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A Seed Shape Mutant of Arabidopsis That Is Affected in Integument Development

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A seed shape mutant of Arabidopsis was isolated from an ethyl methanesulfonate-treated population. Genetic analysis revealed that the heart-shaped phenotype was maternally inherited, showing that this is a testa mutant. This indicated the importance of the testa for the determination of the seed shape. This recessive *aberrant testa shape* (*ats*) gene was located at position 59.0 on chromosome 5. A comparison was made between ovules and developing and mature seeds of the wild type and of the mutant using light and scanning electron microscopy. We showed that the mutant seed shape is determined during the first few days after fertilization, when the embryo occupies only a very small part of the seed. The integuments of *ats* ovules consisted of only three rather than five cell layers. In double mutants, the effect of *ats* was additive to other testa mutations, such as *transparent testa*, *glabra* (*ttg*), *glabrous2* (*gl2*), and *apetala2* (*ap2*). The *ats* mutation resulted in a reduced dormancy, which was maternally inherited. This effect of a testa mutation on germination was also seen in *ttg* seeds, in which the outer layer of the testa was disturbed. This indicated the importance of the testa as a factor in determining dormancy in Arabidopsis.

INTRODUCTION

Seeds can be divided into parts of a genetically different origin: an embryo, the endosperm, and the seed coat or testa. The zygote, from which the embryo develops, combines the genotypes of the haploid male and female gametes. The embryo development in Arabidopsis is well documented: it follows the classic *Capsella* variation of the Onagrad type (Misra, 1962; Mansfield and Briarty, 1991). The endosperm is a result of a triple fusion. The two haploid polar nuclei of the central cell fuse, and, subsequently, this nucleus is fertilized by a haploid sperm nucleus, resulting in a triploid endosperm nucleus (Mansfield et al., 1991). This nucleus starts to divide, giving rise to a coenocytic endosperm. Not until the embryo is in the late globular stage does the endosperm begin to cellularize (Mansfield and Briarty, 1990). In mature seeds, the aleurone layer represents the remainder of the endosperm (Müller, 1963). After fertilization, during embryo development and subsequent seed maturation, the integuments undergo morphological changes and become the testa, which accordingly has the maternal genotype. Recently, ovule development in Arabidopsis has been described extensively (Robinson-Beers et al., 1992; Reiser and Fischer, 1993). The initiation of the integuments in *C. bursa-pastoris* has been described by Roth (1957). Bouman (1975) reported on testa development in some Cruciferae.

In Arabidopsis, various mutations in genes controlling different aspects of ovule and seed development have been described. Embryonic mutants can be defective in essential housekeeping genes or in genes involved in the regulation of embryo development (Meinke, 1991). Mutants with specific defects in pattern formation during embryogenesis are valuable tools in the elucidation of early developmental processes (Mayer et al., 1991). Ovule mutations, such as *short integuments* (*sin1*), *bell* (*bel1*) (Robinson-Beers et al., 1992), *ovule mutant-2* (*ovm2*), and *ovule mutant-3* (*ovm3*) (Reiser and Fischer, 1993), result in female sterility. Another category of seed mutants that are maternally inherited but expressed at later stages of seed development are testa mutants. *Transparent testa* (*tt*) mutants lack the brown pigment in the seed coat and often, but not always, lack anthocyanin in their vegetative tissues as well (Koornneef, 1990). In addition to these characteristics, the *transparent testa*, *glabra* (*ttg*) mutant is impaired in its ability to form trichomes and has a disturbed seed surface, which are features also exhibited by the *glabrous2* (*gl2*) mutant (Koornneef, 1981). It has been suggested that the *TTG* gene is a homolog of the *R* locus in maize, which is a transcription factor that activates promoters of biosynthetic genes in the anthocyanin pathway (Lloyd et al., 1992).

Here, we describe the isolation and the genetic, morphological, and physiological characterization of an Arabidopsis testa mutant with seeds that are heart shaped rather than having the normal oblong shape.

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RESULTS

Genetic Characterization

A mutant with heart-shaped seeds was isolated from an M_3 population. Genetic analysis revealed that this mutation was maternally inherited: if a mutant plant was pollinated with wild-type pollen, the F_1 seeds had the mutant phenotype. If a wild-type plant was pollinated with mutant pollen, the F_1 seeds had the normal, elongated seed shape. All F_2 seeds had this wild-type phenotype as well. In an F_2 generation, 186 plants with wild-type seeds and 47 plants with mutant seeds were found. These data fit a 3:1 ($\chi^2 = 2.78$; $P > 0.05$) segregation ratio, indicating that this is a single recessive mutation. This progeny analysis showed that the maternal heredity is not due to cytoplasmatic heredity or maternal imprinting. Therefore, this mutation apparently affects the testa, and the locus was designated *aberrant testa shape* (*ats*). Linkage analysis using F_2 and F_3 data from crosses with the chromosome 5 markers *ttg*, *yellow inflorescence* (*yi*), and *abscisic acid deficient* (*aba*) revealed significant linkage between *ats* and *ttg* and between *ats* and *yi* (Figure 1), locating the *ats* mutation at position 59.0 on chromosome 5.

