LIGHT MICROSCOPICAL STUDY OF ENDOSPERM FORMATION IN *BRASSICA NAPUS* L.A. A. M. VAN LAMMEREN^{*1}, H. KIEFT¹, F. MA^{1,2} and W. L. H. VAN VEENENDAAL¹¹Department of Plant Cytology and Morphology,
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ABSTRACT

The cellularization of the endosperm of *Brassica napus* was investigated with light microscopy after embedment in Technovit 7100. The microtubular cytoskeleton was visualized by immunofluorescence techniques after embedment in butyl methyl metacrylate (BMM). The analyses of sectioned seeds, sampled at various developmental stages, revealed that the endosperm has a nuclear phase up to the early heart shaped stage of the embryo. From the heart shaped stage onwards cells and alveoli are formed in the endosperm. The cellularization of endosperm was preceded by alveolus formation except in the region surrounding the embryo suspensor and in the chalazal zone of the embryo sac where the nuclear endosperm vacuolated and cell walls were formed all around the nuclei. Alveolus formation only occurred when nuclei had attained a dense distribution in the layer of cytoplasm aligning the wall of the central cell. When nuclei divided within the alveoli, cell plate formation resulted in the formation of mononuclear endosperm cells along the embryo sac wall. When the walls of the alveoli grew towards the centre of the embryo sac, alveoli regularly closed leaving space for enlargement of the remaining alveoli. In this way endosperm cells enlarged going from the periphery to the central area of the embryo sac. The microtubular cytoskeleton was visualized in the nuclear, alveolar and cellular endosperm. The pattern observed, showed that the organization and function of the microtubular arrays was as generally found during endosperm development.

KEY WORDS: *Brassica napus*, cellularization, endosperm, microtubule.

INTRODUCTION

Endosperm formation in angiosperms is characterised by a sequence of events leading to a tissue that nourishes the embryo. The development of the endosperm starts with the fusion of a sperm cell with the central cell of the embryo sac but its further development varies among plant species in a way that it might be cellular from the onset of endosperm formation or it might have an initial nuclear phase (Bhatnagar and Sawhney, 1981, Vijayaraghavan and Prabhakar, 1984). In the latter case cellularization is preceded by alveolation of the nuclear endosperm. During this process endosperm nuclei divide several times and attain an even distribution in the cytoplasm which aligns the wall of the former central cell. Then, cell walls are generated between the nuclei in a position anticlinal to the outer wall of the central cell. As a result nuclei are surrounded by cell walls in a honeycomb appearance. The alveoli however still form a coenocyte because their ends towards the central vacuole of the former central cell remain open. Alveolation might happen simultaneously

all around the central cell or it might start in the vicinity of the embryo and then spread towards the antipodal side of the central cell. Once alveoli are formed, their nuclei can divide and after cell plate formation the first layer of endosperm cells bordering the wall of the central cell is formed. Repeated nuclear division and cell plate formation results in the formation of more or less regular files of cells. Recently it was stressed that the formation of the alveolar endosperm is strongly influenced by the microtubular cytoskeleton in a variety of plant species such as *Triticum aestivum* (Van Lammeren 1988), *Ranunculus sceleratus* (XuHan and Van Lammeren 1993), *Phaseolus vulgaris* (XuHan and Van Lammeren 1994) and *Populus nigra* (XuHan et al. 1995). A model of the influence of the microtubules on alveolation and cell formation was postulated.

In a study on the in vivo development of the seed of *Brassica napus* we focused on the development of the endosperm applying light microscopy. The microtubular cytoskeleton in the endosperm was visualised by immunocytochemical staining of embedded and sectioned material.

