GENETIC ANALYSIS OF OVULE DEVELOPMENT AND SEED COAT MUCILAGE PRODUCTION IN *ARABIDOPSIS THALIANA*

by

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ABSTRACT

Recently, many genes involved in the regulation of ovule development have been identified in *Arabidopsis thaliana.* One of these genes is *BELL1 (BEL1)*, which is involved in the production of ovule integuments and is thought to work at least partially through the suppression of the floral organ identity gene, *AGAMOUS (AG).* A new investigation of the genetic interactions between *BEL1* and *AG* has shown that both genes have active roles throughout ovule morphogenesis. *BEL1* and *AG* act early in ovule development in a partially redundant manner to direct ovule identity. The abnormal integument development characteristic of the Bel1 mutant phenotype was found to be dependent on *AG* function, suggesting that *BEL1* and *AG* work in combination to promote normal integument morphogenesis. Finally, BEL1 appears to be required for embryo sac development independent of other aspects of ovule morphogenesis and *AG* function. Another floral organ identity gene that is thought to act in ovule development is *APETALA2 (AP2).* This has been confirmed through quantitation of ovule phenotypes in multiple alleles, which revealed that in the absence of *AP2* function there is sporadic production of multiple types of abnormal ovules. These data suggest that *AP2* may stabilize developmental decisions throughout ovule development.

During seed development, many plants synthesize and secrete a vast quantity of pectin in the form of mucilage between the plasma membrane and outer cell wall of their epidermal cells. The production of mucilage during *Arabidopsis* seed coat development was studied and then exploited in a novel screen for mutants defective in polysaccharide synthesis, deposition, and extrusion. Mutations in five novel genes, *MUCILAGE-MODIFIED (MUM) 1—5* were isolated, and were studied along with mutants defective in four previously identified loci that affect seed coat development (*TRANSPARENT TESTA GLABRA1 [TTG1], GLABRA2 [GL2], ABERRANT TESTA SHAPE [ATS],* and *AP2*). The phenotypes of the mutants suggest that *AP2* and *ATS*

regulate general seed coat development, while *MUM4, TTG1*, and *GL2* affect the epidermal cell structure and mucilage amount. In addition, *MUM3* and *MUM5* control mucilage composition, and *MUM1* and *MUM2* affect its extrusion beyond the outer cell wall.

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ABBREVIATIONS

AG	*AGAMOUS*
AGL	*AGAMOUS-LIKE*
AN	*ANGUSTIFOLIA*
ANT	*AINTEGUMENTA*
AP1	*APETALA1*
AP2	*APETALA2*
AP3	*APETALA3*
ATS	*ABERRANT TESTA SHAPE*
BAG	*BLASIG*
BEL1	*BELL1*
bp	basepair
°C	degrees Celsius
CLS	carpel-like structure
Col	Columbia ecotype
CRC	*CRABS CLAW*
DIC	differential interference contrast
E	einstein
EDTA	ethylenediaminetetraacetic acid, disodium salt dihydrate
EMS	ethylmethane sulfonate
ER	*ERECTA*
F1	first filial progeny
F2	second filial progeny
FAA	formaldehyde: acetic acid: alcohol fixative
GC	gas chromatograph
GL2	*GLABRA2*
GL3	*GLABRA3*
HCl	hydrochloric acid
HLL	*HUELLENLOS*
ILS	integument-like structure
INO	*INNER NO OUTER*
kb	kilobase pair
kV	kilovolt
LAL	*LAELLI*
Ler	Landsberg *erecta* ecotype
LUG	*LEUNIG*
m	metre
M	molar
MADS-box	sequence motif present in *MCM1, AG, DEF, SRF* loci
µg	microgram
mg	milligram
µm	micrometre
mm	millimetre
MOG	*MOLLIG*
MS	mass spectrometer
MUM	*MUCILAGE-MODIFIED*

N	normal
nm	nanometre
NPTII	neomycin phosphotransferase II
PCR	polymerase chain reaction
PGA	polygalacturonic acid
PI	*PISTILLATA*
RG I	rhamnogalacturonan I
RG II	rhamnogalacturonan II
SEM	scanning electron microscope
SIN1	*SHORT INTEGUMENTS1*
SPT	*SPATULA*
STA	*STACHEL*
SSPE	sodium chloride-sodium phosphate-EDTA buffer
SUB	*STRUBBELIG*
TBE	Tris-boric acid-EDTA electrophoresis buffer
T-DNA	transfer DNA of *Agrobacterium tumefaciens*
TE	Tris-EDTA buffer
TEM	transmission electron microscope
TMS	trimethylsilyl
TTG1	*TRANSPARENT TESTA GLABRA1*
TSL	*TOUSLED*
UDP	uridine diphosphate
UCN	*UNICORN*
WS	Wasselevskija ecotype
WT	wild type
ZWI	*ZWICHEL*

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DEDICATION

I dedicate this thesis to the memory of my grandfather, Frederic Western. I hope to live my life as fully and richly as did he.

1. LITERATURE REVIEW

1.1 Introduction

1.1.1 The roles of *AP2, BEL1,* and *AG* during ovule development in *Arabidopsis*

The angiosperm ovule is the site of the development of the female gametophyte and, following fertilization, differentiates into a seed containing the embryo. The morphology, histology and ultrastructure of ovule development has been well-studied in a large number of species. Only recently, however, has the regulation of ovule development been studied using genetics. Many genes involved in ovule development have been identified in the model genetic species *Arabidopsis thaliana (Arabidopsis).* One of these genes is *BELL1 (BEL1),* which is thought to regulate development of the protective integuments that enclose the embryo sac. Several lines of evidence suggest that *BEL1* is an ovule-specific negative regulator of *AGAMOUS (AG),* the floral organ identity gene required for the development of reproductive organs. Another floral organ identity gene, *APETALA2 (AP2),* may affect ovule development by sealing a primordium into its ovule fate. In this thesis, I explored the genetic interactions between *BEL1* and *AG* during ovule development by performing a detailed analysis of the ovule phenotypes of Ag, Bel1, and Ag;Bel1 mutants in a background that allowed ovule development in the absence of *AG. AG* and *BEL1* were found to have partially redundant roles establishing ovule identity past the primordial stage and in directing integument and embryo sac development during later stages. In addition, the role of *AP2* in ovule develoment was investigated by studying the ovule phenotypes in many mutant alleles. My results show that *AP2* plays at most a minor role in the maintenance of ovule fate throughout development. As a background to these experiments, in the first two sections of this literature review I summarize: (1)

the genetics of flower development in *Arabidopsis*, focussing on the roles of *AP2* and *AG*, (2) the structure, function, and development of ovules, and (3) the current knowledge of the genetic regulation of ovule development.

1.1.2 Seed coat development and mucilage production in *Arabidopsis*

Seeds are extremely important as the precursor of the next generation of plants. A seed consists of an embryo and endosperm surrounded by a seed coat. The seed coat is a specialized maternal tissue with roles in embryo protection, regulation of germination, and seed dispersal. In some species, including *Arabidopsis,* the outermost layer of the seed coat consists of secretory cells that produce pectinaceous mucilage. The production of mucilage involves the biosynthesis and secretion of a very large quantity of pectin which is deposited between the outer cell wall and the plasmalemma as a "secondary" cell wall. In this thesis, I have exploited the presence of mucilage in the seed coat epidermis of *Arabidopsis* to investigate the production of pectin during seed coat differentiation and its extrusion under aqueous conditions. As a first step, I studied wild type seed coat development and mucilage production in *Arabidopsis.* I then isolated novel mutants in five genes, named *MUCILAGE-MODIFIED1—5 (MUM1—5),* that have altered mucilage capsules when exposed to water. These five novel mutants plus four previously isolated mutants with defects in seed coat development, Ap2, Aberrant testa shape (Ats), Transparent testa glabra1 (Ttg1), and Glabra2 (Gl2), were studied to determine the defect in their mucilage production. My results suggest that *MUM1* and *MUM2* affect the amount and post-deposition modification of mucilage and/or the outer cell wall, respectively, while *MUM3* and *MUM5* are involved in the provision of sugar for or the composition of mucilage. I also present evidence that *MUM4, TTG1,* and *GL2* may affect epidermal cell structure and mucilage amount via regulation of either the cytoskeleton or

the amount of mucilage produced. Finally, *AP2* and *ATS* appear to regulate general seed coat development, possibly an indirect effect of their requirement for proper ovule development. As an introduction to seed coat development and mucilage production, in the last two sections of the literature review I have summarized: (1) the structure of the seed coat, (2) the role of mucilage and its production, (3) the role of pectin in the plant cell wall, and (4) pectin structure and biosynthesis.

1.2 The *Arabidopsis* flower

The *Arabidopsis* flower arises from the edge of the shoot apical meristem and its development is marked by the production of organs in a well-defined order and positions. The flowers initiate in a spiral phyllotaxy around the inflorescence meristem, forming a raceme. In the last decade a large number of genetic studies have been done regarding several aspects of flower development, including timing of flowering, identity of the floral meristem, and identity of the floral organs (reviewed in Coen, 1991a; Coen and Meyerowitz, 1991b; Weigel and Meyerowitz, 1993; Meeks-Wagner, 1993; Okamuro *et al.*, 1993; Ma, 1994; Haughn *et al.*, 1995; Weigel, 1995; Meyerowitz, 1997; Weigel, 1997; Ma, 1997; Ma, 1998). In this section I will briefly describe the *Arabidopsis* flower and a model for the control of the floral organ identity, then concentrate on the roles and interaction of two floral organ identity genes: *APETALA2 (AP2)* and *AGAMOUS (AG)*.

1.2.1 The *Arabidopsis* flower

The cruciform flowers of *Arabidopsis* are typical of the Brassicaceae and can be considered as consisting of four whorls of organs (Figure 1.1a). The outermost whorl contains four sepals,

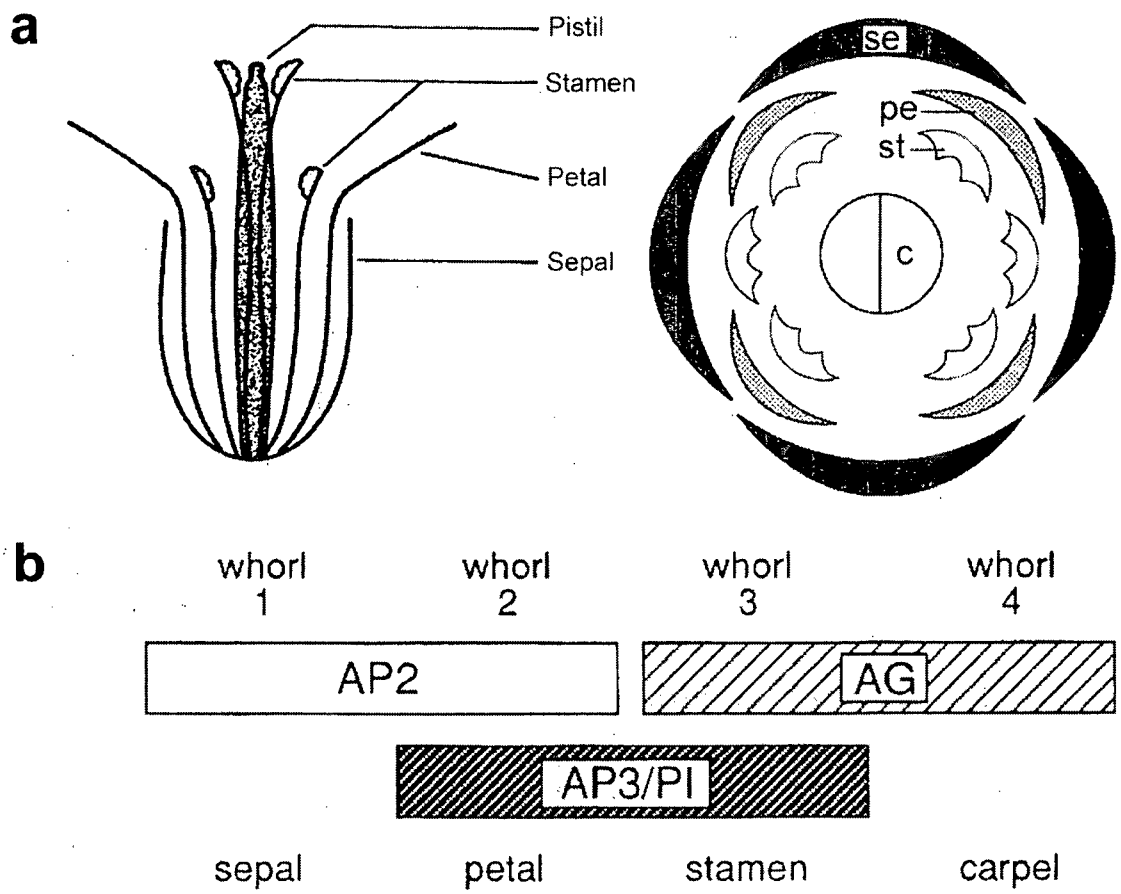


Figure 1.1. Morphology of wild type *Arabidopsis* flowers and the domains of expression of the organ identity genes controlling floral organ identity in these flowers.
(a) Cross section (taken from Haughn and Somerville, 1988) and floral diagrams of a wild type *Arabidopsis* flower showing four concentric whorls of organs. 1st whorl = sepals (se), 2nd whorl = petals (pe), 3rd whorl = stamens (st), 4th whorl = carpels (c).
(b) Diagrammatic representation of the spatial expression of the organ identity gene classes A (*AP1, AP2*), B (*AP3, PI*), and C (*AG*) in four whorls of wild type *Arabidopsis* flowers.

followed by four petals set opposite to the sepals. Six stamens, four long medial and two short lateral, occupy the third whorl. The centre of the flower consists of a syncarpous, bicarpellate gynoecium with ovaries containing 40-60 ovules and topped with a short style and stigma (Müller, 1961; Haughn and Somerville, 1988; Komaki *et al.*, 1988; Kunst *et al.*, 1989; Hill and Lord, 1989; Bowman *et al.*, 1989).

Flower development takes approximately 13 days and has been described in 13 stages up to anthesis (Müller, 1961; Kunst *et al.*, 1989; Hill and Lord, 1989; Smyth *et al.*, 1990). A new flower initiates as a buttress of the inflorescence meristem, quickly growing to become the flower primordium (stage 2). The first organ to initiate is the abaxial sepal, followed shortly by the other three sepals (stage 3). The petals and stamens initiate slightly later, followed by the cylindrical primordium of the gynoecium (stages 5-7). Growth and differentiation of specialized cell types continues for each of the four organ types until anthesis (stages 8-12).

1.2.2 A model for *Arabidopsis* floral organ identity

A model for floral organ identity has been derived for *Arabidopsis* through the study of homeotic mutants defective in flower development. This combinatorial model involves the interaction of three sets of organ identity gene products, termed A, B, and C, each of which function in two adjacent whorls (Figure 1.1b). The class A genes are required for the specification of sepals and petals, the class B genes are responsible for the production at a petals and stamens, and the class C genes are necessary for the reproductive organs, stamens and carpels. Thus, in cells expressing only class A genes, sepals are made, those with both class A and B make petals, a combination of class B and C gives stamens, and class C alone yields carpels. An additional component of this model suggests that the activity of class A and class C gene products are antagonistic, with each preventing the activity of the other in its domain (Haughn and Somerville, 1988; Bowman *et al.*, 1991).

Two each of class A and B genes have been isolated: *APETALA1(AP1)* and *AP2* are class A genes (Komaki *et al.*, 1988; Kunst *et al.*, 1989; Bowman *et al.*, 1989; Irish and Sussex, 1990; Bowman *et al.*, 1991; Schultz and Haughn, 1993; Bowman *et al.*, 1993), while *APETALA3 (AP3)*

and *PISTILLATA (PI)* represent class B function (Hill and Lord, 1989; Bowman *et al.*, 1989; Bowman *et al.*, 1991). Only one Class C gene has been isolated, *AG* (Bowman *et al.*, 1989; Bowman *et al.*, 1991). All of these genes have been cloned and have been found to encode transcription factors. *AP1, AP3, PI,* and *AG* encode proteins containing a MADS box DNA binding domain (Yanofsky *et al.*, 1990; Jack *et al.*, 1992; Mandel *et al.*, 1992b; Goto and Meyerowitz, 1994), while *AP2* codes for a member of a novel class of DNA binding proteins (Jofuku *et al.*, 1994; Okamuro *et al.*, 1997). *In situ* hybridization analyses of developing flowers using anti-mRNA probes for these genes have demonstrated that, with the exception of *AP2,* the transcript patterns match those predicted by the model (Yanofsky *et al.*, 1990; Drews *et al.*, 1991; Jack *et al.*, 1992; Mandel *et al.*, 1992b; Goto and Meyerowitz, 1994; Gustafson-Brown *et al.*, 1994). *AP2* mRNA is found throughout the vegetative portion of the plant and in all floral organs (Jofuku *et al.*, 1994). In addition, the organ identity roles of these genes have been confirmed by constructing transgenic plants where one or more of the organ identity genes have been ectopically expressed (Mizukami and Ma, 1992; Jack *et al.*, 1994; Mizukami and Ma, 1995; Sieburth *et al.*, 1995; Krizek and Meyerowitz, 1996; Sieburth and Meyerowitz, 1997; Jack *et al.*, 1997). A similar model was derived in the distantly-related *Antirrhinum majus* (Scrophulariaceae), where class B (*DEFICIENS* and *GLOBOSA*) and C (*PLENA)* genes act to establish the floral organs (Irish and Yamamoto, 1995; Samach *et al.*, 1997; reviewed in Schwarz-Sommer *et al.*, 1990; Coen, 1991a; Coen and Meyerowitz, 1991b). Orthologous counterparts of the floral organ identity genes also have been found in such species as *Brassica napus*, petunia, maize, cucumber, tomato, and tobacco (Pnueli *et al.*, 1991; Mandel *et al.*, 1992a; Schmidt *et al.*, 1993; Kempin *et al.*, 1993; van der Krol and Chua, 1993; Rasmussen and Green, 1993; Pnueli *et al.*, 1994; Mena *et al.*, 1996; Colombo *et al.*, 1997; Kater *et al.*, 1998).

1.2.3 The reciprocal roles of *AG* and *AP2*

Ap2 mutants have no sepals or petals, they have carpel-like sepals in the outer whorl and the second whorl (and sometimes the third whorl) can consist of carpel-sepals, a few stamens, or no organs (Komaki *et al.*, 1988; Kunst *et al.*, 1989; Bowman *et al.*, 1989; Bowman *et al.*, 1991). The defects in the perianth organs suggest that *AP2* is required for the production of sepals and petals and thus define it as a class A floral organ identity gene (Kunst *et al.*, 1989; Bowman *et al.*, 1991). In addition, the transformation of the sepals and petals into carpels and occasional stamens, respectively, suggests that *AP2* may repress genes that specify reproductive organs (Bowman *et al.*, 1991). A role for *AP2* in the floral initiation process (FLIP) also has been suggested due to the coflorescence-like structures formed under certain growth conditions and when combined in double mutants with other FLIP genes (Schultz and Haughn, 1993; Bowman *et al.*, 1993; Shannon and Meeks-Wagner, 1993). Finally, strong Ap2 mutants appear to have defects in the fourth whorl in the form of defective carpels and/or ovules (see Section 1.3.4; Modrusan *et al.*, 1994). A role for *AP2* throughout the flower has been supported by *in situ* hybridization studies. *AP2* mRNA is strongly expressed in the flower primordium, then in all four whorls of initiating organs early in development (Jofuku *et al.*, 1994). Expression persists at a lower level during organ differentiation, with the exception of expanding petals, the placenta, and developing ovules, where it continues to be strong. In addition, Northern analysis showed that *AP2* transcripts are found throughout the vegetative portions of the plant. Thus, it appears that *AP2* not only is an important player in floral organ identity, but also in flower initiation and the development of ovules as well.

The Ag mutant phenotype consists of nested flowers of the pattern (sepal, petal, petal)$_n$ (Bowman *et al.*, 1989; Bowman *et al.*, 1991). This phenotype suggests that *AG* is a class C gene responsible for the specification of reproductive organs in the innermost two whorls of the flower.

In situ hybridization studies using *AG* anti-mRNA have demonstrated that *AG* is transcribed in the inner part of the floral meristem, predicting the location of the reproductive organs (Drews *et al.*, 1991). *AG* expression continues in the stamen and carpel primodia as they arise and persists during their development, becoming restricted to specific cell types (Bowman *et al.*, 1991). The nested flower phenotype suggests that *AG* also controls floral determinacy (Bowman *et al.*, 1991). The two roles of *AG* have been confirmed through analysis of multiple Ag alleles and transgenic flowers where *AG* is suppressed through antisense RNA constructs. All such mutants are indeterminate but can have varying degrees of reproductive organ identity (Mizukami and Ma, 1995; Sieburth *et al.*, 1995). The role of the specification of reproductive organs has also been confirmed through ectopic expression of *AG*: over-expression of 35S-*AG* in *Arabidopsis* led to the production of reproductive organs in the outer two whorls (Mizukami and Ma, 1992; Jack *et al.*, 1997). A similar phenotype was also found when *B. napus AG* was over-expressed in tobacco (Mandel *et al.*, 1992a).

The overexpression phenotype of *AG* in *Arabidopsis* and its orthologs in other plants led to the production of reproductive organs in the outer whorls – a phenocopy of Ap2 mutants (Mizukami and Ma, 1992; Mandel *et al.*, 1992a; Kempin *et al.*, 1993; Tsuchimoto *et al.*, 1993; Pnueli *et al.*, 1994). This suggests that *AP2*, in addition to the specification of the perianth organs, suppresses *AG* activity in the outer two whorls of the flower. When *AG* mRNA expression was studied in an Ap2 background, *AG* was found not only in the innermost part of the flower, but also in the outer two whorls, confirming that *AP2* is involved in the repression of *AG* transcription in the outer two whorls (Drews *et al.*, 1991). Recent studies have shown that *LEUNIG (LUG)*, an enhancer of a weak Ap2 allele (Ap2-1), works with *AP2* in the restriction of *AG* transcription to the two inner whorls of the flower (Liu and Meyerowitz, 1995). The identity of the Ag floral organs as the sepals and petals specified by class A genes suggests that the reciprocal case is also true: *AG* suppresses *AP2* function in the centre of the flower (Bowman *et al.*, 1991). Since *AP2* is found throughout the

flower both in wild type and Ag flowers (Jofuku *et al.*, 1994), *AG* suppression of *AP2* must act post-transcriptionally. Conversely, transcription of the other class A gene, *AP1*, has been shown to be suppressed by *AG* in the centre of the flower (Gustafson-Brown *et al.*, 1994). Effects of both class A mutations, however, have been shown in the reproductive organs (e.g. unfused gynoecia; Modrusan *et al.*, 1994a; Haughn *et al.*, 1995), suggesting that the regulatory interactions with *AG* are more complex than previously thought and that the class A genes may play a role in the inner whorls of wild type flowers, perhaps even reiterating the regulation of *AG*.

1.2.4 The role of *AG* in carpel morphogenesis

As a class C gene, *AG* is required for the specification of carpel and stamen identity (Bowman *et al.*, 1989; Bowman *et al.*, 1991). In addition, it appears to play a further role in the development of the these organs. For example, *AG* mRNA is expressed throughout the carpels after their initiation and persists during their development (Bowman *et al.*, 1991). At the time of ovule initiation, however, the transcripts become restricted to the initiating ovule primordia and the stigmatic tissue. *AG* expression in these tissues continues throughout their development, with *AG* becoming progressively delimited in the ovule to the integuments and then the endothelium surrounding the embryo sac (Bowman *et al.*, 1991; Reiser *et al.*, 1995). These results suggest that *AG* may direct later carpel morphogenesis, especially in the ovules and stigmatic tissue. No direct targets of the AG transcription factor in the carpel have been identified, but some candidates have been found amongst the many *AGAMOUS-LIKE (AGL)* MADS box genes that have been isolated from *Arabidopsis* (Ma *et al.*, 1991; Flanagan and Ma, 1994; Savidge *et al.*, 1995; Rounsley *et al.*, 1995; Heck *et al.*, 1995; Purugganan *et al.*, 1995; Flanagan *et al.*, 1996). The *AGL* genes, like the floral organ identity MADS box genes, are thought to play important roles in plant development. Specifically, both

AGL1 and *AGL5* mRNA are expressed only in the gynoecium after *AG* appears (Ma *et al.*, 1991; Savidge *et al.*, 1995; Rounsley *et al.*, 1995; Flanagan *et al.*, 1996). Studies of *AGL5* transcript patterns in wild type, Ag, and 35S-*AG* flowers, and binding of AG to an element in the *AGL5* promoter suggested that it is directly regulated by *AG* (Savidge *et al.*, 1995). Similar studies of *AGL1* transcription in wild type and floral mutant plants have shown that, while downstream of *AG*, it may not be directly regulated by *AG* (Flanagan *et al.*, 1996).

Flowers doubly mutant for Ag and Ap2 have a phenotype of nested flowers made up of (leaf, petal-stamen, petal-stamen)$_n$. Surprisingly, it was found that the edges of the outer whorl leafy organs could have stigmatic papillae and placental tissue bearing ovule-like structures. These results suggested that *AG* is not required for the specification of at least some carpel characteristics. Other genes must work in parallel with *AG* in carpel-production. Two such genes, *CRABS CLAW (CRC)* and *SPATULA (SPT)*, have been briefly described (Alvarez and Smyth, 1993a; Alvarez and Smyth, 1993b; Alvarez and Smyth, 1997). Both triple and quadruple mutants with Ag, Ap2, Crc, and Spt demonstrate that *CRC* and *SPT* are almost entirely responsible for the remaining carpel characteristics found on the outer whorl organs of Ag;Ap2 double mutants (Alvarez and Smyth, 1997). Furthermore, Alvarez and Smyth (1997) propose that *AG* is responsible for the differentiation of the ovary cell walls and the style, while *CRC* and *SPT* work in parallel to promote the longitudinal growth of the carpels and the transmitting tract, respectively. Together, these three genes are responsible for proper gynoecium development.

1.3 Ovule development

The ovule is site of development of the female gametophyte, and, following fertilization, develops into the embryo-containing seed. Ovules develop from the placental tissue of the

gynoecium in a well-defined manner. The genetic regulation of ovule development is an expanding field, especially in *Arabidopsis*. Recently, a large number of mutants defective in ovule development have been described and integrated into a model for the regulation of this process (Gasser, 1998).

1.3.1 Ovule morphology

The angiosperm ovule is an integumented megasporangium. This complex structure is the site of megasporogenesis and megagametogenesis, as well as embryogenesis after fertilization. The structure and development of ovules have been studied in many species (reviewed in Bouman, 1984; Reiser and Fischer, 1993).

A typical angiosperm ovule has a nucellus, integuments, and a funiculus (Figure 1.2a) (Esau, 1977). The nucellus contains both sporogeneous cells from which the megaspore and megagametophyte arise, and vegetative cells which provide nutrition to the developing gametophyte. The region at the distal end of the nucellus that gives rise to the megaspore mother cell is the archesporium. The megaspore mother cell undergoes meiosis to give rise to a tetrad of megaspores, one or more of which undergoes several divisions to form the embryo sac. The nucellus normally is surrounded by one or two protective envelopes known as integuments. The integuments initiate at the proximal end of the ovule, opposite the archesporium, and terminate at the distal end, leaving a hole known as the micropyle through which the pollen tube enters at fertilization. Integuments play a protective role during gametogenesis and later re-differentiate into the seed coat following fertilization. The final structure of the ovule is the funiculus, a stalk which attaches the ovule to the placenta. The funiculus is attached to the nucellus at a region known as the chalaza and vascular bundles in the funiculus allow for transport of water and nutrients to the developing ovule.

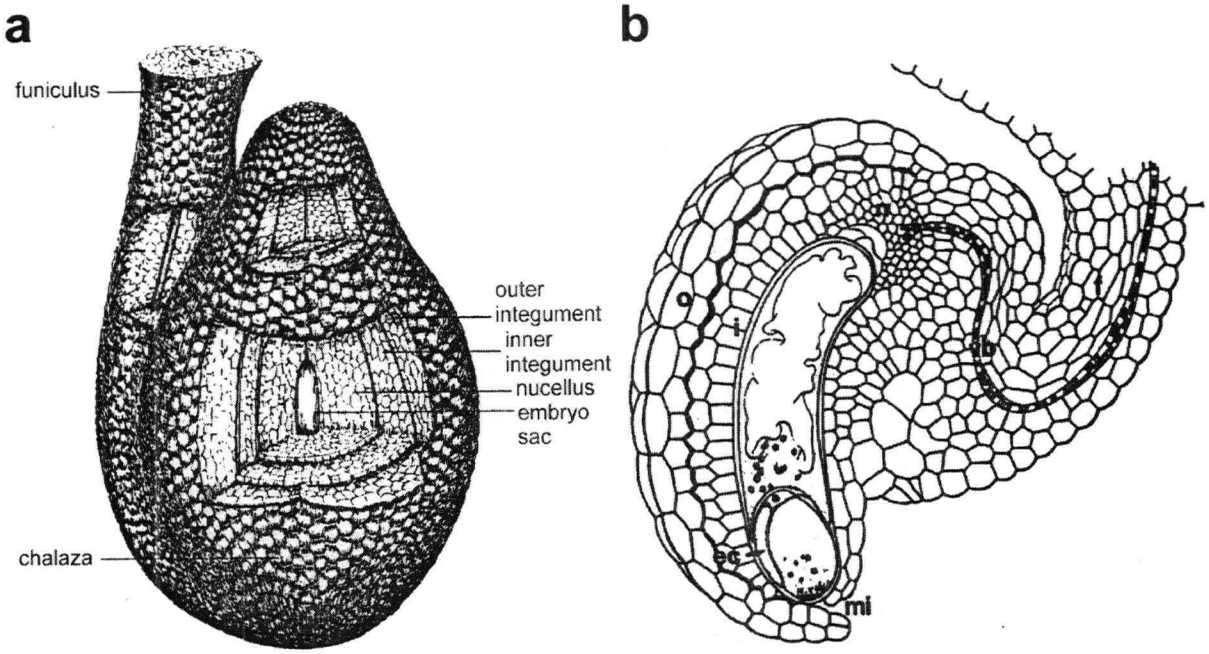


Figure 1.2. The morphology and anatomy of angiosperm ovules.
(a) Structure of the angiosperm ovule consisting of nucellus, inner and outer integuments, and funiculus (taken from Bouman, 1984).
(b) Diagram of *Arabidopsis* ovule with Polygonum-type of embryo sac as seen in a medial longitudinal section (taken from Mansfield *et al.*, 1991). Funiculus (F), nucellus (N), inner integument (I), micropyle (Mi), egg cell (EC), synergids (SC), antipodal cells (AC), central cell (CC), vascular bundle (VB).

The classification of angiosperm ovules is determined by several features, including the number of integuments, the type of archesporium, the number of megaspore mother cells, and the orientation of the ovule and embryo sac with respect to the funiculus (Bouman, 1984; Cronquist, 1988). Ovules can have no, one or two integuments, conditions known as ategmy, unitegmy and bitegmy. Bitegmy is the most common and considered to be the ancestral condition. Nucellar development can be either crassinucellate or tenuinucellate. In the crassinucellate-type, the megaspore mother cell is separated from the nucellar epidermis by one or several parietal layers. The tenuinucellate-type have no parietal layers. As well, angiosperm ovules can have a multicellular or unicellular archesporium that gives rise to a multiple or single megaspore mother cells,

respectively. The norm is a unicellular archesporium; it is considered to be the result of reduction, like the tenuinucellate condition from crassinucellate (Bouman, 1984; Cronquist, 1988).

The orientation of the ovule is an important feature in classification, involving not only the angle of the micropyle with respect to the funiculus, but also the shape of the embryo sac. Orthotropous ovules have the micropyle opposite to the point of attachment, while anatropous ovules are inverted, resulting from a 180° bend in the chalazal region. Both of these types can have a straight embryo sac, or one with a bend; the ones with slight curves are termed campylotropous, while those with larger distortions are known as amphitropous. The anatropous condition (with or without a campylotropous or amphitropous embryo sac) seems to be most common (Bouman, 1984; Gifford and Foster, 1989).

1.3.2 Ovule and embryo sac development

Ovule development commences with the initiation of the primordium from periclinal divisions in the subdermal cell layers and anticlinal divisions in the epidermis of the placental tissue (Bouman, 1984). Growth of the primordium into a radially symmetric, finger-like projection is followed by the initiation of the integument(s). The inner integument is generally derived from the epidermal layer, while the outer integument initiates from both dermal and subdermal layers. The integuments then grow to enclose the nucellus, with the outer integument overgrowing the inner integument. The curvature of the ovule with respect to the funiculus is driven by excess cell division on one side of the outer integument (Reiser and Fischer, 1993). In many species, the nucellus has been completely consumed by ovule maturity, with the exception of the chalazal region where the funiculus and integuments are inserted. These species undergo special differentiation of the

integument layer closest to the embryo sac into an endothelium, which plays a nutritive role (Bouman, 1984).

Concurrent to the initiation and development of the integuments, megasporogenesis and megagametogenesis occur. Early in ovule development, the megaspore mother cell undergoes meiosis to produce a tetrad of megaspores. In the majority of species examined (70%), monosporic gametophyte development of the Polygonum-type occurs (Reiser and Fischer, 1993; Drews *et al.*, 1998). Only the chalazal megaspore goes on to form the embryo sac while the other three degenerate. Three rounds of mitosis and nuclear migration are followed by cellularization to form a seven-celled, eight nuclei embryo sac. The egg cell is found at the micropylar end of the embryo sac, flanked by the synergid cells which facilitate fertilization. The majority of the embryo sac is made up of a diploid central cell which is mostly vacuole. Fertilization of the central cell polar nuclei with the second sperm nucleus leads to the development of the triploid endosperm. The chalazal end of the embryo sac is occupied by the three antipodal cells which may have a nutritive function.

1.3.3 *Arabidopsis* ovule development

Careful analysis of the morphology, histology and ultrastructure of *Arabidopsis* ovules has been performed (Vandendries, 1909; Misra, 1962; Webb and Gunning, 1990; Mansfield *et al.*, 1991b; Robinson-Beers *et al.*, 1992; Modrusan *et al.*, 1994a; Webb, 1994a; Modrusan *et al.*, 1994b; Webb, 1994b; Schneitz *et al.*, 1995). Mature *Arabidopsis* ovules are bitegmic and campylotropous, with a Polygonum-type embryo sac (Figure 1.2b). *Arabidopsis* ovule development also has been closely followed both externally and internally and has been carefully staged both with respect to flower development and between sporophyte and gametophyte development within the ovule (Figure 1.3)

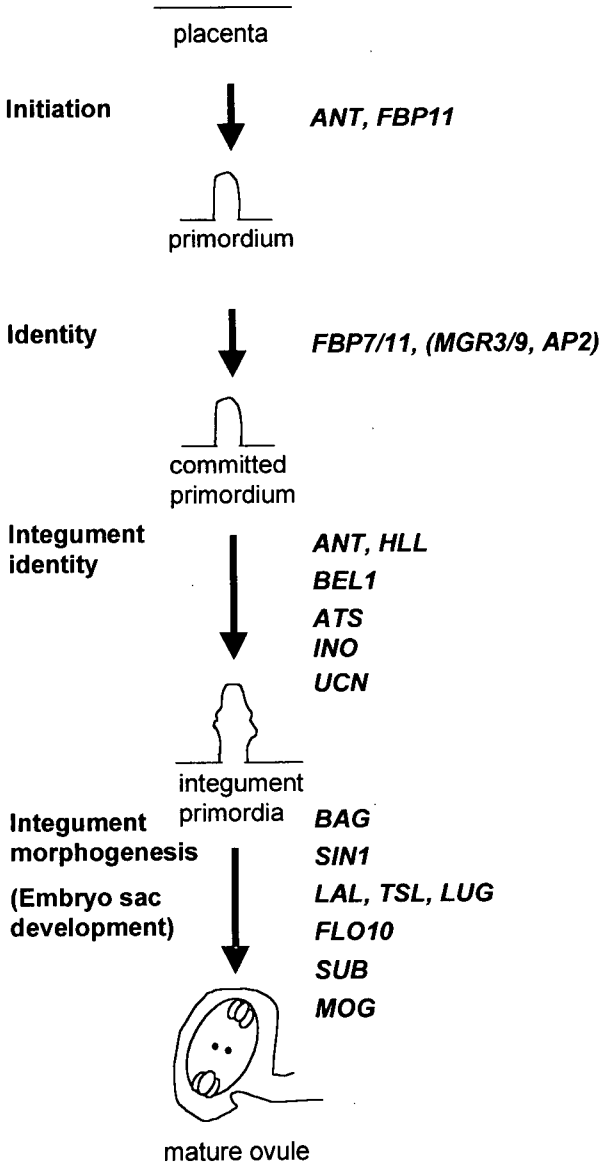


Figure 1.3. A model for the regulation of ovule development.
The stages of the sporophytic portion of ovule development are shown as a linear progression from placenta to mature ovule with the stages listed to the left. The names of genes on right are located at the approximate stage and in the order in which they first affect ovule development.

(Robinson-Beers *et al.*, 1992; Modrusan *et al.*, 1994a; Schneitz *et al.*, 1995). Development follows the basic pattern outlined in the previous section, with both integuments arising from the dermal layer and the nucellus being absorbed during development. The mature *Arabidopsis* ovule thus has a two-cell thick outer integument and a three-cell inner integument that includes the endothelium appressing the embryo sac.

1.3.4 Genetic analysis of ovule development

Ovule development can be thought of as occuring in several steps: initiation of a primordium, specification of ovule identity, initiation of morphogenesis, and morphogenesis (Figure 1.3) (Schneitz *et al.*, 1995; Schneitz *et al.*, 1997; Gasser *et al.*, 1998). Recently, a large number of ovule mutants have been identified that affect development of both the sporophytic and gametophytic tissue (reviewed in Angenent and Colombo, 1996; Drews *et al.*, 1998; Gasser *et al.*, 1998). The mutants affecting sporophytic development can be categorized with respect to the part of ovule development which is affected.

Genes affecting ovule identity. The first step in ovule development after the initiation of a primordium from the placenta is the specification of ovule identity. Several genes affecting ovule identity have been found in *Arabidopsis*, tobacco, and petunia (Figure 1.3).

TOBACCO OVULE MUTANTS. The Mgr9 and Mgr3 mutants of tobacco were found to have some green, spaghetti-like outgrowths in place of ovules (Evans and Malmberg, 1989). Further analysis showed that these outgrowths had carpel-like features, including stigma and style tissue. A similar phenotype was obtained from young tobacco placentas grown in culture (Hicks and McHughen, 1974; Evans and Malmberg, 1989) and led to the hypothesis that the stigmatoid structures resulted from reversion of uncommitted ovule primordia to a carpel fate. Mgr3 and Mgr9

were found as a result of a screen for polyamine pathway mutants and Mgr3 has excess levels of spermidine. The connection between the biochemical lesion and the ovule phenotype is not known. Interestingly, a phenocopy of the spagetti structure was found when *B. napus AG* was transformed into tobacco plants under the 35S promoter (Mandel *et al.*, 1992a). Ectopic expression of *B. napus AG* led to some ovules developing as stigmatic tissue. As discussed in the previous section, *AG* is a class C organ identity gene that, along with other genes (i.e. *CRC, SPT*) (Haughn *et al.*, 1995; Weigel, 1995; Alvarez and Smyth, 1997), is responsible for the production of the carpels from which the placenta and ovules arise. Thus, disturbed function of the carpel genes could explain the occasional reversion in the 35S-*B. napus AG* tobacco plants and possibly the Mgr3 and Mgr9 mutants.

FLORAL BINDING PROTEIN11 (FBP11) and *FBP7.* A search for MADS box genes specific to ovules in petunia led to the isolation of two genes, *FBP7* and *FBP11* (Angenent *et al.*, 1995). These two genes are highly homologous and their transcription is specific to the placenta and ovules. *In situ* hybridization studies have shown that they are first expressed in the placenta, then become delimited to the ovule primordium. Following integument initiation, they are strongly expressed in the developing integument and then, toward the end of ovule development, are found in the endothelium, the layer of the integument closest to the embryo sac. In order to test the role of these genes, cosuppression was performed and carpel-like structures resembling the spaghetti structures of tobacco were found. The frequency of the carpel-like structures was dependent on the extent of down-regulation of *FBP7* and *FBP11,* but the transcription of the petunia class C genes (*pMADS3* and *FBP6*) was not affected. These results suggested that *FBP7* and *FBP11* are involved in the specification of ovule identity rather than just down-regulation of the class C genes. The role of *FBP11* in ovule identity was confirmed through overexpression under the 35S promoter which led to the production of ectopic ovules on the sepals and petals (Colombo *et al.*, 1995). These results also suggested a role in ovule or placenta initiation.

AP2. As described in Section 1.2, Ap2 mutants were first recognized for the aberrant production of the perianth whorls of the flower. Recently, however, Modrusan *et al.* (1994) recognized that flowers resulting from strong *ap2* alleles (*ap2-6, ap2-7*) had a small number of abnormal ovules in their gynoecium. These abnormal ovules either had both carpel and sepal characteristics or were filaments. These results suggested that *AP2*, in addition to its role in floral organ identity, plays a role in the specification of ovule identity.

Genes affecting integument initiation and identity. Following the specification of a primordium to be an ovule, the first step in the production of an ovule is the initiation of the protective integuments. Several *Arabidopsis* mutants have been isolated that affect multiple aspects of integument initiation (Figure 1.3).

AINTEGUMENTA (ANT). Mutations in *ANT* led to variable effects on ovule development, depending on the allele observed. Strong Ant plants made ovule primordia, but no integuments were initiated, only some later thickening of the chalazal region of the nucellus demonstrated that ovule identity had been established (Klucher *et al.*, 1996; Elliott *et al.*, 1996; Baker *et al.*, 1997; Schneitz *et al.*, 1997). In addition, the embryo sac was absent and fewer ovules were present per flower. Weak Ant ovules, however, initiated integuments that could undergo varying degrees of growth. In these mutants, megagametogenesis usually was not completed, but the occasional ovule could finish development and even be fertilized to give a seed (Klucher *et al.*, 1996; Elliott *et al.*, 1996). The Ant phenotype was not restricted to ovules, the floral organs in the outer three whorls were reduced in number and could be abnormal in appearance (Klucher *et al.*, 1996; Elliott *et al.*, 1996). Cloning of *ANT* revealed that it is a an *AP2*-like gene that encodes a putative transcription factor. Like *AP2*, *ANT* is expressed throughout the vegetative organs of the plant (Klucher *et al.*, 1996; Elliott *et al.*, 1996). *In situ* hybridization studies have revealed that, though *ANT* transcripts were present throughout the flower, its expression is highest in initiating and rapidly growing organs,

including the integuments. It has been proposed, therefore, that *ANT* may play a role both in primordia initiation and early growth throughout the flower, including the ovules, and in other, vegetative parts of the plant.

HUELLENLOS (HLL). Hll ovules closely resembled those of Ant mutants. No initiation or growth occurred beyond the first cell division or cell enlargement for either integument and gametogenesis did not proceed past the tetrad stage. Unlike Ant mutants, however, the Hll phenotype was ovule-specific (Schneitz *et al.*, 1997).

BELL1 (BEL1). Mature Bel1 ovules consist of a nucellus surrounded by a single, integument-like structure (ILS) that does not fully enclose the nucellus (Robinson-Beers *et al.*, 1992; Ray *et al.*, 1994; Modrusan *et al.*, 1994a). Developmental analysis of Bel1 ovules revealed that the inner integument did not initiate and the ILS initiated in the place of the outer integument. These results suggested that *BEL1* is responsible for initiation of the inner integument and proper identity and growth of the outer integument. In addition, occasionally Bel1 ILS can grow into carpel-like structures (CLS) after anthesis (Ray *et al.*, 1994; Modrusan *et al.*, 1994a). The CLS had many carpel features, including ovary-like cells, stigmatic papillae, and even secondary Bel1 ovules. This late production of the CLS led to the hypothesis that the *BEL1* product also could be regulating the class C gene *AG* in the ovule. *In situ* hybridization with *AG* anti-mRNA showed that *AG* transcription continued in parts of the ovule from which it was normally restricted in wild type flowers (Ray *et al.*, 1994; Modrusan *et al.*, 1994a). Further support for the role of *AG* in the Bel1 phenotype was provided through ectopic expression of *B. napus AG* in *Arabidopsis.* Ovules of some 35S-*B. napus AG* flowers resembled those of Bel1 flowers, including aberrant ovules and the later growth of ILS into CLS (Ray *et al.*, 1994).

Cloning of *BEL1* revealed that it encoded a putative homeobox transcription factor and that it was transcribed in the ovule (Reiser *et al.*, 1995). *In situ* hybridization analysis showed that *BEL1* is

transcribed throughout the developing ovule primoridia, then it is delimited to the region from which the integuments arise. *BEL1* expression continues in the integuments during their development and is finally restricted to the chalaza in the mature ovule. This expression pattern correlates with a role for *BEL1* in integument initiation and development. The transcription of *AG* in the ovule was also carefully investigated through *in situ* hybridization (Reiser *et al.*, 1995). *AG* transcripts, as mentioned in Section 1.2, are present throughout the reproductive organs. Just prior to ovule initiation, *AG* is strongly expressed in the placenta and continues in the growing primodia. Like *BEL1*, *AG* becomes delimited to the region of the ovule from which the integuments initiate and then is found in the integuments throughout their development. This overlap in expression patterns suggests that if *BEL1* acts to negatively regulate *AG*, it is at a post-transcriptional level (Reiser *et al.*, 1995).

ABERRANT TESTA SHAPE (ATS). Ats seeds are small and heart-shaped with large epidermal cells. Developmental analysis revealed that, unlike wild type, Ats ovules only initiate one integument that undergoes development to give normal-appearing ovules (Léon-Kloosterziel *et al.*, 1994). The inner cell layer of the single integument forms a normal-appearing endothelium and the outer layer can form a relatively normal seed epidermis. Unlike most of the other mutants affecting integument initiation, Ats ovules are fully fertile with a complete embryo sac. Thus, *ATS* may be important in the separation of the two integuments, such that a mutation leads to a single, fused integument (Léon-Kloosterziel *et al.*, 1994; Baker *et al.*, 1997).

INNER NO OUTER (INO). Ovules of Ino mutants initiate both integument primordia, but the outer integument forms on the wrong side of the ovule and does not complete development (Gaiser *et al.*, 1995; Baker *et al.*, 1997; Schneitz *et al.*, 1997). Thus, mature Ino ovules have an inner but no outer integument. In addition, the aberrantly placed outer integument primordium leads to improper curvature of the ovule. Ino plants are semi-sterile, with some ovules being fertilized and

developing into aberrantly shaped seeds. The activity of *INO* appears to only influence the placement of the outer integument primordium and its subsequent growth (Baker *et al.*, 1997).

UNICORN (UCN). Ucn mutant ovules have an unusual protrusion near the chalazal end of the ovule (Schneitz *et al.*, 1997). These plants are semi-sterile with embryo sacs present only in some mature ovules. Developmental analysis revealed that the integuments initiate and grow properly, but, slightly after integument initiation, an extra primordium appears below that of the outer integument that can grow into a few cells or several cell layers. These results suggest that *UCN* may act to suppress extra integument primordia (Schneitz *et al.*, 1997).

Genes affecting integument growth. Growth of integuments from the primordia requires both cell division and cell expansion. Several mutants affecting these processes in both the inner and outer integument have been isolated from mutagenized *Arabidopsis* lines (Figure 1.3). In addition, with one exception, all of the mutants that affect the growth of integuments also affect growth in the whole plant or flower.

BLASIG (BAG). Bag ovules have a very unusual phenotype (Schneitz *et al.*, 1997). The ovules stick together and the integuments are short and extremely ragged. Surprisingly, early Bag ovules appear normal, with two integument primordia and only a few roundish epidermal cells. Later, though, the integuments do not appear to elongate very much and the cells become round and have altered cell surface features that make them sticky. The phenotype of Bag plants is not restricted to the ovules, the plants are also small, with flowers which open prematurely and have glassy petals. *BAG*, therefore, may affect cell growth throughout the plant (Schneitz *et al.*, 1997).

STRUBBELIG (SUB). *SUB* appears to affect elongation growth throughout the plant. Sub ovules have a normal inner integument, but the outer integument does not completely enclose the inner integument and can have abnormal protrusions from the distal tip (Schneitz *et al.*, 1997). The

embryo sac often arrests late in development, leading to very few fertile ovules. In addition, the pistils are wrinkled and twisted and the plants are stubbier than normal.

SHORT INTEGUMENTS1 (SIN1). Sin1 mutants were first described by Robinson-Beers *et al.* (1992). Sin1 ovules undergo normal integument initiation and growth until the time that the outer integument is about to overlap the inner integument, at this point, cell growth stops while cell division continues in both integuments. Further investigations, however, showed that the phenotype was enhanced by the *erecta* mutation (*er*) in the Ler ecotype (Lang *et al.*, 1994; Ray *et al.*, 1996). Backcrosses to an *ER* line were used to isolate plants with a mutation in *SIN1* only. These ovules were still found to have integument development defects, but only the outer integument was found to have retarded growth mid-way through development (Lang *et al.*, 1994). The inner integument, on the other hand, was found to be elongated. In both mutant backgrounds, megagametophyte development was retarded, especially in the Sin1;er double mutant. Sin1 plants, as well, independent of an er background, have shortened internodes. Thus, *SIN1* appears to affect cell elongation in plants, perhaps interacting with *ER* outside of the ovule (Lang *et al.*, 1994; Ray *et al.*, 1996; Gasser *et al.*, 1998).

MOLLIG (MOG). Overall, Mog ovules appear normal, but closer examination reveals that later ovules have large-balloon-shaped cells in the integuments, leading to altered cell layer arrangements (Schneitz *et al.*, 1997). In addition, embryo sac development is aberrant. Similar to the other integument growth mutants listed, Mog mutants also have abnormal vegetative structures. Mog plants have a crowded appearance due to shortened internodes and have aberrant stimatic papillae and stamens. Similar to *BAG* and *SUB*, *MOG* may affect cell differentiation throughout the plant (Schneitz *et al.*, 1997).

