J. Plant Res. **113**: 139-148, 2000

Journal of Plant Research

Histological Study of Seed Coat Development in *Arabidopsis thaliana*

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A detailed analysis of *Arabidopsis* seed coat development using light and transmission electron microscopy revealed major morphological changes associated with the transition of the integuments into the mature seed coat. By the use of a metachromatic staining procedure, cytological events such as the production of phenolic compounds and acidic polysaccharides were followed. Immediately after fertilization, the cells of the inner epidermis of the inner integument became vacuolated and subsequently accumulated pigment within them. This pigment started to disappear from the cytoplasm at the torpedo stage of the embryo, as it became green. During the torpedo stage, mucilage began to accumulate in the cells of the external epidermis of the outer integument. Furthermore, starch grains accumulated against the central part of the inner periclinal wall of these cells, resulting in the formation of small pyramidal domes that persisted until seed maturity. At the maturation stage, when the embryo became dormant and colourless, a new pigment accumulation was observed in an amorphous layer derived from remnants of crushed integument layers. This second pigment layer was responsible for the brown seed colour. These results show that seed coat formation may proceed in a coordinated way with the developmental phases of embryogenesis.

Key words: *Arabidopsis thaliana* — Embryogenesis — Pigmentation — Seed coat

The seed coat often plays an essential role in various processes such as nutrition of the growing embryo, mechanical and chemical protection, dehydration, imbibition, and maintenance of seed dormancy (Boesewinkel and Bouman 1995). It usually develops from one or two integuments, but mature seeds may include funicular tissue or layers of the nucellus and/or the endosperm (Fahn 1990). The inner integument may give rise to a tegmen and the outer integument to a testa (Corner 1976). During seed coat development many histological changes take place. Periclinal and anticlinal cell divisions, combined with cell enlargement, are responsible for the growth of the seed coat. At seed maturity, however, much of the integumental tissue may be degenerated and absorbed by other developing tissues (Fahn 1990). To clarify which parts have taken part in the formation of a seed coat, ontogenetic studies are necessary.

In *Arabidopsis thaliana* (L.) Heynh. various mutations in genes controlling different aspects of ovule and seed development have been described (Klucher *et al.* 1996, Léon-Kloosterziel *et al.* 1994, Reiser and Fischer 1993, Robinson-Beers *et al.* 1992). These studies resulted in detailed analyses of the ovule ontogeny, which mainly focused on integument development. Nevertheless, a description of the subsequent development into the seed coat is entirely lacking.

We performed a detailed analysis of the *Arabidopsis* seed coat development using light and transmission electron microscopy. With a metachromatic staining procedure the accumulation of phenolic compounds and acidic polysaccharides could easily be followed. Based on the obtained anatomical data, we propose a model that describes the main developmental changes in the seed coat after fertilization and explains the structure found in mature seeds. Distinct changes in one or more cell layers coincide with the three main developmental phases of embryogenesis: morphogenesis, maturation including synthesis of reserve products and dormancy and dessication (West and Harada 1993). For a full understanding of the structure of the mature seed coat of *Arabidopsis*, the development of the endosperm, although not belonging to the seed coat proper, needs to be considered as well, because one or two endospermal layers persist and stick to the seed coat.

We will discuss each layer in the seed coat separately, though with an emphasis on the development of the inner epidermis of the inner integument or pigment layer, and on the mucilage-producing outer epidermal layer. The detailed description of embryogenesis in *Arabidopsis* (Jürgens and Mayer 1994) was used to stage the developmental events seen during seed coat formation.

Materials and Methods

Light microscopy

Anatomical changes were recorded on whole-mount preparations as well as on sectioned ovules. For both

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purposes the ovules were dissected from siliques at different stages of development and fixed for 4 hr in a mixture of 45% ethanol, 5% acetic acid, and 5% formaldehyde in distilled water.

To study the initial stage of seed coat development, ovules were isolated from flower buds at stage 13 of flowering as determined by Smyth *et al.* (1990). For whole-mount preparations, the seeds were transferred to a 2:1:1 mixture of chloral hydrate, lactic acid and phenol (chloral lactophenol, CLP). After at least 2 hr of clearing, the ovules were put in a droplet of CLP on a microscope slide and viewed in a Diaplan microscope (Leitz, Wetzlar, Germany) using differential interference contrast optics.

For sectioning, the seeds were dehydrated (upon fixation) and embedded in Technovit 7100 embedding resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Sections (4 μm) were made on a rotary microtome (Supercut 2050) equipped with glass knives (Reichert-Jung, Nussloch, Germany) and stained for 8 min in 0.05% toluidine blue (Toluidine blue O; Merck, Darmstadt, Germany) in an aqueous solution or in an aqueous solution of 1% acid fuchsine and 1% toluidine blue.

Electron microscopy

For scanning electron microscopy, mature dried seeds, without foregoing treatments, were sputter coated with gold and examined with a scanning electron microscope (JEOL Ltd, Tokyo, Japan) at an acceleration voltage of 15 kV. For transmission electron microscopy, seeds were dissected out of the siliques and fixed in a mixture of 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). Mature seeds were put in the same fixative after the seed coat had been carefully pricked with a fine needle under a dissecting microscope. Subsequently, the material was transferred to fresh fixative and kept overnight at room temperature. The next day, the fixative was washed out with three changes of cacodylate buffer (each for 20 min). Postfixation was performed in 2% osmium tetroxide and 1.5% potassium ferricyanide in cacodylate buffer for 2 hr at room temperature. After three washes of 20 min with cacodylate buffer, the material was dehydrated in ethanol. After dehydration to 50% ethanol, the tissue was stained with 1% uranyl acetate. Further dehydration was performed using a graded ethanol series: 70% (overnight), 95% (2 hr) and 100% (2×2 hr). Finally, the samples were gradually infiltrated and embedded in Spurr's resin. Ultra-thin sections (60-90 nm) were cut with an Ultracut microtome (Reichert-Jung, Heidelberg, Germany) using a diamond knife, and collected on collodion-coated copper grids. The grids were poststained in an LKB Ultrastainer for 15 min in uranylacetate at 40 C and 2 min in lead citrate at 20 C. Sections were examined using an Elmiskop 101 transmission electron microscope (Siemens, Karlsruhe, Germany).

