and E = 40 μm ; F to M = 10 μm .

Table 1. Retention times, monosaccharide assignments, and amount of sugar in the major peaks of *Arabidopsis* ecotype Col-2 mucilage

Retention Time	Sugar	Average	SD
		$\mu\text{g}/100$ seeds ^a	
7.09	Rhamnose, Fuc ^b	3.89	0.48
9.51	GalUA	2.37	0.36
10.04	Gal	0.85	0.12
11.00	Gal	2.26	0.32
11.14	GalUA	1.36	0.21
11.24	Glc	0.66	0.20
11.48	Glc	0.23	0.12
13.52	Octadecadienoic acid	0.17	0.37
13.60	Unknown	0.55	0.36
13.79	Unknown	3.41	2.57
13.99	Inositol ^c	48.00	0.00
14.36	Unknown	1.15	0.04
14.54	Unknown	0.74	0.05
16.09	Unknown	0.34	0.29

^a Five samples. ^b Rhamnose and Fuc could not be separated under our conditions. ^c Internal standard.

late developmental events in epidermal cells with other aspects of seed development, it was necessary to study seed development under our growth conditions and with the Col-2 ecotype. Seed development in the *Arabidopsis* ecotype Col-2 takes roughly 16 to 18 d at 20°C to 22°C under continuous light. Clearing in organic solvents was used to determine the size, color, and stage of embryo development for seeds each day from the time of pollination to the completion of seed development (Fig. 2).

Subsequent to pollination the fertilized ovule grew rapidly to a length of 500 μm , the approximate length of a mature seed after which time growth ceased (Fig. 2). By the 5th d, the embryo was in the heart stage and was starting to produce chlorophyll, giving the seed a green appearance. The embryo's cotyledons reached their full size by approximately 12 DAP; after this time, filling of the embryo with storage compounds continued until the time of desiccation. Desiccation was determined as the point at which

seeds started to lose their green color and turn brown. Under our conditions, the seed began to desiccate at approximately 16 DAP and continued for another day or two until the seed was dry (Fig. 2).

Epidermal Cell Development

The development of the epidermal cells of the ovule integument into the mucilaginous cells found in the seed coat was studied to determine the origin of the mucilage and columellae. In addition, these data can be used to establish major stages during epidermal cell differentiation for comparison against mutants defective in mucilage production. Staining of sections of the epidermal cells of the ovule integument with toluidine blue revealed the nucleus, cytoplasm, and a large vacuole that occupied approximately one-half of the cell area (Fig. 1H). A large increase in cell size (3.5- to 4-fold) occurred during the first four DAP and was correlated with an increase in the size of the vacuole. Therefore, the cytoplasm is restricted to the outer margins of the cell (Fig. 1I). Globular, intracellular inclusions were first evident at 3 DAP, and then increased in size and number until 7 DAP (Fig. 1, I and J). In appearance, these inclusions resemble amyloplasts observed in transmission electron microscopic studies of mucilage production in the *Brassica campestris* seed coat and tobacco and *Arabidopsis* root tips (Van Caeseele et al., 1981; Staehelin et al., 1990). To test the hypothesis that the inclusions are amyloplasts that contain starch, we observed developing seeds by light microscopy after staining with a starch-specific stain, I₂-KI, and clearing in organic solvents. The inclusions stain purple-black (Fig. 3, A and B), indicating the presence of starch. In addition, developing seeds of a starchless mutant, *pgm1* (Caspar et al., 1985; Caspar and Pickard, 1989), do not contain the inclusions (Fig. 3, C and D).