LAELLI (LAL). Lal ovules have a similar phenotype to Sin1 ovules. Both integuments are present, but the outer integument is severely reduced, while the inner integument appears to develop

normally (Schneitz *et al.*, 1997). Unlike Sin1 plants, the Lal phenotype is ovule specific. *LAL* may play a role in cell elongation, analogous to *SIN1*, or it may affect cell division in the outer integument (Schneitz *et al.*, 1997).

TOUSLED (TSL). Tsl mutants were originally isolated for their floral phenotype where there was a reduced number of floral organs (Roe *et al.*, 1993). In addition, the floral organs had altered morphology, including a split gynoecium. Recently, however, it has been found that Tsl ovules have a protruding nucellus and inner integument (Roe *et al.*, 1997b). *TSL* encodes an nuclear serine/threonine protein kinase that may affect cell proliferation throughout the plant (Roe *et al.*, 1993; Roe *et al.*, 1997a). *TSL* mRNA expression was not found in ovules, possibly due to weak expression or because the ovule phenotype is the result of abnormal carpel development (Roe *et al.*, 1997b).

LEUNIG (LUG). Lug mutants were isolated first as a gynoecium mutant (Okada *et al.*, 1989) and as an enhancer of the Ap2-1 floral phenotype (Liu and Meyerowitz, 1995). *LUG* is thought to aid *AP2* in the suppression of *AG* in the outer two whorls of the flower. Its ovule phenotype, however, appears to be independent of this earlier regulatory role, as Lug-like abnormal ovules are not found in 35S-*AG* plants (Mizukami and Ma, 1992). The ovule phenotype of Lug mutants is variable, depending on the allele. Lug ovules can be normal or they can have varying degrees of shortening of the outer integument, elongation of the inner integument, and embryo sac defects. Thus, Lug ovules can resemble Tsl, Lal, and Sin1 ovules or have a less severe phenotype (Schneitz *et al.*, 1997; Roe *et al.*, 1997b). Similar to these other genes, *LUG* may affect growth of the integuments or facilitate communication between them (Roe *et al.*, 1997b).

FLO10 (or *SUPERMAN*). FLO10 prevents the expression of class B genes in the innermost whorl of the flower, allowing for development of the gynoecium. The ovules found on Flo10 stamen-carpels are elongated and orthotropous, ranging from perfectly straight to bent at a 45

degree angle, rather than tightly curved (anatropous). The creation of a double mutant with Ino revealed an Ino phenotype, showing that the inner integument is present and normal. This result, along with developmental analysis has shown that the Flo10 phenotype is the result of the lack of asymmetric growth of the outer integument. Double mutant studies with a class B mutant (Ap3) has shown that the Flo10 ovule phenotype is independent of its floral phenotype. Thus, *FLO10* appears to be responsible for the suppression of outer integument growth on one side of the ovule to allow curvature into the anatropous orientation. *FLO10* encodes a putative transcription factor that is expressed around inner edge of the third whorl and in the funiculus in the ovule. The FLO10 protein is hypothesized to create a boundary that prevents expansion of class B activity into the fourth whorl of the flower. Presumably it acts in a similar fashion in the ovule to regulate a factor expressed in the funiculus.

A model for ovule development. Several models for ovule development have recently been proposed (Angenent and Colombo, 1996; Baker *et al.*, 1997; Schneitz *et al.*, 1997; Gasser *et al.*, 1998). I present here a simplified version of these models (Figure 1.3). The development of the sporophytic portion of the ovule, as stated in Section 1.3.3, can be divided into stages: initiation of a primordium, specification of ovule identity, initiation of morphogenesis, and morphogenesis. Only two genes appear to play a role in ovule initiation: *ANT* and the petunia gene *FBP11*. Ant mutants have a reduced number of ovules per gynoecium, while ectopic *FBP11* can cause the development of ovules on the perianth organs. *FBP11* (and *FBP7)* may also play a role in ovule identity. Other genes that may play less direct roles in ovule identity are the tobacco genes *MGR3* and *MGR9,* and *AP2* from *Arabidopsis* (Section 1.3.4).

A number of *Arabidopsis* genes involved in different aspects of integument initiation were described in Section 1.3.5: *ANT, HLL, BEL1, ATS, INO,* and *UCN*. *ANT* appears to work very early for the specification of both integuments, as suggested by the absence of integuments when

mutated and the epistasis of the Ant phenotype over other ovule mutant phenotypes (i.e. Bel1, Sin1, Ino, Flo10) (Baker *et al.*, 1997). Hll mutants are almost identical to Ant mutants, suggesting that *HLL* also works very early in the specification of integument identity. The exact timing of the other gene activities are less well defined. *BEL1* appears to be required quite early for the initiation of the inner integument, then shortly after for the identity of the outer integument. Ats mutants have a single, possibly fused, integument, suggesting that *ATS* is needed to separate the two integument primordia as they arise. *INO* appears to work at approximately the same time for the initiation of the outer integument. Finally, *UCN* may act to suppress the further initiation of organs from the nucellus. Thus, it seems that the initiation of the two integuments is somewhat independent, supporting evolutionary theories for their separate evolution (Herr, 1995; Gasser *et al.*, 1998).

Once the integuments are initiated, several genes are involved in their morphogenesis through cell division and elongation (Section 1.3.6). These *Arabidopsis* genes include *BAG, SUB, SIN1, MOG, LAL, TSL, LUG,* and *FLO10.* With the exception of *LAL*, all of these genes also affect growth in either the flower or the whole vegetative portion of the plant, suggesting that these genes work generally in cell growth and differentiation. *BAG* and *MOG* appear to affect cell character in both integuments, including shape and growth, with *BAG* acting earlier than and having a greater effect than *MOG. SIN1, LAL, TSL,* and *LUG* all are required for the correct growth of the inner integument versus outer integuments. All appear to have an elongated inner integument, and may be responsible for correct elongation growth of the inner and/or the outer integument. *SUB* effects the general growth of the outer integument, while *FLO10* is necessary for asymmetric cell divisions in the outer integument to produce the anatropous orientation of the micropyle.

1.4 Seed coat development

Upon pollination, the pollen tube releases two sperm nuclei into the embryo sac of the ovule. One fuses with the egg cell nucleus, creating a diploid zygote which grows into the embryo. The second sperm nucleus fuses with the two polar nuclei of the central cell, initiating development of the endosperm which provides nutrition for the developing embryo. Double fertilization also triggers re-differentiation of maternal tissue to complete seed development. The integuments undergo both development and degeneration to form the seed coat which houses and protects the embryo during development and in the interim between seed desiccation and germination.

1.4.1 Seed coat morphology

The seed coat is a maternally-derived structure which encloses the embryo and the endosperm at maturity (Figure 1.4a). Seed coat structure, function, and development has been studied in a large number of species (reviewed in (Esau, 1977; Fahn, 1982; Boesewinkel and Bouman, 1984; Kelly *et al.*, 1992; Boesewinkel and Bouman, 1995).

The seed coat plays many roles: mechanical and chemical protection, maintenance of dormancy, nutrition of the developing embryo, and facilitation of dispersal. There is wide variability in the structure of the seed coat amongst different taxa, even within families (Esau, 1977; Fahn, 1982; Boesewinkel and Bouman, 1984; Boesewinkel and Bouman, 1995). The seed coat develops from the maternal tissue of the ovule, the integument(s). Seed coat layers derived from the outer integument are known as the testa, while those that developed from the inner integument are known as the tegmen. Many seed coats are almost entirely testal, with the inner integument having been crushed or consumed during seed coat and embryo development.

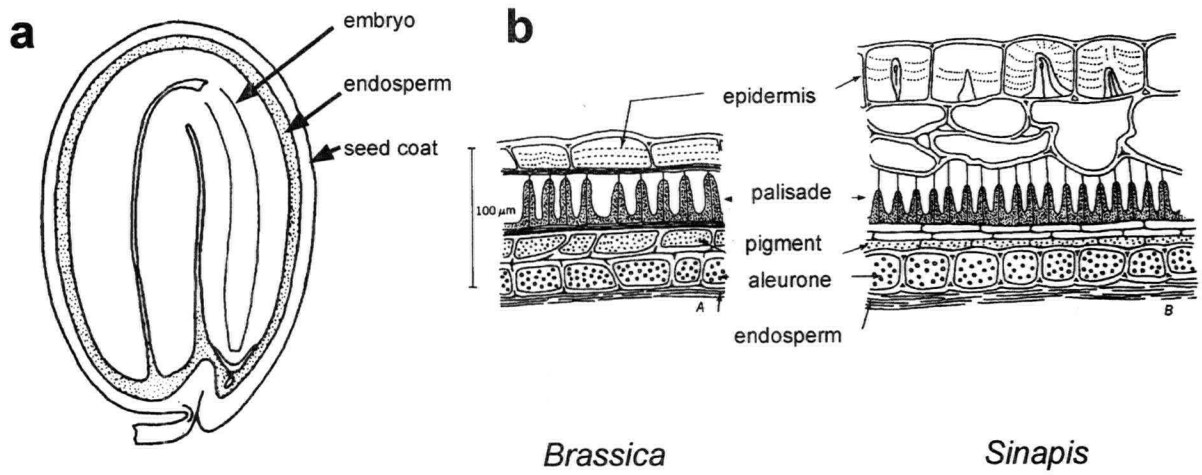


Figure 1.4. The structure of the seed and seed coat in the Brassicaceae.
(a) Diagram of an *Arabidopsis* seed showing embryo, endosperm, and seed coat (adapted from Meinke, 1991).
(b) Diagram of the seed coats of *Brassica napus* and *Sinapis alba*, showing the important layers (adapted from Esau, 1977).

A common property of many seed coats is the re-differentiation of at least one cell layer to provide mechanical strength to the seed coat (Esau, 1977; Fahn, 1982; Boesewinkel and Bouman, 1984; Boesewinkel and Bouman, 1995). These cells are generally known as sclerenchyma and have cell wall thickenings that are often reinforced with lignin. Some species, especially of the Leguminosae, have exaggerated macrosclereids known as Malpighian cells. Corner (1976) divised a classification of seed coat morphology depending upon the location of the important mechanical layer. Exo-, meso-, and endotestal seeds have their structural layer in the outer, middle, or inner layer of the outer integument, while exo-, meso-, and endotegmic seeds are reinforced in the inner integument. While all types of seed coats are found, the majority of seeds are exotestal (e.g. Leguminosae), mesotestal (e.g. Rosaceae), endotestal (e.g. Brassicaceae), or exotegmic (e.g. Voilanae).

The sclereids can also play a protective function from premature hydration and chemicals, which can be substituted for or augmented by the production of polyphenolics such as tannins in

other cell layers. Tannins also impart colour to seeds and may act to reduce edibility and/or digestibility (Boesewinkel and Bouman, 1995). Other cellular adaptations that are commonly found in seed coats are the production of crystals, mucilage, and epidermal hairs. Crystals are most often calcium oxalate and are thought to provide mechanical strength (Esau, 1977; Fahn, 1982; Boesewinkel and Bouman, 1984; Boesewinkel and Bouman, 1995). Mucilage and hairs are most often found in epidermal cells and are thought to aid in seed dispersal or facilitation of germination. There are many other specialized seed coat characters that allow for greater seed dispersal, ranging from an extremely reduced seed coat, to the development of "wings" or air-filled balloon cells to aid wind dispersal, to the production of a fleshy sarcotesta or oily elaiosome to attract animals.

1.4.2 Myxospermy

Plants which produce mucilage in their seed epidermis are termed myxospermous (Boesewinkel and Bouman, 1995). These seeds, when exposed to water, release a pectinaceous slime from the epidermal cells which forms a gel layer around the seed. Seed mucilage has been studied in a large number of species (e.g. Brassicaceae, Solanaceae, Linaceae, Plantanginaceae – for a comprehensive list, see Grubert, 1981). Mucilage found on seeds is predominantly made of pectic substances which may or may not be supported with cellulose microfibrils (Muhlethaler, 1950; Frey-Wyssling, 1976). Indeed, mucilage is a catch-all term for pectinaceous substances secreted by plants outside of the cell wall during normal development (in contrast to gum which refers to pectin produced in response to wounding) (Frey-Wyssling, 1976). Two other parts of the plant that commonly have mucilage include the root cap and the transmitting tract of the gynoecium. Root cap mucilage is made in the peripheral cells and is transported out of the cell and through the cell wall to form a sheath around the root tip. Root cap mucilage is thought to act to protect the root

cap during its movement through the soil and serve as an absorbing surface for the exchange of ions and nutrients (Esau, 1977; Rougier and Chaboud, 1989). In the transmitting tract of many species, mucilage forms part of an extracellular matrix through which the pollen tube grows and may provide sugar for pollen tube production (Esau, 1977; Wilhelmi and Preuss, 1997).

Myxospermy is found in a large number of species, including members of the Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae (Frey-Wyssling, 1976; Grubert, 1981; Boesewinkel and Bouman, 1995). There are many hypotheses on the role of mucilage, including adhesion to soil so that the seed will not be dislodged by wind and rain, adhesion to the coats of animals/birds for transport, protection from stomach acid, retention of water to aid in germination, and inhibition of germination under water-logged conditions by preventing the passage of oxygen into the seed (Edwards, 1968; Swarbrick, 1971; Frey-Wyssling, 1976; Esau, 1977; Hsiao and Chuang, 1981; Fahn, 1982; Boesewinkel and Bouman, 1984).

The cytology of mucilage production has been studied in detail in the seed epidermis of *Plantago ovata* (Hyde, 1970) and *Brassica campestris* (Van Caeseele *et al.*, 1981). In both cases, the epidermal cells first underwent growth, then starch granules accumulated. This was followed by a change in appearance of Golgi stacks, revealing thickened cisternea, and there were many large vesicles carrying a substance that accumulated between the outer cell wall and the plasmalemma. During the production of the mucilage, the starch granules disappeared and shortly thereafter the cytoplasm appeared disorganized as the cells dehydrated. Because mucilage consists of cell wall components and is deposited during seed development between the plasmalemma and the primary cell wall of the epidermal cells, it can be considered as a "secondary" cell wall (Fahn, 1982; Boesewinkel and Bouman, 1995). Upon hydration, the hydrophilic pectin swells and ruptures the primary cell wall to create a gel-like layer around the seed.

1.4.3 The seed coat of the Brassicaceae

The seed coats of the Brassicaceae have been well studied due to their stature as economically important vegetable, condiment, and oilseed crops (Thompson, 1933; Roth, 1957; Hirst *et al.*, 1965; Tyler, 1965a; Tyler, 1965b; Grant *et al.*, 1969; Vaughan, 1970; Vaughan and Whitehouse, 1971; Vose, 1974; Bouman, 1975; Norton and Harris, 1975; Theander *et al.*, 1977; Woods and Downey, 1980; Buth and Ara, 1981; Van Caeseele *et al.*, 1981; Van Caeseele *et al.*, 1982; Schans *et al.*, 1982; Mills *et al.*, 1982; Siddiqui *et al.*, 1986; Buth *et al.*, 1987; Van Caeseele *et al.*, 1987; Daun and DeClercq, 1988; Harris, 1991; Cui and Eskin, 1993; Cui *et al.*, 1994; Rashid *et al.*, 1994; Naczk *et al.*, 1994; Simbaya *et al.*, 1995). Vaughan and Whitehouse (1971) performed a comprehensive survey of the mature seed of 90 genera and 200 species of the Brassicaceae, detailing the seed coat features. In addition, seed coat development has been studied in *B. campestris, Brassica nigra, Sinapis alba (Brassica hirta), Sinapis arvensis, Lunaria annua, Raphanus sativus,* and *Capsella bursa-pastoris* (Roth, 1957; Edwards, 1968; Bouman, 1975; Van Caeseele *et al.*, 1981; Harris, 1991). There is a large amount of variability in the seed coat morphology, but the majority of species are characterized by the presence of a thickened, lignified palisade layer which developed from the inner layer of the outer integument and a pigmented layer which is derived from the endothelium. In addition, the epidermal cells may be mucilaginous and/or have intracellular cellulosic structures (Figure 1.4b).

Numerous studies have been done on the properties of the mucilage of yellow mustard, *S. alba (B. hirta),* and rape seed, *B. campestris* and *B. napus*, to determine possible food emulsifier or industrial uses (Hirst *et al.*, 1965; Tyler, 1965a; Grant *et al.*, 1969; Vose, 1974; Woods and Downey, 1980; Siddiqui *et al.*, 1986; Van Caeseele *et al.*, 1987; Cui and Eskin, 1993). These studies have demonstrated that the chemical composition of the mucilage consisted of glucose, galactose, and

galaturonic acid as major components, and mannose, arabinose, xylose, and rhamnose as minor components. These components suggest that the mucilage is not only composed of pectin but also of cellulose, as suggested by some reports of strands of cellulose embedded in the mucilage of some Brassicaceae (Muhlethaler, 1950; Edwards, 1968; Frey-Wyssling, 1976).

1.4.4 Genetic analysis of seed coat development in *Arabidopsis*

As discussed in Section 1.3, ovule development in *Arabidopsis* has been studied (Webb and Gunning, 1990; Mansfield *et al.*, 1991b; Robinson-Beers *et al.*, 1992; Modrusan *et al.*, 1994a; Schneitz *et al.*, 1995). Seed coat development, however, has not been directly studied. The mature seed coat was briefly described by Vaughan and Whitehouse (1971) in their survey of the Brassicaceae: the epidermal cells contain mucilage and a large solid column (columella) on the inner tangential wall, there is a palisade layer with thickening of the inner tangential walls, and a pigmented layer next to the endosperm. At maturity, the *Arabidopsis* ovule has five cell layers in its integuments, three in the inner and two in the outer (Léon-Kloosterziel *et al.*, 1994; Schneitz *et al.*, 1995). By analogy to the seed development of other Brassicaceae, including *C. bursa-pastoris, B. campestris, S. arvensis,* and *S. alba,* the mucilagenous cells are the outer layer of the outer integument, while the palisade layer is the inner layer of the outer integument. The pigmented layer is the endothelium, with the outer layers of the inner integument being crushed during seed development (Edwards, 1968; Bouman, 1975; Esau, 1977; Van Caeseele *et al.*, 1981).

Very little genetic analysis has been performed on seed coat development. Only four genes are known to affect seed coat morphology. *ATS*, as described in Section 1.3.4, is thought to be required for the production of two distinct integument primordia. Ats mutant ovules have a single, three cell thick integument that undergoes relatively normal development. The seed, however, has

an unusual heart shape and the epidermal cells are irregular and enlarged (Léon-Kloosterziel *et al.*, 1994). In addition, Ats seeds make less mucilage than wild type seeds and have reduced dormancy. The epidermal layer and the pigmented layer are both made from a single integument, suggesting that the information to make both seed coat characters was present. This abnormal integument, however, also could be responsible for the improper epidermal cell growth and mucilage production during seed coat development (Léon-Kloosterziel *et al.*, 1994). Another gene that affects seed coat development is *AP2*. As shown in Sections 1.2 and 1.3, *AP2* affects multiple processes including flower and ovule development. Seeds of at least two alleles of Ap2 (Ap2-1 and Ap2-10) have an abnormal heart shape, lack mucilage, and have flattened, rectangular epidermal cells rather than normal hexagonal cells with raised columellae (Jofuku *et al.*, 1994; Léon-Kloosterziel *et al.*, 1994; Bowman and Koornneef, 1994a).

The other two genes known to be required for a normal seed coat are *TRANSPARENT TESTA GLABRA1 (TTG1)* and *GLABRA2 (GL2)* (Szymanski *et al.*, 1998). Both Ttg1 and Gl2 mutants were isolated as trichome mutants: Ttg1 mutants completely lack leaf hairs, while Gl2 mutants have reduced trichomes or epidermal cells enlarged in the plane of the leaf (Koornneef, 1981; Haughn and Somerville, 1988; Rerie *et al.*, 1994). Ttg1 mutants also lack anthocyanins in the plant and seed coat, and both Ttg1 and Gl2 have more recently been found to have an increased number and improper placement of root hairs (Koornneef, 1981; Galway *et al.*, 1994; Masucci *et al.*, 1996; Di Cristina *et al.*, 1996). The seed epidermis phenotype is identical in both Ttg1 and Gl2 mutant plants, no mucilage is released when seeds are hydrated and the columellae are reduced or absent (Koornneef, 1981; Rerie *et al.*, 1994; Bowman and Koornneef, 1994a). *GL2* encodes a putative homeodomain protein, while *TTG1* also is thought to code for a transcription factor (Rerie *et al.*, 1994; Di Cristina *et al.*, 1996; Szymanski *et al.*, 1998). The phenotypes of the Ttg1 and Gl2

mutants and their activity as transcription factors suggest that they play a general role in controlling epidermal differentiation, especially aspects involving cell outgrowth.

1.5 Pectin and plant cell walls

The cell wall is an extremely important component of plant cells. Far from being a simple box in which the protoplast sits, the cell wall is a complex structure with many functions. The cell wall surrounds and protects cells as well giving the cell its shape and retaining its structural integrity in the face of turgor pressure. As well, cell walls are the extracellular matrix of plant cells and play many roles in interactions amongst cells and between cells and their environment. Structurally, cell walls are made up of cellulose microfibrils embedded in a matrix of pectin, hemicellulose, and protein. In this section I will focus on pectins, their role in the plant cell wall, and their biosynthesis.

1.5.1 The primary cell wall of dicots

The primary cell wall is the initial wall laid down around the cell plate after cytokinesis. Many cells only have a primary cell wall, while others lay down a thicker, secondary cell wall internal to the first one (Brett and Waldron, 1990; Carpita and Gibeaut, 1993). Both primary and secondary cell walls are complex structures with different characteristics, depending on the cell and the stage of growth. Primary cell walls, in particular, are capable of going through expansion during cell growth.

The primary cell wall is usually envisaged as cellulose microfibrils embedded in a matrix, like steel rods in reinforced concrete. While the true structure is not totally understood; the cell wall is more complex and dynamic than this analogy suggests. Dicot primary walls are made up of

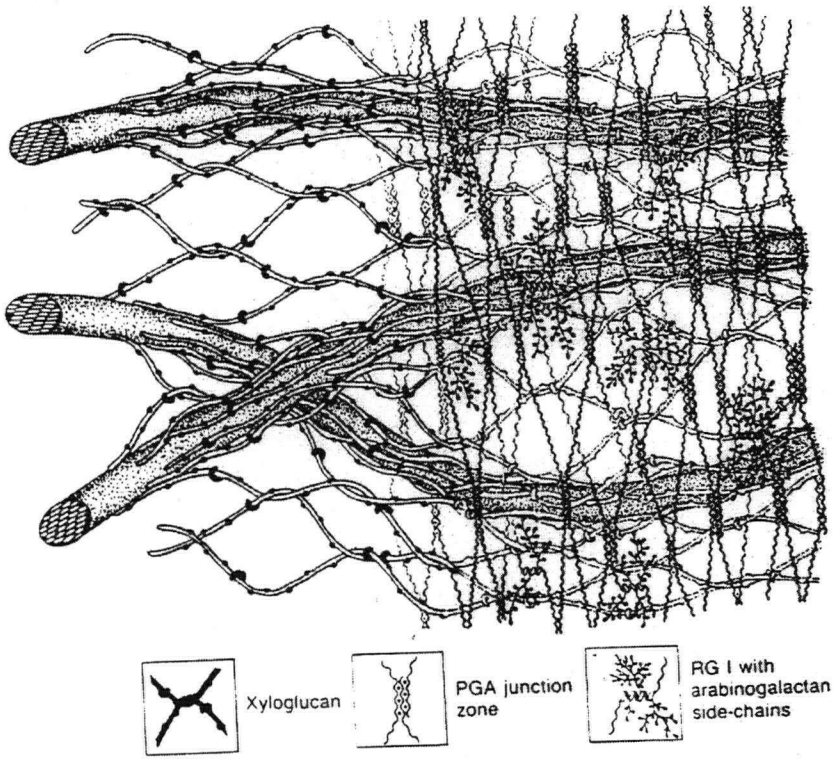


Figure 1.5. A model for dicot cell wall structure.
Cellulose microfibrils are surrounded by an interlinking net of xyloglucan chains and both are embedded in a matrix of pectin (RG I = rhamnogalacturonan I and PGA = polygalacturonic acid) (taken from Carpita and Gibeaut, 1993).

cellulose, hemicellulose, pectin, protein, and water (Figure 1.5) (Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997). Cellulose is an unbranched β1,4-glucan that aggregates into extremely long, semi-crystalline bundles known as microfibrils. The hydrogen bonding within and between cellulose molecules in microfibrils make them extremely strong, with a tensile strength similar to that of steel. Most of the strength of plant cell walls is thought to come from the microfibrils coiled around the cell (Carpita and Gibeaut, 1993). Hemicelluloses are one of the two important carbohydrate components of the matrix. In dicots, the most common hemicelluloses are xyloglucans, which have a β1,4-glucan chain that is substituted with xylose and short chains of xylose, galactose, and fucose. Xyloglucans are able to hydrogen bond to cellulose through long stretches and are thought to create crosslinks between microfibrils. Other hemicelluloses include

galactomannans and β1,3glucans (callose). Pectin refers to a heterogenous group of largely acidic matrix polysaccharides that are characterized by the presence of uronic acids and rhamnose. The role of pectin is thought to be that of a gel in which the other components are embedded, holding them in place. It has also been suggested that pectin can control the porosity of plant cell walls and may also regulate expansion capability (Carpita and Gibeaut, 1993; Cosgrove, 1997). Both structural and catalytic proteins are found in plant cell walls. Structural proteins include hydroxyproline-rich glycoproteins, such as extensin, and arabinogalactan proteins, both of which may be involved in cross linkages within the cell wall. A new class of cell wall proteins, known as expansins, may be involved in facilitatng wall loosening during growth (Cosgrove, 1997). Enzymes present in the cell wall include ones that modify the polysaccharides, for example, endoglucanases, pectinases, pectin methyl esterases, and xyloglucan endotransglycosylases. Other enzymes present play a defensive role and act on the cell wall of potential bacterial and fungal pathogens.

1.5.2 Pectins

Pectins are a set of heterogeneous, acidic, highly-hydrated polysaccharides that take up the majority of the cell wall volume and form a gel in which cellulose and hemicellulose are found. Pectins are also important, along with callose, in the production of the cell plate during cytokinesis (Samuels *et al.*, 1995). In addition, pectins can be secreted in the form of mucilage in the transmitting tract, root cap and during seed development (see Section 1.4). The two major pectins found in dicot cells are polygalacturonic acid (PGA) and rhamnogalacturonan I (RG I) (Figure 1.6a) (Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997). PGA is an unbranched chain of α1,4-linked galacturonic acid residues, while RG I is a branched polysaccharide with a backbone

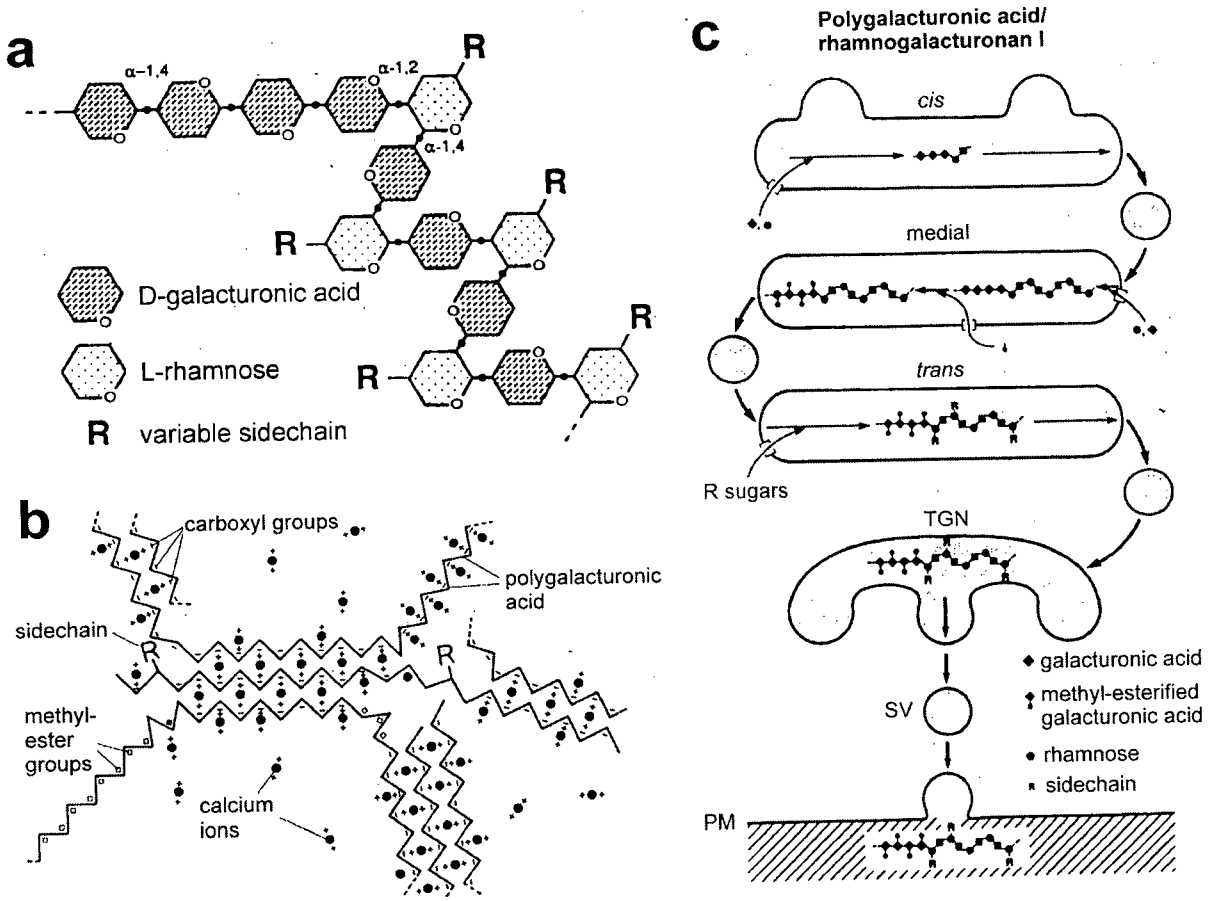


Figure 1.6. The structure, linkages, and biosynthesis of pectins.
(a) A cartoon showing the basic structure of a linked molecule of polygalacturonic acid (top) and rhamnogalacturonan I (right) (adapted from Moore *et al.*, 1991).
(b) Junction zones showing calcium^{++} linkages between adjacent polygalacturonic acid molecules. Note bridges can only form between unbranched molecules that are de-esterified (taken from Brett and Waldron, 1990).
(c) A model of the biosynthetic pathway of polygalacturonic acid/rhamnogalacturonan I in the Golgi apparatus of sycamore maple suspension culture cells as elucidated from immunolocalization studies. TGN = *trans* Golgi network, SV = secretory vesicle, PM = plasma membrane. (taken from Staehelin and Moore, 1995).

of alternating α1,4-linked galacturonic acid and α1,2-linked rhamnose. The RG I side chains are variable and are attached to the rhamnose residues. It has been suggested that PGA and RG I are covalently linked *in muro*, with "smooth" blocks of homogalacturonan broken by "rough", highly-substituted RG I (Figure 1a,b) (Brett and Waldron, 1990). PGA molecules are largely responsible for the gel properties of pectin, due to their ablity to form junction zones (Figure 1.6b). Junction zones are complexes of ajacent PGA chains through ionic bonding between their carboxyl groups

and divalent calcium ions. The fluidity of the cell wall matrix is largely dependent on the degree of bonding between PGA molecules, which in turn is determined by the number of free carboxyl groups and interruptions of homogalacturonan chains with RG I (Brett and Waldron, 1990; Carpita and Gibeaut, 1993). The strength of the pectin gel thus can change over time due to the degree of methyl-esterification and acetylation of the carboxyl groups of galacturonic acid. PGA is secreted into the cell wall as neutral methyl-esters and then is de-esterfied by pectin methyl esterases *in muro*. A decrease of esterification may play a role in the cessation of cell growth by stiffening the matrix against expansion. Covalent diferulic acid linkages between pectins have also been proposed in the formation of the gel.

Cell wall character in general can be affected by its composition, including the amount and types of pectin molecules present. Pectin composition can change not only between cells, but over time (Carpita and Gibeaut, 1993; Staehelin and Moore, 1995). Besides PGA and RG I, other common pectins include rhamnoglacturonan II (RG II), arabinans, glactans, and arabinogalactan I. RG II is a very hetrogeneous, complex molecule with galacturonic acid, rhamnose, arabinose, and galactose in a ratio of 10:7:5:5, and smaller amounts of other sugars. Arabinans and galactans are homopolymers of arabinose and galactose, respectively, and arabinogalactan I consists of a backbone of β1,4-linked galactans with arabinose side chains. These last three polysaccharides may be attached to RG I as side chains or exist independently in the cell wall (Carpita and Gibeaut, 1993; Cosgrove, 1997).

1.5.3 Pectin biosynthesis and secretion

Both pectins and hemicelluloses are known to be synthesized in the endomembrane system, specifically the Golgi stacks. The various sugars for the synthesis of complex polysaccharides are

generally UDP-linked sugars derived from the UDP-glucose pathway by epimerases (Brett and Waldron, 1990). These sugar nucleotides are then transported into the Golgi stacks via membrane transporters for the use of the polysaccharide synthetases (Brett and Waldron, 1990; Carpita *et al.*, 1996). A number of such synthetases or glycosyltransferases have been isolated in membrane extracts and their substrates and polysaccharides formed have been identified through feeding experiments (e.g. Rodgers and Bolwell, 1992; Piro *et al.*, 1993; Carpita *et al.*, 1996). Very few of these enzymes, however, have been fully purified or the genes cloned in plants. Studies have shown that these enzymes are sugar specific and thus a number of enzymes are required to synthesize a complex polysaccharide. In addition, it is not known whether addition of sugars to form side chains occurs in a precise, tightly regulated fashion, or is loosely-controlled, adding a degree of randomness (Brett and Waldron, 1990). Different enzymes have been localized to certain cisternae of the Golgi stacks, suggesting that regulation is at least partially controlled by the location of the transglycosylases, and some polysaccharides have been shown to be sequestered to different regions of the Golgi (Northcote, 1986; Zhang and Staehelin, 1992; Driouich *et al.*, 1993; Staehelin and Moore, 1995).

The biosynthesis of PGA and RG I have been followed in the Golgi stacks through the use of antibodies to various epitopes of these sugars in *Vicia villosa* root hairs, mucilage-producing onion and clover root tips, and cultured sycamore maple cells (Figure 1.6c) (Moore *et al.*, 1991; Zhang and Staehelin, 1992; Sherrier and VandenBosch, 1994; Staehelin and Moore, 1995). Plant Golgi stacks are made up of four morphologically distinct structures: the *cis*-, medial-, and *trans*-cisternae, and *trans*-Golgi network. Biosynthesis of all polysaccharides in the Golgi stacks (sugar side chains of glycoproteins, pectins, and hemicelluloses) appear to occur in a *cis* to *trans* direction, exiting the Golgi stacks through vesicles budding from the *trans*-Golgi network (Staehelin and Moore, 1995). The antibody studies mentioned above have shown that PGA/RG I is synthesized in all three cisternal units. The synthesis of the PGA/RG I backbones appears to commence in the *cis*-cisterna

and continue in the medial cisternae. The glacturonic acid residues become esterified in the medial cisternae and the arabinose-containing side chains are added to rhamnose in the *trans*-cisternae. Interestingly, the degree of esterification of PGA/RG I appears to change between cell types, reflecting differing pectin requirements (Moore *et al.*, 1991; Zhang and Staehelin, 1992; Sherrier and VandenBosch, 1994; Staehelin and Moore, 1995). Once synthesized, the methyl-esterified pectin is packaged into vesicles which are transported to the plasma membrane where the contents are exocytosed. The degree of esterification has been observed to be different between the vesicle contents and the cell wall pectins, with the pectin in the vesicles being neutralized through methyl-esterification and then de-esterification occuring in the cell wall (Sherrier and VandenBosch, 1994).

1.5.4 Genetic analysis of pectin biosynthesis and secretion in plants

Most work that has been performed on cell wall biosynthesis, as described above, has involved isolation and chemical analysis of cell wall polymers, radioactive tracer studies of polysaccharide synthesis, isolation of biosynthetic proteins in membrane extracts, and antibody studies of the location of complex carbohydrates. Only recently has a concerted effort been made to isolate mutants specific to cell wall biosynthesis (reviewed in Reiter, 1994). Of the mutants isolated, only some may affect pectin biosynthesis. A series of Mur mutants with altered levels of neutral monosaccharides in their leaves has been isolated by Reiter *et al.* (1993; 1997). Mur1 mutants lack almost all fucose in their vegetative parts, while Mur2 and Mur3 have reduced levels of fucose (Reiter *et al.*, 1993; Reiter *et al.*, 1997). Mur4 through Mur7 have less arabinose than wild type plants, while Mur8 plants are partially deficient in rhamnose. Mur9 through Mur11 have altered levels of multiple monosaccharides (Reiter *et al.*, 1997). *MUR1* has been found to encode a GDP-*D*-mannose-4,6-dehydratase that is involved in *de novo* synthesis of fucose, thus leading to a lack of

fucose and the partial substitution of galactose for fucose on the terminus of xyloglucan side chains (Zablackis *et al.*, 1996; Bonin *et al.*, 1997). Similar biosynthetic proteins or regulatory proteins could be encoded for by the other *MUR* loci. Any change in the levels of monosaccharides and/or their transglycosidases could lead to altered pectin structure.

A mutant that affects pectin secretion rather than biosynthesis has been found in maize. Ageotropic was first recognized as an agravitropic mutant and later it was realized that the gravitropism defect in the primary root was due to a lack of mucilage surrounding the root cap (Moore *et al.*, 1990). Ultrastructural studies of Ageotropic mucilage-secreting peripheral cells revealed that the cytoplasm was full of unusually large vesicles and the Golgi cisternae were grossly distended (Miller and Moore, 1990). These results suggested that, while mucilage was being made and packaged into vesicles, it was not being exocytosed beyond the plasma membrane (Miller and Moore, 1990). Vesicle transport and docking is a complex process, involving a number of proteins (Staehelin and Moore, 1995), most likely one of these proteins is defective in this mutant. In addition, a smaller amount of mucilage appeared to be made, perhaps due to negative feedback of biosynthetic enzymes in the engorged Golgi apparatus (Miller and Moore, 1990).

1.6 Thesis objectives

My thesis research had several objectives. The first two regard the regulation of ovule development in *Arabidopsis*. One objective was to understand the roles of *AG* and *BEL1* during ovule development. Ag mutants lack carpels and the associated ovules. Flowers doubly mutant for *AG* and *AP2*, however, have carpel-sepals which bear ovules. Thus the roles of *AG* and *BEL1* were investigated through a comparison of Ag, Bel1, and Ag;Bel1 ovule phenotypes in an Ap2 background. A second goal was to determine what role, if any, *AP2* plays in ovule development, through the study of ovules in multiple Ap2 mutants.

The second set of objectives regard the study of mucilage production in the *Arabidopsis* seed coat as a model for pectin biosynthesis. As a basis for the isolation of mutants defective in complex polysaccharide synthesis, wild type seed coat development and mucilage production had to be characterized. Once mucilage and its production was well described in wild type seeds, my goal was to isolate mutants defective in this process. These novel mutants plus mutants at previously isolated loci affecting seed coat development were then characterized in order to determine the nature of their defects and the role of these genes in mucilage biosynthesis and/or seed development.

2. MATERIALS AND METHODS

2.1 Arabidopsis nomenclature

I will use standard *Arabidopsis* genetic nomenclature throughout this thesis. Wild type genes are indicated by uppercase, italicized letters (e.g., *APETALA2*), however, a two or three letter abbreviation is normally used (e.g., *AP2*). In cases where several genes result in a similar phenotype they are differentiated using a number (e.g., *AP1, AP2, AP3*). Mutant alleles are written in lowercase, italics (e.g., *ap2*). Multiple alleles of a single gene are distinguished by hyphenated numbers (e.g. *ap2-1* allele was isolated before *ap2-2*). Mutant phenotypes are denoted by the same abbreviation used for the corresponding gene, having the first letter uppercase, no italics (e.g., Ap2-1). Proteins are indicated by uppercase, non-italicized letters (e.g. AP2).

2.2 Plant material and growth conditions

2.2.1 Plant material

Lines of *Arabidopsis thaliana* used for the ovule work were: Ag-1, Ap2-1 (Landsberg *erecta* [Ler] ecotype; gift from M. Koornneef, Department of Genetics, Wageningen Agricultural University, The Netherlands), Ap2-2, 2-7, 2-8, 2-9 (Ler; gift from E. Meyerowitz, California Institute of Technology, Pasadena, CA), Ap2-3, 2-4 (Ler; gift from K. Okada, National Institute for Basic Biology, Okazaki, Japan), Ap2-6 (Columbia-2 [Col-2] ecotype; Kunst *et al.*, 1989), Bel1-1 (Ler; gift from C. Gasser, University of California, Davis CA), Bel1-3 (Wassilewskija [WS] ecotype), and Ap2-6;Bel1-3 (Col-2 and WS; Modrusan *et al.*, 1994).

Additional lines used for the mucilage work obtained from the *Arabidopsis* Biological Resource Centre at Ohio State University were Pgm-1, Ats, Ttg1-1, and Gl2-1.

2.2.2 Growth conditions

Seeds were stratified at 4°C for 3 days on Terra-Lite Redi Earth prepared soil mix (W.R. Grace and Co. Canada Ltd., Ajax, Ontario, Canada) and then transferred to growth chambers at 20°C under continuous light (CL: 90-120 μE m-2 sec-1 photosynthetically active radiation [PAR]) conditions. Other growth regimes, 12 hours light/12 hours dark (100-150 □E m-2 sec-1 PAR) or 28°C, were used as indicated.

During screening for mucilage mutants, defective seeds stained with Ruthenium red were germinated on AT medium before transfer to soil at the two true-leaf stage.

AT minimal medium: 5 ml/l 1M KNO_3, 2.5 ml/l 1M KH_2PO_4, 2.5 ml/l 20mM FeEDTA, 2 ml/l 1M $MgSO_4$, 2 ml/l 1M $CaNO_3$x4 H_2O, 1 ml/l micronutrients (70mM H_3BO_3, 14mM $MnCl_2$x4 H_2O, 0.5mM $CuSO_4$, 1mM $ZnSO_4$x7 H_2O, 1.2 mM $NaMoO_4$, 10mM NaCl, 0.01mM $CoCl_2$x6 H_2O).

2.2.3 Staging of flower age

The time of pollination was determined phenotypically as the time at which the long stamens started to grow over the gynoecium. Each day for five days, flowers at this stage were marked with a different color of non-toxic, water-soluble paint. This allowed me to go back and select flowers at a certain age.

2.3 Construction of double/triple mutants

2.3.1 Ap2-6;Ag-1 and Ap2-2; Ag-1

Wild type plants from a population segregating Ag plants were used as female parents in a cross to Ap2-6 flowers. Seeds harvested from individual F1 plants formed separate F2 populations which were screened for plants yielding Ap2;Ag flowers (Bowman *et al.*, 1991). Ap2-6 phenotype flowers from these populations were used as female parents in crosses to Ap2-6;Bel1-3 flowers to construct the triple mutant as described below. Ap2-6;Ag-1 double mutant plants were isolated from triple-segregating populations to control for ecotype effects. Ap2-6;Ag-1 phenotype plants from the triple-segregating population that were homozygous for *BEL1* were identified by screening for plants where the T-DNA marker gene, *neomycin phosphotransferase II* (*NPTII*), could not be amplified by polymerase chain reaction (PCR) (see Section 2.4.2). Ag-1;Ap2-2 double mutants were constructed in the same manner, but, since Ag-1, Ap2-2, and Bel1-1 are all of the Ler ecotype, it was not necessary to isolate Ap2-2;Ag-1 double mutants from the triple-segregating population.

2.3.2 Ap2-6;Bel1-3 and Ap2-2;Bel1-1

Ap2-6;Bel1-3 double mutants have been previously constructed (Modrusan *et al.*, 1994), thus it was only necessary to cross Bel1-1 and Ap2-2. Since Bel1 flowers are female sterile, pollen from Bel1-1 flowers was used to fertilize Ap2-2 plants and the F1 was allowed to self fertilize. Both Ap2-6;Bel1-3 and Ap2-2;Bel1-1 double mutants were identified in their respective F2 populations (Modrusan *et al.*, 1994) by screening Ap2 phenotype plants for flowers which produced Bel1 rather than normal ovules. The appearance of immature, two-integumented ovules seen in some Ap2-

6;Bel1-3 plants led to the growth of more plants whose homozygosity for *bel1-3* was tested using molecular methods. The *bel1-3* allele resulted from the insertion of T-DNA (Modrusan *et al.*, 1994a; Reiser *et al.*, 1995), thus this molecular tag was used to differentiate between *ap2-6/ap2-6;BEL1/BEL1*, *ap2-6/ap2-6;BEL1/bel1-3*, and *ap2-6/ap2-6;bel1-3/bel1-3* plants. Putative Ap2-6;Bel1-3 mutants identified in a PCR screen were confirmed through Southern hybridization using a probe that gave different banding patterns for *BEL1* and *bel1-3* alleles (see Section 2.4.2). All plants identified as Ap2-6;Bel1-3 double mutants at the phenotypic level were found to be homozygous for *bel1-3*.

2.3.3 Ap2-6;Ag-1;Bel1-3 and Ap2-2;Ag-1;Bel1-1

Pollen from Ap2-6;Bel1-3 flowers was used to fertilize several Ap2-6 phenotype flowers from an Ap2-6;Ag-1 segregating population, 2/3 of which should be heterozygous for *ag-1*. Selfseed from the maternal plants was grown and the resulting populations screened for the presence of Ap2;Ag plants to determine if the maternal plant was heterozygous for *ag-1*. Analogous to Ap2-6;Bel1-3 flowers, Ap2-6;Ag-1;Bel1-3 flowers were predicted to be identical to Ap2-6;Ag-1 flowers with the possible exception of the ovules, thus the F2 progeny from crosses to known *ap2-6/ap2-6;AG/ag-1* heterozygotes were grown and screened for Ap2-6;Ag-1 plants. The *bel1-3* allele resulted from the insertion of T-DNA (Modrusan *et al.*, 1994a; Reiser *et al.*, 1995), thus this molecular tag was used to differentiate between *ap2-6/ap2-6; ag-1/ag-1;BEL1/BEL1*, *ap2-6/ap2-6; ag-1/ag-1;BEL1/bel1-3* and *ap2-6/ap2-6; ag-1/ag-1;bel1-3/bel1-3* plants. PCR was initially used to differentiate between *bel1-3/bel1-3* homozygotes and the others by amplifying the region surrounding the insertion site, which gave no band in the presence of two copies of the *bel1-3* allele having the T-DNA insertion (see Section 2.4.2). These putative triple mutants were then confirmed through Southern hybridization

using a probe that gave different banding patterns for *BEL1* and *bel1-3* alleles (see Section 2.4.2). Through these analyses, 16 triple mutants were isolated and confirmed.

Ag-1;Ap2-2;Bel1-1 plants were generated in the same fashion. The *bel1-1* allele resulted from a point mutation (Reiser *et al.*, 1995) which created a new *Tai*I (New England Biolabs) restriction site. A region surrounding the site of the point mutation was amplified using PCR and then incubated with *Tai*I (see Section 2.4.2). Those plants where the fragment was completely cleaved were identified as *bel1-1*.

2.4 Molecular techniques

2.4.1 General chemicals and techniques

Restriction enzyme (Gibco-BRL) digestion and agarose gel electrophoresis were performed as described in Sambrook *et al.* (1989). The Geneclean (Bio101) and Random priming kits (Gibco-BRL) with their enclosed protocols were used for recovery of DNA from agarose and radioactive labelling (^{32}P-dCTP) of DNA probes, respectively. Amplification of certain DNA fragments was done by PCR (Innis *et al.*, 1990), using *Taq* polymerase (Gibco-BRL) and a DNA thermocycler (Model 480, Perkin-Elmer). All frequently used buffers and reagents, including Tris, EDTA, TE, TBE, and SSPE, were made and used as described in Sambrook *et al.* (1989).

2.4.2 Detection of *BEL1/BEL1*, *bel1-3/bel1-3* and *bel1-1/bel1-1* plants

Crude DNA for PCR reactions was isolated from single *Arabidopsis* leaves (Edwards, 1991). The *bel1-3* allele was created through insertional mutagenesis with T-DNA containing *NPTII*

(Modrusan *et al.*, 1994a; Reiser *et al.*, 1995). Thus, the absence of *NPTII* was used to identify plants homozygous for *BEL1* in a population segregating *bel1-3*. *NPTII* was detected via PCR using right and left oligonucleotide *NPTII* primers (gift from R. Datla, Plant Biotechnology Institute, Saskatoon, SK). A thermal cycler (Perkin-Elmer) was used to amplify this sequence using the following program: 1st=95°C for 5 min, 2nd=40°C for 5 min, 3rd=94°C for 45 sec, 4th=55°C for 1 min, 5th=72°C for 1 min, 6th=35 cycles of steps 3, 4, and 5, 7th=70°C for 10 min, 8th=4°C for unspecified time.

For the detection of the *bel1-3* allele, PCR was performed using oligonucleotide primers on either side of the approximately 10kb T-DNA insertion. The 5' primer, 5BEL, started 195 bp downstream of the *BEL1* transcription start site (5'-ATGGCAGGGTCATGATCACC-3'), while the 3' primer, R2, started at nucleotide –1055 (5'-TTGCATAGTCTCATGGCAG-3'). Amplification was performed using the following program: 1st=95°C for 5 min, 2nd=40°C for 5 min, 3rd=94°C for 45 sec, 4th=54°C for 1 min, 5th=72°C for 1 min, 6th=35 cycles of steps 3, 4, and 5, 7th=70°C for 10 min, 8th=10°C for unspecified time. *BEL1/BEL1* or *BEL1/bel1-3* plants gave a band of 860 bp, while *bel1-3/bel1-3* plants gave no product. A second 5' primer, BELAT2, starting at nucleotide –750 (5'-AGACATGGCAAGAG-3'), was used with R2 under the same conditions as a control for the quality of the DNA preparations.

