Embryogenesis in *Zea mays* L.
A structural approach to maize caryopsis development in vivo and in vitro

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Embryogenesis in *Zea mays* L. A structural approach to maize caryopsis development in vivo and in vitro

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 8 mei 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

I

Een vergelijkende structurele analyse van de ontwikkeling van de maisvrucht in vivo en in vitro draagt in hoge mate bij aan doordacht veredelingswerk.

(Dit proefschrift)

II

Het endosperm, ontstaan na dubbele bevruchting, is een organisme dat als zodanig meer aandacht verdient.

III

Voor de immunocytochemische localisatie van eiwitten in plantaardige weefsels is het maken van coupes een verantwoorde en aan te bevelen methode.

(Van Lammeren, A.A.M., C.J. Keijzer, M.T.M. Willemse & H. Kieft, Planta 165: 1-11, 1985. Van Lammeren, A.A.M., H.Kieft, E.Provoost & J.H.N. Schel, Acta Bot. Neerl. 36 (2) in press 1987)

IV

Kaliumpermanganaat heeft in combinatie met glutaaraldehyde als fixatief nog niet afgedaan bij de elektronenmicroscopie.

V

De gewoonte van veel promovendi aan de Landbouwuniversiteit om bij hun proefschrift meerdere stellingen te wijden aan daarin beschreven resultaten, getuigt van een geheel eigen interpretatie van het promotiereglement.

(Promotiereglement LUW, maart 1987)

VI

Een goed practicumprogramma is niet vervangbaar door "efficientere" leermethoden zoals hoorcolleges, thuisstudie of audio-visuele media.

(Capaciteitsproblematiek in brief van Faculteitsbestuur 86/1849 - 4 fr A dd 25-8-1986)

VII

Kennis van de maatschappij en haar verhoudingen is niet een eerste vereiste voor een docent maatschappijleer aan het middelbaar onderwijs.

VIII

Het opeenvolgend gebruik van woorden zoals modieus, eigentijds en trendy etaleert hoezeer de commercie van mening is dat niet slechts uiterlijkheden maar ook de aanprijzing ervan dienen te veranderen.

IX

Gezien de huidige behandeling van onze natuurlijke omgeving kan men in het gezegde "Boompje groot, plantertje dood" de zelfstandige naamwoorden beter verwisselen.

X

Echte doe-het-zelvers kunnen het niet laten.

Wageningen, 8 mei 1987 A.A.M. van Lammeren

aan mijn vader
aan mijn moeder
aan Ineke

Dit proefschrift is tot stand gekomen op de vakgroep Plantencytologie en -morfologie van de Landbouwuniversiteit Wageningen.

De tekening op de omslag toont het micropylaire deel van een zaadbeginsel van maïs met daarin het embryo in het zygotisch stadium (240x). De afbeelding aan de onderzijde is een rasterelektronenmicroscopische opname van een maïsembryo op 12 dagen na het ontstaan van de zygote (130x).

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Gaarne wil ik van de gelegenheid gebruik maken een dankwoord te richten tot een ieder die direct of indirect bijgedragen heeft aan de totstandkoming van dit proefschrift.

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GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1. INTRODUCTION - aims of the study.

Sexual reproduction is an essential part of the life cycle of plants and has been the subject of many genetical, physiological and morphological studies (see Maheshwari, 1950, 1963; Raghavan, 1976; Johri, 1982, 1984; Willemse and Van Went, 1985). Double fertilization in higher plants gives rise to a new sporophyte, the embryo, and to a nutritive tissue, the endosperm. Maize has often been chosen as the object of research on this process. In the past, the morphological aspects of maize embryogenesis were well documented by light microscopical techniques (Miller, 1919; Avery, 1930; Randolph, 1936; Kiesselbach, 1949; Cooper, 1951; Sass, 1955; Van Lammeren and Schel, 1983). In addition, more cytological information was obtained by applying scanning and transmission electron microscopy (Diboll, 1964, 1968a, b; Diboll and Larson, 1966; Russell, 1979; Van Lammeren, 1981; Van Lammeren and Kieft, 1983; Schel et al., 1984). There is, however, no report dealing with an ultrastructural investigation of maize embryogenesis covering the whole area from progamic stage to mature embryo.

In biological research, experimental data and comparative studies widely enlarge the knowledge of plant regulation and plant development. This knowledge is of great value since higher plants form the main food source in human consumption. Especially the Gramineae such as rice, wheat, maize, rye and barley contribute to that for the major part. After wheat and rice maize is the third most important crop plant in the world and therefore it has often been the object of experimental biological research. In the study of the initial stages of plant development the experimental embryology has greatly expanded. There is a functional approach which aims at understanding the growth of embryos and at the ways of influencing its mechanisms. The induction of regeneration and the production of somatic embryos from plant cells open ways to overcome sexual reproduction and to multiply individual plants. In many Gramineae, including maize, the initiation of plant regeneration and somatic embryogenesis appears to be complicated (Johri, 1982; Sheridan, 1982; Vasil, 1982; Bright and Jones, 1985). In maize there are serious

problems with obtaining protoplast cultures capable of regenerating cell walls and producing totipotent callus (Harms, 1982) although at least a few genotypes are available for longer-term callus and cell cultures (Tomes, 1985). Endosperm cultures of maize are hard to regenerate, too (Shannon, 1982).

In agriculture several varieties of maize are used among which the starchy, the sugary and the waxy are most important. In the present report two inbred lines were studied. One is called Black Mexican Sweet corn (BMS) and the other A188 which is a starchy line. The BMS is an old standard variety that has been commercially available for over one hundred years. It was one of the two strains which formed suspension cultures in the experiments of Sheridan (1975). Strain A188 was chosen because of the regenerative potency of its immature embryos in experimental conditions in vitro with respect to callus formation and somatic regeneration (Green and Phillips, 1975).

In this study both morphological and cytological changes during maize embryogenesis in vivo and in vitro are emphasized in order to improve the understanding of the developmental processes and to determine the influence of external conditions. Structural aspects of the differentiation of both embryo and endosperm are compared to reveal embryo-endosperm interrelationships. Final goal is to contribute to the knowledge of embryonic morphogenesis and seed formation based on (sub) microscopical observations of cytodifferentiation and tissue interaction in vivo and in vitro.

In Chapter 1 the structure of the ovules of two maize inbred lines is compared before and after fertilization. The shapes of the micropyles and the positions of the megagametophytes (embryo sacs) are investigated in order to compare pollen tube entry in the two lines. In the cells of the megagametophytes structural changes were observed after fertilization. These changes are discussed with respect to the initiation of embryogenesis.

In Chapter 2 the development of the proembryo is presented. The expression of polarity within the proembryo and the factors influencing shape development are emphasized.

The developmental morphology and cytology of the embryo proper are investigated in Chapter 3. The symmetry of the embryo changes, apical meristems are formed and the scutellum develops. The factors

which might induce these phenomena are discussed.

In Chapter 4 the results of some in vitro experiments are presented. Pollination and fertilization of excised pistillate spikelets and the subsequent development of the caryopsis are investigated and compared with the in vivo development. The germination of excised immature embryos and the regeneration of callus and embryoids on such embryos is studied under various experimental conditions.

Interactions between embryo and endosperm are described in Chapter 5. Emphasis is put upon cytological features indicating on the uptake of nutrients by the endosperm from the ovary and the release of nutrients from the endosperm towards the embryo.

The differentiation of the pericarp and endosperm is investigated in Chapter 6. Special attention is paid to the development of outer cell layers of the endosperm; to the accumulation of storage products and to the distribution of microtubules which appear to influence the morphogenesis of the cells.

Finally the results of the foregoing chapters are brought together in Chapter 7 which presents a general view on maize embryogenesis. An attempt is made to show how and when embryo development is influenced by endogenous and exogenous factors.

2. MORPHOLOGY OF THE PISTILLATE FLOWER

2.1. Inflorescences of maize

After the maize plant had been brought to Europe in the sixteenth century, Gerarde (1597) described the localization of the flowers: "At the top of the stalks grow idle or barren tufts like the common Reede..." and "Those ears which are fruitful do grow upon the sides of the stalks among the leaves which are thicke and great..." (cited by Weatherwax 1955). Maize or corn, as a member of the Gramineae family, bears its flowers in spikelets, the characteristic building blocks of the inflorescence of all grasses. It belongs to the tribe Maydeae of the subfamily Panicoideae in which the flowers are either staminate or pistillate. The two kinds of flowers are produced either in different inflorescences, as is regular with Zea mays, or in

different parts of the same inflorescence as with Tripsacum sp. The male spikelets are the units of the tassel which arises from the primary shoot meristem. The female spikelets are born on a thick axis, the rachis of the cob, which is placed in a leaf axil protected by several husks.

2.2. Differentiation and morphology of the pistillate spikelet

Cob development, including the differentiation of unisexual female flowers from bisexual initials, has been investigated by light microscopy (Miller, 1919; Kiesselbach, 1949 and Weatherwax, 1955) and by scanning and transmission electron microscopy (Cheng et al., 1983).The development of the cob begins with an axillary bud meristem. First a prophylum and a number of leaflike husks are initiated, and then rows of spikelet-pair primordia are formed in acropetal sequence along the inflorescence meristem (Cheng et al., 1983). The primordia give rise to primary branches on each of which two spikelets will develop. Within each spikelet usually the top flower comes to maturity but the stamens stay rudimentary or abort (Fig. 1). For a detailed report on the initial phase of spikelet morphogenesis see Cheng et al. (1983), for the later stages of growth of the spikelet see Weatherwax (1916), Miller (1919), Bonnet (1948) and Kiesselbach (1949).

The morphology of the pistillate spikelet in maize has been described by Wigand (1854), True (1893), Guignard (1901), Weatherwax (1917, 1955), Miller (1919), Stratton (1923), Randolph (1936), Kiesselbach (1949), Bonnet (1940) and Cheng et al. (1983). The functional upper flower on the short spikelet axis or rachilla consists of a pistil, three rudimentary stamens, two conspicuous but apparently functionless lodicules, a palea superior and a palea inferior (lemma). The two flowers of the pistillate spikelet are partly enclosed by a pair of empty glumes in the positions as indicated in Fig. 1. The pistil ends in an elongated 'silk', which may properly be referred to either as a stigma or a style, since it is receptive throughout its entire length. In most varieties of corn the lower flower aborts but in a few cases it quite regularly has a functional pistil, too (Kempton, 1913; Steward, 1915; Weatherwax, 1916).

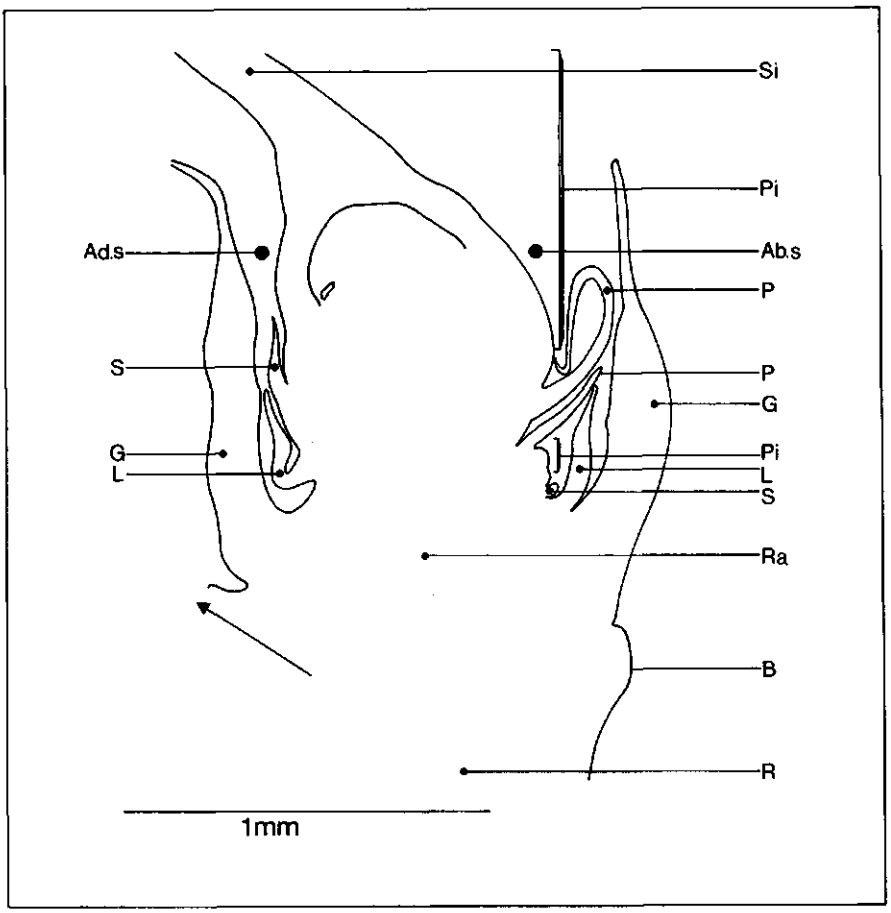

Fig. 1. Median section of an immature pistillate spikelet of Zea mays, strain BMS. The adaxial and abaxial sides of the ovary are indicated with respect to the rachis of the cob (the arrow points towards the apex of the cob). Ab. s = abaxial side; Ad. s = adaxial side; B = bract substending a pair of spikelets; G = glume; L = lemma (palea inferior); P = palea superior; Pi = pistil; R = rachis; Ra = rachilla; S = stamen; Si = silk.

2.3. The pistil

The morphology of the maize pistil has been described by, amongst others, Schleiden (1837), Wigand (1854), Eichler (1875-1878),

Weatherwax (1916, 1955), Miller (1919), Randolph (1936), Kiesselbach (1949) and Cheng et al. (1983). Most of these authors have emphasized the tricarpellate nature of the pistil. The unilocular ovary consists of three carpels, two of which are inserted lateral of the ovule, the third often being a rudiment. Cheng et al. (1983) show that the ovary wall development begins with a ridge on the abaxial surface of the apical meristem. The ridge encompasses the meristem as a ring which is generally considered to represent three undiverged carpels. Through faster growth at the side from which the ridge is initiated the silk emerges. The vascular bundles that pass into the silk are the midrib bundles of the two lateral carpels. So the single style is a structure presumably formed by the fusion of the two lateral carpels. In the initial stage of ovary development an overgrowth of the shoot apex by the ring of tissue leads to the formation of the stylar canal which can often be detected as a slight protuberance in the mature ovary as indicated in Fig. 2. The single ovule is attached at the base of the ovary. It is supplied by the fused marginal bundles of the two lateral carpels (Randolph, 1936). During its development it first grows upward in the cavity of the ovary. By the time of fertilization the ovule has become completely inverted because the integuments and nucellus grew rapidly on the side which is oriented toward the palea but only very slowly on the opposite side (Fig. 2). In form, however, the ovule position is definitely unlike any standard type and represents an extremely modified condition variously referred to as semi-anatropous or modified campylotropous (Randolph, 1936). The sessile ovule has two integuments, a very broad insertion region in the placental tissue and no well-defined funiculus. The inner integument completely surrounds the ovule except at the micropylar orifice. The outer integument does not completely surround the ovule. It is absent in a limited oval-shaped area extending from the micropyle in the direction of the silk-attachment region to the crest of the ovule (Randolph, 1936). The insertion region of the ovule on the placenta is co-extensive with the chalaza which is the location where nucellus and integuments of the ovule are united. Because there is neither a funiculus nor a raphe the insertion region is called the placento-chalazal region.

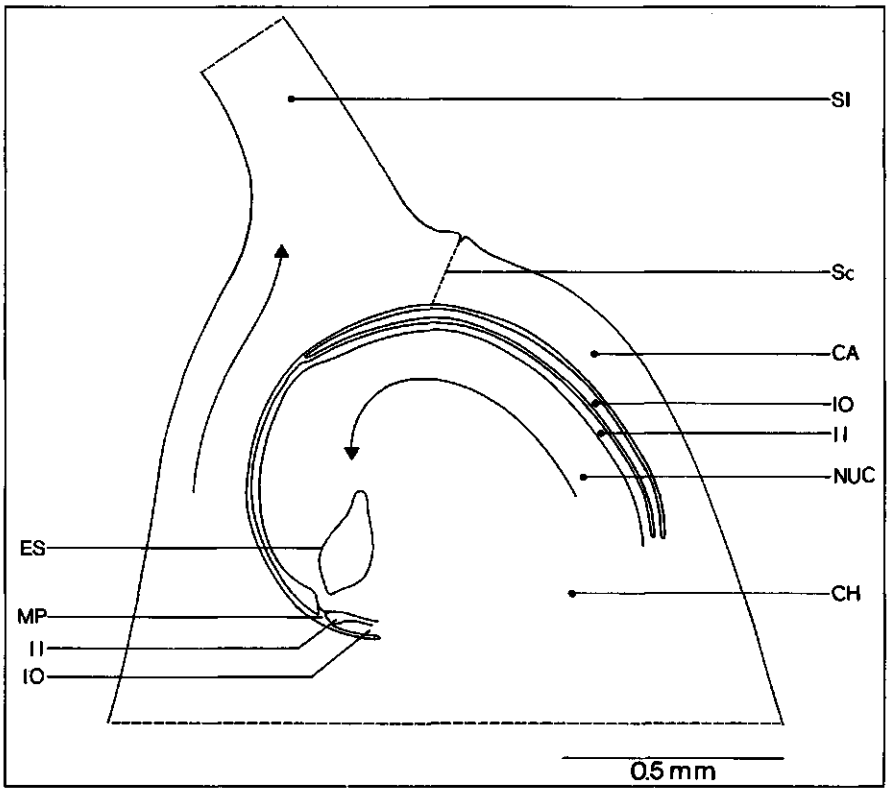

Fig. 2. Median section of a mature ovary with a part of the silk of Zea mays, strain BMS (the arrows indicate the directions of growth). CA = carpel; CH = chalaza; ES = embryo sac; II = inner integument; IO = outer integument; MP = micropyle; NUC = nucellus; Sc = stylar canal; SI = silk.

2.4. Nucellus and embryo sac

In the young nucellus the archesporium is a hypodermal cell overlaid with a single nucellar layer of epidermal cells. The embryo sac development accords with the Polygonum type (Maheshwari, 1950). Through meiosis a linear tetrad of megaspores is formed. The three megaspores most close to the micropyle soon degenerate and the fourth develops into an embryo sac. The cellularization of the coenocytic embryo sac occurs after the three successive mitoses of the megaspore. At first the embryo sac consists of the usual seven cells but

then the three antipodal cells divide resulting in a mass of 20 or more cells, many of which have more than one nucleus. The mature megagametophyte is shown in Fig. 3. The large egg cell and the two synergids occupy the micropylar part of the mature embryo sac and in the remaining central cell there are two polar nuclei adjacent to the egg apparatus. According to Miller (1919) and Weatherwax (1919, 1955) these nuclei do not fuse but remain in close contact until fertilization.

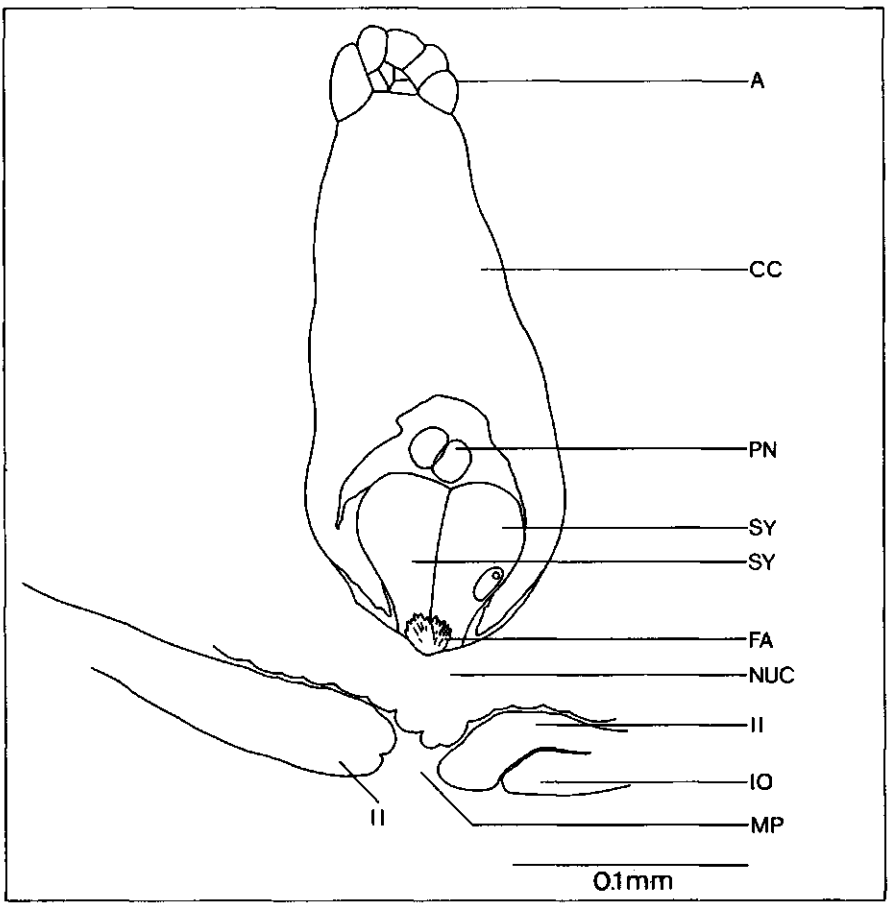

Fig. 3. Median section of a mature embryo sac and the micropylar region of the nucellus of Zea mays, strain BMS. The egg cell is not in the plane of sectioning. A = antipodals; CC = central cell; FA = filiform apparatus; II = inner integument; IO = outer integument; MP = micropyle, NUC = nucellus; PN = polar nucleus; SY = synergid.

During the interval of meiosis and embryo sac formation the nucellar epidermis near the micropyle divides periclinally to form a layer of five or six cells (Kiesselbach, 1949).

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CHAPTER 1

A comparative ultrastructural study of the megagametophytes in two strains of *Zea mays* L. before and after fertilization

A.A.M. van Lammeren

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A COMPARATIVE ULTRASTRUCTURAL STUDY OF THE MEGAGAMETOPHYTES IN TWO STRAINS OF *ZEA MAYS* L. BEFORE AND AFTER FERTILIZATION

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SUMMARY

Two inbred lines of *Zea mays* L. are compared with respect to general morphology and fine structure of the cells of the megagametophyte before and after fertilization. In both inbred lines the semi–anatropous sessile ovules contain one multicellular megagametophyte in a tenuinucellate nucellus. In contrast to the inbred line Black Mexican Sweet corn (BMS) the nucellus cells near the micropyle of strain A188 are not arranged in regular rows. Here the micropyle is formed by a lobed inner integument and hence pollen tube penetration is more cumbrous in strain A188. At the moment of fertilization both synergids may still be intact but the one which will be penetrated by a pollen tube has often partly degenerated. There is a comparable and distinct distribution of organelles in the synergids of the two inbred lines. The overall structure of the egg cell cytoplasm of strain BMS and A188 show many similarities such as the location of the cytoplasm, the distribution, frequencies of occurrence and occupation rates of most organelles and the structural changes initiated by fertilization. Plastids in BMS, however, are smaller and occur in significantly higher frequencies and occupation rates in BMS. In the zygotes of BMS and A188 a polarity is established by a shift of the nucleus and the cytoplasm towards the antipodal cell side. The metabolic activity increases after fertilization as can be deduced from increasing amounts of RER and higher densities of polysomes and dictyosomes. The composition of the cytoplasm of the central cell of BMS is comparable to that in A188. Plastids, however, differ in having more and smaller starch grains in BMS. Before fertilization the polar nuclei and the major part of the central cell cytoplasm are always found at the antipodal side of the egg apparatus. The abundance of well developed organelles points to a high metabolic capacity. Fertilization evokes an increase of cellular activity and a shift of the endosperm nucleus and cytoplasm towards the lateral cell side. Antipodals contain much cytoplasm and numerous well differentiated organelles. The ultra structure of the cytoplasm points to a high synthesis and secretion of organic compounds.

INTRODUCTION

Growth, differentiation and senescence are the characteristics of the complex morphogenesis in the life cycle of plants. When haploid and diploid generations alternate, cell differentiation leads to divergent cell functions in the gametophyte and sporophyte. In Spermatophytae, the cells of the mature micro– and megagametophytes represent an end phase of cell differentiation whereas the zygote is the onset of a new sporophytic generation. The development of the zygote to a heterotrophic young sporophyte offers the opportunity to study the principles of morphogenesis; in a short period of time the initiation of polarity, mer-

istem formation and organogenesis gives rise to a relatively small organism. The onset of embryogenesis has been studied in detail in plants of various families of both the Dicotyledonae and the Monocotyledonae (MAHESHWARI, 1950, 1963; JOHRI, 1984). The details of fertilization, the developmental pathways and the final organization of the embryos vary widely among families of the Monocotyledonae and even within the family of the Poaceae such as barley (CASS and JENSEN, 1970;NORSTOG, 1972, 1974;CASS, 1981), wheat (CHANDRA and BHATNAGAR, 1974; HU, 1964; SMART and O'BRIEN, 1983) and Texas wildrice (EMERY and GUY, 1979).

In maize the shape of the embryo sac, the organelle distribution within the embryo sac and the shape of the developing embryos appear to vary between different strains as has been observed by means of light microscopy (MILLER, 1919; AVERY, 1930; RANDOLPH, 1936; KIESSELBACH, 1949; COOPER, 1951; SASS, 1955) and electron microscopy (DIBOLL, 1964, 1968a, b; CHEBOTARU, 1970; RUSSELL, 1979; VAN LAMMEREN, 1981; VAN LAMMEREN and KIEFT, 1983; VAN LAMMEREN and SCHEL, 1983). Unlike in many other plants (MAHESHWARI, 1950), the first cell divisions of the zygote and the young embryo seem to lack a clearly defined sequence and orientation although, within a strain, the eventual shapes of the embryos are quite similar (RANDOLPH, 1936).

To study the initial phase of maize embryogenesis an inventory was made of the cell shapes and organelle distributions within the embryo sac. Two inbred lines were chosen for the present study which is introductory for the experimental in vitro studies on callus formation and somatic embryogenesis. One inbred line is the sweet corn Black Mexican Sweet (BMS). The second is the starchy corn A188. These strains have been selected because of their favorable properties for experimental manipulation in vitro (SHERIDAN, 1977; GREEN and PHILLIPS, 1975).

The present paper describes the changes in the fine structure of the embryo sac just before fertilization. Then, the interactions of the pollen tubes and the tissues of the pistil including the ovule will be presented. Thirdly the post–fertilization events which occur in the embryo sac will be regarded from a structural and functional point of view. The two inbred lines are compared to detect intraspecific variations in cytology.

MATERIALS AND METHODS

The plant material used in this study was obtained from the *Zea mays* L. inbred lines Black Mexican Sweet (BMS) and A188 which were kindly provided by R.J. Lambert, University of Illinois, Illinois, USA and by C.E. Green, University of Minnesota, St. Paul, USA respectively. Plants were grown under greenhouse conditions; before emergence of the silks, cobs were masked with small bags to prevent uncontrolled pollination. Ovaries were dissected either from unpollinated plants or at defined intervals after hand–pollination. Sagittal

sections of the ovaries containing the whole embryo sac were fixed with 2,5–6% glutaraldehyde in 0,1 M Na–cacodylate buffer, pH 7.0, for 2 hours at room temperature. Sections were rinsed in the buffer and postfixed in a saturated aqueous $KMnO_4$– solution for 5–15 minutes or in a 1% OsO_4– solution in cacodylate buffer, pH 7.2, for 2–4 hours at room temperature. After rinsing, sections were dehydrated in a series of ethanol, ranging from 30% to 100%, followed by a graded series of propylene oxide. The material was then transferred to a propylene oxide – Epon 812 (40:1) mixture and kept overnight at a relative humidity of 30% to permit the propylene oxide to evaporate slowly. Finally, sections were transferred to fresh resin in gelatin capsules. Polymerization occurred for 16 hours at 35 °C, for 8 hours at 45 °C and for 24 hours at 60 °C. Ultrathin sections were cut on an LKB Ultrotome III and, in the case of OsO_4 fixation, poststained in lead citrate for 2–5 minutes and in uranyl acetate for 5–25 minutes. Sections were observed using a Philips EM 301 transmission electron microscope at 60 kV. For the detection of pollen tubes in silks and ovary cavities, sagittal sections (8–50 μm) of freshly frozen pistils were cut with a microtome–cryostat (Daman/IEC division, Mass., USA) at minus 18 °C. Two percent 'Wasserblau' (Merck, Darmstadt, FRG) in 20% aqueous solution of K_3PO_4 was added to the thawed sections to obtain fluorescence of callosic substances. The observations were recorded on Agfachrom 50L.