Morphology of Ovules and Seeds

Scanning electron microscopy and light microscopy were used to compare the development of ovules and seeds of the wild type with that of the *ats* mutant. Figures 2A and 2C illustrate that in wild-type ovule primordia, two distinct rims of cells appear, developing into the inner and outer integument. Figures 2B and 2D show that the development of the integuments of *ats* ovules is irregular. During the development of *ats* ovules, no clear distinction between the developing inner and outer integument can be seen. However, mutant and wild-type ovules have the same overall shape (Figures 2E and 2F).

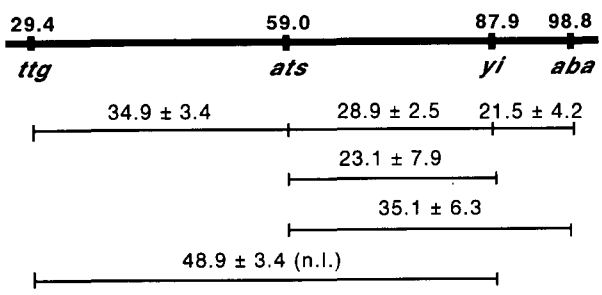

Figure 1. Location of *ats* on the Chromosome 5 Map.

The map locations on chromosome 5 of *ttg*, *ats*, *yi*, and *aba* (in centimorgans) are indicated on the top bar. The estimates of recombination percentages with standard deviations between *ats* and the morphological markers *ttg*, *yi*, and *aba* are shown below the top bar. n.l., no linkage.

Both in wild-type and in mutant ovules, rapid expansion of the endosperm immediately after fertilization was observed (Figures 2G and 2H). The expansion of the testa was merely caused by cell elongation and not by cell division, because the number of cells of the integuments did not increase. During this process, occurring during the first 4 days after anthesis, the wild-type seeds became oval, but *ats* seeds maintained their roundish shape. Figure 3 shows that seeds at 4 days after anthesis have reached the shape of a mature seed and have nearly reached their ultimate size. At this stage, the embryo was in the globular stage and occupied only a very small part of the seed. No differences in the shape of the embryos between the wild type and the *ats* mutant were observed during the first few days after anthesis.

In Figure 4, cleared ovules and scanning electron microscopy sections of ovules at 3 days after anthesis show the cell layers of the integuments. When the embryo is in the globular stage, the endosperm cellularization has not yet begun, so all visible layers are testa layers (Mansfield and Briarty, 1990). In wild-type ovules, the outer two cell layers represent the outer integument, which has overgrown the shorter inner integument, thereby forming the micropyle. The inner three cell layers represent the inner integument. The innermost cell layer of the inner integument ultimately becomes the pigmented layer of the mature seed (Bouman, 1975). In contrast to the wild type, in which the testa consisted of five cell layers at the apical end (Figure 4B), the testa of *ats* seeds consisted of only three cell layers at this position (Figure 4D). Apparently, two cell layers are absent in mutant seeds. In cleared *ats* ovules, the structure of the innermost cell layer was very similar to this corresponding layer in wild-type ovules and because *ats* seeds were normally pigmented, this indicated that this layer was unaffected in *ats* ovules.

The surface of Arabidopsis wild-type seeds consists of polygonal structures with a central elevation, the columella (Figures 5A and 5B). Wild-type seeds excrete a layer of mucilage upon contact with water; this layer can be visualized by staining the mucilage with ruthenium red. Figures 5C and 5D show that polygonal structures can be easily recognized on mature *ats* seeds. However, they were irregularly shaped and larger than those of wild-type seeds. Because these structures represent cells of the outer layer, this implies that this layer is present but that these cells are larger in *ats* than in wild-type seeds, as well as being reduced in number. *ats* seeds produced very little mucilage.

