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Structural analysis of embryogenesis and endosperm formation in celery-leafed buttercup (*Ranunculus sceleratus* L.)

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SUMMARY

Embryo and endosperm development were studied in celery-leafed buttercup (*Ranunculus sceleratus* L.) by light and electron microscopy. The first pollen tubes entered embryo sacs within 8 h after pollination. The two first divisions of the zygote were transversal and the three-celled pro-embryo was linear. Its basal cell gave rise to a multicellular suspensor with the hypophysis, and its middle and upper cell formed the embryo proper. Because of this pattern of cell division, the embryogenic pattern of *R. sceleratus* differs from the Onagrad Type to which Ranunculus supposedly belongs. The suspensor showed limited growth and similarly the embryo proper remained in the early torpedo stage in the mature seed. Endosperm was nuclear initially. Cellularization was preceded by alveolation and coincided with the accumulation of storage products in lipid droplets and amyloplasts. Starch grains gradually disappeared whereas protein bodies and lipid droplets accumulated during further development. At maturity, the endosperm occupied the greater part of the seed and its cytological features varied around the embryo. The endosperm cells surrounding the embryo suspensor persisted whereas those surrounding the embryo proper degenerated. The site-specific degeneration occurred after the establishment of the protoderm and points to the interaction between embryo proper and endosperm. 'Multivesicular structures' were observed in the endosperm at the alveolation stage. They may be involved in the transport of metabolites between the apoplast and symplast.

Key-words: embryo, endosperm, multivesicular structure, *Ranunculus sceleratus*, seed development.

INTRODUCTION

Seed development and embryogenesis of celery-leafed buttercup (*Ranunculus sceleratus* L.) have long been recorded (Souèges 1913; Singh 1936; Vijayaraghavan & Bhat 1982;

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Cardemil & Jordan 1982; Bhandari & Chitralekha 1989; Chitralekha & Bhandari 1991; XuHan & Van Lammeren 1994; see also Johri *et al.* 1992 for review). The pattern of embryo development was investigated in a considerable number of species in Ranunculus and is described to be of the Onagrad Type, as represented in *Myosurus minimus* L. In this type the zygote divides transversally first. Following, the basal cell also divides transversally. The lower two cells of the linear embryo give rise to the hypophysis and to the suspensor by repeated transversal and/or longitudinal divisions. The apical cell of the 2-celled embryo divides longitudinally and gives rise to almost the whole embryo (Johansen 1950; Natesh & Rau 1984). As embryogenic patterns can be irregular in Ranunculus (Johansen 1950) and embryogenesis of the Onagrad type was not observed in somatic embryogenesis of *R. sceleratus* by Konar & Nataraja (1965), Konar *et al.* (1972), Thomas *et al.* (1972) and Dorion *et al.* (1984), we reinvestigated the *Ranunculus* embryogenic pattern.

Endosperm development in *R. sceleratus* is characterized by the transition from a free nuclear phase, via an alveolar phase, to a cellular phase. This transition was described recently (XuHan & Van Lammeren 1994). Since further development of the endosperm was not studied in detail, we present (ultra)structural data concerning endosperm development and accumulation of storage products. Although of major importance for the understanding of embryo nutrition, little is known of the interaction between embryo and endosperm during embryogenesis in *R. scleratus*. In this paper, we therefore present structural data of embryogenesis and endosperm development, and analysed the region of interaction between embryo and its surrounding endosperm.

