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The ultrastructure of seed coat development in *Ranunculus sceleratus*

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SUMMARY

Seed-coat development in *Ranunculus sceleratus* L. has been studied by electron microscopy. Three layers developed from the single integument. The outer epidermis consisted of elongated and flattened cells that were always well attached to each other. The cells were characterized by thin walls, the presence of chloroplasts and small vacuoles. The cells of the middle layer were originally closely packed. Gradually, extensive intercellular spaces were formed. The cells of the inner epidermis elongated initially, until they became cubic and developed a thick wall with numerous wall ingrowths at the side bordering the nucellus. Thus, they give rise to a mechanical layer protecting the inner part of the seed.

The elongation of cells, the thickening of cell walls, the formation of wall ridges, and the formation of intercellular spaces each coincided with characteristic configurations of microtubules. Plasmodesmata were originally found between all cells of the integument but their number decreased drastically during development, especially between the three developing seed-coat layers. Well-differentiated chloro-amyloplasts, present in all cells of the developing seed-coat, pointed to autotrophy during development.

Maturation eventually led to the disappearance of cytoplasm in all cells, the compression of the cells of the outer epidermis and middle layers, and the formation of a mechanical layer from the inner epidermis.

Key-words: cell ageing, integument, intercellular space, microtubule, plasmodesma, *Ranunculus sceleratus*, seed-coat.

INTRODUCTION

Seed-coats in angiosperms have been studied from structural as well as physiological points of view (Boesewinkel & Bouman 1984). As the seed of *Ranunculus sceleratus* L. develops, several cytomorphogenic processes occur in the seed-coat which eventually lead to cell death and the disappearance of cytoplasm. Thus, the development of the seed-coat includes processes of cell growth, differentiation, and cell ageing.

Recently, endosperm formation has been investigated in *R. sceleratus* (XuHan & Van Lammeren 1993) but no detailed data have been recorded on seed coat development and

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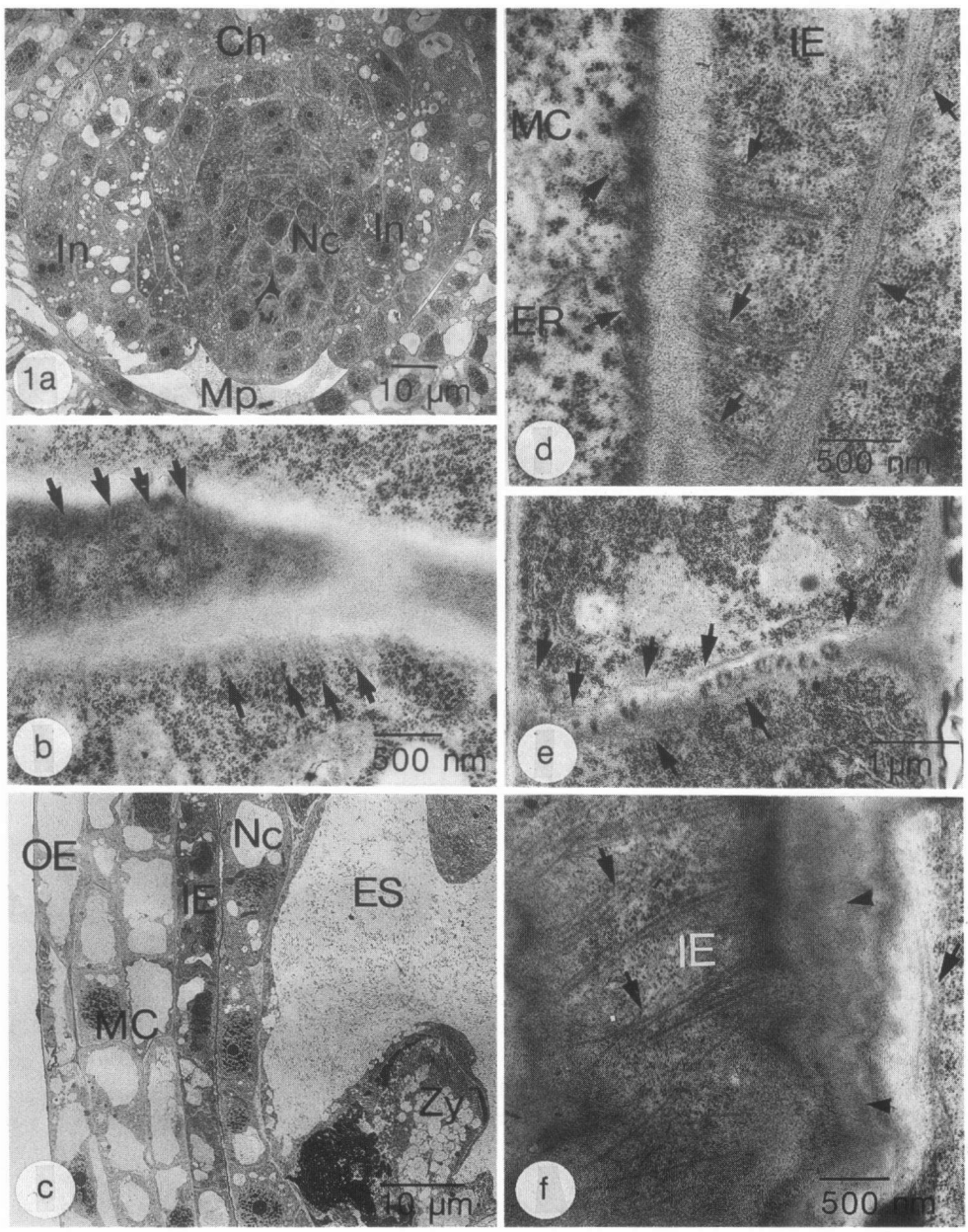
maturation in the genus *Ranunculus* (Sing 1936; Boesewinkel & Bouman 1984). In the study presented here, the general anatomy as well as the cytology of the developing seed coat was analysed in ovules and developing seeds. In particular, attention was paid to the distribution of plasmodesmata, the arrangement of microtubules (MTs), the thickening of cell walls, and the formation of cell wall ingrowths. These developmental