Interaction with Other Testa Mutants

The mutations *gl2* (Figures 5E and 5F) and *ttg* (Figures 5I and 5J) also result in an aberrant seed coat. In *gl2* and *ttg* seeds, the outer layer is affected because the columellas and the mucilage are absent (Koornneef, 1981). In the *ats,gl2* and *ats,ttg* double mutants, an additive phenotype could be observed (Figures 5G, 5H, 5K, and 5L), indicating that the *ats* mutation affects the testa in a different process than do *ttg* and *gl2*. The *apetala2* (*ap2-1*) mutation has a pleiotropic effect on the seeds:

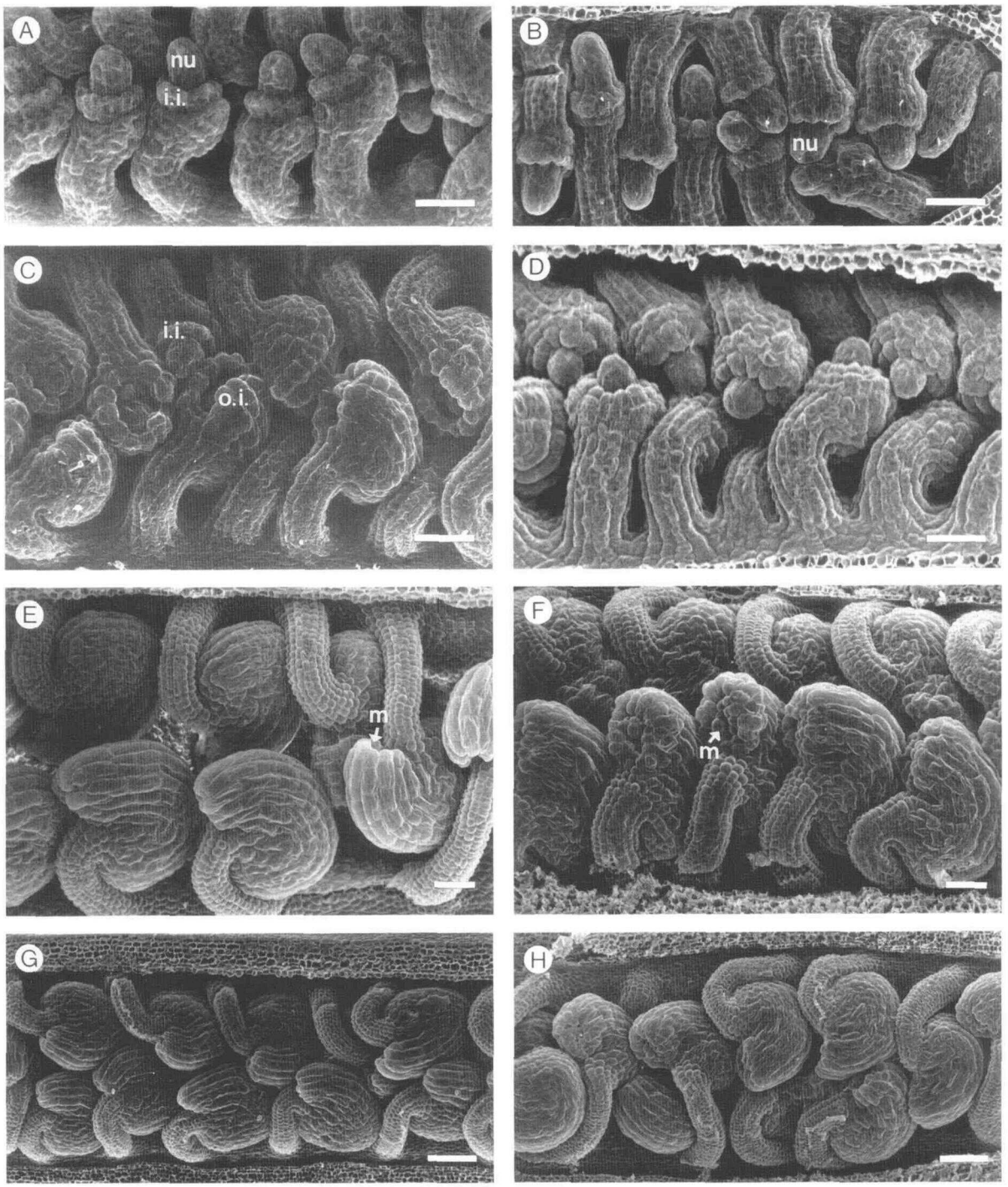

Figure 2. Scanning Electron Micrographs of Developing Wild-Type and Mutant Ovules.

Developmental stages refer to stages as described by Smyth et al. (1990). **(A)**, **(C)**, **(E)**, and **(G)** show wild-type ovules; **(B)**, **(D)**, **(F)**, and **(H)** are micrographs of *ats* mutant ovules.

(A) and **(B)** Stage 11. i.i., inner integument; nu, nucellus. Bars = 25 μm.

(C) and **(D)** Stage 12: early. i.i., inner integument; o.i., outer integument. Bars = 25 μm.