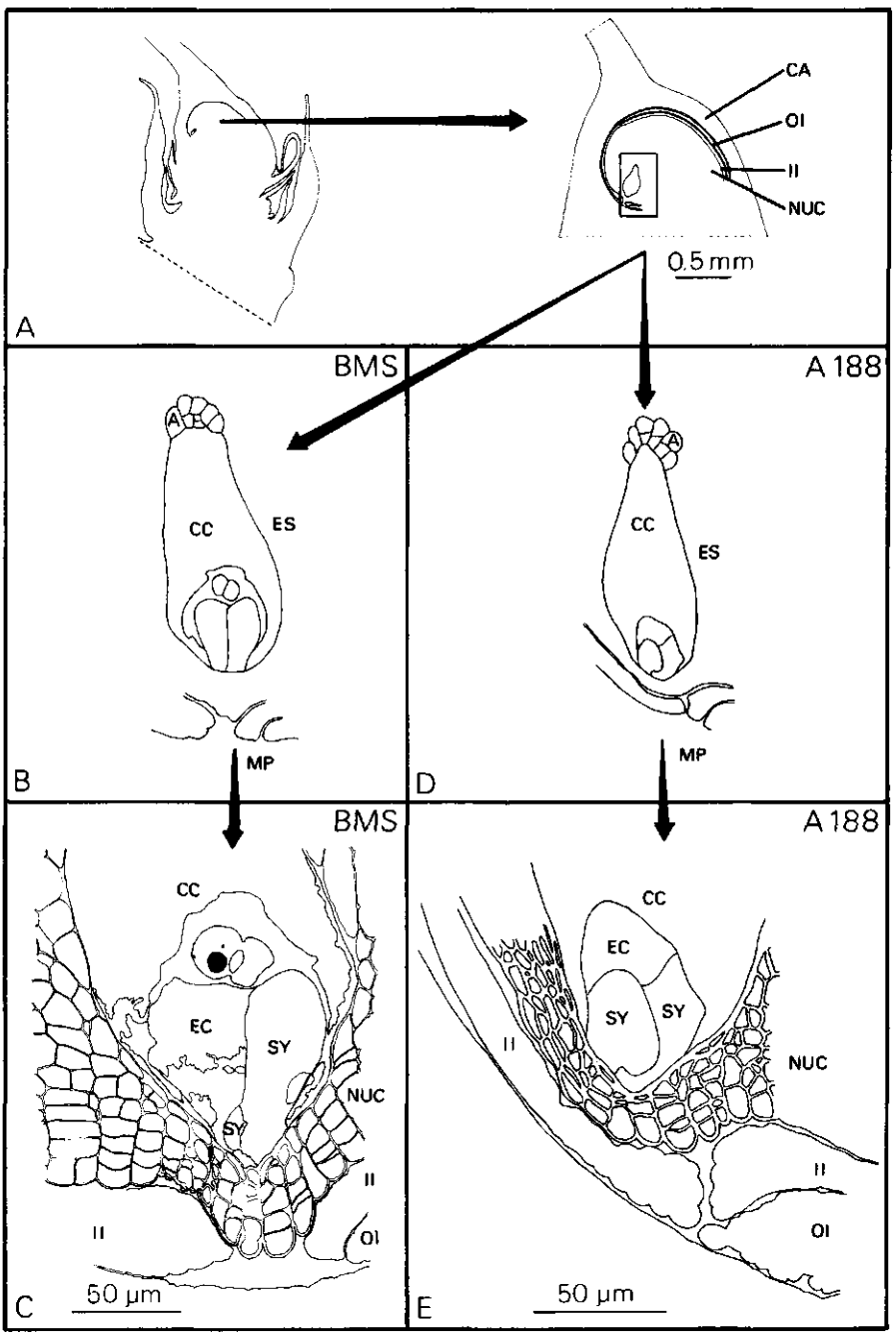
Results

The position of the embryo sac in the ovule

The position of the embryo sac in the nucellus with respect to the micropylar entrance is not similar for the two strains (Fig. 1). In comparison with BMS the embryo sac of strain A188 has a more oblique position towards the micropyle and fewer nucellus cells are in between the embryo sac and the inner integument.

In both strains the nucellus cells of the micropylar region divide several times but more regular rows are formed in BMS (c.f. Figs. 2a and b). In BMS, division and enlargement of nucellus cells also occur in a more symmetrical fashion in the micropylar region. In strain A188, cell division and enlargement are less intensive both near the base of the embryo sac and at the side directed towards the single integument. Therefore, the thickness of the nucellus covering the embryo sac is unequal at the two sides of the egg apparatus. Several nucellus cells which border on the mature embryo sac are flattened because of the enlargement of the embryo sac. Here a total collapse of the cells is preceded by a process of cytoplasmic desintegration (Fig. 1c).

When a pollen tube penetrates the ovule of A188 there appears not to be one straight way towards the embryo sac like in BMS (Figs. 9a and c). Serial sections of the micropylar region of A188 demonstrate the existence of a lobed micropyle (Fig. 10a). One of the folds in the inner integument lies opposite the egg apparatus forming the passage for the pollen tube (Fig. 10a 5, arrow).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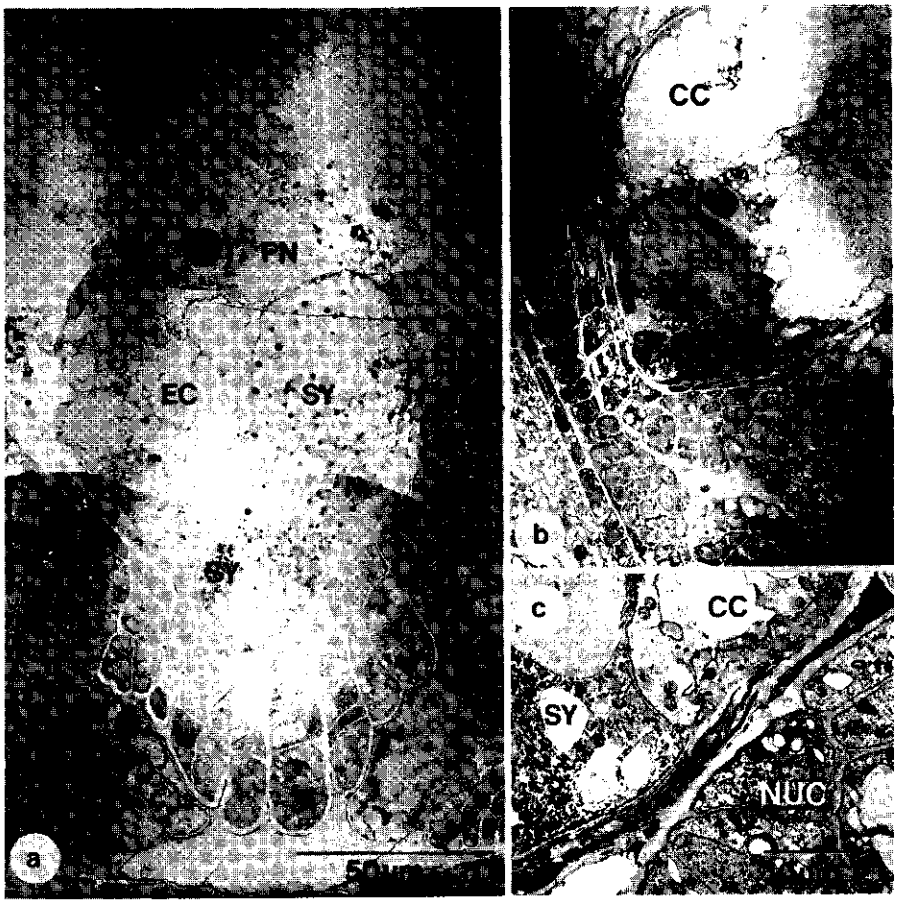
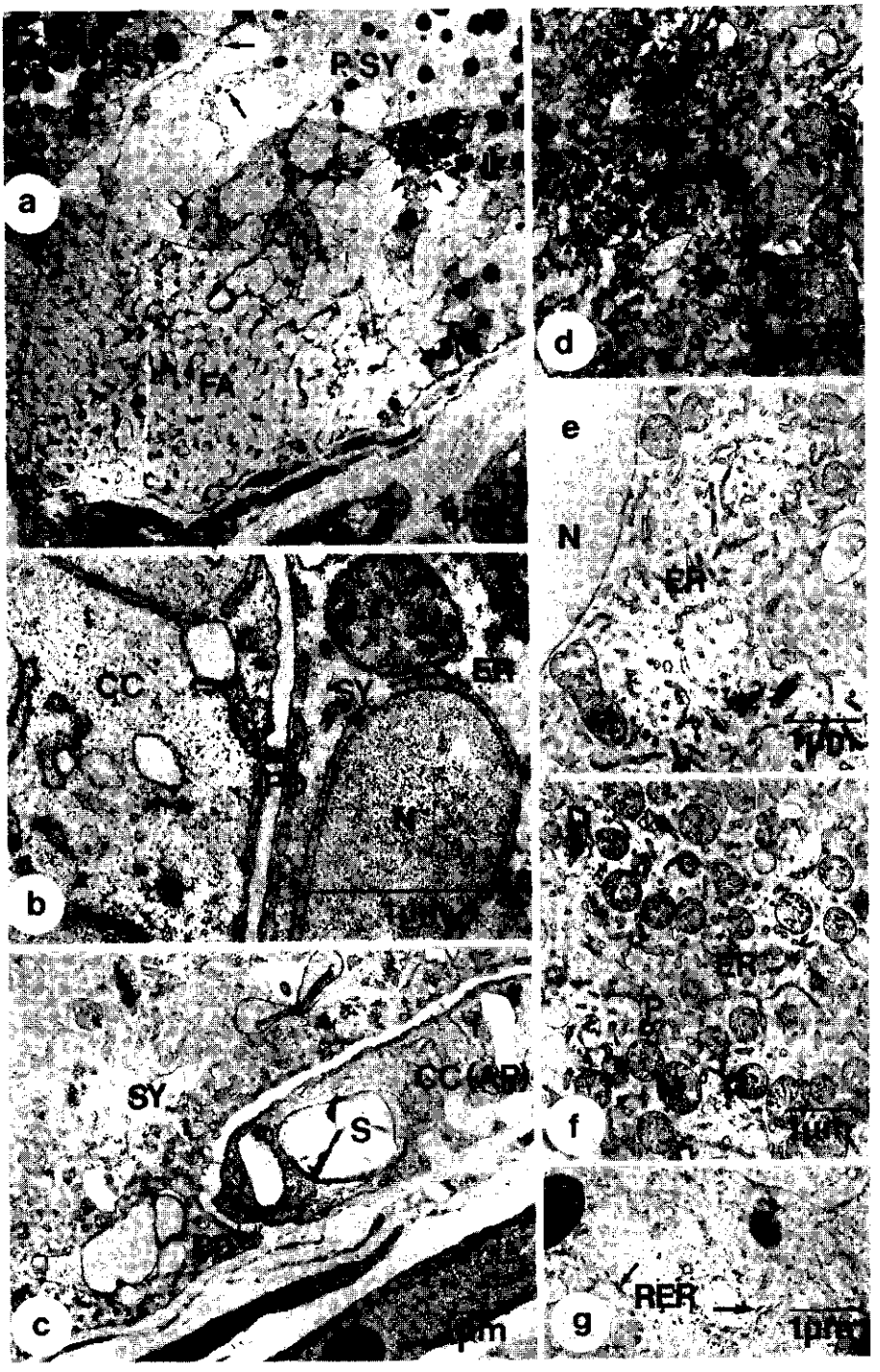


FIG. 2. Median sections through the micropylar parts of the megagametophytes of *Zea mays*, strain BMS (**a**) and A188 (**b,c**). The polar nuclei of the central cell are surrounded by cytoplasm and lie on top of the egg apparatus. In contrast to the synergids the egg cells are highly vacuolated. Their cytoplasm is found in the micropylar half. Note the regular arrangement of the nucellus cells near the micropyle of BMS (**a**). Several nucellus cells collapse because of the enlargement of the embryo sac.

The cells of the embryo sac before fertilization

Intact synergids At the time of fertilization both synergids may still be intact. Sometimes, however, one synergid degenerates before fertilization. This synergid will receive the pollen tube. Synergids are about as tall as the egg cell.

FIG. 1. Schematic representation of the megagametophyte positions in the ovaries of *Zea mays*, strain BMS and A188. **A** The pistilate spikelet and the ovary with its modified campylotropous ovule. The outer integument does not completely surround the ovule. **B, D** The embryo sac consists of approximately 20 antipodals, a large central cell and the egg apparatus. **C, E** With regard to the micropyle there is a difference in position of the egg apparatus in BMS and A188.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CC (AP)
S
1μm
g
RER
1μm

At their micropylar side, where they are attached to the wall of the embryo sac, wall protrusions invade the cytoplasm forming the filiform apparatus (Fig. 3a). Some wall protrusions are also formed at the micropylar part of their joint cell wall. Here, the scarcely spread plasmodesmata give cytoplasmic contact. Plasmodesmata leading to the central cell occur frequently in the apical pockets as indicated in Fig. 3c. In other regions there is less cytoplasmic contact with the central cell and plasmodesmata have only rarely been observed leading to the egg cell. Cell walls are clearly formed at the micropylar half of the synergids but at the upper half, cell walls are rudimentary or absent. The distribution of the organelles is not random in the intact synergids and for most organelles the distribution appears similar in the two strains studied. Vacuoles, however, occur more frequent in strain BMS where they occupy the major part of the cell volume (Fig. 2a). In both strains, the nuclei of the synergids always appear to be adjacent to the cell wall in the mid–region or the micropylar half of the cell (Figs. 2a, 4a). They can be surrounded by strains of ER (Fig. 3b). The organelle distribution within one synergid of strain A188 is shown in Figs. 3d,e,f. The majority of the spherical mitochondria accumulates near the filiform apparatus (Fig. 3f), whilst in the middle and upper parts of the cell the tubular endoplasmic reticulum (TER) is more extensive. Proper fixation reveals the existence of rough ER (Fig. 3g). Polysomes are spread over the cytoplasm. Large plastids are dominantly located in the upper half of the cell but small ones are also found near the filiform apparatus. The accumulation of starch has not been detected. Dictyosomes are spread over the whole cytoplasm although more are found at the base of the cell. Lots of granules bud off at the periphery of the cisternae. Osmiophilic droplets occur in all cells of the megagametophyte but they are most numerous in the synergids. Here they are a concomitant feature of cytoplasmic desintegration and they can be found as large spherical droplets in the cytoplasm and as small ones in the cell membrane (Fig. 3a–arrows) and in the outer membranes of the mitochondria. The quantification of the sizes of nuclei, mitochondria and plastids is summarized in Table I.

Egg cell At maturity the megagamete is a highly vacuolized cell surrounded by the two synergids and the central cell (Figs. 2 and 4a). At its micropylar side it is attached to the wall of the embryo sac next to the filiform apparatus. Here, plasmodesmata give cytoplasmic contact with the central cell as illustrated for strain A188 in Fig. 4b. Only few plasmodesmata lead to the synergids. At the antipodal side a clear cell wall is absent. The position of the cytoplasm and the organelles within the egg cell is very characteristic at the unfertilized stage.

FIG. 3. Longitudinal sections through various parts of the pre– fertilization synergids of *Zea mays*, strain BMS (**a,g**) and A188 (**b–f**). Wall protrusions forming the filiform apparatus invade the cytoplasm (**a**), which shows free (L) and membrane bound (arrows) osmiophilic droplets as a sign of an initiated degeneration. Plasmodesmata lead towards the central cell (**b**) especially in the apical pockets (**c**). Note the distribution of plastids, ER and mitochondria in the antipodal (**d**), mid (**e**) and micropylar (**f**) region of one synergid. Profiles of RER are detected frequently (**g**, arrows).

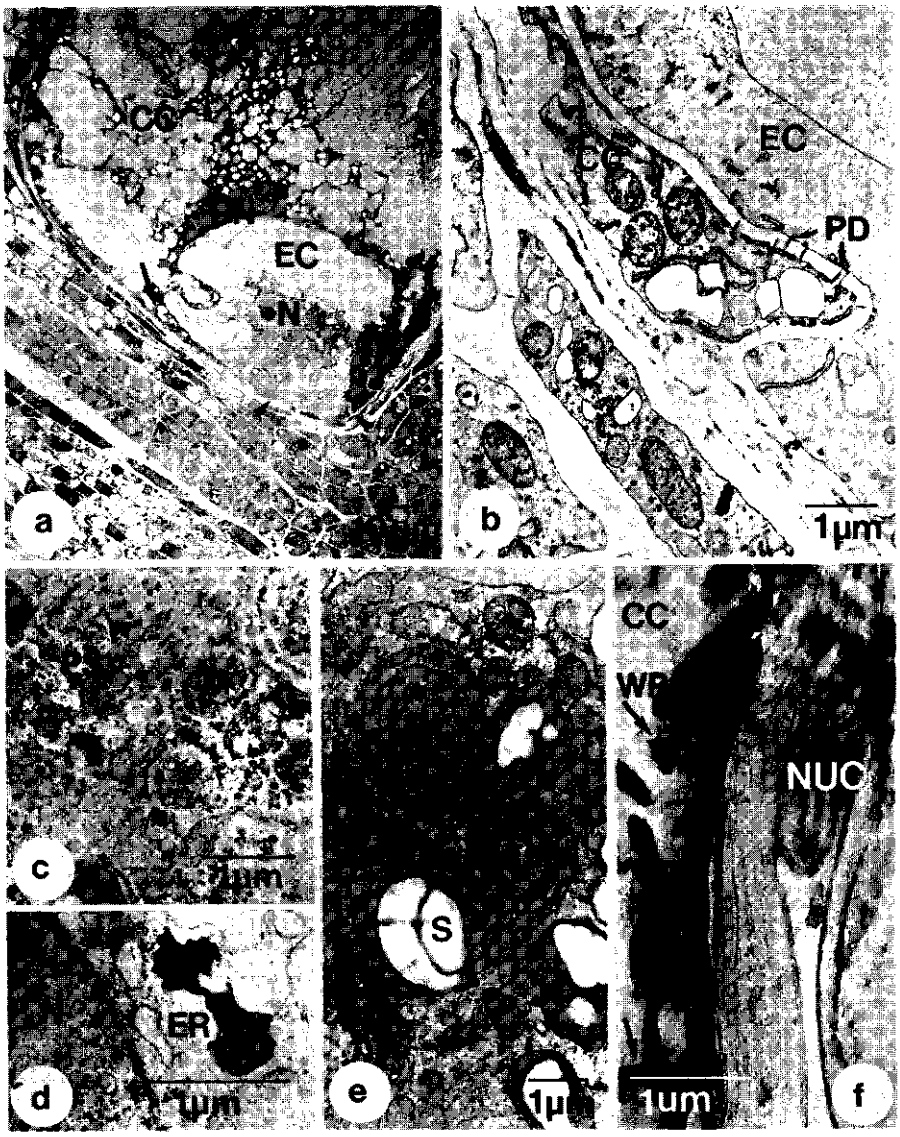


FIG. 4. Longitudinal sections through the micropylar halfs of the embryo sac of *Zea mays*, strain BMS (**c,d,f**) and A188 (**a,b,e**). At its micropylar side the egg cell is connected with the wall of the embryo sac (**a**, arrow–heads). Plasmodesmata from the egg cell towards the central cell are restricted to the apical pockets (**b**). Polymorphic mitochondria, polysomes and starch containing plastids dominate in the egg cell cytoplasm (**c,e**); the ER is in contact with the nuclear membrane (**d**). Numerous wall protrusions (arrows) enlarge the surface of the cell membrane of the central cell. The electron–dense staining results from a selective periodic acid oxidation and the thiocarbohydrazide–silver proteinate reaction after Thiéry (1967) to detect polysaccharides.

In both strains the larger part of the cytoplasm and organelles surrounds the spherical nucleus and can often be found near the wall of a synergid in the basal, micropylar half of the cell. Vacuoles of various sizes occupy the upper, antipodal half. A shift of the cytoplasm and the nucleus towards the apex of the cell results in a basal vacuolation and is an early consequence of fertilization as will be shown later on.

Among the organelles mitochondria are most striking in the egg cell cytoplasm. They are often found solitary but data obtained from serial sections show that they can also be arranged around each other, like shells forming a globular structure with a spherical mitochondrion in its centre (Figs. 4c and e). The latter phenomenon is only observed in this cell of the embryo sac and it occurs most frequent in strain A188. Many ribosomes and polysomes lie between those mitochondria. Smooth ER and RER are scattered throughout the cytoplasm and the ER makes contact with the outer nuclear membrane (Fig. 4d). The ER may have swollen cisternae in both strains. Polysomes and monosomes occur regularly but dictyosomes contribute for a minor part in the composition of the cytoplasm. There are also few plastids. They are large, contain several starch grains and sometimes they tend to be clustered.

Diagram 1 gives a schematic representation of the organelle shapes in both the egg cells and the zygotes of strain BMS and A188. Diagram 2 and Table II summarize the quantification of sizes, frequences and occupation rates of plastids and mitochondria in the egg cell. It appears that mitochondria and plastids do not differ in average sizes significantly although the graphic presentation of sizes demonstrates the existence of larger mitochondria and plastids in strain A188 (c.f. Diagram 2, BMS **1,5**; A188 **1,5**). The occupation rates of the mitochondria and the plastids, as presented in Table II were calculated from organelle surface measurements within a defined area of cytoplasm in which the surfaces of the nucleus and vacuoles were not included. In both strains, about 35% of the cytoplasm is occupied by mitochondria. The rates measured for plastids were significantly lower: for BMS 20 $\pm$ 3%, for A188 12 $\pm$ 4%.

Central cell The cytology of BMS and that of A188 central cells is similar in many aspects. The central cell encompasses the major part of the egg apparatus. The cell wall which borders on the nucellus is well developed, especially in the micropylar region (Fig. 4a, arrows) where it bears small polysaccharide containing protrusions (Fig. 4f, arrows). At the antipodal side of the egg apparatus, however, the cell walls between synergids, egg cell and central cell are often incomplete or undetectable. Here the male gamete is supposed to leave the synergid to fuse with the central cell. In the pre–fertilization phase of the mature megagametophyte the two polar nuclei and the major part of the cytoplasm are always found at the antipodal side of the egg apparatus (Fig. 2a,b; 5a). The nuclei are always close to each other and might already be partly fused before fertilization (Figs. 5b,c). There is continuity between the nuclear envelope and the ER (Fig. 5d). The major part of the central cell is occupied by vacuoles of various sizes. A thin layer of cytoplasm covers the cell membrane adjacent

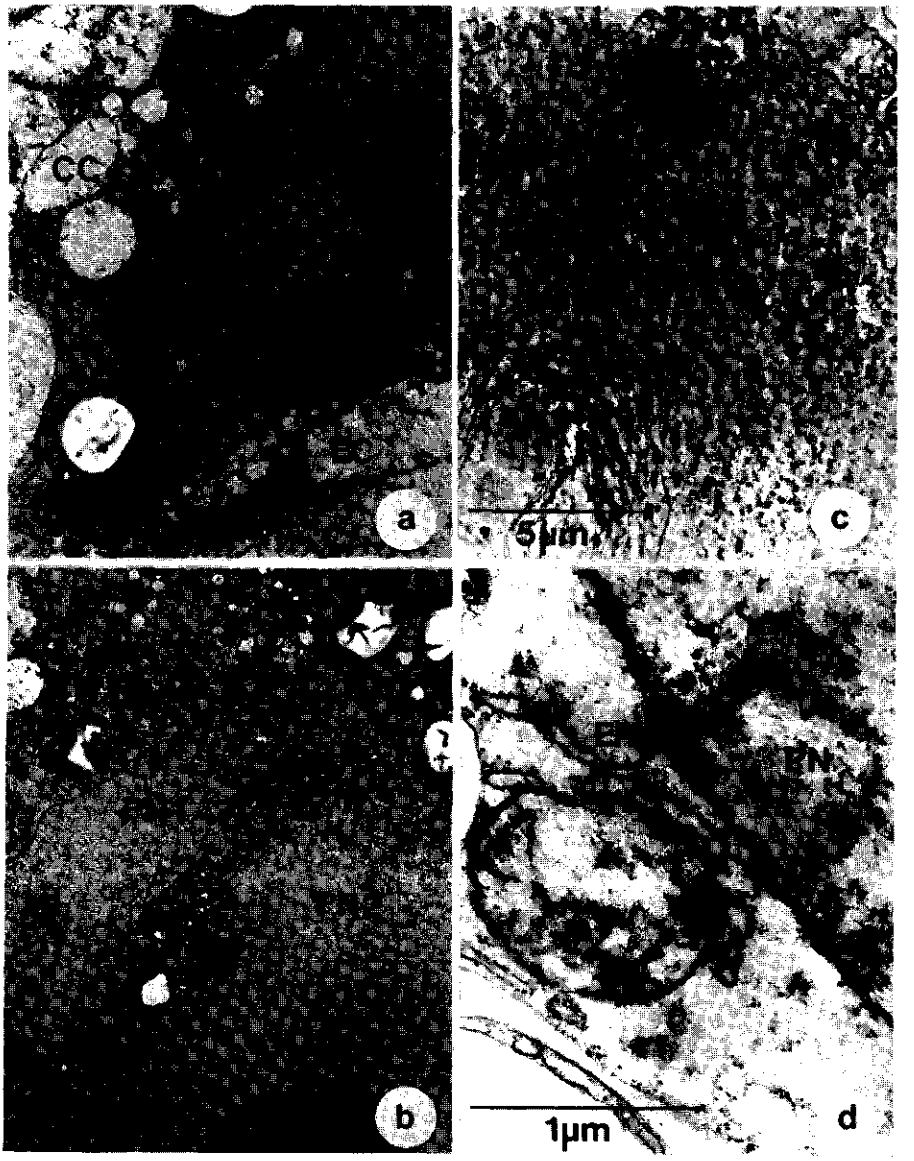


FIG. 5. Polar nuclei in the central cells of *Zea mays*, strain BMS (**c,d**) and A188 (**a,b**) are always at the antipodal side of the egg cell (**a**). The nuclei are close to each other (**b**) and can be partly fused before fertilization (**c**). There is a continuity between the nuclear envelope and the ER (**d**).

to the cell wall of the embryo sac that borders on the antipodal and nucellus cells. Plasmodesmata occur regularly between the central cell and the other cells of the megagametophyte but not between the central cell and the surrounding nucellar tissue.