MATERIALS AND METHODS

Plants of *Ranunculus sceleratus* L. (Ranunculaceae) were collected in the field and transplanted to the greenhouse. Embryo and endosperm development were studied on sectioned material by light and transmission electron microscopy. Fixation and embedding procedures were essentially the same as described previously (XuHan & Van Lammeren 1993, 1994). Briefly, developing achenes (dry fruits containing one seed each) were excised from plants at various developmental stages ranging from anthesis till maturity, from 0 until 25 days after anthesis (DAA). Twenty samples of each stage were fixed in 4% glutaraldehyde for 6 h and then in 1% OsO_4 for 6 h, both in phosphate buffered saline (PBS: 135 mM NaCl, 2·7 mM KCl, 1·5 mM KH_2PO_4, 8 mM Na_2HPO_4; pH 7) at room temperature. They were rinsed, dehydrated in an ethanol series, and embedded in low viscosity resin (Spurr 1969). Three to 10 seeds of each stage were cut and semi-thin sections were stained with 0·3% toluidine blue O and examined with bright field microscopy. For the detection of starch grains in embryo and endosperm, fresh achenes were sectioned and either analysed with polarisation microscopy or stained with IPI (2 g KI+0·2 g I_2 in 100 ml H_2O). Lipids were stained with Sudan IV. 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. Pollen tube entry in ovules was visualized in hand-cut sections of five fresh ovaries by staining the callose of pollen tubes with 1% aniline blue in PBS, pH 9, and observation with a UV fluorescence microscope.

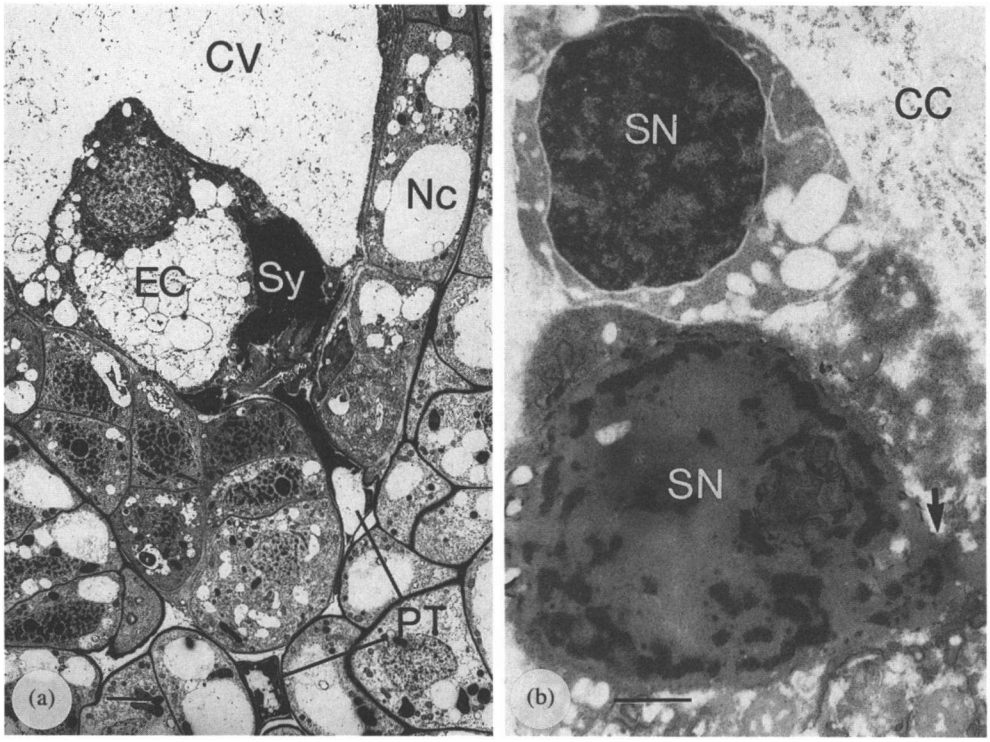


Fig. 1. (a) Electron micrograph of the ovule of *Ranunculus sceleratus* L. showing the growth of the pollen tube (PT) through the micropyle and its entrance into a synergid (Sy) 8 h after pollination. The PT released its contents into that synergid. CV, central vacuole of central cell; EC, egg cell; Nc, nucellus. Bar=5 μm. (b) Electron micrograph of an adjacent section of (a) showing two released sperms in the synergid. Note the thin layer of cytoplasm around the sperm nuclei (SN). The lower sperm shows a tail-like structure (arrow). CC, central cell. Bar=1 μm.