Results

The ovule wall before fertilization

In principle, the start of seed coat development coincides with the onset of embryogenesis. To study the initial stage of seed coat development, ovules isolated from flower buds at 0 hr after flowering (see Materials and Methods) were sectioned and examined. The mature ovule is amphitropous (Robinson-Beers *et al.* 1992) with the micropyle positioned near the insertion of the funiculus with a pronounced curvature of both integuments and embryo sac (Fig. 1a). At this stage, the nucellus is nearly completely resorbed, except for a group of cells at the base of the embryo sac. The inner and outer integuments completely enclose the mature embryo sac. The zone between the chalazal and micropylar pole of the embryo sac is called the curving zone or basal body (Bouman 1975) (Fig. 1a2).

Although the outer and inner integuments are both entirely of epidermal origin (Schneitz *et al.* 1995), the constituent cell layers will be treated here as separate entities because they differ in various characteristics during seed coat formation. To document the seed coat development and to clarify which layers of the integuments take part in the formation of the seed coat, the terms outer and inner integuments as well as their constituting cell layers (abbreviated oi1, oi2, ii1, ii1′, and ii2) will be used instead of testa and tegmen (Corner, 1976). The outer integument (oi) consisted of two cell layers, an inner (oi1) and an outer (oi2) epidermis, both of which were composed of large and vacuolated cells at the micropylar and the chalazal pole, but of smaller cells in the curving zone (Fig. 1a).

The inner integument (ii) contained two cell layers at the micropyle (ii1 and ii2) but became three-layered in the curving zone (ii1, ii1′ and ii2), both at the adaxial and abaxial sides (Figs. 1a and 1b). The ii1′ was only present in the curving zone whereas the inner integument at the micropylar and chalazal poles remained two-layered during seed maturation. Thus, at the beginning of its development the seed coat was composed of five cell layers (Fig. 1b), except in the micropylar and chalazal poles. With the exception of the cells at the micropylar pole, the ii1 layer became darkly stained by toluidine blue before fertilization (Fig. 1a). This layer, usually called an "endothelium", is composed of small isodiametric cells containing small vacuoles, thus resembling meristematic cells (Fig. 2a).

The pigment layer or endothelium

Immediately after fertilization, at the one-cell stage of the embryo (i.e., after the first zygotic division, when the embryo is composed of one small apical and one large basal cell), the cells of the ii1 layer became vacuolated (Fig. 2b). The inner cell wall of ii1, seen on the surface facing the embryo sac, was bordered by an electron-dense layer that reacted positively to osmium tetroxide (arrow, Fig. 2b), suggesting its lipid nature. We considered it as the original cuticle of the inner integument. Such a cuticle was only found on the surface of cells in direct contact with the embryo sac and not on the other integument layers. This cuticle was observed in electron microscopic sections until the mature-embryo stage.

From the two-cell stage of the embryo onwards, the cells of the ii1 layer showed a remarkable change in staining