(E) and **(F)** Stage 13: ovules at the day of anthesis. m, micropyle. Bars = 25 μm.

(G) and **(H)** Ovules 1 day after anthesis. Bars = 50 μm.

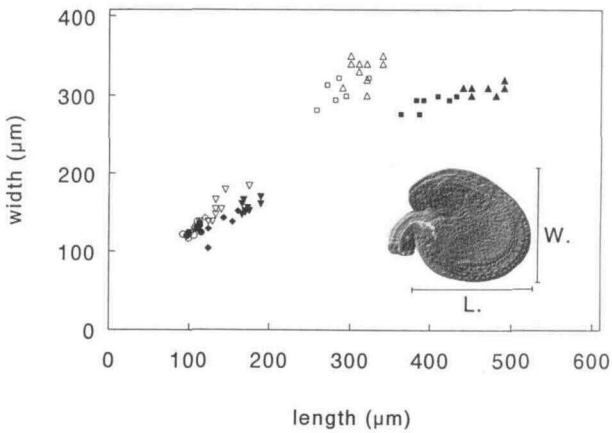

Figure 3. Length–Width Relation of Ovules and Seeds.

Lengths are plotted against widths of mature seeds (△, ▲) and of ovules at the day of anthesis (○, ●) and at 1 day (◇, ♦), 2 days (▽, ▼), and 4 days (□, ■) after anthesis. The closed symbols show the wild type, and the open symbols show the *ats* mutant. W., width; L., length.

ap2-1 seed shape could vary from the normal oblong shape to a variety of aberrant shapes (Figure 5M). Therefore, some *ap2-1* seeds resembled *ats* seeds. On most *ap2-1* seeds, the surface pattern was not normal (Figure 5N). The polygonal structures were only present on parts of the seed surface and lacked the columella. Seeds of the *ats,ap2-1* double mutant also showed an additive phenotype, because the seed malformation was more extreme (Figures 5O and 5P).

Seed Germination Characteristics and Fertility

Viable Landsberg *erecta* seeds, which are freshly harvested, do not germinate under conditions of sufficient oxygen, water, and light supply: they are dormant. Mutants with a transparent seed coat (*ttg*) are known to have a reduced seed dormancy (Koornneef, 1981). Because dormancy is relieved during dry storage of seeds, the germination percentage and germination speed of *ats*, *ttg*, and *ats,ttg* double mutant seeds were determined after seeds were stored for different periods. This allowed us to distinguish the germination characteristics of those genotypes; the results are given in Figure 6. Wild-type and *aba* seeds served as dormant and nondormant controls. Seeds of the *aba* mutant lack dormancy because of the reduced level of abscisic acid (Koornneef et al., 1982).

Three days after harvest, wild-type seeds were fully dormant (0% germination after 7 days of incubation), whereas the nondormant *aba* seeds germinated within 3 days (Figure 6A). Figure 6A shows the severely reduced seed dormancy of *ttg* seeds; *ats* seeds had a slightly reduced dormancy. The germination behavior of *ats,ttg* double mutants is indicative of the additive effect of both mutations at the physiological level. Release from dormancy was faster in *ats* seeds than in wild-type seeds. After 10 days of storage, 80% of the *ats* seeds had germinated after 7 days of incubation, whereas none of the wild-type seeds had germinated (Figure 6B). Seventeen days after harvest of the seeds, all mutants germinated within 3 days, while a large part of the wild-type seeds had also been released from dormancy (Figure 6C). Thus, *ats* has a reduced seed dormancy. Figure 7 shows that the reduced seed dormancy was maternally inherited, indicating that this is determined by characteristics of the testa and not by characteristics of the embryo.

In addition to the seed characteristics, no pleiotropic effect on the *ats* plants was observed, except that *ats* plants had shorter siliques than did wild-type plants. As shown in Table 1, the number of ovules in *ats* siliques was not different from that of wild-type siliques, but the number of mature seeds was lower in *ats* siliques. Because there is a correlation between seed number and silique length (Barendse et al., 1986), the shorter siliques of *ats* plants were the result of a lower seed number per silique. Reciprocal crosses did not indicate that male fertility of the *ats* mutant was affected (data not shown).