Plants that did not amplify the 5BEL-R2 fragment after several trials were selected as putative *bel1-3/bel1-3* homozygotes and were confirmed through Southern hybridization. Single plant DNA preparations for genomic Southern hybridizations were isolated using the CTAB method of Dean *et al.* (1992) scaled down for use with a few leaves. Genomic DNA was digested with *Hin*dIII, resolved in a 0.8% agarose gel and transferred to Hybond N+ (Amersham, Arlington Heights, IL) according to the method of the membrane manufacturer. The probe was derived from the wild type BELAT2-R2 PCR fragment described above using random primer labelling (Gibco-

BRL). The BELAT2-R2 fragment overlaps the 3' end of a 2.9 kb *Hin*dIII fragment within *BEL1*, and a 4-5 kb fragment in *bel1-3*. After hybridization and washing, the filter was exposed to Kodak XAR-5 film.

Unlike *bel1-3*, *bel1-1* resulted from a point mutation (Reiser *et al.*, 1995). Fortuitously, this point mutation created a new restriction site for *Taq*I (New England Biolabs). A region surrounding the site of the point mutation was amplified using PCR and then incubated with *Taq*I. Those plants where the fragment was completely cleaved were identified as *bel1-1*. The PCR 5' and 3' primers used were located at –749 (AT3: 5'-GAGAGACATGGCAAGAGATCAG-3') and –1229 (WT3': 5'-GAGCATGGAGAGCAACTTGG-3'), respectively. PCR amplification was performed under the following conditions: 1st=95°C for 5 min, 2nd=40°C for 5 min, 3rd=94°C for 45 sec, 4th=58°C for 1 min, 5th=72°C for 1 min, 6th=35 cycles of steps 3, 4, and 5, 7th=70°C for 10 min, 8th=10°C for unspecified time.

2.5 Microscopy

2.5.1 Screening for mucilage mutants with Ruthenium red

Seed were stained using a solution of 0.01% Ruthenium red (Sigma) in distilled water. Ruthenium red is a basic dye that is specific for free carboxyl groups of acidic polysaccharides. Since the major acidic polysaccharides found in plant cells are galacturonic acid containing pectins, Ruthenium red is considered to be a more-or-less pectin specific dye. For mutant screening, seeds were first placed in water in microfuge tubes, then shaken for 15 minutes to 1 hour. This was followed by the replacement of the water with Ruthenium red dye and further shaking for 1-1½ hours. Seeds were then rinsed with distilled water and observed under a dissection microscope.

Under these conditions, wild type seeds are surrounded by a pink-staining capsule of pectinaceous mucilage. The absence or reduction in the size of the mucilage capsule was used to isolated mutants defective in seed coat development and/or mucilage production.

2.5.2 Clearing and differential interference contrast optics

Flowers were fixed and cleared in organic solvents by the method of Schneitz *et al.* (1995) for whole-mount squashes of ovules. Ovules were photographed under differential interference contrast (DIC) optics using a Leitz DRB (Leica, Wetzlar, Germany) light microscope with Kodak Ektachrome 160 ASA film (Eastman Kodak, Rochester, New York).

Developing seeds stained for starch were first stained in I_2-KI (Caspar *et al.*, 1985), then placed in a quick clearing solution of chloral hydrate, glycerol, and water (Léon-Kloosterziel *et al.*, 1994) before observation with DIC optics.

2.5.3 Resin embedding for brightfield 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% glutaraldehyde (J.B EM Services) in 0.5 M sodium phosphate buffer at pH 7. After incubation at 4°C overnight, samples were washed with phosphate buffer and postfixed for 1-2 hours in 1% osmium tetraoxide in 0.5M phosphate buffer. Samples were then dehydrated through an ethanol series. Alternatively, samples were fixed in FAA (4% paraformaldehyde [J.B. EM Services], 15% acetic acid, 50% ethanol) then directly dehydrated without post-fixation. All samples were then changed into propylene oxide and slowly infiltrated with Spurr resin (J.B. EM Services). For brightfield microscopy 0.2-0.5□m sections were cut using

glass knives on a Reichert microtome, mounted on glass slides, and stained with 1% Toluidine blue O in 1% sodium borate (pH 11). Sections were photographed using a Leitz DRB (Leica, Wetzlar, Germany) light microscope with Kodak Gold Plus or Royal Gold 100 ASA film (Eastman Kodak, Rochester, New York). In preparation for electron microscopy, thin sections (silver to gold) were cut using a diamond knife on an Ultracut E microtome (Reichert) and collected onto formivar-coated, carbon-coated, nickel grids. Sections were stained in 1-2% uranyl acetate for 30 minutes, followed by 15 minutes in lead acetate. Specimens were observed and photographed on a Zeiss 10C transmission electron microscope, which was operated at an accelerating voltage of 60 or 80 kV.

2.5.4 Scanning electron microscopy

Samples were either dry mounted on stubs or fixed 3% glutaraldehyde in 0.02 M phosphate buffer pH 7.0 overnight at 4°C. The fixed samples were rinsed several times with buffer and dehydrated through a graded ethanol or acetone series before being critical point dried in liquid CO_2. Seed samples were then mounted on stubs, while, for the ovule work, the gynoecia or outer whorl carpel sepals were dissected from the flowers before mounting. All samples were coated with gold or gold-palladium in a Nanotech SEMPrep2 sputter coater and observed using a Cambridge model 250T scanning electron microscope (Leica) with an accelerating voltage of 20 kV.

2.6 Gas chromatography and mass spectrometry

Mucilage was isolated from samples of 100 intact seeds by incubating in 0.2% ammonium oxalate with vigourous shaking for 2 hours at 30°C. 10 µl of internal standard (4.8 mg/ml myo-inositol) was added prior to precipitation with 5 volumes of absolute ethanol. Derivitization of trimethylsilyl ethers was adapted from Chaplin (1986). Samples were hydrolyzed overnight at 70°C in

4:1 1N methanolic HCl:methyl acetate. After transfer to Reactivials, samples were precipitated with ¼ volume 2-methyl-2-propanol and dried under nitrogen gas. Acetylation of amino sugars was performed by a 15 minute incubation in 10:1:1 methanol:pyridine:acetic anhydride and then samples were dried down under nitrogen gas. The monosaccharides then were trimethylsilylated for 1 hour using Tri-Sil reagent (Pierce), dried under nitrogen gas, then resuspended in hexane. Samples were run on a Hewlett-Packard 5890A gas chromatograph on a DB-5 fused silica column (30m by 0.25mm internal diameter, df=0.10 μm) with helium as the carrier gas. The temperature program was 140°C for 2 minutes, then increasing 8 °C/minute up to 240°C, followed by 5 minutes at 240°C. Samples from both wild type and the mutants yielded a consistant pattern of peaks. The peaks were initially identified by comparison to the retention times of reference sugar standards, then confirmed through gas chromatography-mass spectrometry. The gas chromatography-electron impact mass spectrometry was performed by the UBC Chemistry Mass Spectrometry Centre.

Isolation of cell wall components from whole seeds was performed by grinding 100 seed in 0.2% ammonium oxalate. Derivitization 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. Individual sugar standards and a composite standard were made from the following monosaccharides: myo-inostitol (used as an internal standard), fucose, mannose, galactose, glucose, arabinose, rhamnose, xylose, glucuronic acid, and galacturonic acid (Chaplin, 1986).

2.7 Statistical analyses

For SEM analyses of Ap2, Ag, Bel1 mutant combinations, carpel-sepals were removed from several flowers per plant and mounted together on stubs, thus the average number of ovules per flower was calculated for each stub and treated as a random sample (for the Ap2-6, Ag-1, Bel1-3

mutant set: Ap2-6=6 stubs, Ap2-6;Bel1-3=16 stubs, Ap2-6;Ag-1=21 stubs, Ap2-6;Ag-1;Bel1-3=16 stubs; For the Ap2-2, Ag-1, Bel1-1 mutant set: Ap2-2=9 stubs, Ap2-2;Bel1-1=8 stubs, Ap2-2;Ag-1=11 stubs, Ap2-2;Ag-1;Bel1-1=3 stubs). The data set obtained for each of the four ovule types in the four mutants was subjected to a single factor Analysis of Variance (ANOVA) followed by Tukey pairwise comparisons using SYSTAT 4.1 (Wilkinson, 1988) to determine if the sample means were identical.

3. THE RELATIVE ROLES OF *AG*, *BEL1*, AND *AP2* IN *ARABIDOPSIS* OVULE DEVELOPMENT

3.1 Introduction

As discussed in Chapter 1, morphological, genetic, and molecular studies of ovule mutants are beginning to yield understanding in the regulation of ovule development in *Arabidopsis*. Ovules can be considered substructures of flowers, so it is intriguing that two floral organ identity genes may play a late role in the development of ovules. One of these genes, *AP2*, was initially identified by its defects in the perianth organs and has also been suggested to play a role in floral meristem initiation. Recently, in our lab, Modrusan *et al.* (1994) observed that abnormal ovules could be found in the gynoecium of two strong *ap2* alleles, *ap2-6* and *ap2-7*. I have followed up on those observations and have investigated the role of *AP2* in ovule development through the quantitation of the ovule types found in the gynoecia of many Ap2 alleles.

Molecular and genetic analyses have demonstrated that the *Arabidopsis* gene *BEL1* is required for proper morphogenesis of the ovule integuments. Several lines of evidence suggest that *BEL1* may act, at least in part, to repress the function of the organ identity gene *AG* during ovule development. The mechanism by which *BEL1* influences *AG* in the ovule is not known. Evidence indicates, however, that the negative regulatory effect of *BEL1* on *AG* is post-transcriptional (Reiser *et al.*, 1995). Whether *BEL1* is needed primarily to regulate *AG* function or is required more directly for ovule development, or both, needs to be determined. Whether *AG* has a specific role in ovule development or not is unclear. *AG* is transcribed in the ovule in a complex temporal and spatial pattern (Bowman *et al.*, 1991; Reiser *et al.*, 1995), suggesting that it may be involved in several aspects of ovule morphogenesis. Ag mutants lack carpels and do not make associated ovules. Flowers

doubly mutant for *AG* and *AP2*, however, have carpel-sepals which bear ovules (Bowman *et al.*, 1991). Some of these Ag;Ap2 ovules have a wild type morphology, suggesting that *AG* is not required for ovule development. To clarify the roles of *BEL1* and *AG* during ovule development, I performed a detailed analysis of the ovule phenotypes of Ag, Bel1, and Ag;Bel1 in an Ap2 background.

3.2 The ovules of Ap2 mutants

3.2.1 The ovules of Ap2 gynoecia

The flowers of strong Ap2 mutants (e.g. Ap2-6, Ap2-2) have carpel-sepal organs in the outer whorls, followed by stamens or stamen carpel-organs, and a bicarpellate gynoecium that is sometimes unfused (Figure 3.1a). Ap2 outer whorl carpel-sepals are marked by the presence of stigmatic papillae and by placental tissue on their margins, which can give rise to structures resembling ovules (Figures 3.1a, 3.2h) (Komaki *et al.*, 1988; Kunst *et al.*, 1989; Bowman *et al.*, 1991). A previous study has suggested that *AP2* may play a role in ovule development (Modrusan *et al.*, 1994a). In order to investigate further the role of *AP2* in ovule development, I have quantitated the ovule types found in the gynoecia of a strong allele, Ap2-6. I have focussed on fully-fused gynoecia to avoid the possible effects of improper carpel development.

There was only a small proportion of abnormal ovules in Ap2-6 gynoecia (5.4%) (Table 3.1). They occurred sporadically; not every flower had abnormal ovules, while some had several. These numbers were increased when improperly fused carpels were observed (data not shown; see Modrusan *et al.*, 1994). Four types of abnormal ovules were commonly seen (Figure 3.1; Table 3.1):

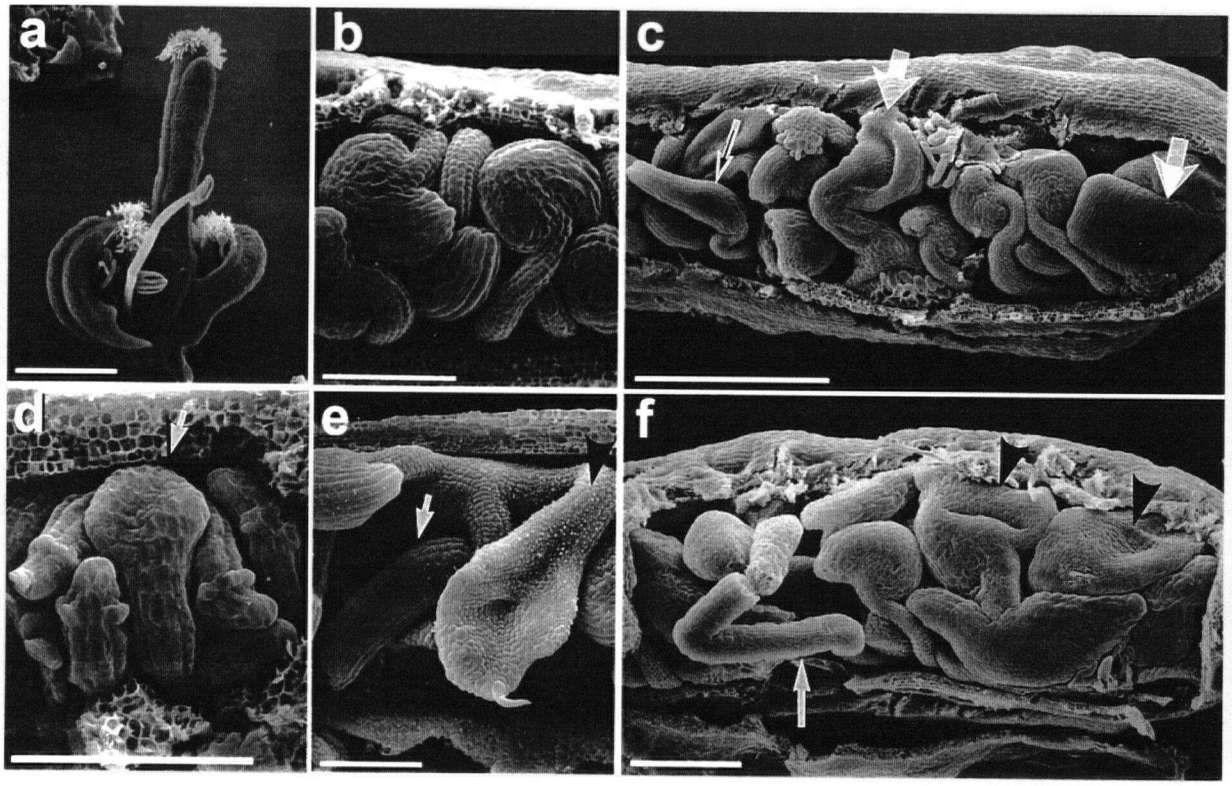


Figure 3.1. Scanning electron micrographs of the ovule types found in Ap2-6 flowers.
(a) Ap2-6 mature flower.
(b) Morphologically normal ovules.
(c) Base of gynoecium showing location of planar ovules (arrow heads) and a Flo10-like ovule (arrow).
(d) Ovules at time of integument initiation, note developing planar ovule (arrow).
(e) Conical ovule in foreground (arrowhead) and Flo10-like ovule in background (arrow).
(f) Base of gynoecium showing location of planar ovules (arrow heads), note broken filamentous ovule to left (arrow).
Magnification bars: (a) = 1mm; (b),(d),(e) = 100μm; (c) = 400μm; (d) = 200μm.

Planar ovules. The majority of abnormal ovules (4.2%) were large, flat, leaf-like structures that sometimes had stigmatic papillae at their tips (Figure 3.1c,f). Examination by SEM showed that these planar ovules were composed of cells similar to those of ovaries, which, in combination with the stigmatic papillae, suggested a carpel-like character. Planar ovules were normally found at the base of the gynoecium where ovules first start to form (Figure 3.1c,f). Observation of developing Ap2-6 ovules revealed that spade-like structures could be found at a

Table 3.1. Percentage of ovule types found in wild type and Ap2 gynoecia at 20°C.[a,b,c]

Allele	Number of flowers	Wild type morphology	Planar	Filamentous	Conical	Flo10	Other
Col-2	77	100 (4389)	0	0	0	0	0
Ap2-5	69	98 (4746)	1 (60)	<1 (5)	<1 (3)	<1 (6)	<1 (10)
Ap2-6	172	95 (11715)	4 (524)	<1 (31)	<1 (12)	1 (97)	<1 (5)
Ap2-7	93	87 (3386)	11 (429)	1 (40)	<1 (6)	1 (25)	<1 (20)
Ler	63	100 (3717)	0	0	0	0	0
Ap2-1	100	99 (6683)	<1 (2)	<1 (4)	0	<1 (11)	0
Ap2-2	58	95 (3100)	1 (36)	<1 (16)	<1 (5)	3 (85)	<1 (6)
Ap2-3	34	92 (2209)	2 (58)	2 (56)	<1 (11)	3 (80)	0
Ap2-4	35	93 (2047	1 (20)	3 (56)	<1 (9)	3 (73)	0
Ap2-8	21	93 (1348)	2 (28)	2 (35)	<1 (3)	2 (35)	0
Ap2-9	16	92 (1072)	2 (24)	2 (26)	<1 (1)	4 (45)	0

[a]Number of ovules in parentheses.
[b]Counted with dissection microscope, both locules observed.
[c]Absolute number of abnormal ovules given; total number ovules used to calculate percentages was determined from an average number ovules per flower for each allele (4-38 pistils counted).

time when the other, normal ovules were initiating their integuments (Figure 3.1d), suggesting that the planar ovules deviate very early in development.

Filamentous ovules. Comprising 0.3% of the ovules, these consisted of a long filamentous structure that could be plain, or topped with an ovule or stigmatic papillae (Figure 3.1f). These were found throughout the gynoecium, most often near the top.

Conical ovules. These resembled planar ovules, but had a more three-dimensional appearance (0.1%). Examination by SEM revealed that they may be outgrowths of the integuments and that the cells resemble those of carpels, including the presence of stigmatic papillae (Figure 3.1e).

Flo10-like ovules. A small number (0.8%) of the ovules had an elongated appearance, resembling those of Flo10 (Superman) flowers (Figure 3.1c,e; unpublished observations; Gaiser *et al.*, 1995). Similar to Flo10 ovules, these most likely resulted from symmetric growth of the outer integument. They were found throughout the gynoecium.

The small number of abnormal ovules suggests that the role of *AP2* is relatively minor. The variable types of abnormal ovules, however, seems to imply that it affects multiple aspects of ovule development.

3.2.2 The effect of different alleles and ecotypes

In order to more thoroughly investigate the role of *AP2*, the types of ovules found in nine alleles from two ecotypes were compared (including Ap2-6) (Table 3.1). Wild type gynoecia of both ecotypes (Col-2 and Ler) had all normal ovules, while abnormal ovules were found in all Ap2 alleles investigated. The proportion ranged from 0.2-13% with some correlation between the strength of the allele and the number of abnormal ovules. The two lowest percentages were found in the two alleles that have the weakest floral phenotype, Ap2-1 and Ap2-5, while Ap2-7, the second strongest Col-2 allele, had the largest number of abnormal ovules.

Ecotype-specific effects were also evident. In the Ler alleles (Ap2-1, -2, -3, -4, -8, -9), the majority of abnormal ovules were Flo10-like, with smaller numbers of filamentous and planar ovules. The Col-2 alleles (Ap2-5, -6, -7), in contrast, had a preponderance of planar ovules—from five to ten times the number of Flo10-like or filamentous ovules.

3.2.3 Environmental effects

Growth conditions are known to affect the growth of Ap2 mutants, with higher temperatures leading to more severe floral phenotypes (Bowman *et al.*, 1989; Bowman *et al.*, 1991). In order to determine if there was a similar effect of growth conditions on ovule development, a weak and strong allele of each ecotype was grown at 28°C (Table 3.2). Increased temperature led to

Table 3.2. Percentage of ovule types found in wild type and Ap2 gynoecia at 28°C.[a,b,c]

Allele	Number of flowers	Wild type morphology	Planar	Filamentous	Conical	Flo10	Other
Col-2	20	100 (980)	0	0	0	0	0
Ap2-5[d]	24	99 (1670)	0	0	0	1 (10)	0
Ap2-6	20	76 (725)	20 (195)	2 (22)	<1 (5)	1 (12)	<1 (1)
Ler	31	99 (1425)	0	0	0	<1 (2)	0
Ap2-1	53	97 (2302)	1 (35)	<1 (5)	<1 (3)	1 (33)	<1 (7)
Ap2-2	4	75 (54)	13 (9)	8 (6)	0	0	4 (3)

[a]Number of ovules in parentheses.
[b]Counted with dissection microscope, both locules observed.
[c]Absolute number of abnormal ovules given; total number ovules used to calculate percentages was determined from an average number ovules per flower for each allele (4-28 pistils counted)[d].
[d]Average number ovules per flower from 20°C was used.

a five-fold increase in the proportion of abnormal ovules for both strong alleles (Ap2-6 and Ap2-2). In addition, both alleles showed the Col-2 pattern of the majority of the abnormal ovules being planar. This effect was still stronger in Ap2-6 versus Ap2-2. An increase in the number of abnormal ovules was also seen in Ap2-1, but not in Ap2-5. These results demonstrated that, not only does increased temperature lead to more abnormal ovules, but that the phenotype of the abnormal ovules became more severe, with greater numbers of carpel-like planar ovules.

3.3 Genetic analysis of the interaction between *AG* and *BEL1*

To determine the relative roles of *AG* and *BEL1* during ovule development, the ovule phenotypes of Ap2;Ag, Ap2;Bel1, and Ap2;Ag;Bel1 mutant flowers were examined using scanning electron microscopy (SEM) (Figure 3.2). Ap2 ovule phenotypes were studied to determine those aspects of the phenotype specific to *AP2*. In addition, to control for differences in background, two similar sets of double and triple mutants were constructed differing in the *ap2* and *bel1* alleles used (*ap2-6*, *bel1-3*, *ag-1* versus *ap2-2*, *bel1-1*, *ag-1*). Since similar results were observed for both sets of

mutants, I will describe in detail only those for one set (Ap2-6, Ap2-6;Ag-1, Ap2-6;Bel1-3, and Ap2-6;Ag-1;Bel1-3 – Tables 3.3, 3.5; Figure 3.4). The data for the second set (Ap2-2, Ap2-2;Ag-1, Ap2-2;Bel1-1 and Ap2-2;Ag-1;Bel1-1) can be found in Tables 3.4 and 3.6, and Figure 3.5. The ovule phenotypes found in each of these mutants were extremely variable. I thus classified them based on their severity into four basic categories which overlap those found for Ap2 gynoecia (Figure 3.2; Table 3.3). Since the double and triple mutants examined had ovule types from all four categories, I will first describe the ovule types and then their frequencies in the different mutant combinations mentioned above.

3.3.1 Mutant ovule morphology

Mature Ovules. Ovules with well-developed integuments and nucellus were termed mature ovules. Included in this category are ovules with a wild type morphology (Figure 3.2a) and ovules with symmetric integuments resembling those of Flo10 (Superman) flowers (Figure 3.2b; unpublished observations; Gaiser *et al.*, 1995).

Bel1 Ovules. All ovules lacking an inner integument and having an abnormal outer integument similar to those of Bel1 mutant flowers, were catagorized as Bel1 ovules (Figure 3.2c).

Immature Ovules. Structures which, when observed by SEM, resemble ovules with integuments arrested at various developmental stages were classified as immature ovules (Figure 3.2d,e). Some of the structures with two integuments had an asymmetric outer integument that did not enclose the inner integument on the underside of the ovule (Figure 3.2e).

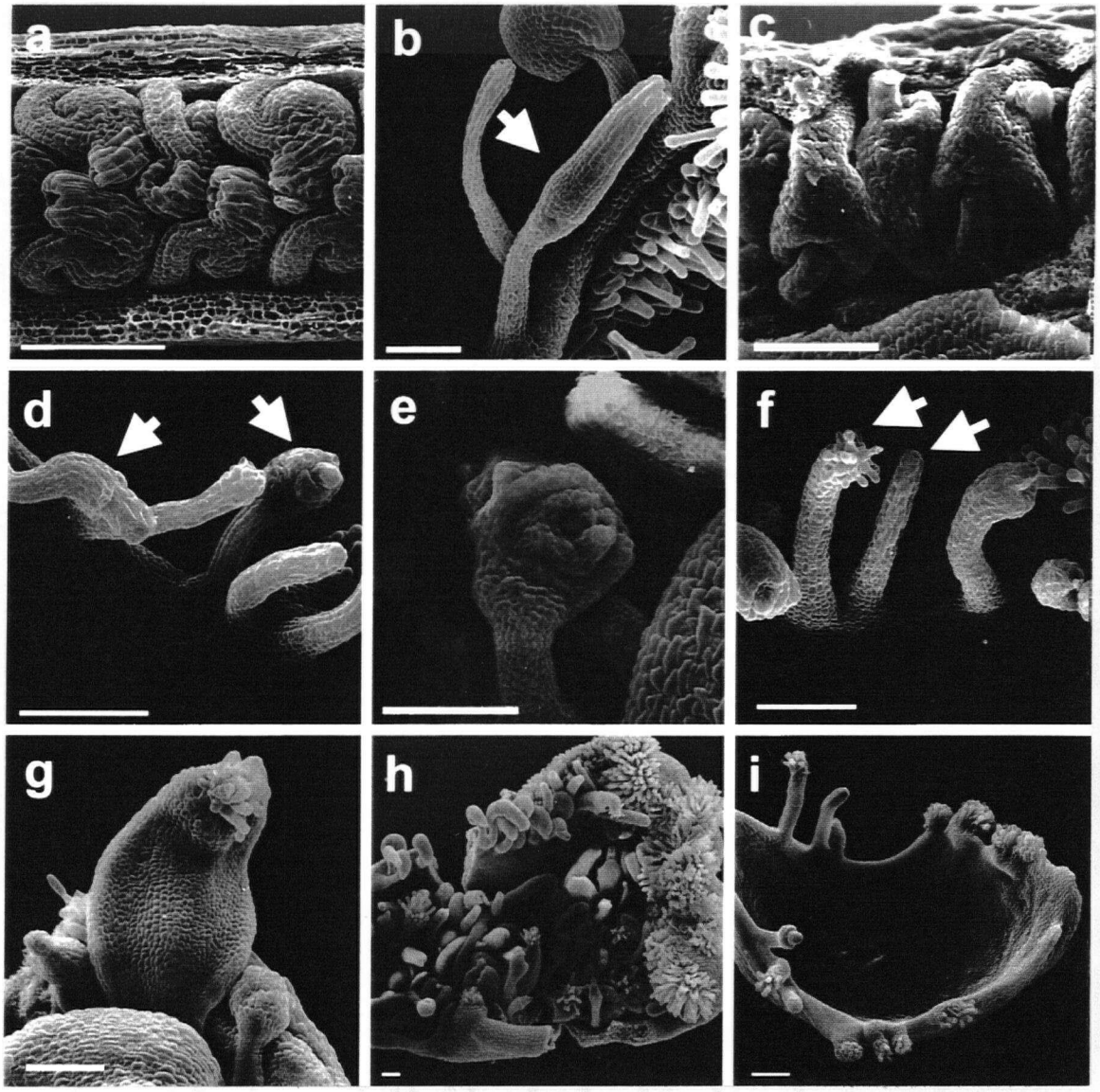


Figure 3.2. Scanning electron micrographs of the ovule types found in Ap2-6, Ap2-6;Bel1-3; Ap2-6;Ag-1 and Ap2-6;Ag-1;Bel1-3 flowers.

(a) Wild type ovules.

(b) A Flo10 ovule (arrow) on a Ap2-6;Ag-1 carpel-sepal.

(c) Bel1 ovules in an Ap2-6;Bel1-3 gynoecium.

(d) Immature ovules (arrows) on an Ap2-6;Ag-1 carpel-sepal.

(e) Immature ovule on an Ap2-6;Bel1-3 carpel sepal, note 2 integuments present on only one side of nucellus.

(f) Projection ovules on an Ap2-6;Ag-1;Bel1-3 carpel sepal.

(g) Planar ovule on an Ap2-6;Bel1-3 carpel-sepal.

(h) An Ap2-6 carpel-sepal. Note the variety of structures.

(i) An Ap2-6;Ag-1;Bel1-3 carpel-sepal.

Magnification bars equal 100μm.

Table 3.3a. Percentage of ovule types found on the carpel-sepals of Ap2-6, Bel1-3, and Ag-1 mutant combinations.[a,b]

Mutant	Number of Flowers	Ovule Type: Mature		Bel1	Immature	Primordial Outgrowths	
		Wild type	Flo 10			Projection	Planar
Ap2-6	34	49 (650)	6 (81)	0	5 (66)	16 (209)	24 (320)
Ap2-6;Bel1-3	59	0	0	6 (95)	31 (470)	30 (449)	32 (484)
Ap2-6;Ag-1[c]	63	33 (618)	3 (68)	0	6 (119)	54 (1021)	3 (63)
Ap2-6;Ag-1;Bel1-3	70	1 (20)	0	0	11 (272)	76 (1832)	12 (300)

[a]SEM data.
[b]Number of ovules are in parentheses.
[c]Isolated from the triple-segregating population.

Table 3.3b. Percentage of ovule types found in the gynoecia of Ap2-6 and Ap2-6;Bel1-3.[a]

Mutant	Number of Flowers	Ovule Type: Mature		Bel1	Immature	Primordial Outgrowths	
		Wild type	Flo10			Projection	Planar
Ap2-6[b]	172	94 (11715)	1 (97)	0	0	<1 (31)	4 (541)
Ap2-6;Bel1-3[c]	23	0	0	70 (460)	26 (173)	0	4 (25)

[a]Number of ovules are in parentheses.
[b]Dissection microscope, includes both locules.
[c]SEM data, one locule per flower.

Table 3.4. Percentage of ovule types found on the carpel-sepals of Ap2-2, Bel1-1, and Ag-1 mutant combinations.[a,b]

Mutant	Number of Flowers	Ovule Type: Mature		Bel1	Immature	Primordial Outgrowths	
		Wild type	Flo 10			Projection	Planar
Ap2-2	36	45 (722)	9 (144)	0	10 (169)	23 (365)	13 (218)
Ap2-2;Bel1-1	23	0	0	1 (8)	42 (258)	39 (240)	18 (115)
Ap2-2;Ag-1	33	8 (89)	1 (8)	0	20 (234)	68 (781)	3 (32)
Ap2-2;Ag-1;Bel1-1	8	0	0	0	6 (16)	90 (238)	4 (10)

[a]SEM data.
[b]Number of ovules are in parentheses.

Primordial Outgrowths. Structures which had no ovule features but arose from the placental tissue fell into two groups. Finger-like projections of cells arising from the placental tissue in the position of ovules were termed projection ovules (Figure 3.2f). Some projection ovules were short with domed tips similar to ovule primordia while others were longer with stigmatic papillae covering a blunt tip. The large, flat, carpel-like planar ovules seen in Ap2 gynoecia were also included in this group (Figure 3.2g).

3.3.2 The ovules of Ap2 outer whorls

While the Ap2-6 gynoecia had 95% mature, morphologically normal ovules and 4% primordial outgrowths, the ovules arising from the placental tissue of the carpel-sepals were much more variable. SEM revealed three categories of ovules (mature, immature, and primordial outgrowths) (Table 3.3a; Figure 3.4). Most of the ovules on carpel-sepals were either mature (55%) or primordial outgrowths (40%), with only a smaller proportion of immature ovules (5%). In addition, there were fewer ovules arising from the outer whorl placenta than found in each carpel of the gynoecium (average 20 per carpel-sepal versus 36 per locule of the gynoecium). To confirm that the mature ovules seen on carpel-sepals were histologically normal, clearing was performed. The majority of ovules had both inner and outer integuments and embryo sacs (94%) (Figure 3.3a), while a few had underdeveloped embryo sacs (6%) (16 ovules counted). Clearing of immature ovules revealed that most had a megaspore or developing embryo sac (80%) while a few (20%) did not (5 ovules counted).

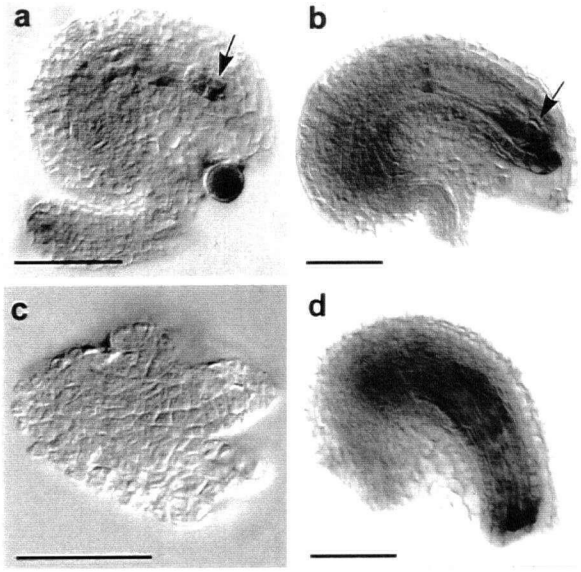


Figure 3.3. Wild type and mutant ovules cleared in organic solvents photographed under differential interference contrast optics.
(a) Wild type ovule with distinct embryo sac (arrow).
(b) Mature ovule from Ap2-6;Ag-1 leaf-carpel with distinct embryo sac (arrow).
(c) Bel1-3 ovule, note lack of embryo sac.
(d) Mature ovule from Ap2-6;Ag-1;Bel1-3 leaf-carpel. Note space with files of cells where embryo sac should be.
Magnification bars equal 50μm.

3.3.3 The ovules of Ap2;Bel1 double mutants

Ap2;Bel1 flowers resemble Ap2 flowers with Bel1 ovules in place of mature ovules (compare Figures 3.2a,c; Modrusan, 1994), thus they were identified in a segregating population by examining the gynoecia of Ap2 phenotype flowers for Bel1 ovules. The genotype was confirmed through molecular methods (see Chapter 2).

Ap2-6;Bel1-3 flowers have been previously characterized (Modrusan *et al.*, 1994a). To determine the effect of the loss of *BEL1* function on ovule phenotypes in an Ap2 background, I analyzed the ovule types in these flowers in more detail (Table 3.3). In the gynoecium, there were 70% Bel1, 25% immature, and 4% primordial outgrowths (Table 3.3b). Relative to Ap2, the

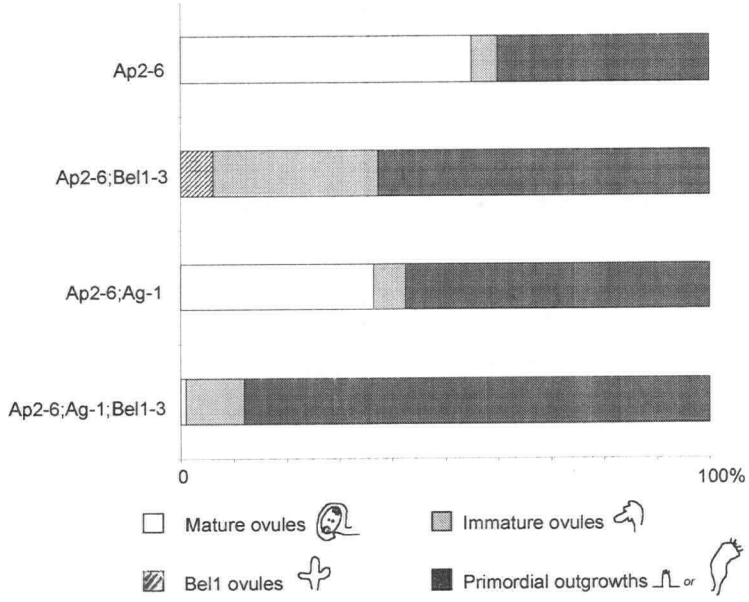


Figure 3.4. Percentage of ovule types found on the carpel-sepals of the Ap2-6, Bel1-3, Ag-1 mutant combinations.

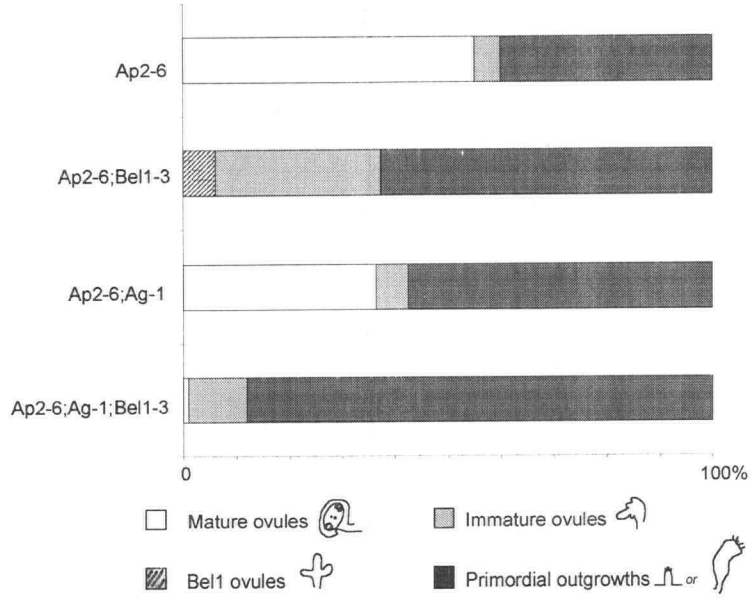


Figure 3.5. Percentage of ovule types found on the carpel-sepals of the Ap2-2, Bel1-1, Ag-1 mutant combinations.

Ap2;Bel1 gynoecium had a lower percentage of mature ovules and a higher percentage of immature ovules (Table 3.1b). This contrasts with the phenotype of Bel1 mutants where only Bel1 ovules were seen (Robinson-Beers *et al.*, 1992; Modrusan *et al.*, 1994a). Interestingly, the gynoecial immature ovules were seen in Ap2-6;Bel1-3 but not in Ap2-2;Bel1-1 (data not shown), suggesting that this aspect of the phenotype may be due to allele or background specific effects. Ap2-6;Bel1-3 carpel-sepals had 6% Bel1, 31% immature, and 62% primordial outgrowths. A comparison between Ap2 and Ap2;Bel1 carpel-sepals (Figure 3.4, Tables 3.3a, 3.5) indicates that, as in the gynoecium, the loss of *BEL1* function results in a higher proportion of immature ovules to mature ovules. The appearance of Bel1 and immature ovules, but not normal ovules, in Ap2;Bel1 mutants suggest that the *BEL1* gene is required for integument development (Figure 3.6), as has been suggested previously (Robinson-Beers *et al.*, 1992; Modrusan *et al.*, 1994a).

3.3.4 The ovules of Ap2;Ag double mutants

Ap2;Ag double mutant flowers consist of leafy organs followed by two whorls of petal-stamen organs, then inner flowers which follow the same pattern. The outer whorl leafy organs, like Ap2 carpel-sepals, can have stigmatic papillae and placental tissue giving rise to structures resembling ovules (Figure 3.2h,i; Bowman *et al.*, 1991). To determine the role of *AG*, we examined both inner and outer whorl carpel-sepals of Ap2;Ag flowers and compared them to Ap2 carpels and carpel-sepals (Tables 3.3a and 3.5; Figure 3.4). Carpel-sepals of the inner and outer whorls of Ap2;Ag flowers were almost indistinguishable from each other with respect to ovule number and type (data not shown), thus ovule data from all carpel-sepal organs were combined (Table 3.3a). All the ovule types seen on Ap2 outer whorls were also found on Ap2;Ag carpel-sepals (mature,

Table 3.5. Results of ANOVA and Tukey pairwise comparisons to determine if Ap2-6, Bel1-3, and Ag-1 mutant combinations have same number of different ovule types per flower[a,b].

Mature and Bel1 Ovules				
	Ap2-6	Ap2-6;Bel1-3	Ap2-6;Ag-1	Ap2-6;Ag-1;Bel1-3
Ap2-6	-			
Ap2-6;Bel1-3	P<0.01	-		
Ap2-6;Ag-1	P<0.01	P<0.01	-	
Ap2-6;Ag-1;Bel1-3	P<0.01	same[c]	P<0.01	-

Immature Ovules				
	Ap2-6	Ap2-6;Bel1-3	Ap2-6;Ag-1	Ap2-6;Ag-1;Bel1-3
Ap2-6	-			
Ap2-6;Bel1-3	P<0.01	-		
Ap2-6;Ag-1	same	P<0.01	-	
Ap2-6;Ag-1;Bel1-3	same	P<0.01	same	-

Primordial outgrowths				
	Ap2-6	Ap2-6;Bel1-3	Ap2-6;Ag-1	Ap2-6;Ag-1;Bel1-3
Ap2-6	-			
Ap2-6;Bel1-3	same	-		
Ap2-6;Ag-1	same	same	-	
Ap2-6;Ag-1;Bel1-3	P<0.01	P<0.01	P<0.01	-

[a]Outer whorls only.
[b]Average number of outer whorl carpels examined per flower: Ap2-6=1.8, Ap2-6;Bel1-3=1.9, Ap2-;Ag-1=6.1, Ap2-6;Ag-1;Bel1-3=5.2. Total number of ovules per flower not significantly different (data not shown).
[c]Any comparison where P>0.01

Table 3.6. Results of ANOVA and Tukey pairwise comparisons to determine if Ap2-2, Bel1-1, and Ag-1 mutant combinations have same number of different ovule types per flower[a,b].

Mature and Bel1 Ovules[c]				
	Ap2-2	Ap2-2;Bel1-1	Ap2-2;Ag-1	Ap2-2;Ag-1;Bel1-1
Ap2-2	-			
Ap2-2;Bel1-1	P<0.01	-		
Ap2-2;Ag-1	P<0.01	same[d]	-	
Ap2-2;Ag-1;Bel1-1	s.d.	same	s.d.	-

Immature Ovules				
	Ap2-2	Ap2-2;Bel1-1	Ap2-2;Ag-1	Ap2-2;Ag-1;Bel1-1
Ap2-2	-			
Ap2-2;Bel1-1	P<0.01	-		
Ap2-2;Ag-1	same	same[e]	-	
Ap2-2;Ag-1;Bel1-1	same	P<0.01	same	-

Primordial outgrowths				
	Ap2-2	Ap2-2;Bel1-1	Ap2-2;Ag-1	Ap2-2;Ag-1;Bel1-1
Ap2-2	-			
Ap2-2;Bel1-1	same	-		
Ap2-2;Ag-1	P<0.01	P<0.01		
Ap2-2;Ag-1;Bel1-1	P<0.01	P<0.01	same	-

[a]Outer whorls only.
[b]Total number of ovules per flower significantly smaller for Ap2-2;Bel1-1 and Ap2-2;Ag-1 versus Ap2-2, rest not significantly different (data not shown).
[c]No mature or Bel1 ovules were found on Ap2-2;Ag-1;Bel1-1 carpel-sepals, thus the comparisons amongst the other three mutants were done by ANOVA and Tukey test and the comparisons with Ap2-2;Ag-1;Bel1-1 were done through comparing the mean and standard deviation.
[d]Any comparison where P>0.01.
[e]P<0.05.

immature, and primordial outgrowths), but there were two to three times fewer ovules present per carpel-sepal (average 8 for Ap2-6;Ag-1, versus 20 for Ap2-6). This decrease in the number of primordia initiated from the placental tissue compared with Ap2 carpel-sepals suggests that *AG* is involved in the initiation of ovule primordia. In addition, when compared with Ap2 carpel-sepals, the proportion of mature ovules was lower (36% in Ap2-6;Ag-1 compared with 55% in Ap2-6), while the number of primordial outgrowths was larger (57% instead of 40%) (Table 3.3a). There were a similar number of immature ovules. Statistical analyses support these results as there was a significant decrease in the number of mature ovules, especially when a partial loss of function of *AG* in Ap2 carpel-sepals is considered, leading to a proportional increase in the number of immature ovules and primordial outgrowths (Table 3.5). Clearing confirmed that the mature and immature ovules were histologically normal and most contained mature or developing embryo sacs, respectively (89%; 55 ovules examined) (Figure 3.3b). The lower percentage of mature ovules coupled with an increase in the proportion of immature ovules and primordial outgrowths indicates that *AG* plays a role in ovule morphogenesis following initiation of ovule primordia (Figure 3.6).

While the carpel-sepals of both Ap2;Bel1 and Ap2;Ag double mutants had a lower percentage of mature ovules and a higher percentage of primordial outgrowths compared with Ap2, the two double mutant phenotypes were distinct (Figure 3.4). The mature ovules of the Ap2;Bel1 double mutant are all Bel1-like while those of Ap2;Ag double mutants have a wild type morphology. In addition, the Ap2;Bel1 double mutants have a much higher ratio of immature to mature ovules compared to that of the Ap2;Ag double mutants. The differing effects of the loss of *AG* and *BEL1* suggest that, while both *AG* and *BEL1* play roles in ovule development, they are working at different times and influence ovule development to different extents (Figure 3.6).

3.3.5 The ovules of Ap2;Ag;Bel1 triple mutants

To further investigate the roles of *AG* and *BEL1* in ovule development and to determine if there are any interactions between these two genes, Ap2;Ag;Bel1 triple mutants were constructed and the ovule phenotype compared to that of Ap2;Bel1 and Ap2;Ag double mutants. The floral phenotype of the triple mutants was similar to that of Ap2;Ag double mutants and molecular methods were required for their identification (see Chapter 2).

SEM studies of the ovule types on the carpel-sepals of the triple mutants revealed all the ovule types found on Ap2 and Ap2;Ag carpel-sepals although the relative proportions of ovule types changed (Table 3.3a, Figure 3.4, Table 3.5). Ap2-6;Ag-1;Bel1-3 mature ovules with a wild type morphology were examined more closely by clearing in organic solvents. When examined in this way (Figure 3.3d) it was clear that the triple mutant mature ovules were not entirely normal. The outer integument did not always grow to cover the inner integument to the same extent as seen in normal ovules and the majority of mature and immature ovules of the triple mutant lacked mature or developing embryo sacs (80%, 113 ovules were examined).

The ovule phenotypes of Ap2;Ag;Bel1 and Ap2;Ag carpel-sepals were compared to yield information about the role of the *BEL1* gene. The proportion of mature ovules with a wild type morphology was significantly lower in Ap2-6;Ag-1;Bel1-3 (1%) compared with Ap2-6;Ag-1 (36%). In addition, the percentage of primordial outgrowths was significantly higher (88% instead of 57%) and there were more immature ovules (Figure 3.4; Tables 3.3a, 3.5). The increase in the severity of the Ap2;Ag phenotype suggests that *BEL1* may be working in a partially redundant manner with *AG* to establish ovule identity following the initiation of ovule primordia as well as to promote integument development. The lack of an embryo sac in ovules of the Ap2;Ag;Bel1 triple mutant was similar to the mature ovule phenotype of Bel1 and Ap2;Bel1 mutants (Figure 3.3c,d), but unlike

those of Ag;Ap2 mature ovules (Figure 3.3b), suggesting that *BEL1* but not *AG* gene function is required for normal embryo sac development independent of development of the integuments (Figure 3.6).

The ovule phenotypes of Ap2-6;Ag-1;Bel1-3 and Ap2;Bel1 carpel-sepals were compared to yield information about the role of *AG*. The Ap2;Bel1 double mutant had a significantly lower percentage of primordial outgrowths (62% compared with 88%; Figure 3.4; Table 3.5) relative to the triple mutant. These data are consistent with the idea that *AG* is required to establish ovule identity. In addition, it is important to note that the Bel1 ovules of the Ap2;Bel1 double mutant were lacking in the triple mutant while the mature ovules with a wild type morphology of the triple mutant were not present in the Ap2;Bel1 double mutant. These data suggest that in the absence of *BEL1* activity, *AG* activity is detrimental to the development of normal integuments by promoting the formation of mature Bel1 ovules at the expense of a more normal pattern of development.

3.4 Discussion

3.4.1 Diverse ovule phenotypes suggest multiple defects in ovule development

The large variety of ovule phenotypes observed on Ap2, Ag, Bel1 single, double and triple mutants suggests that mutations in these genes can result in defects in several stages of ovule development as shown in Figure 3.6. As well, the decrease in the number of primordia initiated from the placental tissue of mutants homozygous for *ag* suggests a defect in the initiation of ovule primordia.

Both types of primordial outgrowths, apart from their position on the placenta, had no ovule features except for an enlarged cell resembling a megaspore in a few projection ovules. More

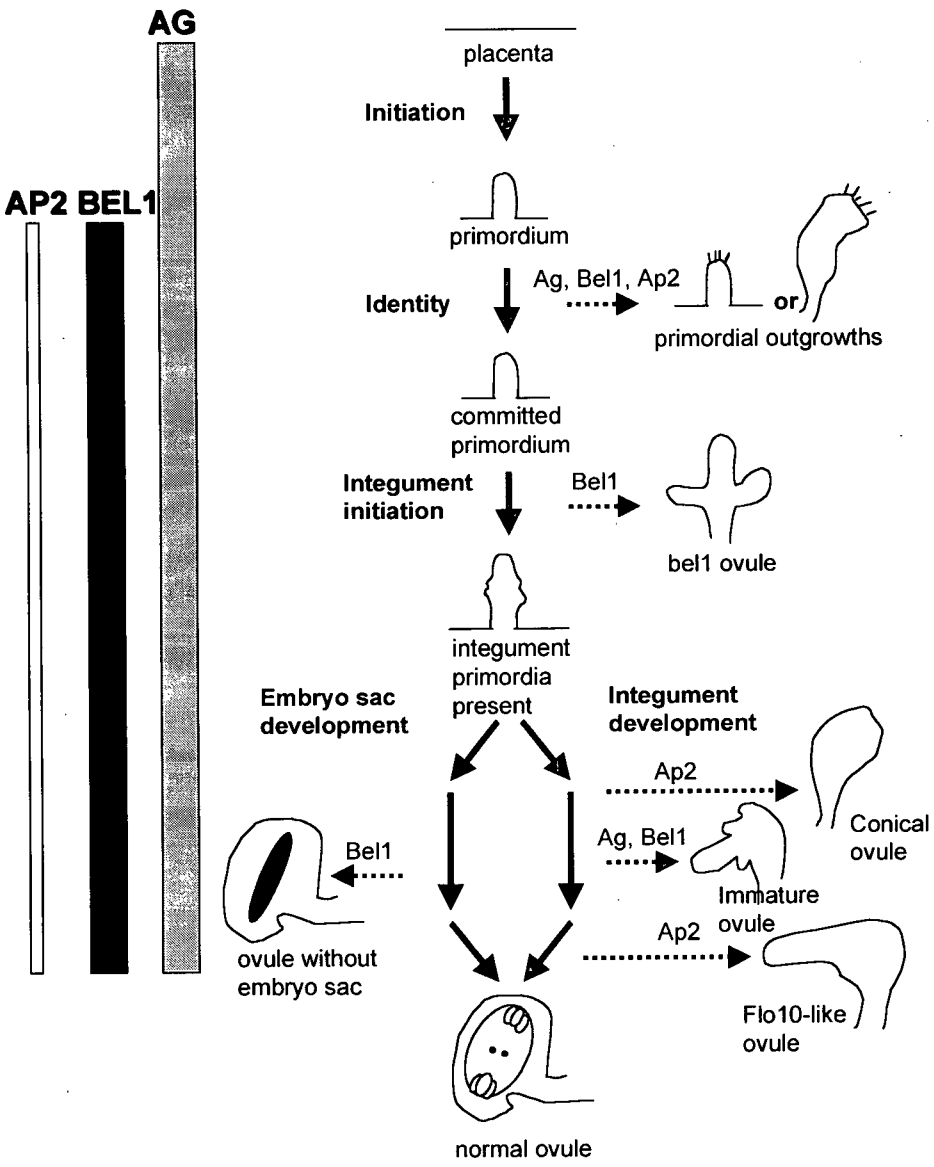


Figure 3.6. Model showing the timing and roles of *AG*, *BEL1* throughout ovule development and the effects of their absence.