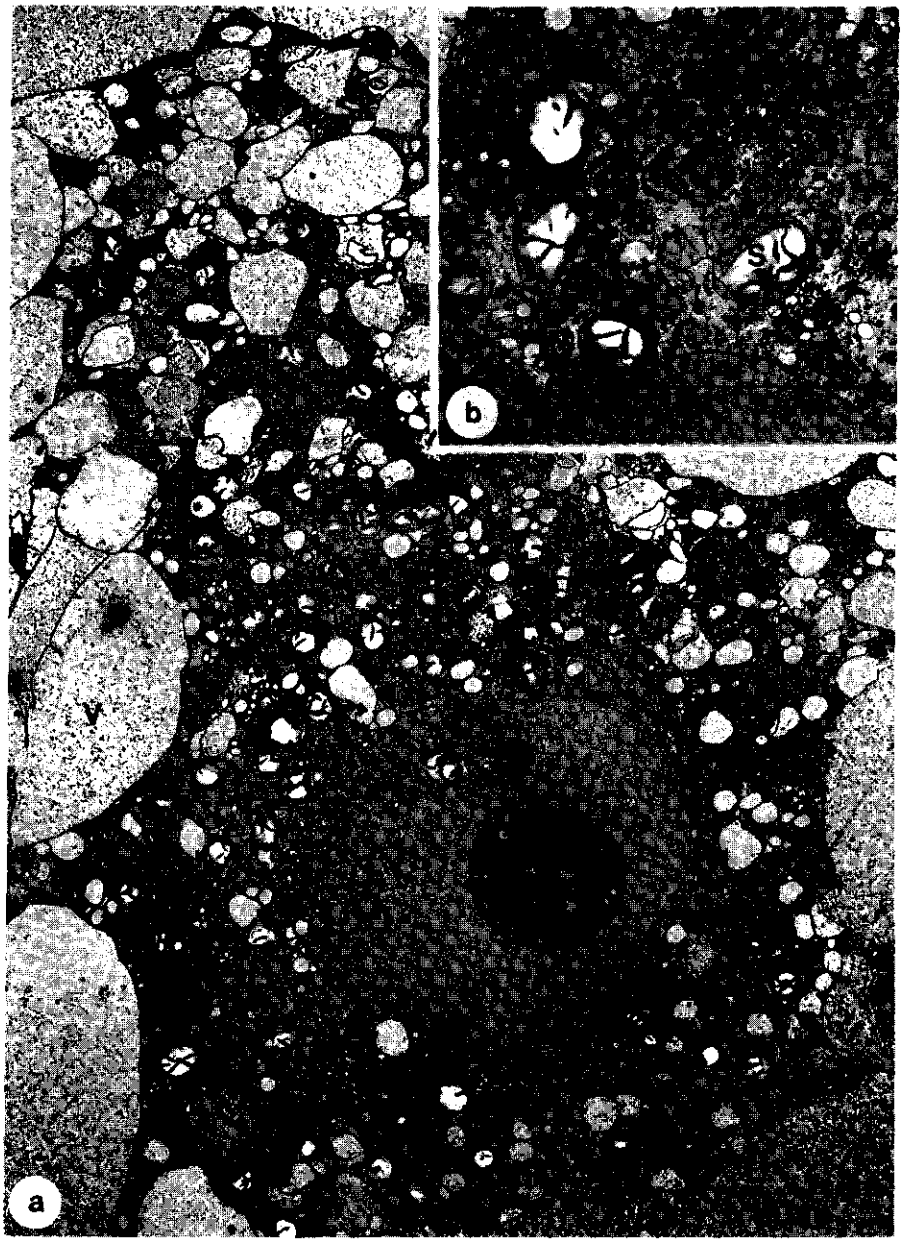


FIG. 6. An overall picture and a detail of the majority of the central cell cytoplasm of *Zea mays*, strain A188 (**a**,**b**). Mitochondria have well developed cristae; the ER is often tubular; dictyosomes have small vesicles and the polymorphic plastids often contain starch.

For both strains there is a high density of organelles in the cytoplasm around the nuclei as is shown in Fig. 5 and 6. Mitochondria are sometimes polymorphic but mostly ellipsoid and have well developed cristae. The endoplasmic reticulum appears to be mainly tubular in strain A188 whereas in strain BMS long profiles of ER have often been seen in sections. Dictyosomes have small vesicles and are never numerous at this stage. Plastids vary considerably in size and shape and often contain starch grains in strain A188. The organelles of the central cell are schematically represented in Diagram 3. Diagram 2 and Table II summarize the quantification of sizes, frequences and of the occupation rates of mitochondria and plastids in a part of the cytoplasm near the egg apparatus. The mitochondria of the BMS and A188 central cells have about the same size. There are no differences in cytoplasmic occupation rates. For plastids significant differences can neither be detected in mean organelle sizes nor in cytoplasmic occupation rates. When, however, central cells are compared with the egg cells it appears that the occupation rate of mitochondria is significantly higher in the egg cell of strain A188 than in the central cell of that inbred line.

Antipodals Multiplication of the three antipodal cells results in approximately 20 antipodals which form part of the full-grown embryo sac (Figs. 1b, 1d). Several cells contain more than one nucleus because complete cell separation was not realized after mitosis. Cell walls, adjacent to the nucellar tissue, may

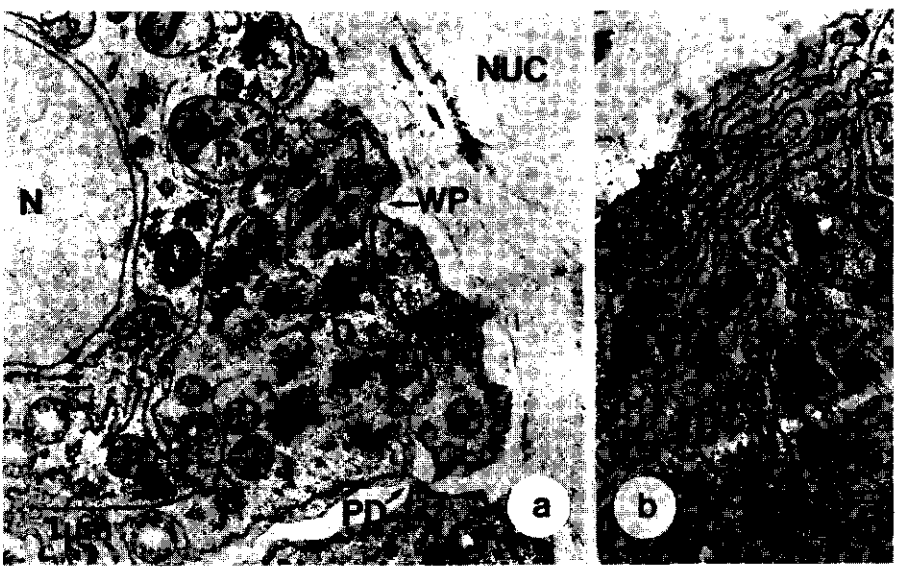


FIG. 7. Antipodals of *Zea mays*, strain BMS (**a,b**) contain numerous organelles among which an abundance of ER, dictyosomes and mitochondria. The ER is frequently arranged in long, interconnected sheets parallel to each other (**b**). Dictyosomes can be in close association with the ER. They have many cristae but secretory granules do not bud off frequently.

have small protrusions (Fig. 7a) as observed in the central cell, too. Plasmodesmata allow contact with other antipodals and with the central cell but there is no cytoplasmic contact with the nucellus cells. Some antipodals have large vacuoles, others have only small ones. The cytoplasm contains numerous organelles among which an abundance of ER, dictyosomes and mitochondria (Fig. 7a). In both strains the ER is frequently arranged in long interconnected sheets parallel to each other (Fig. 7b). Intra–cisternal continuity throughout the cytoplasm from the viscinity of the outer nuclear membrane up to the periphery of the cell is obvious. Nuclei may be spherical or invaginated. They are partly enclosed by sheets of ER. Plastids have small thylakoid membranes and may contain starch grains as detected in BMS. In both strains, BMS and A188, a high number of polysomes, many large, well developed dictyosomes, and mitochondria with well developed cristae were observed.

The quantification of the sizes of some organelles is summarized in Table I. The antipodal nuclei are the smallest nuclei in the embryo sac. Antipodal mitochondria and plastids are small, too, like in the synergids. Due to the quantification procedure high standard deviations occur (see legend Diagram 2) and therefore, despite different average sizes, significant distinctions could only be demonstrated incidentally. Only the plastids of BMS antipodals are significantly smaller than the plastids in the egg cell and central cell.

Penetration of the pollen tube into the pistil and the embryo sac

The pollen tube growth is initiated after the contact of the pollen grain with the multicellular, receptive hairs located on two opposite sides of the silk. The pollen tube may penetrate the body of the style directly but it usually enters by way of one of these stigmatic hairs (Fig. 8a).

Once inside the tissue of the style, pollen tubes grow intercellularly towards a transmitting tissue which is associated with the strands of vascular tissue and then between the cells of that tissue down the silk towards the ovary (KROH et al. 1979; HESLOP–HARRISON et al. 1985). Pollen tubes are traced by the detection of fluorescent callose deposits in the pollen tube (Fig. 8b,c,d). In these studies about 7 pollen tubes were detected in a cryosection (25 μm) of an ovary. The pollen tubes follow the inner surface of the ovary wall until the micropyle is reached (Fig. 8b,c). Not all the pollen tubes grow straight towards the micropyle. Some were observed between the integuments and others at the opposite side of the ovule but eventually all come to the micropyle (Fig. 8d). The pollen tubes are detected by the staining of callose deposits, with 'Wasserblau' but when a pollen tube penetrates the nucellus, callose can no longer be detected.

In the ovules of strain BMS the micropyle consists of only one opening in the inner integument through which one or more pollen tubes have to reach the nucellus. Only one pollen tube was observed to pass the cuticle of the nucellus. It penetrates the nucellar tissue and grows directly towards the egg apparatus. No cells of the nucellus are damaged when the pollen tube grows through the cell walls and the intercellular spaces (Figs. 9a,c). The pollen tube penetrates the embryo sac by growing into the filiform apparatus through the joint cell

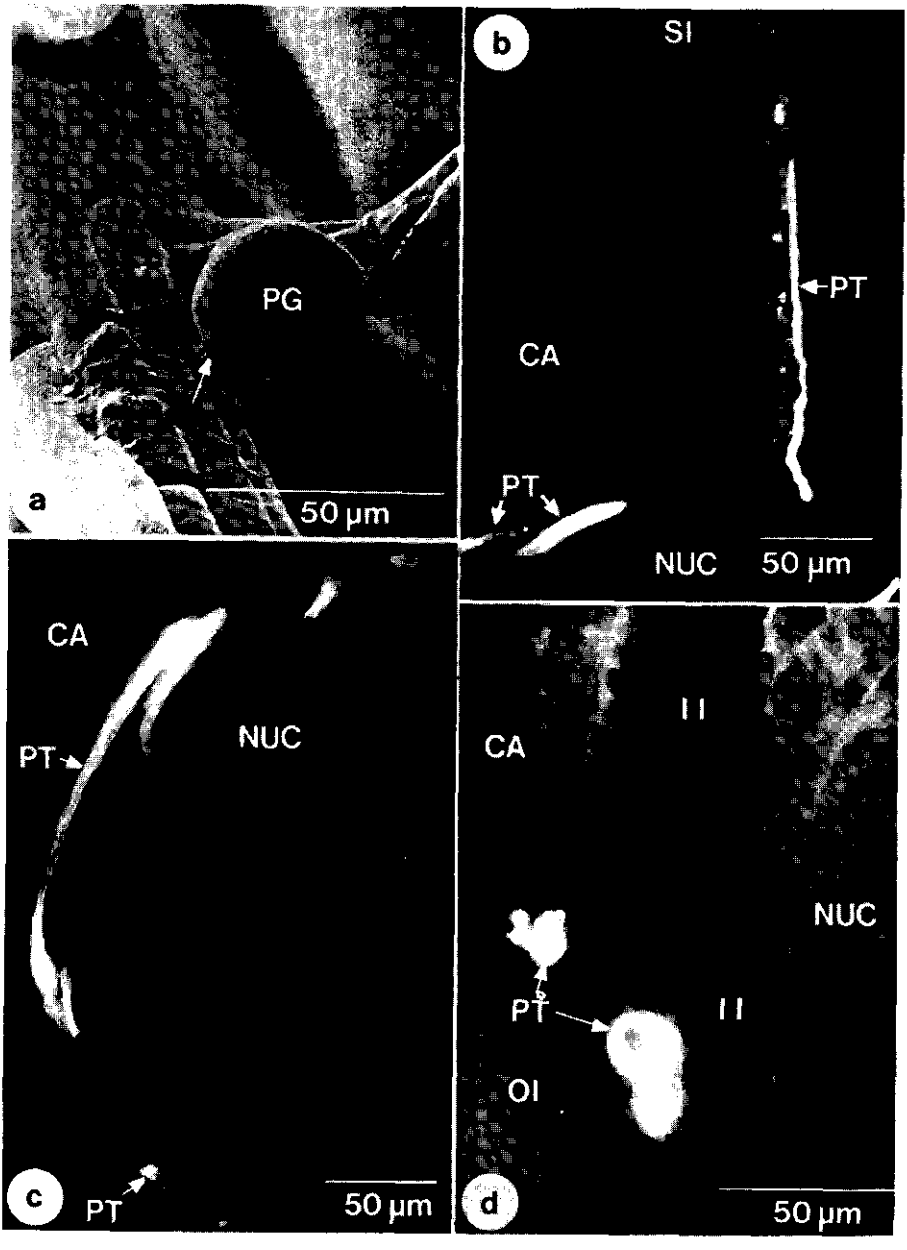


FIG. 8. A pollen grain (PG) of *Zea mays*, strain BMS, adheres to a stigmatic hair. It has germinated and the pollen tube penetrated the body of the silk directly (**a**, photograph courtesy Dr.Ir. H.J. Wilms). Then pollen tubes grow through the transmitting tissue of the silk into the ovular locule (**b,c**) towards and between the integuments at the micropyle (**c,d**).

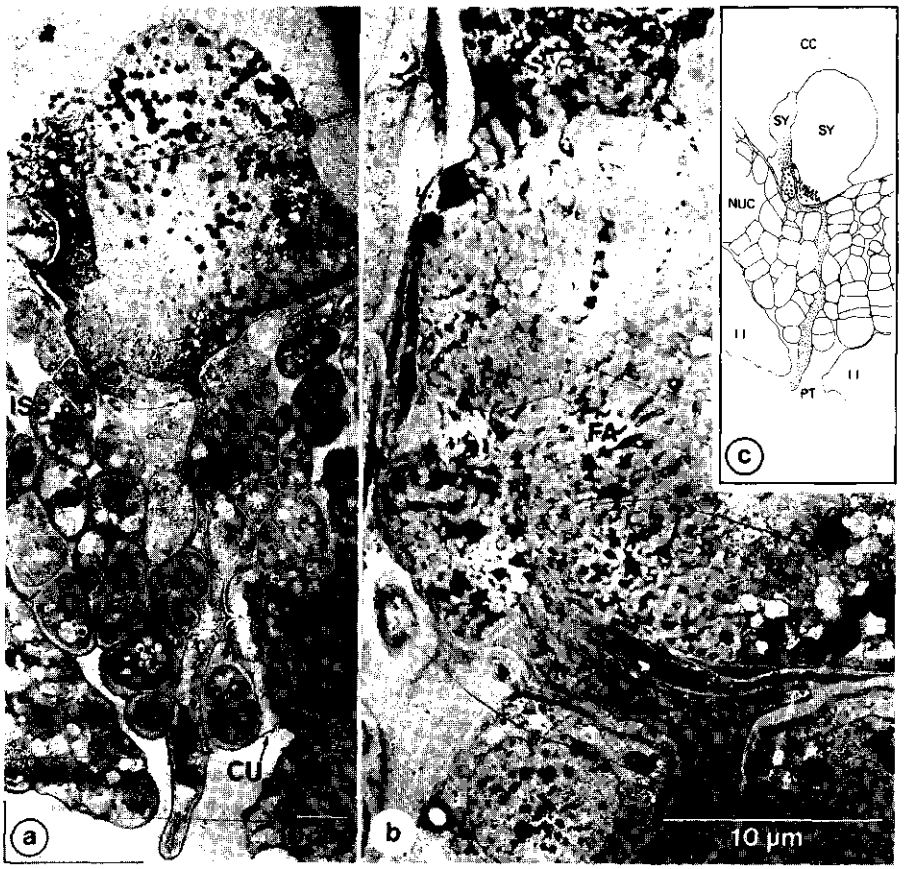
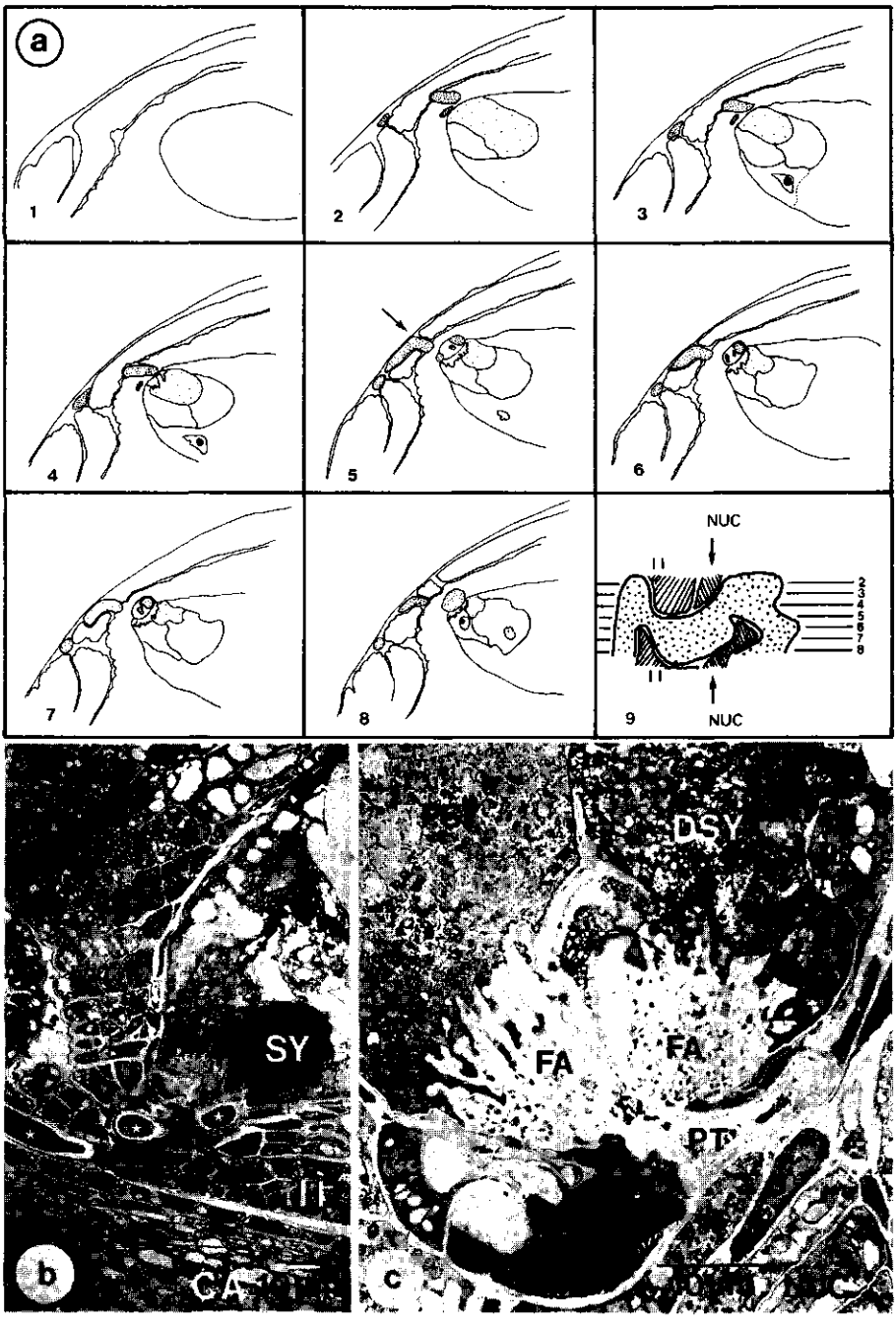


FIG. 9. Penetration of the nucellus and embryo sac by the pollen tube in *Zea mays*, strain BMS; overall view (**a**), detail (**b**) and drawing (c). The pollen tube penetrates the cuticle of the nucellus, then it grows through intercellular spaces into the nucellus and eventually it enters the filiform apparatus through the joint cell wall of the two synergids. Pollen tube cytoplasm has been discharged into one synergid.

wall of the two synergids. Then the pollen tube enters a synergid along side the joint cell wall (Fig. 9b). It stops growing and discharges a part of its contents into the synergid which gets filled up completely. When the synergid is penetrated it may have been degenerated already.

When a pollen tube penetrates the ovule of A188 there isn't one straight way towards the embryo sac like in BMS. Serial sections of the micropylar region of A188 demonstrate the existence of a lobed micropyle which has several openings (Fig. 10a,b). The pollen tube winds its way and cannot be observed in one plane of sectioning. One of the folds in the inner integument lies near to the egg apparatus and forms the eventual passage for the pollen tube (Fig.10a,5, arrow). Once in the nucellus, the pollen tube appears to bend again searching its way to the embryo sac. Reaching the base of the filiform apparatus the pollen


a
1
2
3
4
5
6
7
8
NUC
II
2
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NUC
SY
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PT
c

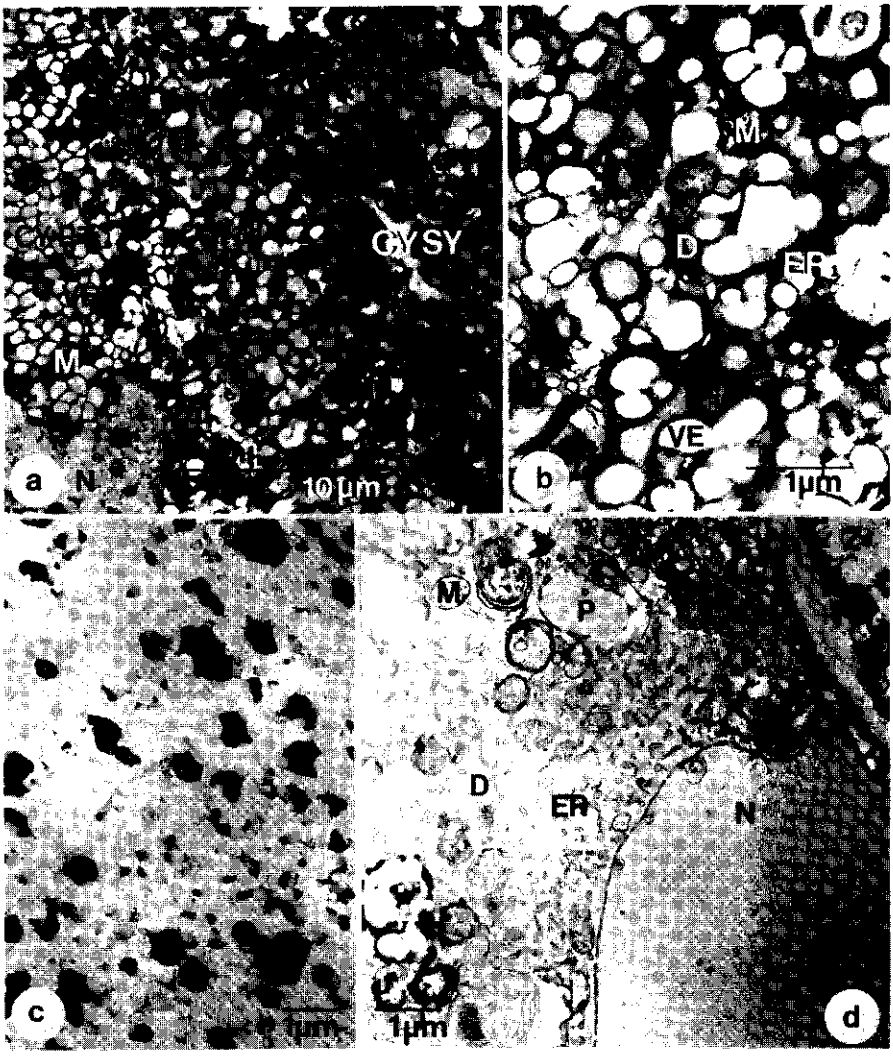
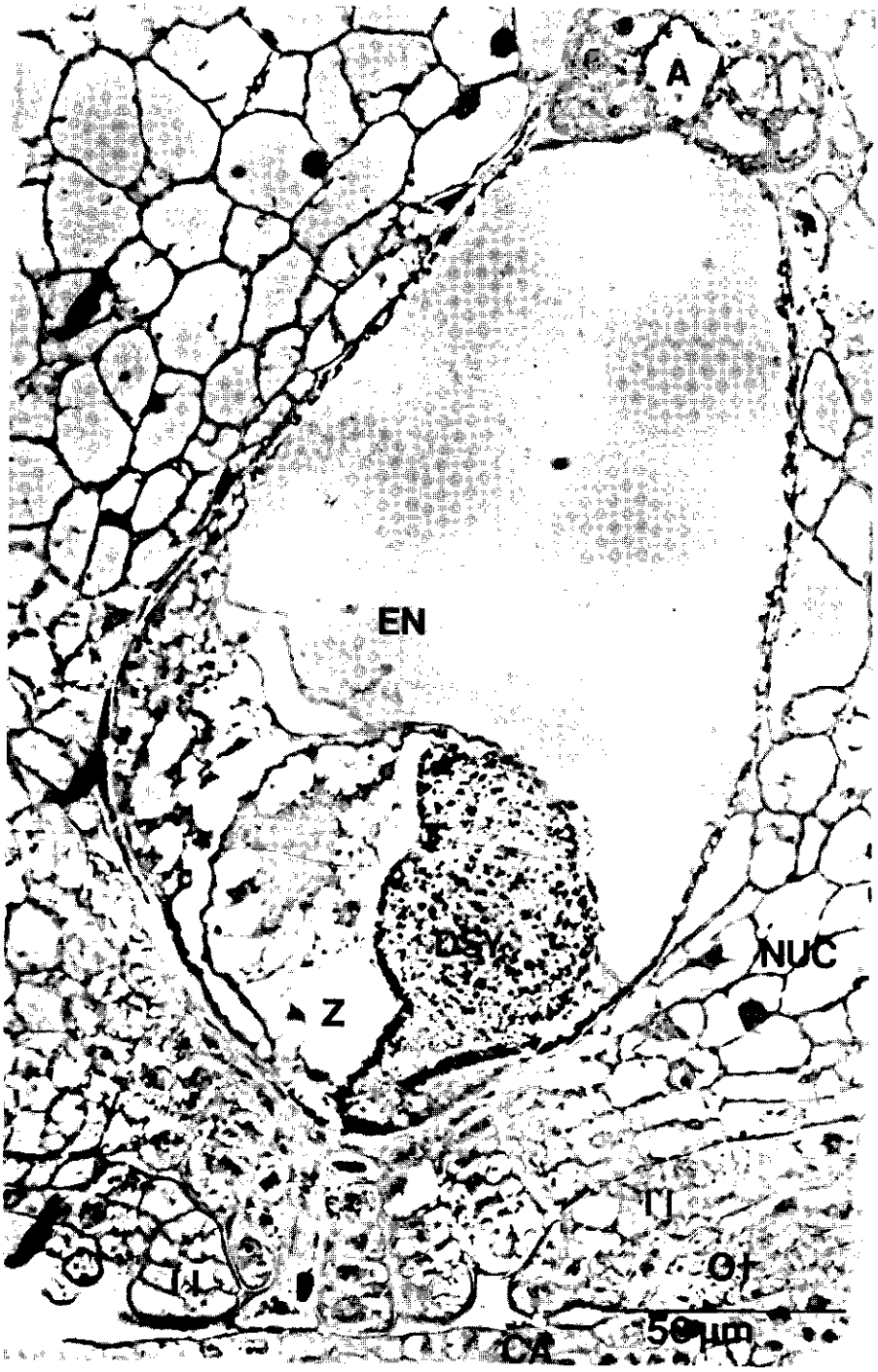


FIG. 11. Post-fertilization structure of the synergids of *Zea mays*, strain A188. Except for a small region, the pollen tube cytoplasm and the vegetative nucleus do not mix with the cytoplasm of the degenerated synergid (**a**). The cytoplasm mainly consists of dictyosomes, vesicles and mitochondria (**b**). Within a few hours, that cytoplasm degenerates and osmiophilic droplets and vesicles remain (**c**) whereas the persistent synergid is still intact (**d**).

FIG. 10. Penetration of the pollen tube into the ovule of *Zea mays*, strain A188.**a**) Serial, longitudinal sections through the micropylar region visualize the course of the pollen tube (**a 1–8**, pollen tube dotted) which runs up and down through the inner integument and nucellus. The arrow indicates the site of pollen tube entry; **a–9** gives the two-dimensional compilation of the drawings. **b–c**) Electron micrographs of micropylar region with pollen tube. Note the bending of the pollen tube (**b** asterisks), the penetration of the filiform apparatus (**c**) and the sub-terminal opening of the pollen tube (**c**-arrow).


A
EN
DSY
NUC
Z
CA
50 µm

tube sometimes enlarges and eventually penetrates a synergid like in strain BMS (Fig. 10b,c)

For strain A188 it takes about 21 hours from pollen tube germination up to penetration of the embryo sac and about 26 hours for strain BMS. However, the periods mentioned are largely dependent on the greenhouse conditions.