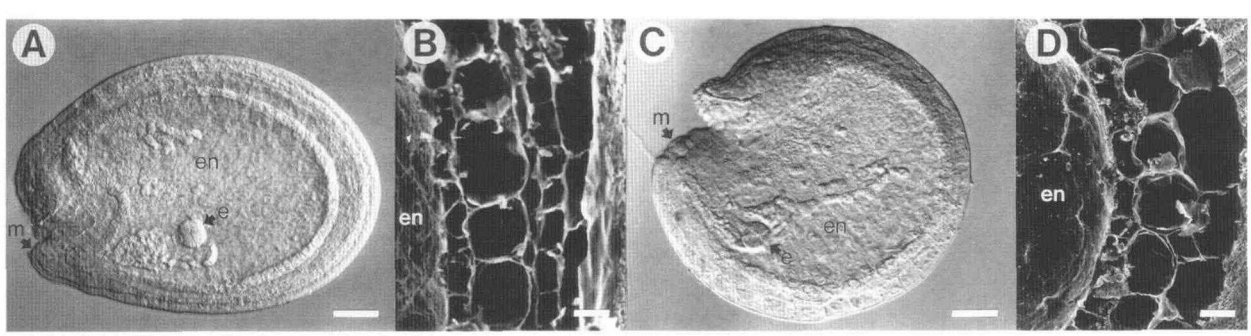

Figure 4. Light Micrographs and Scanning Electron Micrographs of Ovules at 3 Days after Anthesis.

(A) and **(B)** Wild-type ovules.
(C) and **(D)** Ovules from an *ats* plant.
Bars in **(A)** and **(C)** = 50 μm; bars in **(B)** and **(D)** = 10 μm. e, embryo; en, endosperm; m, micropyle.

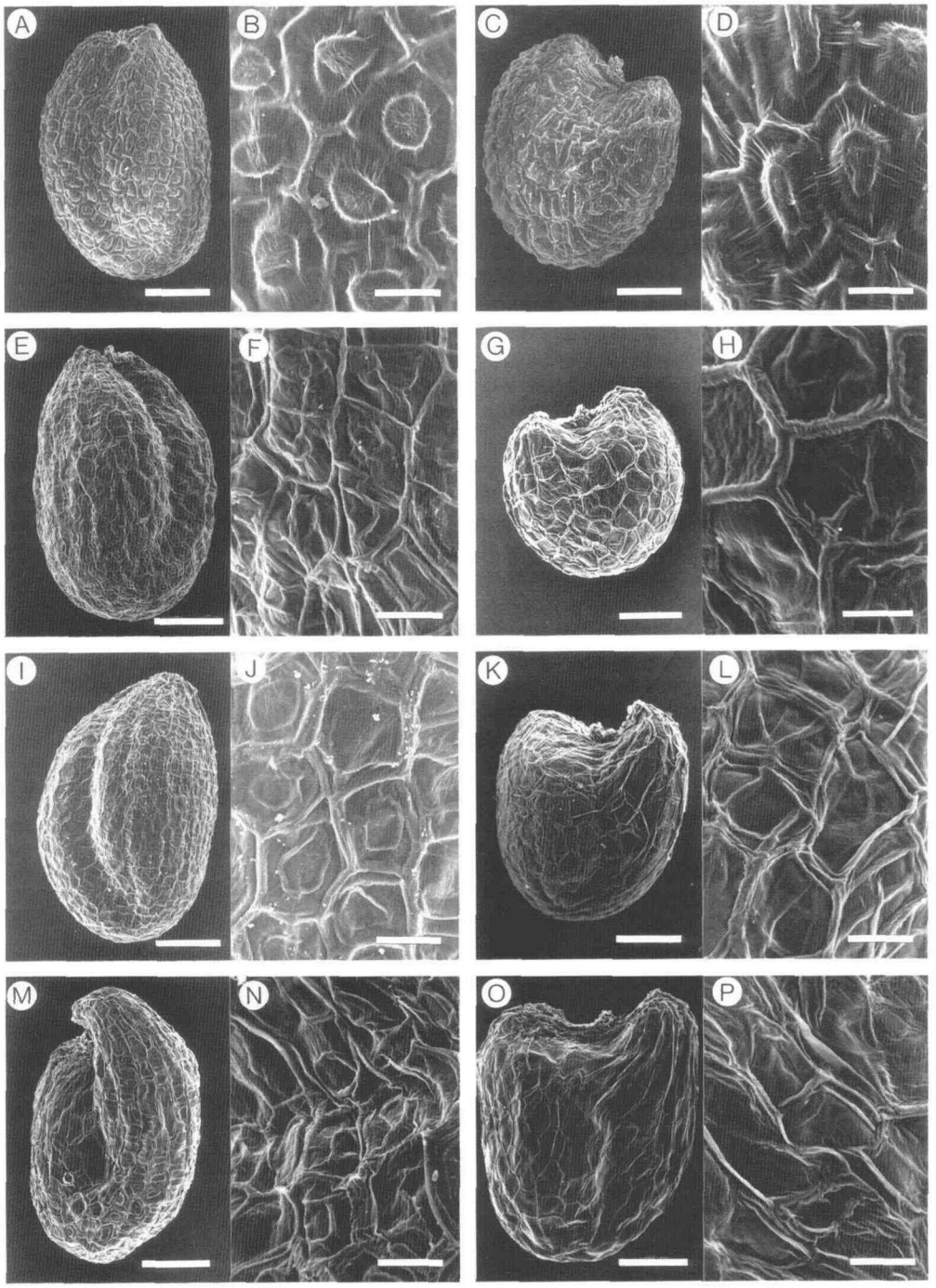

Figure 5. Scanning Electron Micrographs of Mature Seeds.