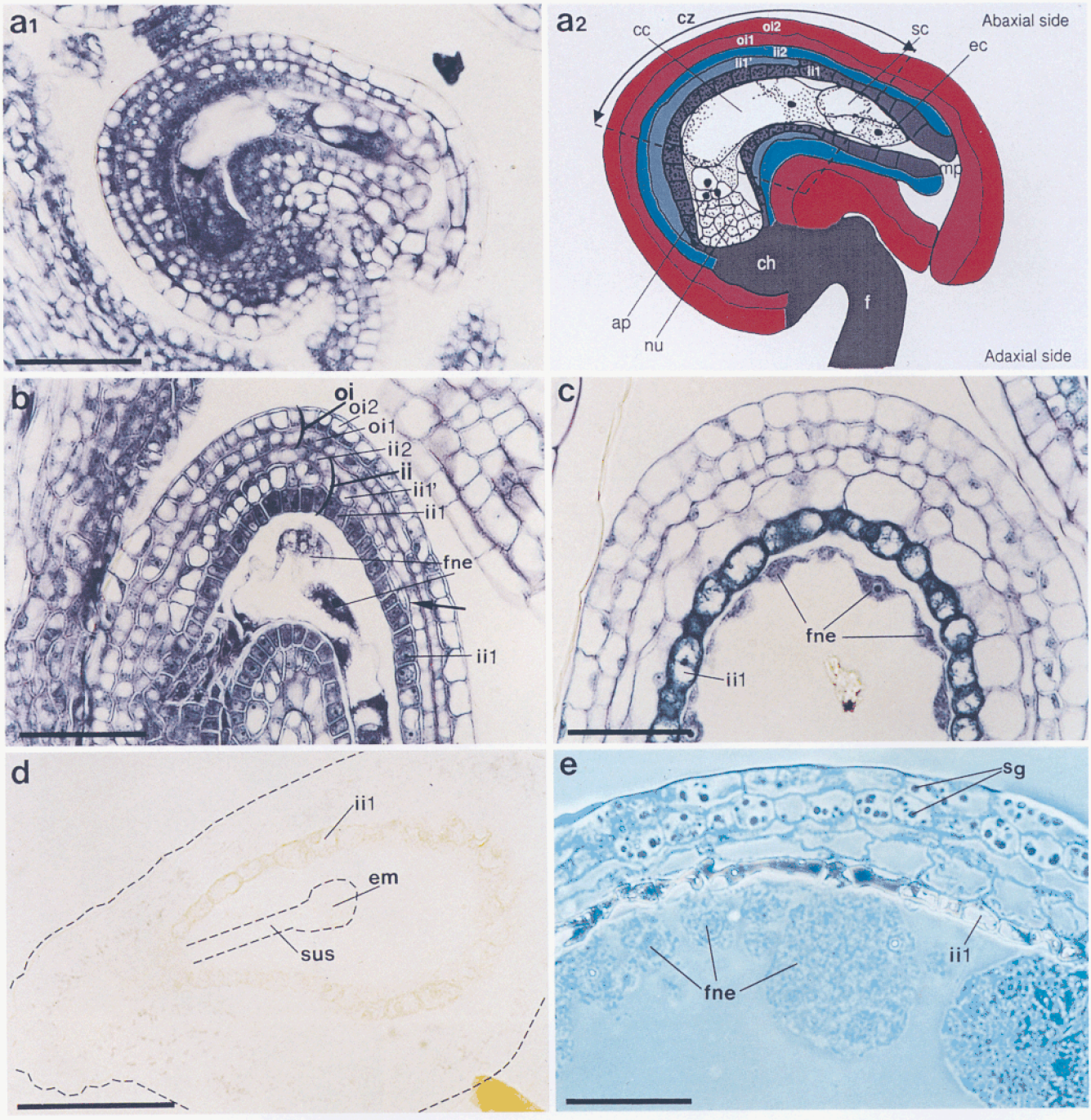
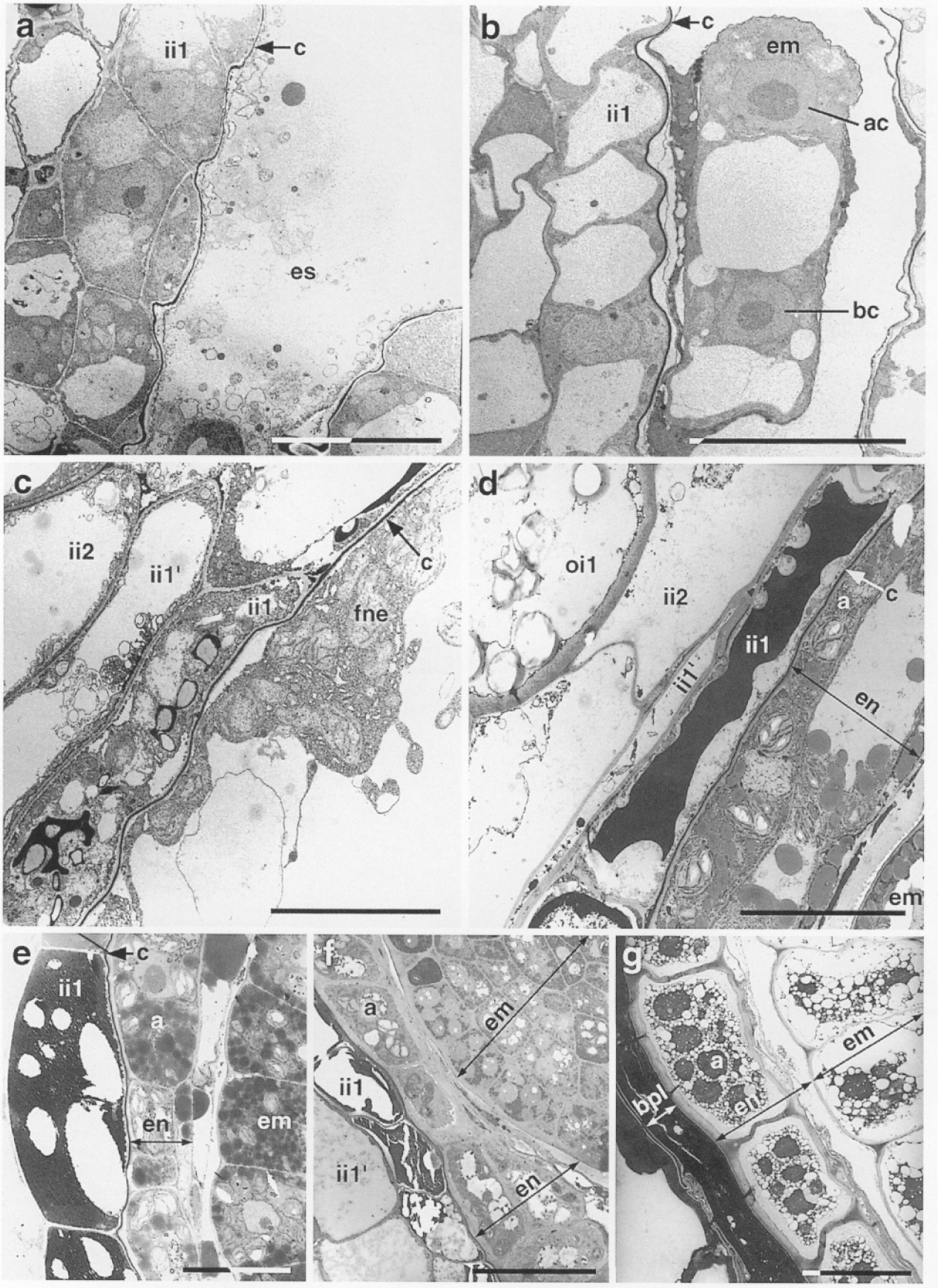