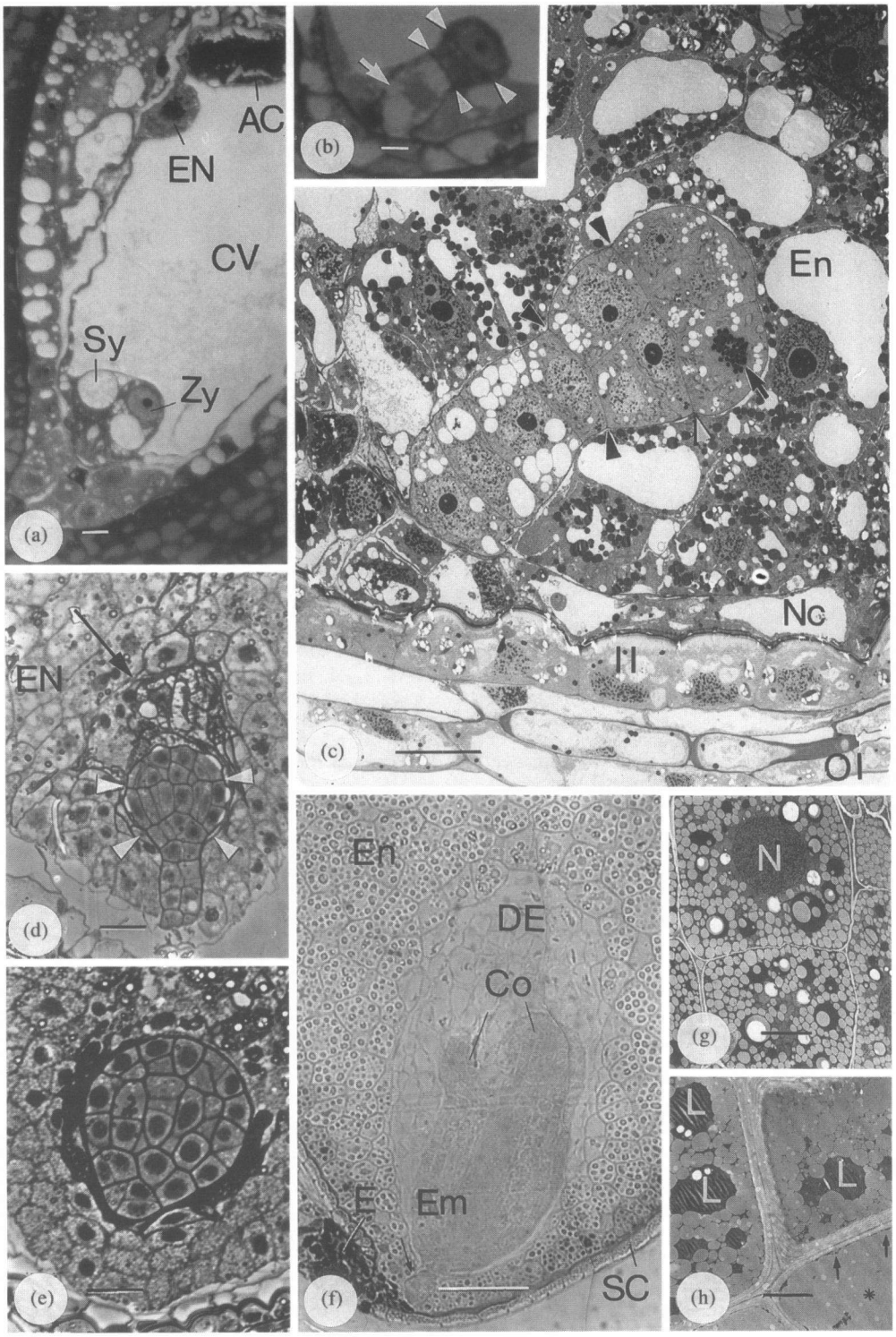
RESULTS

Progamic phase

The earliest penetration of pollen tubes into the ovule was observed 8 h after pollination. Electron microscopy showed that the pollen tube penetrated via the micropyle and released its contents into a synergid (Fig. 1a). At that stage mature embryo sacs consisted of the egg cell with its nucleus at the chalazal side, two synergids, three antipodal cells, and a central cell with either fused or unfused polar nuclei. Serial sectioning of ovules showed that the two sperms were released in the middle area of the synergid (Fig. 1b), whereas two x-bodies, the vegetative and synergid nucleus, were positioned in the micropylar part of the synergid. Tail-like appendices of sperms were observed (Fig. 1b). The cytoplasm of all embryo sac cells contained extensive arrays of endoplasmic reticulum (ER). Microtubules were observed throughout the cytoplasm of the cells of the embryo sac.