At 6 to 7 DAP, as the starch granules become larger, pink-staining acidic polysaccharide is seen throughout the cells. From d 7 to 9 the intensity of the pink stain increases toward the external surface of the cell and the amyloplasts appear to be delimited to a column in the center of the cell

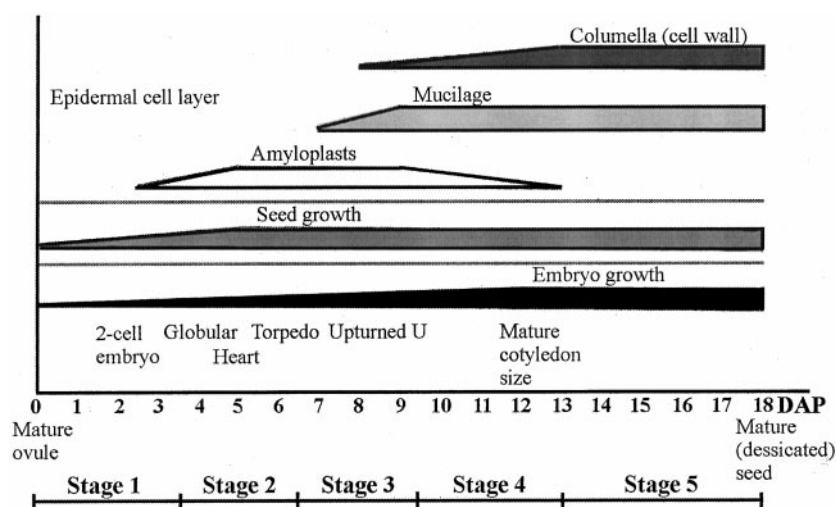
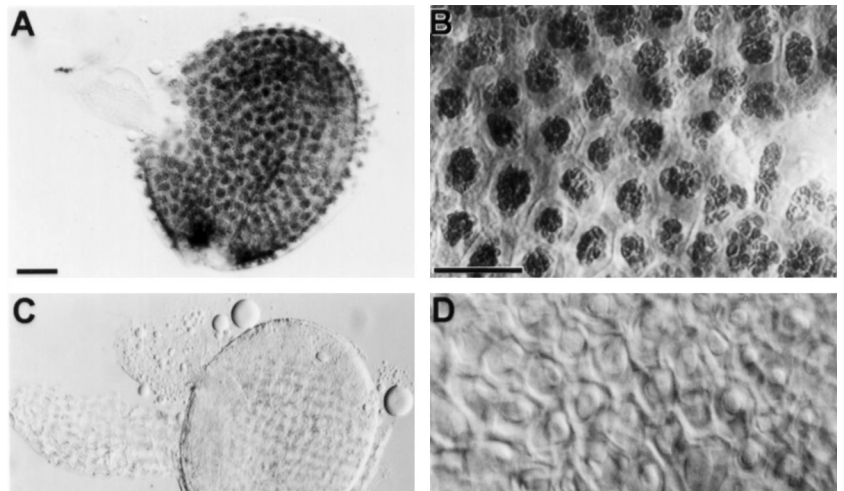


Figure 2. A diagram showing the correlation between events in seed coat development, embryo development, and seed growth following pollination. The thickness of the shaded bars associated with each process represents the cumulative number of amyloplasts or amount of columella cell wall, seed growth, or embryo growth that has occurred by the indicated stage of development. Seed coat epidermal differentiation has been divided into five stages based on the major cellular events described in the text.

Figure 3. Wild-type and *pgm1* seeds stained with iodine. Whole-mount seeds were stained in I₂KI, then cleared with organic solvents and photographed with differential interference contrast optics. A, Wild-type seed at 6 DAP showing globular inclusions that stain purple-black with I₂-KI. B, Close-up of wild-type seed at 8 DAP showing stained globular inclusions in the epidermal cells. C, *pgm1* seed at 9 DAP showing no globules. D, Close-up of *pgm1* seed at 7 DAP showing no stained globules. Scale bars: A and C = 100 μ m; B and D = 50 μ m.