The major stages of *Arabidopsis* ovule development are shown as a linear pathway from placenta to mature ovule. In bold are the processes occurring in between each of these stages. At the far left are bars showing the timing of activity of *AG* (grey), *BEL1* (black), and *AP2* (white) – the thinness of the *AP2* bar indicates its minor role. Our results suggest that *AG* is involved in ovule development from the time of ovule initiation throughout integument development, while both *BEL1* and *AP2* act from shortly after ovule inititation until maturity, including embryo sac development. *AG* appears to have a greater role early in development, while *BEL1* has a stronger role at the later stages; *AP2* appears to act throughout development. To the right and left of the developmental progression are diagrams of the various ovule types found when these genes are mutated, placed at the stage of ovule development at which they deviate from the norm.

frequently, the projection and planar ovules showed carpel characteristics including stigmatic papillae and ovary cells. Thus the primordial outgrowths could be considered to be the result of a defect in the establishment of ovule identity following the initiation of primordia from the placenta (Figure 3.6). This hypothesis is consistent with the data and conclusions of Evans and Malmberg (1989) who cultured wild type tobacco placentas at different stages of carpel development. Culturing placentas bearing ovule primordia that had not yet undergone ovule specific morphogenesis resulted in the primordia adopting a carpel-like fate.

Bel1 ovules appear to have committed to an ovule fate but were unable to elaborate the normal pattern of integument initiation and morphogenesis. Finally, the mature ovules lacking embryo sacs, the immature ovules with underdeveloped integuments, and Flo10 ovules appear to result from defects in growth late in ovule development.

3.4.2 *AP2* may stabilize ovule fate throughout ovule development

AP2 negatively regulates *AG* in the flower, preventing the production of carpels and other reproductive structures in the outer whorls. In Ap2 mutants, the outer whorl carpel-sepals, which result from ectopic activity of *AG* and other reproductive genes (e.g. *CRC* and *SPT*; Alvarez and Smyth, 1997), display abnormal ovules which also could be due to the aberrant activity of these genes (Bowman *et al.*, 1989; Drews *et al.*, 1991; Bowman *et al.*, 1991). Similar carpel-sepals revealing even greater proportions of abnormal ovules, however, are seen on flowers lacking *AG* (Ap2; Ag), suggesting that the milder ovule phenotype of Ap2 carpel-sepals is also the result of a reduction in *AG* activity. Indeed, in Ap2 mutants, complete transformation of the sepals to carpels has never been observed suggesting that expression of the carpel program in the outer whorls is incomplete.

Thus, in the outer whorl, *AP2* appears to affect ovule development indirectly through its regulation of *AG*.

In Ap2 mutants, planar ovules and long filaments of cells are seen at a low frequency in the gynoecium. While the appearance of abnormal ovules in the gynoecium where activity of reproductive genes is likely to be normal suggests that *AP2* does play a role in ovule development, their infrequent appearance implies that it is minor one. The carpel-like phenotype of a large proportion of the abnormalities suggests that *AP2* affects ovule fate, but since the abnormal ovules are often found at the base of the gynoecium where ovules first initiate, *AP2* may be acting, not to activate ovule fate, but to sharpen the switch from carpel to ovule development. This activity as a maintainer of ovule development could also explain the sporadic appearance of other ovule abnormalities arising at several different points during ovule development (Figure 3.6). A role for *AP2* in ovule development is supported by its expression in the ovule (Jofuku *et al.*, 1994). Conversely, analogous to the outer whorls, the role of *AP2* in ovule development may be an indirect one, as it is required to influence the expression of *AG* and other, independent carpel genes at the time of ovule development.

3.4.3 *AG* controls ovule development

Beyond its role in determining carpel identity, *AG* has not been considered a direct player in ovule development since normal ovules can develop in its absence (Bowman *et al.*, 1991) and its function can be detrimental to ovule development (Ray *et al.*, 1994). In contrast, my results suggest that *AG* acts throughout ovule development promoting ovule initiation, identity, and integument morphogenesis (Figure 3.6). The role of AG function during ovule development is not necessarily a direct one. Ovule defects observed in an Ap2; Ag background could be an indirect result of the

plant's inability to develop a normal gynoecium. However, the *AG* gene is expressed in the placental tissue prior to ovule initiation (Bowman *et al.*, 1991) and in the developing ovule primordium in a pattern similar to that of *BEL1* (Reiser *et al.*, 1995), as expected for a gene playing a direct role in ovule initiation, identity, and integument morphogenesis. In addition, the dependency of normal ovule development on the level of *AG* activity relative to that of *BEL1* (Figure 3.4; see also the discussion below; Ray *et al.*, 1994) is consistent with the idea that *AG* functions in the developing ovule. Taken together, these data suggest that the role of AG in ovule development is direct.

3.4.4 *BEL1* and *AG* interact to control ovule development

Previous studies of the Bel1 phenotype have suggested that the gene is required for ovule integument morphogenesis (Robinson-Beers *et al.*, 1992; Ray *et al.*, 1994; Modrusan *et al.*, 1994a; Reiser *et al.*, 1995). My data confirm and extend these conclusions. I have shown that the distinctive Bel1 integument morphology observed in Bel1 loss-of-function mutants is dependent on *AG* activity. A similar phenotype was observed in transgenic *Arabidopsis* lines where *B. napus AG* gene was over-expressed (Ray *et al.*, 1994). Thus excess *AG* activity appears to have a dominant negative effect on integument development. Taken together these results suggest that the relative ratio of *BEL1* to *AG* activities is critical for proper development of the integuments. What could be the role of such BEL1-AG interactions in integument development? As has already been suggested (Ray *et al.*, 1994; Modrusan *et al.*, 1994a; Reiser *et al.*, 1995), BEL1 may act as a negative regulator of *AG* function during integument development, with *AG* serving no role at that stage of ovule development. Even if this hypothesis is correct, however, the negative regulation of *AG* cannot be the only role of *BEL1* in integument development since most Bel1 integuments do not develop

normally even in the absence of *AG*. An alternative hypothesis is that *AG* has an active role in integument development but requires *BEL1* activity to function correctly at that stage.

Both *AG* and *BEL1* gene products are putative transcription factors (AG = MADS domain protein; BEL1 = homeodomain protein) that are transcribed in the young ovule primordium and later more specifically in the integument primordia (Yanofsky *et al.*, 1990; Reiser *et al.*, 1995). Thus any interactions that occur between the two genes must be post-transcriptional (Reiser *et al.*, 1995). The simplest model for *AG* and *BEL1* interaction would be via formation of a heterodimeric protein complex. Indeed interactions between MADS and homeodomain proteins have been documented in yeast (Vershon and Johnson, 1993). Independent yeast two-hybrid cDNA library screens for proteins which directly interact with either AG (S. Kohalmi and B. Crosby, personal communication) or BEL1 (A. Samach, Z. Modrusan, S. Kohalmi, B. Crosby, and G. Haughn, unpublished results) have failed to identify BEL1 and AG proteins as interacting partners. An alternative hypothesis for interaction is suggested by the ability of AG and its *Antirrhinum* orthologous counterpart PLENA to interact with other MADS box proteins (AGLs in *Arabidopsis*) which are expressed in the ovules (Huang *et al.*, 1996; Davies *et al.*, 1996; Fan *et al.*, 1997; S. Kohalmi, and B. Crosby, unpublished results). The BEL1 protein could activate transcription of an *AGL* gene (*AGLX*) whose protein product could in turn interact with AG (Figure 3.7b). In the event of a high AG/AGLX protein ratio, AG may function as a homodimer or with alternative partners to disrupt development in a dominant negative fashion.

3.4.5 *AG* and *BEL1* act in parallel to control ovule identity

In addition to its roles in integument and embryo sac development, my results suggest that *BEL1*, like *AG*, has a role in the specification of ovule identity in the early stages of ovule

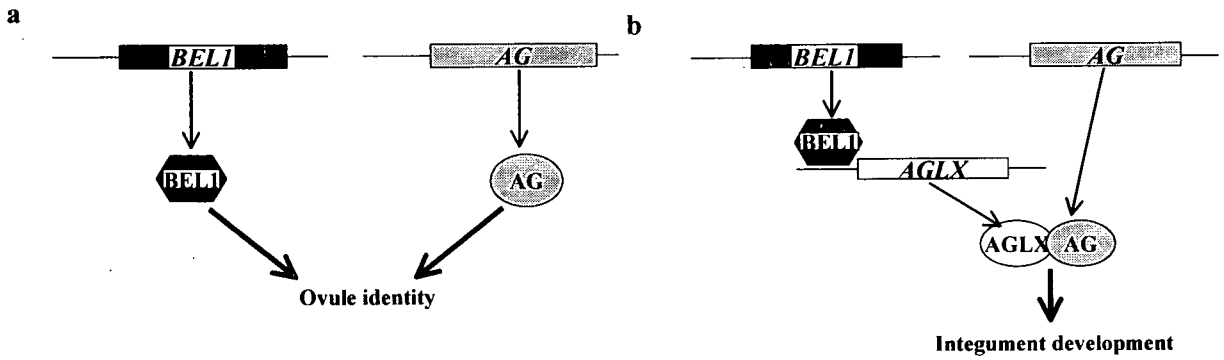


Figure 3.7. A molecular model for the interaction between *AG* and *BEL1*.
(a) Early ovule development. The transcription factors encoded by *AG* and *BEL1* work in parallel to specify ovule identity.
(b) Late ovule development. The *BEL1* protein product activates transcription in an *AGL* gene (*AGLX*) whose protein product could in turn interact with AG as an AG-AGLX heterodimer in order to regulate integument development.

development (Figure 3.4 and Tables 3.3 and 3.5, compare Ap2;Ag and Ap2;Ag;Bel1 phenotypes), a hypothesis supported by the presence of *BEL1* transcript throughout the newly initiated ovule primordium (Reiser *et al.*, 1995). This role could only be observed in the absence of *AG* function, suggesting that *AG* activity is sufficient to promote ovule identity even in the absence of *BEL1* function (e.g. in Bel1 or Ap2;Bel1 mutants). In contrast, the primordial outgrowths among Ap2;Ag ovules suggests that *BEL1* activity alone is insufficient to promote identity in all developing ovules.

A comparison of the ovule types found on Ap2 carpels and the Ap2;Ag and Ap2;Ag;Bel1 double and triple mutant carpel-sepals suggests that *AG* and *BEL1* promote ovule identity independently of one another. Thus with respect to ovule identity, *AG* and *BEL1* could be considered to function in parallel pathways (Figure 3.7a).

3.4.6 *BEL1* and *AG* have overlapping but nonidentical roles in ovule development

Figure 3.6 illustrates the relative roles of *BEL1* and *AG* in ovule development. Both *AG* and *BEL1* control ovule identity and integument development, with AG playing the dominant role in ovule identity and BEL1 having a stronger effect on integument development. In addition, *AG* appears to be uniquely involved in initiation while *BEL1* is uniquely involved in embryo sac development. Thus *AG* exerts its major influence early in ovule development while *BEL1* does so at later stages.

3.4.7 Multiple genes control ovule fate

My results suggest that *AG, BEL1*, and *AP2*, influence the specification of ovule fate. The control of ovule fate has parallels with the process controlling shoot fate in the *Arabidopsis* inflorescence (the Floral Initiation Process or FLIP; Schultz and Haughn, 1993; Bowman *et al.*, 1993; Shannon and Meeks-Wagner, 1993; Haughn *et al.*, 1995). Both processes are controlled via the activity of several genes working together in a partially redundant manner to specify an important developmental switch. In addition, individual genes exert different degrees of control and have roles beyond the determination of ovule or meristem identity.

The rare appearance of mature ovules in the absence of *AG, BEL1*, and *AP2* activities suggests that there is at least one additional gene that functions in a redundant manner to specify ovule identity. Another gene known to act in the determination of ovule identity is the *P. hybrida* MADS-box gene *FBP11* (Colombo *et al.*, 1995; Angenent *et al.*, 1995). Cosuppression of *FBP11* and closely-related *FBP7* resulted in large numbers of carpel-like structures in place of ovules, while ectopic expression of *FBP11* alone led to the formation of ovules on sepals and petals. The

cosuppression phenotype was similar to that resulting from the absence of *AG* and/or *BEL1* in an Ap2 background. Thus an *Arabidopsis* gene orthologous to *FBP11* could be a fourth gene acting to promote ovule identity. One candidate for an *FBP11* gene in *Arabidopsis* is *AGL11*, an *AG*-like MADS box gene that is specifically expressed in the ovules and surrounding placental tissue (Rounsley *et al.*, 1995; Angenent and Colombo, 1996).

4. MUCILAGE PRODUCTION IN THE SEED COAT OF *ARABIDOPSIS*

4.1 Introduction

Cell walls are extremely important for the function of plant cells. In order to play their multiple roles, cell walls are complex, made up of cellulose, hemicelluloses, pectins, proteins, and water. While the polysaccharides of plant cell walls have been well characterized chemically and their production has been studied using antibodies and isolation of cell fractions, very few genes for biosynthetic enzymes have been isolated. Recently, several screening strategies have been initiated in an attempt to isolate genes involved in the production of cell wall polysaccharides.

Many plants deposit pectinaceous mucilage in the seed epidermis during seed development. These mucilage cells synthesize and secrete large amounts of pectin, which is deposited between the cytoplasm and the outer cell wall. I have exploited the presence of seed mucilage in the plant model system *Arabidopsis* in a novel strategy to search for mutant plants defective in pectin production and/or secretion. Prior to using mutants to dissect mucilage synthesis and secretion, it is important to understand the timing and cell biology of its production in wild type *Arabidopsis* seeds. In this chapter I describe the development of the seed coat in *Arabidopsis*, focussing on the histology and cell biology of mucilage-producing epidermal cells. In addition, I present the sugar composition and staining characteristics of *Arabidopsis* mucilage.

4.2 The *Arabidopsis* seed coat

4.2.1 Mucilage of the mature seed

The mature *Arabidopsis* seed of the Col-2 ecotype is small, reddish-brown, and roughly 500 μm in length. SEM studies revealed that the seeds are covered by roughly isodiametric, hexagonal cells approximately 35 μm across (Figure 4.1a). The cells have thickened radial cell walls and in the center of each cell is a raised plateau known as the columella. The area enclosed by the thickened cell walls surrounding the columella consists of a depression, presumably due to the dehydration of the seed.

When *Arabidopsis* seeds are placed in water, a gel-like capsule of mucilage appeared within seconds to envelop the whole seed. When placed in a 0.01% solution of Ruthenium red, a dye which stains acidic polysaccharides (and normally thought to be specific to pectin) (Frey-Wyssling, 1976), seeds had a pink-stained capsule with two distinct layers (Figure 4.1c). The outer layer was quite cloudy and diffuse, extending outwards from the seed surface approximately a seed width, while the inner capsule resembled a bright pink halo directly around the seed. If seeds were shaken in water before staining in Ruthenium red, the outer layer was absent (Figure 4.1d). Upon closer examination, the inner capsule appeared to have rays radiating out from the columellae of the cells below (Figure 4.1d). Ruthenium red not only stained mucilage around the seed, but also highlighted the thickened radial cell walls and the columellae in bright red.

When wetted seeds were air dried and then examined using SEM, the seeds appeared to sit in a pool of white film, presumably dried mucilage, and the tips of the columellae were often coated with a small glob of similar white film, also presumably mucilage (data not shown). In addition, the outer tangential wall appeared to be missing, as the depression around the columella appeared to be

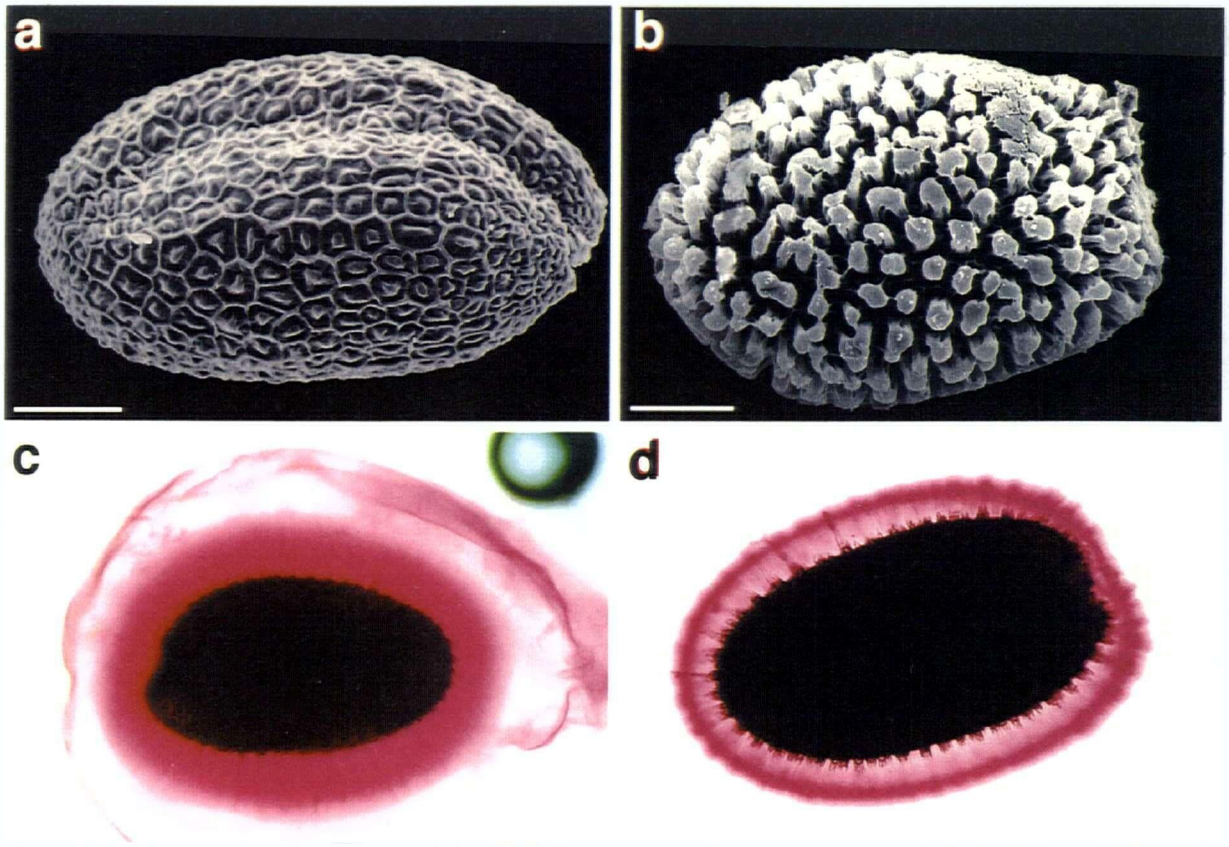


Figure 4.1. Mature wild type seeds.
(a) SEM of dry seed. Note the hexagonal epidermal cells with thickened radial cell walls and columellae in the centre.
(b) SEM of seed that has been fixed in aqueous glutaraldehyde and critical point dried. Spikes of mucilage arise from each cell.
(c) Seed stained in Ruthenium red without agitation. Two layers of mucilage are present, an outer cloudy layer and a darkly staining inner capsule.
(d) Seed stained in Ruthenium red after first shaking in water, only the inner capsule is present. Note the "rays" radiating from each columella.
Magnification bars: (a),(b)=100 μm, (c),(d)= 75x.

much sharper and no wrinkled cell wall was present over from thickened radial cell walls. This suggests that the release of mucilage correlated with rupturing of the cell wall at the time of seed hydration. When seeds were fixed in an aqueous solution of 3% glutaraldehyde, dehydrated through an ethanol series, then critical point dried, the mucilage, instead of forming a pool beneath the seed, formed solid white spikes radiating out from the columellae, similar to the rays observed with

Ruthenium red staining (compare Figure4.1b and d). Aldehyde fixatives are known to crosslink proteins (O'Brien and McCully, 1981), thus this result suggests that protein may be found in the mucilage. A control experiment was done using seeds that were first hydrated in water, then dehydrated through an ethanol series and critical point dried. These seeds had reduced spikes, suggesting that the retention of the spike may be at least partially due to keeping the seeds in liquid until instantaneous drying.

The nature of both the mucilage and the rays were further investigated in collaboration with an Honours student, Yeen Ting Hwang. To confirm that the mucilage was pectin, seeds were treated with pectinase, an enzyme which acts as an endo-polygalacturonase to produce soluble oligogalacturonic acids (Frey-Wyssling, 1976). Treated seeds were stained with Ruthenium red and were found to be missing both mucilage layers, suggesting that they, indeed, were pectin. The fibrous rays, however, were still present. The rays may be remnants of the outer tangential cell wall still attached to the columella. To test this hypothesis, seeds were treated with cellulase, an endo-β-1,4-glucanase, which digests cellulose. Cellulase-treated seeds were found to not only be missing the rays, but also both layers of mucilage. These results suggest that the rays have a cellulose component and could be the remains of the cell wall, and that the mucilage attachment is dependent on cellulose.

4.2.2 Chemical analysis of *Arabidopsis* mucilage

Enzymatic analysis with pectinase and specific staining of acidic polysaccharides by Ruthenium red and Toluidine blue suggested that *Arabidopsis* seed mucilage is largely composed of pectin. Chemical analysis of *Arabidopsis* mucilage has been performed by Goto (1985) for the Sendai

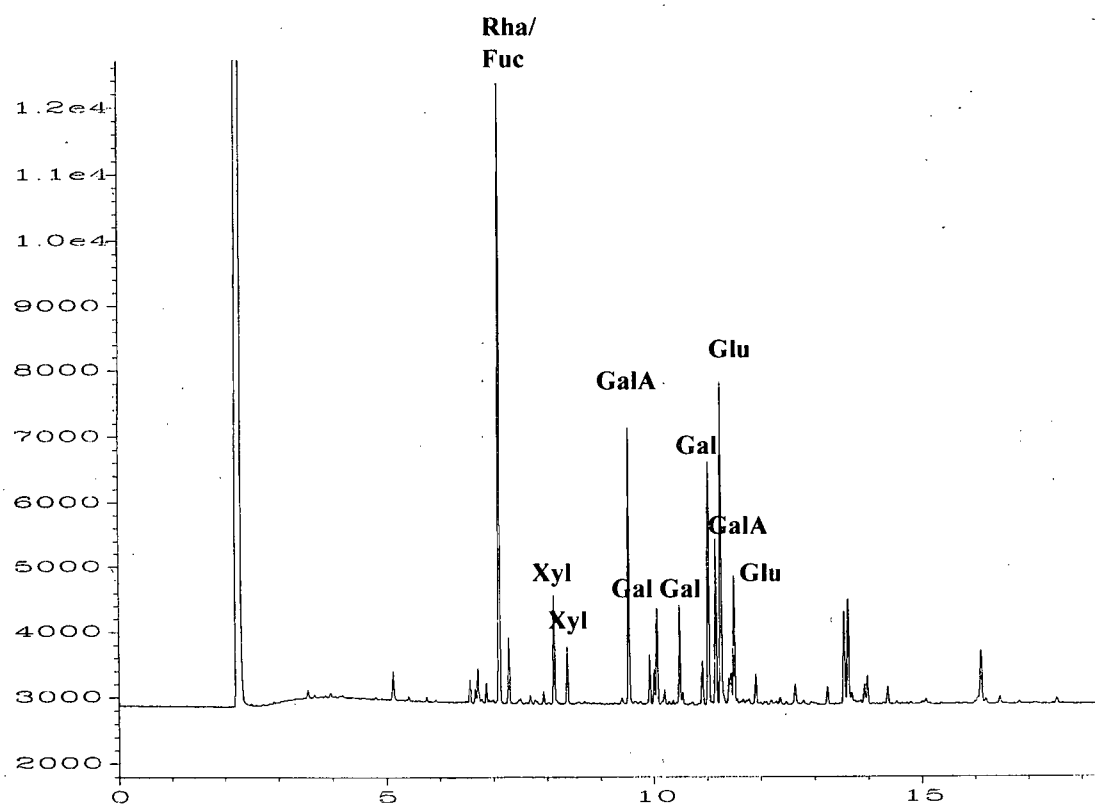


Figure 4.2. A sample gas chromatogram showing common peaks found in isolated mucilage. Bottom axis represents retention time on column in minutes. Rha=rhamnose, Fuc=fucose, Xyl=xylose, GalA=galacturonic acid, Gal=galactose, Glu=glucose.

ecotype, showing that it is a pectic substance made up of uronic acids and neutral monosaccharides. In order to confirm the composition of Col-2 mucilage, and for comparison to mutants, the monosaccharide composition of wild type mucilage was determined using gas chromatography (GC) of trimethylsilyl ethers (Chaplin, 1986). Mucilage was isolated from seeds by shaking in ammonium oxalate (Goto, 1985) and, after derivatization, was subjected to GC. The resulting peaks were initially identified through comparison to the retention times obtained with individual sugar standards, and then confirmed through gas chromatography-mass spectrometry (GC-MS) analysis to identify the components in individual peaks. A consistent sugar profile was obtained for Col-2 mucilage which revealed quantities of rhamnose and galacturonic acid, the major components of the

Table 4.1. Retention times, monosaccharide assignments, and amount of sugar in the major peaks of Col-2 mucilage.

Columbia mucilage		ug/100 seeds[a]	
Retention time	Sugar	Average	Standard deviation
7.09	Rhamnose, Fucose[b]	3.89	0.48
9.51	Galacturonic acid	2.37	0.36
10.04	Galactose	0.85	0.12
11.00	Galactose	2.26	0.32
11.14	Galacturonic acid?	1.36	0.21
11.24	Glucose	0.66	0.20
11.48	Glucose	0.23	0.12
13.52	Octadecadienoic acid	0.17	0.37
13.60	?	0.55	0.36
13.79	?	3.41	2.57
13.99	Inositol[c]	48.00	0.00
14.36	?	1.15	0.04
14.54	?	0.74	0.05
16.09	?	0.34	0.29

[a]5 samples
[b]Rhamnose and fucose could not be separated under our conditions.
[c]Internal standard

Table 4.2. Retention times, monosaccharide assignments, and amount of sugar in the major peaks of whole Col-2 seeds.

Col Whole Seed		ug/100 seeds[a]	
Retention time	Sugar	Average	Standard deviation
6.66	Ribose	2.15	0.82
6.72	Arabinose, Ribose[b]	6.40	2.17
6.87	Arabinose	3.45	1.03
7.11	Rhamnose, Fucose[b]	9.04	2.62
7.29	Rhamnose, Arabinose[b]	2.00	0.55
8.14	Xylose	3.17	0.74
8.38	Xylose	1.79	0.40
9.54	Galacturonic acid	3.94	0.40
9.92	Mannose	1.40	0.16
10.02	Galactose	3.76	0.71
10.06	Galacturonic acid	1.72	0.16
10.48	Galactose, Mannose[b]	11.66	1.80
10.92	Galactose	5.10	0.75
11.03	Galactose	4.22	0.31
11.17	Galacturonic acid?	3.71	1.68
11.27	Glucose	19.11	2.08
11.42	?	2.52	0.19
11.51	Glucose	7.76	0.97
13.55	Octadecadienoic acid	8.92	2.44
13.63	?	10.76	1.57
13.82	?	6.23	6.19
14.00	Inositol[c]	48.00	0.00
16.12	?	4.92	1.60

[a]4 samples
[b]These sugars could not be separated under our conditions.
[c]Internal standard

pectins rhamnogalacturonan I and polygalacturonic acid (Figure 4.2). Other neutral monosaccharides, including glucose and fucose were also found. Monosacccharide analysis was also carried out for whole seeds, yielding an even wider spectrum of neutral sugars. The five replicates for isolated mucilage and four replicates for whole seeds showed reproducible profiles that would allow comparison to mutants (Tables 4.1 and 4.2).

4.2.3 The mature seed coat

The seed coat of *Arabidopsis* has been briefly described by Vaughan and Whitehouse (1971) in their survey of the Brassicaceae. As the first step of studying seed coat development, mature seeds of Col-2 ecotype were embedded in plastic and thick sections were stained with Toluidine blue O, a polychromatic dye which stains different cell components different colours (O'Brien *et al.*, 1964). Similar to other Brassica species and to the observations of Vaughan and Whitehouse (1971), the mature *Arabidopsis* seed coat consists of three discernible cell layers: the epidermal[1], palisade, and pigmented layers (Figure 4.3a). The epidermal layer, when viewed in seeds that had been fixed in a non-aqueous fixative (FAA), showed 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 the cell which stained similar to the cell walls (Figure 4.3a,c). The outer cell wall appeared to be draped over the columellae and between the columella and the radial cell walls was pink-staining acidic polysaccharide – mucilage. In cells which have been fixed with an aqueous solution of 3% glutaraldehyde, the inner cell wall thickenings and the columellae were still present, but the polysaccharide was absent and the outer cell wall appeared to have ruptured, with cell wall remnants attached to the top of the columella (Figure4.3b,d). The next cell layer was the palisade layer that

[1] Throughout this thesis I shall refer to outermost layer of the seed coat as the seed epidermis or epidermal layer.

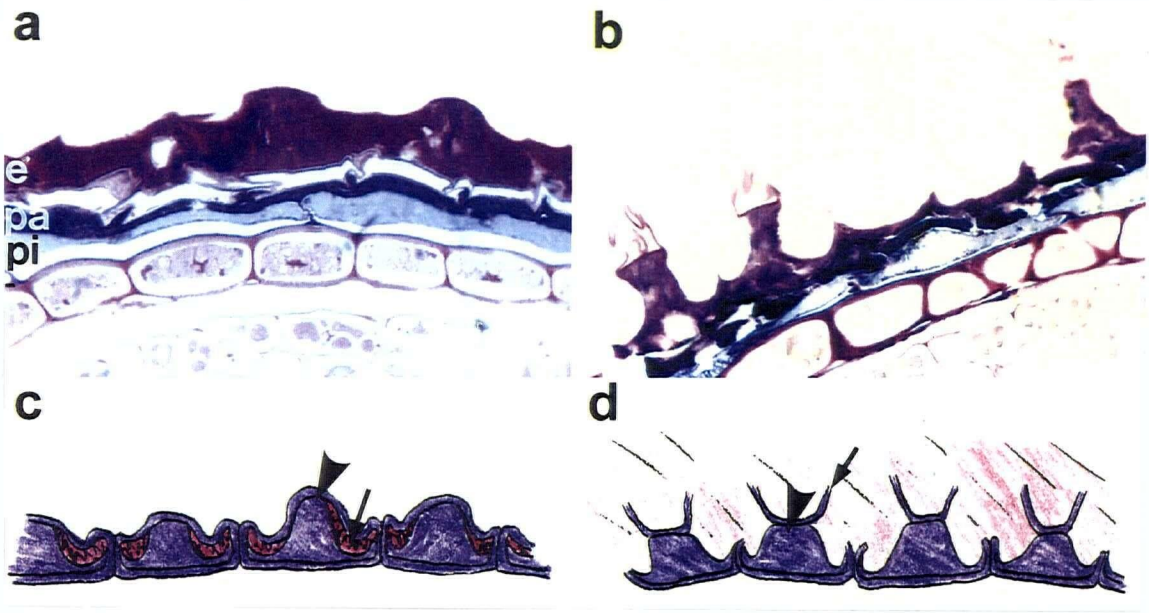


Figure 4.3. Cross sections of mature wild type seeds stained with Toluidine blue.

(a) The seed coat when fixed under non-aqueous conditions. Mucilage is retained in the epidermal layer (e), surrounding the columella. 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. (680x)
(b) The seed coat when 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 columellae. (700x)
(c) Cartoon of epidermal layer shown in (a). A columella is indicated by an arrowhead, while the mucilage is shown in pink (arrow).
(d) Cartoon of epidermal layer shown in (b). A columella is indicated by an arrowhead, while the mucilage is shown in pink. The remnants of the outer cell wall are attached to the columellae (arrow).

had thickened inner tangential cell walls that stained blue, suggesting the presence of lignin. The final cell layer that was evident was the pigmented layer, which stained the blue-green expected with polyphenols and contained the condensed tannins which give the seed its red-brown colour.

4.3 Seed development in *Arabidopsis*

4.3.1 Outline of seed development in *Arabidopsis*

Seed development has been studied by various groups interested in embryo development, using different ecotypes and growth conditions (Meinke and Sussex, 1979; Mansfield and Briarty, 1991a; Mansfield *et al.*, 1991b; Bowman *et al.*, 1994b). In order to correlate developmental events of 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 days at 20 to 22°C under continuous light. Progress during the seed development was followed by using clearing in organic solvents to determine the size, color, and stage of embryo development for seeds of each day after fertilization from the time of pollination to the completion of seed development.

Seed growth from a pollinated ovule to a seed of the mature size of 500 μm long took approximately five days. At this point, the embryo was in the heart-stage and was starting to produce chlorophyll, giving the seed a green appearance. The embryo cotyledons reached their full size by approximately 12 daf (days after fertilization), following which embryo filling 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, desiccation commenced at approximately 16 daf and continued for another day or two until the seed was dry (see Figure 4.9).

4.3.2 Palisade and pigmented layer development

The *Arabidopsis* ovule has two integuments—the outer integument consists of two cell layers, while the inner integument has three cell layers (Schneitz *et al.*, 1995). By contrast, the seed coat that develops from the integuments has only three cell layers (Figure 4.3). During seed coat development, the outer layer of the outer integument differentiates into the seed epidermis while the inner layer of the outer integument becomes the palisade layer. The uppermost two layers of the inner integument are crushed and the inner layer of the inner integument, the endothelium, develops into the pigmented layer. In this first section I will briefly discuss the development of the palisade and pigmented layers, following which I will describe the development of the epidermal cell layer, which is the focus of my studies.

The palisade layer of the seed coat develops from the inner layer of the outer integument, which is the cell layer directly below the epidermis. The outstanding feature of the palisade layer is that it has a thickened inner tangential cell wall that is reinforced with lignin (Figure 4.3a,b). At 0 daf the plastic sectioned cells stained with Toluidine blue had a similar appearance to those of the epidermal layer with a well-defined cytoplasm and some vacuole (Figure 4.4a). During the period of cell growth the major change was the enlargement of the vacuole and delimitation of the cytoplasm to the edges of the cell. Similar to the epidermal cells, globules started to accumulate at 3 daf and continued until a peak at 7 daf (Figure 4.4b,c). At 8 daf thickenings in the lower tangential cell wall started to become obvious and this thickening continued until approximately 13 daf. At first, the thickenings stained a similar color to the primary cell walls around them and the new cell walls in the epidermal layer, but by 12 daf the thickened lower tangential cell wall of the palisade layer appeared to take on a slightly different color, dark blue rather than purple (Figure 4.4 d-f), suggesting the presence of lignin (O'Brien, 1964). This may suggest that new cellulose was laid down first,

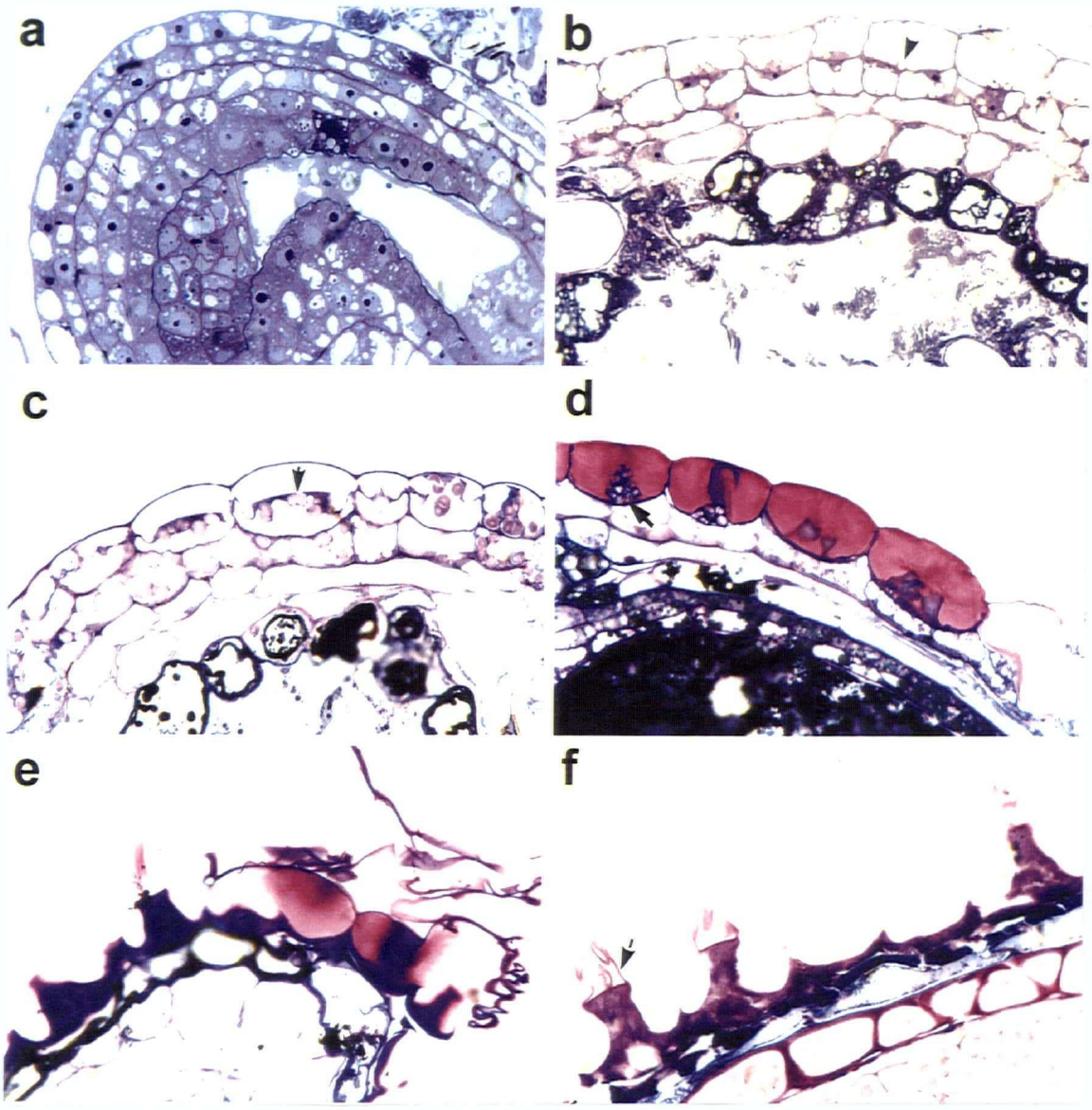


Figure 4.4. Wild type seed coat development.
Samples were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue.
(a) Mature ovule (0 days after fertilization [daf]). (750x)
(b) Seed at 4 daf. Note small globules (arrow) in cells. (540x)
(c) Seed at 7 daf. The globules (arrow) are larger and found in centre of the epidermal cells, which also have faint pink staining. (420x)
(d) Seed at 10 daf. Intense pink staining polysaccharide is in epidermal cells, globules are small and are found at the bottom of cell (arrowhead). Purple staining cell wall material can be seen in centre of some cells. Thickening of the inner tangential cell wall is also apparent in the palisade (sub-epidermal cell layer). (430x)
(e) Seed at 13 daf. 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 are completely filled in. (430x)

(f). Seed at 18 daf. 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 columellae. Directly below the base of the columellae are the dark blue, thickened cell walls of the palisade layer. The contents of the pigmented layer cells stains pale blue. (680x)

followed by the deposition of lignin. The change in staining was accompanied by a decrease in globule size, starting at 11 daf (see Figure 4.9).

The two outermost layers of the inner integument, similar to the epidermal and palisade layers, became more vacuolated during the growth phase of the seed coat. Very few or no globules, however, accumulated in these cells and by 10 daf the cells were beginning to be crushed and were not easily discernible by 13 daf, depending on the area of the seed observed (Figure 4.4). The fifth original layer of the integuments is the endothelium that becomes the pigmented layer of the seed coat. The endothelium is a cell layer that differentiates in the ovules very shortly before pollination and differs from the other layers of the integuments in that they are densely cytoplasmic (Schneitz *et al.*, 1995) (Figure 4.4a). This density of the cytoplasm was still apparent after pollination and during development of the seed coat. The staining properties of the endothelium during development were unusual, revealing osmiphilic (black-staining) and grainy contents of unknown origin (Figure 4.4b-d). This cell layer, however, remained intact. Similar to the epidermis and palisade layer, the cytoplasm appeared to degrade around 12 daf and nothing was seen until maturity when the contents of these cells stained blue-green (Figure 4.4f). The blue-green colour characteristic of polyphenols when stained with Toluidine blue is consistent with the presence of condensed tannins in this cell layer, as seen in other Brassicaceae (O'Brien *et al.*, 1964).

4.3.3 Epidermal cell development

Epidermal cell development was studied both externally using SEM and internally using approximately 0.2μm thick plastic sections stained with Toluidine blue. First I will describe the SEM data collected by both an Honours student, Debra Skinner, and me (Figure 4.5). During the first four to five days after fertilization the ovule underwent a shape change such that it became an elongated oval and grew rapidly in size from roughly 100 μm to 500 μm in length (Figure 4.5a,b). This growth was achieved through both cell division and cell expansion. Measurement of cell size and number versus total length of 10 ovules and 10 seeds revealed that approximately 25% of the length increase was due to cell division while 75% was due to an increase in cell elongation. As well, during the first three days the cells underwent a change in shape from elongated rectangles to hexagonal cells that were roughly isodiametric. Starting at 4 daf, globular structures were seen under the outer cell wall (Figure 4.5c). The globules increased in size and moved from throughout the cell into a ring in the center of the cell by day 6 (Figure 4.5d). At 8 or 9 daf columellae became apparent, first at the micropylar end of the seed, then over the whole seed by 10 days (Figure 4.5e). About the same time, the thickenings appeared on the radial cell walls. Mucilage was apparent surrounding the seed starting with a small amount at about 9 or 10 daf and more at later stages when seeds were fixed in 3% aqueous glutaraldehyde (Figure 4.5 f,g). The appearance of fixed seeds did not change after approximately 13 daf, and appeared more inflated than dry seeds at maturity due to the hydration in fixative.

The epidermal cells of the ovule, when viewed in section, had an obvious nucleus and cytoplasm, with a large vacuole occupying approximately half of the cell area (Figure 4.4a). As observed by SEM, there was a large increase in cell size during the first four days after fertilization which was reflected by the cells becoming almost completely filled by the vacuole with the

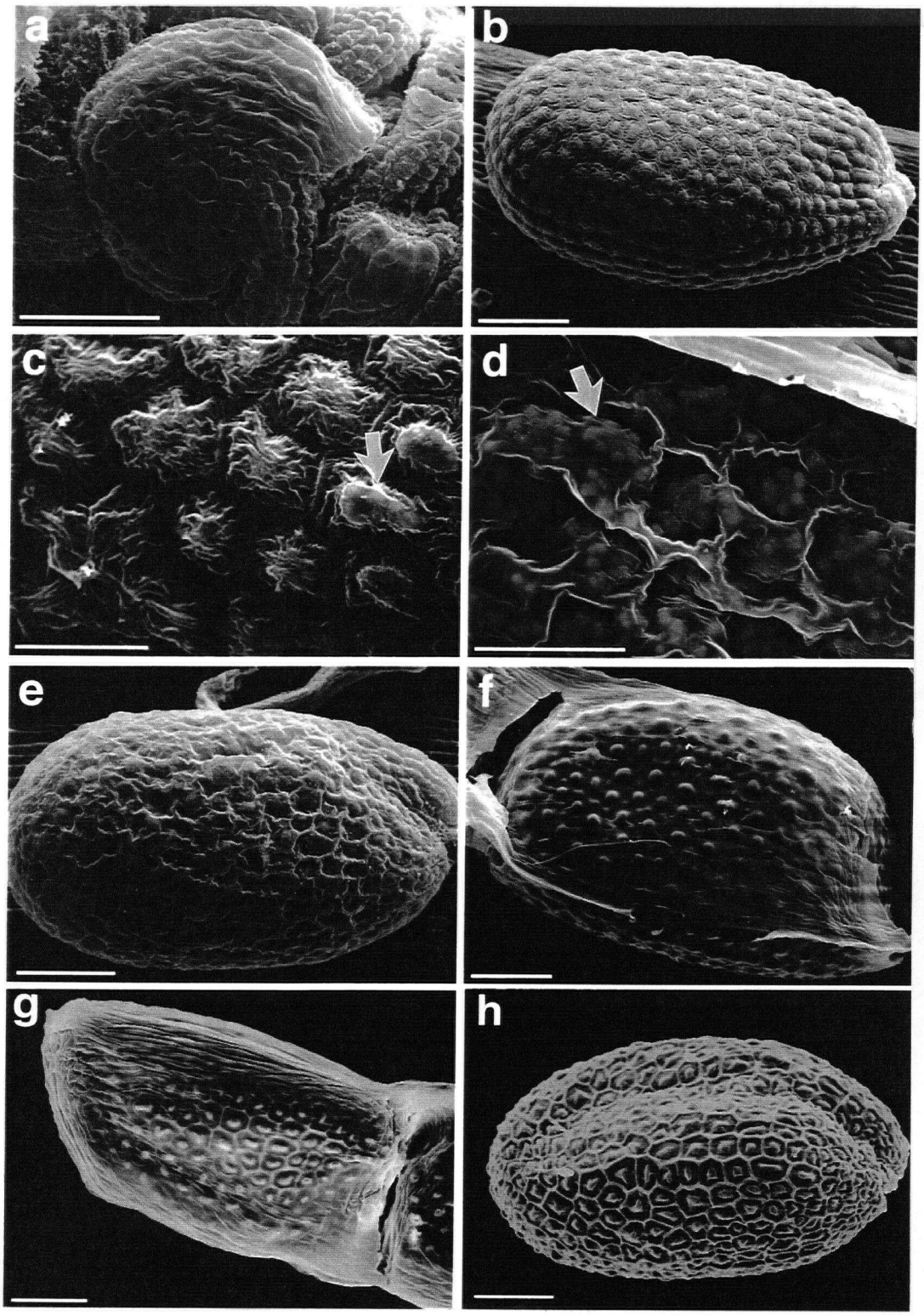


Figure 4.5. Scanning electron micrographs of wild type seed development. All seeds except (h) were fixed in the silique in aqueous 3% glutaraldehyde.

(a) Mature ovule (0 daf).
(b) Seed at 4 daf. The developing seed has increased in size and has changed in shape compared with the mature ovule. Hexagonal cells are apparent.
(c) Close-up of epidermal cells at 4 daf. Some of the hexagonal cells contain globules (arrow).
(d) Close-up of epidermal cells at 6 daf showing ring of globules in centre of cells (arrow).
(e) Seed at 7 daf.
(f) Seed at 10 daf. Columellae are apparent in centre of cells, the seed is covered in a small amount of white-staining mucilage.
(g) Seed at 13 daf. There is more mucilage surrounding the seed than at 10 daf, however, the thickened radial cell walls and columellae of the epidermal cells are apparent.
(h) Dry mature seed. Note the hexagonal epidermal cells with thickened radial cell walls and columellae in the centre.
Magnification bars: (a),(c),(d)=40 μm; (b),(e)-(h)=100 μm.

cytoplasm visible only around the edges (Figure 4.4b). The intracellular globules were first evident at 3 daf, following which they increased in size and number until 7 daf (Figure 4.4b,c). In the appearance, the globules resembled amyloplasts observed in TEM studies of mucilage production in the *B. campestris* seed coat and *Nicotiana tobaccum* and *Arabidopsis* root tips (Van Caeseele *et al.*, 1981; Staehelin *et al.*, 1990). To test the hypothesis that the globules were starch-containing amyloplasts, Debra Skinner stained developing seeds with a starch specific stain, I_2-KI, and observed them cleared in organic solvents. She found that the globules, indeed, stained purple-black (Figure 4.6a,b), indicating the presence of starch. As well, developing seeds of a starchless mutant, Pgm1 (Caspar *et al.*, 1985; Caspar and Pickard, 1989), did not contain starch globules (Figure 4.6c,d).