Embryo

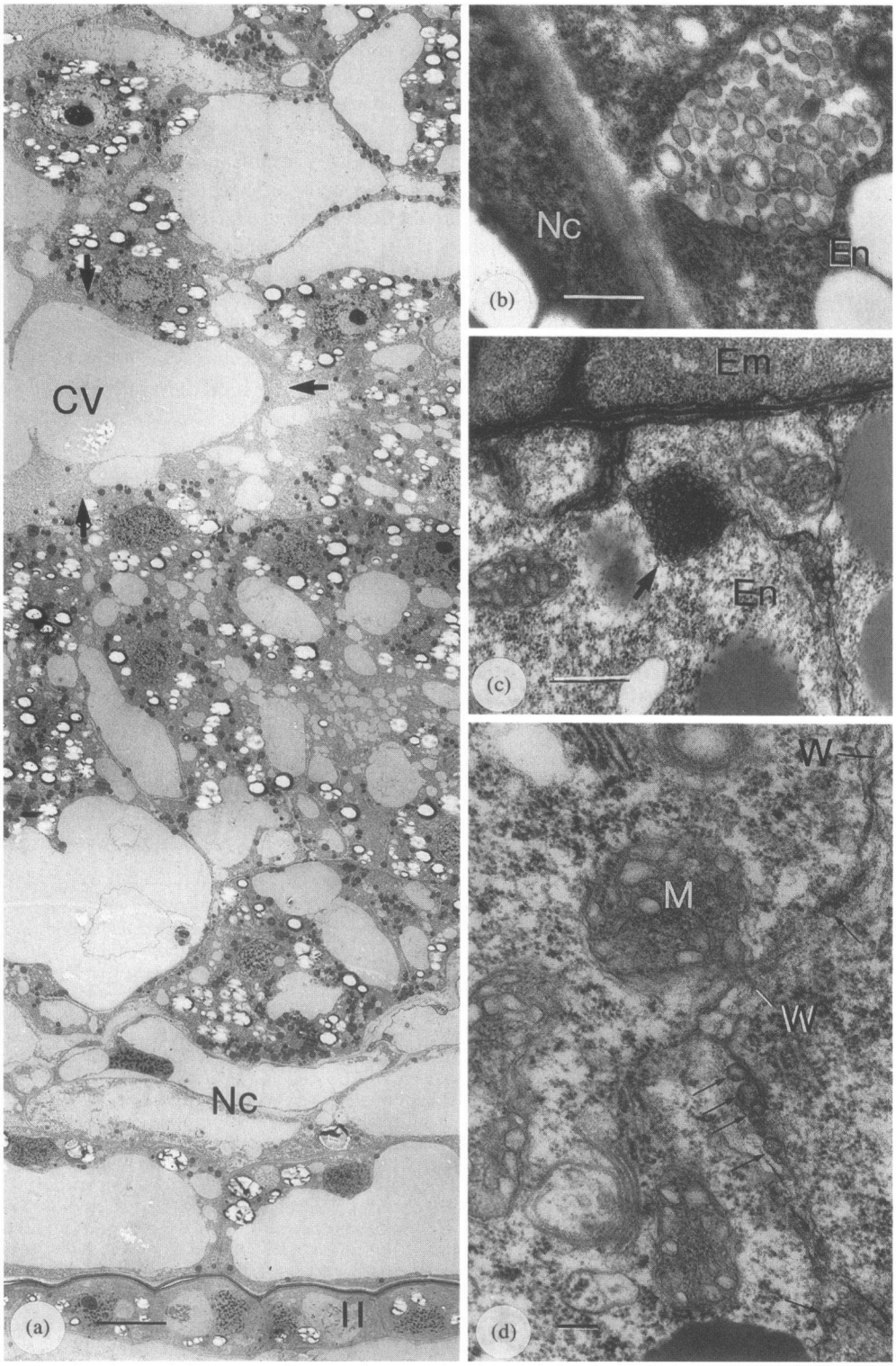
After fertilization, the zygote still had its nucleus positioned at the chalazal side (Fig. 2a). At 1–2 days after anthesis (DAA) the primary endosperm nucleus had divided