(Fig. 1, J and K). The maximum intensity of pink stain appears to be reached at 9 DAP after which time the pink color of intact cells remains unchanged. By 10 DAP, cells prematurely hydrated in aqueous fixative can break open and release mucilage, while a new cell wall (stained dark purple) begins to be deposited around the cytoplasmic column (columella) and form the column to part way up the radial axis of the cell. At this stage, the amyloplasts begin to stain darker and appear smaller (Fig. 1K). The cell walls of the columellae are much thicker by 12 DAP, and fuse with thickenings at the base of the radial cell walls. Cytoplasm is reduced but still apparent under the columellae and most of the cells break open to release the mucilage when hydrated. By 13 DAP, the columellae appear to consist entirely of cell wall material (Fig. 1L). From 13 to 18 DAP there is little difference in the structure of the cells. Once cells are mature, hydration leads to mucilage release, resulting in cells with a protruding columellae surrounded by a space where the mucilage had been and lacking an outer tangential cell wall. In addition, after breaking to release the mucilage, portions of the outer cell wall can remain attached to the edges of the columella, contributing to the ray-like appearance of the extruded mucilage (Fig. 1, G and M).

The Presence of Starch Is Correlated with Mucilage Production

As described in the previous section, amyloplasts accumulate prior to the production of mucilage and are present in their highest quantity during the time of mucilage synthesis, following which their number and size decreases (Figs. 1, 2, and 3, A and B). This correlation of the appearance of amyloplasts with mucilage production has also been noted in previous studies of mucilage synthesis (Van Caesele et al., 1981; Staehelin et al., 1990) and suggests that starch may be necessary for mucilage production. The timing of the amyloplast accumulation may also be correlated with the production of the new cell wall of the columellae.

The requirement for starch in mucilage and columellae formation was tested by studying a starchless mutant (*phosphoglucomutase 1 [pgm1]*). Plants homozygous for the *pgm1* mutation are unable to make the enzyme phosphoglucomutase and have been found to completely lack starch (Caspar et al., 1985; Caspar and Pickard, 1989). The seeds of *pgm1* plants were compared with wild-type (ecotype Col-2) plants under two different growth conditions. When both wild type and *pgm1* are grown under continuous light, both produce normal seeds with columellae and mucilage (data not shown). This suggests that starch, itself, is not necessary for seed epidermal cell development.

It has been shown that *pgm1* mutants grown under continuous light accumulate pools of soluble sugars in the cytoplasm (Caspar et al., 1985). Since pectin production involves UDP-sugars made in the cytoplasm (Brett and Waldron, 1990), it is not surprising that mucilage can still be made by *pgm1* seed coats. When *pgm1* mutants are grown under a light-dark cycle of 12 h of light/12 h of dark, the pools of sugars become depleted (Caspar et al., 1985). In order to test the hypothesis that this sugar pool was used to make mucilage in *pgm1* mutants grown in continuous light, both wild-type and *pgm1* plants were grown under a regime of 12 h of light and 12 h of darkness. Under these conditions, both wild-type and *pgm1* plants still produce normal seeds with mucilage and columellae. The *pgm1* plants, however, instead of making the full complement of 50 to 60 seeds, make only two to three seeds per silique (data not shown). It is possible that Arabidopsis aborts seed development under conditions when carbohydrate reserves are low, thus investing available resources in a few progeny. While our results demonstrate that starch itself is not necessary for seed coat differentiation, it is still possible that starch is the primary source of carbon during development of mucilage cells in wild-type seeds.

Secretion of Mucilage

Transmission electron microscopy was used to determine the cytological events leading to the secretion of

mucilage in the developing seed coat. Epidermal cells of developing seeds at 4, 7, and 10 DAP were examined for the secretory apparatus (Fig. 4). At 4 DAP, cells were largely vacuolated, with the cytoplasm, including some amyloplasts, appearing around the edges of the cell (Fig. 4A). At this stage, there was very little evidence of mucilage. In contrast, at 7 DAP, the ultrastructure of the cells was quite different. The vacuole was smaller and there was an extracellular space between the plasma membrane and the outer cell wall (Fig. 4C), which contained dark fibrils (Fig. 4D). A smaller number of fibrils was also found in the rest of the cell, often bounded by membranes. The appearance of the fibrillar material correlated in time and space with the pink-staining acidic polysaccharides observed in thick sections, suggesting that it was mucilage.