aspects are of particular importance to cell to cell contact, cell morphogenesis and the mechanical properties, respectively, and thus to the ultimate function of the developing seed-coat.

MATERIALS AND METHODS

Plants of *Ranunculus sceleratus* L. were grown in the glasshouse. Developing seeds were excised from ovaries at five developmental stages from 1 day before anthesis (Stage I), when seeds contained a zygote (Stage II), a proembryo (Stage III), a globular to heart-shaped embryo (Stage IV) and a mature embryo (Stage V, i.e. 25 days after anthesis (DAA). For electron microscopy, the seeds were fixed in 4% glutaraldehyde for 6 h and then in 1% osmium tetroxide for 6 h, both in phosphate-buffered saline (PBS), pH 7. Samples were dehydrated, and embedded in a low-viscosity resin (Spurr 1969). Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with a JEM-1200 EXII transmission electron microscope operating at 80 kV.

RESULTS

Stage I (− 1 DAA)

One day before anthesis, ovules were at the megaspore stage. As shown in Fig. 1a, the single integument grew from the chalazal part of the ovule towards the micropylar part. It consisted of an outer epidermis, one to three middle layers, and an inner epidermis. The cytoplasm of the various cells of the integument had a comparable appearance, but the cell walls of the two epidermes had a cuticle and differed from the middle layer in this respect. The cytoplasm of the integumentary cells contained small and medium-sized vacuoles and numerous organelles, such as mitochondria, plastids, endoplasmic reticulum (ER), dictyosomes and spherosomes. Intercellular spaces were not observed at this stage. All the cells of the integument showed plasmodesmata to adjacent cells. Cortical MTs were observed in all cells. They formed a helical network under the cell membrane of slightly elongated cells (Fig. 1b) and were arranged in more random patterns in isodiametric cells.