The cells of the embryo sac after fertilization

Synergids When the pollen tube has penetrated a synergid and discharged its contents into that synergid, the cytoplasm of both cells is not mixed (Fig. 11a). In some parts of the cell the synergid cytoplasm can still be detected. The cytoplasm coming out of the pollen tube reaches up to the tip of the synergid and can be discerned by its characteristic appearance (Fig. 11b). Translucent vesicles, originating from the dictyosomes, predominate in the cytoplasm. Mitochondria have long cristae but the accumulation of small osmiophilic droplets which are often attached to the cristae and inner membranes of the envelope indicates the onset of degeneration. Plastids, however, are still intact and some strands of ER are scattered throughout the cytoplasm.

Soon after fertilization the discharged cytoplasm degenerates to such an extent that organelles can hardly be detected (Fig. 11c). Large, osmiophilic droplets and numerous small vesicles are the main constituents of the cytoplasm. The persistent synergid may be structurally intact until the division of the zygote (Fig. 11d) whereas even before fertilization signs of degeneration were observed (Fig. 3a).

Zygote Just after fertilization the egg cell and zygote are not remarkably different in cell size or in shape and yet they are easily distinguished by observing the location of the cytoplasm. As shown previously for the egg cell, the majority of the cytoplasm surrounds the nucleus in the micropylar half of that cell (Fig. 2). As a striking result of fertilization the cytoplasm and the nuclei of the zygotes of both strains BMS and A188 are translocated towards the antipodal side of the cell (Fig. 12,13,17a). The syngamy proper and the karyogamy, however, were not observed and organelles of the male gamete were not distinguished. Fertilization causes no significant change in size of the zygote but in the cytoplasm an increasing complexity is observed (Diagram 1). This holds for the ER, the dictyosomes and the polysomes rather than for the plastids and mitochondria of which the sizes (Diagram 2), occupation rates and frequences (Table II) do not change significantly. As was observed in the egg cells of BMS and A188, the mitochondria are often stacked, forming globular structures. In the zygotes they have large cristae (Figs. 14a,b). The ER tends to concentrate in some areas (Fig. 14c).

FIG. 12. Phase contrast light micrograph of a longitudinal section through the embryo sac of *Zea mays*, strain BMS. After fertilization the prophase nucleus and the cytoplasm are located in the antipodal half of the zygote whereas the endosperm cytoplasm shifted lateral.

FIG. 13. Longitudinal section of the zygote of *Zea mays*, strain A188 with the nucleus and the surrounding cytoplasm at the antipodal side of the cell. Note the abundance of well differentiated mitochondria. The pollen tube cytoplasm in the synergid has already degenerated.

By the time of mitosis, i.e. about 8–10 hours after fertilization, the distribution of organelles changes in the zygotes of both strains. During early prophase ribosomes occur frequently as monosomes, polysomes or they are attached to the ER. At prophase the larger part of the cytoplasm surrounds the nucleus (Fig.

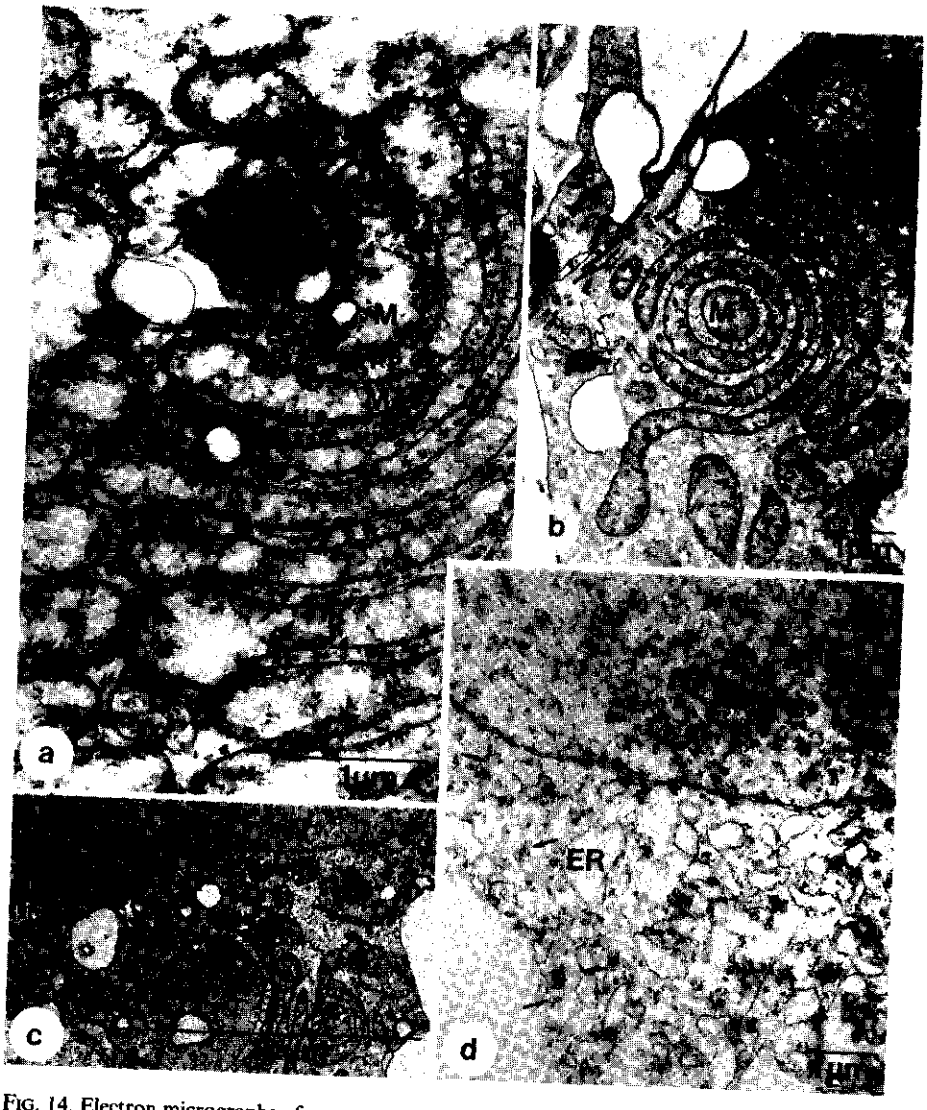


FIG. 14. Electron micrographs of some parts of the cytoplasms of the zygotes of *Zea mays*, strain BMS (**a,d**) and A188 (**b,c**). In both strains the mitochondria can stack, forming globular structures (**a,b**). Proper fixation (glutaraldehyde–OsO_4) reveals the existence of numerous polysomes between the mitochondria (**a**); The ER concentrates in some areas and has a tubular appearance (**c**); it dilates at prophase (**d**). Than, microtubules occur regularly (**d**, arrows; ultra–thin section made after reimbedding of the semi–thin section of fig. 12).

12). Most types of organelles except MTs, ER and ribosomes recede from the vicinity of the nuclear membrane (Fig. 14d). Serial sections reveal the existence of dilated sheets of ER forming a network around the nucleus. Both plastids and mitochondria decrease in size and increase in number then. During pro–

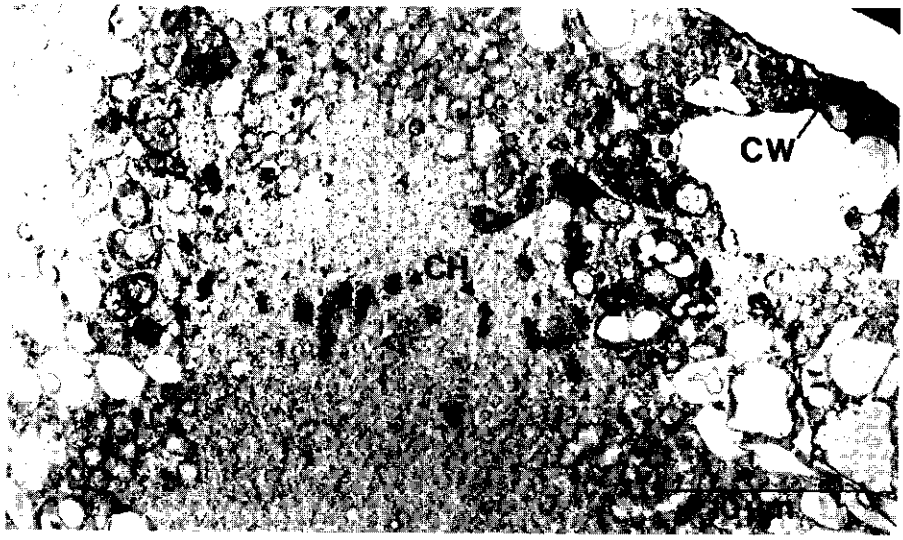


FIG. 15. The zygote of *Zea mays*, strain BMS at mitosis i.e. approximately 8 hrs after fertilization. The metaphase plate is located at the site of the nucleus near the antipodal side of the cell.

metaphase the phragmoplast is formed perpendicular to the long axis of the zygote and at or near the former site of the nucleus (Fig. 15). Just after telophase, the cell segregation is initiated from the cell plate and it proceeds towards the cell membrane. So division results in two unequal cells: an apical cell which is small and has only few vacuoles and a large basal cell in which vacuoles occupy the major part of the cell volume. Figure 16 summarizes some of the early events of fertilization and embryogenesis in the embryo sac of strain BMS up to the two–cellular stage of the embryo.

Endosperm The fusion of the male gamete with the central cell is the impulse for a series of events through which the endosperm will develop. As was observed in both strains fertilization soon evokes a shift of the first endosperm nucleus surrounded by the major part of the cytoplasm towards the lateral and micropylar part of the cell (Fig. 17a). Nuclear divisions occur fast and frequently in the young endosperm of both strains resulting in a coenocyt with eight nuclei at the moment of zygote division, i.e. about 10 hours after fertilization. In the early stage of endosperm formation the nuclei divide synchronously. They lie predominantly near the outer cell wall in the micropylar half of the former central cell. The cytoplasm is concentrated around the nuclei as is drawn in Figs. 16b and c. The position of the organelles near such a nucleus is shown in Figs. 17b and c.

It appears that the plastids and the mitochondria of BMS and A188 do not change in size significantly after fertilization (see Diagram 2: c.f. 3,4 and 7,8). The large plastids which are found in the cytoplasm of the central cell, however,

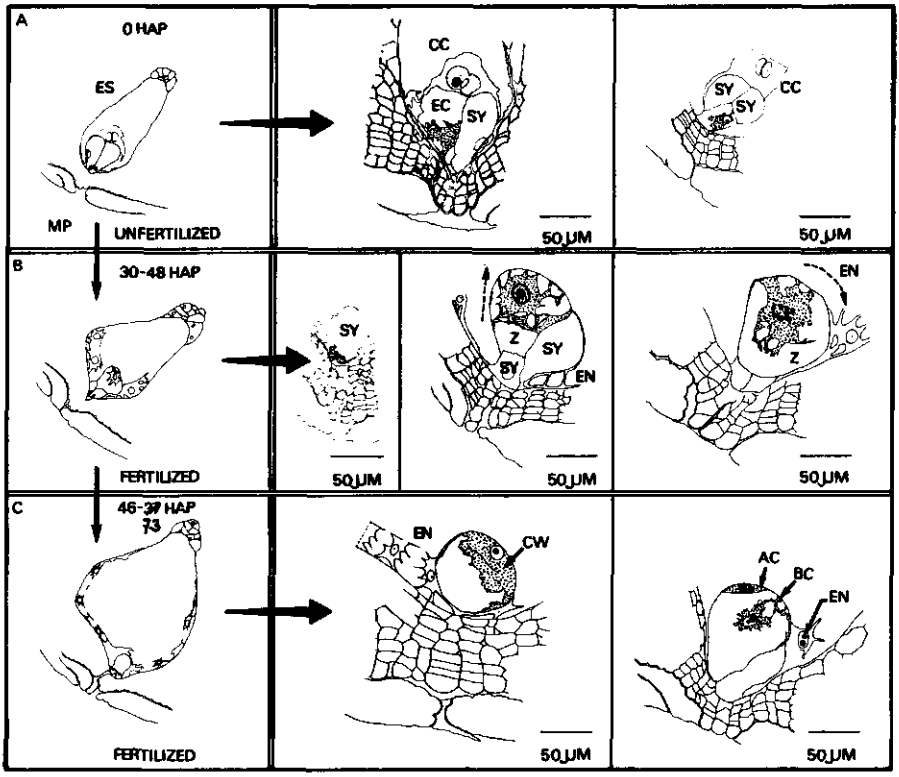


FIG. 16. Summary of the early events of fertilization and early embryogenesis in *Zea mays*, strain BMS. Note the characteristic positions of the cytoplasms of the egg cell and central cell at 0 hours after pollination (**A**) and the shift of the cytoplasms after fertilization (**B**, striated arrows). Mitosis results in a small apical cell and a large vacuolated basal cell (**C**). Than the endosperm is multinucleate.

are not found in the endosperm. The occupation rates of mitochondria and plastids of BMS and A188 do not differ significantly in the central cell and the endosperm (Table II). When the mitochondria of the central cell and the endosperm of A188 are compared with the mitochondria of the egg and the zygote respectively, there is no significant difference in size (Diagram 2: c.f. 3,4 and 1,2), but there is a significant difference in occupation rates being twice as high in the egg and zygote (Table II). As a result of fertilization, dictyosomes increase their complexity and a higher concentration of polysomes was detected in the young endosperm of BMS and A188 (Diagram 3). Long profiles of ER were found running parallel to the cell membrane and near the nuclei.

Antipodals Fertilization has no notable influence on the size and shape of the antipodals of BMS and A188 (Fig. 18a). Plasmodesmata between antipodals

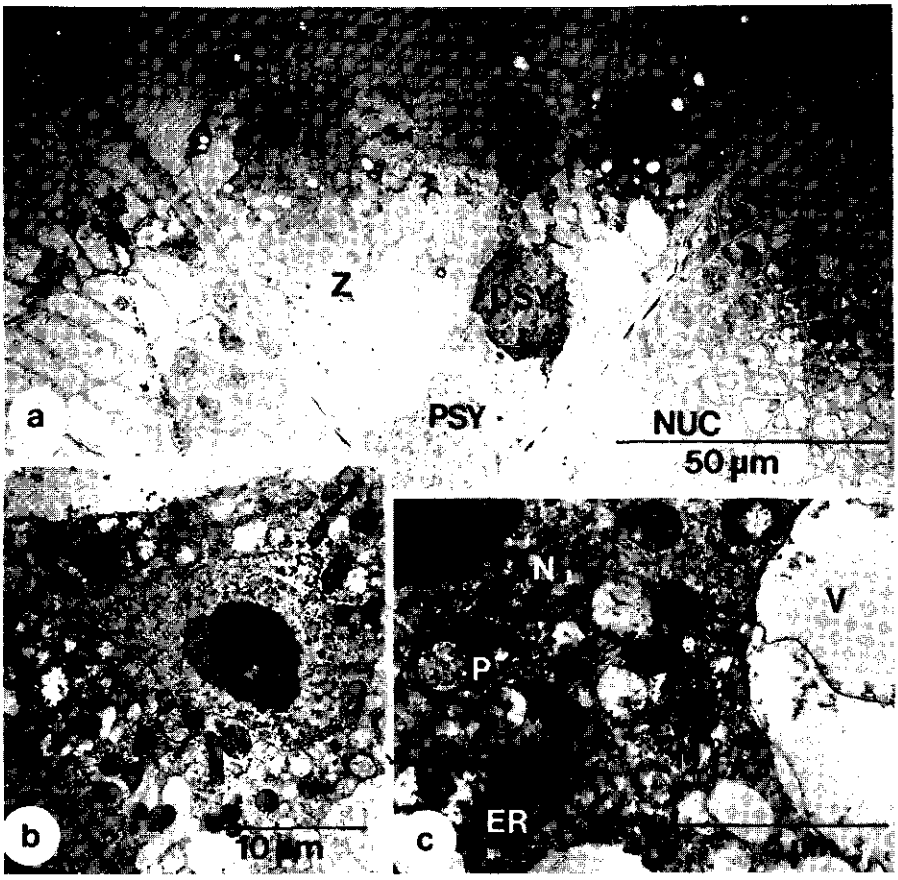


FIG. 17. Post–fertilization cytology of the embryo sac in *Zea mays*, strain A188 (**a**) and BMS (**b,c**). The nucleus of the zygote is located at the antipodal side of the highly vacuolated cell. The persistent synergid is still intact but shows increasing amounts of lipid droplets indicating the onset of degeneration. The endosperm is nucleate at this stage. The majority of the cytoplasm which contains well differentiated organelles surrounds the nuclei (**b,c**).

and leading towards the central cell still exist. Vacuoles can be prominent or nearly absent. The nuclei of the cells are sometimes lobed, even after fertilization. The texture of the chromatin in areas with euchromatin and heterochromatin is most clear in these cells of the megagametophyte. There are no signs of degeneration in the antipodals during the first period of endosperm development. Proper fixation reveals high amounts of ribosomes either bound to proliferating ER of free in the cytoplasm (Fig. 18b). Dictyosomes appear to bud off amounts of vesicles (Fig. 18c). The overall structure of the cytoplasm points at a high activity in the tissue.

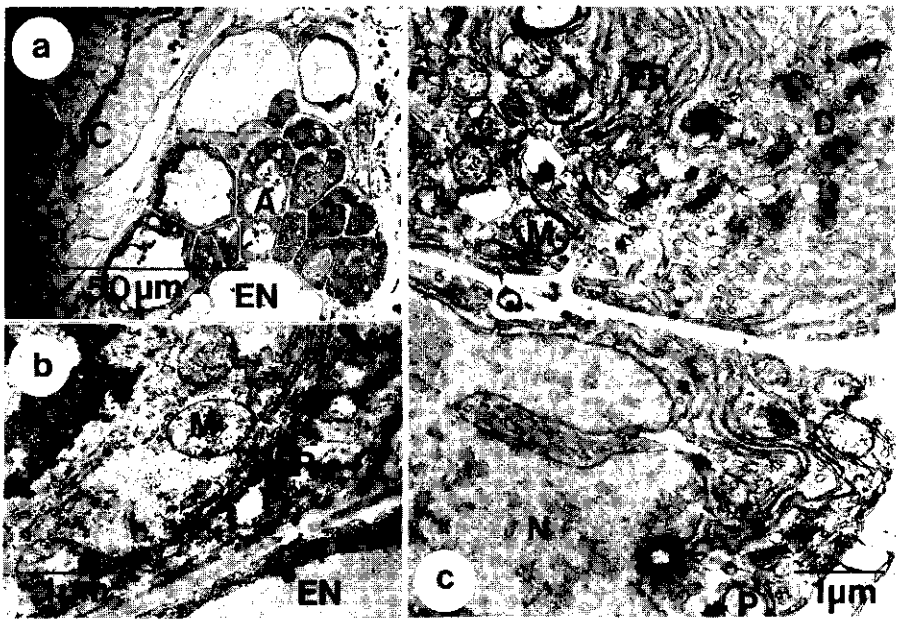


FIG. 18. Antipodals of *Zea mays*, strain A188 (**a,c**) and BMS (**b**) after fertilization. Well developed dictyosomes (**c**) and high amounts of ribosomes either bound to intensively proliferating ER (**b,c**) or free in the cytoplasm point to a high activity in the tissue.

	cells / organelles	Synergids	Egg cell	Central cell	Antipodals
BMS	Nuclei	70	185	310	30
	Mitochondria	0.36 ± 0.07	0.78 ± 0.66	0.62 ± 0.34	0.42 ± 0.07
	Plastids	1.23 ± 0.6	2.79 ± 1.64	4.75 ± 4.17	1.24 ± 0.39
A188	Nuclei	141	200	680	23
	Mitochondria	0.22 ± 0.07	1.60 ± 1.15	0.84 ± 0.65	0.24 ± 0.07
	Plastids	1.57 ± 1.06	5.38 ± 2.93	5.00 ± 3.15	0.68 ± 0.35

TABLE I. Average sizes* of nuclei, mitochondria and plastids in the cells of the unfertilized megagametophyte of *Zea mays*, strain BMS and A188. Measurements are performed with an image analyser (Kontron, MOP–30) and expressed in μm^2. *See note in legend of diagram 2.

cell types / Organelles		Egg cell	Zygote	Central cell	Endosperm
Mitochondria	BMS	*36 ± 12%* 56 ± 21	*25 ± 7%* 32 ± 25	*27 ± 13%* 40 ± 20	*24 ± 3%* 39 ± 24
	A188	*33 ± 14%* 19 ± 11	*24 ± 5%* 15 ± 11	*16 ± 1%* 17 ± 6	*14 ± 4%* 17 ± 4
Plastids	BMS	*20 ± 3%* 5.5 ± 2.1	*12 ± 5%* 2.8 ± 0.8	*16 ± 8%* 7.3 ± 5.0	*10 ± 3%* 6.4 ± 3.5
	A188	*12 ± 4%* 1.7 ± 0.5	*22 ± 7%* 3.1 ± 1.8	*23 ± 7%* 4.6 ± 1.2	*23 ± 11%* 7.8 ± 4.6

TABLE II. Occupation rates (italics) and frequencies of occurrence (capitals) of mitochondria and plastids in the egg cells, zygotes, central cells and endosperm of *Zea mays*, strain BMS and A188. Each value and its standard deviation are based on five independent measurements. Frequencies of occurrence are expressed by the 100 μm^2.

DISCUSSION

Prefertilization and fertilization phase

After pollination and the germination of the attached pollen, the pollen tubes grow through the cortex of the silk towards one of the two parenchyma layers which are associated with the two vascular bundles of the silk (KROH et al., 1979; HESLOP–HARRISON et al., 1985). The parenchyma layers are interpreted as being a transmitting tissue because the pollen tubes grow further down the silk through the intercellular spaces of the fusiform cells of that tissue. Intercellular growth of pollen tubes has also been reported for other members of the Poaceae (see RANDOLPH, 1936; CHO, 1956: BONNET, 1961; BATYGINA, 1966; CHANDRA and BHATNAGAR, 1974). In maize, not all the pollen tubes which grow down in the transmitting tissue reach the ovule. The late–entering tubes are eliminated either at a stigma abscission zone on the base of the silk or at a constricted zone of the transmitting tracts in the upper ovary wall (HESLOP–HARRISON et al., 1985). Once inside the ovary the pollen tubes penetrate the inner epidermis and the cuticle of the ovary wall and they grow in between an integument and the ovary wall towards the micropyle as has also been observed with *Hordeum* (CASS and JENSEN, 1970). During the penetration of cell walls and cuticles the pollen tube probably excretes enzymes to digest cellulose, hemicellulose, pectine and cutin. At the same time it is dependent on metabolites provided by the sporophyte. Because the pollen tube growth, which will result in porogamy, is clearly directed towards the micropyle, a chemoattraction has often been suggested (see VAN WENT and WILLEMSE, 1984) The starch which was observed to accumulate in the micropylar region of the integuments and in the micropylar region of the nucellus might function in that pollen tube attraction. The carbohydrates are excreted into the intercellular spaces to provide the pollen tube with energy.

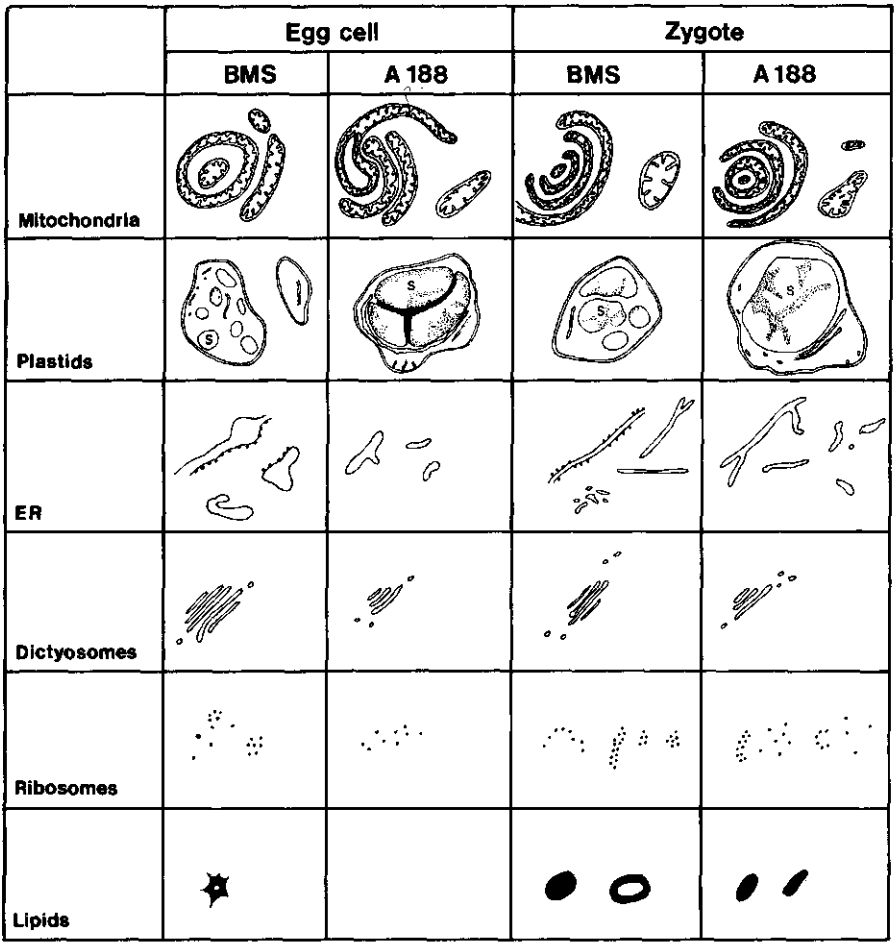


DIAGRAM 1. Schematic representation of organelles in the egg cell and zygote of *Zea mays*, strain BMS and A188.

SINGH and MALIK (1976) propose that metabolites, such as carbohydrates, are also passed on to the filiform apparatus which serves as an entry for metabolites towards the embryo sac. In both strains of *Zea* only one pollen tube entered each ovule. Multiple pollen tube entry into the ovule has been observed in *Triticum* (CHANDRA and BHATNAGAR, 1974), in a remote hybridization of *Triticum* (BATYGINA, 1966) and in *Oryza* (CHO, 1956). The entry of only one maize pollen tube in the nucellus might be explained by the relative short period of time which is necessary for the growth of that pollen tube through the thin layer of tissue from the nucellar cuticle to the degenerated synergid (distance: 20– 40 μm; estimated time 1,5–3 min.). As soon as the synergid is penetrated and probably

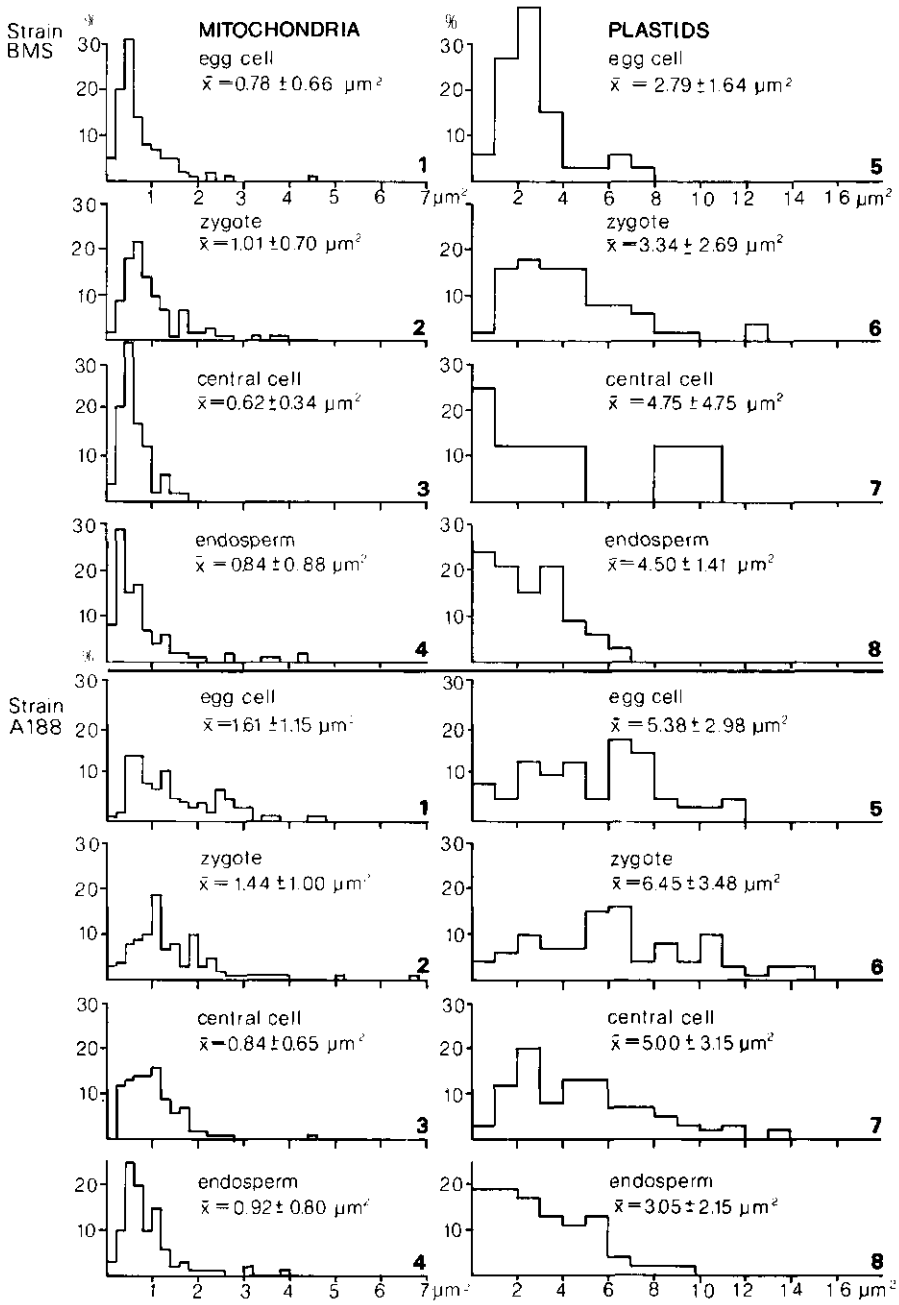


DIAGRAM 2. Size distributions, calculated average sizes and standard deviations of cut areas of mitochondria and plastids in the egg cell–zygote and central cell–endosperm of *Zea mays*, strain BMS and A188. Each histogram is based on measurements in five different areas of cytoplasm.
When organelle surfaces are measured on thin sections one should realize that the organelles can be cut at their maximal diameter and that they can be grazed. Therefore the average sizes do not present real organelle sizes and they coincide with high standard deviations.