The cytoplasm at 7 DAP stained very darkly and was full of vesicles (Fig. 4, compare D with B). Golgi stacks were also apparent, usually surrounded by vesicles with contents having a grainy appearance (Fig. 4, E and F). At 10 DAP, however, depending on the seed studied, these vesicles were either present in lesser numbers or completely absent (Fig. 4I). The fibrillar material in the extracellular space was much denser than that found at 7 DAP (Fig. 4, J and K), and many 10-DAP cells had already ruptured in the aqueous fixative, releasing the fibrillar material (Fig. 4G). This breakage occurred at the upper part of the radial cell wall, where the wall is thinnest (Fig. 4G). These data are consistent with the hypothesis that the mucilage is made in the Golgi stacks, deposited into vesicles, and secreted into the extracellular space between the cytoplasm and the primary cell wall. Once mucilage production is complete, seed hydration leads to the rupture of the original cell wall at its weakest point.

Formation of the Columella

At 7 DAP, the cytoplasm was found in a very distinct column in the center of the cell, spreading at the base over a reduced vacuole (Fig. 4, C and D). The cytoplasmic column at 10 DAP was surrounded by a layer of electron-dense material resembling the primary cell wall, though slightly more diffuse (Fig. 4G). Since the purple-staining columella became apparent around 10 DAP, it appears that its formation results from the deposition of secondary cell wall material around the narrow cytoplasmic column and small vacuole (Fig. 4, G and H). From 10 to 13 DAP, the cell wall increased in thickness until it had filled the entire column (Fig. 4L) except for a small amount of cytoplasm at the bottom of the cell, which disappeared during dehydration. The resulting columella cell wall not only formed a column in the center of the cell but also extended along the bottom of the cell and midway up the new radial cell walls. Therefore, the inner tangential cell wall and the lower part of the radial walls were reinforced (Fig. 4, G and L). The columella wall was closely appressed to the center of the outer tangential portion of the primary cell wall (Fig. 4, K and L).

DISCUSSION

Arabidopsis, like many other species of the Brassicaceae, is a myxospermous plant that extrudes a gel-like layer around its wetted seeds (Vaughan and Whitehouse, 1971). This mucilage has been found to be composed largely of pectin both in Arabidopsis (Goto, 1985; this study) and in other plants with mucilage-containing seeds (Siddiqui et al., 1986; Van Caesele et al., 1987; Cui and Eskin, 1993; Fedeniuk and Biliaderis, 1994). Seed mucilage production in Arabidopsis is a part of a remarkable differentiation process during which the epidermal cells of the mature ovule grow, rearrange their cytoplasm, synthesize and secrete mucilage, and form a secondary cell wall (Fig. 5). These events are triggered by pollination and result in a seed coat epidermis with cells that have large quantities of mucilage located between the outer tangential cell wall and a cellulosic structure known as the columella.

Differentiation of the Epidermal Cells of the Arabidopsis Seed Coat Involves an Active Period of Secretion

After a period of growth, the seed coat epidermal cells undergo changes that are consistent with the idea that the epidermal cells become very active in secretion. There is a dramatic increase in the number of large vesicles such that the entire cytoplasm is filled. Some vesicles are found near both the Golgi cisternae and the plasma membrane. Concomitantly, fibrillar material gradually accumulates between the plasma membrane and the outer tangential cell wall. The role of the increase in secretory activity and the identity of the fibrillar material observed in the extracellular space have not been determined. However, the appearance of the vesicles and fibrillar material correlates closely in time and space with the synthesis and secretion of mucilage observed by light microscopy. In addition, mucilage-producing cells in the root tips of Arabidopsis, tobacco, *Trifolium pratense*, and oat (Staehelin et al., 1990; Lynch and Staehelin, 1992, 1995) and in the seed epidermis of *Plantago ovata* and *B. campestris* (Hyde, 1970; Van Caesele et al., 1981) share similar ultrastructural characteristics. These data suggest that the seed coat mucilage is secreted via the Golgi apparatus.