Fig. 1. Electron micrographs of *R. sceleratus* ovules from 1 day before (a,b) to 1 day after anthesis (c–f). (a) Longitudinal section of the anatropous ovule surrounded by the pericarp of the unilocular ovary. Only one integument was formed. Vacuolization had started in the middle layer cells and the epidermal cells. (b) Detail of a juncture area of the middle layer cells. Note the cortical arrays of MTs (arrows) before intercellular space formation. (c) Longitudinal section showing the integument at fertilization stage (1 DAA). Note the formation of vacuoles in the outer epidermis and middle layers and the deviating differentiation of the inner epidermis which surrounds the nucellus and embryo sac. (d) A part of the seed-coat showing cytoplasmic differences between inner epidermal and middle layer cells. Parallel arrays of cortical MTs (arrows) were found. (e) Detail of inner epidermis showing an anticlinal wall with numerous MTs running parallel to the cell wall (arrows) and plasmodesmata which were darkly stained by the OsO_4. The cytoplasm is darkly stained because of ribosomes. (f) Oblique section of the wall between the inner epidermis of integument and nucellus. Cuticles had fused (arrow head). Wall thickening was mainly observed at the integument side (electron-dense area). Note the extensive arrays of parallel MTs (arrows). Abbreviations: Ch, chalaza; En, endosperm; ER, endoplasmic reticulum; ES, embryo sac; IE, inner epidermis of integument; In, integument; IS, intercellular space; L, lipid droplet; MC, middle layer cells of integument; Mp, micropyle, N, nucleus; Nc, nucellus; OE, outer epidermis of integument; S, starch grain; V, vacuole; W, wall ingrowth; Zy, zygote.

Stage II (1 DAA)

The integument of the mature ovule now consisted of three morphologically distinct layers covering the nucellus with the fertilized embryo sac (Fig. 1c). There were no stomata in the outer epidermis, and plastids had differentiated into chloroplasts.

The two or three layers of cells of the middle layer showed large vacuoles now. Numerous electron-dense microfibres were observed in the cell walls at this stage. Tiny intercellular spaces were frequently observed between the cells of the middle layer at 1 DAA. Cortical MTs were found in parallel arrays in the cells of the inner epidermis and middle layer cells (Fig. 1d).

The cells of the inner epidermis contained much cytoplasm, numerous ribosomes (Fig. 1d,e), small vacuoles and plastids without well developed grana and thylakoid membranes. Plasmodesmata were frequently found in the anticlinal walls (Fig. 1e), not in the outer periclinal walls and only sometimes in the inner periclinal walls. The outer periclinal walls bordering the nucellus thickened and the cuticulae of epidermis and nucellus fused (Fig. 1f). Extensive cortical arrays of MTs were found near the thickening walls.

Stage III (2–5 DAA)

The cells of the integument had enlarged considerably at 5 DAA (Fig. 2a). The outer epidermis consisted of a single layer of elongated and flattened cells that were well attached to each other and characterized by thin walls, the presence of vacuoles and chloro-amyloplasts containing several small starch grains each. From 2 DAA onwards, intercellular spaces enlarged. Cell walls separated from each other at the middle lamella, and the plasmodesmata in the bordering walls disappeared completely. Electron-dense material was regularly found in the corners of the newly formed intercellular spaces, independent of the direction of sectioning (Fig. 2b). Cell walls bordering the intercellular spaces slightly thickened before and during the formation of the intercellular spaces. As the intercellular spaces enlarged, contact between the middle layer cells and the cells of the outer epidermis decreased. In general, less plasmodesmata were observed in the periclinal cell walls of seed-coat cells than in the anticlinal cell walls, and more plasmodesmata were found in the anticlinal walls of the inner epidermis than in those of the outer epidermis and middle layer. Cortical MTs were observed in all cells of the developing seed-coat. In the elongating cells they were all found in hoop-like orientations, parallel to each other and perpendicular to the length axis of the cells. When they were found near the separating walls bordering a developing intercellular space, cortical MTs were observed in a less ordered way (Fig. 2b,c). At the end of the stage, the tonoplasts of the central vacuoles in the middle layer cells and the outer epidermal cells degenerated. In such cells, cortical MTs were observed only incidentally.

The cells of the inner epidermis were still flattened and contained darkly stained cytoplasm, amyloplasts (Fig. 2a) and numerous ribosomes (Fig. 2d). Large plasmodesmata were found in the anticlinal walls and cortical MTs were observed in their vicinity (Fig. 2d).

Stage IV (6–12 DAA)

At about 7 DAA, wall ingrowth and cell degeneration were observed in the young seed-coat (Fig. 3a,b).