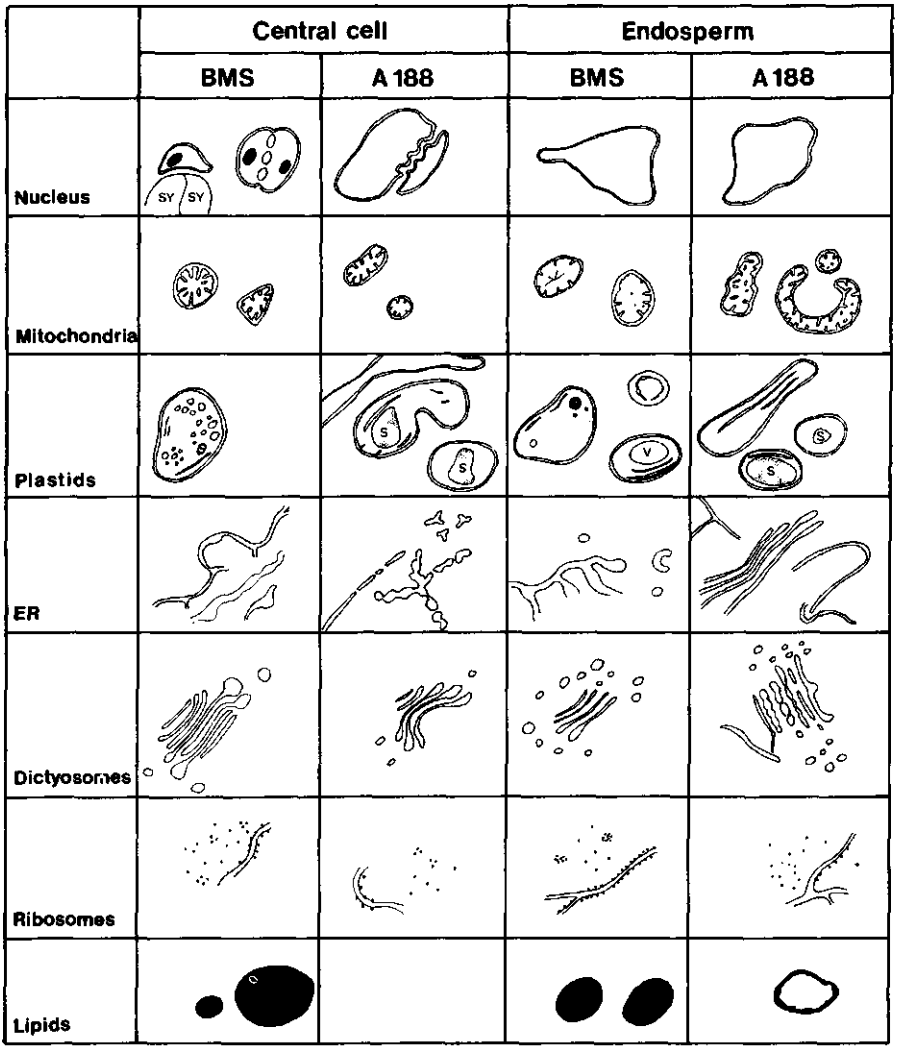


DIAGRAM 3. Schematic representation of organelles in the central cell and endosperm of *Zea mays*, strain BMS and A188.

during nucellus penetration the chemoattraction might disappear and no more pollen tubes will enter the ovule.

When the pollen tube discharges its contents into a synergid, that synergid can be degenerated to some extent as has been observed in various monocot species such as *Sorghum* (VAZART, 1955), *Panicum* (NARANAYANASWAMI, 1955) and *Hordeum* (CASS and JENSEN, 1970). Synergid degeneration has, however, not been observed with *Oryza* (CHO, 1956) and *Triticum* (BHATNAGAR and

CHANDRA, 1975) in which the pollen tube enters the embryo sac between one synergid and the egg cell. With *Hordeum* the degeneration of a synergid starts after pollination but before fertilization (CASS and JENSEN, 1970). In that case the degeneration of the synergid is thought to be necessary for the entry of the pollen tube through that synergid into the embryo sac. The nondegenerated synergid could have a function in nourishing the zygote and the endosperm as was suggested by JENSEN (1964) for *Gossypium*. MAZE and LIN (1975) also suppose that each of the two synergids has specific functions. The synergid which will be penetrated controls the pollen tube growth or the transfer of pollen tube growth directing substances out of that synergid while the persistent synergid and its filiform apparatus function in the transfer of material into the megagametophyte. Synergids might indeed be considered as transfer cells in the sense described by PATE and GUNNING (1972) because the synthesis of the filiform apparatus implies an increase of the plasma membrane area. WILMS (1981) suggested that enzymes are secreted by the synergids because middle lamellae of nucellus cells start to dissolute near the filiform apparatus when the ovule becomes receptive, a phenomenon which was observed in the present study as well. In *Plumbago* there are no synergids in the embryo sac. Here, the egg cell develops filiform cell wall protrusions which are likely to function in pollen tube attraction (RUSSELL, 1982). In relation to pollen tube attraction BROOKS (1963) discusses the function of electrical potentials in physiologically polarized activities. As these potentials require ionic gradients the filiform apparatus might be the source of available carbohydrates for conversion to acids. These acids could function in pollen tube attraction and in fertilization and they could also be involved in the development of the polarity of the embryo.

When the pollen tube grows in the pistel of maize it penetrates only intercellularly until the degenerated synergid is reached and when that synergid is considered not to be a living cell, the pollen tube never enters the symplasts of both the sporophyte and the megagametophyte. In maize the latter two never make contact through plasmodesmata. In the developing caryopsis the plasmodesmata disappear during the development of the megaspore mother cell (DIBOLL and LARSON, 1966) and later on during megasporogenesis, when the three nonfunctional megaspores are degenerated (RUSSELL, 1979). Afterwards, the embryo sac, the developing endosperm and the embryo appear to have exclusively apoplasmic communication with the nucellus, the placento–chalazal region and endosperm respectively (SCHEL et al., 1984). All these phenomena stress the importance of the transmembranal transport of organic and anorganic compounds and minerals and thus the dependence of the heterotrophic gametophytes, embryos and endosperm on the sporophyte.

The growth of the pollen tube through the micropyle and the nucellus is different for the two strains of maize being more straight and regular with strain BMS. This phenomenon can be explained by the existence of only one micropylar opening and a more regular arrangement of the cells between the embryo sac and the micropyle in BMS. This arrangement leads to a continuous gradient in attraction from the micropyle straight to the filiform apparatus. When the

pollen tube reaches the embryo sac at the base of the filiform apparatus it penetrates the cell wall or middle lamellae between the two synergids. This cell wall still has some filiform projections in the micropylar region but at the site where the projections are absent the pollen tube penetrates the degenerated cell. Then its growth ceases and it opens directly in a subterminal pore. The choise to penetrate the degenerated synergid is similar to that in other species such as *Torenia* (VAN DER PLUIM, 1964), *Capsella* (SCHULZ and JENSEN, 1968a,b), *Petunia* (VAN WENT, 1970) and *Hordeum* (CASS and JENSEN, 1970) and might be caused by the excretion of attraction substances by this cell. With respect to attraction calcium is assumed to be the chemotropic agent because it appears to be important in the direction of growth of pollen tubes (MASCARENHAS and MACHLIS, 1964; JENSEN, 1965; REISS and HERTH, 1982; PICTON and STEER, 1983).

The attraction stimulates the pollen tube to penetrate the very base of the filiform apparatus. This might be caused by the high concentration of attractants which are accumulated in the filiform structures of the cell wall. At the top of the synergid the cell wall is incomplete or absent and so attraction will diminish when the tube grows on. It should also be realized that the pronounced polar distribution of mitochondria, dictyosomes and plastids in the synergids can influence the pollen tube growth (see DIBOLL, 1968).

When the pollen tube has discharged its cytoplasm into the synergid, its cytoplasm still appears to be intact. Like in *Cucumis* (VAN WENT et al., 1985) the very active mitochondria and dictyosomes and the profiles of ER give evidence of a high metabolic activity. Soon afterwards, the cytoplasm degenerates; osmiophilic droplets appear, organelles disorganize and electron transparant areas arise. It has been suggested that the latter contain neutral and acid polysaccharid components (DIBOLL, 1968). The function of the pollen tube residue is not yet known, but RNA has been demonstrated in it and also proteins rich in lysine, arginin and histidin; the osmiophilic droplets probably represent lipids (DIBOLL, 1968).

Egg cell–zygote

The overall structures of the egg cell cytoplasms of the two strains, BMS and A188, show similarities in many aspects such as the location of the cytoplasm, the distribution of the organelles and vacuoles in the cells, the frequency of occurrence and the occupation rates of most organelles and, the structural changes initiated by the fertilization. The most remarkable indication for fertilization of maize egg cells is the translocation of the cytoplasm and nucleus from the micropylar side of the cell towards the antipodal side. This reversal of cytoplasmic polarity has been reported for *Zea mays* strain BMS (VAN LAMMEREN, 1981) and *Papaver nudicaule* (OLSON and CASS, 1981). In the zygote, the polarity is now expressed by the dominating presence of vacuoles at the micropylar side and a concentration of cytoplasm surrounding the nucleus at the antipodal side as was also observed in *Capsella* (SCHULZ and JENSEN, 1968a,b). The development of that polarity resulting in a two–celled embryo with a small apical and a large basal cell is widely distributed but not obligatory (SIVARAMAKRISHNA,

1978; JOHRI, 1984). In contrast to many dicots the position of the cell plate resulting from the first zygotic mitosis is not well predictable in the Gramineae family (see WARDLAW, 1955; JOHRI, 1984). Both the cell size and the orientation of the new cell wall may variate. The change in the compartmentation of the zygote results in the polarity. Whether this polarity is developed by an intensive formation of vacuoles at the micropylar side of the cell or by an active translocation of the cytoplasm and nucleus towards the antipodal side remains to be elucidated. The translocation always coincides with the sideward movement of the cytoplasm and nucleus of the fertilized central cell. The existence of a correlation of these phenomena has, however, not been stated up to now. Whether microtubules are involved in the translocation has not been observed because they have only been detected in the zygote during mitosis.

The overall structures of the cytoplasms of the egg cell and zygote indicate a high potency of energy supply. The egg cell is in a state of relatively low activity first. After fertilization, however, the synthesis capacity of the zygote increases as can be deduced from the increasing amount of RER and the higher density of polysomes and dictyosomes (see Table I). Indeed, this cell needs the equipment for high activities such as cell division and increased production of cytoplasm. SINGH and MALIK (1976) observed very feeble enzyme activity in ovules of *Zea mays* and strong reactions for several enzymes prior to, during and after fertilization indicating tempory enhancement of metabolic activity. Studies using radioactive nucleic acid and amino acid tracers show that many animal egg cells are also in a state of metabolic inhibition at maturity (MONROY, 1965). The observation that egg cells of maize are in rest and not meristematic corresponds to observations on the barley egg cell (BUTTROSE, 1963) and to several observations on the maize egg cell (VAZART 1958; DIBOLL and LARSON, 1966). In wheat, equal cytological and cytochemical changes like an increased concentration of starch grains around the nucleus, an increased vacuolation, and a decrease of RNA are regarded as manifestations of life activity at a low level (HU, 1964). With maize, however, mitochondria in the egg cell are well differentiated bearing many cristae. This indicates the potential for the high metabolic rates generally associated with post-fertilization activity (RUNNSTRÖM et al., 1959). Their clustered location points to high and local demands for ATP in both the egg cell and the zygote. After fertilization the increase in size of the mitochondria and plastids in strain A188 corresponds to the observations of DIBOLL (1968) and this can indeed be a sign of the increased metabolic activity in the zygote. At the time of the first mitosis of the zygote, however, the decrease in size and the increase in number of mitochondria suggest that the division of these organelles precedes mitosis. Protein synthesis is restricted to ribosomes and ER and since protein synthesis is ATP dependent, the occurrence of many ribosomes adjacent to the mitochondria supports the suggestion that there is either a high potential capacity or a high rate of protein synthesis. Starch containing plastids were detected in both the egg cells and zygotes of BMS and A188. The cytoplasm of BMS and A188 egg cells can, however, be distinguished because the plastids of BMS are smaller and occur in significantly higher fre-

quencies and occupation rates. Moreover, the plastids of the starchy inbred line A188 contain more starch and larger starch grains in the egg cell and zygote than in the sweet corn BMS. After fertilization there are more yet smaller grains in a plastid and the total amount of starch has decreased, which is related to the increased cell metabolism.

Central cell–endosperm

The composition of the cytoplasm of the central cell in BMS is comparable to that in A188. The two strains can be distinguished by the differences in the structure of the central cell plastids. Black Mexican Sweet corn plastids contain many small starch grains whereas A188 plastids only contain one or a few large starch grains each, which might be related to the starchy nature of this inbred line. Microscopical observations revealed the majority of the central cell cytoplasm to lie characteristically on top of the egg apparatus in both BMS and A188. This position favours an efficient nutrition of the egg cell and zygote. It also permits a fast karyogamy with the polar nuclei. The sequence of the fusion of polar and gamete nuclei appears not to be strictly ordered. MILLER (1911) first noticed a gamete fusion with one polar nucleus and then a fusion with the other nucleus as was also shown in barley (LUXOVA, 1967) and oat (BONNET, 1961) whereas RANDOLPH (1936) has reported a simultaneous fusion of the polar nuclei together with the male gamete nucleus.

The abundance of organelles in the cytoplasm of the central cell and the continuity of the ER with the nuclear membrane (c.f. DIBOLL and LARSON, 1966) indicate an intensive metabolism or at least on a high potency of cell activities such as protein synthesis, secretion and ATP production. Fertilization evokes an increase in cellular activity which is equal for both BMS and A188 as can be deduced from the comparable increase in complexity or size of organelles such as polysomes, RER, dictyosomes and mitochondria. Indeed, these young endosperm cells synthesize cytoplasm and induce mitosis frequently in both inbred lines. The enlargement of the mitochondria coincides with a decrease in occupation rates. The former phenomenon points at higher ATP production, the latter is probably caused by the synthesis of cytoplasm. An increase of enzyme activity was demonstrated histochemically in the cytoplasm of the fertilized central cell by SINGH and MALIK (1976). The central cell and the endosperm might be the most important nutrient resources for the egg cell and zygote respectively. The location of the cytoplasm and the extremely long profiles of ER, aligning the egg cell and zygote indicate that.

The young endosperm is supplied by nutrients coming from the antipodals and nucellus cells (SINGH and MALIK, 1976). There is no cytoplasmic contact with the nongametophytic nucellus cells and thus the uptake of all nutrients must proceed through the cell walls and the cell membranes. The wall papillae in the apical pockets might function in that uptake of metabolites in the young endosperm and indeed soon after fertilization the majority of the endosperm cytoplasm is found there. Metabolites coming from the antipodals may also be essential to the early stages of endosperm development in grasses (BRINK

and COOPER, 1944). The papillate cell walls of the antipodals are probably functional in the movement of large amounts of nutritive materials from adjacent tissues. SCHNEPF (1964) demonstrated that cells which emerge secretory products have papillate cell walls, too. The cytoplasm of the antipodals exhibits a characteristic increase in organelle number and apparent activity after fertilization as was demonstrated both histochemically (SINGH and MALIK, 1976) and structurally (DIBOLL and LARSON, 1968).

In all, it appears that the cytological development in the cells of the megagametophytes show many similarities in the two varieties of *Zea mays*, both before and after fertilization. Principally some remarkable differences in the structure of the ovule and the position of the embryo sac characterize the two inbred lines. Up to now the functions of all the cell types of the megagametophyte can not be determined unambiguously. The subcellular organization, however, clearly indicates the most probable functions. Synergids and micropylar nucellus cells function in pollen tube attraction. The central cell and endosperm nourish the egg cell and young embryo respectively. The antipodals function in the synthesis and secretion of organic compounds made from precursors coming from the nucellus. Before fertilization the cells of the megagametophyte exhibit signs of moderate metabolic activity. This explains the various stages of degeneration of one synergid at the moment of fertilization. After fertilization the metabolic activity increases.

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Abbreviations in the figures

A = antipodals; CA = carpel; CC = central cell; CH = chromosome; CU = cuticle; CW = cell wall; CY = cytoplasm; DSY = degenerated synergid; D = dictyosome; EC = egg cell; EN = endosperm; ER = endoplasmic reticulum; ES = embryo sac; FA = filiform apparatus; II = inner integument; IS = intercellular space; L = lipid; M = mitochondrion; MP = micropyle; N = nucleus; NUC = nucellus; OI = outer integument; P = plastid; PD = plasmodesma; PN = polar nucleus; PSY = persistent synergid; PS = polysomes; PT = pollen tube; RER = rough endoplasmic reticulum; S = starch; SH = stigmatic hair; SI = silk; SY = synergid; V = vacuole; VE = vesicles; WP = wall protrusions; Z = zygote.

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CHAPTER 2

Embryogenesis in *Zea mays* L. Development of structure and polarity during proembryo formation

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EMBRYOGENESIS IN ZEA MAYS L.
DEVELOPMENT OF STRUCTURE AND POLARITY DURING PROEMBRYO FORMATION

ABSTRACT

Early stages (0-7 days after pollination) during the embryogenesis of Zea mays L. were investigated by light microscopy using semi-thin sections of Epon-embedded material. Attention was paid to the development of the proembryo upto the formation of the embryo proper. The polarity within the zygote, which is apparent from the distribution of cytoplasm and vacuoles, is maintained during the whole process of proembryo formation. Further, after an initial enlargement of the proembryo in the endosperm a shift in orientation takes place which strongly directs towards the inner integument. This shift probably is caused by a high activity of endosperm cells at the base of the embryo.

1. INTRODUCTION

Plant embryogenesis can be considered as a process of both formation of the proembryo and differentiation of the embryo proper. Within many plant species, the initial development of the proembryo passes along rather fixed patterns of cell division and orientation (see e.g. Maheswari, 1950). However, in other species such as Zea mays L. first cell divisions seem to lack a defined sequence and orientation although the eventual shape of the proembryo is nearly similar (Randolph, 1936).

In maize, differences in cell size and contents at the base and apex of the proembryo indicate a polarity in structure which maintains upto the initiation of the embryo proper. During its development the proembryo fully depends on nutrients from the endosperm. Moreover, the specific orientation in the endosperm and the shape of the proembryo also determine the morphogenesis of the embryo proper. Therefore, the structure and polarity of the developing proembryo of

Zea mays L. and its orientation in the endosperm were registered in order to get more insight into the initial development of the embryo proper (see also Van Lammeren and Kieft, 1982).

2. MATERIALS AND METHODS

Ovaries (Fig. 1a) of Zea mays L., strains A188 and Black Mexican Sweet (kindly provided by Dr. C.E. Green, Minnesota and Dr. R.J. Lambert, University of Illinois, U.S.A., respectively) were dissected in the greenhouse and fixed with 3% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.0. They were postfixed and stained with a saturated $KMnO_4$ solution in water for 5 min, at room temperature, dehydrated and embedded in Epon.

Sampling times varied from the moment of pollination upto 7 days after controlled pollination (DAP). Semi-thin sections were prepared with glass knives on an LKB 8800 Ultrotome. Drawings were made directly from the sections using a drawing tubus fixed onto a Wild phase-contrast microscope. Also, drawings were prepared from phase-contrast micrographs.

3. RESULTS AND DISCUSSION

As was reported previously (Van Lammeren, 1981), fertilization in Zea mays L. is preceded by the penetration of the pollen tube into the micropylar part of the nucellus and the discharge of its contents into the degenerating synergid. Prior to fertilization the egg nucleus and the majority of the cytoplasm are located near the micropylar side of the egg cell. After plasmogamy and karyogamy the situation is reversed, the cytoplasm and the nucleus being located near the apex of the zygote (Fig. 1b). The degenerated synergid, filled up with cytoplasm from the pollen tube, has a very thin cell wall near the top of the zygote. This might suggest that plasmogamy occurs at the apex of the egg cell.

At about 35 hrs after pollination the first mitosis results in a large basal and a small apical cell. The former has large vacuoles,

the latter is mainly filled with cytoplasm (Fig. 1c). Both cells divide several times but they do not elongate considerably after each division. However, the total amount of cytoplasm in the proembryo increases, most of it being situated in the upper part (Fig. 1d).

At about 3 DAP the proembryo is no longer globular but slightly elongated, mainly caused by division and elongation of cells at the base and mid-region of the embryo (Figs. 1d, e). At this time the endosperm is cellular and surrounds the embryo completely. Where the embryo penetrates the endosperm, cells of the latter are compressed and disappear. The penetration is strongly directed towards the inner integument as is shown in Fig. 1e. This orientation probably is caused by a high rate of division and elongation of endosperm cells at the opposite side (Fig. 1e, arrow head). This process continues for about 24 hrs; then, in the next few days, the proembryo grows straight on and prallel with the integument upto a length of about 500 μm (Figs. 1f-h).

As a result, the proembryo consists of a suspensor with large, highly vacuolated cells and an apical region with small cells containing few vacuoles. At the apical region the outer cells develop an epidermis by anticlinal cell divisions (Fig. 1h).

At about 7 DAP the apical region of the proembryo transforms into the embryo proper which is visualized in Fig. 1i. Some aspects of the development of this embryo proper are reported elsewhere (Van Lammeren and Kieft, 1983).

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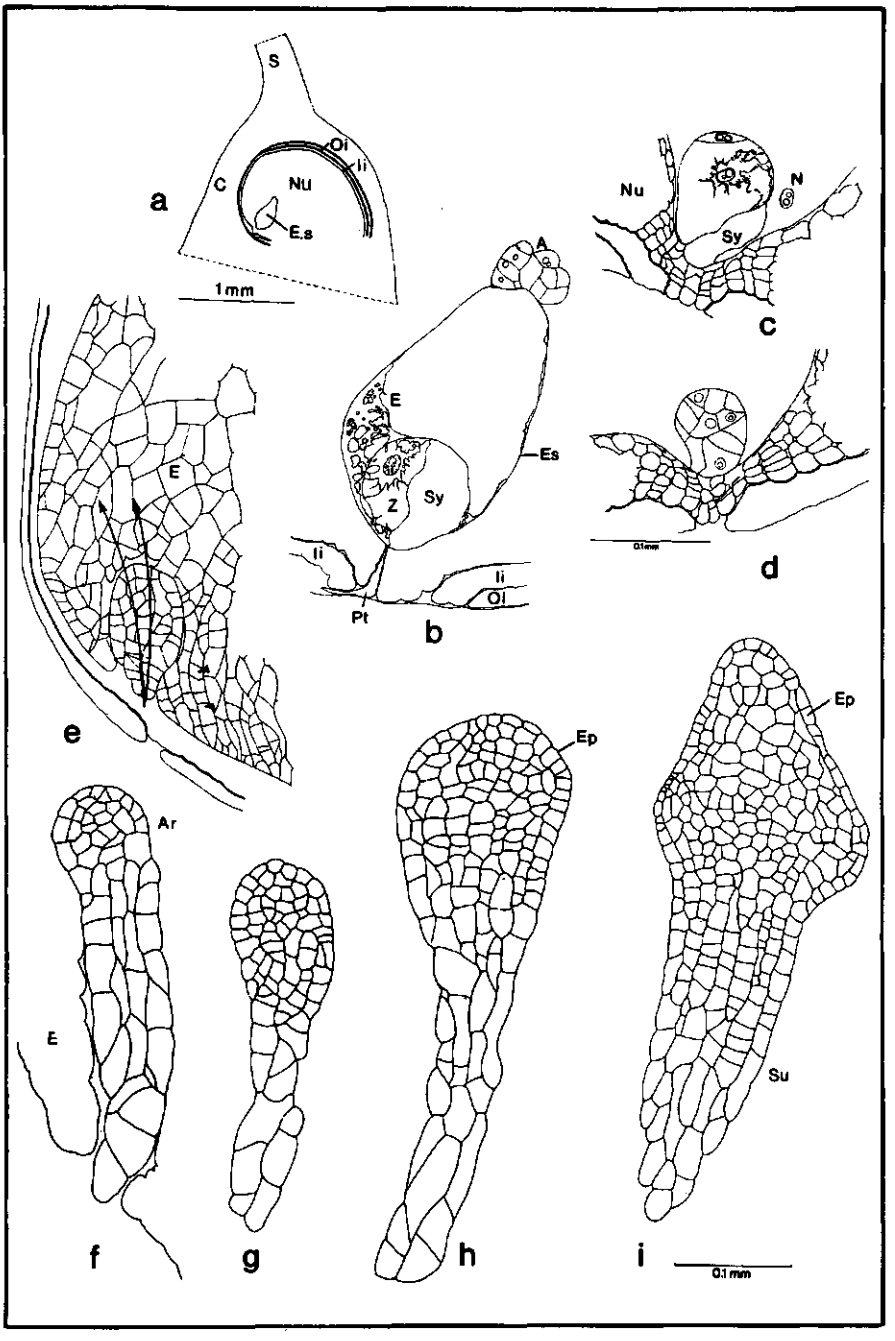
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Fig. 1. Early stages of embryogenesis in Zea mays. Abbreviations: A = antipodal cell; Ar = apical region; C = carpel, E = endosperm; Ep = epidermis: Es = embryo sac: Ii = inner integument; N = nucleus, Nu = nucellus: Oi = outer integument: Pt = pollen tube; S = silk; Su = suspensor, Sy = synergid, Z = zygote.

a. Sagittal section through an ovary with a mature embryo sac.

b. Fertilized embryo sac at 1 DAP. The cytoplasm in the zygote concentrates near the apex.

c. Two-cellular stage of the proembryo at about 1.5 DAP. There is a small apical and a large basal cell.

d. Late globular stage (about 3 DAP) with smaller cells near the top. The endosperm around the proembryo has become cellular.

e. Torpedo-like shape at 4 DAP. The proembryo is strongly directed towards the inner integument (arrow), probably caused by division and elongation of endosperm cells (arrow head).

f,g,h Elongated stages (5-6 DAP). The outer cells of the apical region develop into an epidermal layer.

i. At about 7 DAP the proembryo transforms into a suspensor and the embryo proper.

CHAPTER 3

Developmental morphology and cytology of the young maize embryo (*Zea mays* L.)