Fig. 1. Seed coat formation during early embryogenic stages in *Arabidopsis thaliana.* Longitudinal sections through ovules stained with toluidine blue, except for (d) in which no staining was used. Bright-field optics were used except for (e). a1. Pre-fertilization stage with a mature embryo sac. a2. Schematic drawing of a1. b-e. Details of the integuments at the abaxial side in the curving zone, i.e. the part of the ovule, between the micropyle and the chalaza. Ovular side facing the locule of the silique is called abaxial, whereas the side connected to the placenta via the funiculus is called adaxial. b. One-cell stage. Arrow indicates where ovular coverings become five-layered. Note that the cells of the inner layer of the inner integument (ii1) are stained dark-blue. c. Two-cell embryo stage. The cytoplasm of ii1 or pigment layer cells are stained bluish-green. d. Dermatogen embryo stage. The contours of the ovule, suspensor, and embryo are indicated by dotted lines. The ii1 layer appears light yellow because of an early pigment deposition. e. Globular embryo stage. Picture made using phase-contrast optics to demonstrate numerous starch granules in the layers of the outer integument. ap, antipodal cells; cc, central cell; ch, chalaza; cz, curving zone or basal body; ec, egg cell; em, embryo; f, funiculus; fne, free nuclear endosperm; ii, inner integument; ii1, inner epidermis of inner integument or pigment layer; ii1′, median layer of inner integument; ii2, outer layer of inner integument; mp, micropyle; nu, nucellus, oi, outer integument; oi1, inner epidermis of outer integument; oi2, outer epidermis of outer integument; sc, synergid cell; sg, starch granules; sus, suspensor.. Bars=50 μm (a-c, e), and=100 μm (d).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capacity in light microscopic sections stained with toluidine blue (Fig. 1c). Until the one-cell embryonic stage the cell content stained dark-blue (Fig. 1b), but from the two-cell stage onwards a bluish green staining appeared in this layer. With toluidine blue a similar bluish green colour was normally obtained in lignified cell walls. In the ii1 layer toluidine blue clearly stained the cell content. Even without staining, the accumulation of a light yellow pigment could be observed in these cells (Fig. 1d). Therefore, this layer is generally termed a "pigment" layer.

In electron microscopic sections, we noticed that pigment accumulation was preceded by the formation of a central vacuole (Fig. 2b). In the stages following the one-cell embryo stage a dark electron-dense substance was deposited, first inside the vacuoles (Fig. 2c), gradually filling most of the cells by the early torpedo stage (Fig. 2d). The pigment was also deposited in the cytoplasm; organelles (e.g. nuclei) were found completely embedded in this electron-dense material. From the late-torpedo stage onwards, the pigment disappeared from the central part of the cells and only remained at the cell periphery, leaving a large central cavity at the mature-embryo stage (Figs. 2e and 2f). At the desiccation stage, the pigment layer had become a layer of empty, dead cells (Figs. 3f and 3g) or had disappeared completely, its remnants being incorporated into the brown pigment layer (bpl) (Fig. 2g).

The ii1′ and ii2 layers

At the one-cell stage of the embryo, an extensive vacuolisation in the cells of the ii1′ and ii2 layers at the abaxial side of the seed coincided with the expansion of the ovule along the adaxial-abaxial axis (for terminology of the axis, see Fig. 1a). This expansion gradually intensified during the following stages. Even in cleared preparations of torpedo-stage seeds, large cells could be seen in these layers at the abaxial side (data not shown). Cell width along the adaxial-abaxial axis of ii1′ cells in the abaxial region of the ovule, measured on longitudinal sections, was approximately 5 μm before fertilization, 8 μm at the one-cell stage, and 25 μm at the torpedo stage. Both cell layers were highly vacuolated and their walls only showed weak staining. From the bent-cotyledon stage onwards, shrinkage of both these cell layers seemed to be induced by the growing embryo (Fig. 3d). At the desiccation stage, the ii1′ and ii2 layers were completely crushed and formed the brown pigment layer (bpl) (stained green with toluidine blue) that was responsible for the seed colour (Figs. 2g, 3f, and 3g).

The oi1 and oi2 layers

At the onset of embryogenesis the cells in the outer integument were vacuolated (Fig. 1b). By the globular stage, they were characterized by the presence of starch grains (Fig. 1e). In cleared preparations, some dermatogen-stage seeds showed starch grains whereas others did not, indicating that the accumulation of starch started mainly at this stage.

Electron microscopic micrographs of torpedo-stage seeds showed that the inner periclinal walls of the oi1 became thickened (Figs. 3b and 3c: arrowheads). In light-microscopic micrographs of sections stained with toluidine blue, these walls stained darker than comparable cell walls at previous stages (Fig. 3a: arrowheads). The other cell walls of these cells kept their original thickness. The dark bluish colour indicated that the primary wall became thickened rather than that a secondary wall, accompanied with lignification, was formed, as this would result in a bluish green colour. This observation was confirmed by electron microscopy, revealing a thickened primary inner periclinal cell wall throughout the whole oi1 layer (Figs. 3b and 3c).

At the torpedo stage, a second obvious cytological change was noticed in the seed coat. After toluidine blue staining, the content of the cells composing the oi2 layer turned reddish purple (Fig. 3a). This colour reaction, observed in the middle lamellae of unlignified cells by O'Brien *et al.* (1964), was thought to indicate the presence of pectin substances. In the same layer of mature *Arabidopsis* seeds, Goto (1985) demonstrated the presence of mucilage composed of a pectin substance with rhamnose as its main neutral sugar. The appearance of the reddish staining at the torpedo stage therefore probably marked the onset of mucilage production in the seed coat epidermis. Simultaneously, amyloplasts containing starch grains accumulated centrally against the inner periclinal wall of the epidermal cells, resulting in the formation of small domes (Figs. 3a and 4d-4f). Upon seed desiccation, these heaps persisted and were seen in surface view as centrally located domes (Figs. 4a and 4b). Upon contact with water the mucilage in these cells started to swell and to be excreted, destroying the outer cell walls. When fully hydrated, it formed a sphere of 200 to 300 μm thickness around the seed and could easily be visualized with ruthenium red or toluidine blue (Fig. 4c: mu).