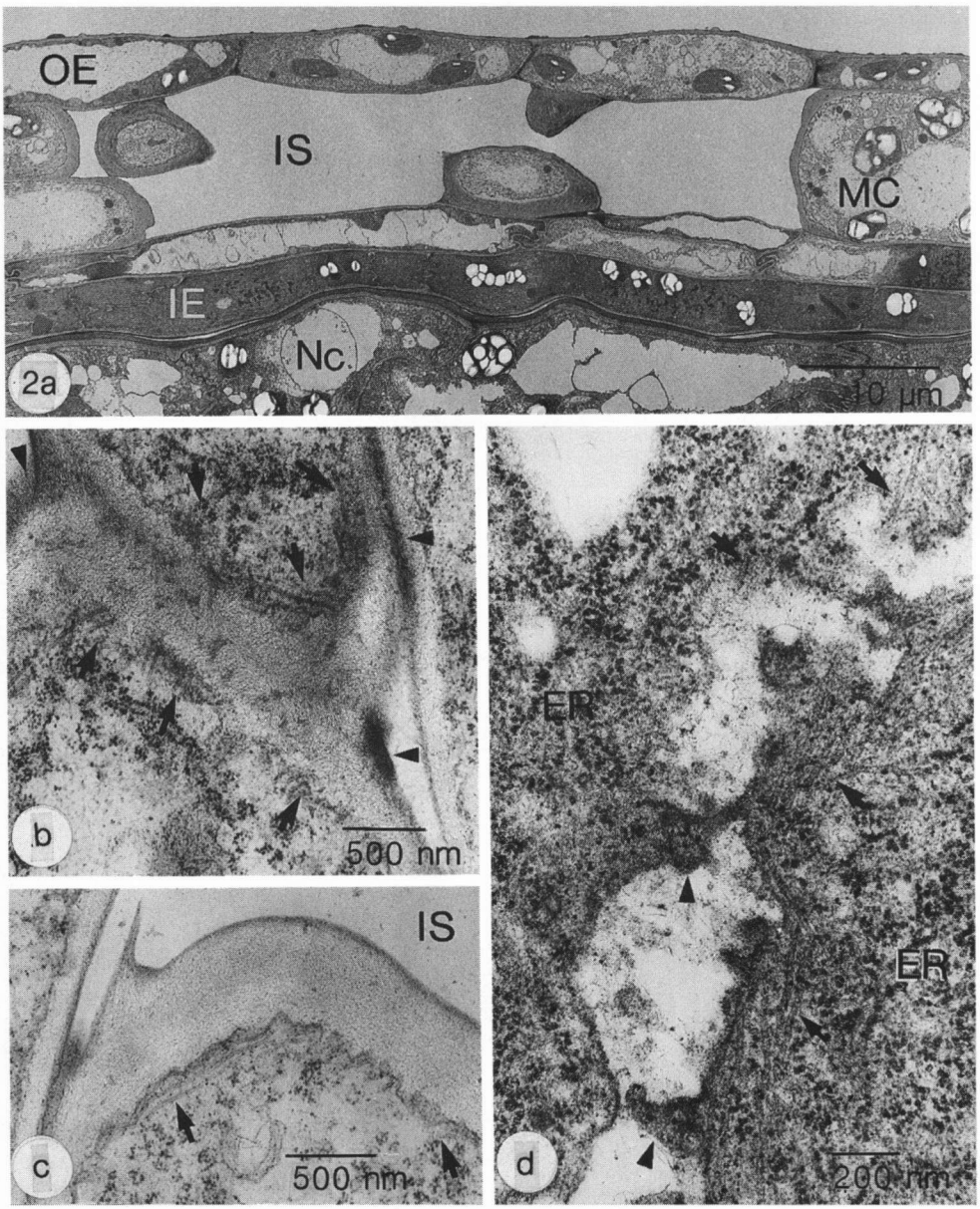


Fig. 2. Electron micrographs of the developing seed-coat of *R. sceleratus* from 4 (d) and 5 days after anthesis (a–c). (a) Longitudinal section of seed-coat. Note the elongated cells of the outer epidermis and the large intercellular spaces in the middle layer. (b–c) Details of the middle layer cells during the formation of intercellular spaces. Electron-dense material was regularly observed in the corners of the enlarging intercellular spaces (arrow head). MTs near the wall exhibted various orientations (arrows). (d) Detail of anticlinal wall of the inner epidermis. Note the large plasmodesmata (arrow heads) and the numerous MTs running near the wall (arrows). See Fig. 1 for explanation of abbreviations.