(A) and **(B)** Wild-type seeds.
(C) and **(D)** *ats* seeds.
(E) and **(F)** *gl2* seeds.
(G) and **(H)** *ats,gl2* double mutant seeds.
(I) and **(J)** *ttg* seeds.
(K) and **(L)** *ats,ttg* double mutant seeds.
(M) and **(N)** *ap2-1* seeds.
(O) and **(P)** *ats,ap2-1* double mutant seeds.
Bars in **(A)**, **(C)**, **(E)**, **(G)**, **(I)**, **(K)**, **(M)**, and **(O)** = 100 μm. Bars in **(B)**, **(D)**, **(F)**, **(H)**, **(J)**, **(L)**, **(N)**, and **(P)** = 20 μm.

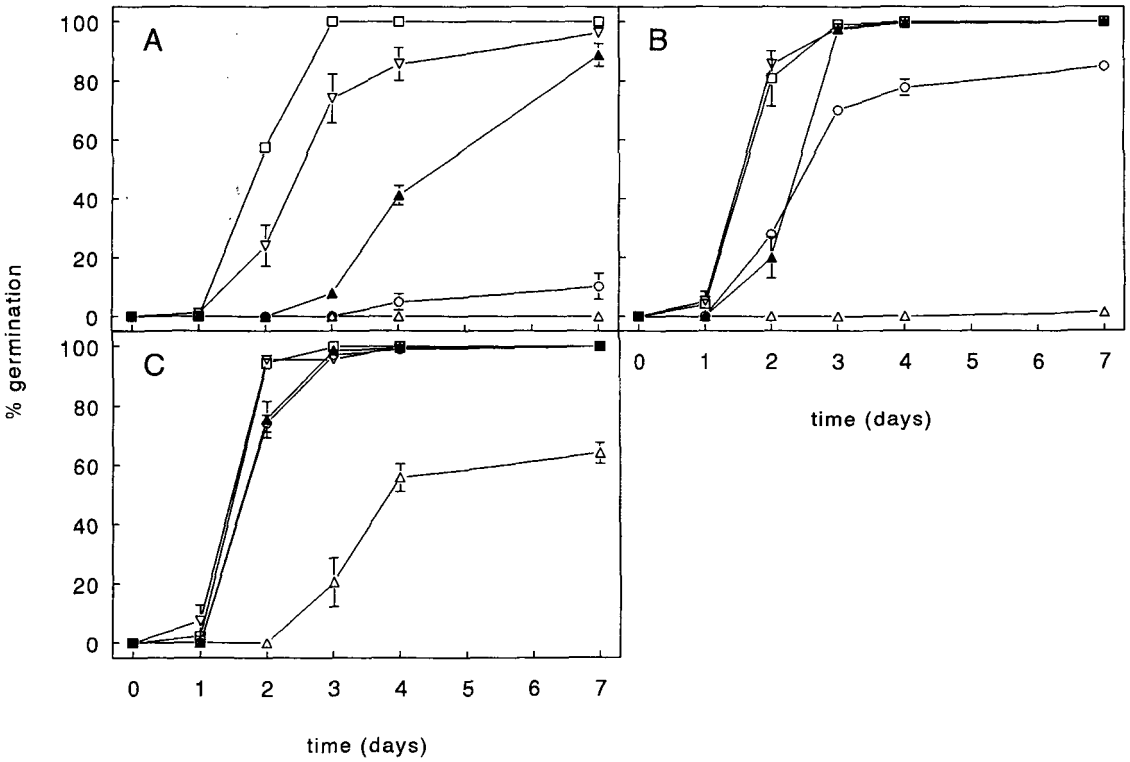

Figure 6. Comparison of Germination and Dormancy of the Wild Type and Testa Mutants.

The time course of germination of wild-type (△), *ats* (○), *ttg* (▲), *ats,ttg* (▽), and *aba* (□) seeds was determined after different storage periods.
(A) Storage period of 3 days.
(B) Storage period of 7 days.
(C) Storage period of 17 days.