It is interesting that secretion of mucilage is polar, such that large quantities of mucilage accumulate only between the plasma membrane and the outer tangential cell wall. Polar secretion has been investigated by researchers studying cells that undergo tip growth, as is the case for pollen tubes. The polar nature of secretion appears to depend on the cytoskeleton, especially actin microfilaments that direct vesicles to a specific region of the plasma membrane (Cai et al., 1997; Fowler and Quatrano, 1997; Dupree and Sherrier, 1998). It is possible that the polar secretion in seed coat epidermal cells is controlled by similar mechanisms.

The Formation of the Columella Occurs through the Manipulation of the Cytoplasm and Cell Wall Production

The structure and function of the columella in the Arabidopsis seed coat are unknown. It has been suggested

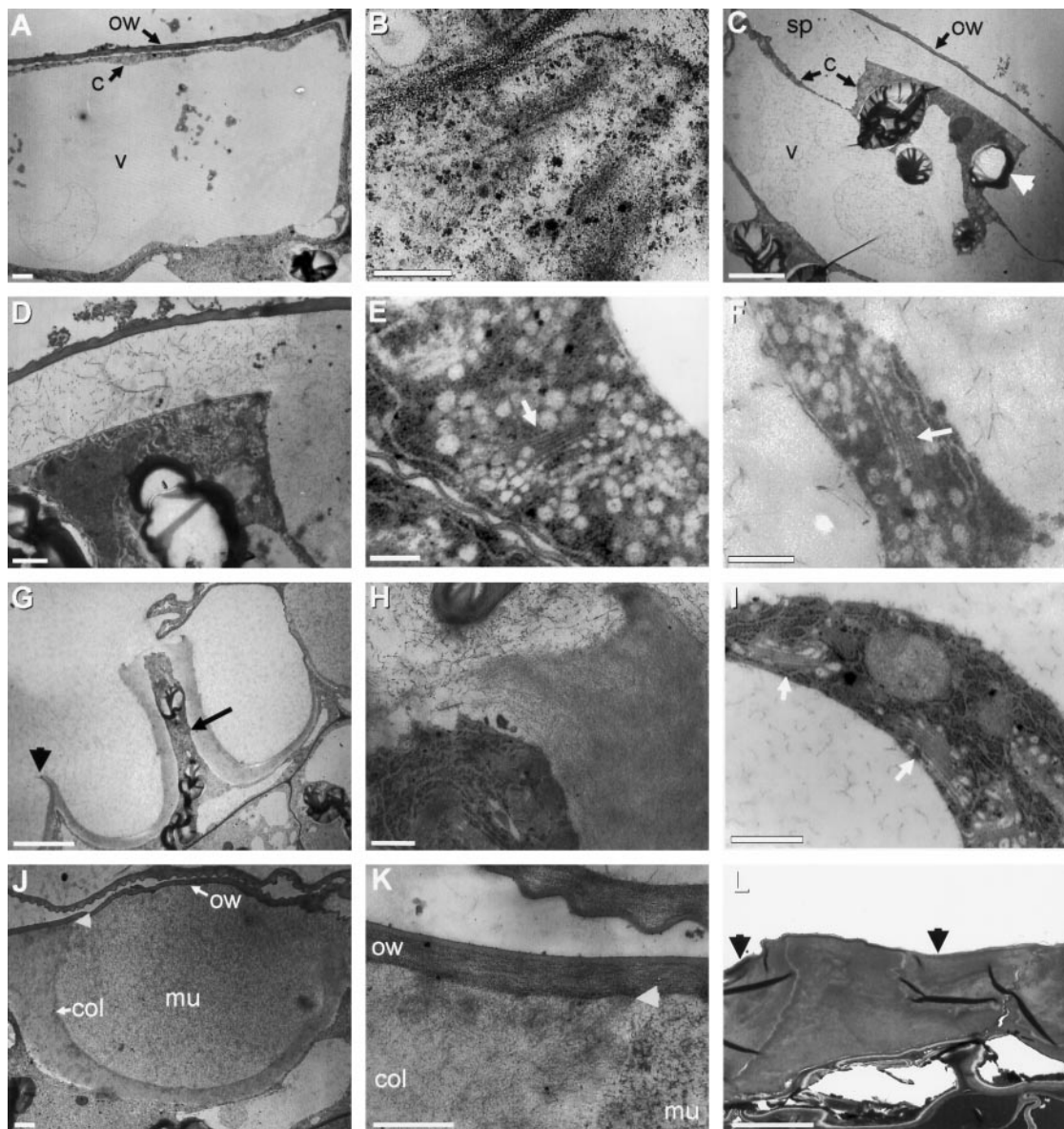


Figure 4. Transmission electron micrographs of developing epidermal cells of wild-type seeds. A, Cell at 4 DAP. Note that the large vacuole (v) and cytoplasm (c) found around the edges of cell close to the outer cell wall (ow). B, Cytoplasmic detail of cell at 4 DAP. Note lack of obvious vesicles. C, Cell at 7 DAP. Most of the cytoplasm (c) is in a column in the center of the cell. There is a space (sp) containing fibrillar material (see D) between the cytoplasm and the outer cell wall (ow), and the vacuole is found under the cytoplasmic column (v). Note very large amyloplasts (arrowhead). D, Structural detail of a cell at 7 DAP showing top corner of cytoplasmic column. The cytoplasmic column is filled with vesicles and tubular clearings. Fibrillar material is present between the cytoplasm and the outer cell wall. E, Detail of cytoplasm from 7-DAP cell. Note large number of vesicles with grainy contents. Arrow indicates Golgi stack. F, Detail