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MATERIALS AND METHODS

Rape plants (*Brassica napus* L. cv. Topas) were grown under greenhouse conditions. Flowers were marked with a label at the beginning of anthesis. At regular intervals from anthesis until 14 days after anthesis, developing seeds were excised from the fruit and processed for light microscopy. Seeds were fixed in either FAA or in a mixture of 3% formaldehyde and 2% glutaraldehyde in microtubule stabilising buffer (MSB: 0.1 M PIPES (1,4-piperazinediethanesulfonic acid), 10 mM EGTA, 10mM MgSO₄, pH 6.9) for 2h at room temperature (RT). Samples were rinsed in MSB two times for 1h and dehydrated through an ethanol series in steps of 30 min for each of the concentrations up to 70%, and in steps of 1h up to 100%. Infiltration of Technovit 7100 (Ademas, Amerongen) was according to the manufacturers suggestion, i.e. samples were put in mixtures of ethanol: Technovit infiltration solution = 3:1, 1:1, 1:3 for at least 1h for each step, and then overnight in 100% infiltration solution, all at RT. Infiltration solution was replaced by the embedding solution and polymerisation took place at 37°C during 1h. Semi-thin sections (2 µm) were affixed to slides at 60°C, stained with 1% Toluidine Blue O in 1% sodium tetraborate (Na₂B₄O₇) and analysed.

Developing seeds were also sampled to visualise the microtubular cytoskeleton. They were fixed in 4% freshly prepared paraformaldehyde in MSB for 2h at RT.

Dehydration and embedding in butyl methyl metacrylate (BMM) were according to Baskin et al. (1992). After rinsing in MSB, seeds were dehydrated through an ethanol series (10, 30 and 50%), 30 min each at RT. Further dehydration through 70, 90 and 100% ethanol was done at 4°C. The ethanol used to prepare the mixtures for both dehydration and infiltration was supplemented with 10mM dithiothreitol (DTT) to preserve the antigenicity of the microtubules (Mts). The seeds were infiltrated with mixtures of ethanol and butyl methyl metacrylate (BMM). The mixtures consisted of ethanol: BMM = 3:1, 1:1 and 1:3, and were applied at 4°C in steps of 2h each. The BMM is a mixture of 40 ml butylmethacrylate, 10 ml methyl methacrylate, 0.5% (w/v) benzoin ethyl ether and 10 mM DTT. These components were mixed by bubbling nitrogen gas through the solution. After a last step in pure BMM overnight at 4°C the seeds were put in gelatine capsules and BMM was polymerised by UV illumination at 4°C for 24 h. After sectioning, processing was done without delay to prevent the loss of antigenicity. Sections of 1 and 2 µm thickness were put on a droplet of water on slides which were cleaned with ethanol, and affixed by drying at 40°C for 1 min. The BMM in the sections was dissolved by putting the slides in pure acetone for 12 min under continuous gentle shaking. Autofluorescence within the tissue was reduced by dipping the slides in a highly diluted solution of the toluidine blue (2 ppm) in water for 30 sec. Microtubules were labelled with the monoclonal anti α tubuline (Sigma, clone DM1-A, dilution 1:200 in phosphate buffered saline (PBS)), supplemented with 0.1% acetylated BSA (BSAac, Aurion, Wageningen) overnight at 4°C. They were then rinsed in PBS-BSAac for 4 x 15 min and labelled with an FITC conjugated secondary antibody (GaM-FITC Mol. Probes, Eugene, dilution 1:200 in PBS-BSAac), for 2 h at RT. Slides were rinsed in PBS and sections were embedded in the antifading reagent Citifluor in glycerol (Citifluor Ltd, London) and observed by epifluorescence microscopy.

RESULTS

Endosperm development in rape proceeds through a nuclear phase. Mitotic activity of the primary endosperm nucleus and its derivatives leads to the formation of a layer of free nuclei bordering the wall of the central cell. In the beginning the nuclei were distantly located and all were encompassed by the cytoplasm which formed a thin layer aligning the cell wall of the central cell. With the increase of nuclear divisions and the proliferation of the cytoplasm, nuclei came closer to one another but still remained free in a band of cytoplasm. Figure 1 presents an overview of a developing seed at 6 days after anthesis showing the embryo at the globular stage and the nuclear endosperm surrounding the central vacuole of the central cell. The free endosperm nuclei were scattered over the cytoplasm, sometimes on distances less than 6 µm. Immunocytochemical labelling of the BMM sections revealed the occurrence of microtubular arrays radiating from the envelope of each interphase nucleus. Some Mts radiating from a nucleus, interconnected with the radiating Mts of the neighbouring nuclei (Fig. 5). Double staining with DAPI showed that a great number of DNA-containing organelles (plastids and mitochondria) occurred in the cytoplasm, especially in spherical zones surrounding the nuclei.