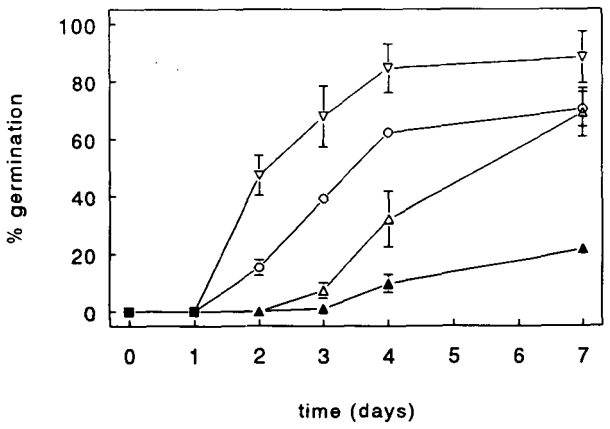

Figure 7. Time Course of Seed Germination.

Seeds were stored for 17 days. Germination was determined for wild-type (△), *ats* (○), F_1 wild-type × *ats* (▲), and F_1 *ats* × wild-type (▽) seeds.

DISCUSSION

In this study, we described the isolation and characterization of an Arabidopsis mutant that has a maternally inherited aberrant seed shape (Figure 5C). In this *ats* mutant, only the seed shape is affected; this is in contrast to *ap2-1*, for example, in which the seeds have a deviant shape (Figure 5M), and the development of flowers is also affected (Bowman et al., 1989).

The maternal inheritance of *ats* showed that the shape of *ats* seeds is determined by the testa and not by the embryo, notwithstanding the fact that in mature Arabidopsis seeds, the embryo fills up the seed completely and that the seed coat seems to follow the contours of the embryo. The testa in Arabidopsis develops from a two-layered outer integument and a three-layered inner integument (Figure 4B). This showed that not only is the embryogenesis of Arabidopsis similar to the embryogenesis of *C. bursa-pastoris* (Mansfield and Briarty, 1991) but that the testa morphology of Arabidopsis

Table 1. Silique Length, Number of Ovules, and Number of Mature Seeds in Siliques of Wild-Type and *ats* Plants

	Average Silique Length (mm)	Average No. Ovules per Silique Half	Average No. Mature Seeds per Silique Half
Wild Type[a]	12.6 ± 0.7	32.2 ± 3.1	30.9 ± 3.2
ats[a]	10.4 ± 0.7	32.2 ± 2.5	26.2 ± 3.4

[a] n = 23; means are given ± SD.

is also the same as has been observed in *C. bursa-pastoris* (Bouman, 1975).

Light and scanning electron microscopy revealed that *ats* produces ovules in which the integuments do not develop properly. In wild-type ovule primordia, two distinct rings of cells appear, developing into the inner and outer integument (Figure 2A; Robinson-Beers et al., 1992; Reiser and Fischer, 1993). This is also similar to the situation in *C. bursa-pastoris*: Roth (1957) reported that first the inner and, subsequently, the outer integument develop from two different rings of dermal cells. This clear distinction between the developing integuments is absent in *ats* ovules (Figures 2B and 2D), and results in ovules in which the embryo sac is surrounded by three rather than five cell layers (Figures 4B and 4D). A possible explanation for these missing layers is that in *ats* ovule primordia only one ring of dermal cells develops or that the two integument primordia are fused and give rise to one integument, which has characteristics of both the inner and outer integument. The aberrant shape of *ats* seeds is established in the first few days following anthesis, when a rapid expansion of the ovules occurs. As a consequence of deviant cell divisions, leading to missing layers, the integuments also may not exhibit proper cell expansion, which results in the aberrant shape.

The *ats* mutant is a valuable addition to the other Arabidopsis ovule mutants *bel1*, *sin1* (Robinson-Beers et al., 1992), *ovm2*, and *ovm3* (Reiser and Fischer, 1993). These mutants are impaired in the formation of normal integuments and embryo sacs. It is hypothesized that the inner integument is missing from the ovules of the *bel1* mutant. Because no linkage data have been published for *bel1*, we cannot rule out the possibility that *ats* is a leaky allele of the *bel1* locus. However, the fertility of *ats* plants was only slightly reduced and was not due to a reduced pollen fertility. This indicates that *ats* may be a new locus that is required only for integument initiation and not for megagametogenesis because *ats* is not female sterile. Segregation of F_2 plants bearing *ats* seeds fitted a 3:1 ratio, indicating that the viability of *ats* embryos was not affected. The additive effect of *ats* with the other testa mutations *ttg*, *gl2*, and *ap2-1* indicated that the formation of the integuments is a complex process requiring several genes to determine aspects of cell division planes and cell differentiation.