of cytoplasm from 7-DAP cell. This small portion of cytoplasm is full of vesicles. Some vesicles are adjacent to a Golgi stack (arrow). G, Cell at 10 DAP. The cytoplasm (arrow) is found in the center of the cell and at the bottom surrounded by electron dense material. Note small vacuole at the bottom of the cell and the ruptured outer cell wall. The cell wall has broken at a point directly above where the secondary wall ends (arrowhead). H, Top of column in center of cell in G. The electron-dense material around the cytoplasm resembles the outer cell wall but is more diffuse. The cytoplasm contains fine tubules. I, Cytoplasm at the base of a cell at 10 DAP. Note Golgi stacks (arrows) and reduced number of vesicles compared with 7 DAP (see E and F). J, Portion of intact cell at 10 DAP. The new cell wall of the developing columella (col) appears to fuse with the original outer cell wall (ow), and the space between the intracellular column and the outer cell wall is filled with densely packed fibrils of mucilage (mu). The arrowhead indicates the junction between the columella cell wall and the outer tangential cell wall. K, Top of the columella (col) from the cell in J showing appression of original outer cell wall (ow) and the new, secondary cell wall (arrowhead) surrounding the cytoplasm (mu = mucilage). Note that the position of the arrowhead is the same as the one in J. L, Cell of 14-DAP seed that has been fixed in 50% (v/v) ethanol. The columella is surrounded on either side with mucilage (arrowheads) within the outer cell wall. Scale bars: A, D, and J = 1 μ m; B, H, and K = 500 nm; E, F, and I = 250 nm; C, G, and L = 5 μ m.

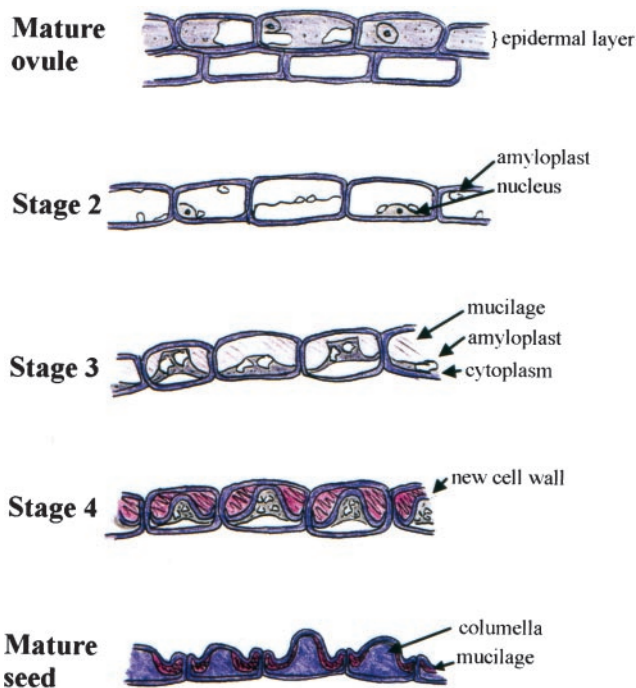


Figure 5. Drawing showing the stages in the production of mucilage and columellae in the epidermal cells of wild-type Arabidopsis seeds. See text for details.