From the torpedo stage until the mature embryo stage, the oi1 layer remained discernible by its thickened inner periclinal walls (Figs. 3a, 3d, and 3e). At the desiccation stage, this layer is mostly collapsed (Fig. 3f) but may persist in some parts of the seed coat (Fig. 3g). The inner periclinal cell wall of oi1 contributed to the brown pigment layer (bpl) together

Fig. 2. Transmission electron micrographs showing development of the ii1 (or pigment) layer during embryogenesis in *Arabidopsis thaliana*. a. Pre-fertilization stage. ii1 cells with dense cytoplasm and large nuclei. b. One-apical-cell stage. ii1 cells with large central vacuoles. c. Octant stage. Onset of accumulation of electron-dense substance (pigment) in the vacuoles of ii1 cells. d. Torpedo stage. ii1 cells completely filled with pigment. Aleurone layer becomes distinct. e. Bent-cotyledon stage. f. Mature embryo stage: pigmentation in ii1 gradually less dense. g. Desiccation stage. a, aleurone; ac, apical cell; bc, basal cell; bpl, brown pigment layer; c, cuticle; em, embryo; en, endosperm; es, embryo sac; fne, free nuclear endosperm; ii1, innermost layer of the inner integument or pigment layer; ii1′, median layer of inner integument; ii2, outer layer of inner integument; oi1, inner epidermis of outer integument. Bars=10 μm.

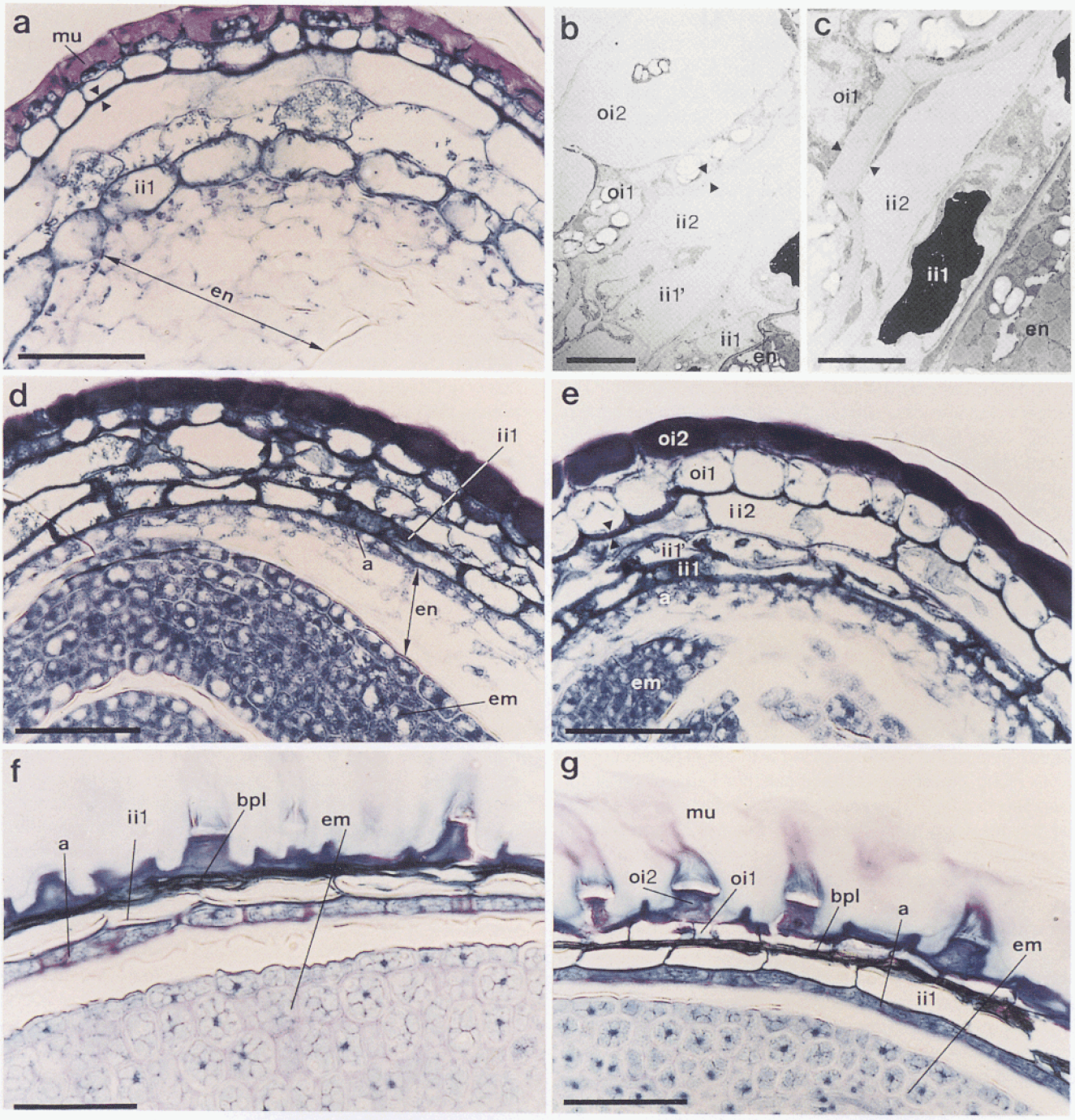

Fig. 3. Seed coat formation during late embryonic stages in *Arabidopsis thaliana*. a, d–g, light-microscopic micrographs of sections stained with toluidine blue; b, c, transmission electron microscopic micrographs. a. Torpedo stage. Purple staining of the oi2 cells indicates the presence of mucilage. Arrowheads show the thickened inner periclinal cell wall of the oi1 cells. b. Detailed view of the integumentary layers at the early torpedo embryo stage. The oi1 layer shows a thickened inner periclinal cell wall (arrowheads). c. Detailed view of the thickened inner periclinal cell wall (arrowheads) at the same stage as in (b). d. Bent-cotyledon stage. ii1′ and ii2 cells and cellular endosperm become crushed. Aleurone layer becomes distinct. e. Mature embryo stage. Shrinkage of the ii1′ and ii2 layers is intensified. The oi1 layer is still discernible by its thickened inner periclinal walls (arrowheads). f. Mature seed with the oi1 layer largely crushed. g. Mature seed with the oi1 layer persisting. a, aleurone; bpl, brown pigment layer; em, embryo; en, endosperm; ii1, inner epidermis of the inner integument or pigment layer; ii1′, median layer of the inner integument; ii2, outer epidermis of the inner integrument; mu, mucilage; oi1, inner epidermis of the outer integument; oi2, outer epidermis of the outer integument. Bars=50 μm in a, d, f, and g; 20 μm in e; 5 μ in b and c.