In the micropylar region of the embryo sac, again a thin layer of cytoplasm and nuclei surrounded the embryo proper, but around the embryo suspensor a mass of cytoplasm existed containing a cluster of nuclei not aligned in a monolayer (Fig. 2).

The distance between nuclei in the section ranged from 6 µm until 100 µm indicating an uneven distribution over the cytoplasm at this early nuclear stage. In the chalazal region, the endosperm consisted of a region of dense cytoplasm with regularly spaced nuclei (Fig. 4). Here, short arrays of Mts were distributed throughout the cytoplasm (Fig. 6) and sometimes bundles of Mts radiated from the surface of the nuclei forming a three dimensional network in the cytoplasm.

Referring to the developmental stage of the embryo it was found that the nuclear endosperm phase persisted until the early heart shaped stage of the embryo proper, that was until about 12 days after anthesis. Cellularization had started in the vicinity of the embryo (Fig. 3). The mass of endosperm cytoplasm found here in the nuclear stage, vacuolated, but the nuclei remained surrounded by cytoplasm. Cell wall formation started in the thin layer of cytoplasm surrounding the embryo proper and in the layer of cytoplasm bordering the wall of the former central cell. In the micropylar region endosperm nuclei were surrounded by these cell walls completely. Thus real endosperm cells were formed here. In the thin layer of cytoplasm surrounding the embryo proper and in the layer of cytoplasm bordering the wall of the former central cell, however, only anticlinal cell walls developed resulting in the formation of alveolar endosperm. Alveolus formation generally started when nuclei were closer than 30 µm.

The first alveoli had a diameter of about 20 µm on section. At the chalazal side the endosperm was still nuclear and contained regularly spaced nuclei.

In the phase that endosperm cells, alveoli and free nuclei existed simultaneously, all endosperm types had their specific microtubular configurations. Concomitant with the vacuolation in the endosperm cytoplasm, Mts pointing to the central vacuole bent sideways and ran immediately underneath the tonoplast of the central vacuole. Cellularization in the area of the alveolar endosperm was first seen when the embryo differentiated to the late heart or early torpedo shaped stages. Cell walls grew inward and were associated with microtubules.

Fig. 1. Topographic overview of median section of a rape seed at 6 days after anthesis showing a globular embryo and nuclear endosperm. The endosperm cytoplasm forms a thin layer surrounding the embryo and the greater part of the central vacuole of the central cell, and forms a mass of cytoplasm at the chalazal region. Bar represents 200 μ m.

Fig. 2. Light micrograph of a median section of a Technovit-embedded rape seed at 6 days after anthesis. Note the free nuclei of the endosperm scattered throughout the cytoplasm aligning the suspensor, the embryo proper and the wall of the central cell. Bar represents 100 μ m.

Fig. 3. Light micrograph of a median section of a Technovit-embedded rape seed at 9 days after anthesis. Note the vacuolation and the onset of cellularization of the endosperm surrounding the suspensor. The embryo proper is still surrounded by a layer of nuclear endosperm. Bar represents 100 μ m.

Fig. 4. Light micrograph of a median section of a Technovit-embedded rape seed at 6 days after anthesis showing the nuclear endosperm at the chalazal region of the embryo sac. Note the regular spacing of the nuclei throughout the cytoplasm. Bar represents 100 μ m.

Fig. 5. Fluorescence micrograph of the microtubular (Mt) cytoskeleton in sectioned alveolar endosperm of rape at 12 days after anthesis. Note the fluorescent bundles of microtubules surrounding the endosperm nuclei (arrows). Bar represents 20 μ m.