twice and the zygote divided transversally, giving rise to a small apical cell and a large basal cell. At 3 DAA, 3-celled linear pro-embryos were formed with an apical cell, a small middle cell, and a vacuolated large basal cell (Figs 2b, 4). Only such linear embryos with two small upper cells and one large basal cell were found. At 5 DAA, the 3-celled pro-embryos further developed into 4-celled pro-embryos by longitudinal division of the apical cell, or into 5-celled pro-embryos by longitudinal division of both the apical and sub-apical cell. At 6 DAA, the upper cells of the pro-embryos divided longitudinally once more, the basal cell divided transversally. At 7 DAA the suspensor and embryo proper could be distinguished (Fig. 2c); the upper part of the embryo proper was derived from the upper cell of the former 3-celled embryo proper by longitudinal and or transversal division, and the sub-apical cell of the former 3-celled embryo had divided longitudinally and transversely and formed the middle part of the embryo proper; the basal cells of the former three celled embryo had divided transversally and now formed a file of four suspensor cells, of which the upper most cell was the hypophysis (Fig. 2c). During the early developmental stages vacuoles and small starch grains were found in all cells of the embryo. Although lipid droplets were present in all embryo cells, they were much smaller and less abundant than in the surrounding endosperm cells.

Comparing embryos of 10 DAA with those at younger stages, the original cell lineage could still be recognized (Fig. 2d). The four suspensor cells had divided longitudinally once. They formed a 2-cell-thick suspensor which hardly enlarged or elongated (Fig. 2d). Cells of the embryo proper showed more cell divisions than the suspensor cells, but the size of the embryo proper only slightly increased before reaching the globular stage. The endosperm surrounding the embryo proper degenerated, but the endosperm surrounding the suspensor persisted. Further cell divisions of the outer cells of the globular embryo proper now resulted in the development of the protoderm and coincided with enlargement of the embryo proper (Fig. 2e). At 25 DAA the mature seed contained an embryo which had a length of about 1/3 the seed length. It remained at an early cotyledon stage (Fig. 2f). The cells of the small cotyledons contained many semi electron-translucent vesicles, darkly stained lipid droplets (Fig. 2g) and some starch

Fig. 2. (a) Longitudinal section of ovule of *R. sceleratus* at 1–2 days after anthesis (DAA) showing the zygote (Zy), an endosperm nucleus (EN) and antipodal cells (AC) in the embryo sac. The persistent synergid (Sy) adjacent to the zygote is still visible. CV, central vacuole of nuclear endosperm. Bar=10 μm. (b) Low magnification micrograph of longitudinal section through a 3-celled pro-embryo at 3 DAA. Note the vacuolated basal cell (arrow), and the smaller middle and apical cells. Arrowheads point to the two transversal walls. Bar=10 μm. (c) Proglobular embryo at 7 DAA, showing the embryo proper and the 4-celled suspensor. Chromosomes are indicated by an arrow. Arrowheads point to the two transversal walls of the former 3-celled embryo. Endosperm (En) surrounding the apical part of the embryo has not yet degenerated. II, inner integument; Nc, nucellus; OI, outer integument. Bar=10 μm. (d) Globular embryo and cellular endosperm (EN) at 10 DAA. Note the degeneration of the cellular endosperm (arrow) surrounding the embryo proper, and the two cell thick suspensor. Arrowheads point to the two transversal walls of the former 3-celled embryo. Bar=20 μm. (e) Older stage of globular embryo. Note the accumulation of reserve materials in the endosperm cells and cell degeneration at the apical and lateral sides of the embryo. Bar=20 μm. (f) Mature seed at 25 DAA. The embryo was at the early cotyledon stage with a small suspensor (arrow). The persistent endosperm (En) occupied the largest part of the seed and accumulated many lipid droplets and protein bodies. Note the absence of storage products and the degeneration of the endosperm cells (DE) near the embryo (Em). Co, cotyledons; E, epistase; SC, seed coat. Bar=100 μm. (g) Electron micrograph of cotyledon cells of the embryo shown in (f). Note the accumulation of lipid droplets, and semi electron-translucent vesicles. N, nucleus. Bar=5 μm. (h) Electron micrograph of endosperm cells of the seed shown in (f). Detail at the transition area with cells that contain lipoprotein inclusions with an electron dense matrix (L) and empty endosperm cells (*) surrounding the embryo. Arrows indicate the thin layer of degenerating cytoplasm in the empty endosperm cells. Bar=5 μm.