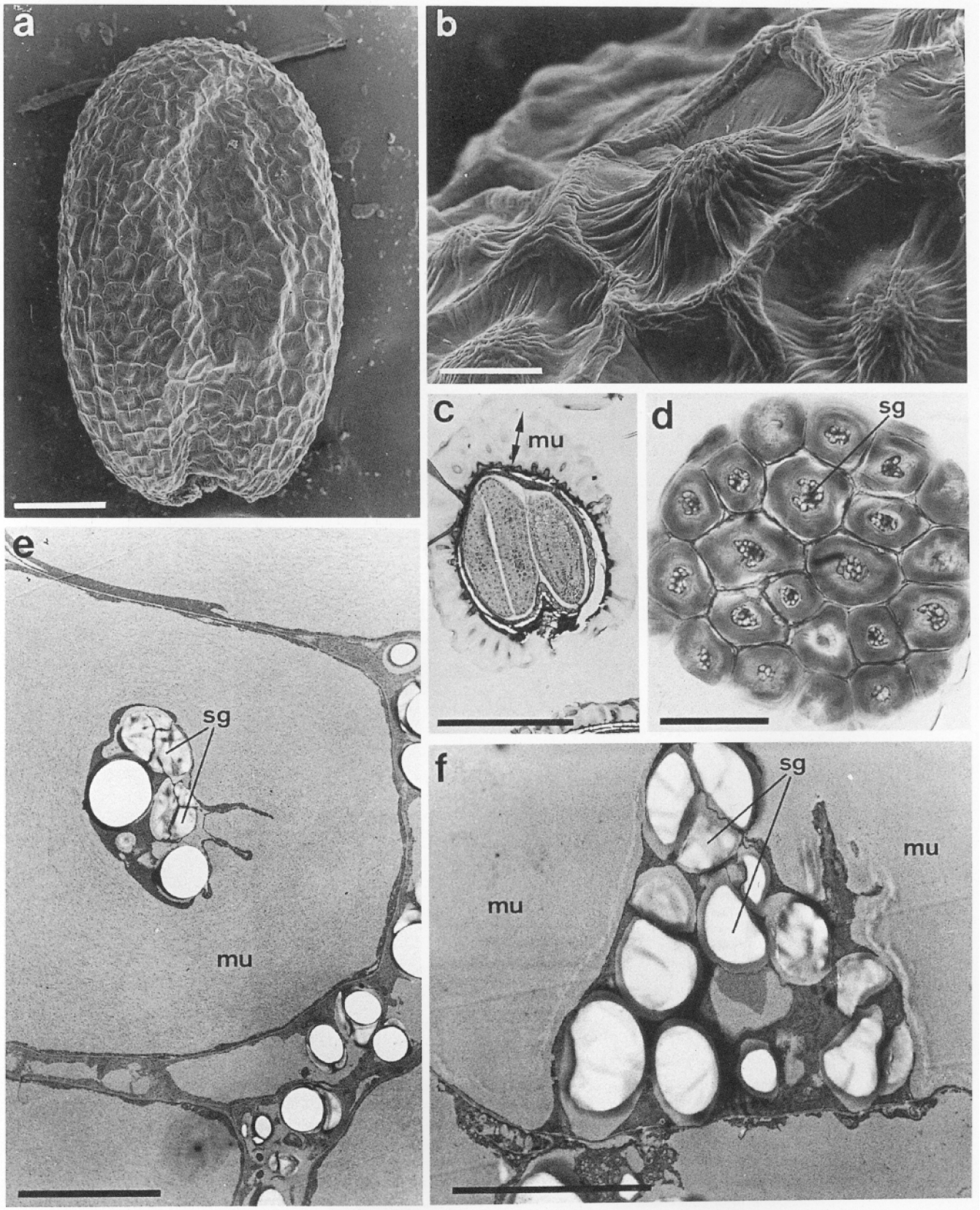

Fig. 4. The oi2 layer at seed maturity in *Arabidopsis thaliana*. a and b: scanning electron microscopy micrographs. c and d: light-microscopic micrographs of sections stained with toluidine blue. e and f: transmission electron microscopic micrographs. a. Surface view of mature seed. b. Close-up showing pyramidal domes. c. Longitudinal section of imbibed seed showing surrounding sphere of mucilage. d. Paradermal section through oi2 layer showing polygonal cell shape and centrally located starch granules. e. Same as in d. f. Longitudinal section through a central pyramidal dome composed of starch granules. mu, mucilage; sg, starch granules. Bars= 100 μm in a; 10 μm in b, e, and f; 250 μm in c; 50 μm in d.

with the remnants of the ii1' and ii2 (Figs. 3f and 3g) and in some cases also of the ii1 layer (Fig. 2g).

The endosperm

Endosperm development was analyzed in detail by Mansfield and Briarty (1990a, b) and is characterized by an initial period of free-nuclear endosperm formation lasting until the late-globular stage, a subsequent period of endosperm cellularization completed at the torpedo stage, followed by a period of partial degeneration of the endosperm. During the last period, much of the endosperm is crushed and absorbed by the expanding embryo.