The cells of the outer epidermis and the middle layers ceased growing and the cytoplasm became electron-dense and degenerated (Fig. 3b). Degeneration in outer epidermis and middle layers preceded that of the inner epidermal cells. Deterioration of

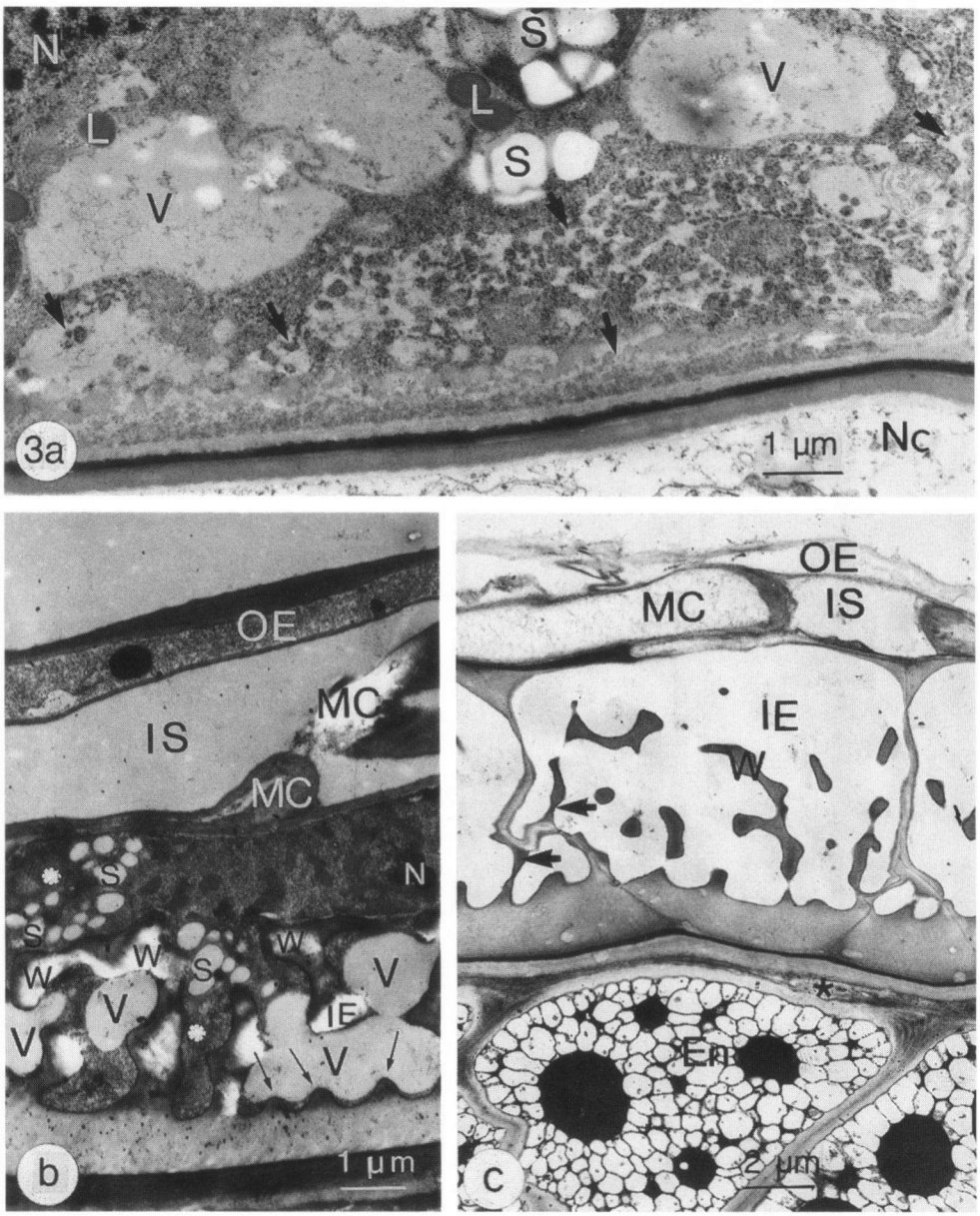


Fig. 3. Electron micrographs of the young seed-coat of *R. sceleratus* from 7 (a, b) and 25 days after anthesis (c). (a) Onset of wall ingrowth formation at the outer periclinal wall of an inner epidermal cell. Note the membraneous vesicle-tube complex (arrows) giving rise to the wall ingrowths. (b) Seed-coat showing degenerating outer epidermis and middle layer cells. The inner epidermis formed a thick outer cell wall bearing well developed wall ingrowths (W) surrounding areas with cytoplasm (*). The ridges in the cell wall (arrows) are covered with a thin layer of cytoplasm, in which bundles of parallel MTs were observed at higher magnification. (c) Part of mature seed-coat and endosperm. Note the absence of cytoplasm in all cells of the seed-coat and the prominent wall ingrowths in the cells of the inner epidermis. Some wall ingrowths are connected to the anticlinal wall (arrows). *Degenerated nucellus. See Fig. 1 for explanation of abbreviations.

the tonoplast of the central vacuole preceded that of the cell membrane, and starch grains, nuclei and ER were the last recognizable cell components. Microtubules were not found in the degenerated cells of the outer epidermis and middle layer.

The outer periclinal walls of the inner epidermis continued to thicken and at its inner side cell wall ingrowths were formed (Fig. 3b). Initially, amorphous membraneous vesicle-tube complexes appeared in the cytoplasm (Fig. 3a). These vesicle-tube complexes fused with each other and with the plasmalemma. They extended deep into the cell and enlarged, resulting in the formation of many finger-like and smooth-surfaced profiles (Fig. 3b). This occurred as long as, eventually degenerating cytoplasm in the inner epidermal cells was observed, i.e. up to 20 DAA. Plasmodesmata were not found in the profiles. Microtubules were observed along the vesicle-tube complex in a random order but there was no association between the membrane complexes and the MTs.