At 6 to 7 daf, as the starch granules became larger and more apparent, pink-staining acidic polysaccharide began to become evident in the cells. From days 7 to 9 the pink stain became darker and the amyloplasts appeared to be delimited to a column in the center of the cell (Figure 4.4c,d). The maximum intensity of pink stain appeared to be reached at 9 daf, as the colour of intact cells was consistent at times thereafter. By 10 daf, some cells, if hydrated, would break open, releasing mucilage, and the columella became apparent as purple-staining material surrounding the cytoplasmic column. At this stage, the amyloplasts began to stain darker and look smaller (Figure 4.4d). The cell walls of the columellae were much thicker by 12 daf, and also appeared to fuse with

the thickenings at the base of the radial cell walls. Cytoplasm was reduced but was still apparent under the columellae and most of the cells had broken open to release the mucilage. By 13 daf, the columellae appeared to be entirely cell wall and the majority of cells had released their mucilage (Figure4.4e). From 13 daf to 18 daf there was little difference in structure of the cells. The cells apparently had finished development and mucilage was extruded from the cells. It appeared that, after breaking to release the mucilage, the outer cell wall remains attached to the edges of the columella, likely producing the rays seen around wetted seeds (Figure 4.4f).

Epidermal cell development can be summarized as follows (see Figure 4.9). First there is cell growth, followed by the accumulation of amyloplasts starting at 3 daf and peaking at 5 daf. Mucilage is apparent in the cells at 7 daf and continues to be deposited until at least 9 daf. After most of the mucilage has been made, the amyloplasts begin to disappear and the new cell wall forming the columella appears between 10 and 13 daf.

4.3.4 Starch is correlated with mucilage production, but is not necessary

As described in the previous section, the amyloplasts accumulated prior to the production of mucilage and appeared to be present in their highest quantity during the time of mucilage production, following which their number and size decreased (Figures 4.4 and 4.6a,b). This correlation of the appearance of amyloplasts with mucilage production has also been noted by Van Caeseele *et al.* (1981) in their study of *B. campestris* mucilage production, and by Staehelin *et al.* (1990) in their study of root cap mucilage production in *N. tobaccum* and *Arabidopsis,* and suggests that starch may be necessary for mucilage production. The timing of the amyloplast accumulation also appears to be correlated with the production of the new cell wall of the columella.

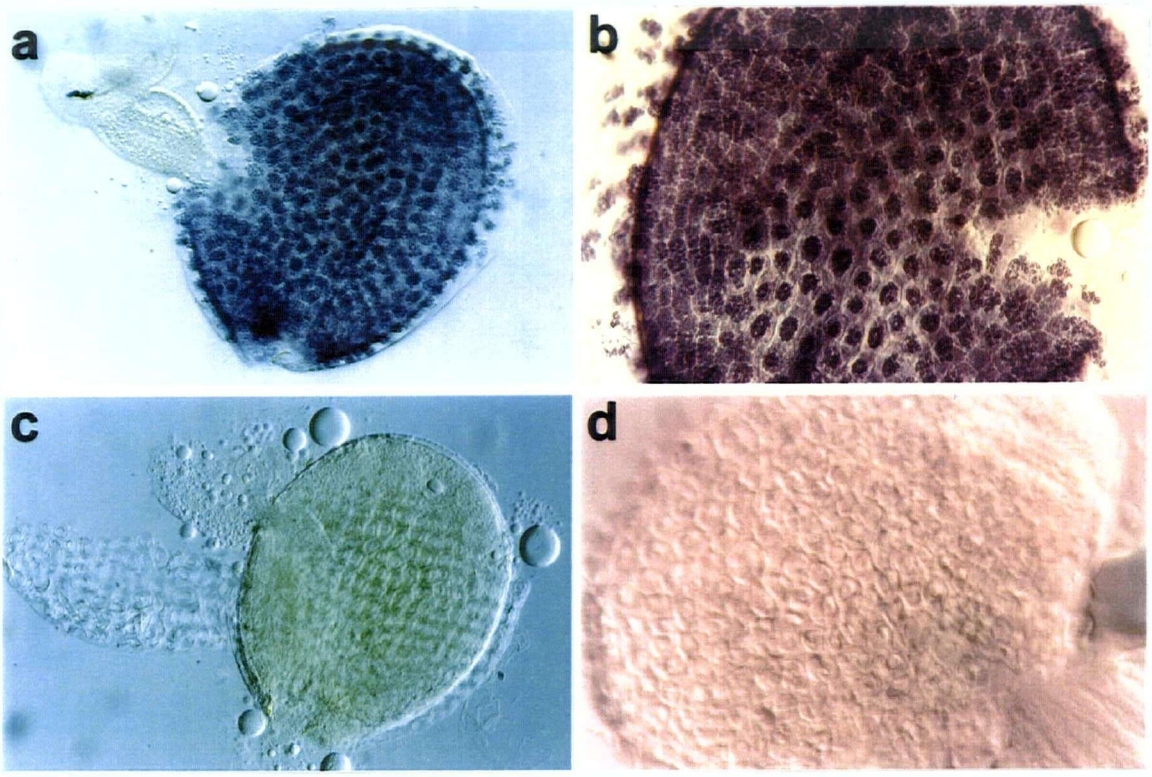


Figure 4.6. Wild type and Pgm1 seeds stained with iodine.
Whole mount seeds were stained in I_2KI, then cleared with organic solvents and photographed with DIC optics.
(a) Wild type seed at 6 daf, showing purple-black staining of globules for starch. (75x)
(b) Close-up of wild type seed at 8 daf, showing stained globules in the epidermal cells. (140x)
(c) Pgm1 seed at 9 daf, showing no globules. (60x)
(d) Close-up of Pgm1 seed at 7 daf, showing no stained globules. (130x)

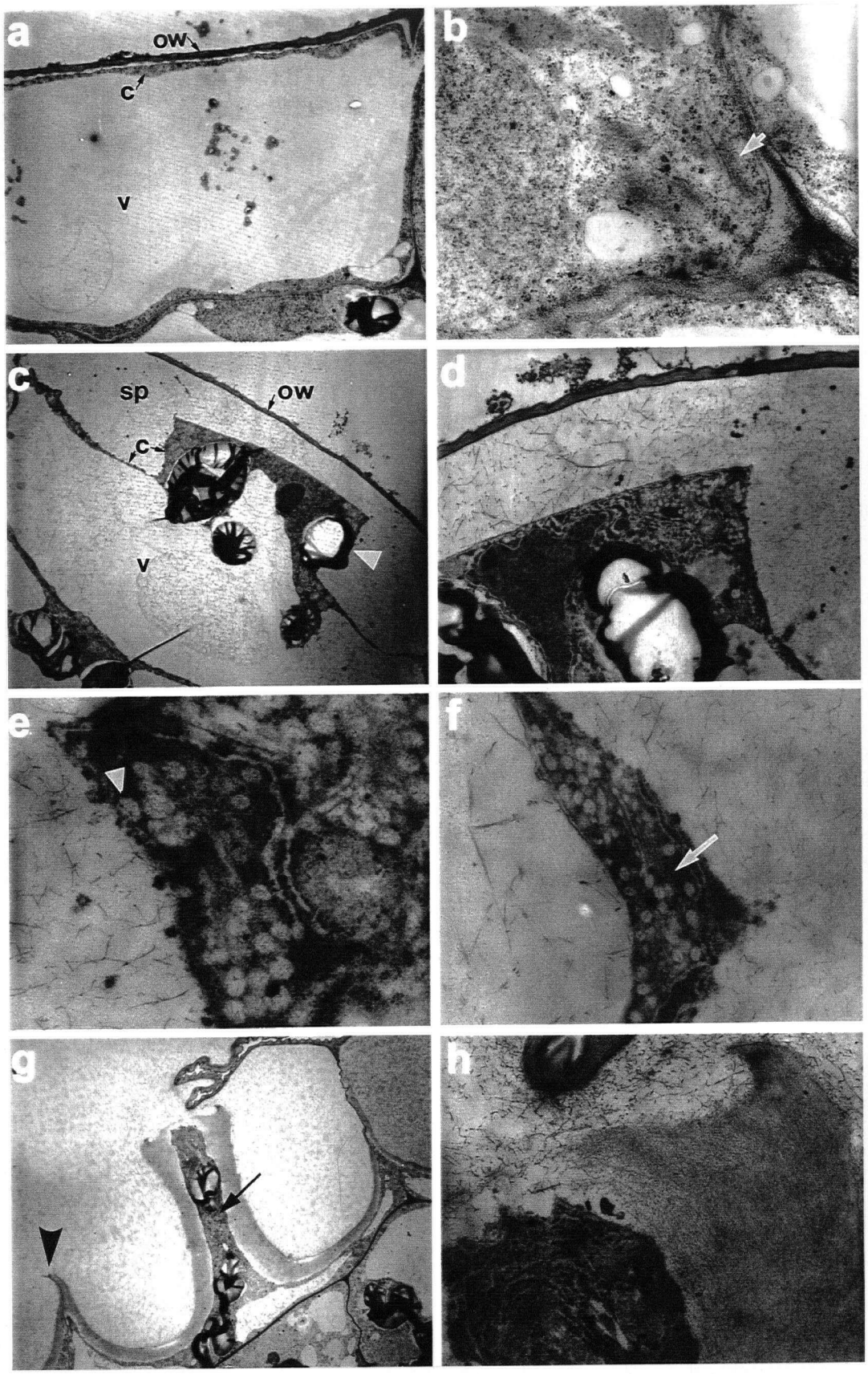
The requirement for starch to make mucilage was tested in collaboration with Debra Skinner by studying a starchless mutant, Pgm1. Pgm1 plants are unable to make the enzyme phosphoglucomutase and have been found to completely lack starch (Caspar *et al.*, 1985; Caspar and Pickard, 1989). The seeds of Pgm1 plants were compared to wild type plants under two different growth conditions. When both wild type and Pgm1 were grown under continuous light, both produced normal seeds with columellae and mucilage. This suggests that starch, itself, is not necessary for proper seed epidermal cell development.

It has been shown that Pgm1 mutants 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 hours of darkness 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 hours light and 12 hours darkness. Under these conditions, both wild type plants and Pgm1 plants produced normal seeds with mucilage and columellae. The Pgm1 plants, however, instead of making the full complement of 50-60 seeds, made only 2-3 per silique in 12 hours light/12 hours dark.

4.4 Cytology of epidermal cell development

4.4.1 Secretion of mucilage

In order to understand the events leading to the secretion of mucilage at the cytological level, TEM of several stages of wild type epidermal cell development was performed (Figure 4.7). At 4 daf, as seen by light microscopy, the cells were largely vacuolated, with the cytoplasm, including some amyloplasts, appearing around the edges of the cell (Figure 4.7a). At this stage there was very little evidence of mucilage and Golgi stacks had thin cisternae (Figure 4.7b). By contrast, at 7 daf the ultrastructure of the cells looked quite different. The vacuole was smaller and the space between the plasma membrane and the outer cell wall was full of short, dark fibrils (Figure 4.7c,d). A smaller amount of fibrils was also found in the rest of the cell, sometimes bounded by membranes. The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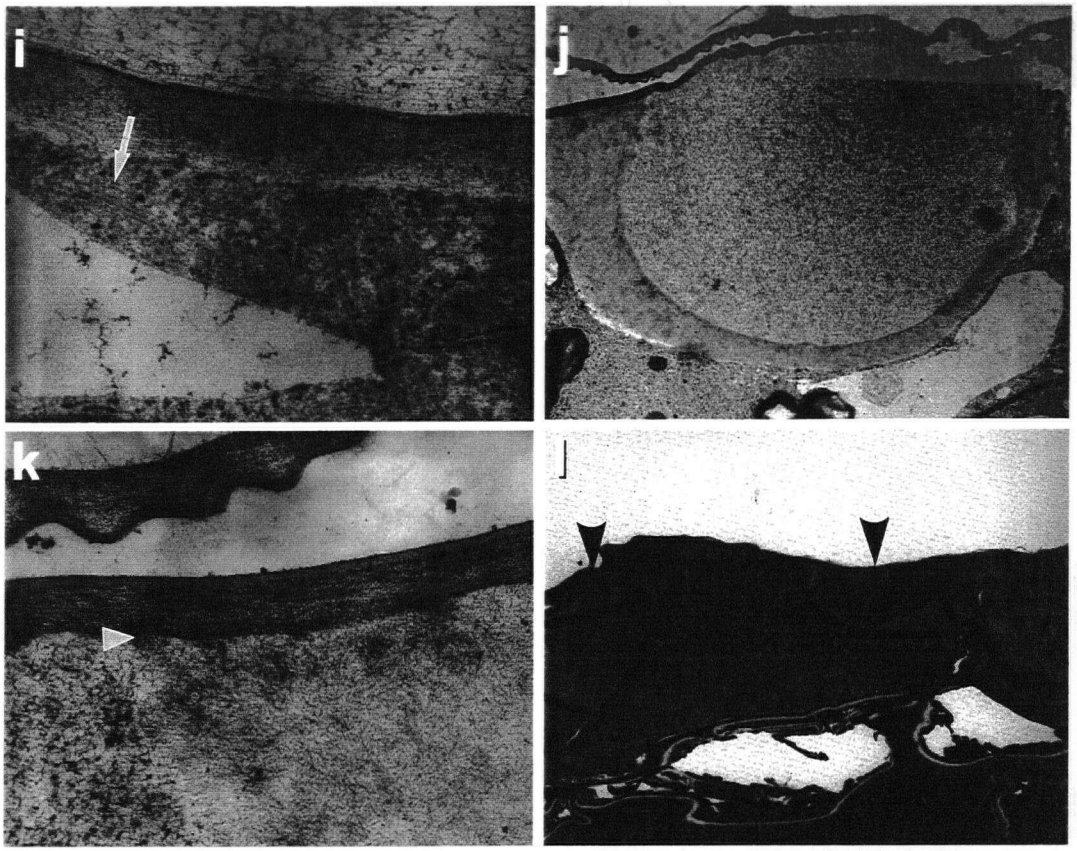

Figure 4.7. Transmission electron micrographs of developing epidermal cells of wild type seeds.
(a) Cell at 4 daf, note large vacuole (v), and cytoplasm (c) found around the edges of cell close to the outer cell wall (ow). (4000x)
(b) Close-up of cytoplasm at 4 daf, arrow indicates a Golgi body. (19,200x)
(c) Cell at 7 daf. Most of the cytoplasm (c) is in a column in the centre of the cell. There is a space (sp) containing fibrillar material (see (d)) between the cytoplasm and the outer cell wall (ow), and a smaller vacuole is found under the cytoplasmic column (v). Note very large amyloplasts (arrowhead). (2200x)
(d) Close-up of cell at 7 daf showing top corner of cytoplasmic column. The cytoplasm is filled with vesicles and tubular clearings. Fibrillar material is present between the cytoplasm and the outer cell wall. (6700x)
(e) Increased magnification of cytoplasm in (d). Fibrils are in vesicles, some of which (arrowhead) are found at the edge of the cytoplasm. (33,800x)
(f) Close-up of cytoplasm from 7 daf cell. This small portion of cytoplasm is full of vesicles and they can be seen budding off a Golgi stack (arrow). (26,400x)
(g) Cell at 10 daf. The cytoplasm (arrow) is found in the centre 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 reinforcement with electron dense material ended (arrowhead). (2400x)
(h) Top of column in centre 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. (17,100x)

(i) Cytoplasm at the base of a cell at 10 daf. Note Golgi stack with thin cisternae on left (arrow), and absence of large vesicles throughout cytoplasm. (24,500x)
(j) Portion of intact cell at 10 daf. The new cell wall of the developing columella appears to fuse with the original outer cell wall and the space between the intracellular column and the outer cell wall is filled with densely-packed fibrils. (3700x)
(k) Top of the columella from the cell in (j) showing appression of original outer cell wall and the new, secondary cell wall (arrowhead) surrounding the cytoplasm. (32,000x)
(l) Cell of mature seed that has been fixed under non-aqueous conditions. The columella is surrounded on either side with mucilage (arrowheads) within the outer cell wall. (3200x)

cytoplasm stained quite dark and was very full of vesicles containing fibrillar material (Figure 4.7d-f). The fibrillar material seen both in the vesicles and in between the cytoplasm and the cell wallcorrelated in time and space with the pink staining acidic polysaccharides identified in thick sections, suggesting that the fibrillar material is pectin. The fibrillar appearance may be due to the attraction of positive lead to the negative polygalacturonic acid backbones of the pectin. The fibrillar material appeared to be outside of the cytoplasm, though, unfortunately, the plasma membrane was difficult or impossible to see in most of the sections (Figure 4.7c,d). Golgi stacks were quite obvious at 7 daf and were enlarged compared to 4 daf. Large vesicles containing fibrillar substance were observed budding off the Golgi stacks (Figure 4.7e,f). Vesicles also could be seen exocytosing (Figure 4.7e,f). At 10 daf, depending on the seed studied, these vesicles were either present in lesser numbers or completely absent (Figure 4.7g-i), with the Golgi stacks assuming an appearance similar to that seen at 4 daf (Figure 4.7i). The fibrillar material found in 10 daf cells was much denser than that found at 7 daf (Figure 4.7j,k). Indeed, many of the 10 daf cells had already ruptured, releasing the fibrillar material outside of the cell (Figure 4.7g). The breakage of the cell wall occurred at the upper part of the radial cell wall where the cell wall was the thinnest (Figure 4.7g). These data suggest that pectin is made in the Golgi stacks, deposited into vesicles and secreted into the extracellular space between the cytoplasm and the primary cell wall.

4.4.2 Production of the columella

As mentioned in the previous section, at 4 daf the cytoplasm was between the cell wall and the edges of a very large vacuole (Figure 4.7a). At 7 daf, however, depending on the part of the cell sectioned through, the cytoplasm was seen as a very distinct column in the center of the cell that spread out at the bottom of the cell over a reduced vacuole (Figure 4.7c,d). The very sharp edges of this cytoplasmic column and its presence in multiple cells correlated with the delimitation of the amyloplasts to a ring in the center of the cell seen both with SEM and clearing, and the column of amyloplasts seen with thick sections. This suggests that at this stage the cytoplasm is actively organized into a cylinder shape.

The cytoplasmic columns were also seen at 8 and 10 daf, but at 10 daf the cytoplasm was surrounded by electron dense material resembling the primary cell wall, though slightly more diffuse (Figure 4.7g). Since the columella is known to be taking shape around 10 daf, it appears that its formation results from the laying down of secondary cell wall material around a further-constricted cytoplasmic column and small vacuole (Figure 4.7h,k). At later stages, this cell wall progressively filled in the entire column except for a small amount of cytoplasm at the bottom of the cell that disappeared during dehydration (Figure 4.7l). The columella cell wall material closely appressed that of the primary cell wall at the top of the cell, otherwise there was no obvious connection between the two cell walls (Figure 4.7k,l). Since the cytoplasm took on a volcano shape, the columella cell walls not only created a column in the center of the cell but they also lined the bottom of the cell and ran midway up the new radial cell walls, thus reinforcing the inner tangential cell wall and the lower part of the radial walls (Figure 4.7g,l).

4.5 Discussion

4.5.1 Seed coat development in *Arabidopsis*

Arabidopsis, like many other species of the Brassicaceae, is a myxospermous plant which has a gel-like layer around its wetted seeds (Vaughan and Whitehouse, 1971). This mucilage has been found to be composed of pectin (Goto, 1985; this research), as seen in other plants with mucilage-containing seeds (Hirst *et al.*, 1965; Tyler, 1965a; Tyler, 1965b; Vose, 1974; Wright and Northcote, 1974; Siddiqui *et al.*, 1986; Van Caeseele *et al.*, 1987; Cui and Eskin, 1993; Benhura and Marume, 1993; Cui *et al.*, 1994; Fedeniuk and Biliaderis, 1994; Karihaloo and Malik, 1996). *Arabidopsis* seed mucilage is made in the outermost cell layer where it is found between the outer tangential wall and a volcano-shaped structure known as the columella.

Mucilage production has been studied in the seeds of several plants, including *B. campestris, P. ovata*, and *Cydonia oblonga* (Hyde, 1970; Van Caeseele *et al.*, 1981; Abeysekera and Willison, 1988). Not surprisingly, mucilage production in *Arabidopsis* seeds (Figures 4.8 and 4.9) is quite similar to that of its relative, *B. campestris*. During rapid seed growth, the epidermal cells become increasingly vacuolar and start to accumulate amyloplasts. A few days later, while the cytoplasm is drawn into the center of the cell, mucilage appears. Mucilage is laid down for several days, and as its production decreased, new cell wall is being laid down around the cytoplasm to form the columella. Cell wall continues to fill in the columella and the remaining cytoplasm may degrade during seed desiccation. When seeds are placed in water, the mucilage swells, forcing the side cell walls to break, releasing the mucilage and leaving the remnants of the tangential wall attached to the columella as rays.

While mucilage production is similar between *B. campestris* and *Arabidopsis*, there are several differences. *B. campestris* does not have columellae or any secondary cell wall formation in the

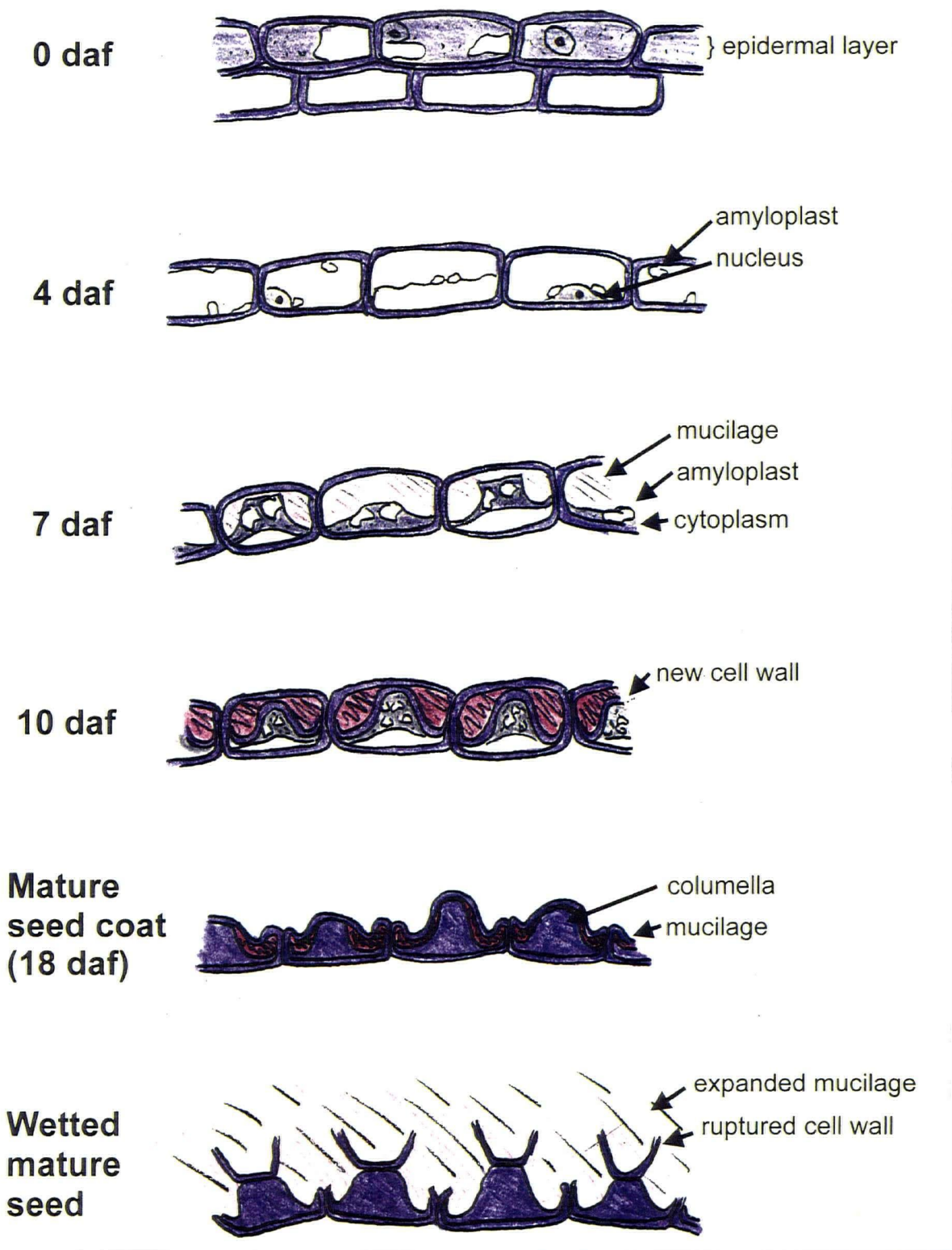

Figure 4.8. A model showing the production of mucilage and columellae in the epidermal cells of wild type *Arabidopsis* seeds.

epidermal cells (Van Caeseele *et al.*, 1981). The function of the columellae in *Arabidopsis* is not known, but various forms of intracellular protrusions have been found in many species of the Brassicaceae, including *Capsella bursa-pastoris* (Vaughan and Whitehouse, 1971). Possible functions of the columellae in *Arabidopsis* could be to decrease the volume in the cells to allow for the deposition of less mucilage and still have enough pressure to burst the radial cell walls, or to provide a large surface/volume ratio for rapid mucilage deposition.

The two other layers of the seed coat, the palisade and pigmented layers, develop concurrently with the epidermal cell layer, while the other two original layers of the inner integument are crushed. Development of the inner layer of the outer integument into the palisade layer occurs in a similar fashion in both *Arabidopsis* and *B. campestris* (Van Caeseele *et al.*, 1982). Amyloplasts accumulate in the cell, followed by the development of the thickened inner radial cell wall. The laying down of the thickened cell wall is then followed by the deposition of lignin (Figure 4.9). The development of the innermost layer of the inner integument into the pigmented layer, however, is less clear. Unusual staining characteristics made it difficult to determine what was happening, but the cells appeared to be largely vacuolar and do not show the blue-green staining typical of polyphenols until the late stages of desiccation corresponding with the red-brown colour appearing in the seeds. It is likely that the reduced tannins are made at an earlier stage, and then a change in intracellular conditions, such as pH, possibly due to degradation of the cytoplasm, leads to the change of colour.

During *Arabidopsis* seed development, the first event is the rapid growth of the seeds in the first four to five days (Figure 4.9). About the time that seed growth is finished the amyloplasts have accumulated and mucilage production in the epidermal cells and new cell wall deposition in the palisade cells commences. The production of mucilage and columella in the epidermal cells, and lignin deposition in the palisade cells appears to be finished at about the time that the embryo

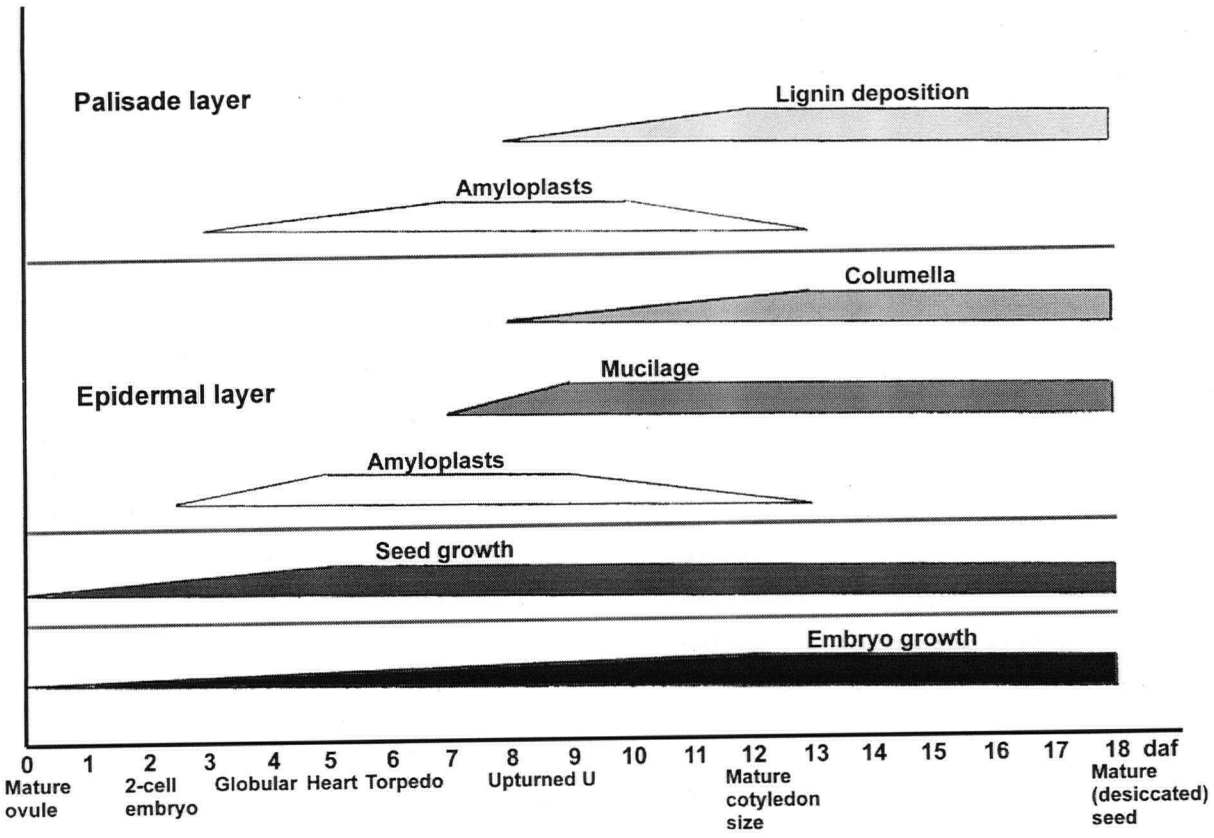

Figure 4.9. A model comparing the timing of events in embryo growth, seed growth, and epidermal and palisade layer development.

reaches its mature cotyledon size. The developing seeds may direct their energy into different developmental events at different times (Figure 4.9). First there is seed growth, followed by the development of the seed coat, during which the embryo is undergoing cell divisions and cell growth. Seed growth and seed coat development is finished at the time that the embryo is ready for rapid filling with energy reserves which continues until the time of desiccation.

4.5.2 The epidermal cells of the seed coat are significant secretory cells

The appearance of a large number of fibrils between the plasma membrane and the outer cell wall of the epidermal cells correlates with the time at which pink-staining acidic polysaccharides are found in the thick plastic sections, suggesting that the fibrillar material is mucilage. At the same time, the cytoplasm is full of vesicles containing similar fibrils. These vesicles can be seen both attached to Golgi stacks and the cell membrane, suggesting that the fibrillar substance seen outside of the cytoplasm is produced in the Golgi stacks. These data show that a vast amount of pectin synthesis and secretion is occurring in the epidermal cells as pectin is both a major component of mucilage and known to be made in the Golgi stacks (Esau, 1977; Brett and Waldron, 1990; Staehelin and Moore, 1995). Similar results were found by Staehelin and coworkers while studying mucilage production in the root tips of clover, onion, sycamore maple, tobacco, and *Arabidopsis* (Staehelin *et al.*, 1990; Moore *et al.*, 1991; Zhang and Staehelin, 1992). They used antibodies to show that two pectin components, rhamnogalacturonan I and polygalacturonic acid, were present in the Golgi stacks and vesicles of pectin-producing cells.

Some fibrils are also seen at 4 daf, but at very low amounts and they appeared to be enclosed in a membrane within the space thought to be vacuole. This suggests that some mucilage production is occurring at this early stage. At 7daf, the majority of the mucilage is found between the cytoplasm and the outer tangential cell wall, but a small amount is also seen within the vacuole, sometimes appearing to be membrane bound. This mucilage within the plasma membrane could simply be a sectioning artifact, with the plane running through an invagination of the plasma membrane, or it may be an intracellular collection of mucilage that will later be secreted and/or degraded as suggested in *P. ovata* (Hyde, 1970; Fahn, 1982). This is supported by the lack of mucilage within the vacuole at 10 daf.

The majority of the mucilage, however, is found between the cytoplasm and the outer tangential cell wall, suggesting that polar secretion is occurring. Polar secretion is involved in many processes, including cell wall production in the tip-growth of pollen tubes and root hairs in plants (Fowler and Quatrano, 1997; Cai *et al.*, 1997).

4.5.3 Formation of the columella through manipulation of the cytoplasm and cell wall production

At the early stages of seed coat development (e.g. 4 daf), the cytoplasm is found around the edges of the cell, surrounding a extremely large vacuole. At 7 daf, however, when mucilage production is at full swing, the cytoplasm is found in a centrally located column which has quite sharp, square edges at the top, and curves outward at the bottom (Figure 4.8). This rearrangement of the cytoplasm is correlated with the creation of a large extracellular space at the expense of the vacuole. Secretion of the mucilage could be responsible for the production of this space and the reduction of the vacuole. Secretion alone, however, would be expected to force the cytoplasm to the bottom of the cell as seen in *B. campestris,* rather than leading to the production of a sharply defined cytoplasmic column in the centre of the cell (Van Caeseele *et al.*, 1981). In addition, the shape is established early, prior to the production of a large percentage of the mucilage. There are several possibilities as to how this shape could be established. One is that the cytoplasm is pulled into the centre of the cell by the cytoskeleton. The well-defined borders of the cytoplasm suggests that the shape of the cell was actively established. An analysis of the cytoskeleton during this period could provide evidence in support of such an hypothesis. Another possibility is that there is a specific, strong linkage between the plasma membrane and the center of the outer cell wall, such that only the edges are pushed down by the accumulation of mucilage. The cytoplasmic column,

however, has not always been observed to reach the top of the cell, but this may be due to the location of the sections observed.

The second step in the production of the columella is the synthesis of a large amount of cell wall material which is laid down around the cytoplasmic column, pushing the cytoplasm farther down to the bottom of the cell (Figure 4.8). At this time the majority of the vesicles involved in mucilage production have disappeared and the cytoplasm appears to be packed with a large amount of rough endoplasmic reticulum and a few Golgi stacks with a 4 daf appearance. The different morphology the Golgi stacks and the appearance of lots of ribosome-coated ER may reflect the need for a large amount of protein in order to make the new cell wall.

4.5.4 A role for starch in seed coat development

Amyloplasts accumulate in the epidermal cells prior to mucilage deposition and then tail off at approximately the time that mucilage production finishes. A similar phenomenon was also seen in several other mucilage producing cells including the epidermal cells of *B. campestris* seeds, and *N. tobaccum,* and *Arabidopsis* root tips (Van Caeseele *et al.*, 1981; Staehelin *et al.*, 1990). This correlation suggests that the amyloplasts are supplying the sugar for the production of the pectin. Amyloplasts have also been found to accumulate in the developing palisade cells of both *Arabidopsis* and *B. campestris* (Van Caeseele *et al.*, 1982). In these cells, the starch may be used for the production of the large amount of new cell wall material. The requirement for starch for the production of mucilage was tested by observing the seeds of the of starch deficient mutant Pgm1, which lacks phosphoglucomutase (Caspar *et al.*, 1985; Caspar and Pickard, 1989). The Pgm1 seeds were found to have mucilage, suggesting that starch is not necessary for mucilage production. Pgm1 plants, however, are known to accumulate pools of soluble sugars, presumably in place of starch, so this is

may be supplying sugar for the production of mucilage and other new cell wall material seen in the cells. Thus, while starch is not necessary for the production of new cell wall material in the seed coat, it is still possible that it is present to supply sugar for their manufacture.

5. MUCILAGE-MODIFIED MUTANTS DEFECTIVE IN SEED COAT DEVELOPMENT

5.1 Introduction

Once the histology and cell biology of mucilage production is known, then the process can be investigated at the biochemical level. To do this, genes involved in the biosynthesis and secretion of pectin may be identified via the isolation of mutants defective in mucilage production. Mucilage can be eliminated without lethality to *Arabidopsis* plants as there are four well-characterized genes that affect seed coat development and mucilage production: *TTG1, GL2, AP2, ATS* (Koornneef, 1981; Rerie *et al.*, 1994; Jofuku *et al.*, 1994; Léon-Kloosterziel *et al.*, 1994; Bowman and Koornneef, 1994a). All of these genes, however, regulate several processes. *TTG1* and *GL2* were identified as genes which alter the production of the trichomes and were also found to influence other epidermal cell features, such as root hairs and seed epidermis (Koornneef, 1981; Rerie *et al.*, 1994; Bowman and Koornneef, 1994a). *AP2* is involved in both flower and ovule development, and *ATS* affects ovule development (Jofuku *et al.*, 1994; Léon-Kloosterziel *et al.*, 1994; Bowman and Koornneef, 1994a). In order to investigate mucilage production fully, it would be useful to have many mutants, especially some that are specific to seed epidermal cell development. Thus, in addition to studying these four mutants, I have performed a screen for new mutants with aberrant amounts of mucilage. In this chapter I describe the isolation of mutants affected in five novel genes (*MUM1—5)* and the initial characterization of Ttg1, Gl2, Ap2, Ats, and Mum1—5 mutant seed coats.

5.2 Screen for seed coat mutants

As mentioned in Chapter 4, when wild type *Arabidopsis* seeds are placed in a 0.01% solution of Ruthenium red, a pink layer of mucilage is found around the seed and the hexagonal cell walls and columellae of the epidermal cells are sharply outlined (Figure 5.1a). This specific staining was quite obvious under a dissection microscope and was used as the basis of a screen for mutants defective in mucilage production, secretion, and extrusion, and seed coat development. For screening, seeds were first shaken in water to hydrate the mucilage, and then they were stained in Ruthenium red and observed. In this manner, approximately 1000 ethylmethane sulfonate (EMS) mutagenized plant lines and 3060 T-DNA (Feldmann, 1991) lines were screened for mucilage and seed coat defects by Dr. Joanne Burn and myself. The 11 EMS mutants that remained stable throughout several generations were studied (Table 5-1). The one T-DNA mutant that was isolated has had preliminary analysis done and has been passed on to another student.

5.3 Genetic analysis

5.3.1 Complementation tests

In order to determine if these 11 EMS mutants were alleles of the same gene, complementation tests were performed by intercrossing the mutants. The final results of these crosses are outlined in Table 5.1. Five complementation groups were established. With the exception of the complementation group containing RW15 and K14-1, where there was a phenotypic difference between the two mutants, all mutants in a group were considered the same allele since they were isolated from the same mutagenesis batch. In addition, the mutant 3(34) was

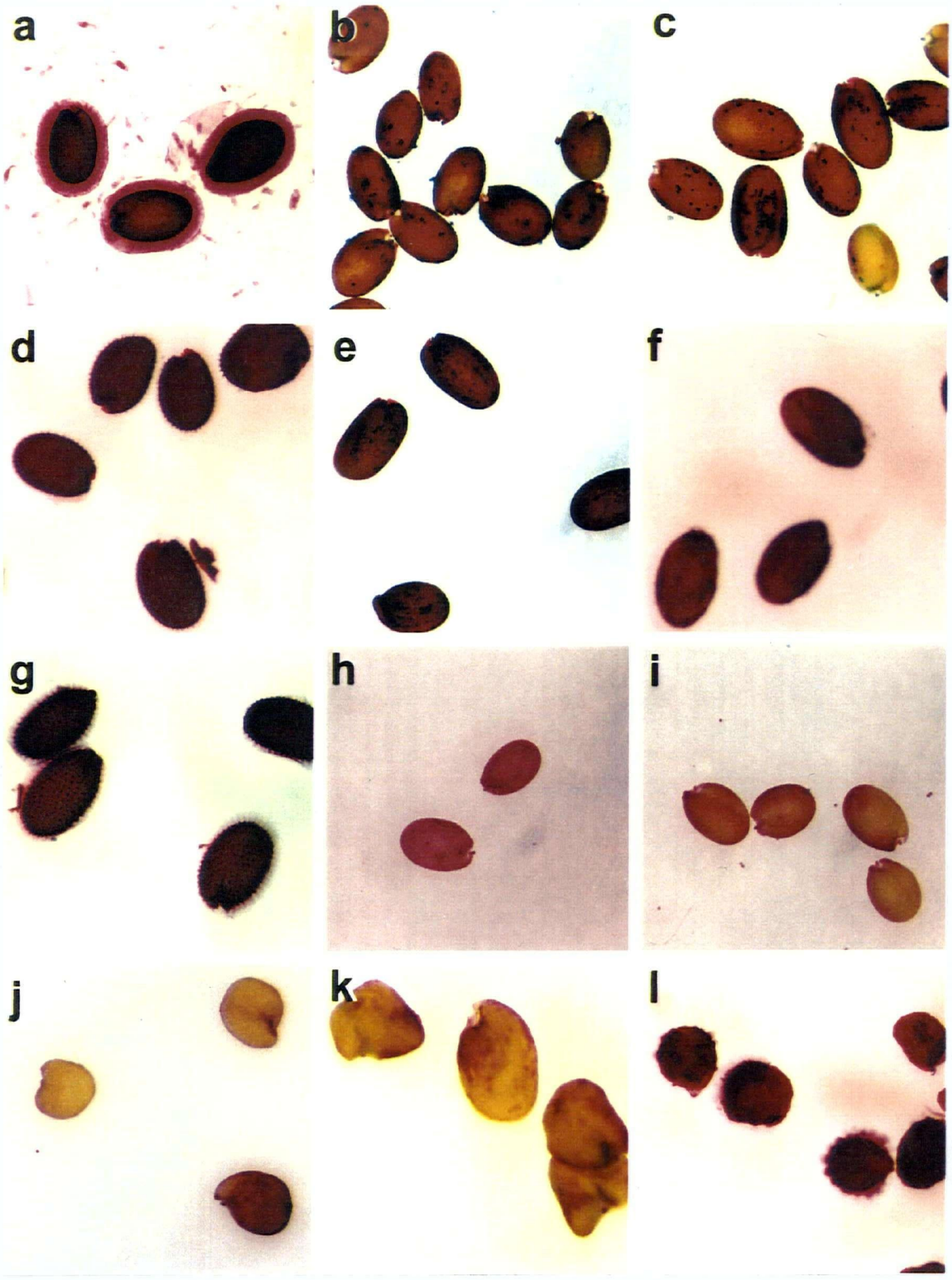

Figure 5.1. Wild type and mutant seeds staining with Ruthenium red after shaking in water.

(a) Wild type seeds with dark-pink mucilage capsule.
(b) Mum1-1 seeds. Note lack of mucilage capsule and red staining.
(c) Mum2-1 seeds. Note lack of mucilage capsule and red staining.
(d) Mum3-1 seeds with thin layer of mucilage directly around the seeds.
(e) Mum4-1 seeds. Note lack of mucilage capsule and red staining.
(f) Mum4-2 seeds with thin layer of mucilage directly around the seeds.
(g) Mum5-1 seeds with thin layer of mucilage directly around the seeds.
(h) Ttg1-1 seeds. Note lack of mucilage capsule and pink staining of seed coat.
(i) Gl2 seeds, showing lack of mucilage capsule and some red staining.
(j) Ap2-1 seeds. Note lack of mucilage capsule, heart shape, and red stain only on one of the three seeds shown.
(k) Ap2-6 seeds. Note the large size, abnormal shape, and lack of mucilage capsule and staining of seed coat.
(l) Ats seeds showing round shape and patches of mucilage.
Magnification (a)-(l) = 28x.

isolated from a separate mutagenesis batch and thus was also given a new allele designation. Complementation tests performed between representative mutants of the four known loci (Ttg1, Gl2, Ap2 and Ats) and the five new complementation groups suggested that the latter were novel genes (Table 5.1). These novel genes have been named *MUCILAGE-MODIFIED1—5 (MUM1—5)*.

5.3.2 Backcrosses

Reciprocal backcrosses to wild type plants were performed in order to determine whether: (a) the seed phenotype was, as expected, maternally determined, (b) the mutant allele was dominant or recessive to wild type, and (c) the phenotype was determined by a single or multiple genes (Table 5.2). Staining of the F1 seed demonstrated that the mother plant determined the phenotype for each of the five genes. In each case, the F2 seeds were wild type, showing that the mutant alleles were recessive. The F3 segregation pattern was determined for Mum1-1, Mum2-1, Mum4-1, and Mum5-1. In each case a 3:1 ratio of wild type:mutant seed phenotype was observed and was found to be

Table 5.1. List of mutants defective in seed coat development and their mucilage and seed phenotypes.

Mutant (alleles studied)	Isolates[a]	Mucilage phenotype (Ruthenium red)[b]	External features of seed coat (SEM)
Col-2		pink capsule with rays	hexagonal cells, round columellae
Mum1-1	K24, L1	no mucilage, red stained seed	irregularily shaped cells and columellae
Mum2-1	K34, K7-3, K26-1, K20-2	no mucilage, red stained seed	wild type
2-2	3(34)[c]	no mucilage, red stained seed	wild type
Mum3-1	CQF-1	thin layer mucilage directly around seed	wild type
Mum4-1	RW15	no mucilage, some with red stained seed	no or reduced columellae
4-2	K14-1	no mucilage or thin layer mucilage directly around seed	reduced columellae
Mum5-1	H1, G3	thin layer mucilage directly around seed	wild type
Ttg1-1		no mucilage, pink stained seed	no or reduced columellae
Gl2-1		no mucilage, no staining of seed	no or reduced columellae
Ap2-1		no mucilage, no staining of seed	heart-shaped seed, elongated cells with no or reduced columellae
2-6		some wild type, some no mucilage	some wild type, some heart shaped with rectangular cells and no columellae
Ats		reduced mucilage ring	round seed, large cells

[a]Since the majority of the Mum mutants were isolated from one mutagenesis batch, multiple isolates could not be considered to be independent alléles unless an altered phenotype was observed (as seen for RW15 and K14).

[b]Shaken first in water, then stained.

[c]Isolated by D.J. Skinner

Table 5.2. Results of reciprocal backcrosses to wild type plants and segregation analysis.

	Mum1-1		Mum2-1		Mum3-1		Mum4-1		Mum5-1	
	x WT	WT x	x WT	WT x	x WT	WT x	x WT	WT x	x WT	WT x
F1 seed	muc-	+	muc-	+	red. muc	+	muc-	+	red. muc	+
F2 seed	+	+	+	+	+	+	+	+	+	+
F3 seed	3:1 WT:mut (188:50) (X^2=2.0224, 0.5>P>0.1)[c]		3:1 WT:mut (98:33) (X^2=0.0025, 0.9>P>0.5)		in progress[b]		3:1 WT:mut (147:37) (X^2=2.348, 0.5>P>0.1)		3:1 WT:mut (142:41) (X^2=0.6576, 0.5>P>0.1)	

[a]WT & + = wild type, muc- = no mucilage, red. muc = reduced mucilage

[b]relatively recent isolate

[c]degrees of freedom = 2, cutoff = P=0.05

significant at a P level of 0.05 (Table 5.2). These results showed that single genes determined these phenotypes.

5.4 Basic phenotype of mutants

I studied the external seed phenotype of Ttg1, Gl2, Ap2, Ats, and Mum1—5 using both Ruthenium red staining and SEM (Table 5.1; Figures 5.1 and 5.2). Ruthenium red staining after shaking in water has divided the mutants into three categories depending on their mucilage capsule phenotype: (a) no mucilage capsule, Mum1, Mum2, Mum4-1, Ttg1, Gl2, and Ap2 (Figure 5.1b,c,e,h-l), (b) a very reduced mucilage capsule seen just around the seed periphery, Mum3, Mum4-2, and Mum5 (Figure 5.1d,f,g), and (c) reduced amount of mucilage, Ats (Figure 5.1l). Further analysis when seeds were added directly to Ruthenium red without shaking revealed that Mum3 and Mum5 have a mucilage capsule but their mucilage fails to stain properly, thus defining a fourth group (see Chapter 8). These categories have been further refined by SEM of dry seeds done in collaboration with Debra Skinner[1] (Table5-1; Figure 5-2). Mum2-1, Mum2-2, Mum3-1, and Mum5-1 had wild type seed coats with thickened hexagonal cell walls enclosing round columellae (Figure 5.2a-d), while Mum1-1 epidermal cells and columellae were slightly irregular. Mum4-1, Gl2-1 and Ttg1-1 have similar seed phenotypes where the columellae were absent or reduced (Figure 5.2e,f). Mum4-2, corresponding to its weaker mucilage phenotype, had reduced columellae. Ap2-1 seeds were heart-shaped with some elongated epidermal cells with no or reduced columellae, while Ap2-6 seeds were either much more severe than Ap2-1 or wild type (Figure 5.2i,j). Finally, Ats seeds were round with large and slightly irregular epidermal cells (Figure 5.2 k,l).

[1]SEM of all Mum mutants with the exception of Mum3-1 was performed by D. J. Skinner, while SEM of the remaining mutants was performed by myself. SEM of Ttg1-1, Gl2-1, Ats, Ap2-1, and Ap2-10 also has been described previously (Koornneef, 1981; Bowman, 1994; Jofuku, 1994; Leon-Kloosterziel, 1994; Rerie, 1994).