At the torpedo stage, the outermost layer of the endosperm became distinct (Figs. 2d and 3a), being composed of elongated cells that contained numerous protein bodies and a few chloroplasts. During the following stages, protein bodies accumulated whereas chloroplasts were converted into starch grain-containing amyloplasts (Fig. 2e). Such a layer, also reported in other members of the Brassicaceae, was frequently considered an aleurone layer (Groot and Van Caeseele 1992, Vaughan and Whitehouse 1971). In contrast to layers derived from the integuments, it remained virtually intact as an aleurone layer after desiccation and had not collapsed in mature seeds (Figs. 2g, 3f, and 3g). A hyaline layer was usually present between the embryo and the aleurone layer, but no cell content could be demonstrated within its cells.

Discussion

Our study of the development of the *Arabidopsis* seed coat reveals major structural changes that allow the compilation of the model presented in Fig. 5. Most of the histological changes observed are in agreement with earlier descriptions for the related *Capsella bursa-pastoris* (L.) Med. (Bouman 1975, Dale and Scott 1943). However, in *Capsella*, the coloured inner epidermis of the inner integument (ii1) also becomes crushed and resorbed after the two outermost layers of the inner integument (ii1', ii2) have disappeared. In *Arabidopsis thaliana*, we observed that, at least in the majority of the

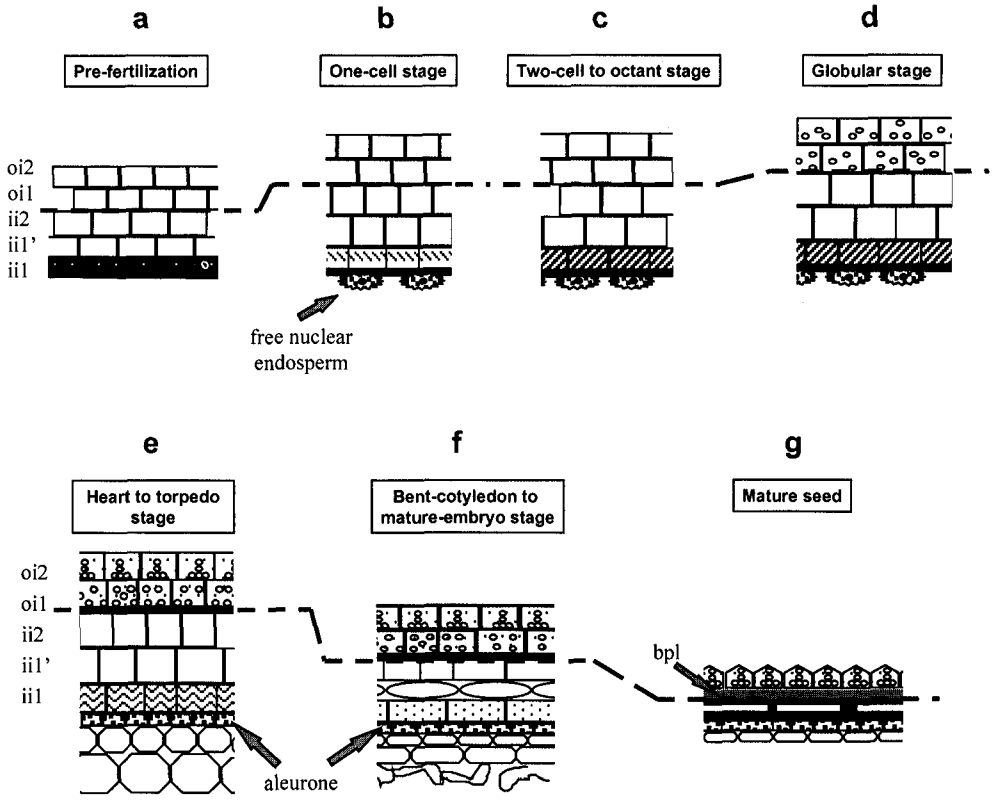

Fig. 5. Diagrams showing major anatomical events during seed coat development in *Arabidopsis thaliana*. Drawings refer to abaxial part of the ovular coverings where it consists of five cell layers. The dotted line indicates the boundary between inner and outer integuments. a. ii1 cells with dense cytoplasm. b. ii1 cells become vacuolated; formation of free nuclear endosperm. c. ii1 cells with pigment accumulation. d. oi2 cells with starch accumulation. e. Less pigment in ii1. Mucilage production in oi2 and starch grains grouped on inner periclinal wall. Inner tangential wall of oi1 thickened (collenchyma). Aleurone layer differentiated. f. Pigmentation gradually disappearing from the cytoplasm of ii1 cells. ii1' becoming crushed. Endosperm, except for the outermost layers, becomes gradually consumed by the growing embryo. g. ii1 remains as layer of empty thick-walled cells. ii1', ii2, oi1 collapsed, forming a brown amorphous layer (bpl, brown pigment layer). Two layers of endosperm remain: hyaline layer (surrounding the embryo) and aleurone layer (in close contact with the ii1 layer of the seed coat).

seeds analyzed, this layer persists as a thick-walled non-pigmented layer in the mature seed coat.

In their extensive work on the seed structure of some 200 species of the Brassicaceae, Vaughan and Whitehouse (1971) reported the occurrence of a palisade layer, derived from the inner layer of the outer integument, in the mature seed coat of *Arabidopsis thaliana*. We found that this layer (oi1) is present before the desiccation stage and has a thickened primary inner periclinal cell wall. It can be considered as a collenchymatous layer as was previously described for *Sinapis alba* and *Brassica nigra* seeds by Bouman (1975). In desiccated seeds, it becomes crushed in most parts of the seed coat and, together with wall material from the two outermost cell layers of the inner integument (ii1′, ii2), its remains contribute to the thick amorphous brown pigment layer (bpl) that gives the brown colour to the mature seed.

Before seed coat development starts, the inner epidermis of the inner integument (ii1) becomes distinct by its staining characteristics and its isodiametric cell shape.