Fig. 6. Fluorescence micrograph of the microtubular cytoskeleton in the nuclear endosperm of sectioned rape seeds at 6 days after anthesis. The detail is in the chalazal region where the free nuclei are regularly spaced in a mass of cytoplasm. Note the short arrays of fluorescent Mts distributed throughout the cytoplasm. Bar represents 10 μ m.

Abbreviations: AE – alveolar endosperm; Ch – chalazal region of endosperm; CE – cellular endosperm; CV – central vacuole of central cell; En – endosperm, Em – embryo proper; II – inner integument; M – micropyle; Mt – microtubule; OI – outer integument; N – nucleus; NE – nuclear endosperm; Su – suspensor; V – vacuole

Fig. 7. Topographic overview of a median section of a rape seed at 14 days after anthesis showing a torpedo shaped embryo and cellular endosperm. Note that the central region of the central cell is still occupied by the central vacuole. Bar represents 200 μm.

Fig. 8. Light micrograph of median section of a Technovit-embedded rape seed at 14 days after anthesis showing cellular endosperm and the base of the embryo. Bar represents 100 μm.

Fig. 9. Detail of cellular endosperm at 14 days after anthesis. The outer cell layers of the endosperm exhibit cell divisions. Note the presence of arrays of Mts in mitotic spindles (arrows). Bar represents 10 μm.

Fig. 10. Detail of cellular endosperm at 14 days after anthesis. The outer cell layers of the endosperm exhibit cell divisions. Note the presence of arrays of Mts at the outer margins of the phragmoplast which forms the cell plate (arrows). Bar represents 10 μm.

Fig. 11. Light micrograph of a median section of a Technovit-embedded rape seed at 14 days after anthesis showing the area of alveolar endosperm bordered by cellular endosperm and the central vacuole. Arrays of Mts radiate from the nuclear surfaces and sometimes point to the place where freely growing wall-ends are being formed. Bar represents 10 μm.

Abbreviations: AE – alveolar endosperm; Ch – chalazal region of endosperm; CE – cellular endosperm; CV – central vacuole of central cell; En – endosperm, Em – embryo proper; II – inner integument; M – micropyle; Mt – microtubule; Ol – outer integument; N – nucleus; NE – nuclear endosperm; Su – suspensor; V – vacuole

lar aggregates in the cytoplasm at the wall edge. Cell wall growth continued resulting in the formation of cylindrical alveoli. The nuclei migrated from the center of the alveolus to a position near the central vacuole. The radial Mts remained prominent but pointed preferentially to the tonoplast of the central vacuole and to the ends of the growing anticlinal walls. When alveolar nuclei divided, periclinal walls appeared, and the first layer of uninucleated endosperm cells was formed. They bordered the embryo sac wall, and at the inner side, the layer of alveoli remained. In this way, more layers of endosperm cells were produced. Endosperm cells at interphase exhibited Mts in the cortical cytoplasm aligning the cell walls. Some short bundles of Mts were detected around the flattened nuclei. Cellularization continued until the central vacuole of the former central cell had disappeared. Figure 7 presents an overview of a developing seed at 14 days after anthesis showing the embryo at the torpedo shaped stage. The endosperm is cellular for the greater part (Fig. 8). In the central zone of the seed the central vacuole of the former central cell persisted. It was still aligned by a layer of alveolar endosperm but the nuclei of the alveoli were less densely distributed than at 6 days after anthesis.

Immunocytochemical labelling of the BMM sections revealed that the organization of the microtubular cytoskeleton in the alveoli had not changed at this stage.

Endosperm cells were not only formed by divisions in the alveoli. Existing endosperm cells, divided too, especially those in the outer cell layers of the endosperm. These cells exhibited various stages of karyokinesis and cytokinesis.

Technovit embedding showed the microtubular spindles at various stages of mitosis (Fig. 9) and Mts associated with the ends of new cell plates (Fig. 10). In the alveolar zone of the endosperm many nuclei were surrounded by arrays of Mts radiating from the nuclear surface. Figure 11 shows an oblique section of the alveolar endosperm.