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DEVELOPMENTAL MORPHOLOGY AND CYTOLOGY OF THE YOUNG MAIZE EMBRYO (ZEA MAYS L.)

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SUMMARY

Young embryos of maize were analyzed with light and electron microscopical techniques to determine the sequence of morphological, anatomical and cytological events resulting in the onset and initial development of the embryo axis and the scutellum.

The proembryo stage ends at 5 days after pollination (DAP). The radial symmetry of the proembryo changes into a bilateral symmetry, probably caused by the excentric position of the embryo apex within the endosperm. Scutellum formation starts at the apex and posterior side of the embryo proper and is characterized cytologically by different types of cell growth and multiplication in epidermis and mesophyl. From anatomical observations it is concluded that the coleoptile arises as a protuberance of the scutellum.

Shoot meristem formation initiates in the protoderm at the anterior side of the embryo and is characterized cytologically by decreasing cell sizes, by vacuolation and by an increase of cytoplasm. The lateral orientation of the shoot meristem is likely caused by the failure of a second cotyledon to develop.

Root meristem formation is somewhat retarded. Only vacuolized cells at the base of the embryo proper were detected at 5 DAP but at 7 to 8 DAP the root meristem was identified as a group of dividing cells with few vacuoles and much cytoplasm. The meristem is not located at the exterior of the embryo but at the base of the embryo proper, in direct line with the suspensor. The coleorhiza is formed adjacent to the seminal root. Its exogenous character was not established.

The new axis from root to shoot meristem deviates from the axis of the proembryo as a result of the lateral position of the shoot meristem. Lateral outgrowth of the shoot is, however, restricted by mechanical forces from outside and growth of tissues inside the embryo.

In the determination of the location of the meristems next to morphological, also physiological and genetical factors are important. Metabolic gradients and physiological sinks are established already before the zygote divides and expression of polarity is present in all cormophyta.

1. INTRODUCTION

The sexual reproduction of maize has been the object of extensive genetical, physiological and structural research (SPRAGUE 1977, SHERIDAN 1982 and JOHRI 1984). Embryogenesis, which is a part of this process, starts with fertilization and includes both proembryo formation as well as development of the embryo proper. Questions related to developmental morphology made it necessary to visualize differentiation of the embryo with the aid of optical techniques. Many early workers studied fertilization and embryogenesis in maize by light microscopy (LM) (e.g. MILLER 1919; WEATHERWAX 1919, RANDOLPH 1936 and KIESSELBACH 1949). More recently, electron microscopical (EM) techniques have been applied by DIBOLL (1964, 1968), CHEBOTARU (1970), VAN LAMMEREN (1981, 1986) and SCHEL et al. (1984). Recent work on the embryo region of other grass

caryopses has been that of NORSTOG (1972) on barley and of SMART & O'BRIEN (1983) on wheat.

Morphological data on the embryogenesis of maize were reported by AVERY (1930), who compared maize with several other monocots, and by RANDOLPH (1936), who gives a detailed account of the developmental history of the caryopsis (see also SASS 1977). The first phase of maize embryo development is characterized by the formation of a proembryo in which the epidermis and the apical meristems of the root and the shoot are not yet differentiated.

Cytological studies of proembryo formation in maize have been reported recently (VAN LAMMEREN 1981, VAN LAMMEREN & SCHEL 1983). During the second phase of embryo development a scutellum and two apical meristems differentiate. Examinations on this phase, particularly those concerned with the organization of the system of primary meristems (protoderm, procambium and ground meristem) and of the apical meristem are rather limited in number in monocots and dicots (ARNOTT 1962, BUELL 1952, MILLER & WETMORE 1945, REEVE 1948, SHAH 1982, 1983 and VAN LAMMEREN & KIEFT 1983). In particular, the cytological aspects of primary meristem formation and scutellum development in maize, have only been investigated incidentally. However, the subsequent development of the maize shoot has not only been studied in embryos (AVERY 1930, RANDOLPH 1936, ABBE & STEIN 1954, CLOWES 1978b) but in seedlings as well (AVERY, 1930, ABBE et al. 1951. LEDIN 1954, CLOWES 1978b). ABBE et al. (1951) reported on the cytological aspects of cell proliferation in the shoot meristem and CLOWES (1978b) determined mitotic frequencies in the region of the embryo axis. The root growth in maize seedlings was investigated by, amongst others, CLOWES & JUNIPER (1964) and CLOWES (1978a) but little attention was paid to the initial development of the seminal root in the developing embryo. Moreover, a report on meristem initiation in which fine structure is combined with LM observations lacks, although it is felt to be of great value to compare the histogenesis in somatic embryos with the in vivo development (VASIL et al. 1985). The cytology of meristem formation in vivo was only scarcely studied in detail with EM techniques (VAN LAMMEREN & KIEFT 1983) and the following questions can still be posed. What determines the location of meristem formation? How do the developmental patterns of cells in a meristem proceed?

Therefore the present report focusses upon the morphological and fine structural changes during the onset of primary meristem formation to analyse the sequence of events taking place during this early phase of embryogenesis. The maize inbred line A188 is used because of its favourable response in experimental conditions for embryogenesis in vitro (GREEN & PHILLIPS 1975). Especially the cytology of the formation of the root and shoot meristems and the resulting embryo axis are analysed using light and electron microscopical techniques.

2. MATERIALS AND METHODS

Maize plants of strain A188 (kindly provided by Dr. C. E. Green, University

of Minnesota, St. Paul, USA) were grown under greenhouse conditions i.e. 16 hrs in the light at 23°C and 8 hrs in the dark at 18°C in a relative humidity of 70–90%. Sampling times varied from 5 up to 15 days after controlled pollination (DAP). For the cytological studies, ovaries were dissected and from each a thick sagittal section containing the median part of the embryo was fixed for 2 hrs in a solution of 5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.0) containing 0.1% $CaCl_2$. The sections were rinsed in the buffer and post-fixed with 1% OsO_4 in buffer for 2 hrs. Alternatively, they were fixed with a saturated, aqueous solution of $KMnO_4$ for 5 to 10 min., all at room temperature. Preparations were rinsed thoroughly in water and dehydrated in a graded series of ethanol. They were transferred to propylene oxid and embedded in Epon. Semi-thin and thin sagittal sections of the median part of the embryo were made using a LKB ultramicrotome equiped with glass and diamond knives respectively. Semithin sections (2 μm) were used for LM photography and 'camera lucida' drawings. Cell sizes were quantified with a Kontron MOP-30 by measuring cell areas on photos of semi-thin section. Thin sections were optionally poststained with uranyl acetate and lead citrate and observed with a Philips 301 EM at 60 kV.

For scanning electron microscopy (SEM), intact embryos were dissected from the caryopses and treated with various fixatives. The adapted method which was eventually used for embryo fixation, is given in the results. After dehydration in a graded series of ethanol, the ethanol was substituted by amylacetate and the embryos were critical point dried in a "Balzers Union" equipment, coated with gold in a "Polaron E 5100" sputter coater and examined in a JEOL ISM-35C scanning electron microscope.

3. RESULTS

3.1. Preparation of maize embryos for SEM

In order to study the embryo development by SEM, procedures for specimen preparation were evaluated (see also VAN LAMMEREN & MARTI 1983). Observa-

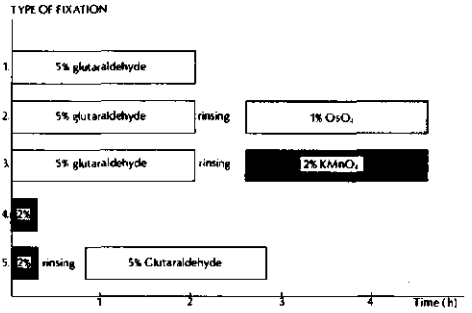

Fig. 1. Survey of 5 fixation procedures for scanning electron microscopical observation of immature embryos of *Zea mays* L. For additional information see Materials and Methods.

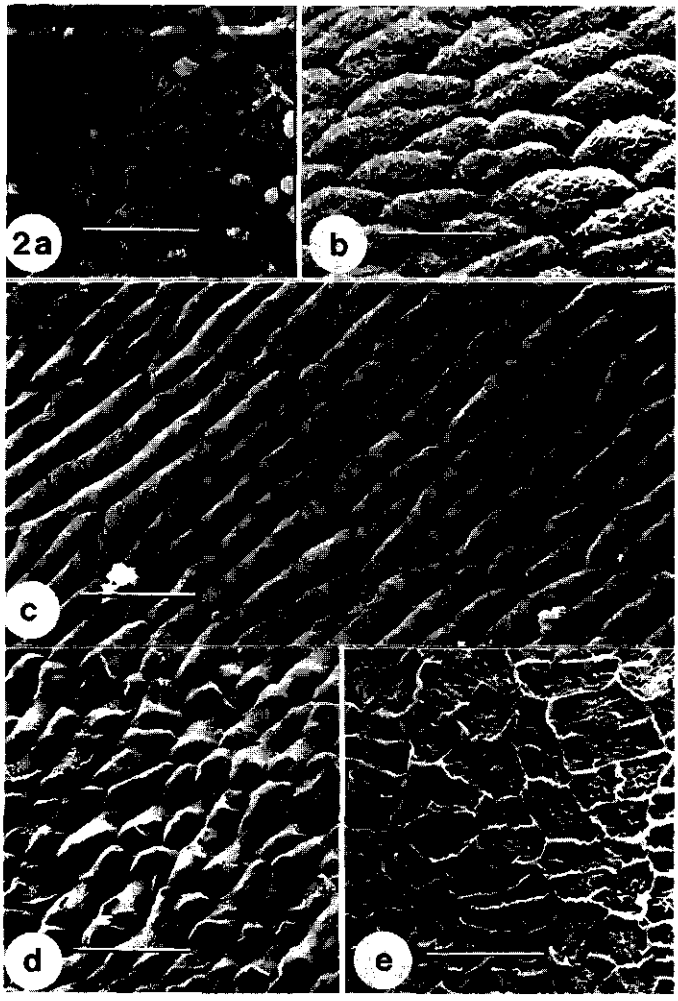

Fig. 2. Epidermal cells of maize embryos seen after the application of 5 different types of fixation. The micrographes a, b, c, d and e show the results of fixation types 1, 2, 3, 4 and 5 respectively (for fixation types 1–5, see *fig. 1*). Note the good quality of surface preservation with fixation type 3 (glutaraldehyde-$KMnO_4$). The bars represent 10 μm.

Abbreviations in the figures: C = coleoptile, CA = calyptra, CG = calyptrogen, CPD = critical point drying, cw = cell wall, cy = cytoplasm, D = dictyosome, DAP = days after pollination, EA = embryo axis, ER = endoplasmic reticulum, LP = leaf primordium (1, 2, 3), M = mitochondrion, N = nucleus, P = plastid, PB = peribleme, PD = plasmodesma, PL = plerome, PRD = protoderm, PS = polysome, RER = rough endoplasmic reticulum, RM = root meristem, S = starch, SC = scutellum, SM = shoot meristem, SN = scutellar node, SU = suspensor, V = vacuole, VE = vesicle.

tions on fresh material with SEM coincided with rapid distortion of the tissue and contamination of the electron microscope. The initial shape, however, appeared to be quite natural. Special attention was then paid to the effect of fixation on the shape of the epidermal cells. The various fixation procedures which were tested are shown in *fig. 1*. *Fig. 2* represents epidermal cells of maize embryos seen after various types of fixation. Fixation type 3 with glutaraldehyde and $KMnO_4$ gave best results with respect to the preservation of the cell shape (*fig. 2c*). Artificial shrinkage of the embryos was, however, caused by processing and was quantified (*fig. 3*). With fixation type 3 the critical point drying and especially the duration of the decompression phase appeared to influence the size of the embryos markedly. When a slow decompression was applied, shrinkage could be minimized, but not prevented. Best results were obtained with glutaraldhyde-$KMnO_4$ fixation and therefore this procedure was selected for the present study.

3.2. Morphology of the developing embryo proper

The morphological aspects of the transition of the proembryo to the embryo proper are shown in *fig. 4*. Proembryo formation took about five days in which a club-shaped embryo with a long cylindrical suspensor and a hemispherical apex reached a length of about 270 μm. The apex of the proembryo had a diameter of 100 μm. From about 5 DAP onward it gave rise to the embryo proper the side of which directed towards the ovary wall is called the anterior side.

The initial growth at the apex of the proembryo results in a trowel-shaped cotyledon, called the scutellum, on the epidermis of which the cell pattern indicates the direction of cell elongation (*figs. 4b* arrow and *5a1* arrow). Already at 6 DAP one can distinguish the region of the shoot meristem which is initiated

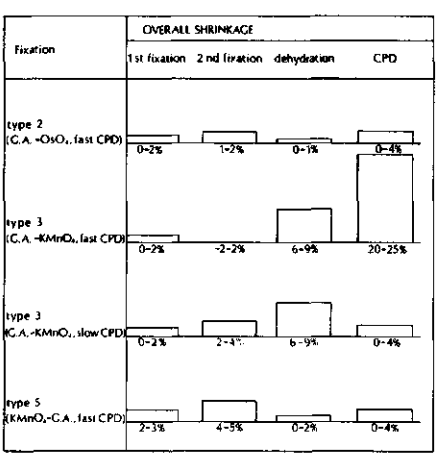

Fig. 3. Shrinkage of whole maize embryos as a result of various procedures of fixation, dehydration and critical point drying (CPD). For the types of fixation, see *fig. 1*. Fast CPD includes a gradual decompression from 77 to 1 bar in 12 min. Slow CPD includes a gradual pressure decrease from 77 to 65 bar in 30 min. followed by a decompression to 1 bar in 7 min.

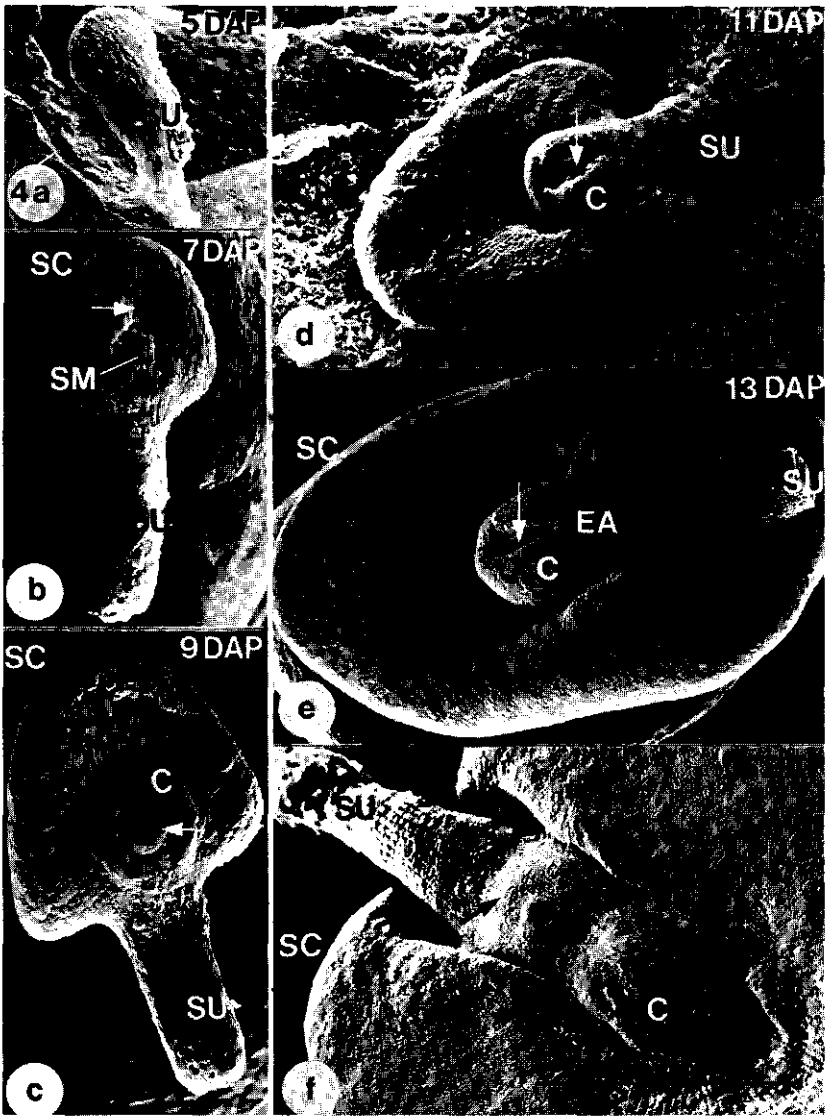

Fig. 4. Morphology of the developing maize embryos from 5 up to 15 days after pollination (DAP). The adaxial side of the scutellum is faced in every picture. The development of the proembryo (a) to a well differentiated embryo (f) is marked by the initiation (b) and rapid enlargement of the scutellum and the concommitant exogenous formation of the shoot meristem. Note the differentiation of the coleoptile from 7 DAP and the formation of the first leaf primordium from 9 DAP (c, d and e, white arrows). Details of b, c and e are given in *figs. 5b1, 5c1* and *6b* respectively. The bar represents 100 μm for all pictures.

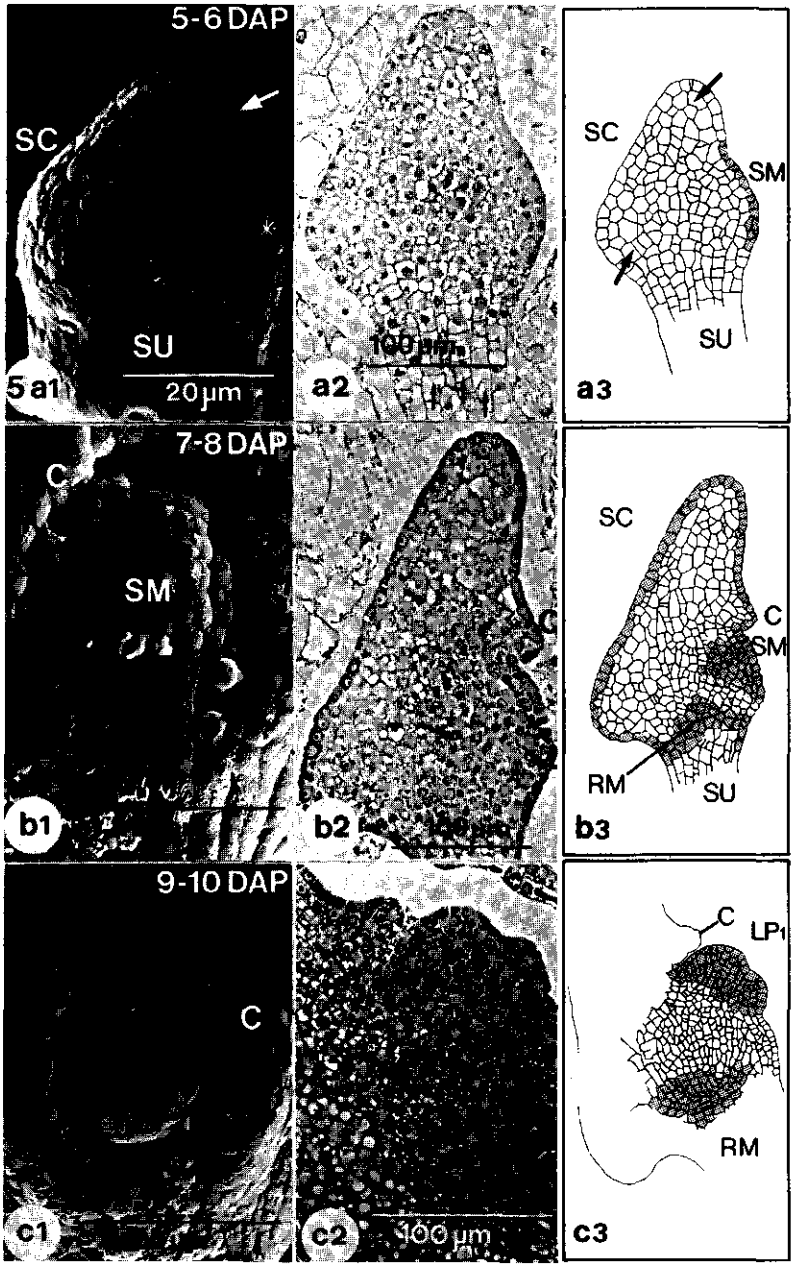

Fig. 5. The developing embryo proper of *Zea mays* L. from 5 to 10 days after pollination. The SEM pictures show the appearance of the shoot meristem. In the longitudinal sections the anterior side of the scutellum is directed to the right. Note the development of the shoot meristem and coleoptile in b and the appearance of the root meristem in c. The stippled areas in the drawings accentuate the regions where cells have a meristematic appearance.

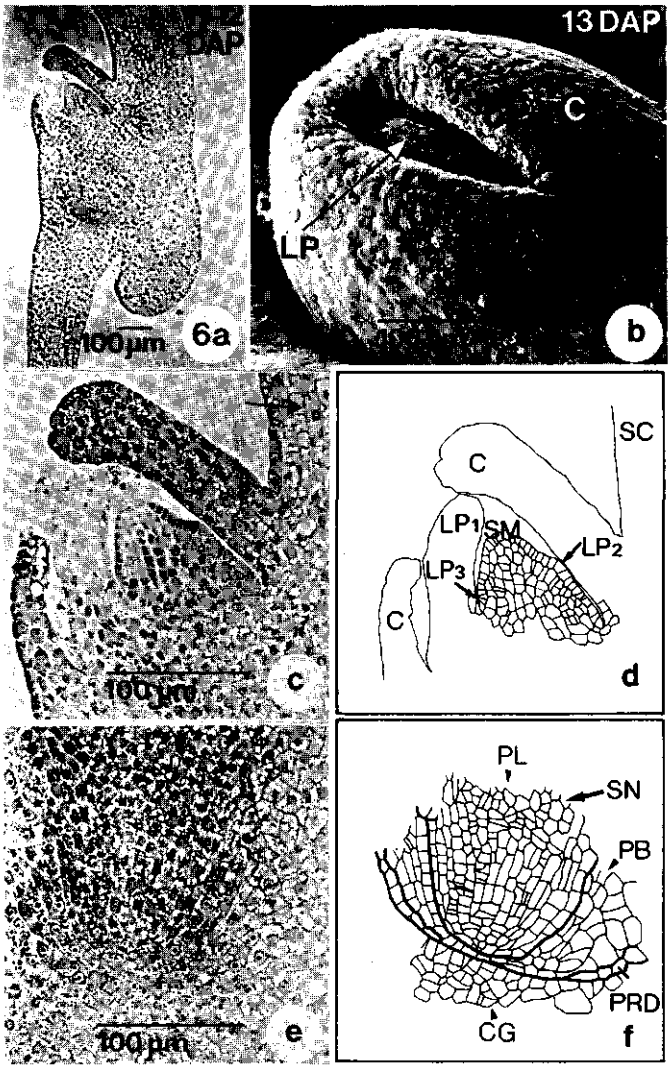

Fig. 6. Longitudinal sections and drawings of a maize embryo at 11 to 12 DAP show the anatomical differentiation of the shoot and the root meristems. The SEM micrograph presents the face view of the shoot of a 13 DAP embryo which is, however, in the same stage of development.

as a small protuberance consisting of relatively small cells at the anterior side near the apex of the embryo (*figs. 4b* and *5a1*, asterisk). Enlargment and elongation of the scutellum continues and the promeristem of the shoot is clearly demarcated by a furrow at 7 DAP (*figs. 4b* and *5b1*). Because of that furrow the shoot meristem will get a hemispherical shape as is shown in *fig. 9a*. From 9 up to 12 DAP the scutellum still enlarges and the coleoptile which is generated

as a ridge of tissue surrounding the shoot meristem envelopes the whole meristem eventually (see *figs. 4 c–e* and *fig. 6b*). Starting at 13 DAP and clearly visible at 15 DAP the lateral sides of the scutellum enlarge and partly overgrow and enclose the embryo axis (*fig. 4e, f*).

At about 9 DAP the shoot meristem itself initiates the development of the first leaf primordium at circa 40 μm from its tip (*fig. 4c*, arrow). In the following period of development the first leaf overgrows the apex of the shoot meristem (*fig. 4d, e*, arrows). The leaf itself is then enclosed by the differentiated coleoptile.

The root meristem is not located at the exterior of the embryo and hence it can not be detected morphologically. The embryo axis, consisting of the two meristems and the tissue in between, elongates and can be discerned from about 13 DAP. The root meristem lies in direct line with the suspensor and it can be noted as a small swelling at the anterior side as indicated in *fig. 4f*, arrow.

3.3. Cytology of the developing embryo proper

The transition of the proembryo to the embryo proper is shown in *fig. 5* and *6* and *table 1* with respect to overall shape and size of the embryo and to location, size and contents of the composing cells. The development of the scutellum, its coleoptile and the embryo axis will be treated hereafter.

Elongation of the suspensor is the dominating feature of proembryo formation. Sub-apical cells divide once or more and elongate, creating regular rows of cells in which new cell walls are perpendicularly arranged to the long axis of the suspensor. However, at a length of about 500 μm the proembryo changes shape. The growth of the suspensor decreases considerably and only some cells in the upper part of the suspensor still divide once or more. Then, the spherical apex of the club-shaped proembryo transforms into the embryo proper by the formation of the trowel-shaped scutellum primordium and the promeristem of the shoot of an embryo at 6–7 DAP.

3.3.1. Development of scutellum and coleoptile

Scutellum formation is initiated at the abaxial side of the hemispherical apex

Table 1. Cell sizes in scutellum and apical meristems of maize embryos at various stages of development. The cell areas are expressed in μm^2. The standard deviations are high because many cells are not sectioned at their maximal width and length. Number of counted cells is 10–25 for each value. (1) anterior side of scutellum, (2) posterior side of scutellum, (3) peribleme, (4) plerome.