grains, but unlike the endosperm the embryo never formed large storage bodies throughout its development. The small suspensor persisted in the mature seed (Fig. 2f).

Endosperm

The endosperm was nuclear at the zygote (Fig. 2a) and pro-embryo stages (Fig. 2b). Cellularization of the nuclear endosperm started at the proglobular embryo stage and was preceded by alveolation of the nuclear endosperm, a process described in detail elsewhere (XuHan & Van Lammeren 1993).

At the globular embryo stage the endosperm was cellular throughout the embryo sac. Most endosperm cells accumulated starch grains and osmiophylic droplets (Fig. 3a). Endosperm cells contained fewer starch grains but were filled with osmiophylic droplets in the vicinity of the embryo proper (Fig. 2c). The endosperm cells surrounding the suspensor showed the droplets in cytoplasm and vacuoles. Initially, the increase in size of the globular embryo proper did not coincide with the degeneration of endosperm cells bordering the apical part of the embryo (Fig. 2c), but the surrounding endosperm cells had degenerated after the embryo proper had become multicellular. Endosperm cells surrounding the suspensor, however, were still intact but differed from the majority of the endosperm cells in size, had fewer large vacuoles, only some starch grains, but contained numerous small vacuoles (Fig. 2d,e). At maturity when the early torpedo stage was reached, the greater part of the cytoplasm of the endosperm cells was filled with large inclusions (Fig. 2f). These inclusions were neither stained by IPI nor by Sudan IV. Electron microscopy revealed that they consisted of a dark matrix enclosing one or more electron translucent spherical regions (Fig. 2h). They were surrounded by small semi electron-translucent vesicles that stained red with Sudan IV in sections of fresh material.

Degeneration of endosperm cells close to the embryo started at the globular stage (Fig. 2d) and continued at the later developmental stages when the embryo proper already occupied a part of the space originally filled by the endosperm. However, degeneration remained restricted to the region close to the embryo proper (Fig. 2e,f). Finally, endosperm cells at the apical and lateral sides of the embryo proper differed from the other endosperm cells. The degenerated cells had lost their original contents or were completely compressed (Fig. 2f,h). Endosperm cells surrounding the suspensor did not exhibit degeneration (Fig. 2d,f).

During endosperm cellularization, multivesicular structures were observed in the cells of the endosperm. These structures consisted of many membranous vesicles which gathered apoplasmically into groups enveloped by a membrane (Fig. 3b). Sometimes parts of the envelope exhibited a double membrane appearance. Such multivesicular structures were frequently present in various regions of the endosperm. They were found at the cell walls bordering the nucellus (Fig. 3b), at the cell walls between endosperm cells, and at the cell walls bordering the embryo (Fig. 3c). The vesicles were regularly

Fig. 3. (a) Electron micrograph of the middle part of the seed of *R. sceleratus* at the final stage of endosperm cellularization showing the accumulation of reserve materials i.e. starch and lipid droplets. Arrows point to the central vacuole (CV) of the former central cell. II, inner integument; Nc, nucellus. Bar=10 μm. (b) Electron micrograph of a multivesicular structure of an endosperm cell (En) bordering the nucellus (Nc) The multivesicular structure is attached to the embryo sac wall. Bar=500 nm. (c) Electron micrograph showing a multivesicular structure (arrow) in the endosperm cell (En) bordering the embryo (Em). Bar=500 nm. (d) Electron micrograph of endosperm alveoli showing accumulation of vesicles (arrows) in the newly formed wall (W). Note the connection of a mitochondrion (M) to the wall. Bar=200 nm.

observed at the cell walls between endosperm alveoli (Fig. 3d). Darkly stained material, found in the embryo sac wall, was connected with the multivesicular structures. They might originate from degenerating nucellus cells. Golgi bodies, ER, plastids and mitochondria were often observed close to multivesicular structures. Sometimes mitochondria appeared to be connected to the cell membrane at sites where vesicles of multivesicular structures accumulated (Fig. 3d).