The Mts of four neighboring alveolar nuclei interacted at the sites where the cell walls of the alveoli are being formed.

DISCUSSION

Analyzing the successive stages of endosperm development in *B. napus* it appeared that there existed large differences between ovules of the same age. These differences probably depended on the position of the ovules in the fruit and the position of the fruit in the inflorescence. However, the stages of development of endosperm and embryo were related.

Cellularization of the nuclear endosperm started at the early heart shaped stage, and either occurred directly as seen in the endosperm surrounding the embryo suspensor, or it was mediated by alveolation, a process first described by Hodcent (1969) in which the microtubular cytoskeleton is highly involved (XuHan 1995). Alveolation might occur simultaneously throughout the embryo sac as seen in the peripheral type of endosperm formation found in e.g. wheat (Fineran et al. 1982, Van Lammeren 1988), or cellularization varies depending on the site in the embryo sac. With *B. napus* cellularization occurred immediately in the area surrounding the suspensor, it was preceded by alveolation along the longitudinal walls of the embryo sac, and retarded at the chalazal site. Thus, the cellularization of the endosperm of *B. napus* has characteristics as described in the lingering type also found in e.g. bean (Yeung and Cavey 1988, XuHan and Van Lammeren 1994).

The asynchrony of the development might be due to the different distribution of cytoplasm and nuclei over the embryo

sac. The micropylar area and the chalazal zone of the embryo sac are cone shaped, likely caused by the differentiation of the integument at the micropylar region and by the elongation of the embryo sac at the cost of nucellus tissue in the chalazal region. Here the cytoplasm did not form a thin layer lining the wall of the embryo sac, but a mass of cytoplasm enabled the free nuclei to accumulate in more than a monolayer. Once cell wall formation is initiated it will only result in the formation of alveoli at sites where a layer of nuclei borders a large vacuole. The remaining nuclei in the cytoplasmic mass are surrounded by neighbouring nuclei and will be fully surrounded by cell walls, and thus become cells without an alveolar phase.

First signs of cell wall formation were observed in the micropylar region. This might indicate that the signal for cellularization, if any, is located here. It is unlikely that the density of the population of nuclei causes cell wall formation alone, because in the chalazal zone there was a high density of nuclei, too.

Alveolus formation was preceded by a redistribution of the free endosperm nuclei, because at the late globular stage we still observed an irregular distribution of nuclei over the cytoplasm. As development proceeded, nuclei became regularly spaced. It is suggested that the regular spacing of the nuclei is caused by Mts which radiate from the nuclear surface in a way reported for e.g. wheat (Van Lammeren 1988).

Alveolation is the result of cell plate formation, elongation, and cell wall fusion (XuHan and Van Lammeren 1994). The formation is likely dependent on the presence of a high density of phragmoplast Mts found after karyokinesis, as well as on the presence of dense arrays of Mts between neighbouring non-daughter nuclei in a way as was proposed by XuHan (1995). High densities of microtubular arrays are only obtained when nuclei are densely packed in the monolayer, that is after repeated mitotic divisions. Consequently, the high density of nuclei, needed for the onset of cell wall formation also results in the formation of alveoli with relatively small diameters.

Therefore the initial cell files at the periphery of the endosperm were slender. The increase in size of the more centripetal endosperm cells is a result of the reduction of the number of alveoli which continue to grow inwardly. As a result the distances between the nuclei of neighbouring alveoli increase.

Cell formation in alveoli is caused by nuclear division and cell plate formation in a plane perpendicular to the walls of the alveoli in a way described earlier for e.g. wheat (Van Lammeren 1988). Once endosperm cells of *B. napus* are formed, they are still able to divide. Especially we observed cell divisions at the outer region of the endosperm. Various stages of the mitotic cycle were found as were the microtubular configurations associated with the various stages of karyokinesis and cytokinesis.

Simultaneously cells are formed in the alveoli until the anticlinal walls of alveoli at opposite sides of the central vacuole meet and fuse. Thus the central vacuole of the former central cell is replaced by cellular endosperm.