Stage (DAP)	Scutellum apex		Shoot Meristem		Root Meristem
	Epdermis	Mesophyl	Protoderm	Inner cells	
5– 6	175 ± 77	253 ± 61	81 ± 15	149 ± 25	156 ± 56
7– 8	97 ± 36	139 ± 63	57 ± 19	56 ± 25	82 ± 27
9–10	67 ± 40 (1) 147 ± 55 (2)	380 ± 146	41 ± 16	52 ± 25	62 ± 27
11–12	69 ± 7 (1) 155 ± 62 (2)	342 ± 80	38 ± 11	60 ± 23	111 ± 32 (3) 94 ± 49 (4)

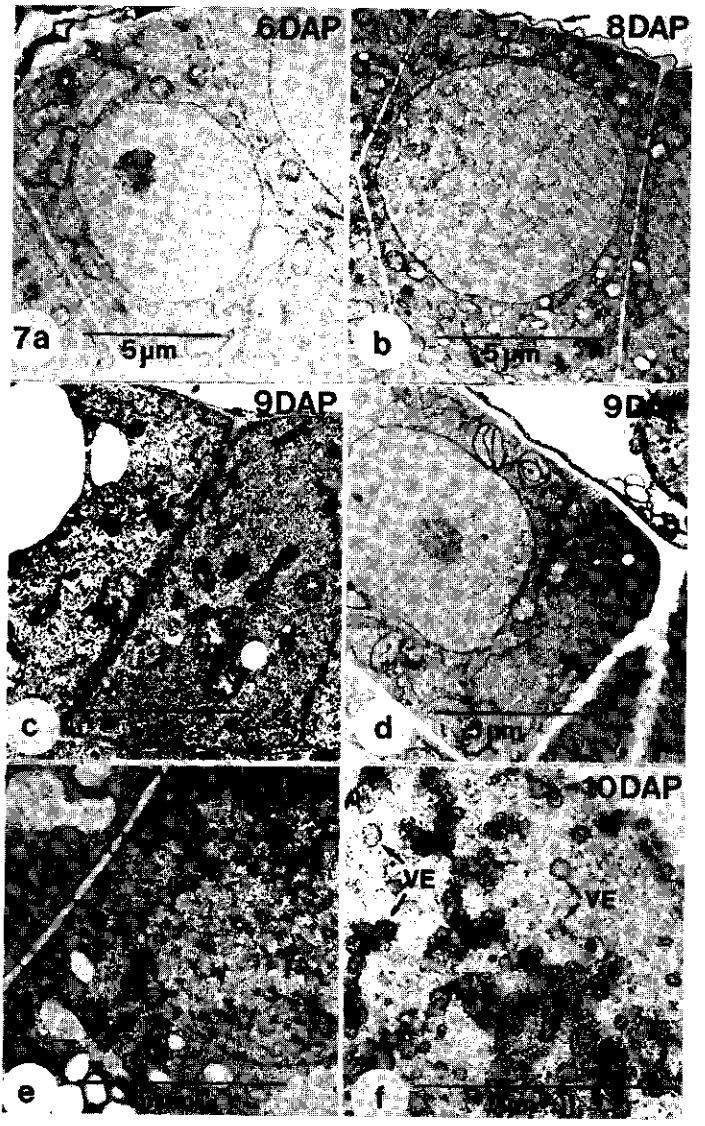

Fig. 7. Fine structure of epidermal cells of the scutellum of immature embryos of maize at 6, 8, 9 and 10 days after pollination. Spherical nuclei occupy a large part of the cell volume. Fixation with $KMnO_4$ reveals the existence of various membranous organelles (a, b, d, e, f) whereas numerous ribosomes are detected after OsO_4 fixation (c). a, b, c, e are cells at the anterior side, d up to f are cells at the posterior side.

of the proembryo. Already at 6 DAP three zones were distinguished anatomically in the young scutellum; the adaxial protoderm, the mesophyl and the abaxial protoderm. The protoderm is the primary meristematic tissue that give rise to the epidermis. The *figs. 5a2* and *5a3* show the scutellum primordium in a

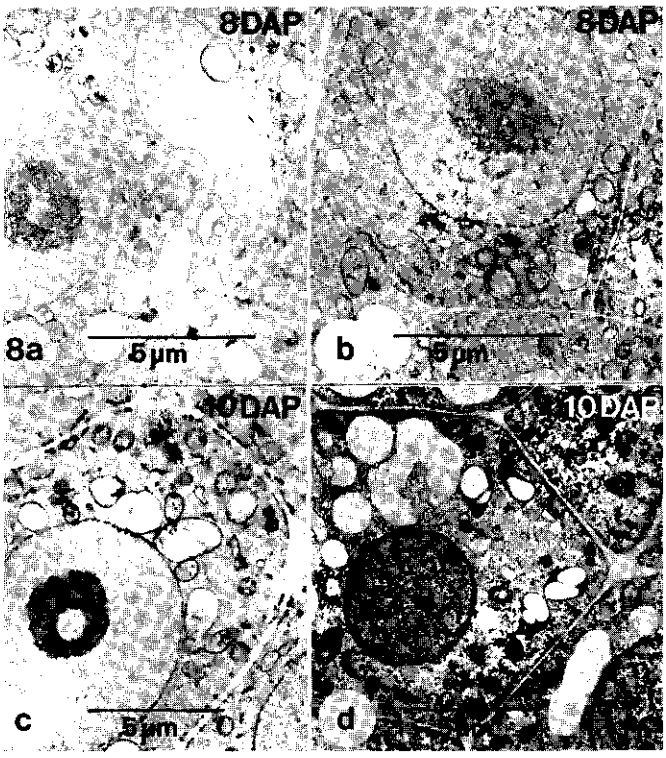

Fig. 8. Fine structure of inner cells of the embryo proper of *Zea mays* L. at 8 and 10 days after pollination after glutaraldehyde-$KMnO_4$ (a, b, c) or glutaraldehyde-OsO_4 (d) fixation. Cells a, c and d are located in between the root and shoot meristem. Cell b takes part of the root meristem. Note the presence of amyloplasts at 10 DAP.

median section at this stage. The stippled area accentuates the region where cells have a meristematic appearance. In the protoderm anticlinal planes of cell division were detected preferentially at this stage. As compared with the region of the shoot meristem, the initial enlargement of the scutellum apex is caused by cell stretching rather than by an increase of cell number (see *fig. 5a3* arrows). The subsequent elongation of the scutellum, which can clearly be seen at 8 DAP (*fig. 5b*), is, however, mainly caused by growth and multiplication of cells in the upper part of the scutellum. The new cell walls are often perpendicular to the long axis of the scutellum and during further development the processes of cell division and cell elongation resulted in regular rows of cells in the epidermis and mesophyl (see *fig. 6c* arrow).

Epidermal and mesophyl cells appeared to differ in structure from the onset of development. Some epidermal cells of the developing embryo are shown in *fig. 7*. From 6 DAP onward the greater part of the cell volume of these relatively small cells is occupied by a large nucleus and the cytoplasm. The nucleus has a central position and the other organelles are spread over the cytoplasm. Mito-

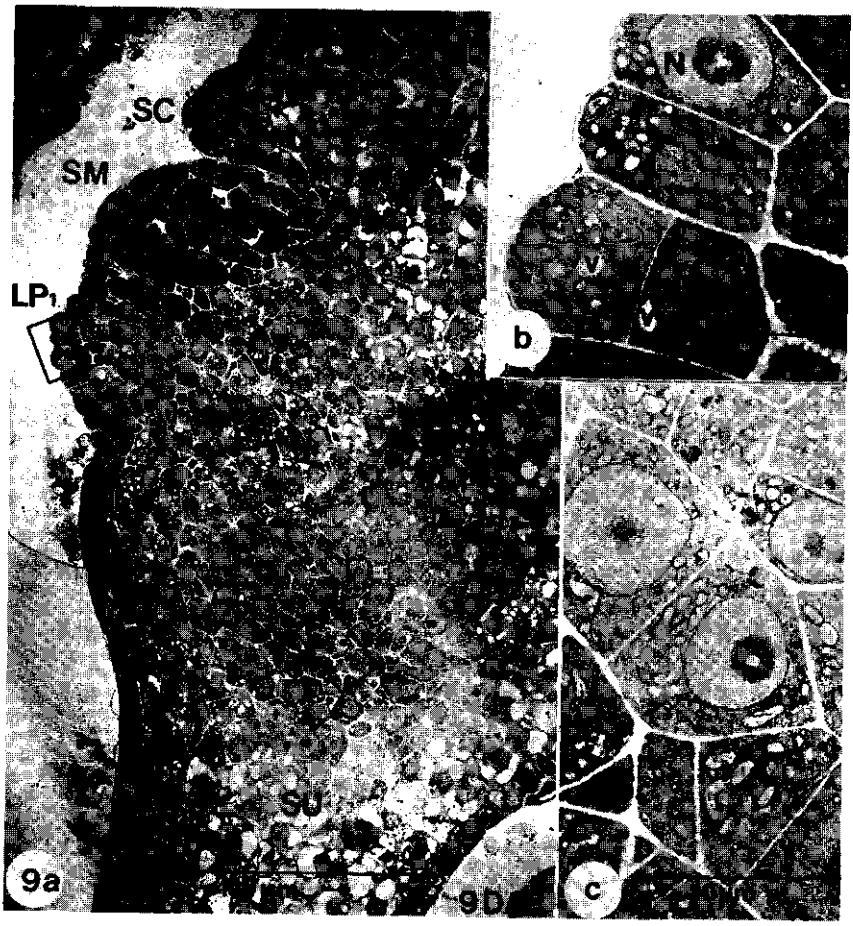

Fig. 9. Meristem formation in the embryo proper of *Zea mays* L. at 9 DAP. The root meristem develops endogenous at the base of the embryo proper in direct line with the suspensor. The shoot meristem develops at the outside with an angle of about 40 degrees to the long axis of the embryo proper. Note the high degree of vacuolation in suspensor, scutellum and embryo axis. The meristematic cells of the leaf primordium (inset b) have less vacuoles and the cells of the developing root meristem (inset c) contain few vacuoles, too. Note the periclinal division in the protoderm.

chondria and plastids have comparable sizes and can be spherical or elongated. Starch was not found in these cells. Few vacuoles and some strands of RER are scattered throughout the cytoplasm but polysomes occurred frequently as shown in OsO_4-fixated material (*fig. 7c*). Only few cytological changes were observed in the subsequent days. A cuticle was already formed at 8 DAP (*fig. 7b*, arrow) and at 10 DAP, epidermal cells slightly increased the number of dictyosome vesicles (*fig. 7f*). Most mesophyl cells are larger than the epidermal cells (*table 1*). They tend to vacuolize in the apical and abaxial zones of the scutellum (see *fig. 5b2*) and they form regular rows in the elongating part of the scutellum as indicated by arrows in *fig. 9a*.

Near the shoot meristem and in direct line with the suspensor, cells are relatively small and contain only few vacuoles. These cells will take part in the formation of the embryo axis (*figs. 5b2* and *9a*) and do not belong to the scutellum.

Coleoptile formation started at 7 DAP in the epidermal and subepidermal scutellum cells which border the shoot meristem (*fig. 5b*). The epidermal cells divided anticlinally and subepidermal cells divided in a restricted zone and formed new cell walls perpendicular to the direction of cell growth. At 9 DAP the division and enlargement in distinct directions gave rise to a ridge of tissue, the coleoptile primordium (*figs. 5c* and *9a*). The coleoptile developed fast and at 12 DAP it was an elongated, leafy structure that partly enveloped the shoot meristem and the developing primary leaves (*fig. 6*). Its epidermis was still connected with the scutellum epidermis and its mesophyl, which was 3 to 4 cell layers thick, was linked up with the subepidermal scutellum cells.

3.3.2. Development of the embryo axis

When the trowel-shaped scutellum primorium is formed at 5 to 6 DAP there is no random distribution of cell divisions in the embryo proper. Most mitoses were found in and near the protoderm at the anterior side at about 100 μm from the apex as is indicated with the stippled area in *fig. 5a3*. This appears to be the location where the shoot meristem will differentiate. A typical cell of that region is depicted in *fig. 7a*. Its ultrastructure points to an organization which is common for a meristem. The epidermal cells had large nuclei, small proplastids which never accumulated starch, only few vacuoles and thin cell walls. The subepidermal cells in that region were larger (*table 1*), contained many more vacuoles and were about isodiametric (*fig. 5a2*).

At 7 DAP the location of the shoot meristem was marked by the indentation and the coleoptile formation (*fig. 5b*). The epidermal cells of the meristem region still had the meristematic appearance like in the protoderm of the proembryo. In the proembryo, however, the outer cells of the apical region divided periclinally as well as anticlinally but as soon as the coleoptile primordium was formed, the epidermal cells of the meristem gave rise to a cell layer in which periclinal divisions were not encountered anymore. Subepidermal cells dedifferentiated from 6–8 DAP. The amount of cytoplasm increased, cells divided and formed smaller cells (*table 1*) which took part in the formation of the shoot meristem.

In direct line with the suspensor a group of cells at the base of the embryo proper dedifferentiated, too (*fig. 5b*). These cells are the forerunners of the root meristem. Initially they were large (*table 1*), isodiametric and contained many vacuoles. Thereafter the vacuoles disappear and the cells divide once or more. In the cells close to the suspensor, the orientation of the new cell walls is mainly parallel to the axis of that suspensor. From these relatively large cells the calyptra develops. Cells which are adjacent to them at the side of the embryo proper are smaller and will give rise to the other constituents of the root meristem (*fig. 5c*). A typical cell of the root meristem at 8 DAP is shown in *fig. 8b*. The regions of the shoot meristem and the root meristem are separated by a zone of cells which have a higher degree of vacuolation (*figs. 5b2*, arrows and *8a*) as compared

to the cells of the meristems. This zone will form the scutellar node of the embryo axis (see *fig. 6f*).

At 9 DAP the shoot meristem was hemispherical and it consisted of many small epidermal and subepidermal cells (*table 1*) which still had large nuclei, much cytoplasm but hardly any vacuoles. The first leaf primoridum is formed at its anterior side (see *figs. 5c* and *9*). The ultrastructure of some epidermal cells of the primordium is shown in *fig. 9b*. Except for the tendency of increasing vacuolation there is no striking difference in appearance of these cells and the protoderm cells of the shoot meristem. However, periclinal divisions in the outer cells, as depicted in *fig. 9b*, were only detected in leaf primordia and not in the protoderm of the meristem. In the zone between the meristems the vacuolized cells clearly enlarged and elongated by which the distance between the meristems increased. The cells of the middle zone of the embryo axis accumulated starch and contained many ribosomes (*fig. 8c, d*). Moreover, cells which were formed by the root meristem, elongated, too, and thus contributed to the growth of the embryo axis as well (*fig. 9a*). At 9 DAP one can not yet clearly distinguish the various histogenes within the root meristem, because the differentiation is not that far already (see *fig. 9a* and *c*).

The developmental stage of the embryo axis at 12 DAP is shown in *fig. 6*. The shoot meristem is enveloped by the coleoptile. The first leaf primordium (LP_1) had elongated and a second leaf primordium (LP_2) had generated at the opposite side of the meristem. The location of the third leaf primordium (LP_3) is marked in *fig. 6d*. The shape of the shoot meristem has changed from hemispherical to pointed but the ultrastructure of its cells is comparable to that in the previous stages. Now the root meristem is composed of well distinguisable tissues as is indicated in *fig. 6f*. One tier of meristematic cells forms the peribleme. A central group of cells forms the plerome (see *fig. 6f*) and a cluster of cells forms the root cap. Some root formation was observed too. Root formation at 13 DAP is based on an increase of cell size (*table 1*) and cell number.

During the development from 6 up to 13 DAP the orientation of the shoot meristem changed. With reference to the future root meristem or the axis of the suspensor, the shoot meristem was found at an angle of 90° at 5 DAP. Gradually this angle changed to about 105° at 8 DAP and to about 145° at 10 and 11 DAP. The change of angle is caused by several factors. The wall of the caryopsis exerts a mechanical force on the embryo. This restricts the lateral development when the embryo axis widens. The development of the primary leaf (LP_1) restricts the direction of growth of the shoot meristem as well and when the scutellar node develops, the elongation and multiplication of cells at the anterior side of the embryo axis is accelerated as compared to the opposite side.

4. DISCUSSION

In this report the developmental stages of embryos are related to the number of days after pollination (DAP). The real age of the embryos is about one day

less because that is the time the programic phase takes. Comparing embryos of different ages is only valid when the embryo-bearing plants are grown under identical conditions. Physical conditions such as humidity, temperature, light intensity and duration of irradiation strongly influence the rate of embryogenesis. The greenhouse conditions applied in these experiments probably caused the faster embryo development as compared to the descriptions of AVERY (1930), RANDOPH (1936), KIESSELBACH (1949) and ABBE & STEIN 1954) who studied embryogenesis under field conditions.

4.1. Development of bilateral symmetry and of the single cotyledon

During the differentiation of the embryo proper we questioned what determines the changes of radial symmetry to bilateral symmetry and what determines the locations of the apical meristems and the scutellum. In a study of grass embryogenesis SOUÈGES (1924) found evidence that the origin of organs in Poaceae could be assigned to specific tiers in the young embryo. Like RANDOLPH (1936) we were not able to distinguish such specific tiers in the embryo of maize whatever stage we observed.

In a previous report it was noticed that during the elongation of the proembryo there is a curving growth of the proembryo when it protrudes the endosperm at 4 and 5 DAP (VAN LAMMEREN & SCHEL 1983). During this developmental stage the apex of the proembryo nears the endosperm epidermis. Because of its new position in the endosperm the embryo apex is encompassed by endosperm cells that are different among themselves: small epidermal cells are found at the anterior side of the embryo apex and larger inner cells covered the rest of the embryo apex. With reference to the club-shaped proembryo, which itself has a radial symmetry, the endosperm environment has a bilateral symmetry by which that embryo is influenced directly. Exogenous influences like metabolites, minerals, osmotic values or electric potentials (RYCZKOWSKI et al. 1985) might stimulate the embryo to develop a bilateral symmetry which is first expressed by the location of the developing cotyledon that develops into the scutellum.

If a strict bilateral symmetry in the embryo proper exists, the scutellum primordium can only be initiated in the median plane of the embryo which coincides with the median plane of the ovule. Thus a two-ranked leaf sequence can develop. In fact, in many monocots and dicots, e.g. *Triticum*, *Capsella* and *Ricinus*, the attachment of the cotyledons on the embryo axis lies in the median plane of the ovule as well. The epiblast which is found in several monocots is then interpreted as the second but rudimentary cotyledon (see NEGBI & KOLLER 1962). In maize there is no epiblast and the remaining cotyl, the scutellum, is appressed to the inner endosperm from which it will be supplied with nutrients. In dicots the shoot meristem is located at the apex of the embryo and the two cotyledons differentiate lateral. When the cotyledons elongate, the differentiation and growth in the shoot meristem is greatly retarded. In monocots, scutellum formation precedes meristem activation as well. The single cotyledon in

monocotyledons sometimes appears terminal and the apical meristem lateral. This situation was observed in maize, too.

The relation of the single cotyledon of monocotyledons to the apex of the embryo is discussed by ESAU (1965, 1977) as a matter of controversy (see also BROWN 1960 for references). According to one view the cotyledon is terminal in origin, the shoot apex is lateral, and the whole plant is a sympodium of lateral shoots (SOUÈGES 1954 and GUIGNARD 1975). Other authors consider the terminal positions of the cotyledons to be only apparent: the lateral position of the apical meristem results from its displacement by the cotyledon (HACCIUS 1952 and BAUDE 1956). Evidence from dicotyledons which develop only one cotyledon supports the view that the cotyledon is a lateral structure. It has been suggested that several species of the monocotyledon *Dioscorea* have two cotyledons, one of which has an absorbing function and remains within the endosperm while the other emerges and functions as a leaf (LAWTON & LAWTON 1967). In the embryo of wheat there is still a rudiment of the second cotyl and one finds the shoot meristem in between that epiblast and the scutellum. It might be argued that the longitudinal symmetry, which is found in proembryos of monocots and dicots and in embryos of dicots, is disturbed in monocots because of the absence or rudimentary development of the second cotyledon. So the lateral position of the apex is caused by the unbalance in development of the two cotyledons. This is already expressed during early embryo development in the lateral location of the shoot meristem due to development and enlargement of only one cotyledon. It is remarkable, however unexplained, that the ontogenesis of the ovary, the ovule and the embryo all lead to bilateral symmetry. With respect to the spikelet axis, the adaxial side of the ovary grows faster resulting in a lateral position of the silk. In the ovule the unequal growth results in the semi-anatropous position. Even the megagametophyte sometimes grows towards the abaxial side as well, and in the embryo the lateral position of the meristem and the scutellum enlargement point to the same phenomena.

There has been much controversy about the status of the coleoptile. Some interpret the coleoptile as the first leaf (GUIGNARD 1961), and BROWN (1960) assumes that the coleoptile is new aqcuisition without homologues in other embryos. We regard the coleoptile as a protuberance of the scutellum, the scutellar sheath, rather than that it would be a product of the apical meristem as was suggested by amongst others PANKOW & VON GUTTENBERG (1957).

4.2. Development of the embryo axis

The formation of the shoot meristem starts soon after the development of the protoderm which is generated by periclinal divisions in the upper part of the proembryo. Protoderm formation is often regarded as the termination of the proembryo stage (see ESAU 1977) and at the anterior side of the proembryo it gradually passes into shoot meristem formation. The first cytological features of the shoot meristem formation in epidermal and subepidermal cells are the enlargement of the nucleus, the increase in amount of cytoplasm and dictyosomes, and the decrease of vacuolation. Subsequently the cells divide in deter-

mined directions e.g. the anticlinal divisions in the epidermal cells which form the tunica. CLOWES (1978b) studied the development of the shoot apex of maize by stathmokinesis and DNA labeling to find rates of cell proliferation in the regions of the developing shoot apex. It was found that the tunica which contains about half the cells in the apex, contributes a decreasing fraction of the total cell production as the embryo ages. This finding is in agreement with our observations that meristem formation first means a substantial enlargement of the meristem by an increase in cell number in corpus and tunica. Thereafter the meristem enlarged only slowly, it became pointed and cells with a meristematic appearance were predominantly found at its base where leaf primordia were formed. Between the developing meristems of the root and the shoot of 7 DAP embryos we observed cells which neither belong to the meristems nor to the scutellum. They form a procambial plate which is held to be the first node of the young plant (WARDLAW 1955). Because of their specific cytological differentiation we suggest that they already function in the transport of metabolites in an early phase of embryogenesis.

The development of primary roots in lower vascular plants is initiated exogenously in the protoderm. The radicula is a strictly lateral organ with respect to the main vertical axis but its place and time of development is variable (FOSTER & GIFFORD 1959 and VON GUTTENBERG 1964). In dicotyledons, primary root development is exogenously, too. A dermacalyptrogen, which forms the rhizodermis and the calyptra, develops directly from the protoderm of the proembryo. Most monocotyledons have a real radicula, as well, because it was inititated in the protoderm of the proembryo. They have, however, a secundary dermatogen which is descended from the root peribleme. In the Gramineae the seminal root is not a primary root. The extant root meristem develops endogenously as is regular for adventitious roots. The coleorhiza is often considered to be the rudiment of that primary root (VON GUTTENBERG 1964) but we could not establish the exogenous character of the coleorhiza.

In most seed plants the first root is found at a fixed location in the basal part of the embryo proper, in line with the suspensor. With respect to that fixed root position, VON GUTTENBERG (1964) argues that embryos of seed plants are bipolar without exception. The basipolar position of the root might facilitate germination. The identical position of the adventitious root in the Gramineae embryo strengthens this explanation. MCCALL (1934) stresses that the positional relationships of organs in adult plants and in embryos are essentially similar. The axial polarity from shoot apex to root apex often coincides with the polarity of the proembryo. Thus it is important to understand the polarity development in proembryos. Like in the majority of the archegoniate plants, the apex of the young seed plant embryo faces inwardly towards the gametophytic tissue of the embryo sac and away from the neck of the archegonium. This is called endoscopic polarity (FOSTER & GIFFORD 1959). In the proembryo of maize it is already established before the zygote divides (VAN LAMMEREN 1986) and probably it is maintained by metabolic gradients during proembryo development. According to amongst others WARDLAW (1968) and SCHEL et al. (1984) it may reasona-

bly inferred from visual evidence that nutrients are mainly taken up by the tissues in the basal region of the proembryo and are translocated to the apex. Cells in the apex are specially active, divide repeatedly and so constitute a "physiological sink" to which a general flow of metabolic materials is directed.

The influence of the nutrition flow towards and within the embryo proper can be considered as an important morphogenic factor. As long as the suspensor elongates, it brings the embryo proper in intimate contact with the endosperm. Soon the anterior side of the embryo proper is but covered by a persisting endosperm epidermis whereas the developing scutellum is in contact with the degenerating inner endosperm cells. Hence the uptake of nutrients will be highest at the posterior or scutellar side and nutrients will be transported to the "physiological sinks". During further development the scutellum enlarges fast at the cost of the endosperm at its posterior side. Moreover the uptake by and transport of nutrients in the embryo proper can be deduced from the cytological features observed such as the vacuolation and elongation of the future transport elements in the scutellum and in the embryo axis, and the accumulation of starch and the differentiation of vascular elements in several regions.

The cause of the precise location of root meristem formation on the transition region of the embryo proper and suspensor remains to be elucidated. In most embryos of seed plants the bipolarity of the proembryo remains unchanged in the embryo proper because of the basipolar location of the root meristem. Growth regulators already generated in the shoot apex at the proembryo stage and transported basipetally might evoke the very initiation.

In conclusion it can be stated that there is an ordered sequence of events resulting in the initiation and activation of the meristems and the development of the scutellum. Vacuolation and starch accumulation are early cellular features of differentiation whereas an increase of cytoplasm a decrease of cell size and vacuolation precede meristem formation. The endosperm environment of the embryo can initiate the shift to the bilateral symmetry. The failure of the second cotyledon to develop, causes an apparent change of the orientation of the shoot meristem. The coleoptile and the coleorhiza are not considered to be parts generated by the eventual shoot and root meristems. The former is considered to be a part of the scutellum, the latter probably the rudimentary radicula.

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CHAPTER 4

Structural development of excised ovaries and embryos of maize (*Zea mays* L.) cultured in vitro

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(submitted for publication)

STRUCTURAL DEVELOPMENT OF EXCISED OVARIES AND EMBRYOS OF MAIZE (ZEA MAYS L.) CULTURED IN VITRO

SUMMARY

The morphological and cytological aspects of embryogenesis, premature germination, callus formation and plant regeneration in vitro were investigated by light and electron microscopy. Embryo development was induced either by in vivo pollination or by the in vitro pollination of excised pistils. It was found that the in vivo pollination results in the highest fertilization rates. Pollination in vitro was most effectively when the pollen attachment on silks is close to the ovary.

The in vitro development of excised immature embryos is strongly influenced by the composition of the nutrient medium, the presence of endosperm, the orientation on the medium and the genotype of the embryo. In fluid media containing 2,4D, the embryos of the inbred strains A188 and BMS gave rise to suspended cells which were formed by root tip-like aggregates. Cell divisions were not observed in the suspended cells. Culture on solidified medium containing 2,4D resulted in e.g. local cell degeneration, differentiation of chlorenchyma,collenchyma and vascular tissue and in cell multiplication, especially in the coleorhizal end of the scutellum. Callus formation and generation of adventitious shoots was observed, provided the axis side of the embryo is in contact with the medium. Regeneration capacity of strain A188 is highest. Up to 100% of the embryos regenerated up to 15 adventitious shoots each. Germination of immature embryos and maturation were achieved from 9 days after pollination onwards, provided the scutellum was in contact with the nutrient medium.

1. INTRODUCTION

Seed formation is a developmental process in which embryo growth, organ differentiation and the interaction between embryo and endosperm are of major importance. In vitro techniques are often

applied when deficiencies in development lead to low seed setting or embryo abortion. In vitro pollination often overcomes incompatibility barriers (Gengenbach 1977) and the in vitro culture of immature sexual embryos prevents embryo abortion in hybrid seed formation when endosperm development retards or fails (Sheridan et al. 1978). Moreover, in vitro embryo culture provides a tool for the initiation of callus cultures which might give rise to somatic embryos or adventitious roots and shoots (Street 1977, Sheridan 1982, Evans et al. 1983, Vasil 1984).

With maize, the culture of excised immature embryos resulted in callus formation and the development of somatic embryos (see, amongst others, Green & Phillips (1975), Vasil et al. (1983, 1984), Duncan et al. (1985)). The formation of callus and somatic embryos from protoplasts and cell suspensions has been realized, too (Potrykus et al. 1977, 1979, Vasil 1982, Green et al.1983, Imbrie-Milligan & Hodges 1986) The structural aspects of the development of maize embryos in vitro e.g. the cytological events of premature germination, the callus formation and the embryoid differentiation were studied by, amongst others, Springer et al. (1979), Vasil (1982) and Vasil et al. (1985). Submicroscopical data, however, are scarce and relations to the in vivo development are drawn only incidentally. In a series of studies on the sexual reproduction of maize, cytological and morphological data of the in vivo development were presented earlier (Schel et al. 1984, Van Lammeren 1986 a,b).

In this report the in vitro pollination and the resulting caryopsis development, the callus formation and plant regeneration in vitro will be presented and compared with the morphologic development in vivo. The nature and the significance of the cytological and morphological differences which are caused by embryo excision and in vitro culture will be discussed in relation to environmental conditions. The field corn inbred A188 was selected because of its potency to form differentiating cultures and to regenerate plants in vitro (Green & Phillips 1975). Based on the data of Sheridan (1975,1977) who obtained cell suspensions in only two varieties of the inbred strains studied, the standard variety Black Mexican Sweet corn was chosen because of its

potency to produce callus and to initiate good cell suspensions.

2. MATERIALS AND METHODS

Plant material was obtained from the Zea mays L. inbred lines Black Mexican Sweet (BMS) and A188 which were kindly provided by R.J. Lambert, University of Illinois, Illinois, USA and by C.E. Green, University of Minnesota, St Paul, USA respectively. Plants were grown under greenhouse conditions, with 16 hrs light at 23°C and 8 hrs darkness at 18°C with a relative humidity of 70-90 %. Cobs were masked with small bags before the emergence of the silks.

Preparatory to in vitro pollination and ovary culture, the cobs were surface sterilized with 70% ethanol, the husks were removed in a laminar flow chamber and the silks were shortened to about 8 cm. Sectors of the cobs, bearing several spikelets each, were dissected and placed in Petri dishes on solidified culture medium according to Green & Phillips (1975) in which the minerals are according to Murashige and Skoog (1962), supplemented with thiamin (0.5 mg/l), L-asparagin (0.35 g/l), sucrose (20 g/l) and agar (7 g/l). The medium is henceforth referred to as GP medium and can be supplemented with various concentrations of 2,4 dichlorophenoxy acetic acid (2,4D). The silks were cross-pollinated either on the part inside the Petri dish or on the part outside the dish.