previously that the columella is a receptacle for mucilage (Koorneef, 1981). Our results, however, suggest that the columella is an elaborate secondary cell wall produced subsequent to mucilage secretion. The columella is produced in a two-step process involving the initial production of a cytoplasmic column in the center of the cell, followed by deposition of a secondary cell wall.

The first stage of columella production is intracellular reorganization. The cytoplasm is drawn in from the margins of the cell to a well-defined column in the center of the cell. This cellular morphogenesis is coupled with a reduction in vacuolar size and the creation of an extracellular space (Fig. 5). Despite the fact that morphogenesis is correlated with the beginning of mucilage production, it is unlikely that such reorganization is merely a consequence of mucilage accumulation in the extracellular space. Rather, the sharp, well-defined edges of the cytoplasmic column suggest that the cell actively forms this column through a carefully regulated program of cellular morphogenesis. Columella production continues through further compression of both cytoplasmic column and vacuole, followed by the formation of a secondary cell wall. The switch from mucilage biosynthesis and secretion to secondary cell wall production is reflected by changes in cell ultrastructure. The vesicles are reduced in number and/or are no longer large and obvious. In addition, the cytoplasm appears to be packed with a large amount of rough endoplasmic reticulum (data not shown). The rough endoplasmic reticulum may be necessary for the production of new enzymes and other proteins needed to form the secondary cell wall. The tight correlation of mucilage production, intracellular rearrangement, and secondary cell wall for-

mation suggests a highly regulated system involving not only the sequential production of abnormally high amounts of varied cell wall materials, but also the possible involvement of the cytoskeleton in cell shape changes and directed secretion.

The function of either the cytoplasmic column or the columella in Arabidopsis is unclear. The columella is found in many species of the Brassicaceae, including *Capsella bursa-pastoris* (Vaughan and Whitehouse, 1971). However, other species such as *B. campestris* fail to form either a cytoplasmic column or a secondary cell wall in the epidermal cells (Van Caesele et al., 1981), indicating that columella formation is not an essential characteristic of mucilage secretory cells. The cytoplasmic column provides a large surface-to-volume ratio that may allow for an increased rate of exocytosis and, therefore, more rapid mucilage deposition. A function for the columella itself may be to provide a rigid surface to assist in the rupture of the outer cell wall during mucilage expansion. Alternatively, the columella may be necessary for maintaining the structural integrity of the seed coat following extrusion.

Mucilage Extrusion through Mucilage Expansion

Hydration of Arabidopsis seeds leads to the immediate release of mucilage, an event correlated with breakage of the outer tangential cell wall of the epidermal cell. Pectins are extremely hydrophilic (Frey-Wyssling, 1976), suggesting that mucilage extrusion results from the rapid expansion of dried mucilage upon hydration, leading to breakage of the cell wall. Interestingly, the rupture occurs at the top of the radial cell walls, above the point of reinforcement by the secondary cell wall (Figs. 1, F and M, and 4G). Thus, the formation of the secondary cell wall may influence the position of cell wall breakage. Cell wall remnants can remain attached to the columella (Fig. 1G), possibly contributing to the appearance of dark-staining rays in hydrated seeds. In addition, Ruthenium red staining of mucilage reveals two layers: a dark pink layer close to the seed and a cloudy layer farther away (Fig. 1A). The dark-staining layer likely consists of more compact mucilage, which in turn would result in increased Ruthenium red binding (Sterling, 1970). The retention of mucilage close to the seed may be due to the association of the mucilage with the columella and outer cell wall remnants.