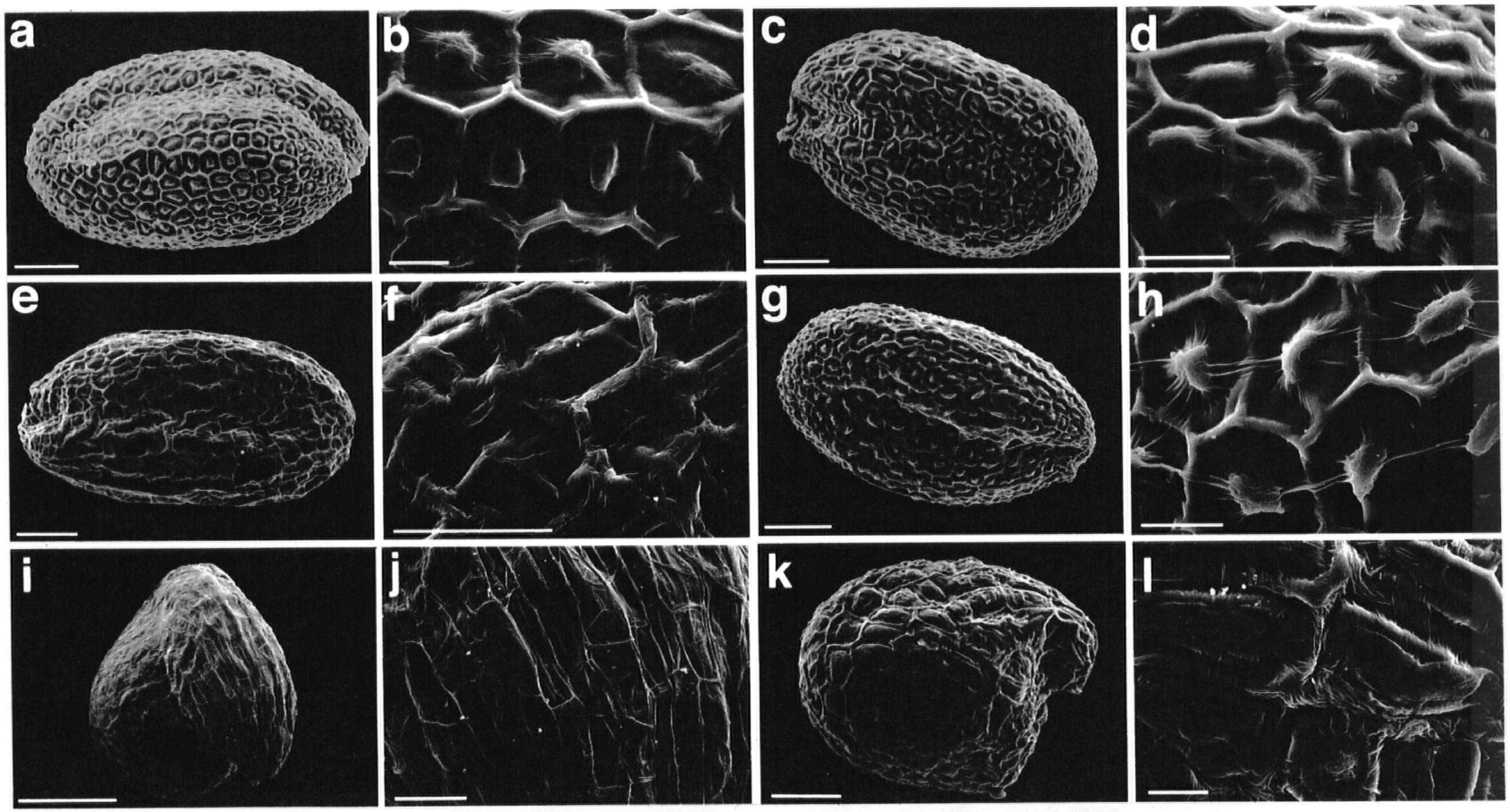
a
b
c
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Figure 5.2. Scanning electron micrographs of mature wild type and mutant seeds.
(a) Wild type seed, showing hexagonal epidermal cells with columellae.
(b) Close-up of epidermal cells of wild type seed showing thickened cell walls and columellae.
(c) Mum2-1 seed, showing normal shape and appearance of epidermal cells.
(d) Close-up of epidermal cells of Mum2-1 seed.
(e) Mum4-1 seed. Note lack of columellae.
(f) Close-up of Mum4-1 seed, showing epidermal cells sunken between the thickened radial cell walls.
(g) Mum5-1 seed, showing normal shape and appearance of epidermal cells.
(h) Close-up of epidermal cells of Mum5-1 seed.
(i) Ap2-6 seed. Note abnormal shape and lack of hexagonal cells with columellae.
(j) Close-up of Ap2-6 rectangular, thin-walled epidermal cells.
(k) Ats seed. The slightly irregular epidermal cells are enlarged. Note heart shape of seed.
(l) Close-up of Ats epidermal cells showing large columellae.
Magnification bars: (a),(c),(e),(g),(k)=100 μm; (b),(d),(h),(l)=20 μm; (f),(j)=40 μm; (i)=200 μm.

Thus, Ttg1, Gl2, Ap2, Ats, and Mum1—5 can be divided into four groups based on their Ruthenium red and external seed coat defects: (a) no mucilage capsule (Mum1, Mum2), (b) no mucilage capsule and reduced columellae (Mum4, Gl2, Ttg1), (c) abnormal mucilage staining or composition (Mum3, Mum5), and (d) no or reduced mucilage and aberrant ovule and/or seed coat development (Ap2, Ats).

5.5 Discussion

This screen for mutants with aberrant mucilage amounts led to the identification of five novel genes that affect mucilage amount in *Arabidopsis*. *MUM1—5,* unlike the previously known loci that affect mucilage production *(TTG1, GL2, AP2,* and *ATS)* (Koornneef, 1981; Rerie *et al.*, 1994; Jofuku *et al.*, 1994; Léon-Kloosterziel *et al.*, 1994; Bowman and Koornneef, 1994a), may act specifically in the seed epidermis.

Mutants in *MUM1—5* were isolated in a screen of only approximately 1000 EMS lines. The fact that plants mutant in five genes affecting this process were found in such a small screen suggests that there may be many genes affecting this process. Indeed, this preliminary screen failed to find

mutants defective in any of the four previously known loci. A more extensive screen should yield many more mutants. In addition, it would be useful to screen populations mutagenized with either T-DNA or transposons. These methods of insertional mutagenesis result in gene disruption with a known piece of DNA that allows easy isolation of the flanking pieces of the gene of interest that, in turn, can be used as probes to isolate the complete gene from a wild type genome.

Phenotypic analysis of Ttg1, Gl2, Ap2, Ats, and Mum 1—5 seeds revealed several categories of defects. *MUM1* and *MUM2* appear to be involved in the production of mucilage, while *MUM4, GL2,* and *TTG1* affect both the production of mucilage and columellae. *MUM3* and *MUM5* appear to affect mucilage composition. Both Ap2 and Ats mutants have aberrant ovule development and unusually shaped seeds with abnormal amounts of mucilage (Jofuku *et al.*, 1994; Léon-Kloosterziel *et al.*, 1994; Bowman and Koornneef, 1994a), which may be a result of their integument defects. These possibilities will be explored in the following chapters of this thesis.

6. MUTANTS AFFECTING MUCILAGE EXTRUSION

6.1 Introduction

In Chapter 5, Mum1—5 and the mutants at previously isolated loci (Ttg1, Gl2, Ap2 and Ats) were roughly placed into four phenotypic groups. One of these groups consisted of Mum1 and Mum2 and was characterized both by the absence of a mucilage capsule when placed in water and a relatively normal seed coat. In this chapter these two mutants are examined further in order to discover the role of these two genes, with an emphasis on Mum2.

6.2 Mum1 and Mum2 mutants

6.2.1 Seed and mucilage phenotype

In order to determine better the defect in Mum1 and Mum2 plants, mature seeds were fixed under aqueous conditions and sectioned. The results for Mum1-1, 2-1, and 2-2 were identical. In each case, the columella was surrounded by pink-staining acidic polysaccharide within an intact outer tangential wall, resembling wild type seeds fixed under non-aqueous conditions (Figure 6.1a-d). Thus it appears that both Mum1 and Mum2 mutants are able to make and secrete acidic polysaccharides, but the outer tangential cell wall is not ruptured upon hydration of the seed. In addition, examination of the palisade and pigmented layers at this level showed that both were present (Figure 6.1a-d).

Mum2-1 seeds were subjected to various chemical and physical treatments to determine if the "mucilage" was being retained by a strengthened cell wall. Scarifying was attempted using three

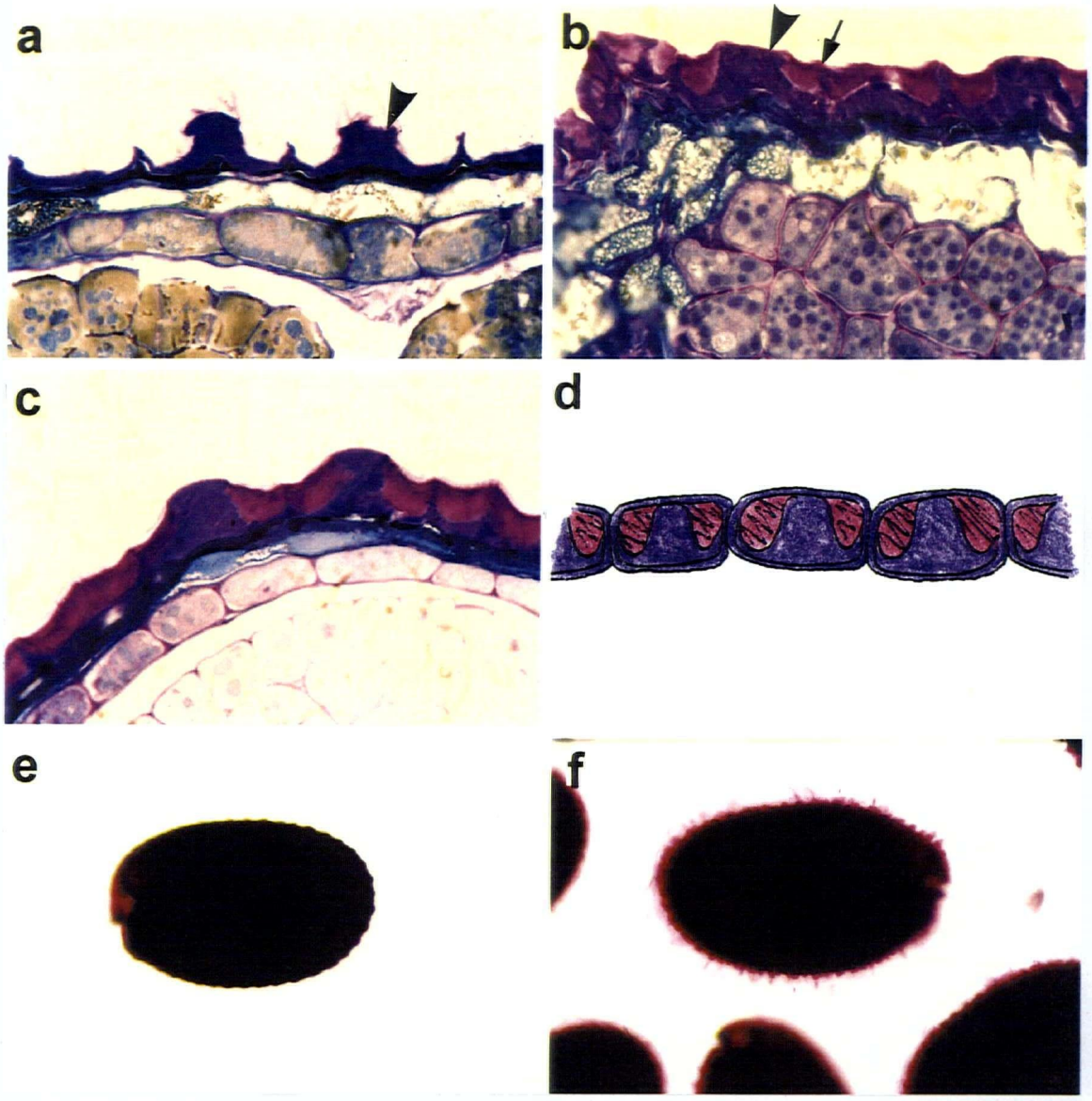

Figure 6.1. Cross sections of wild type, Mum1-1, and Mum2-1 seeds, and Mum2-1 seeds treated with EDTA.
All sections were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue. Mum2-1 seeds (e), (f) were stained with Ruthenium red.
(a) Wild type seed coat. The epidermal cells have burst open to release mucilage, so only bare columellae (arrowhead) are visible. (680x)
(b) Mum1-1 seed coat showing intact epidermal cells containing pink-staining mucilage (arrow), surrounding the columellae (arrowhead). (680x)
(c) Mum2-1 seed coat showing intact epidermal cells containing pink-staining mucilage. (690x)
(d) Cartoon of Mum1-1 and Mum2-1 epidermal cell phenotype showing mucilage retained between outer cell wall and columella.
(e) Mum2-1 seed after shaking in water and staining with Ruthenium red. Note lack of mucilage and columellae which are not visible in intact cells (d). (70x)

(f) Mum2-1 seed after shaking in EDTA and staining with Ruthenium red. The columellae are apparent and a small amount of pink-staining mucilage surrounds the seed. (70x)

techniques: slicing of seeds with razor blades, grinding seeds in a mortar and pestle, and treating seeds in 2M HCl. The results obtained by scarifying were equivocal. It was difficult to determine if mucilage was released from the small number of cells damaged by chopping and grinding, while acid treatment had no obvious effect. When Mum2 seeds were treated with a 0.5M solution of the chelator EDTA, however, a number of seeds were able to extrude mucilage (Figure 6.1e,f). This suggests that ionic bonds between Ca^{++} ions and the carboxyl groups of polygalacturonic acid may be involved in retaining the mucilage within the epidermal cells of Mum2 mutants.

6.2.2 Plant phenotypes

To determine if the recessive mutations in *MUM1* and *MUM2* caused pleiotropic effects in parts of the plant other than the seed, the phenotypes of different parts of the plant were determined. Trichomes and root hairs were investigated to see if they had other epidermal cell defects (like Ttg1 and Gl2), while the general plant phenotype, flowers, and ovules were studied to determine if the mutation led to more general development defects. In addition, the presence of root cap mucilage was investigated to determine if mucilage production was disrupted in another portion of the plant. Mum1-1, 2-1, and 2-2 plants were quite robust and had normal three-prong trichomes on their rosette leaves. In addition, all three had root cap mucilage and root hair number similar to wild type plants. Mum1-1 had normal flowers, ovules, and elongated siliques. More than half of both Mum2-1 and 2-2 plants, however, had some shortened siliques (26/35 and 5/6 plants, respectively), suggesting reduced fertility (Bouttier and Morgan, 1992; Modrusan *et al.*, 1994a). This was confirmed during fixation of developing Mum2-1 seeds where it was found that, in most

siliques, only a small number of seeds continued to develop past 4-5 daf. Investigation of the flowers and ovules of Mum2-1 plants showed that some plants with reduced siliques also had some abnormal ovules with short integuments (10/13), and/or aberrant flowers with shortened petals and stamens (4/13). Mum2-2 plants also had similar floral and ovule abnormalities. The appearance of these defects in two independently isolated Mum2 alleles suggests that reduced fertility and/or the inability to develop past early seed development are pleiotropic effects of altering MUM2 activity.

6.3 Seed coat development in Mum2-1

6.3.1 Seed coat development

Mum2-1 was chosen as a representative of this group to be studied in detail throughout development. SEM was used to follow external changes in collaboration with Debra Skinner. The external features of seed coat development differed very little between Mum2-1 and wild type (Figure 6.2; see Chapter 4.3.2). In both cases, the ovule underwent a shape change and growth during the first four to five days (Figure 6.2a-d). Some degenerating seeds and ovules were observed at these early stages (data not shown). At roughly 4 to 5 daf the hexagonal shape of the epidermal cells became apparent and amyloplasts were observed under the surface of the outer tangential cell wall (Figure 6.2c,d). The amyloplasts were later pulled into the center of the cells and disappeared as the columella formed around 9 to 10 daf (Figure 6.2e-h). In wild type seeds, mucilage became apparent surrounding the seeds at approximately 10 daf, which was also seen for some Mum2-1 seeds. At later stages, however, while the amount of mucilage increased around wild type seeds, it was no longer seen on Mum2-1 seeds under aqueous conditions (Figure 6.2i,j).

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c
d
e
f
g
h
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k
l

Figure 6.2. Scanning electron micrographs of developing wild type and Mum2-1 seeds.
All seeds were fixed in the silique in aqueous 3% glutaraldehyde, except (k) and (l), which were fixed after harvesting from the siliques.
(a) Mature (0 daf) wild type ovule.
(b) Mature (0 daf) Mum2-1 ovule.
(c) Wild type seed at 4 daf. The developing seed has increased in size and has changed in shape compared with the mature ovule. Hexagonal cells are apparent.
(d) Mum2-1 seed at 4 daf. Similar to wild type, the developing seed has increased in size and has changed in shape compared with the mature ovule.
(e) Wild type seed at 7 daf.
(f) Mum2-1 seed at 7 daf.
(g) Wild type seed at 10 daf. Columellae are apparent in centre of cells, the seed is covered in a small amount of white-staining mucilage.
(h) Mum2-1 seed at 10 daf. Note lack of mucilage.
(i) Wild type seed at 13 daf. There is more mucilage surrounding the seed than at 10 daf, however, the thickened radial cell walls and columellae of the epidermal cells are apparent.
(j) Mum2-1 seed at 13 daf. Unlike wild type, no mucilage is present, though the epidermal cells look normal.
(k) Wild type seed at maturity showing spikes of mucilage arising from each cell.
(l) Mum2-1 seed with no mucilage.
Magnification bars: (a),(b)=40 μm; (c)-(l)=100 μm.

Thick sections of developing Mum2-1 seeds also revealed little differences compared with wild type (Figure 6.3; see Chapter 4.3.2). In both cases, the epidermal cells underwent a large increase in size during the first four to five days, becoming largely vacuolar, with the cytoplasm around the edges of the cells (Figure 6.3a-d). Amyloplasts were apparent by 3 daf and became larger until 7 daf where they were found in the centre of the cell surrounded by pink-staining acidic polysaccharides (Figure 6.3c-f). "Mucilage" continued to accumulate in Mum 2-1 until 10 daf and stained a similar intensity to that of wild type cells (Figure 6.3g,h). Columellae were apparent in both wild type and Mum2-1 at approximately 10 daf and were more-or-less completely filled in by 13 daf (Figure 6.3g-j). At this time, however, the "mucilage" was retained inside the outer tangential wall of Mum2-1 epidermal cells while it was released from almost all wild type cells (Figure 6.3i,j). A similar phenotype was seen at maturity where wild type epidermal cell walls had burst open to release the mucilage, while in Mum2-1 the cell walls remained intact, holding the "mucilage" within the outer tangential cell wall (Figure 6.3k,l).

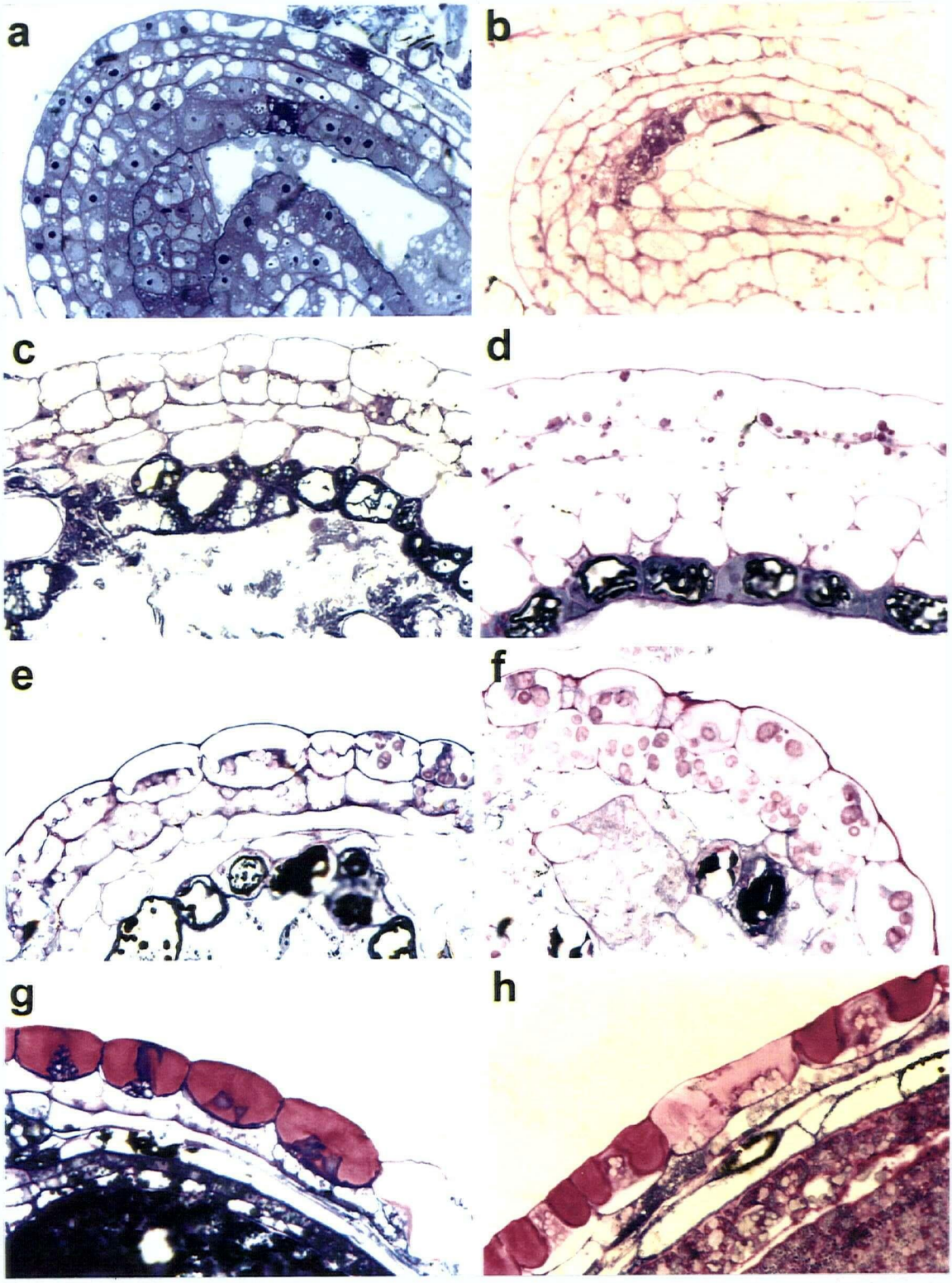
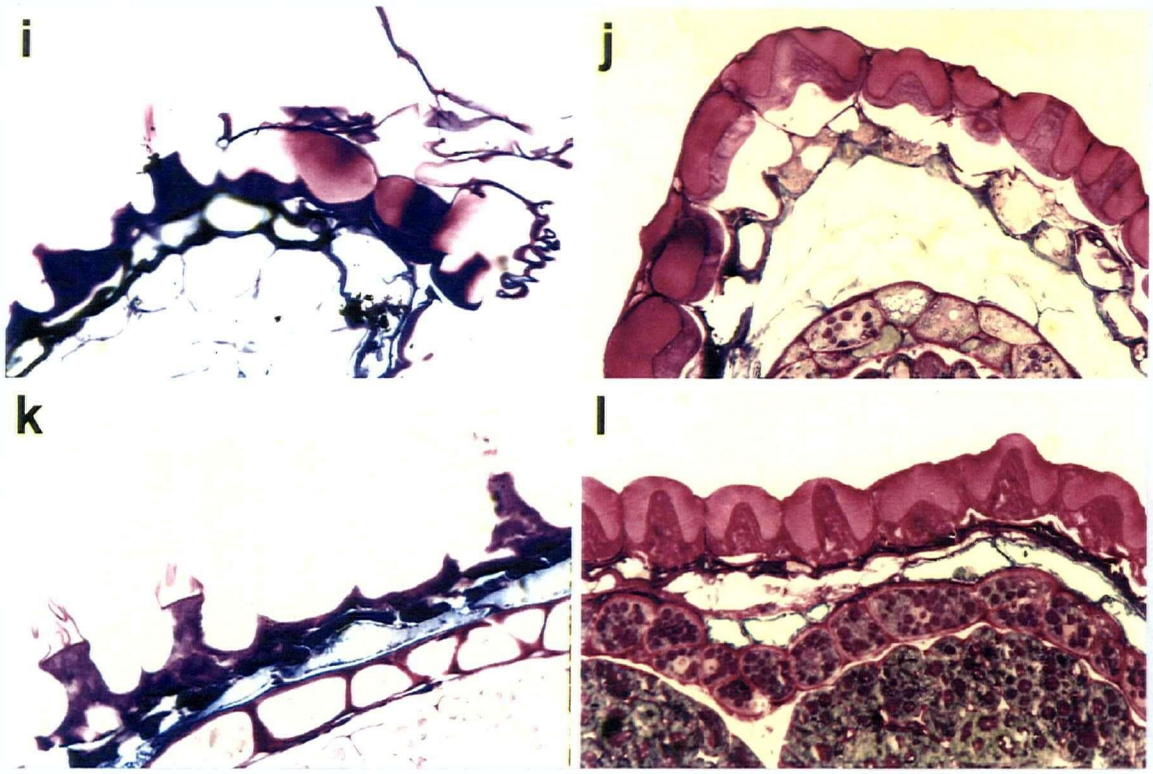


Figure 6.3. A comparison of wild type and Mum2-1 seed coat development.
Samples were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue.

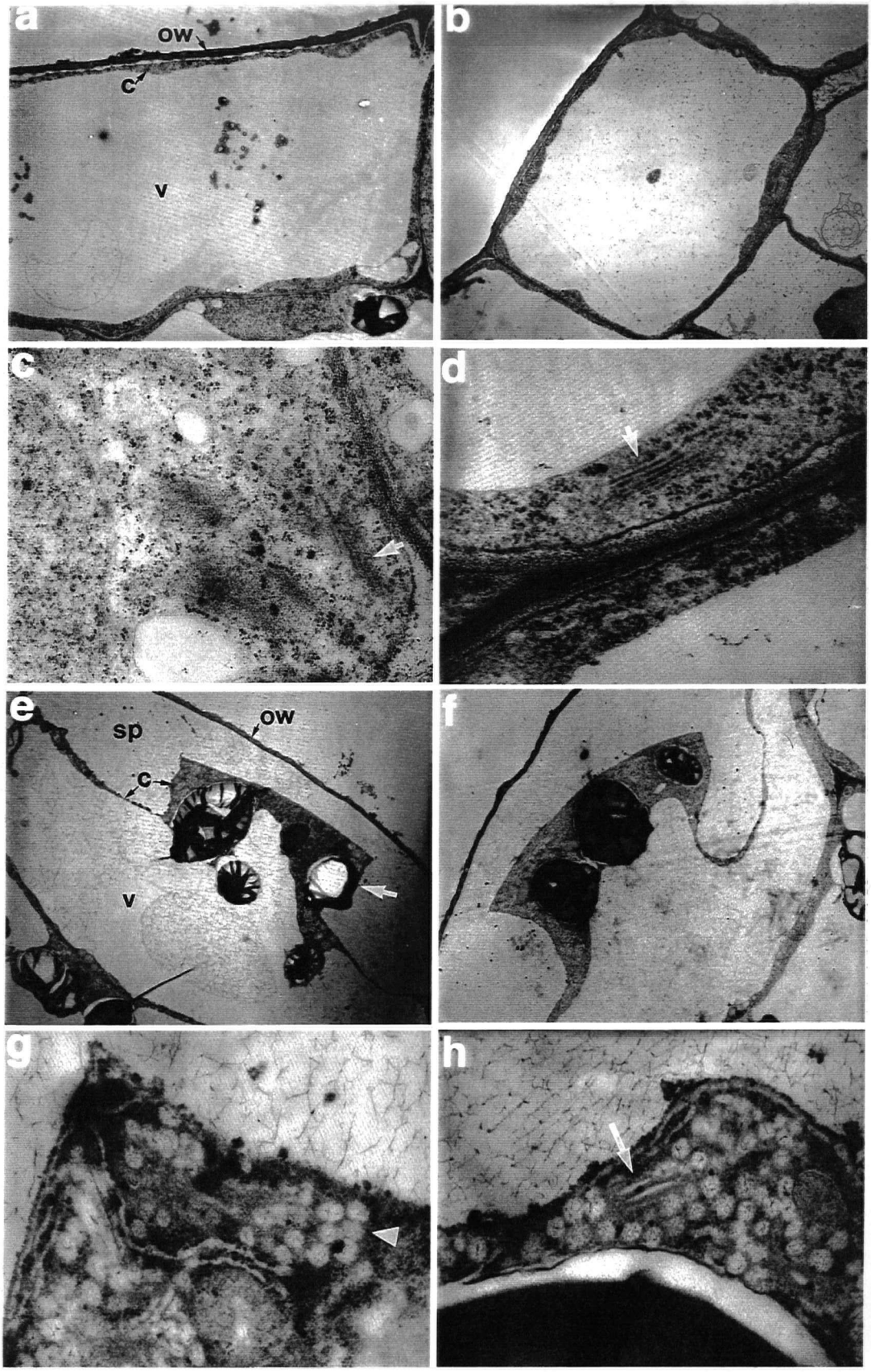


(a) Wild type mature ovule (0 days after fertilization [daf]). (750x)
(b) Mum2-1 mature ovule (0 daf). (720x)
(c) Wild type seed at 4 daf. Note small amyloplasts in cells. (540x)
(d) Mum2-1 seed at 4 daf. Similar to wild type, there are large vacuoles and amyloplasts are starting to accumulate. (690x)
(e) Wild type seed at 7 daf. The amyloplasts are larger and found in centre of cell. Faint pink stain is apparent throughout cell. (420x)
(f) Mum2-1 seed at 7 daf. Mucilage also stains faintly throughout the epidermal cells. (430x)
(g) Wild type seed at 10 daf. Intense pink staining polysaccharide in epiderml cells and amyloplasts have reduced in size and are at the bottom of cell. Purple staining cell wall material can be seen in centre of cell. (430x)
(h) Mum2-1 seed at 10 daf. Intensely-staining mucilage is also present and amyloplasts can be seen in a column in the centre of the cell. (410x)
(i) Wild type seed at 13 daf. The outer cell wall of most epidermal cells has burst, releasing mucilage. In the cells that are still partially intact, dark pink mucilage is present. The columellae are completely filled in. (430x)
(j) Mum2-1 seed at 13 daf. Cells are intact and mucilage can be seen on either side of the columellae. (440x)
(k) Wild type seed at 18 daf. 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 columellae. (680x)
(l) Mum2-1 seed at 18 daf. Unlike wild type, the outer epidermal cell walls have remained intact, holding mucilage around the columellae. (430x)

The development of the palisade layer in Mum2-1 also seemed to correlate with that of wild type (Figure 6.3; see Chapter 4.3.4). Amyloplasts gathered starting at 4 daf and increased until 7 daf, at which point there appeared to be some thickening of the lower tangential cell wall of the palisade cells (Figure 6.3c-f). The thickening was increased at 10 daf and then appeared to undergo a colour change to reflect the presence of lignin (Figure 6.3g-j). Similarly, the pigmented layer appeared to undergo normal development in Mum2-1 seeds (Figure 6.3; see Chapter 4.3.4). These results suggest that at the level of the tissue, seed coat development is normal in Mum2-1.

6.3.2 Cytology of epidermal cell development

The retention of "mucilage" within the outer tangential cell wall of the epidermis of Mum2 mutants may result from a change in the composition or amount of mucilage. Either deficiency could lead to hydrated mucilage yielding an insufficient amount of pressure to the burst the cell wall. Thus, one important question regarding the phenotype of Mum2-1 was whether the pink-staining acidic polysaccharide was mucilage and, if so, was it found in a similar amount to wild type mucilage. One technique used to investigate this was to study mucilage production using TEM. A developmental analysis of ultrastructure could reveal if the polysaccharide was synthesized and secreted like wild type mucilage, and if there was a gross change in the amount of mucilage fibrils and secretion machinery. Epidermal cell development in Mum2-1 was very similar to that that of wild type (Figure 6.4; see Chapter 4.4.1), but there appeared to be more variability in the stages, possibly due to the problems with fertility. As seen in wild type, at 4 daf the cytoplasm was found around the edges of the cell, the Golgi that were present had thin cisternae, and some fibrils were seen in the vacuole (Figure 6.4a-d). At 7 daf, when pink-staining acidic polysaccharides were present in the thick sections, the large number of the fibrils seen in wild type were also present in Mum2-1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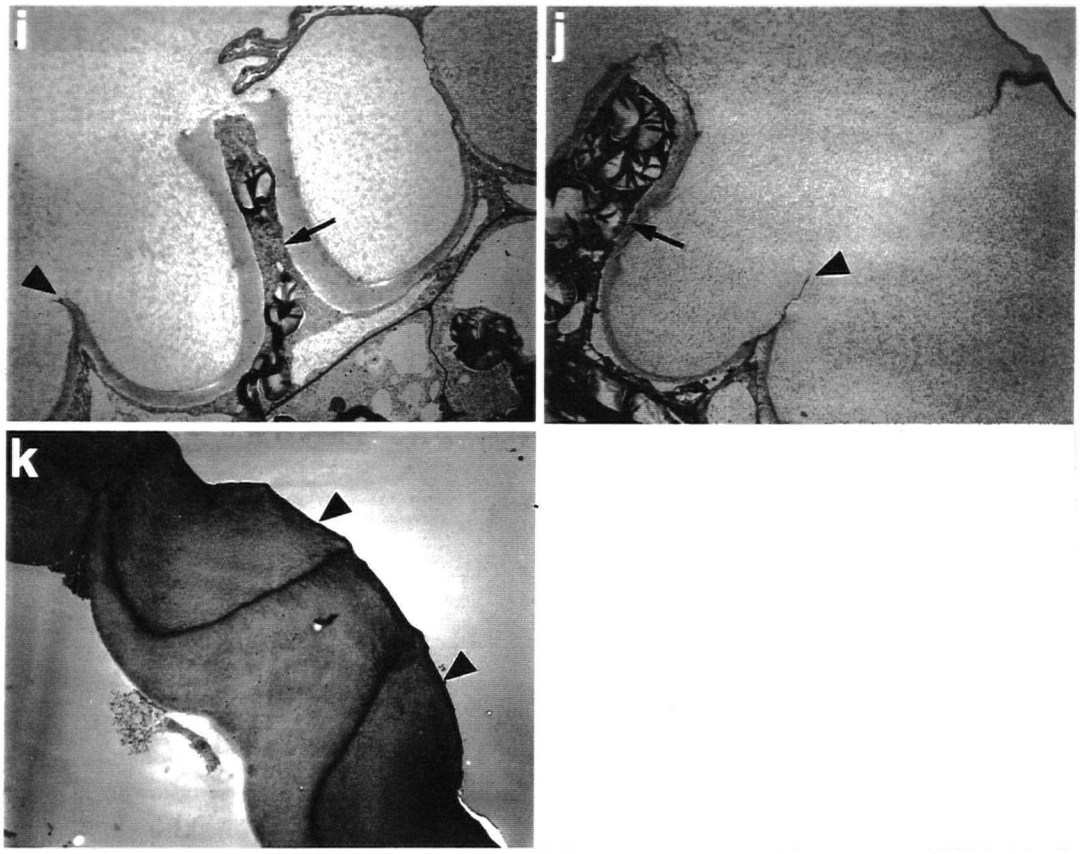


Figure 6.4. Transmission electron micrographs of developing epidermal cells in wild type and Mum2-1 seed coats.
(a) Wild type epidermal cell at 4 daf, note large vacuole (v) and cytoplasm (c) found around the edges of the cell (ow = outer cell wall). (4100x)
(b) Mum2-1 epidermal cell at 4 daf. (2500x)
(c) Wild type: close-up of cytoplasm at 4 daf with arrow showing a Golgi stack. (26,700x)
(d) Mum2-1 4 daf cytoplasm at base of cell showing Golgi stack (arrow). (36,400x)
(e) Wild type cell at 7 daf. Most of the cytoplasm (c) is in a column in the centre of the cell. The space (sp) between the cytoplasm and the outer cell wall (ow) is filled with fibrillar material (see (g)) and below the cytoplasm is a vacuole(v). Note very large amyloplasts (arrow). (2200x)
(f) Mum2-1 seed at 7 daf. Note cytoplasmic column and amyloplasts have very similar appearance to those in wild type cells. (2200x)
(g) Close-up of wild type cell at 7 daf showing top corner of cytoplasmic column. The cytoplasm is filled with vesicles (arrowhead) and tubular clearings. (33,100x)
(h) Cytoplasm in 7 daf Mum2-1 cell, showing a large quantity of fibril-containing vesicles and an enlarged Golgi stack (arrow). (32,800x)
(i) Wild type cell at 10 daf. The cytoplasm (arrow) is found in the centre of the cell and at the bottom, surrounded by new cell wall. 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 reinforcement with electron dense material ended (arrowhead). (2100x)

(j) Mum2-1 cell at 10 daf. Similar to wild type cells, a column of cytoplasm (arrow) surrounded by a new cell wall is present. The outer cell wall has ruptured at a thin point above the secondary cell wall (arrowhead). (2100x)
(k) Mum2-1 cell at 14 daf showing the retention of mucilage (arrowheads) between an intact outer cell wall and the columella. (2100x)

epidermal cells. The cytoplasm was also in a column in the center of the cell and was filled with the vesicles containing fibrillar material (Figure 6.4e-h). These vesicles appeared to be budding off Golgi stacks and exocytosing to release their contents into the space between the plasma membrane and the outer tangential cell wall (Figure 6.4g,h). Thus, by analogy to wild type, it appears that some form of pectin is being made in the Golgi stacks and secreted in Mum2-1. In both wild type and Mum2-1 seeds there was an increased number of fibrils during development, reflecting the accumulation of mucilage. Thus, a crude quantitation of the amount of "mucilage" was performed by counting the number of fibrils in a 2.0 x 2.2 cm square (magnification = 40,000x) in four cells each of wild type and Mum2-1 at 7 daf. There was little difference in the number of fibrils counted in this manner (105 +/- 24 and 116 +/- 17, respectively), suggesting that there might not be much difference in the amount of mucilage produced in Mum2-1 seeds.

The retention of mucilage within the epidermal cells of Mum2-1 might also result from a change in the radial cell walls where breakage occurs to allow mucilage extrusion in wild type (Figure 6.4i). These radial cell walls may be thicker in Mum2-1 than in wild type and thus the normal amount of mucilage might not create enough pressure to rupture the wall. A study of the thickness of the radial cell walls at the approximate location of the breakage was undertaken both in wild type and Mum2-1 at 7 and 10 daf. There was very little difference in cell wall thickness between wild type and Mum2-1 cells at both of these stages (7 daf=75.0 +/- 27.5 nm and 82.5 +/- 20.0 nm, respectively; 10 daf=42.5 +/- 5.0 nm and 50.0 +/- 15.0 nm, respectively, n=5 or 6 cells each), suggesting that if there is a change in the cell wall it is not obvious at this level. While observing 10 daf seeds, it was found that, as seen in the SEM studies, the epidermal cells of 10 daf Mum2-1 outer

tangential cell walls can burst to release the mucilage (Figure 6.4j). By 14 daf, however, this ability to extrude mucilage was lost in Mum2-1 seeds (Figure 6.4k).

As expected from the normal appearance of the seed coat of Mum2-1 seeds as viewed by SEM, columella production appears to occur normally in the Mum2-1 epidermal cells (Figure 6.4; see Chapter 4.4.2). At 7 daf the cytoplasm is found in a centrally localized column with sharp edges, while at 10 daf this column became surrounded with new cell wall material to form the columella (Figure 6.4e,f,i,j). At 14 daf the columella was more-or-less completely filled in with cell wall material (Figure 6.4k).

6.4 Chemical analysis of Mum1 and Mum2 mucilage

Gas chromatography was used in order to determine if there was a change in the amount or monosaccharide composition of the acidic polysaccharides of both Mum2-1 and Mum1-1. Due to the retention of the "mucilage" within the epidermal cells under aqueous conditions, whole seeds were ground and hydrolyzed with acid to isolate monosaccharides for derivitization with TMS. The comparison of the monosaccharide composition of Mum2-1 seeds with wild type seeds demonstrated that there was no significant difference in the amount of each of the components studied (Table 6.1; Figure 6.5). These data suggest that Mum2-1 makes normal mucilage in the usual quantity.

When Mum1-1 seeds, however, were compared with wild type the amount of almost all components increased 1.5 to 2 fold (Table 6.1; Figure 6.6). Mum1-1 seeds were not significantly larger than wild type seeds (length= 472 +/- 34□m [n=4] and 502 +/- 26□m [n=10], respectively). Thus, these results suggest that Mum1-1 seeds manufacture an increased amount of both mucilage and/or other cell wall components per cell.

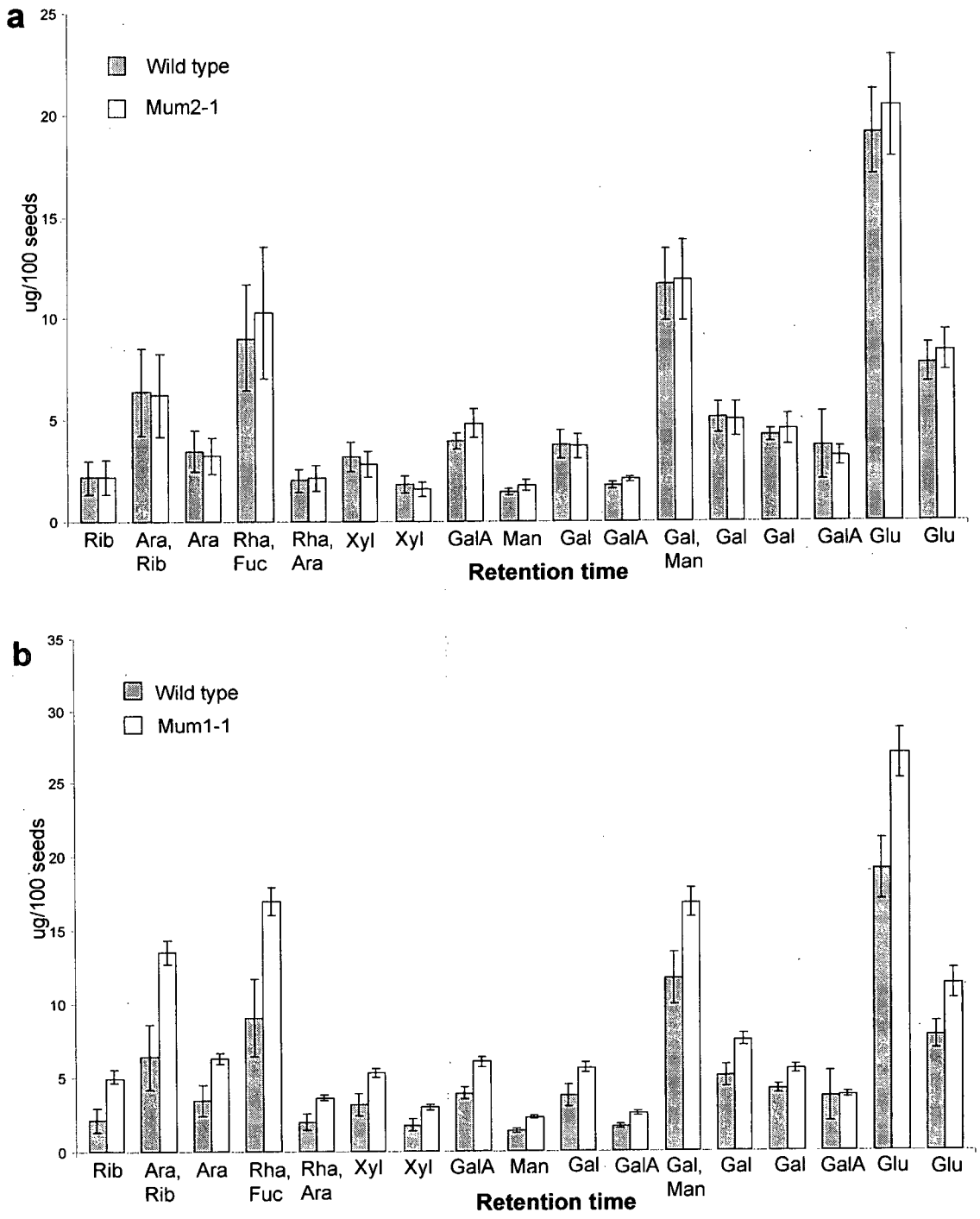


Figure 6.5. A comparison of the major peaks observed using gas chromatography for Col-2, Mum1-1, and Mum2-1 whole seeds.
(a) Mum2-1 versus wild type (number of samples = 3 and 4, respectively).
(b) Mum1-1 versus wild type (number of samples = 3 and 4, respectively).
Rib=ribose, Ara=arabinose, Rha=rhamnose, Fuc=fucose, Xyl=xylose, GalA=galacturonic acid, Man=mannose, Gal=galactose, Glu=glucose.

Table 6.1. A comparison of the values of major peaks observed using gas chromatography for Col-2, Mum1-1, and Mum2-1 whole seeds.[a]

		Col-2		Mum1-1		Mum2-1	
Retention time	Sugar	Average (ug/100 seeds)	Standard deviation	Average (ug/100 seeds)	Standard deviation	Average (ug/100 seeds)	Standard deviation
6.66	Ribose	2.15	0.82	4.98	0.35	2.16	0.86
6.72	Arabinose, Ribose	6.40	2.17	13.50	0.80	6.22	2.06
6.87	Arabinose	3.45	1.03	6.26	0.36	3.22	0.89
7.11	Rhamnose, Fucose	9.04	2.62	16.99	0.97	10.31	3.27
7.29	Rhamnose, Arabinose	2.00	0.55	3.65	0.19	2.11	0.62
8.14	Xylose	3.17	0.74	5.31	0.27	2.78	0.64
8.38	Xylose	1.79	0.40	3.00	0.19	1.56	0.35
9.54	Galacturonic acid	3.94	0.40	6.02	0.33	4.81	0.71
9.92	Mannose	1.40	0.16	2.32	0.08	1.72	0.26
10.02	Galactose	3.76	0.71	5.63	0.32	3.67	0.61
10.06	Galacturonic acid	1.72	0.16	2.56	0.14	2.04	0.30
10.48	Galactose, Mannose	11.66	1.80	16.85	1.01	11.86	2.02
10.92	Galactose	5.10	0.75	7.49	0.43	5.01	0.83
11.03	Galactose	4.22	0.31	5.55	0.32	4.53	0.76
11.17	Galacturonic acid?	3.71	1.68	3.80	0.20	3.15	0.48
11.27	Glucose	19.11	2.08	26.99	1.72	20.40	2.51
11.42	?	2.52	0.19	2.80	0.15	2.74	0.26
11.51	Glucose	7.76	0.97	11.31	0.58	8.38	1.02
13.55	Octadecadienoic acid	8.92	2.44	12.21	0.48	10.54	1.14
13.63	?	10.76	1.57	14.80	0.50	13.03	1.34
13.82	?	6.23	6.19	15.42	9.13	6.88	5.88
14.00	Inositol	48.00	0.00	48.00	0.00	48.00	0.00
16.12	?	4.92	1.60	6.92	0.38	6.21	0.63

[a]Samples numbers: Col-2=4, Mum1-1=3, Mum2-1=3)

6.5 Discussion

6.5.1 *MUM2* may regulate cross-linkages in mucilage or cell walls

The only observable difference between the epidermal cells of wild type and Mum2 mutants is the absence of mucilage extrusion under aqueous conditions upon the approach of maturity. Gas chromatographic analysis of the mucilage from Mum2-1 seeds showed that the monosaccharide composition of the mucilage and its amount fall within the normal range. The lack of mucilage extrusion thus could be due to increased cross linkages within the mucilage that curtail its expansion.

Alternative explanations include strengthening of the epidermal cell walls, such that they require greater pressure to break, or the absence of programmed weakening of the epidermal cell walls. The latter explanation appears to have a precedent in the active weakening of epidermal cell walls in tomato seeds in order to make seed hairs (Hayward, 1967). The ability of Mum2-1 seeds to release mucilage around 9 to 10 daf but not after 13 daf suggests, however, that is not the case; rather, something is occurring at 12 to13 daf to prevent mucilage extrusion after this time. This result also suggests that increased thickness of the radial walls would not be expected as this change is occurring after the synthesis of the columella and it would be unlikely for a cell to transport extra cell wall material through a secondary cell wall. Strengthening of the outer wall, however, could be effected by increased cross-linking between the microfibrils through xyloglucan bridges (Carpita and Gibeaut, 1993; Cosgrove, 1997; Levy *et al.*, 1997). Conversely, as noted above, the mucilage may have increased cross-linking and this is supported by the release of mucilage in Mum2-1 seeds placed in EDTA. EDTA is a heavy metal chelator that can disrupt the calcium bridges between polygalacturonic acid chains (Frey-Wyssling, 1976; Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997). Thus, if Mum2-1 seeds have an increased number of these ionic bonds between pectin molecules that prevent proper mucilage expansion, these would be expected to be disrupted by treatment with EDTA, allowing greater mucilage swelling and breakage of the outer cell wall. Strengthening of the outer cell wall, however, cannot be ruled out since EDTA may also loosen it and/or allow enough expansion of normally cross-linked mucilage to break a stronger cell wall. *MUM2* thus may code for a negative regulator of xyloglucan cross-linking of cellulose, or a negative regulator of pectin methyl esterases which de-esterify pectin to allow for more calcium bridges (Frey-Wyssling, 1976; Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997; Levy *et al.*, 1997).

6.5.2 *MUM2* also regulates ovule development

The reduced number of mature seeds suggests that Mum2 plants are partially sterile. The appearance of occasional ovules with shortened integuments suggests that this could be due to a problem in ovule development with reduced expressivity. This may result in a mixture of fertile ovules, ovules with megagametophyte defects, and ovules with both overtly abnormal integuments and embryo sacs. A direct connection between the seed coat phenotype and reduced fertility is not obvious. The seed coat defects could be a downstream effect of abnormal integuments or *MUM2* could be acting at two different times as seen for many developmental regulators (Bowman *et al.*, 1991; Haughn *et al.*, 1995; Sieburth *et al.*, 1995).

6.5.3 Mum1 mutants have excess cell wall material

Investigation of the mature seed phenotype of Mum1 showed that, similar to Mum2, mucilage was made but not extruded from the epidermal cells. This appears to be a seed specific phenotype as no other gross defects were seen in these plants. Chemical analysis of Mum1 demonstrated that there was a 1.5 to 2 fold increase in almost all of the cell wall components extracted from the seeds relative to wild type seeds. A comparison of the sugars of wild type mucilage with that of whole wild type seeds showed that at least 40-60% of the galacturonic acid present in whole seeds was found in mucilage, while only 3% of glucose was derived from mucilage (Chapter 4.2.3). Since both components increase by the same proportion, Mum1 seeds thus appear to make not only increased mucilage but also more cellulose or other glucans. Mum1 seeds are not significantly larger than wild type seeds, suggesting that these components have been incorporated into normal-sized cells. The increased polysaccharide deposition may have occurred throughout

development, leading to thicker cell walls everywhere, including the epidermal cells and the "secondary" cell wall of mucilage. These thickened cell walls then could account for the lack of mucilage extrusion. Investigation of cell wall thickness in the seed epidermis and other tissues of the plant may reveal that *MUM1* is a negative regulator of cell wall polysaccharide biosynthesis.

Conversely, the extra sugars could be derived solely from the epidermal cells of the seed. Under this hypothesis, the whole cell wall production and modification machinery may be excessively up-regulated during epidermal cell differentiation, not only leading to extra pectin and cellulose biosynthesis during mucilage and columella production, but also increased amounts of cross-linking enzymes. Thus, the explanation for mucilage retention itself would be similar to that of Mum2 mutants.