Corticle MTs along the outer wall were observed at particular sites, i.e. at the tips of the undulating cell membrane as indicated by arrows in Fig. 3b (MTs are not visible due to the low magnification). Oblique sections revealed that the MTs ran parallel to these ridges. At the end of the stage, cortical MTs disappeared from the cells of the inner integument. The tonoplasts and other membraneous structures degraded and disappeared in the cytosol, and remnants of cytoplasm and degenerated nuclei were observed in the cells. At this developmental stage the nucellus was replaced by endosperm.

Stage V (13–25 DAA)

At the final stage of seed development, the seed-coat eventually covered the endosperm directly because the nucellus was compressed and consumed by the endosperm (Fig. 3c). The outer epidermis and the middle layers were stretched and pressed between the inner epidermis and the pericarp. All cytoplasm and plasmodesmata disappeared, leaving only cell walls in the seed-coat. However, intercellular spaces remained in the middle layer. The inner epidermis of the integument showed well developed wall ingrowths. It became the mechanical layer of the seed coat, the most prominent structure at this stage (Fig. 3c).

DISCUSSION

The differentiation of outer epidermis, middle layer and inner epidermis of the integument of *R. sceleratus* resulted in three morphologically and functionally different cell layers of the seed-coat. As summarized schematically in Fig. 4, morphological changes coincided with various cytological changes such as the change in distribution of plasmodesmata, the change in configuration of MTs, the separation of cells by the formation of intercellular spaces, and the local thickening of cell walls.

The differentiation of plant cells depends on the position of the cell in the tissue and on the presence of symplasmic contact with neighbouring cells by plasmodesmata (Robards & Lucas 1990). Fransz & Schel (1991) reported that during somatic embryogenesis in maize, the embryogenic clusters lack plasmodesmatal connections to the surrounding callus. Symplasmically isolated cells can undergo a different development, rather auto-controlled than imposed by surrounding tissue, such as that known from studies on embryo-sac formation, pollen development and embryo–endosperm interactions (see e.g. Van Lammeren 1986a, b).