DISCUSSION

Cell patterning during early embryogenesis

A considerable number of species in *Ranunculus* have the *Myosurus* variant of the Onagrad type of embryo development in which the apical cell of the 2-celled embryo divides longitudinally and the basal cell divides transversally (Johansen 1950). The embryo proper for its greatest part is then formed by the apical cell. The very base of the embryo proper is formed by the hypophysis which in turn is derived from the basal cell of the 2-celled embryo. Presently, we only observed 3-celled embryos of *R. sceleratus* in which the upper cell formed the upper part of the embryo proper whereas the subapical cell gave rise to the middle part of the embryo proper. The very base of the embryo proper was formed by the hypophysis which itself is derived from the basal cell of the former 3-celled embryo. Apparently, the developmental pattern in *R. sceleratus* differs from the Onagrad type. Figure 4 provides a schematic representation of the early development of *R. sceleratus* in comparison with an embryo of the Onagrad type. Although Johansen (1950) mentions irregular pro-embryo stages in *Ranunculus,* the present results imply that large differences in embryogenesis exist within the Ranunculaceae.

It could not be determined with certainty whether the middle cell of the pro-embryo derived from the basal cell, as in *Myosurus minimus*, or from the apical cell of the embryo, although the close position of the nuclei of the upper two cells, the similar shape of the upper two cells and the common fate in the formation of the embryo proper all point to their daughter nature. A common descent from the apical cell is also known in the Caryophyllad type (Johansen 1950). As the middle cell of the 3-celled pro-embryo does not contribute to suspensor formation, the suspensor is only derived from its basal cell. This change of contribution, compared to the Onagrad type, might also explain the limited growth of the suspensor, characteristic for *R. sceleratus*.

It was found that somatic embryos in *R. sceleratus* show neither the Onagrad pattern (Konar & Nataraja 1965; Konar *et al.* 1972; Thomas *et al.* 1972; Dorion *et al.* 1984), nor the pattern described above, but such deviation is more often observed during somatic embryogenesis, e.g. in the microspore or pollen derived embryos of *Brassica napus* L. (Hause *et al.* 1994).

Endosperm development

Besides the cytochemical studies by Vijayaraghavan & Bhat (1982), the present study showed the formation of lipid bodies and large inclusions and changes in the number of starch grains in the endosperm. The appearance of starch was one of the earliest signs of accumulation of storage products. Starch was only present in the first phase of development, and did not occur in the mature endosperm. Simultaneously, we observed electron dense droplets in the endosperm, probably representing lipid droplets as