The ii1 layer has received different names by different authors: pigment layer (Bouman 1975, Léon-Kloosterziel *et al.* 1994), endothelium (Schneitz *et al.* 1995), endothecium (Kuang *et al.* 1995) or integumentary tapetum (Bowman 1994, Robinson-Beers *et al.* 1992). A similar cell layer was recorded in 65 families of dicotyledons and reviewed by Kapil and Tiwari (1978). Although this layer shows considerable variation throughout the families with regard to its differentiation, morphology, and extent of coverage of the embryo sac, it can be "characterized" as follows: a layer of initially isodiametric cells with dense cytoplasm, differentiated during embryo sac formation, showing meristematic activity at least during a certain stage of its development, and separated from the nucellus and embryo sac by a cuticle. The same characteristics were found in our study.

In many publications, a nutritive role has been attributed to the ii1 layer (Kapil and Tiwari 1978, Maheshwari 1950). However, further investigations are needed to prove that ii1 supplies nutrients to the embryo sac and embryo in *Arabidopsis*. Consequently, we do not propose to use the term "integumentary tapetum" for this layer in the *Arabidopsis* ovule until such evidence has been found, but rather the name pigment layer.

The abrupt deposition of an electron-dense product in the vacuoles of the pigment layer shortly after fertilization and its gradual disappearance during later stages are remarkable. Until now, the exact timing of this early pigment accumulation has not been mentioned elsewhere. Recently, Albert *et al.* (1997) reported the isolation of a seed coat mutant (*banyuls* or *ban*) in *Arabidopsis* that accumulates a high level of red pigments in the pigment layer at the (pre)globular stage. However, these authors did not perform an anatomical analysis of the early stages and overlooked the early pigmentation we found in wild-type seed coat formation by staining with toluidine blue and by transmission electron microscopic analysis. Therefore, the *ban* mutant is perhaps only affected in its level and not in its timing of pigment accumulation as stated by Albert *et al.* (1997).

In *Arabidopsis*, several mutations, collectively named the *transparent testa* or *tt* mutations, result in yellow or pale brown seeds because of the absence or reduced levels of pigments in the seed coat (Koornneef 1990). The yellow colour of the mature seeds is mainly caused by the weak pigmentation of the cotyledons whereas the seed coat itself is transparent. As several of these *tt* mutants also had no, or a reduced, anthocyanin content in their leaves, it seems plausible that the pigments produced in the seed coat may have a similar precursor to anthocyanins (although yellow seeded mutants with a normal anthocyanin content exist; Koornneef 1981). Meanwhile, three of these genes have been cloned and appear to encode proteins that control different enzymatic steps in the flavonoid biosynthetic pathway, whereas biochemical evidence indicates the involvement of other TT loci in the same pathway (reviewed by Shirley *et al.* 1995). Studies on two of these mutants, *tt4* and *tt5*, which appear to be completely deficient in flavonoids in all tissues, have provided evidence that flavonoid compounds are essential in protecting plants from UV radiation (Li *et al.* 1993). Therefore, we hypothesize that the described early accumulation of a phenolic compound in the cells of the pigment layer shortly after fertilization serves as a protection for the young embryo against UV-radiation.

The disappearance of this pigment during later stages is probably necessary to allow the differentiation of chloroplasts in the embryo. Interestingly, the observed pigment loss seems to coincide with the greening of the *Arabidopsis* embryo, which is completed by the end of the late-heart stage or by the beginning of the torpedo stage (Jürgens and Mayer 1994).

After seed desiccation, our histological analyses, based on the similar staining characteristics of toluidine blue, showed the presence of phenolic compounds in the cell walls of the outer crushed cell layers. Because phenolic compounds have been suggested to strengthen cell walls by cross-linking cell wall components (Yeung and Cavey 1990), their accumulation during seed desiccation might be the prerequisite for the establishment of the hard brown seed coat.

In the oi2 layer, mucilage production starts at the torpedo stage. We found that the elevations seen in surface view on scanning electron micrographs are caused by the aggregation of starch grains on the central part of the inner periclinal wall of each epidermal cell. Vaughan and Whitehouse (1971), reviewing the previous literature on the seed structure in the Brassicaceae, noticed that in the initial studies the mucilage is found to be formed from the starch grains. Likewise, from studies on amyloplast-containing root cap cells it has become clear that starch granules appear to be the primary source of sugars for the synthesis of mucilage (Rougier 1981).

The production of a surface layer of mucilage on seeds or fruits is widespread. Generally, two main ecological functions are ascribed to this layer: assistance to both seed or fruit dispersal and germination (Swarbrick 1971). The mucilaginous seeds of *Juncus bufonius* L., adapted for distribution via the legs and feathers of waterfowl, clearly illustrate the dispersal function. On the other hand, the presence of a mucilage layer would be an adaptation

ensuring the continuity of water supply to the embryo during critical stages of germination (Swarbrick 1971).

In conclusion, the data presented here show that the process of seed coat formation is characterized by distinct morphogenetic events. The following three stages, characterized by particular changes in one or more layers, can be detected: (i) pigment deposition in ii1 and starch accumulation in oi2 (quadrant to globular stage), (ii) pigment vanishing in ii1 and mucilage production from oi2 (heart to torpedo stage), and (iii) pigment accumulation in oi2 and shrinkage of cell layers (bent-cotyledon stage to desiccation stage). These three stages coincide with the three main developmental phases of embryogenesis: morphogenesis, maturation including synthesis of reserve products, and dormancy and desiccation (West and Harada 1993). How and why these events are so precisely coordinated and how they are related to embryogenesis is still unknown. The present study may serve as a basis for future genetic and molecular analyses to unravel these questions.

We thank an anonymous reviewer, Prof. Dr. Hugh Dickinson, and Dr. Melissa Spielman (University of Oxford, U.K.) for helpful comments on the manuscript and advice concerning the electron microscopy, Martine De Cock for help in preparing the text, and Rebecca Verbanck, Karel Spruyt, Stijn Debruyne, and Peter Chaerle for figures and photographs.

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(Received May 25, 1999; accepted February 10, 2000)