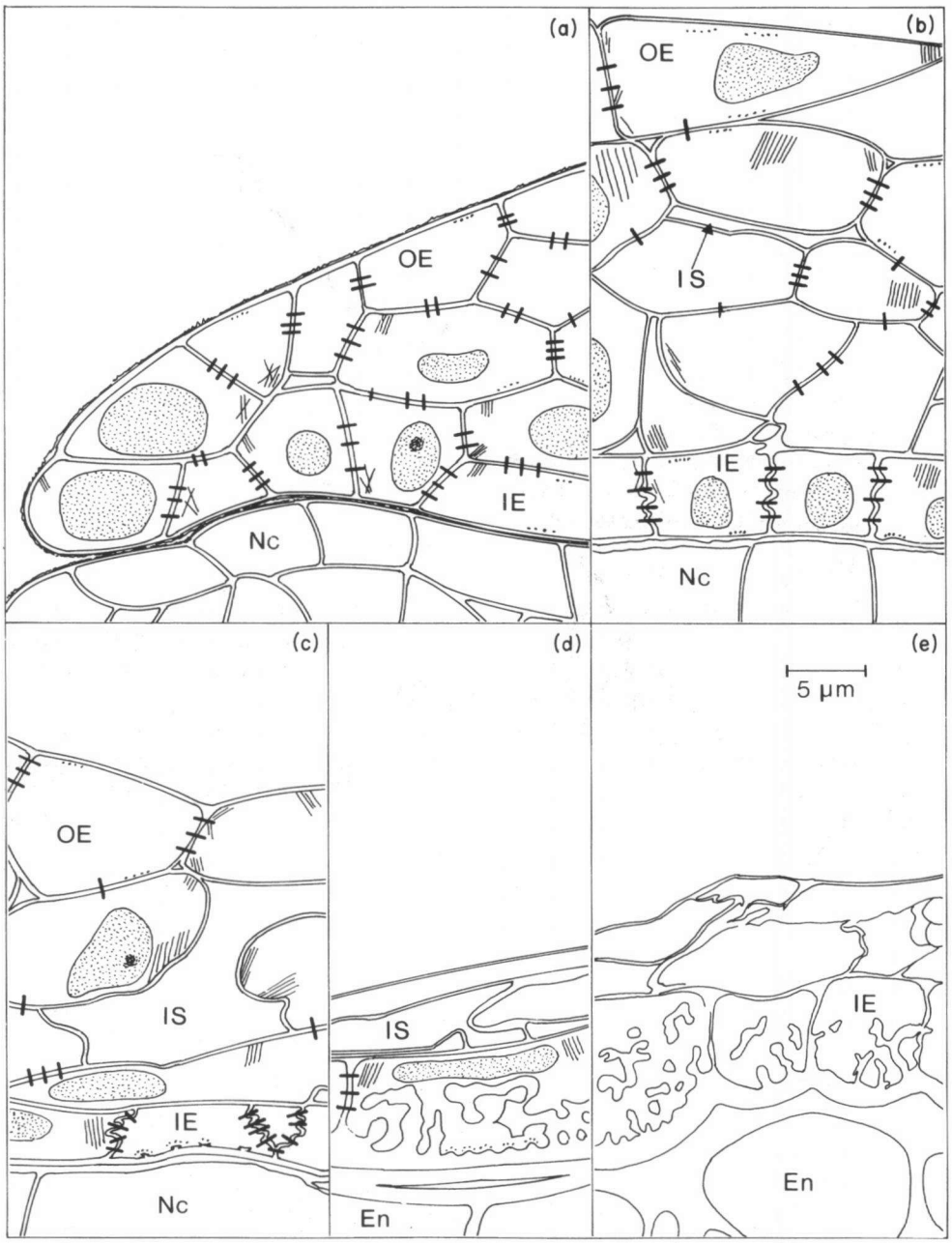


Fig. 4. Schematic representation of seed-coat development in *R. sceleratus* with plasmodesmata in cell walls and cortical MTs in cytoplasm from 1 day before anthesis up to 25 days after anthesis. (a) Stage I; 1 day before anthesis. (b) Stage III (early); 3 DAA. (c) Stage III (late); 5 DAA. (d) Stage IV (late); 12 DAA. (e) Stage V; 25 DAA. Thin short lines in the cytoplasm and dots close to the plasmalemma represent the position and the orientation of microtubules, and thick lines represent the positions and relative frequencies of plasmodesmata in the cell walls. See Fig. 1. for explanation of abbreviations.