In conclusion, this study enabled us to identify three stages of endosperm development in which the site within the embryo sac, the density of the population of nuclei, and the configurations of the microtubular cytoskeleton were parameters of major importance for this particular endosperm development.

LITERATURE CITED

- BASKIN T.I., BUSBY C.H., FOWKE L.C., SAMMUT M., GUBLER F. 1992. Improvements in immunostaining samples embedded in methacrylate: Localization of microtubules and other antigens throughout developing organs in plants of diverse taxa. *Planta* 187: 405-413.
- BHATNAGAR S.P., SAWNEY V. 1981. Endosperm – Its morphology, ultrastructure, and histochemistry. *Intern. Rev. Cyt.* 73: 55-102.
- FINERAN B.A., ZILD D.J.C., INGERFELD M. 1982. Initial wall formation in the endosperm of wheat *Triticum aestivum*: a reevaluation. *Can. J. Bot.* 60: 1776-1795.
- HODCENT E. 1969. Recherch sur les facteurs entrant en jeu lors de l'alvéolization des prothalles femelles de l'*Araucaria araucana*. *Rev. Cytol. Biol. Vég.* 32:155-164
- VAN LAMMEREN A.A.M. 1988. Structure and function of the microtubular cytoskeleton during endosperm development in wheat: an immunofluorescence study. *Protoplasma* 146: 18-27
- VIJAYARAGHAVAN M.R., PRABHAKAR K. 1984. The endosperm. In: Johri, B.M. (ed.) *Embryology of Angiosperms*. Springer-Verlag, Berlin, Heidelberg, pp. 319-376.
- XUHAN X. 1995. Seed development in *Phaseolus vulgaris* L., *Populus nigra* L., and *Ranunculus sceleratus* L. with special reference to the microtubular cytoskeleton. Doctoral thesis, Wageningen
- XUHAN X., VAN LAMMEREN A.A.M. 1993. Microtubular configurations during the cellularization of coenocytic endosperm in *Ranunculus sceleratus* L. *Sex. Plant Reprod.* 6: 127-132
- XUHAN X., VAN LAMMEREN A.A.M. 1994. Microtubular configurations during endosperm formation in *Phaseolus vulgaris*. *Can. J. Bot.* 72: 1489-1495.
- XUHAN X., SOUVRE A., GRANIER C., PETITPREZ M. 1995. An improved immunolabelling method for microtubular cytoskeleton in poplar (*Populus nigra* L.) free nuclear endosperm. *Biotech. Histochem.* (accepted).
- YEUNG E.C., CAVEY M.J. 1988. Cellular endosperm formation in *Phaseolus vulgaris* I. Light and scanning electron microscopy. *Can. J. Bot.* 66: 1209-1216.

ROZWÓJ BIELMA U *BRASSICA NAPUS* L.

STRESZCZENIE

Zbadano zakładanie ścian w bielmie *Brassica napus* w środkowej fazie wzrostu zarodka, od stadium sercowatego do stadium U. Cytokinezy rozpoczynają się w rejonie mikropylarnym dając tam kompletne komórki. Proces tworzenia ścian postępuje ku biegunowi chalazalnemu poprzez stadium alweoli. Alweole powstają w monowarstwie jądrowej przy ścianie woreczka zalążkowego, potem mitozy i cytokinezy zabudowują przestrzeń ku środkowi, redukując centralną wakuolę. W stadium globularnym zarodka jądra w monowarstwie rozmieszczone są nieregularnie. Tworzenie alweoli poprzedza równomierne rozmieszczanie jąder przy udziale cytoszkieletu. Dla tworzących się alweoli charakterystyczny jest astralny układ mikrotubul wokół jądra. Rozwój bielma jest zróżnicowany w różnych miejscach woreczka zalążkowego i zależy od zagęszczenia populacji jąder oraz architektury cytoszkieletu mikrotubularnego.

SŁOWA KLUCZOWE: *Brassica napus* L., bielmo, mikrotubule, zakładanie ścian komórkowych.