7. MUTANTS AFFECTING MUCILAGE EXTRUSION AND COLUMELLA PRODUCTION

7.1 Introduction

As seen in Chapter 5, Mum4 mutants had a similar mucilage and seed phenotype to Gl2 and Ttg1. All three lacked mucilage extrusion when placed in water and SEM of their seeds revealed reduced or absent columella. These mutants are more fully investigated in this chapter.

7.2 Mum4, Ttg1, and Gl2 mutants

7.2.1 Seed and mucilage phenotype

To further characterize the mature seed phenotypes of Mum4, Gl2, and Ttg1, sections stained with Toluidine blue were compared with wild type seeds (Figure 7.1). As mentioned in Chapter 5, all of the Mum mutants were isolated from Col-2, while Ttg1-1 and Gl2-1 were found in Ler. Under aqueous conditions, wild type seeds of both ecotypes released mucilage from their epidermal cells, leaving behind bare columellae (Figure 7.1a). The mature seed phenotypes of Mum4-1, Gl2-1, and Ttg1-1 were similar, with intact cells containing little or no "mucilage" around flattened columellae (Figure 7.1b-d). By contrast, some Mum4-2 seeds released their "mucilage" and the columellae more closely resembled wild type in shape (Figure 7.1e). The release of "mucilage" from some Mum4-2 seeds and the larger columellae suggest that it is a weaker allele than Mum4-1. Thus, as a group, these mutants appear to make at least some "mucilage" in their epidermal cells, but lack the ability to extrude it or form full-sized columellae (Figure 7.1f). With the exception of Ttg1

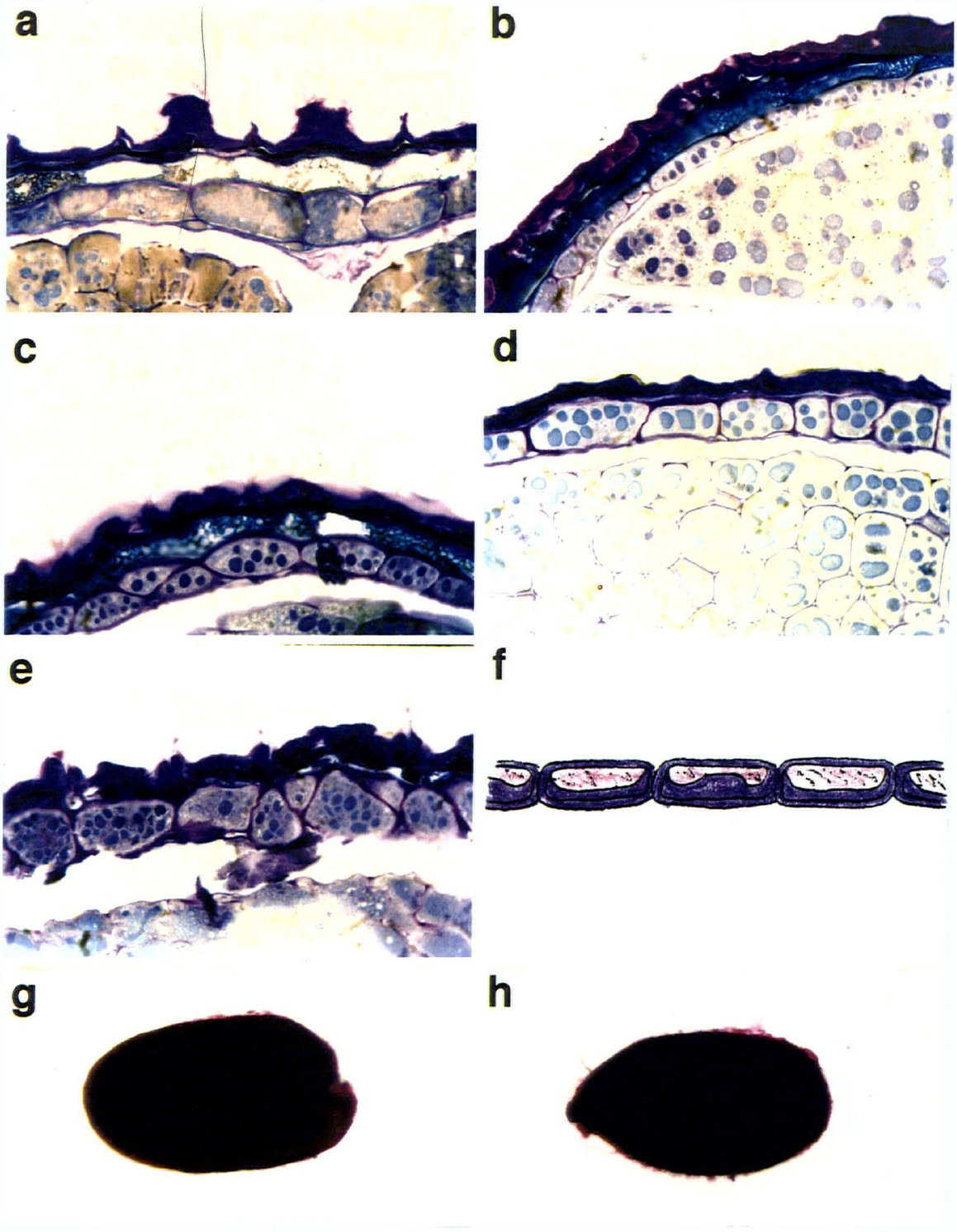


Figure 7.1. Cross sections of wild type, Mum4-1, Mum4-2, Ttg1-1, and Gl2 seeds, and Mum4-1 seeds treated with EDTA.
All sections were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue. Mum4-1 whole seeds (g), (h) were stained with Ruthenium red after treatment with EDTA.

(a) Wild type seed coat. The epidermal cells have burst open to release mucilage, so only bare columellae are visible. (680x)
(b) Mum4-1 seed coat showing intact epidermal cells containing pink-staining mucilage and flat columellae. (460x)
(c) Gl2-1 seed coat showing reduced columellae and faint, pink-staining mucilage. (700x)
(d) Ttg1-1 seed coat showing very reduced columellae, no mucilage is apparent. (690x)
(e) Mum4-2 seed coat showing flattened columellae, epidermal cells have burst open to release mucilage. (690x)
(f) Cartoon of Mum4-1, Gl2, and Ttg1 epidermal cell phenotype showing intact cells with flat or reduced columellae and a small amount of mucilage.
(g) Mum4-1 seed after shaking in water and staining with Ruthenium red. Note lack of mucilage and rays are not visible in intact cells. (70x)
(h) Mum4-1 seed after shaking in EDTA and staining with Ruthenium red. A few rays are apparent and very little pink-staining mucilage is seen in these areas. (70x)

mutants, which are known to lack anthocyanins in the pigmented layer of the seed (Koornneef, 1981), both palisade and pigmented layers appeared normal in these four mutants (Figure 7.1a-e).

In order to determine if the "mucilage" retention in Mum4-1 was due to strengthening of the outer tangential cell wall, the seeds were subjected to both physical and chemical scarification. As seen for Mum2-1 (see Chapter 6.2.1), the results were equivocal. Slicing or grinding of the seed possibly released some mucilage but this was difficult to confirm due to the small number of cells involved, while the acid treatment led to no mucilage release. Similar to Mum2-1, however, treatment of seeds with the chelator EDTA led to some release of mucilage, suggesting that ionic bonds may be involved in the retention of mucilage within the cell wall (Figure 7.1g,h).

7.2.2 Plant phenotypes

Both Ttg1 and Gl2 mutants were initially isolated for their trichome abnormalities: Ttg1 mutants completely lack trichomes, while Gl2 plants can have either reduced trichomes or enlarged epidermal cells (Koornneef, 1981; Haughn and Somerville, 1988; Rerie *et al.*, 1994; Hülskamp *et al.*, 1994). In contrast to their trichome phenotypes, both Ttg1 and Gl2 make too many root hairs

(Galway *et al.*, 1994; Masucci *et al.*, 1996) and Ttg1 mutants lack anthocyanin pigments (Koornneef, 1981). Other than these epidermal cell abnormalities, both of these mutants are normal plants (Koornneef, 1981; Rerie *et al.*, 1994).

Mum4 plants were studied to determine if mutations in *MUM4* led to seed specific or pleiotropic defects. Mum4-1 plants were robust with normal trichomes, root hairs, and root cap mucilage. An initial survey revealed that 22/23 plants had normal, extended siliques, suggesting normal ovule and seed fertility, which was confirmed in dissection for seed development studies. The phenotype of Mum4-1 thus appears to be seed specific. Mum4-2 plants were also normal for their trichomes, root hairs, root cap mucilage, flowers, and extended siliques. The majority of Mum4-2 plants, however, had reduced or very reduced apical dominance, leading to short plants with multiple inflorescences (5/6), while the remaining plant was quite spindly. Since reduced apical dominance was not observed in Mum4-1 and the Mum4-2 mutant was not backcrossed, this additional phenotype of Mum4-2 plants was likely due to a background mutation.

7.3 Seed coat development

7.3.1 Seed coat development in Mum4-1, Ttg1-1, and Gl2-1

Seed coat development was studied in Mum 4-1, Ttg1-1, and Gl2-1 using Toluidine blue-stained sections. All three gave similar results, thus only Mum4-1 will be described in detail (Figure 7.2). In this experiment, the timing of Mum4-1 seed development lagged behind that of wild type. This lag, however, was not seen in another population of Mum4-1 plants, suggesting that it was due to slow growth in the set of plants used for sectioning. During the first four days, Mum4-1 development was identical to wild type, with enlargement and vacuolization of the epidermal cells

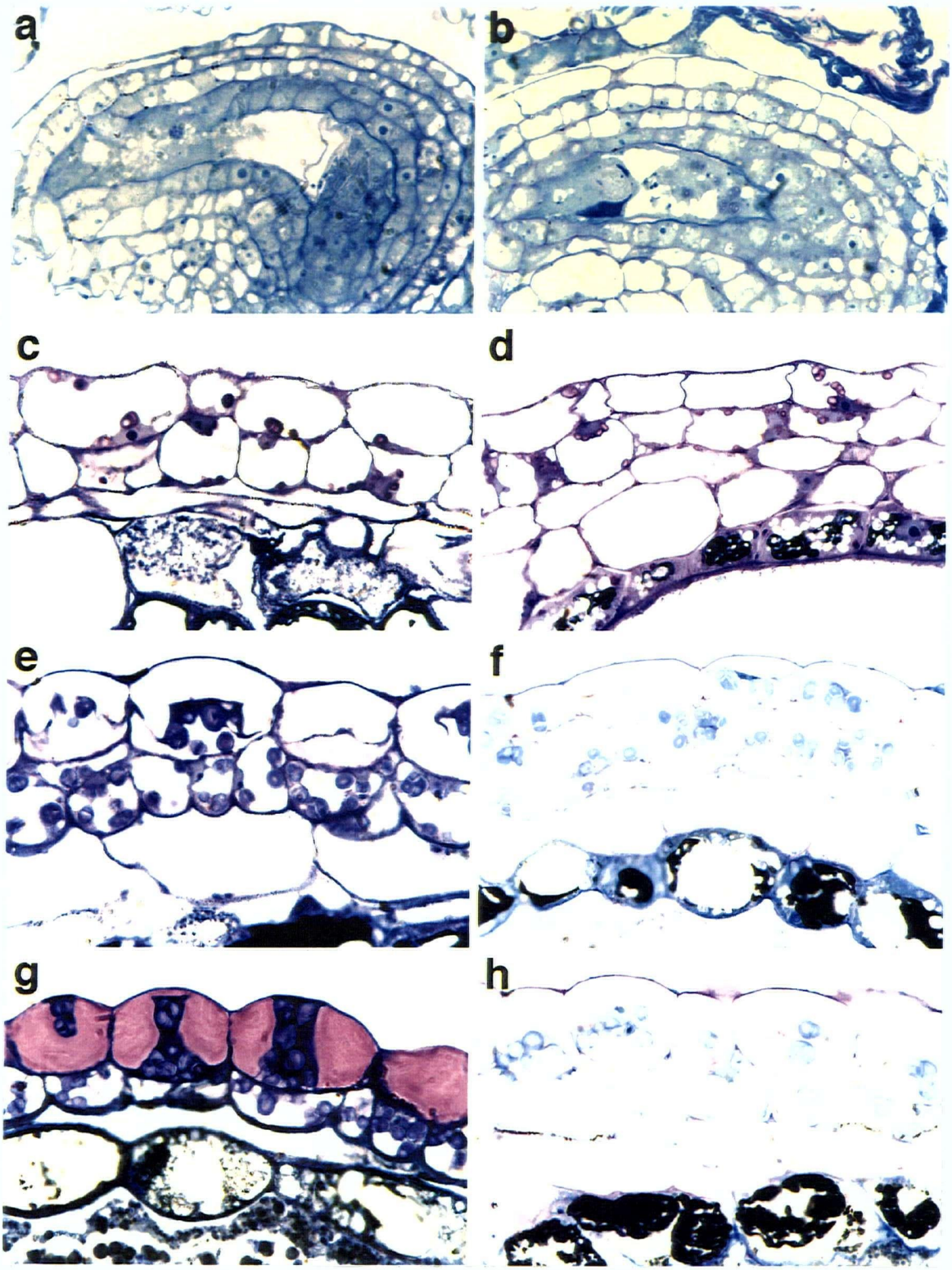
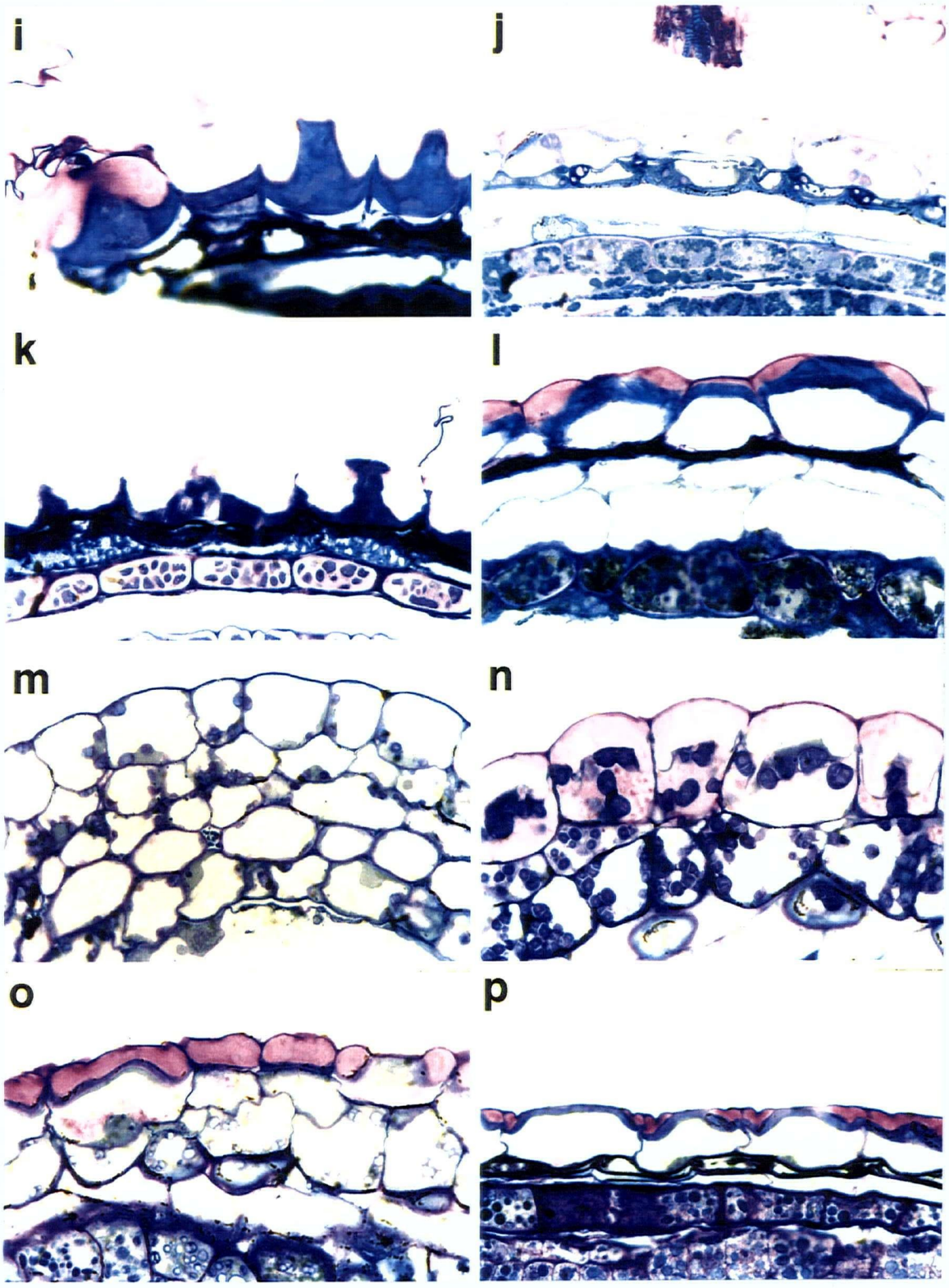


Figure 7.2. A comparison of wild type, Mum4-1, and Ttg1-1 seed coat development.
Samples were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue.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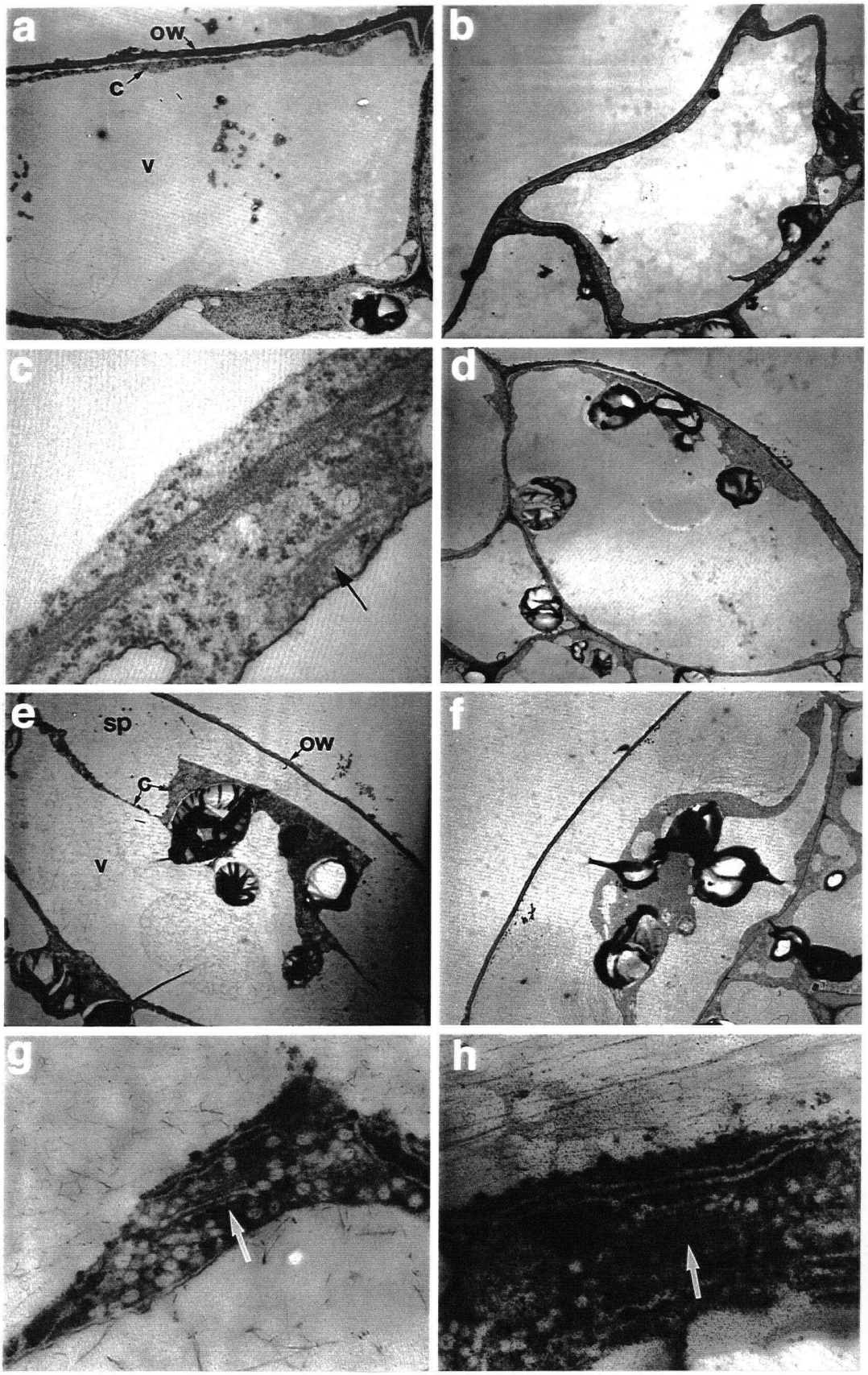
(a) Wild type mature ovule (0 days after fertilization [daf]). (670x)
(b) Mum4-1 mature ovule (0 daf). (690x)
(c) Wild type seed at 4 daf. Note small amyloplasts in cells. (660x)
(d) Mum4-1 seed at 4 daf. Similar to wild type, there are large vacuoles and amyloplasts are starting to accumulate. (680x)
(e) Wild type seed at 7 daf. The amyloplasts are larger and found in centre of cell epidermal cells, which also have faint pink staining. (670x)
(f) Mum4-1 seed at 7 daf. No mucilage yet apparent, though amyloplasts are larger and cytoplasm is now found in line across centre of epidermal cells. (680x)
(g) Wild type seed at 10 daf. Intense pink staining polysaccharide in cell and amyloplasts are in a column in the centre of epidermal cells. (690x)
(h) Mum4-1 seed at 10 daf. A small amount of mucilage is also present and amyloplasts can be seen in the centre of the epidermal cells. (680x)
(i) Wild type seed at 13 daf. The outer cell wall of epidermal cells has burst, releasing mucilage. In the cell that is still partially intact, dark pink mucilage is present. The columellae are completely filled in. (690x)
(j) Mum4-1 seed at 13 daf. Epidermal cells are intact and new cell wall material is present in a dome over the cytoplasm and a large vacuole. Some mucilage is present between the new cell wall and the outer cell wall. (690x)
(k) Wild type seed at 18 daf. The outer cell wall of the epidermal cells has burst and mucilage has been released to surround the seed. (670x)
(l) Mum4-1 seed at 18 daf. Unlike wild type, the outer epidermal cell walls have remained intact, holding mucilage around the flattened columella. A large white space is present in the bottom of the cell, probably due to the seed not being fully dehydrated. (690x)
(m) Ttg1-1 seed at 4 daf. Similar to wild type and Mum4-1, there are large vacuoles and amyloplasts are starting to accumulate. (700x)
(n) Ttg1-1 seed at 7 daf. Mucilage is starting to accumulate in the epidermal cells and amyloplasts are found mostly in the centre of the cells. (670x)
(o) Ttg1-1 seed at 10 daf. Like Mum4-1, the epidermal cells appear to have a large vacuole above which the secondary cell wall is forming. Mucilage is also present within the intact outer cell wall. (690x)
(p) Ttg1-1 seed at 13 daf. Some mucilage and a flattened columella are present above a large white space, presumably the vacuole. (700x)

paired with the accumulation of amyloplasts (Figure 7.2a-d). This was followed by enlargement of the amyloplasts and accumulation of pink-staining mucilage (Figure 7.2e-h). Differences between wild type and Mum4-1 development became evident when new cell wall laid down around the cytoplasm formed a peaked dome over a large vacuole in Mum4-1 mutants, rather than a volcano-shaped columella (Figure 7.2i,j). "Mucilage" was also present at the very top of the cell on either side of the domed columella and the outer tangential cell wall was intact. 13 and 18 day old wild type epidermal cells had burst open to release the mucilage and tall, upright columellae were readily

apparent (Figure 7.2i,k). By contrast, 18 daf Mum4-1 seeds had one of two phenotypes. Some resembled the mature seeds, with "mucilage" surrounding reduced columellae in intact cells (Figure 7.1b), while others had both the columellae and "mucilage" at the top of the cell with a large white space underneath, suggesting that the seeds might not have fully desiccated at the time of fixation (Figure 7.2l). In both cases, the columellae were quite short and the "mucilage" found in the narrow space around them stained a similar intensity to that found in intact wild type cells. These results suggest that several processes are defective in developing Mum4-1 (Figure 7.2 a-l), Ttg-1 (Figure 7.2m-p), and Gl2 (data not shown) epidermal cells. First, a reduced amount of an acidic polysaccharide may be made, second, a large vacuole is retained, and third, while a columella is made, it is lower and dome-shaped rather than upright as in wild type.

7.3.2 Cytology of epidermal cell development in Mum4-1

The results of light microscopy suggested that a reduced amount of "mucilage" was produced in these mutants. TEM of Mum4-1 epidermal cells was used to determine if there was a difference in mucilage production in these cells (Figure 7.3). Similar to wild type, at early stages, the cytoplasm was found around the edges of the cells and Golgi stacks, when present, appeared to have thin cisternae with no obvious vesicles (Figure 7.3a-d). A much larger amount of fibrillar material was present a few days later, where it was found at the top and sides of a cytoplasmic column (Figure 7.3e,f). The fibrils, unlike in wild type cells, appeared almost to be in layers and some were also present in the large vacuole below the cytoplasmic column. A closer examination of the cytoplasm revealed enlarged Golgi and vesicles carrying fibrils in some cells, while other cells had Golgi with flattened cisternae and few obvious vesicles (Figure 7.3h,i). The cells containing lots of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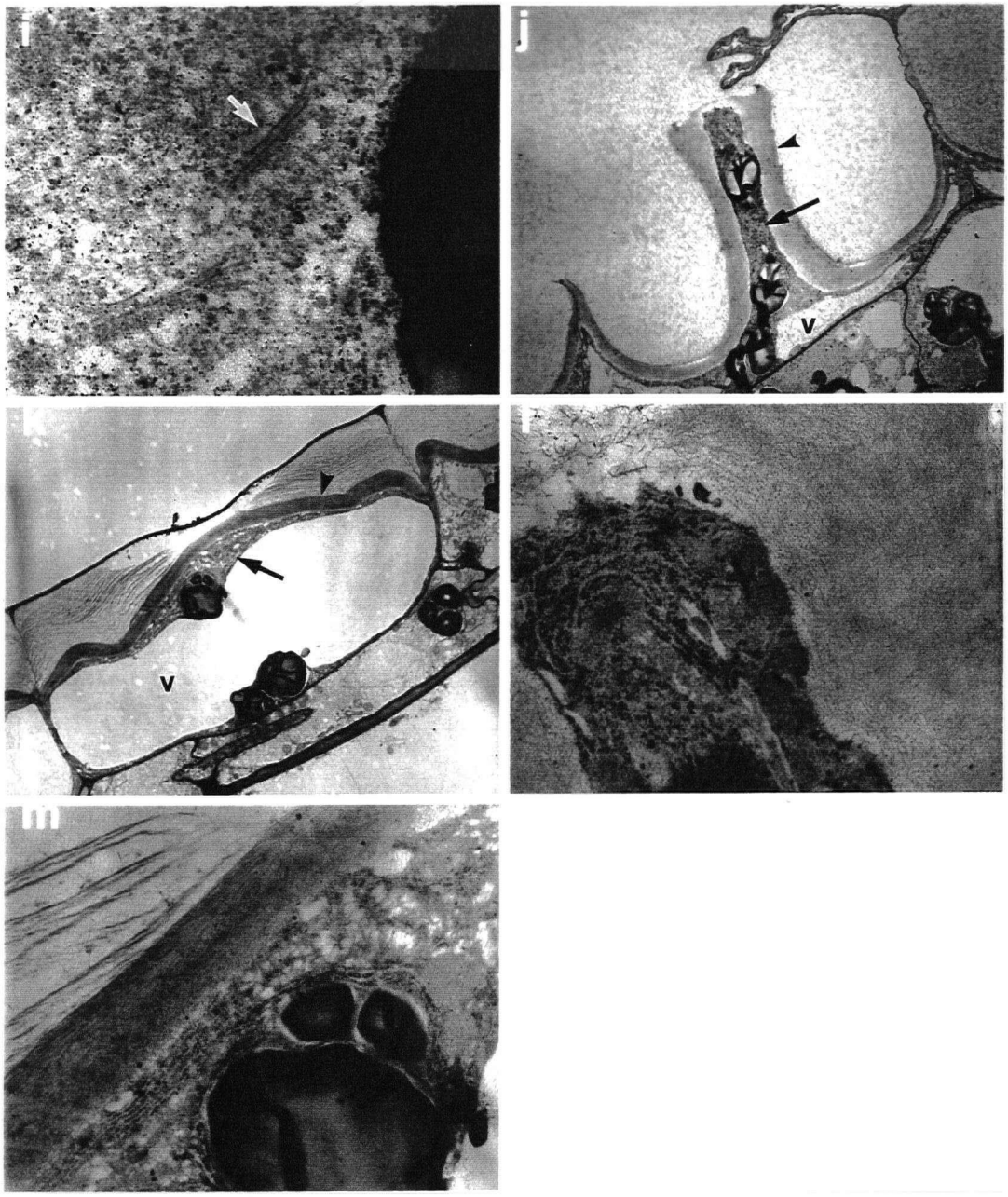


Figure 7.3. Transmission electron micrographs of developing epidermal cells of wild type and Mum4-1 seed coats.

(a) Wild type epidermal cell at 4 daf, note large vacuole (v) and cytoplasm (c) found at edges of the cell (ow = outer cell wall). (4100x)

(b) Mum4-1 epidermal cell at 4 daf. (2600x)

(c) Close-up of Mum4-1 cytoplasm at base of cell showing Golgi stack (arrow). (35,700x)

(d) Mum4-1 cell at 7 daf, note large amyloplasts. (1700x)

(e) Wild type cell at 7 daf. Most of the cytoplasm (c) is in a column in the centre of the cell. The space (sp) between the outer cell wall (ow) and the cytoplasm is filled with fibrillar material (see (g)), and below the cytoplasm is a vacuole (v). Note very large amyloplasts. (2100x)

(f) Mum4-1 seed at 10 daf. The cytoplasmic column is present, but the edges are not as sharp as seen in wild type cells. (2100x)
(g) Close-up of wild type cell at 7 daf showing cytoplasm filled with vesicles and a Golgi stack (arrow). (32,200x)
(h) Cytoplasm in 10 daf Mum4-1 cell, showing a large quantity of fibril-containing vesicles and a Golgi stack (arrow). (28,200x)
(i) Close-up of cytoplasm in Mum4-1 cell at 10 daf, showing lack of vesicles and presence a smaller Golgi stack (arrow). (38,000x)
(j) Wild type cell at 10 daf. The cytoplasm (arrow) is found in the centre of the cell and at the bottom, surrounded by new cell wall (arrowhead). Note small vacuole (v) at the bottom of the cell and the ruptured outer cell wall. The cell wall has broken at a point directly above where the reinforcement with electron dense material ended. (1700x)
(k) Mum4-1 cell at 13 daf. Similar to 10 daf Mum4-1, with rounded cytoplasmic column (arrow) over a large vacuole (v). Fibrillar material is seen above a new secondary cell wall (arrowhead) being laid down around the cytoplasm. (2200x)
(l) Close-up of wild type 10 daf cell showing top of columella. Note the cytoplasm contains lots of thin tubules that probably represent rough endoplasmic reticulum. (17,600x)
(m) Cytoplasm in a Mum4-1 cell at 13 daf. Tubules like those seen in wild type at 10 daf are present, as are some vesicles. Note new cell wall present above the cytoplasm. (18,300x)

vesicles were very similar to wild type cells in the midst of mucilage production (Figure 7.3g), while the others more closely resembled cells following its production (Figure 7.3l,m). The relative absence of active secretion machinery suggests either that these cells produce less "mucilage" or that due to the altered staging of the Mum4-1 seeds, these cells have finished producing "mucilage". It was not clear whether Mum4-1 cells at 13 daf have more acidic polysaccharide than cells at 10 daf, as the "mucilage" and cytoplasm were compressed at the top of the cell. A comparison of the amount of "mucilage" found in Mum4-1 versus wild type cells was not feasible due to the layered rather than diffuse appearance of the Mum4-1 "mucilage" and the differences in timing of development.

The second defect of Mum4-1, Ttg1-1, and Gl2-1 seeds was the improper development of the columellae. This was also investigated through TEM of Mum4-1 epidermal cells. As stated above, similar to wild type, the cytoplasm was first found around the edges of the cell and then was drawn into the centre of the cell (Figure 7.3a,b,d-f). Wild type cells had a very well defined cytoplasmic column with sharp edges located over a large vacuole (Figure 7.3e). The cytoplasmic

column of Mum4-1 cells was similar, but the edges were not as sharp as in wild type (Figure 7.3f). In moving from 7 to 10 daf in wild type cells, the cytoplasm was constricted into a very narrow column in the centre of the cell with edges that spread at the bottom of the cell over a small vacuole. New cell wall material was apparent bordering the cytoplasm to form a sharp, volcano-shaped columella that also ran part way up the sides of the cell. By contrast, the arrangement of the cytoplasm differed very little between the equivalent stages (10 and 13 daf) of Mum4-1 epidermal cells (Figure 7.3f,k). At 13 daf, where new cell wall material was evident, the cytoplasm was still found in the top half of the cell, with a small column at the top and a large vacuole below. The new cell wall material being laid over the cytoplasm was in the form of an arch. Thus, it appears that further constriction of both the vacuole and the cytoplasm is not occurring in Mum4-1 mutants, resulting in a flattened columella.

7.4 Chemical analysis of Mum4-1, Ttg1, and Gl2 seeds

Gas chromatography was used in order to determine if the amount and chemical composition of mucilage was the same in each of these mutants compared with their respective wild type seeds. Due to the retention of the "mucilage" within the epidermal cells of Mum4-1, Ttg1-1, and Gl2-1, whole seeds were crushed and used for derivitization. When Mum4-1 seeds were compared with Col-2 it was found that all the components were present, but all three peaks for galacturonic acid were significantly reduced. As well, the amount of glucose and mannose increased slightly (Table 7.1; Figure 7.4). A similar trend was seen when Ttg1-1 and Gl2-1 were compared with Ler. In these two mutants not only were all three galacturonic acid peaks substantially reduced, but so were a peak for rhamnose and fucose, and all of the galactose peaks (Table 7.2; Figure 7.5). Thus, all three of mutants had a decrease in the monosaccharides (galacturonic acid, rhamnose) that

Table 7.1. A comparison of the values of major peaks observed using gas chromatography for Col-2 and Mum4-1 whole seeds.[a]

		Col-2		Mum4-1	
Retention time	Sugar	Average (ug/100 seeds)	Standard deviation	Average (ug/100 seeds)	Standard deviation
6.66	Ribose	2.15	0.82	2.64	0.53
6.72	Arabinose, Ribose	6.40	2.17	7.43	1.05
6.87	Arabinose	3.45	1.03	3.96	0.42
7.11	Rhamnose, Fucose	9.04	2.62	6.58	0.69
7.29	Rhamnose, Arabinose	2.00	0.55	1.86	0.2
8.14	Xylose	3.17	0.74	3.45	0.23
8.38	Xylose	1.79	0.40	1.97	0.11
9.54	Galacturonic acid	3.94	0.40	2.12	0.2
9.92	Mannose	1.40	0.16	1.92	0.1
10.02	Galactose	3.76	0.71	4.12	0.17
10.06	Galacturonic acid	1.72	0.16	1.16	0.05
10.48	Galactose, Mannose	11.66	1.80	12.46	0.47
10.92	Galactose	5.10	0.75	5.45	0.22
11.03	Galactose	4.22	0.31	2.31	0.12
11.17	Galacturonic acid?	3.71	1.68	1.74	0.09
11.27	Glucose	19.11	2.08	25.13	0.36
11.42	?	2.52	0.19	2.62	0.1
11.51	Glucose	7.76	0.97	9.8	0.19
13.55	Octadecadienoic acid	8.92	2.44	10.19	0.61
13.63	?	10.76	1.57	11.79	0.75
13.82	?	6.23	6.19	6.52	2.04
14.00	Inositol	48.00	0.00	48	0
16.12	?	4.92	1.60	6.16	0.46

[a]Sample sizes: Col-2=4, Mum4-1=3.

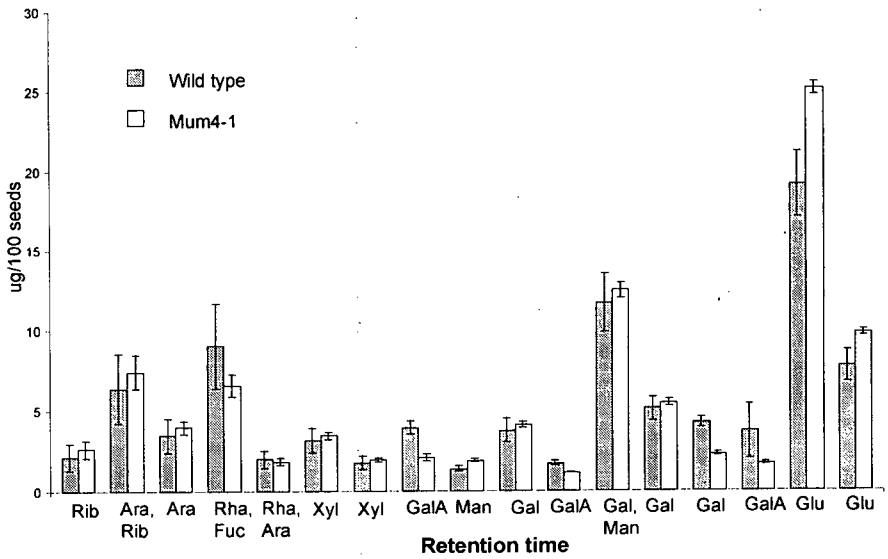


Figure 7.4. A comparison of the major peaks observed using gas chromatography for Col-2 and Mum4-1 whole seeds.
Rib=ribose, Ara=arabinose, Rha=rhamnose, Fuc=fucose, Xyl=xylose, GalA=galacturonic acid, Gal=galactose, Man=mannose, Glu=glucose.

Table 7.2. A comparison of the values of major peaks observed using gas chromatography for Ler, Ttg1-1, Gl2-1, and Ap2-1.[a]

		Ler		Ttg1-1		Gl2-1		Ap2-1	
Retention time	Sugar	Average (ug/100 seeds)	Standard deviation	Average (ug/100 seeds)	Standard deviation	Average (ug/100 seeds)	Standard deviation	Average (ug/100 seeds)	Standard deviation
6.66	Ribose	3.41	0.41	3.91	0.45	3.02	0.40	1.56	0.52
6.72	Arabinose, Ribose	9.46	1.11	8.96	0.75	7.69	1.18	4.02	1.3
6.87	Arabinose	4.86	0.60	4.63	0.44	3.97	0.61	2.25	0.62
7.11	Rhamnose, Fucose	11.92	1.25	5.07	0.51	5.76	0.90	2.51	0.75
7.29	Rhamnose, Arabinose	2.77	0.29	2.15	0.20	1.88	0.22	1	0.28
8.14	Xylose	4.12	0.50	3.68	0.26	3.07	0.48	2.28	0.5
8.38	Xylose	2.47	0.31	2.20	0.23	1.82	0.26	1.4	0.32
9.54	Galacturonic acid	5.80	0.56	1.59	0.12	2.11	0.52	1.11	0.19
9.92	Mannose	1.61	0.15	1.18	0.08	1.22	0.14	0.93	0.22
10.02	Galactose	4.12	0.36	2.86	0.19	2.83	0.22	1.96	0.41
10.06	Galacturonic acid	2.49	0.25	0.99	0.07	1.09	0.13	0.80	0.16
10.48	Galactose, Mannose	12.45	1.13	8.24	0.56	8.36	0.82	5.81	1.23
10.92	Galactose	5.39	0.50	3.58	0.26	3.65	0.33	2.55	0.55
11.03	Galactose	5.48	0.49	1.54	0.12	2.28	0.42	1.38	0.27
11.17	Galacturonic acid?	3.71	0.30	1.41	0.26	1.65	0.29	1.08	0.21
11.27	Glucose	27.99	2.67	24.04	1.47	20.09	2.23	22.9	0.91
11.42	?	2.47	0.22	5.79	5.55	2.61	0.22	2.04	0.78
11.51	Glucose	11.91	1.07	10.30	0.73	8.40	0.31	9.23	0.52
13.55	Octadecadienoic acid	10.44	1.28	24.65	23.03	9.06	1.87	8.94	3.47
13.63	?	15.99	1.60	33.81	32.04	12.43	2.53	13.77	5.38
13.82	?	9.70	7.16	4.99	3.64	6.15	5.79	4.25	1.22
14.00	Inositol	48.00	0.00	48.00	0.00	48.00	0.00	48	0
16.12	?	7.85	1.60	17.97	18.81	5.63	1.06	5.98	4.05

[a]Sample sizes were all n=3.

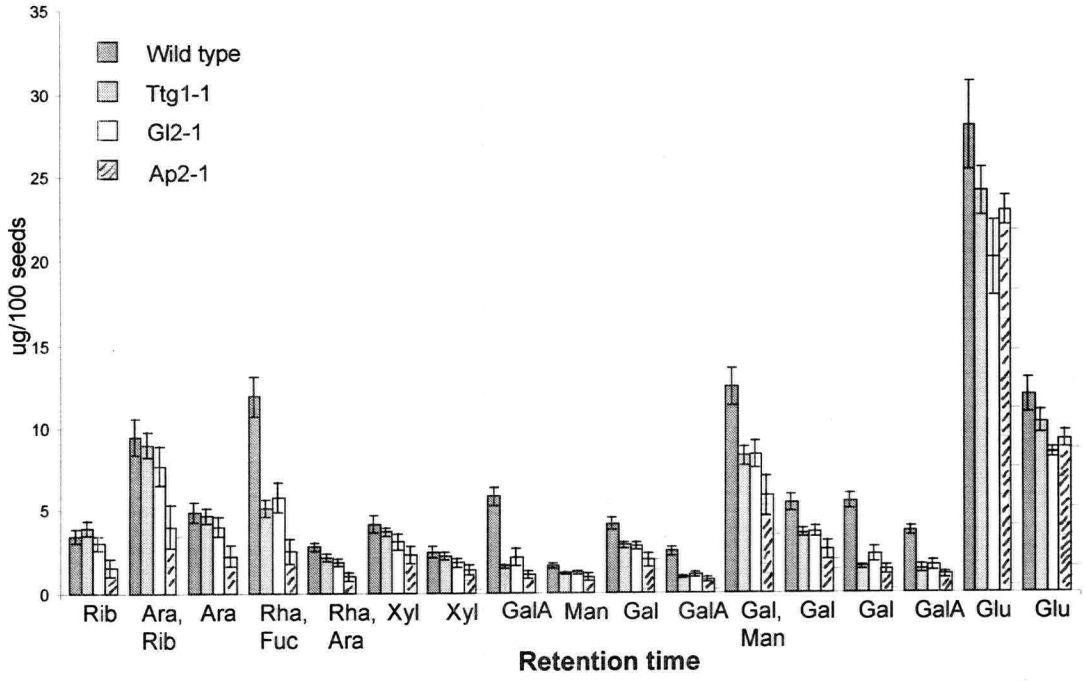


Figure 7.5. A comparison of the major peaks observed using gas chromatography for Ler, Ttg-1, Gl2-1, and Ap2-1 whole seeds.
Rib=ribose, Ara=arabinose, Fuc=fucose, Rha=rhamnose, Xyl=xylose, GalA=galacturonic acid, Gal=galacose, Man=mannose, Glu=glucose.

are major components of pectin (Frey-Wyssling, 1976; Brett and Waldron, 1990; Carpita and Gibeaut, 1993), suggesting less mucilage may be made in these mutants.

As a control, chemical analysis was performed on Ap2-1 seeds. Ap2-1 seeds, as discussed in Chapter 9.2.1, did not make any mucilage in a large number of their cells, thus they were used as a control for seeds lacking mucilage, but not normal cell wall pectin. Ap2-1 seeds had an even more severe decrease in the amount of the sugars in their seeds compared with Mum4-1, Gl2-1 and Ttg1-1, showing that a similar monosaccharide profile resulted from seeds completely lacking mucilage in most cells.

7.5 Discussion

7.5.1 *MUM4, TTG1,* and *GL2* are required for normal mucilage and columella production

A comparison of epidermal cell development in Mum4-1, Ttg1-1, and Gl2-1 seeds with wild type showed that these mutants make a reduced amount of pectin, which may account for the lack of mucilage extrusion in these mutants. A limited amount of extrusion was seen when seeds were treated with a high concentration of the chelator EDTA, which may have resulted from loss of calcium bridges in the pectin and consequent increased swelling of the reduced amount of mucilage (Frey-Wyssling, 1976; Brett and Waldron, 1990; Carpita and Gibeaut, 1993). Some epidermal cells of developing Mum4-1 mutants appeared to have a reduced amount of secretion machinery, but this apparent lack may have been due to the differences in timing in the wild type and Mum4-1 seeds observed. In addition, while a columella is made in these mutants, it is in the form of a shallow dome rather than an upright volcano. This appeared to result from a lack of full constriction of the cytoplasm and/or subtending vacuole prior to cell wall production to form the columella.

7.5.2 *MUM4* is a seed-specific regulator of epidermal cell structure

Both Gl2 and Ttg1 mutants were identified through their lack of trichomes and were later found to have an increased number of root hairs and an abnormal seed coat (Koornneef, 1981; Haughn and Somerville, 1988; Rerie *et al.*, 1994; Hülskamp *et al.*, 1994; Galway *et al.*, 1994; Masucci *et al.*, 1996). These multiple roles suggest that *TTG1* and *GL2* play general roles in epidermal cell development throughout the plant. By contrast, *MUM4* appears to be acting in a seed-specific manner. *MUM4* could be a downstream target of TTG1 or GL2, or, conversely, MUM4 could interact with them in the seed coat to regulate epidermal cell structure. Double mutant analysis may clarify this relationship.

7.5.3 MUM4, TTG1, and GL2 may work through the regulation of the cytoskeleton

Production of the columella involves the initiation of a cytoplasmic column, followed by the further constriction of the cytoplasm and vacuole to form a narrow columella in the centre of the cell. In Mum4, Ttg1, and Gl2 mutants, the initial cytoplasmic column appears to be formed, but no further changes appear to occur prior to the production of the secondary cell wall. In addition, these mutants produce a reduced amount of mucilage. Since the cytoskeleton may be responsible for the constriction of the cytoplasm to form the columella (see Chapter 4.5.3), these three genes may be involved in the regulation of the cytoskeleton. Improper arrangement of the cytoskeleton could also explain the reduced amount of mucilage produced by these mutants. In wild type seeds, mucilage is made and then secreted in a polar manner to the outside of the plasma membrane at the top of the cell. Polar secretion is carried out by the movement of vesicles along the cytoskeleton, often involving actin microfilaments (Drubin, 1991; Chant and Pringle, 1991; Fowler and Quatrano,

1997; Cai *et al.*, 1997; Asada and Collings, 1997; Fowler and Quatrano, 1997). A lack of polar movement of vesicles may result in less secretion beyond the cytoplasm, the retention or breakdown of mucilage within the cytoplasm, or possibly reduction of the amount of mucilage produced as seen in the maize mutant Ageotropic (Miller and Moore, 1990). In addition, constriction of at least some of the cytoplasm to a certain area of the cell by the cytoskeleton may also be necessary for the polar growth of plant cells to form trichomes and root hairs (Oppenheimer *et al.*, 1997; Wang *et al.*, 1997). Thus, a role for MUM4, TTG1, and GL2 in the regulation of the cytoskeleton may also explain the linkage between the trichome, root hair, and seed coat phenotypes of Gl2 and Ttg1 plants.

An alternative hypothesis is that the reduced amount of mucilage is directly responsible for the partial cytoplasmic column formation and retention of a large vacuole. While the initial set up of the cytoplasmic column would still be done by the cytoskeleton (see Chapter 4.5.3), pressure created by and/or the cell volume taken up by the mucilage may be enough to further compress the vacuole and form the cytoplasm into a thinner column in the centre and bottom of the cell. This hypothesis would suggest that since the initial cytoplasmic column appears to be formed in these mutants, *MUM4, GL2,* and *TTG1* may only influence the amount of mucilage produced. Both hypotheses seem to fit the current data equally, with the exception that the latter cannot easily explain the connection with the trichome and root hair phenotypes seen in Gl2 and Ttg1 plants. An analysis of the cytoskeleton during seed coat differentiation may help to determine which hypothesis is correct.

7.5.4 The biochemical role of MUM4

GL2 has been found to encode a homeodomain protein (Rerie *et al.*, 1994), while *TTG1* encodes a protein predicted to have a WD40 protein interaction domain (Szymanski *et al.*, 1998). The possible activity of GL2 and TTG1 as transcription factors correlates with their multiple roles

throughout plant development. The biochemical role of MUM4 is not yet known, but one possibility is that it is also a transcription factor, interacting with TTG1 or GL2 in the seed coat. Alternatively, MUM4 could be a protein regulating cytoskeletal events that is a seed epidermis-specific target of TTG1 or GL2. Determination of the true role of the protein must await cloning of *MUM4*.

8. MUTANTS AFFECTING MUCILAGE STAINING

8.1 Introduction

As discussed in Chapter 5, Mum3 and Mum5 both were isolated as reduced mucilage mutants that had wild type seeds and appeared only to have a small layer of mucilage. A change of the staining protocol revealed that normal mucilage layers were present. The abnormal staining characteristics of Mum3 and Mum5 mucilage are further investigated in this chapter.

8.2 Mum3 and Mum5 Mutants

8.2.1 Seed and mucilage phenotype

As seen in Chapter 5 with SEM analysis, both Mum3 and Mum5 have normal epidermal cells in their seed coat. This was also observed with cross-sections of mature seeds fixed under aqueous conditions where the mucilage was absent and prominent columellae were observed (Figure 8.1a-d). The pigmented layer was also obvious in both of the seed coats, however, the palisade layer was difficult to discern in these sections due to poor embedding (Figure 8.1b,c).