Mucilage Composition

Mucilage is a general term for pectinaceous compounds extruded by plants under normal growth. The chemical composition of Arabidopsis seed mucilage was initially characterized by Goto (1985) using the Sendai ecotype. His results showed that it is largely composed of GalUA and lesser amounts of neutral monosaccharides. Our GC analysis using trimethylsilyl derivatives of multiple seed samples of the Col-2 ecotype suggested a similar composition and demonstrated a consistent monosaccharide profile for wild-type seeds. The pectinaceous nature of Arabidopsis mucilage has also been suggested both by specific staining with Ruthenium red and toluidine blue O (O'Brien et al.,

1964; Frey-Wyssling, 1976), corresponding to that expected for an acidic polysaccharide, and by its loss when seeds were treated with pectinase, an endo-polygalacturonase (Frey-Wyssling, 1976). A precise structure of *Arabidopsis* mucilage has yet to be determined.

Staging of Epidermal Cell Differentiation in the Non-*Arabidopsis* Seed Coat

We have correlated the key features of epidermal cell differentiation (presence of amyloplasts, mucilage, and cell wall of columellae) with other aspects of seed development, including overall seed growth and embryo development (Fig. 2). These results will be valuable for comparison of mutants isolated in different ecotypes and under different growth conditions. Our studies have allowed us to divide epidermal cell differentiation into five stages (Figs. 2 and 5). Stage 1 immediately follows fertilization and consists of a period of cell growth driven by expansion of the vacuole, forcing the cytoplasm to the margins of the cells. In stage 2, the amyloplasts accumulate and grow and the cytoplasm is rearranged such that strands of cytoplasm can be seen across the center of the cells. Once the cells have reached their final size and amyloplasts have accumulated to their full extent, mucilage production can occur. During stage 3, a cytoplasmic column is formed in the center of the cell, the vacuole is reduced in size, vesicles appear throughout the cytoplasm, and mucilage gradually accumulates. In stage 4, mucilage production is completed and a secondary cell wall becomes apparent around the cytoplasmic column, forming the columella. In addition, the amyloplasts shrink and the vacuole becomes limited to a small space under the cytoplasm. During the final stage (stage 5), dehydration occurs, leading to shrinkage of the mucilage such that the outer cell wall drapes over the contours of both the thickened radial cell walls and the columella.

Seed coat differentiation happens simultaneously with embryo growth past the heart stage and ends at approximately the time that the embryonic cotyledons have reached their full size and production of storage compounds becomes the major focus of embryo development. These correlations suggest that the plant might stage events of seed development such that processes requiring high amounts of energy do not occur simultaneously, with seed growth occurring first, followed by the production of the seed coat and, finally, the filling of the embryo with storage compounds.

Arabidopsis Seed Mucilage as a Model System for the Study of Complex Polysaccharide Biosynthesis and Secretion

Differentiation of the *Arabidopsis* seed coat epidermal cells represents an excellent model system for the genetic analysis of several important cellular events including growth, morphogenesis, carbohydrate secretion, and secondary cell wall formation. The tissue is easily accessible and is completely dispensable under normal laboratory conditions. Indeed, loss-of-function alleles of *APETALA2*

completely lack seed coat mucilage cells (Jofuku et al., 1994; T.L. Western and G.W. Haughn, unpublished results), yet germination and embryo viability are not seriously compromised. Other mutants with defects in seed coat epidermal cell differentiation have also been identified previously (*transparent testa glabra*, *glabra2*, and *aberrant testa shape*; Koornneef, 1981; Bowman and Koornneef, 1994; Jofuku et al., 1994; Léon-Kloosterziel et al., 1994; Rerie et al., 1994). A screen for additional mutants defective in the biosynthesis, secretion, or extrusion of mucilage has led to the identification of at least five novel loci, *MUCILAGE-MODIFIED (MUM)* 1 to 5 (T.L. Western and G.W. Haughn, unpublished results). Our characterization of the structure and differentiation of the wild-type seed coat mucilage cells and mucilage composition represents an important prerequisite for determining the defects in these mutants.

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