Plasmodesmata show a clear distributional change during seed coat development of *R. sceleratus* (Fig. 4); from an even distribution in the young integument (Stages I and II), to an uneven distribution in the young seed-coat (Stages III and IV) and the complete disappearance at maturity (Stage V). The cells of the outer epidermis maintained intensive symplasmic contact by plasmodesmata whereas the contact of these cells with the middle layer cells reduced because of the formation of intercellular spaces. It was also found that the inner epidermis became isolated symplasmically from adjacent middle layer cells. Thus, the three layers became isolated from each other to a certain degree and then showed a different development and fate. Because the symplasmic isolation coincided with characteristic development, the distribution of plasmodesmata might serve as an early indicator for cell differentiation.

As a consequence of symplasmic isolation, all cell layers had to be more or less self-supporting. The precursors for the synthesis of the wall ingrowths in the inner epidermis should come from the inner epidermis itself, probably from the starch resources, which indeed disappeared during maturation, and by increased transport from cell to cell within the epidermis. The enlarged plasmodesmata in the anticlinal cell walls of the inner epidermis point to such a possibility. However, it is possible that apoplasmic and symplasmic transport of storage products from the outer part of the seed-coat still existed to some extent.

The extensive intercellular spaces, developed in the middle layer of the seed-coat, form a closed system because there are no stomata or other openings in the epidermis, which itself was covered by a cuticle. Little is known about the physical and biochemical characteristics as well as the function of non-stomatal intercellular spaces (Woolley 1983). In mature seeds, intercellular spaces and dead cells function in seed dispersal on water by the reduction of specific gravity of the seeds. At earlier stages of seed-coat development, the intercellular space of the photosynthetically active middle layer probably functions as an air/gas exchange and storage accommodation likely filled with a.o. O_2, CO_2 and, in later stages, probably ethylene. The electron-dense material found in various corners of intercellular spaces, might represent remnants of wall material, in this case the middle lamella.

Observations on the cytoskeleton revealed that MTs are found in all seed-coat cells until degeneration occurs (Fig. 4). Three typical configurations of cortical MTs were distinguished.

(1) Parallel arrays of MTs were observed perpendicularly to the length axis of elongating epidermal cells. This phenomenon was also observed, after immunolabelling of MTs in semi-thin sections (unpublished), and probably relates to the method of cell wall formation and expansion, although the literature is controversial (see Derksen *et al.* 1990; Seagul 1991).

(2) Extensive arrays of MTs were found at places where cell walls thickened, e.g. the outer wall of the inner epidermis and cell walls of the middle layer that thickened before intercellular space formation. In the latter case their orientation was less ordered.

(3) The bundles of parallel running MTs found on the tips of the undulating outer cell wall of the inner epidermis might well function in the formation of these wall ridges, too, as was shown during the formation of wall thickening in annular vessels of the xylem and the cell wall thickenings in the mesophyll cells of maize (Apostolakos *et al.* 1991).

Cortical MT configurations remained unchanged during the intercellular space formation in *R. sceleratus*. It thus seems probable that they also assist cell wall thickening at early stages of intercellular space formation and probably do not function directly in space-enlarging, for which the physical force responsible may be generated by the growing epidermes and by the production of metabolic gas which cannot escape from the seed-coat.

The extensive cortical MTs, present under the thickening outer walls of the inner epidermis, did not show a clear association with the fusing vesicle-tube complexes of the developing wall ingrowths. At later stages of wall ingrowth formation, cortical MTs were no longer present, although thickening of the wall ingrowths continued until starch grains and cytoplasm had disappeared. Based on these observations, we suggest that cell walls develop in various patterns: Cortical MTs do function when regular wall thickening occurs. However, when wall ingrowths are formed and when wall thickening continues in partly degenerating cells the MTs do not function in the process.

Cell ageing in higher plant cells has drawn some attention but, up to now, most of the biochemical processes involved in plant cell ageing are unknown (Rodriguez & Sanchez 1990). Dawidowicz-Grzegorzewska & Podstolski (1992) observed a decrease of contrast in all cellular membranes in the transmission electron microscope, and regarded this as a symptom of age-induced membrane deterioration. This phenomenon was also observed in the ageing cells of the seed-coat of *R. sceleratus*. When the inner epidermal cells degenerated, the ER, nuclei and starch grains were still present in the degrading cytoplasm. The thickening of the profiles of the cell wall ingrowths, however, continued until almost all remnants of the cytoplasm disappeared. This implies that the ageing process is asychronous for normal cell processes and wall synthesis.

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