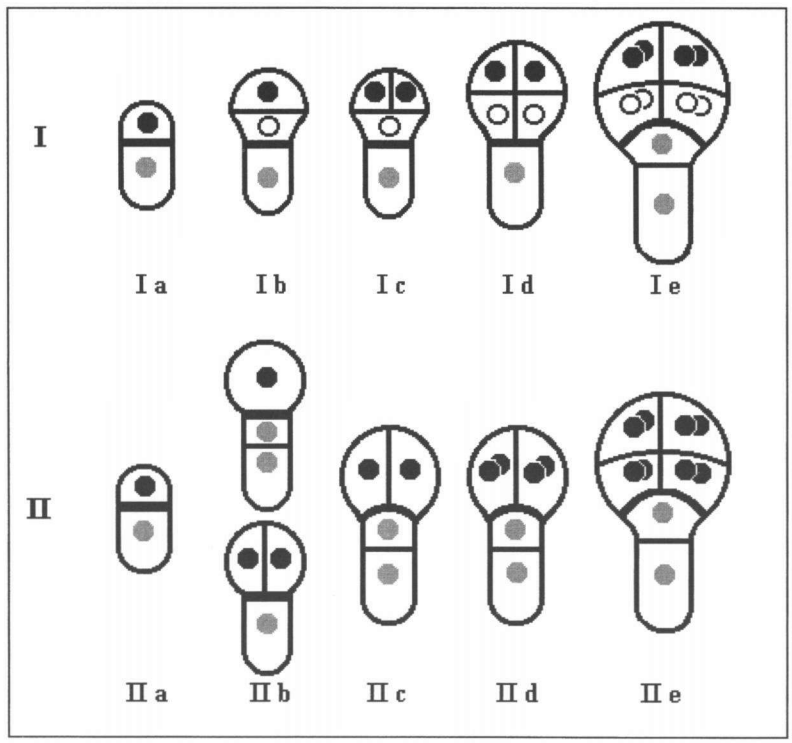


Fig. 4. Schematic representation of the early stages of the embryogeny of *R. sceleratus* (I) compared with the Onagrad type (II) showing that in *R. sceleratus* the middle cell of the 3-celled embryo is involved in the formation of the embryo proper. (Ia) 32 h after pollination; (Ib) 3 days after anthesis (DAA); (Ic) 4 DAA; (Id) 5 DAA; (Ie) 6 DAA. Black nuclei are derived from the upper cell of the 2-celled embryo; grey nuclei from the basal cell, and white nuclei probably from the apical cell. IIa–e are the corresponding developmental stages of the Onagrad type of embryo development.

indicated by Sudan IV staining. They had disappeared at the mature stage, but then numerous Sudan IV positive but semi electron translucent vesicles were found. This points to a change in lipid composition in the endosperm.

The absence of storage products in embryos might relate to the presence of a persistent endosperm storing lipids, starch and proteins.

The large inclusions in the mature endosperm observed by light microscopy were the electron dense granules with electron translucent spherical regions as observed by transmission electron microscopy. They were not stained by Sudan IV and were not seen in the polarizing microscope. These dense granules are probably protein bodies. They have already been described by Vijayaraghavan & Bhat (1982). Because the starch grains and the darkly stained lipid droplets disappeared when protein bodies and vesicles accumulated, their contents might well be used as a source of energy for the synthesis of these structures.

Membranous, multivesicular structures were observed in almost all newly formed endosperm cell walls, and in some parts of the embryo sac wall. Similar structures, named *multivesicular bodies*, *multivesicular endosomes* when present in the cytoplasm, or *paramural bodies*, *plasmalemmasomes* and *lomasomes* when observed extracellularly, are also reported in cells of other plant and animal species. Although extracellular vesicles

are sometimes considered as artefacts, the origin of the multivesicular bodies is presumed to be from the plasma membrane or organelles, and their function is suggested to be related to endocytosis or exocytosis (see review Marchant & Robards 1968; Tanchak & Fowke 1987). Ohoue & Makita (1989) reported ultrastructural proof for the mitochondrial origin of multivesicular structures of adipose cells of the chick embryo. In our study we also observed that mitochondria connected to the cell membrane close to the places where vesicles of the multivesicular structure accumulated. This might suggest a mitochondrial origin of the multivesicular structure. In *R. sceleratus*, the behaviour of the multivesicular structures cannot be related with either endocytosis or exocytosis, as these processes do not involve the translocation of intact vesicles from the apoplast to the symplast or vice versa. We observed whole intact vesicles or multivesicular structures in or against the cell wall. The vesicles of the multivesicular structure might function in apoplastic transport. Possibly, the vesicles release enzymes to digest apoplastic materials.

When the embryo is still at the proglobular stage and protoderm is not yet formed, the endosperm cells surrounding the embryo are still intact. When the protoderm has formed, the endosperm cells surrounding the embryo proper degenerate. The suspensor does not develop protoderm, and remains surrounded by endosperm cells which do not degenerate. The coincidence of the development of the protoderm and the degeneration of its surrounding endosperm in *R. sceleratus* points to a functional differentiation of the outer cell layer of the embryo proper, already in the seed which might correspond to a difference in gene expression (Sterk *et al.* 1991) as shown for carrot embryos.

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