The Ruthenium red staining characteristics of these two mutants are shown in Figure 8.2. When placed directly into Ruthenium red stain without shaking, wild type seeds developed two pink layers, an outer, diffuse layer and an inner, more darkly-staining, pink capsule (Figure 8.2a). Similar staining was seen in both Mum3 and Mum5 seeds under these conditions. Both had an outer, cloudy layer which was more easily dispersed than the wild type layer under normal handling, andthe inner capsule which was smaller than in wild type (Figure 8.2b). When seeds were shaken directly in

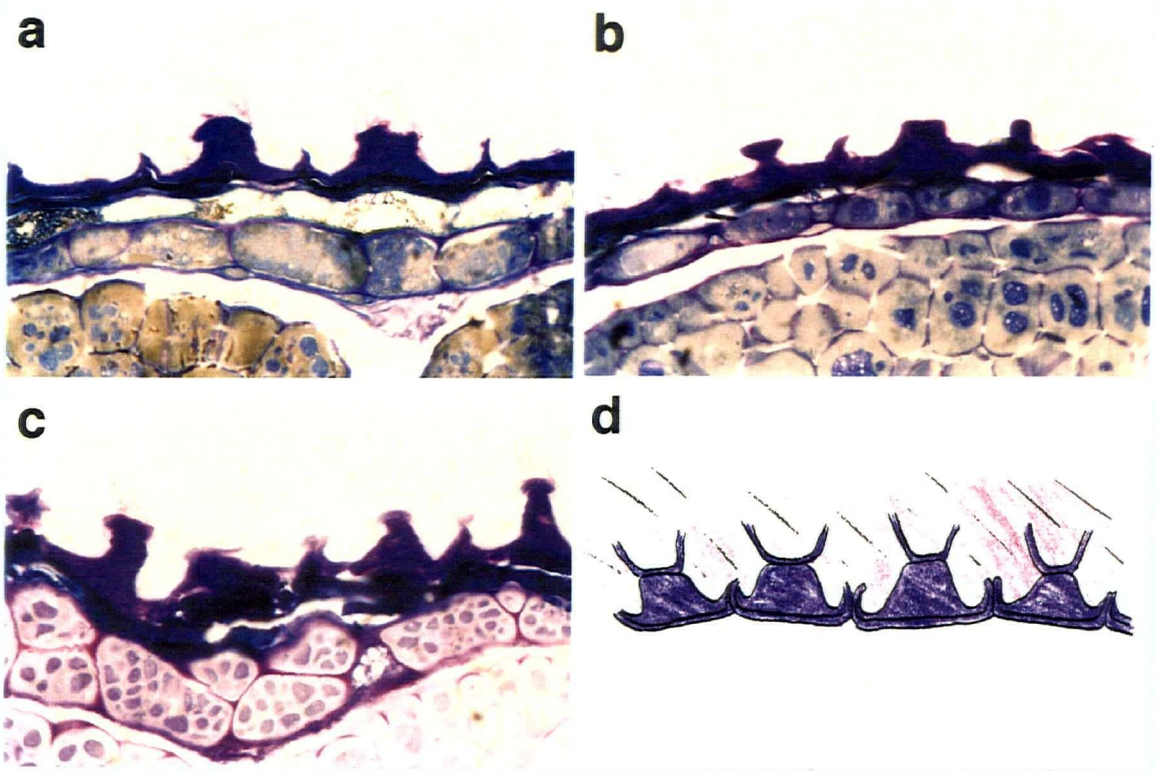


Figure 8.1. Cross sections of wild type, Mum3-1, and Mum5-1 seeds.
All sections were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue.
(a) Wild type seed coat. The epidermal cells have burst open to release mucilage, so only bare columellae are visible. (680x)
(b) Mum3-1 seed coat showing similar result to wild type. (670x)
(c) Mum5-1 seed coat showing cells that have broken open to release mucilage. (660x)
(d) Cartoon of Mum3-1 and Mum5-1 epidermal cell phenotype showing cells that have burst to release mucilage.

Ruthenium red, only the inner capsule was visible on wild type seeds and both Mum3 and Mum5 seeds showed no capsule, only a thin layer of pink-staining mucilage directly around the seed (Figure 8.2c,d). Finally, when seeds were shaken in water for 15 minutes prior to staining in Ruthenium red with shaking, wild type seeds once again had only the inner mucilage capsule while Mum3 and Mum5 seeds had columellae but no mucilage capsule (as mentioned in Chapter 5) (Figure 8.2e,f). These mucilage characteristics may result either from the mucilage being less tightly attached to the seed than in wild type or from an altered mucilage chemical composition.

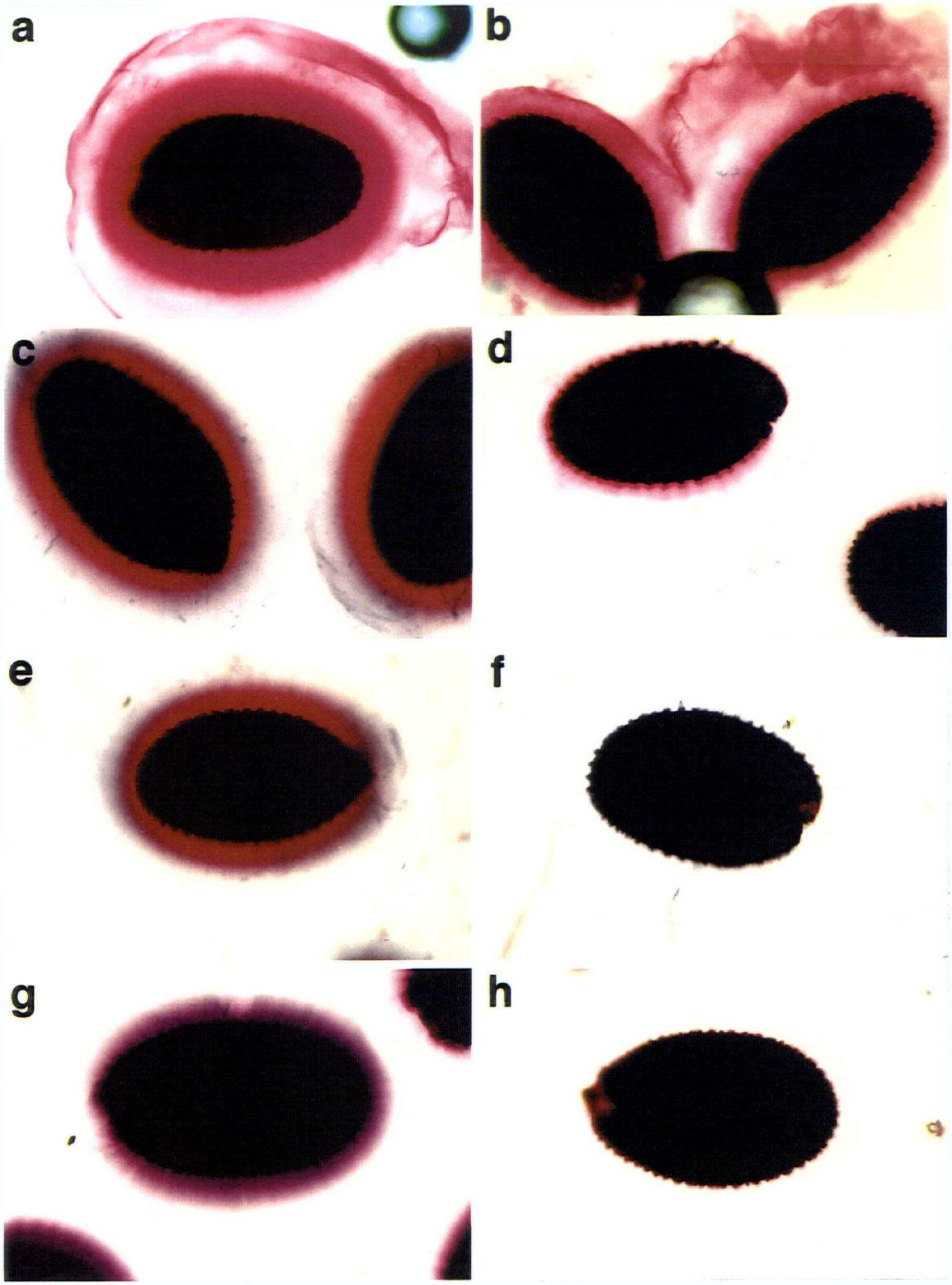


Figure 8.2. Wild type and Mum5-1 seeds stained with Ruthenium red under different treatments.

(a) Wild type seed stained directly in Ruthenium red with no agitation. Note two layers of mucilage, an outer, diffuse layer and an inner, intensely staining capsule.
(b) Mum5-1 seed stained directly in Ruthenium red with no agitation. Similar to wild type, there are two layers of mucilage, an outer, diffuse layer and an inner, intensely staining capsule. The layers, however, are thinner than seen in wild type.
(c) Wild type seed shaken in Ruthenium red showing a red inner capsule and a faint, smokey-grey outer layer.
(d) Mum5-1 seed shaken in Ruthenium red, showing a thin, pink inner capsule only.
(e) Wild type seed shaken first in water, then stained in Ruthenium red with shaking. Note red inner mucilage capsule.
(f) Mum5-1 seed shaken first in water, then stained in Ruthenium red with shaking. There is no obvious mucilage, though the cells have burst and the columellae are visible.
(g) Wild type seed shaken first in water, then stained in Ruthenium red with shaking, showing only the mucilage capsule.
(h) Wild type seed shaken first in EDTA, then stained in Ruthenium red with shaking. Similar to Mum5-1 (f), there is no obvious mucilage, though the cells have burst and the columellae are visible.
Magnification: (a)-(h) = 70x.

In order to test whether the mucilage of Mum3 and Mum5 seeds was more loosely attached to the seed than wild type mucilage, staining with India ink was used (Rerie *et al.*, 1994). Wild type, Mum3, and Mum5 seeds were shaken in water and then placed in a dilute solution of India ink (data not shown). India ink, a colloidal liquid, cannot easily penetrate the mucilage and thus wild type seeds appear to have a clear gel layer surrounding the seed. A similar capsule was seen for both Mum3 and Mum5 seeds, suggesting that the inner layer of mucilage was equally well attached for them as for wild type. When investigating the effect of 0.5 M EDTA on Mum2-1 and Mum4-1 seeds (see Chapters 6.2.1 and 7.2.1), wild type seeds were used as a control. When wild type seeds were treated with EDTA, the mucilage still came out but it did not stain with Ruthenium red (Figure 8.2g,h). Thus, the treatment of wild type seeds with the chelator EDTA led to a phenocopy of Mum3 and Mum5 seeds shaken before or during the staining. These results suggest that the linkages between pectin molecules may be affected in these two mutants.

8.2.2 Plant phenotype

In order to determine if Mum3 and Mum5 mutations resulted in seed specific phenotypes, other aspects of the plants were studied. Mum5 plants were completely wild type, being robust plants with normal trichomes, root hairs, root cap mucilage, flowers, and siliques. Mum3 plants, on the other hand, while they had normal root hairs and root cap mucilage, they had two-prong trichomes rather than the wild type three-prong trichomes, and the plants had very reduced apical dominance or were extremely spindly. In addition, some of the flowers had reduced stamens. Unfortunately, since the segregation analysis of Mum3 was not complete, it cannot be ruled out that the trichome and apical dominance phenotypes were the result of mutation(s) unrelated to the one causing the mucilage phenotype.

8.3 Chemical analysis of Mum3-1 and Mum5-1 mucilage

Gas chromatography of isolated mucilage was used to determine if the composition and amount of mucilage of Mum3 and Mum5 differed from wild type (Table 8.1, Figure 8.3). For both mutants, all of the mucilage components were present, but the peak representing rhamnose and fucose was reduced. In addition, Mum5 mucilage had a slightly reduced amount of galactose and galacturonic acid. A reduction in the amount of rhamnose or fucose, both important components of *Arabidopsis* mucilage, may be responsible for the altered staining phenotype of Mum3 and Mum5 seed mucilage.

Table 8.1. A comparison of the values of major peaks from gas chromatography of Col-2, Mum3-1, and Mum5-1 mucilage.[a]

		Col-2		Mum3-1		Mum5-1	
Retention time	Sugar	Average (ug/100 seeds)	Standard deviation	Average (ug/100 seeds)	Standard deviation	Average(ug/100 seeds)	Standard deviation
7.09	Rhamnose, Fucose	3.89	0.48	2.28	0.47	1.76	0.39
9.51	Galacturonic acid	2.37	0.36	2.16	0.09	1.80	0.03
10.04	Galactose	0.85	0.12	0.77	0.02	0.65	0.03
11.00	Galactose	2.26	0.32	2.09	0.11	1.74	0.02
11.14	Galacturonic acid	1.36	0.21	1.35	0.05	1.09	0.01
11.24	Glucose	0.66	0.20	0.91	0.51	1.77	1.62
11.48	Glucose	0.23	0.12	0.27	0.28	0.69	0.70
13.52	Octadecanoic acid	0.17	0.37	0.00	0.00	0.00	0.00
13.60	?	0.55	0.36	0.20	0.03	0.17	0.02
13.79	?	3.41	2.57	4.55	1.63	4.22	0.55
13.99	Inositol	48.00	0.00	48.00	0.00	48.00	0.00
14.36	?	1.15	0.04	1.00	0.05	1.08	0.02
14.54	?	0.74	0.05	0.79	0.06	0.81	0.02
16.09	?	0.34	0.29	0.14	0.24	0.00	0.00

[a]Samples sizes: Col-2=5, Mum3-1 & Mum5-1=3.

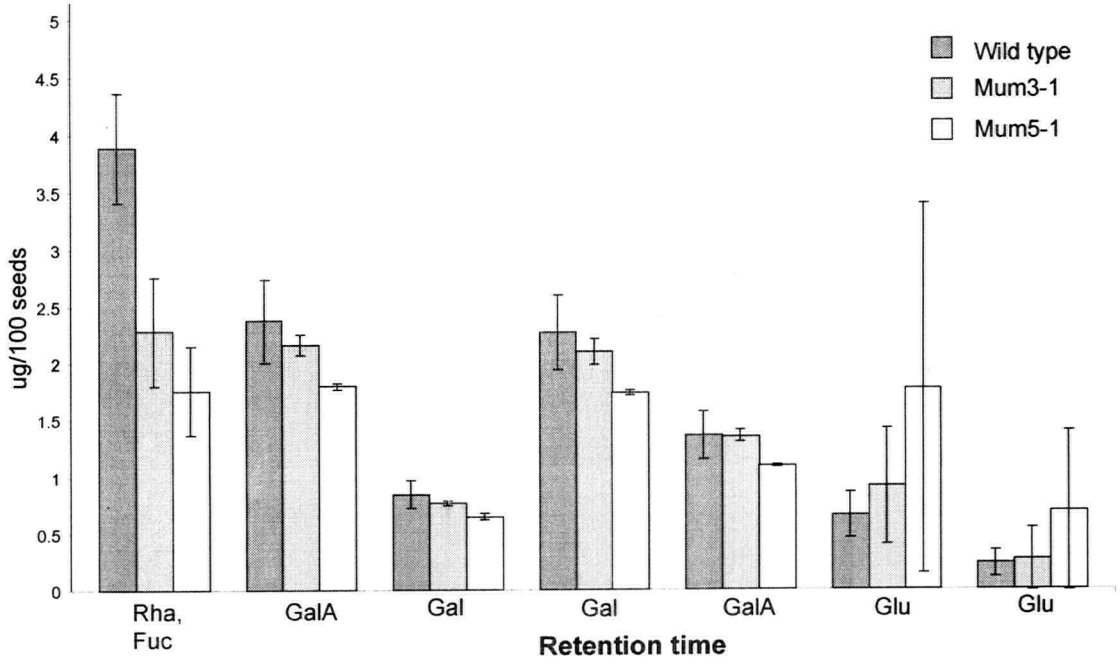


Figure 8.3. A comparison of the major peaks observed using gas chromatography for Col-2, Mum3-1, and Mum5-1 mucilage.
Rha=rhamnose, Fuc=fucose, GalA=galacturonic acid, Gal=galactose, Glu=glucose.

8.4 Discussion

8.4.1 *MUM3* and *MUM5* may regulate mucilage composition and cross-linking

Both Mum3 and Mum5 mutant seeds had abnormal staining characteristics – the mucilage loses its ability to stain with Ruthenium red when the seeds are shaken in an aqueous environment. This phenotype was mimicked in wild type seeds when they were placed in EDTA prior to staining with Ruthenium red. As discussed in Chapter 6, EDTA is a heavy metal chelator that could disrupt calcium bridges between adjacent pectin molecules, thus allowing greater expansion of the pectin (Frey-Wyssling, 1976; Brett and Waldron, 1990; Carpita and Gibeaut, 1993). Thus it appears that there may be looser or fewer cross-linkages between the pectin molecules in the mucilage of Mum3 and Mum5. Ruthenium red has been reported to stain pectin only when the charged polygalacturonic acid chains are a certain distance apart (Gahan, 1984), which would explain why the staining was lost in Mum3, Mum5, and EDTA-treated wild type seeds.

Gas chromatographic analysis of the monosaccharide composition of Mum3 and Mum5 mucilage demonstrated that both have a reduced peak for rhamnose and fucose. Unfortunately, these two sugars cannot be resolved under our conditions. The reduction of either of these sugars could be responsible for the reduced cross-linkages within the mucilage. Rhamnose, along with galacturonic acid makes up the backbone of rhamnogalacturonan I and II, two major components of pectin (Brett and Waldron, 1990; Carpita and Gibeaut, 1993; Cosgrove, 1997). If there is less rhamnogalacturonans, this could lead to a looser mesh of pectin, thus allowing for altered staining. Conversely, if fucose was missing this could also lead to a change in staining due to fucose being an important component of rhamnogalacturonan II side chains (Reiter *et al.*, 1993). Recently, the Mur1 mutant of *Arabidopsis* was found to contain less than 2% of the wild type amount of fucose in the

aerial parts of the plant and 60% in the roots (Reiter *et al.*, 1993; Bonin *et al.*, 1997; Reiter *et al.*, 1997). Staining of Mur1 seeds in Ruthenium red after shaking in water revealed no changes from wild type, suggesting that the linkages in the mucilage were not affected in this mutant (data not shown). Reiter *et al.* (1997) have also isolated two more mutants with reduced amounts of fucose (Mur2—3) and one with reduced rhamnose (Mur8). The seed mucilage phenotype of these mutants has not been yet determined and no complementation tests have been performed, thus Mum3 or Mum5 could be allelic to one of them.

8.4.2 Pleiotropic effects of mutations in *MUM3*

Mum3 mutant plants had two pleiotropic phenotypes that may be due to other, background, mutations. One was very reduced apical dominance, while the other was two-prong rather than three-prong trichomes on the leaves. The trichome phenotype is intriguing due to the trichome phenotypes of other seed coat mutants (Ttg1 and Gl2). Several known mutants have trichomes with two branches: Gl2, Glabra3 (Gl3), Angustifolia (An), Stachel (Sta), and Zwichel (Zwi) (Haughn and Somerville, 1988; Hülskamp *et al.*, 1994; Marks, 1997; Oppenheimer *et al.*, 1997; Folkers *et al.*, 1997; Hülskamp *et al.*, 1998). Complementation tests between seed mutants demonstrated that Mum3 was not a weak allele of Gl2 (see Chapter 5). Gl3 mutants have a reduced number of trichomes which have a variable but small number of branches, while Mum3 have only two-branched trichomes, suggesting that Mum3 might not be allelic to Gl3 (Haughn and Somerville, 1988; Hülskamp *et al.*, 1994; Marks, 1997; Folkers *et al.*, 1997; Hülskamp *et al.*, 1998). Angustifolia plants have narrow leaves and twisted stems and siliques (Hülskamp *et al.*, 1994) in addition to trichomes that closely resemble those of Mum3. While the plant phenotypes do not completely overlap, Mum3 might be allelic to An. Alternatively, its trichomes also closely resemble Sta and weak Zwi trichomes

(Hülskamp *et al.*, 1994; Marks, 1997; Oppenheimer *et al.*, 1997; Folkers *et al.*, 1997; Hülskamp *et al.*, 1998). Complementation tests, as well as several backcrosses to wild type plants will have to be performed to determine what is responsible for the trichome phenotype seen in Mum3 and whether it was due to the same gene as the seed mucilage defects.

9. MUTANTS AFFECTING OVULE INTEGUMENT AND/OR TESTA DEVELOPMENT

The final two mutants, Ap2 and Ats, are known to cause defects during ovule development (see Chapter 4; Leon-Kloosterziel *et al.*, 1994; Modrusan *et al.*, 1994). The exact nature of the seed coat defects of Ap2 is examined in this chapter. Due to the limited nature of the mucilage defect in the Ats seeds, little investigation was done beyond what was already published by Leon-Kloosterziel *et al.* (1994).

9.1 Ap2 and Ats Mutants

9.1.1 Ap2 seed development

Several alleles of Ap2 have been found to have abnormal seed shape and seed coat features. As shown in Chapter 5, the strong Columbia allele Ap2-6 and the weak Ler allele Ap2-1 have abnormal seeds. In both cases, the seed takes on a heart shape and the epidermal cells can be flat and rectangular instead of hexagonal with a columella in the centre of the cell (Figure 9.1b-f). Both the rectangular cells and the heart shape are reminiscent of the unfertilized ovule (Figure 9.1a), suggesting that Ap2 seeds are aberrant not only in seed coat differentiation, but also the shape change that occurs early in seed development. Seed coat development was examined in both Ap2 alleles.

Some mature seeds of Ap2-6 were completely normal, including mucilage and hexagonal epidermal cells with columellae, while others were heart-shaped and completely covered with flattened, elongated rectangular cells resembling those of ovules (Figures 9.1c,d and 9.2a). Aberrant

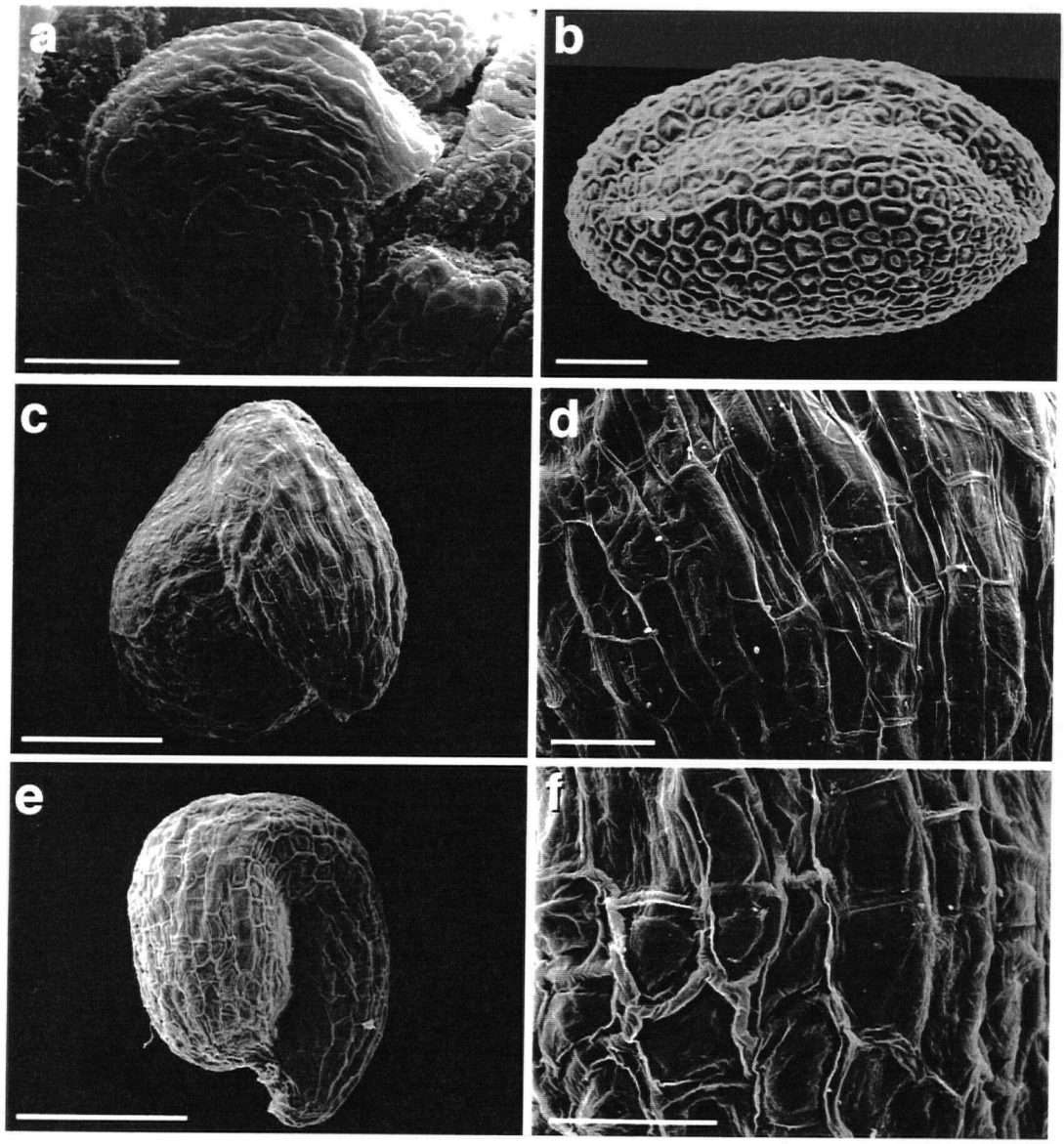


Figure 9.1. Scanning electron micrographs of wild type, Ap2-6, and Ap2-1 seeds.
(a) Mature wild type ovule; note curved shape and elongated, rectangular cells.
(b) Mature wild type seed with oval shape and hexagonal cells with central columellae.
(c) Ap2-6 seed, showing abnormal shape, similar to a mature ovule, and elongated, rectangular cells.
(d) Close-up of the epidermal cells of an Ap2-6 seed, showing thin-walled, rectangular cells.
(e) Ap2-1 seed, showing curved shape and a mixture of hexagonal cells with flattened columellae, and elongated, rectangular cells.
(f) Close-up of Ap2-1 cells, showing the two types present.
Magnification bars: (a),(d),(f)=40 μm; (b)=100 μm; (c),(e)=200 μm

and normal seeds were generally found in different siliques, all arising from flowers with an Ap2 phenotype, but possibly differing in severity of the floral phenotype. When the mature seeds of the

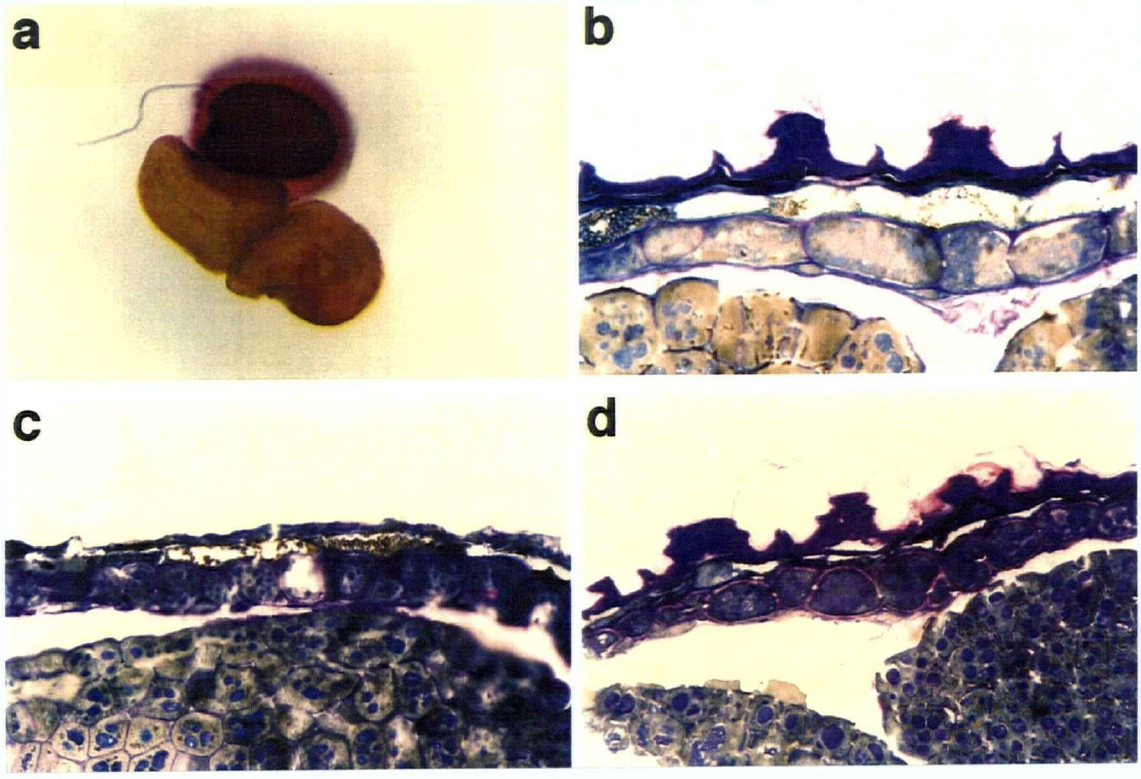


Figure 9.2. Ruthenium red staining of Ap2-6 seeds and cross sections of wild type, Ap2-6, and Ats seeds.
All sections were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue.
(a) Ap2-6 seeds stained with Ruthenium red showing both abnormally shaped seeds lacking mucilage, and a normal seed. (30x)
(b) Wild type seed coat. The epidermal cells have burst open to release mucilage, so only bare columellae are visible. (680x)
(c) Seed coat of abnormal Ap2-6 seed showing lack of differentiated epidermal layer. (430x)
(d) Ats seed coat, showing relatively normal columellae and some mucilage. (430x)

abnormal type were sectioned, the pigmented layer was apparent but the palisade and the epidermal layer were not obvious; only flattened cell layers surrounded the pigmented layer (Figure 9.2b,c). The lack of the palisade layer may explain the inability of Ap2 seeds to survive sterilization through bleach treatment compared with wild type seeds. Development of both normal and aberrant seeds from Ap2-6 flowers has been followed using microscopy and staining with Toluidine blue. The normal seeds appear to undergo development identical to that of wild type (data not shown). The aberrant seeds, on the other hand, appeared to start development normally, with the epidermal cells

enlarging, becoming vacuolated, and accumulating amyloplasts (Figure 9.3 a-d). The cells continued to have this appearance in the outer two layers of the seed coat at 7 daf when mucilage becomes apparent in normal seeds (Figure 9.3e,f). At 10 and 12 daf, when large amounts of mucilage and the columellae, respectively, dominate wild type cells, the majority of the Ap2-6 aberrant seed cells had no obvious contents, not even the amyloplasts seen at earlier stages (Figure 9.3g-j). A few exceptional cells, however, either appeared to have staining of mucilage and/or columella similar to Mum4, Ttg1, and Gl2 seeds, but very faint, or were filled with a dark granules normally only seen in the pigmented layer (Figure 9.3h,j, see Chapter 7). By contrast to the epidermal layer, no palisade layer cells were found to develop normally in the aberrant seeds (Figure 9.3a-j). Thus it appears that Ap2-6 seeds either underwent completely normal seed coat development or fail to re-differentiate the outer integument into the epidermal and palisade layers.

Ap2-1 seeds appeared to have no mucilage when stained with Ruthenium red (see Chapter 5). When the development of the epidermal cells of the seed coat was followed it was found that, like Ap2-6, the pigmented layer underwent normal development while the palisade layer did not develop at all (Figure 9.3k-n). In the epidermal layer, approximately one half of the cells underwent relatively normal mucilage production while the other cells acted similar to the Ap2-6 aberrant seed cells in losing all their contents by 10 daf. The mucilage producing cells in Ap2-1 seeds generally resemble those of Mum4-1, Ttg1, and Gl2 but with a more defined columella (Figure 9.3n, see Chapter 7). The results in both Ap2-6 and Ap2-1 suggest that *AP2* affects both the shape change early in seed development and the re-differentiation of the outer integument into the palisade and epidermal layers.

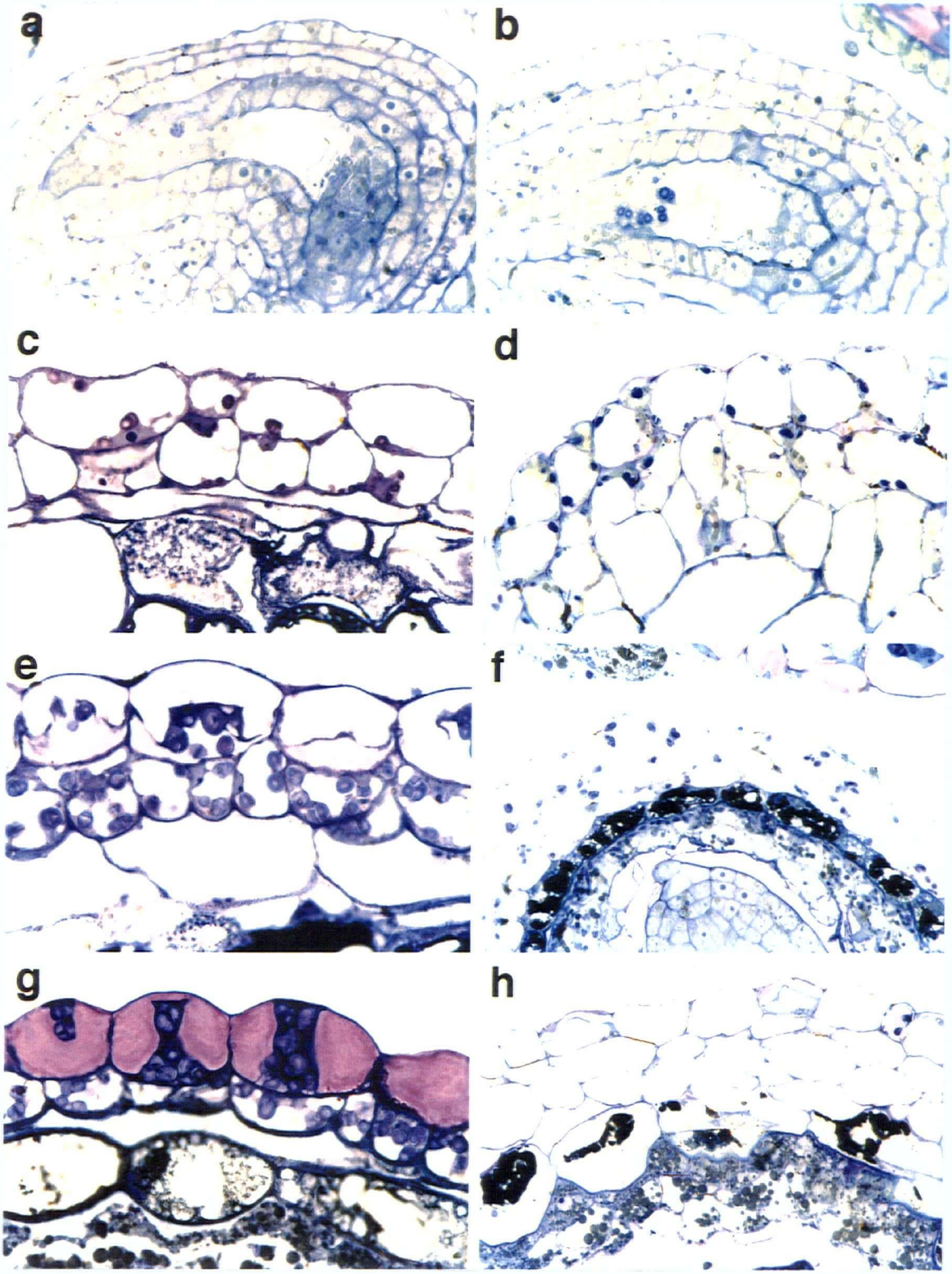
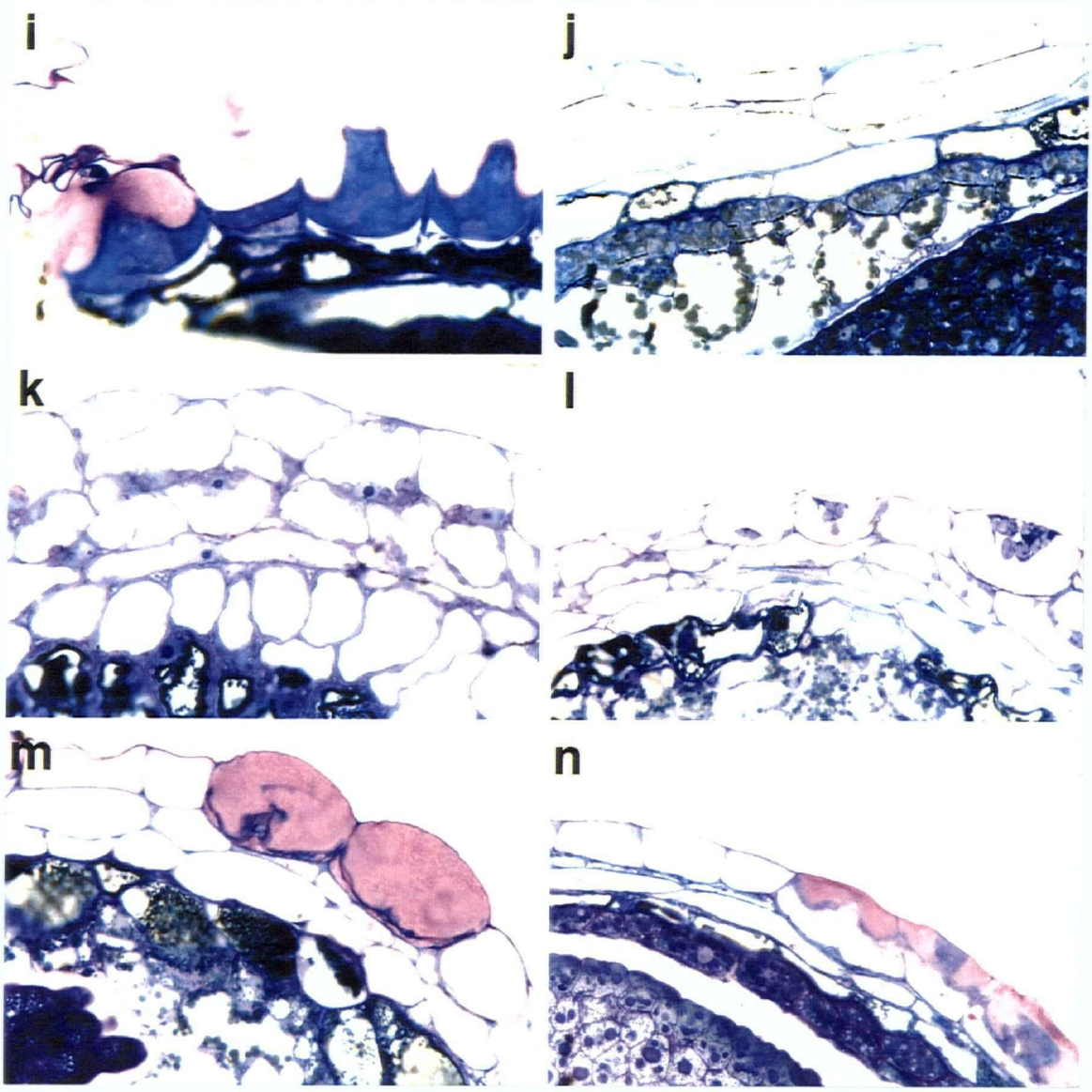


Figure 9.3. A comparison of wild type, Ap2-6, and Ap2-1 seed coat development.
Samples were fixed in aqueous 3% glutaraldehyde and sections were stained with Toluidine blue.



(a) Wild type mature ovule (0 days after fertilization [daf]). (670x)
(b) Ap2-6 mature ovule (0 daf). (670x)
(c) Wild type seed at 4 daf. Note small amyloplasts in cells. (660x)
(d) Ap2-6 seed at 4 daf. Similar to wild type, there are large vacuoles and amyloplasts are starting to accumulate. (700x)
(e) Wild type seed at 7 daf. The amyloplasts are larger and found in centre of the epidermal cells. Faint pink stain is apparent throughout cell. (670x)
(f) Ap2-6 seed at 7 daf. Cells resemble those at 4 daf. (580x)
(g) Wild type seed at 10 daf. Intense pink staining polysaccharide in cell and amyloplasts are in a column in the centre of the epidermal cells. (690x)
(h) Ap2-6 seed at 10 daf. Most epidermal cells are empty, but a few have a small amount of mucilage at the top of the cell. (422x)

(i) Wild type seed at 13 daf. The outer cell wall of the epidermal cells has burst, releasing mucilage. In the cell that is still partially intact, dark pink mucilage is present. The columellae are completely filled in. (670x)
(j) Ap2-6 seed at 12 daf. The epidermal cells are largely empty, except a few that have a very small amount of mucilage at the top. (440x)
(k) Ap2-1 seed at 4 daf, showing few amyloplasts. (700x)
(l) Ap2-1 seed at 7 daf, showing some cells with amyloplasts and some faintly staining mucilage, while other cells appear empty. (470x)
(m) Ap2-1 seed at 10 daf. A few epidermal cells appear normal with dark pink mucilage, while other cells are empty. (460x)
(n) Ap2-1 seed at 13 daf. Some epidermal cells are empty, while others resemble Mum4-1 cells with slightly flattened columellae over a large white space. (490x)

9.1.2 Ats seed phenotype

Ats mutant seeds stained with Ruthenium red appeared to make a reduced amount of mucilage and also stained patchily, with only some cells extruding mucilage (see Chapter 5). This phenotype was further investigated through sectioning of mature Ats seeds. The epidermal cells were quite similar to wild type, with obvious columellae, the mucilage having been extruded (Figure 9.2d). Many cells, however, were intact or only partially expanded. These results suggest that mucilage extrusion was only possible in some cells. The pigmented layer was readily obvious in these cells while the palisade layer was hard to detect due to poor embedding.

9.2 Discussion

9.2.1 *AP2* regulates seed shape and re-differentiation of the outer integument into the mucilaginous epidermis and lignified palisade layers

AP2 appears to be involved in the initiation of both the shape change from ovule to seed and the development of the outer integument into the palisade and epidermal layers. Both Ap2-6

and Ap2-1 mutants can have heart-shaped seeds, the outer cell layers of which failed to undergo re-differentiation into the epidermal and palisade layers of the seed coat. In aberrant seeds of both alleles, the palisade layer was completely undeveloped, while some cells of the epidermal layer were able to make at least some mucilage and a reduced columella. There were also some allele-specific effects. Ap2-6 seeds were variable, having a mixture of completely normal and severely aberrant seeds, while Ap2-1 seeds were all heart-shaped with some epidermal cells having relatively normal mucilage and columella. The severity seen in the seeds is similar to the floral phenotypes of these two mutants where Ap2-1 has a relatively constant phenotype while Ap2-6 has a greater range in severity, even within a plant (Komaki *et al.*, 1988; Kunst *et al.*, 1989; Bowman *et al.*, 1989; Bowman *et al.*, 1991).

Ap2 mutants have been also found to affect ovule development (Chapter3; Modrusan *et al.*, 1994). The seed coat phenotype could be a downstream effect of integument abnormalities in seemingly normal ovules that were not apparent at the level studied during ovule development, or they could be completely independent events. The roles of *AP2* in seed coat development add to the list of activities performed by *AP2*. *AP2* appears to affect almost all aspects of plant growth, from floral initiation to flower development to ovule development to seed coat development. The connection between these events is unclear and the importance of *AP2* to each of these roles varies. The multiple roles of *AP2* appears to be a case of a developmental regulator being used repeatedly (Bowman *et al.*, 1991; Haughn *et al.*, 1995; Sieburth *et al.*, 1995).

9.2.2 *ATS* may affect the amount of mucilage produced

Only some of the epidermal cells of Ats mutants release mucilage and these appear to give out a reduced amount. These results suggest that the amount of mucilage or the strength of the outer cell wall may vary from cell to cell in these mutants. Conversely, the amount of mucilage may be at a threshold level that leads to occasional outer cell wall breakage in a stochastic fashion. The reduced mucilage phenotype may, along with the enlarged and slightly irregular epidermal cell shape and columellae, result as an indirect consequence of the absence of proper integument development. The abnormal shape of Ats seeds and their reduced dormancy has been attributed to production of only one, three-cell layer integument, rather than two integuments with a total of five cell layers (Léon-Kloosterziel *et al.*, 1994). The presence of epidermal cells with a relatively normal phenotype and seed pigment suggested that these two seed coat layers, which normally derive from the outer and inner integument, respectively, can develop (this chapter; Leon-Kloosterziel *et al.*, 1994). The presence of the palisade layer, though, has not been confirmed.

10. CONCLUDING REMARKS AND RECOMMENDATIONS FOR FURTHER INVESTIGATIONS

10.1 Concluding remarks

My studies have taken two tracks. First, I have clarified the roles of *AP2*, *AG*, and *BEL1* during ovule development in *Arabidopsis*. Second, I have exploited mucilage production in the seed epidermis to isolate novel genes affecting pectin biosynthesis, secretion, post-deposition modification, and extrusion, as well as general seed coat development.

10.1.1 The roles of *AP2*, *AG*, and *BEL1* during ovule development in *Arabidopsis*

My studies of multiple Ap2 mutants have shown that *AP2* may play a minor role in the stabilization of developmental decisions throughout ovule development. In addition, my investigation of the hypothesis that *BEL1* may act via control of *AG* has revealed that *AG* plays several roles during ovule development. My data suggest that *BEL1* and *AG* act early in ovule development in a partially redundant manner to direct ovule identity. The abnormal integument development characteristic of the Bel1 mutant phenotype was found to be dependent on *AG* function, suggesting that *BEL1* and *AG* function in combination at later stages to promote normal integument morphogenesis. Finally, *BEL1* appears to be required for embryo sac development independent of other aspects of ovule morphogenesis and *AG* function.

10.1.2 Seed coat development and mucilage production in *Arabidopsis*

My research has shown that mucilage production in the seed epidermis of *Arabidopsis* closely parallels that of *P. ovata* and *B. campestris*. In order to produce vast amounts of pectin, these cells become extremely active biosynthetic and secretory cells. In addition, in *Arabidopsis*, these cells also form an intracellular, volcano-shaped cell wall structure known as the columella in a process that may require both the action of the cytoskeleton and the production of mucilage.

I have used the absence of mucilage as a tag to isolate mutants affected in five novel genes, *MUM1—5*. My results show that these genes, along with four previously isolated loci, *AP2, ATS, TTG1*, and *GL2*, affect all levels of mucilage production, modification, and general seed coat development.

The differentiation of the seed coat from the ovule integuments appears to be regulated by *AP2* and *ATS*. *AP2* may regulate the initiation of almost all aspects of seed coat development, ranging from the initial shape change of the seed to the re-differentiation of both the epidermal and palisade layers of the seed coat. A lesser role is played by *ATS*, which seems to affect both seed shape and epidermal cell re-differentiation, most likely as a downstream effect of abnormal integument development.

MUM4, TTG1, and *GL2* act at the next level down, the regulation of the epidermal cell layer structure. While *TTG1* and *GL2* are general regulators of epidermal cell development throughout the plant, especially of those cells that undergo re-differentiation into a complex shape, *MUM4* works in a seed-specific manner. These three genes may be involved in the modification of cell structure through regulation of the cytoskeleton. Alternatively, they may act to positively regulate mucilage production in the epidermal cells of the seed coat. Another gene, *MUM1,* may also control

the amount of mucilage produced, but as Mum1 mutants have extra cell wall components, *MUM1* may also act as a negative regulator of cell wall production.

MUM4/TTG1/GL2 or *MUM1* may work through the control of *MUM3* and *MUM5,* which appear to act directly in the biosynthesis of pectin. Mutations in both genes led to the reduction of rhamnose and/or fucose, two components of complex polysaccharides. *MUM3* and *MUM5* thus may control the provision of sugar for or the actual composition of mucilage, possibly acting as biosynthetic enzymes.

MUM2, conversely, appears to act after the deposition of the mucilage in the cell wall. It may act as a negative regulator of pectin modifying enzymes found in the cell wall, such as the pectin methyl esterases.

10.2 Recommendations for further investigations

10.2.1 Ovule development

My conclusions regarding the activity of *AG* and *BEL1* during ovule development have been based on a combination of genetic analyses and transcription patterns. This hypothesis should be confirmed at the biochemical level. To ensure that AG and BEL1 are both active in the developing ovules, immunolocalization of both proteins ought to be performed. Since antibodies have not yet been produced for either of these proteins, the isolation of the antibodies would be the first step. In addition, direct interaction between AG and BEL1 should also be tested *in vitro* through gel retardation assays or *in vivo* via two-hybrid work in yeast.

Our hypothesis also suggests that AG may be working with an AGL protein in wild type ovule development. To test this, *in vitro* tests could be used to study possible interactions between AG and ovule specific AGLs.

10.2.2 Seed coat development

Of the novel genes identified through my screen, *MUM4* is particularly interesting due to its potential activity in the regulation the cytoskeleton or positive regulation of pectin biosynthesis. Several experiments could be performed in order to determine the true role of MUM4. First, the *MUM4* gene should be cloned and sequenced to determine if it encodes a protein directly associated with either of these roles (e.g. a cytoskeleton-associated protein). Cloning could either be done by map-based techniques or by screening for a tagged allele in T-DNA or transposon mutagenized lines. Second, a role for the cytoskeleton could be supported by following its changes throughout seed coat development. Third, a screen could be initiated for mutants in which the columella and reduced mucilage production phenotypes have been separated.

MUM3/5 and *MUM2* appear to be involved in mucilage biosynthesis and *in muro* modification, respectively, while *MUM1* may negatively regulate the amount synthesized. It would be useful to clone these four genes to determine if any encode an important protein in these processes. Similar to *MUM4*, cloning would either have to be map based or through the isolation of a tagged allele of the gene.

Finally, these five mutants were found in a preliminary screen of only 1000 EMS lines. Further screening for more mutants affecting mucilage production and secretion in *Arabidopsis* should lead to the identification of other genes that play important roles in these processes. In

addition, these screens should be carried out in lines created through insertional mutagenesis to facilitate the cloning of the genes.

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