The germination behavior of *ats* and *ttg* showed that the degree of dormancy of a seed is determined not only by characteristics of the embryo but also by characteristics of the testa. At germination, the radicle has to penetrate the seed coat, and apparently the structure determining the solidity of the testa is a factor that influences germination. The role of the mucilage must also be considered. Germination of seeds of *Blepharis persica* can be stimulated by removing the mucilage or the seed coat and by increasing the percentage of oxygen to which they are exposed (Witztum et al., 1969). Pricking the seeds or removing the testa also promotes germination of Arabidopsis seeds (Kugler, 1951). A large increase in oxygen uptake rate occurs at the start of germination of seeds of *Sisymbrium officinale*, a species closely related to Arabidopsis (Derkx and Karssen, 1993). Perhaps the absence of mucilage on *ats* and *ttg* seeds allows more oxygen to diffuse into the seed, and this higher oxygen level might be able to release it from dormancy. To explain the additive effect of *ttg* and *ats* on germination, one has to assume that the structure of the testa influences germination.

The *ats* mutant will be useful in the study of integument initiation because specific layers of the integuments and, thereby, the testa are affected. Detailed light microscopy of sections of ovules at the stage of integument initiation will more clearly show how the integuments develop in the *ats* mutant. By using testa mutants, the function of the testa for dormancy and germination can be explored.

METHODS

Mutant Isolation and Genetic Analyses

Mutant lines were generated in *Arabidopsis thaliana* ecotype Landsberg *erecta* (Ler) seeds carrying the *transparent testa, glabra* (*ttg*) mutation by applying 15 mM ethyl methanesulfonate for 24 hr. In a selection program designed to isolate seed dormancy mutants, the seeds from one putative mutant that was selected in the M_2 generation appeared to be heart shaped. This mutant, which had a limited seed set, was backcrossed twice with wild-type Ler. Fertile plants with heart-shaped brown seeds were obtained and used for the microscopic and physiological analysis.

For genetic analysis, the *aberrant testa shape* (*ats*) mutant was reciprocally crossed with the wild type. For mapping, a cross was made between *ats* and a line homozygous recessive for the markers *ttg* and *yellow inflorescence* (*yi*). Subsequently, a cross was made between an *ats,yi* recombinant and a line homozygous recessive for the markers *ttg* and *abscisic acid deficient* (*aba*). F_2 and F_3 populations derived from these crosses were scored for seed shape, together with the marker phenotypes. Recombination percentages were estimated using the RECF2 program (Koornneef and Stam, 1992). The map locations were determined with the JOINMAP program (Stam, 1993) by using the data obtained with the present analyses in combination with the data set for classic genetic markers used by Hauge et al. (1993). Correction for double cross-overs was done with the Kosambi mapping function. Double mutants were constructed by crossing the mutant with lines carrying the mutations *ttg*, *glabrous2* (*gl2*), and *apetala2* (*ap2-1*), respectively. F_2 plants with heart-shaped seeds, but otherwise a wild-type phenotype, were selected. Double mutants were selected from the selfed progeny of these F_2 plants that segregated for *ttg*, *gl2*, or *ap2-1*, respectively.

Microscopy

For scanning electron microscopy, siliques and flowers were immersed in 2% glutaraldehyde for 16 to 20 hr at room temperature and subsequently dehydrated in a graded series of ethanol. Critical-point drying was conducted in liquid carbon dioxide. The siliques and flowers were mounted on stubs, dissected using a special microtome (Keijzer, 1993), and sputter coated with paladium gold. Specimens were examined with a scanning microscope (model JSM 5200; Jeol, Tokyo). For light microscopy, ovules were removed from the pistil or the silique and immersed in a droplet of clearing solution (72% [w/v] chloral hydrate, 17% [w/v] water, 11% [w/v] glycerol) on a slide and covered with a coverslip. The ovules were examined with a microscope (Nikon) equipped with Nomarski optics.

Germination Assay

For germination assays, mature seeds were harvested from dehydrated siliques. After storage for 3, 10, or 17 days at room temperature, the seeds were sown on water-saturated filter paper (No. 595; Schleicher & Schuell) in Petri dishes and incubated at 21°C under continuous white light (Philips TL57 and incandescent bulbs). The seeds were scored for germination every day during a 7-day period.

Culture Conditions

To grow plants, seeds were sown in Petri dishes on water-saturated filter paper and incubated in a growth chamber at 25°C. After 2 days of incubation, germinated seeds were transferred into soil and cultivated in an air conditioned greenhouse (18 to 23°C) with additional light during the winter (Philips HPI-T/400W; 16-hr photoperiod).

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NOTE ADDED IN PROOF

Hybrids of the *ats* and *bel1* mutants were fertile and had seeds with the wild-type phenotype, indicating that both mutants are not allelic.