To study callus formation, embryoid formation and germination of excised immature embryos, the silks were hand-pollinated in situ. Ovaries were dissected at defined intervals from 1 day after pollination (DAP) onwards and they were sterilized according to Dahmen & Mock (1972) by a dip in 70% ethanol, sometimes followed by an incubation in 3% Na-hypochlorite in distilled water during 5 min. They were then thoroughly rinsed in sterile water. Complete embryo sacs, or blocks of endosperm containing the embryos, were dissected when the embryos were too small to manipulate, i.e. from 1 up to 6 DAP. Older embryos were either separated from the endosperm or dissected with some attached endosperm and placed on solidified culture medium. Various nutrient media were tested; the B5 medium of

Gamborg et al. (1968), and the media of Dahmen & Mock (1972), Graebe & Novelli (1966), Green & Phillips (1975) and Monnier (1976). Germlings, developed in vitro, were allowed to mature in a mixture of Sphagnum and sand (1:1) moistened with a 0.1 % solution of chemical manure.

For the morphological, histological and cytological analysis, the embryo development was registrated during the culture period. Some embryos were selected and prepared for scanning and transmission electron microscopy (SEM,TEM) and some for light microscopy (LM) essentially as reported previously (Van Lammeren 1986b). Embryos which were prepared for SEM were post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer pH 7.0.

Cultures in liquid media were started with freshly dissected embryos, cultured embryos, and callus. Various media were tested for the cell culture such as the B5 medium and the media according to Green & Phillips (1975) and Potrykus et al. (1979). Cell suspensions were initiated from embryos and embryonic callus. They were cultured in spinner flasks or in an orbital shaker (140 rev/min. at 27°C and 16 hrs light, 2200 lux).

Viability tests on suspension cells were performed with fluorescein diacetate (FDA, see Heslop-Harrison and Heslop-Harrison 1970), with lissamine green and by observing cytoplasmic streaming. Lissamine green is a selective stain for the cytoplasm of degenerating and degenerated cells because it only permeates disrupted cell membranes. A 0.1% solution of lissamine green in culture medium is prepared and stored at -20°C. It was diluted 1:50 with culture medium just before staining and one drop was added to an equal volume of cell suspension on a slide and observed after 5 minutes.

3. RESULTS

3.1 In vitro pollination and caryopsis development

In order to study the initial phase of embryogenesis under defined conditions, excised pistillate flowers were pollinated in vitro. The in vivo pollination of the mature pistillate flowers in the mid

region of the cob resulted in approximately 100% fertilization, as was deduced from the in situ enlargement of the ovaries and the subsequent caryopsis development. In vitro pollination of excised ovaries, taken from the same region resulted, however, in lower percentages (Table 1). Irrespective of the method of pollination, about 27% of the ovaries had enlarged after 10 days in culture (DIC). This initial enlargement was caused by growth in the basal part of the carpels and, especially with strain BMS, frequently followed by an indentation of the young ovary, resulting from the degeneration and collapse of the underlying nucellus tissue.

NUMBER OF POLLINATED OVARIES AT 10 DIC		OVARY GROWTH AT 24 DIC	EMBRYO&ENDOSPERM DEVEL. AT 32 DIC	GERMINATION
INSIDE PETRI DISH	70	27%	24%	5-6%
OUTSIDE PETRI DISH	84	27%	10%	1-2%

Table 1. Caryopsis development in maize after in vitro pollination either inside or outside the Petri dishes. The percentages express the relative number of ovaries which grow or germinate as a result of pollination. (DIC=days in culture)

After prolonged in vitro culture (24 DIC), there was a clear difference in the number of developing caryopses with regard to the two procedures of pollination. As compared to pollination outside the Petri dishes, pollination inside the dishes resulted more often in full embryo and endosperm development (10 and 24% respectively) and germination(2 and 6% respectively).

3.2. In vitro development of excised immature embryos

3.2.1. Premature germination

Embryogenesis in vivo is characterized by organogenesis and embryo enlargement while germination is suppressed. When immature embryos

of strain BMS were cultured on solid media without growth regulators, the culture resulted in premature germination when the abaxial side of the scutellum was in contact with the nutrient medium. As compared to the in vivo development the growth of the scutellum retarded whereas the development and elongation of roots and shoots accelerated. Depending on the age of the embryo and the composition of the culture medium various abnormalities could occur (see Table 2). The media of Dahmen & Mock and Gamborg were not suited for premature germination. With the first medium severe deformations of the embryo coincided with retarded growth, even at 16 DAP. When cultured on Gamborg's B5 medium, 7 DAP embryos did not develop. At 8 DAP the cultures showed predominantly root formation, and at 9 DAP the embryos only exhibited limited growth of the axis whereas the scutellum did not develop at all. On the other hand, the medium of Graebe & Novelli permitted organ development. It was used for further germination experiments.

When embryos are in the proembryo stage of development, i.e. from 1 to 6 DAP, they are very small and have to be dissected with some surrounding tissue to prevent injury because the endosperm is con-

MEDIUM	EMBRYO AGE (DAP)	ORGAN DEVELOPMENT			CONTINUED DEVELOPMENT
		ROOT	SHOOT	SCUTELLUM	
Dahmen&Mock	16	-	-	-	-
Graebe&Novelli	3	+	-	-	-
,,	5	+	-	-	-
Gamborg-B5	8	+	-	-	-
Graebe&Novelli	8	+	*	+	-
,,	9	+	+	+	+

Table2. Formation and deformation of the root, shoot and scutellum in maize embryos of various ages, cultured on three different nutrient media. Abbreviations: DAP=days after pollination. +=distinct development, *=limited development, -=no development.

nected with the embryo suspensor. Embryos older than 6 DAP which were surrounded by some endosperm, however, exhibited a different type of development as compared to the embryos which were dissected completely. Table 3 shows the in vitro development of embryos which were either excised or embedded in endosperm, and cultured on the medium of Graebe & Novelli. Embryo sacs in sagittal sections of ovaries and surrounded by nucellus cells were set in culture at 1 DAP. They developed callus at the site of the zygote. Callus growth

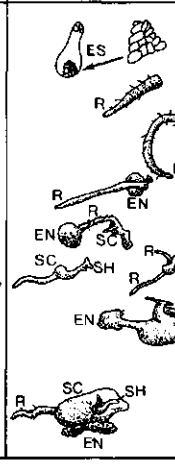
EMBRYO AGE (DAP)	MEDIUM	EMBRYO DEVELOPMENT									
		EMBEDDED IN ENDOSPERM					EXCISED FROM ENDOSPERM				
		R	SH	DEF	IES	DRAWINGS	R	SH	DEF	IES	DRAWINGS
1	G&N	-	-	+	0.1						
3	MONN.	+	-	-	0.2						
5-6	G&N	+	*	-	0.4		+	*	-	0.4	
7-8	"	+	-	+	0.6		+	-	-	0.8	
8-9	G-B5	+	-	+	0.8		*	*	+	1.4	
9	G&N	+	+	+	0.8		+	+	-	1.5	
11	"	+	+	*	1		+	+	+	1	
13	"	-	-	+			+	+	-	0.8	
15-16	"	+	+	+	2		+	+	-	1.5	

Table 3. In vitro development of immature maize embryos (strain BMS) which were either embedded in endosperm or excised from the endosperm and cultured without 2,4 D on the nutrient media of Graebe & Novelli (G&N, 1966), Gamborg et al. (G-B5, 1968) or Monnier (MONN., 1976). The drawings show the results of the culture. 1) An embryo of 9 DAP which was excised and transplanted upon the endosperm of another caryosis developed with less deformation. 2) These embryos were grown to maturity and developed fruit bearing inflorescences. Abbreviations: DAP= age of embryo in days after pollination at the onset of culture, DEF=deformation, EN=endosperm, ES=embryo sac, IES=initial embryo size in mm, R=root, SC=scutellum, SH=shoot, +=distinct development, *=limited development, -=no development.

ceased after 3 days of culture and root nor shoot meristems were formed. When proembryos of 3 DAP were set in culture with some surrounding endosperm cells, they did not have root or shoot meristems yet. Root meristems, however, were formed on the medium of Monnier and roots elongated up to 2 cm. Embryos which were excised at 7 DAP did have root and shoot meristems at the beginning of culture. When embedded in some endosperm they developed a morphologically normal root but only a small defective shoot. Older embryos were also embedded in the endosperm during culture in vitro, they then exhibited defective shoot formation , too.

The culture of embryos which were free from endosperm resulted in less deformations of the root and the shoot, provided they were cultured on the medium of Graebe & Novelli (see Table 3). The embryos of 6-7 DAP showed normal root formation but limited shoot development. From 9 DAP onwards, however, the immature embryos formed roots and shoots, germinated completely and could form plantlets which were grown to maturity.

3.2.2. Callus formation and plant regeneration from embryonic tissues

3.2.2.1. Selection of culture conditions

Callus formation and embryoid formation were most effective when the anterior or axis side of the embryo was in contact with the nutrient medium and when growth regulators were added. Root and shoot formation was almost completely inhibited when 10^{-5} M zeatin was added. Premature germination was also prevented by 10^{-5} M 2,4D which, however, initiated callus formation in roots, shoots and scutellar tissue. Table 4 presents growth responses of the embryos of two maize inbred lines when cultured on the medium of Green & Phillips, supplemented with various concentrations of 2,4D. The highest concentration of 2,4D (2mg/l) induced morphological abnormalities in germination such as the curling of coleoptiles, the retarded growth of roots and the formation of callus.

MAIZE INBRED LINE	2,4 D mg/ml	GERMINATION		CALLUS			CHLOROPLASTS IN SCUTELLUM	ADVENTITIOUS	
		ROOT	SHOOT	R	SH	SC		SHOOTS	ROOTS
BMS	-	+	+	-	-	-	+	-	-
"	0.25	*	+	+	-	-	+	-	+
"	2.0	*	*	+	+	+	+	+	+
A188	-	+	+	-	-	-	+	-	-
"	0.75	*	+	*	-	+	+	+	+
"	2.0	*	*	+	-	+	+	+	+

Table 4. Growth response of excised maize embryos of 16 to 17 DAP on the culture medium of Green & Phillips (1975), supplemented with various concentrations of 2.4D. +=distinct development, *=limited development, -=no development. For abbreviations see Table 3.

3.2.2.2. Callus formation

The morphological development of callus on A188 embryos, cultured over a period of 45 to 160 hours on GP-2 medium, is shown in Fig. 1. Various growth responses were discerned. In some cases the whole scutellum enlarged (Fig. 1a). In other cases a local swelling was formed at the base of the scutellum (Fig. 1b) and incidentally a combination was detected (Fig. 1c). Sometimes only the embryo axis was swollen (Fig. 1d). All these phenomena, however, could appear simultaneously as well (Fig. 1e). At the surface of the scutellum the callus was frequently observed at the abaxial side and the lateral sides. Calli appeared as wart-like structures, as globular aggregates with epidermal-like smooth surfaces, or as loose, friable arrangements (Fig.5a, arrow-heads).

The onset of cell proliferation in embryos starts within 24 hours of culture. Indications of deviating cell growth were seen in the meristems of the root and shoot and in the scutellum. Initially the cells of the root meristems were vacuolized and exhibited nuclei with much heterochromatin (Fig. 2a,b) whereas after 24 hrs of cul-

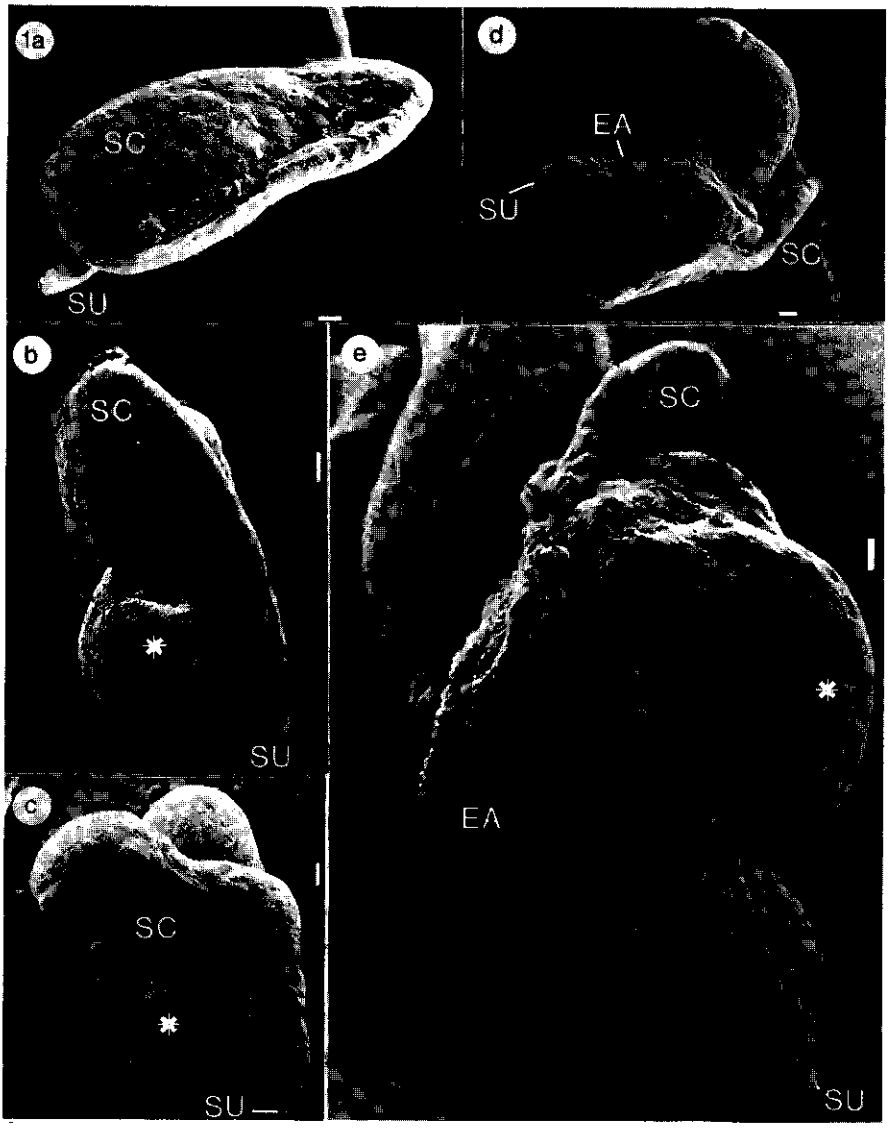


Fig. 1 Morphological development of callus on embryos of maize (strain A188), cultured over a period of 45 to 160 hrs on GP-2 medium. Culture period for a=45 hrs, b-c=68 hrs, d=90 hrs and e=160 hrs. Note the total enlargement of the scutellum and the embryo axis (EA) and the local swelling (*) at the base of the scutellum. The bars represent 100 µm. (SC=scutellum, SH=shoot, SU=suspensor).

ture (Fig. 2c) the vacuoles decreased in size and nuclei became euchromatic. After 45 hrs of culture most vacuoles had disappeared and cells divided frequently (Fig. 2d).

Within the scutellum,a swelling was caused by the growth of the mesophyll cells and by the enlargement of the intercellular spaces

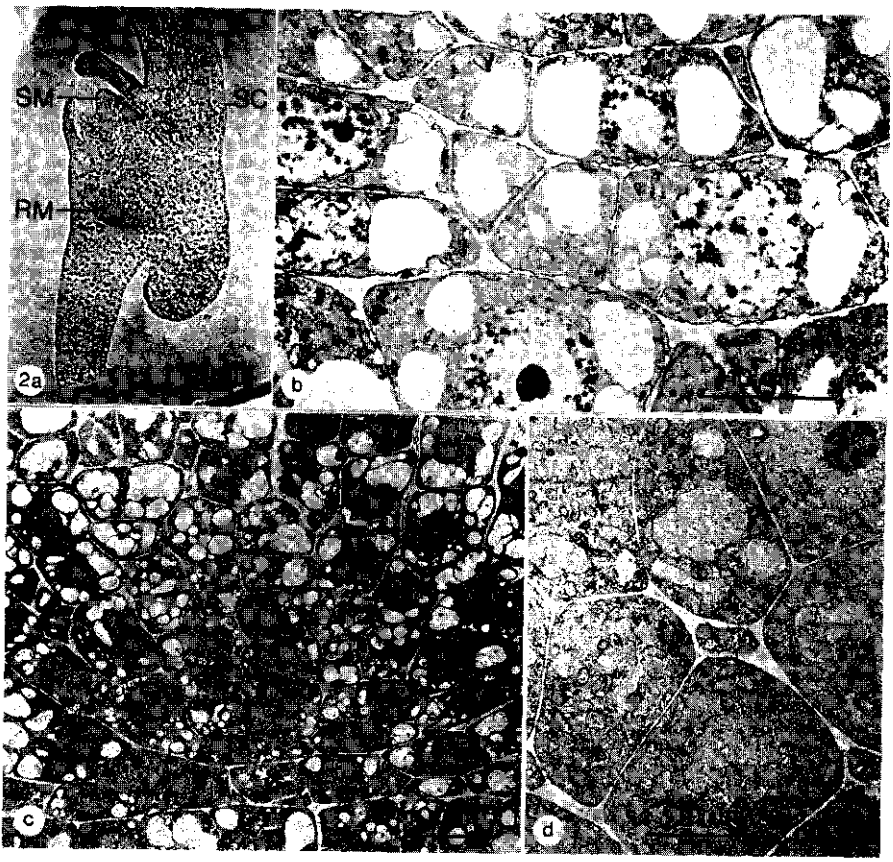
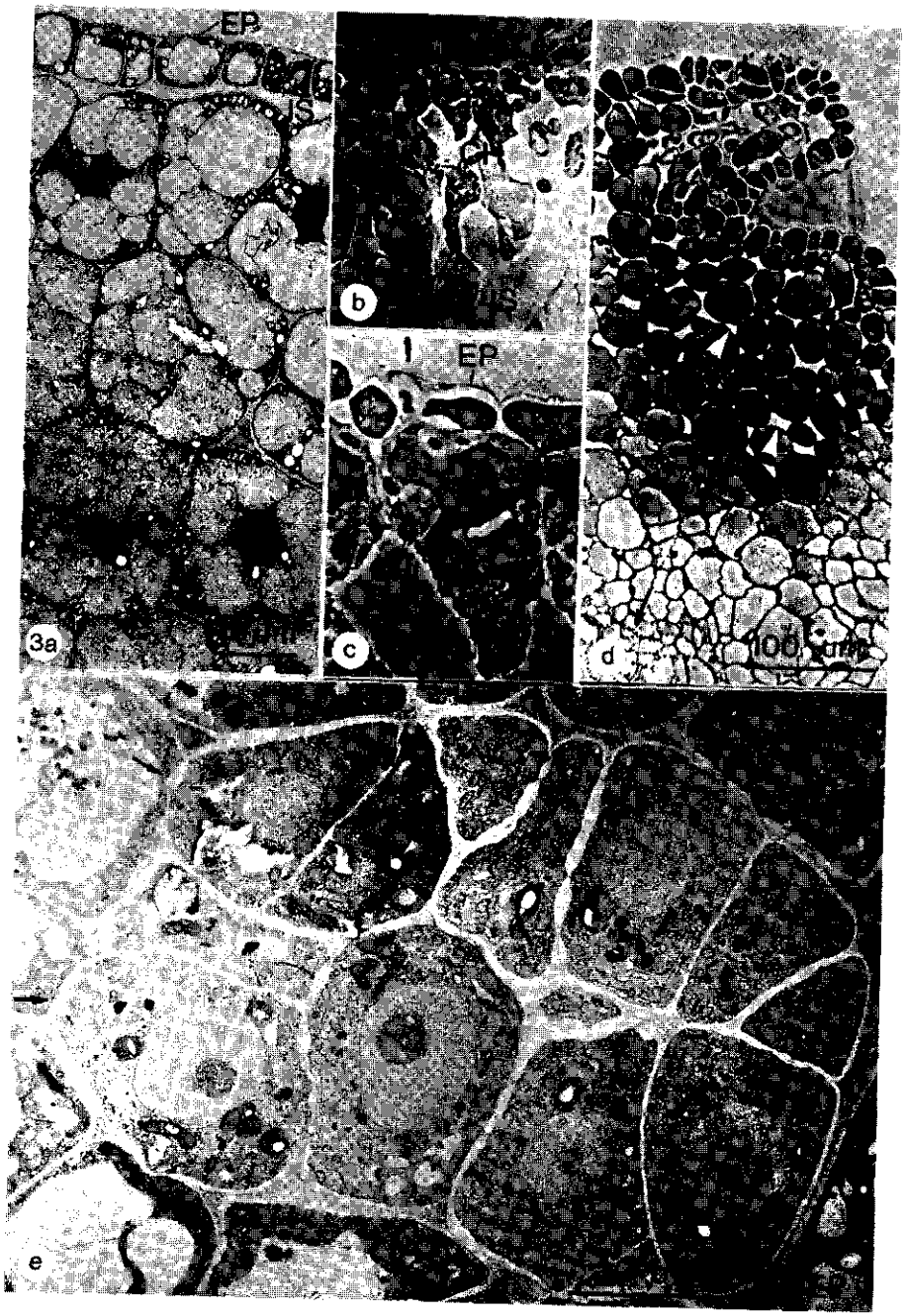


Fig. 2 Activation of root meristem cells of immature A188 maize embryos set in culture on GP-2 medium with the axis side in contact with the medium. a. Light micrograph of a sagittal section of an embryo grown in vivo for 11 to 12 days. b The root meristem of such an embryo before culture. c. A root meristem at 24 hrs. and d. the root meristem after 45 hrs in culture. Note the decrease of vacuolar size, the changes in the nucleoplasm and the increase of cell divisions (arrows).


EP
IS
3a
b
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d
e

as is visualized in Fig. 3a,b. In addition, meristematic regions were formed in the inner part of the scutellum and new strands of vascular tissues were observed as well (Fig. 3d, arrow). Scutellum cells of the subepidermal regions often developed into chlorenchyma (Fig. 3b,c) or collenchyma (Fig. 3d, arrow-heads) and in some regions they showed meristematic activity (Fig. 3e). The latter regions, in which the epidermis is included, were characterized by increased mitotic rates and by relatively small cells which had few vacuoles and many organelles. There was often a marked difference in staining of the various cells in such a region. A cluster of cells with a presumed single cell origin exhibited intensive staining (Fig. 3e, arrows) whereas the organelles of the adjacent cells showed less contrast. The cluster constitutes an embryoid or a shoot primordium; the bordering cells the callus tissue. On the other hand, subepidermal cells at the abaxial scutellum side could also degenerate within 24 hrs of culture (see Fig. 4a) and at 45 hrs in culture, the larger part of the scutellum could then have collapsed (cf. Fig. 1d) because of cell degeneration as visualized in Fig. 4b.

In order to compare the callus-generating ability of the various scutellum regions, some embryos of 21 DAP were cut in several transversal parts and cultured on GP-2 medium. Except for the one containing the apex of the scutellum, all sections had formed callus after 4 days in culture.

Fig. 3 Cytological differentiation in the maize scutellum during in vitro culture on GP-2 medium. Intercellular spaces (a,b), chlorenchyma (b,c), collenchyma (d, arrow-heads), vascular tissues (d, arrow) and meristematic, callus and shoot or embryoid forming zones (e) resulted within 13 days in culture (DIC). a=1 DIC, b-d=11 DIC, e=13 DIC (CH=chloroplast, IS=intercellular space).

Callus on the scutellum grew fast and often had a friable appearance (Fig. 5a, arrow-heads). Callus formation on roots was observed as soon as the roots came into contact with the nutrient medium. The cell proliferation occurred in the epidermis and cortex of the seminal roots. The aggregates of callus branched frequently, due to the deficient formation of lateral roots (Fig. 5b,c). Adventitious roots were generated from the embryo shoot and from the scutellum (Fig. 5a, arrow). They had a normal appearance as long as they were not in contact with the nutrient medium.

3.2.2.3. Plant regeneration

After a culture period of three weeks on solidified GP medium with 2mg 2,4D/l, adventitious organs developed on the scutella of A188 embryos. Embryos of strain BMS formed leafy structures after about 6 weeks. As compared to BMS, strain A188 also formed more adventitious roots and shoots. Up to 100% of the cultured embryos of strain A188 produced up to 15 adventitious organs each in a culture period of three months whereas only up to 20% of the BMS embryos bore adventitious shoots (Fig. 6). It was observed that the wart-like structures often developed into adventitious roots.

The adventitious shoots (Fig. 6a, arrows) and leafy structures (Fig. 6b,c) developed at the coleorhizal end on the abaxial side of the scutellum. Their place of origin was not fixed but often a greening of the site preceded the development. The epidermis at such a site was still an undisrupted structure. The leafy structures bore many epidermal hairs (Fig. 6c) and initially the adventitious shoots often had a scutellum-shaped appearance with a shoot meristem-like structure at the surface. Adventitious roots developed (Fig. 6c, arrows) but were never seen to originate from the adventitious shoots under the applied circumstances.

3.2.3. Culture in liquid media

Embryos of BMS and A188 were dissected at 16 DAP and cultured in liquid GP medium supplemented with various concentrations of 2,4D. When cultured without 2,4D, the embryos formed roots and shoots. Having added 0.75 mg 2,4D/l , the scutellum enlarged, the coleoptile partly developed and root formation was inhibited completely. Some small clusters of cells were detected in the suspension fluid. When

cultured at 2 mg 2,4D/l, there was a slight growth of the embryo axis, the scutellum enlarged and callus was formed within 5 days. At 8 days in culture, individual cells and clusters of cells from both inbred lines were detected in the culture medium. Most suspended cells were elongated, their number increased, and a dense suspension developed. With strain BMS over 90% of the suspension cells exhibited cytoplasmic streaming after 8 days in culture, but with strain A188 hardly any cell showed this tendency although many strands of cytoplasm radiated from the nucleus towards the periphery of the cell (Fig. 7b, arrows). During a culture period of 4 weeks the streaming-based viability decreased to about 40% with BMS and after 7 weeks of culture, streaming was only observed in about 10% of the cells. Based on the lissamine green - FDA tests, however, a viability of about 30% was still detected in those cells. From the

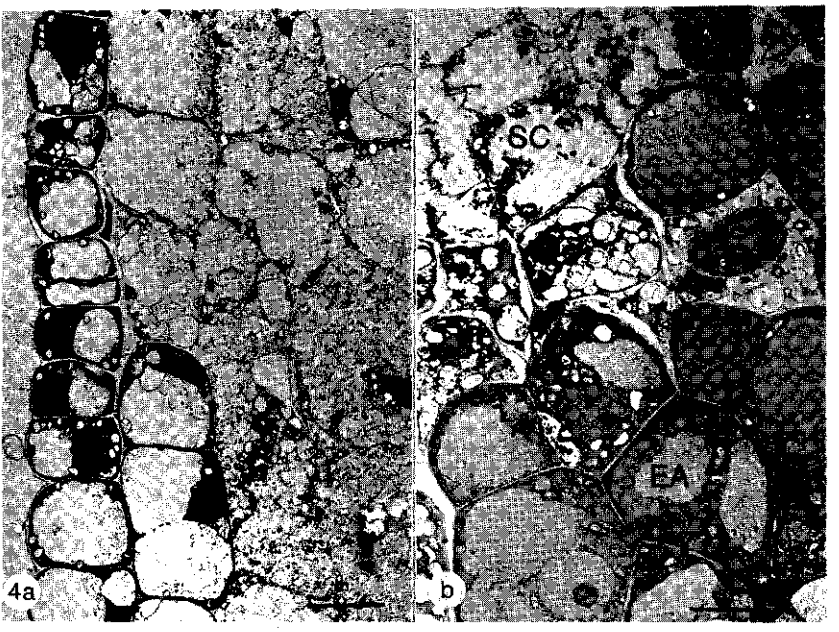

Fig. 4 Degeneration of subepidermal (a) and central scutellum (SC) cells (b) of immature A188 maize embryos after the initiation of in vitro culture at 25 hrs (a) and 45 hrs (b) on GP-2 medium. The swelling of the cells of the embryo axis (EA) resulted in the enlargement of the axis. The bars represent 10 um.

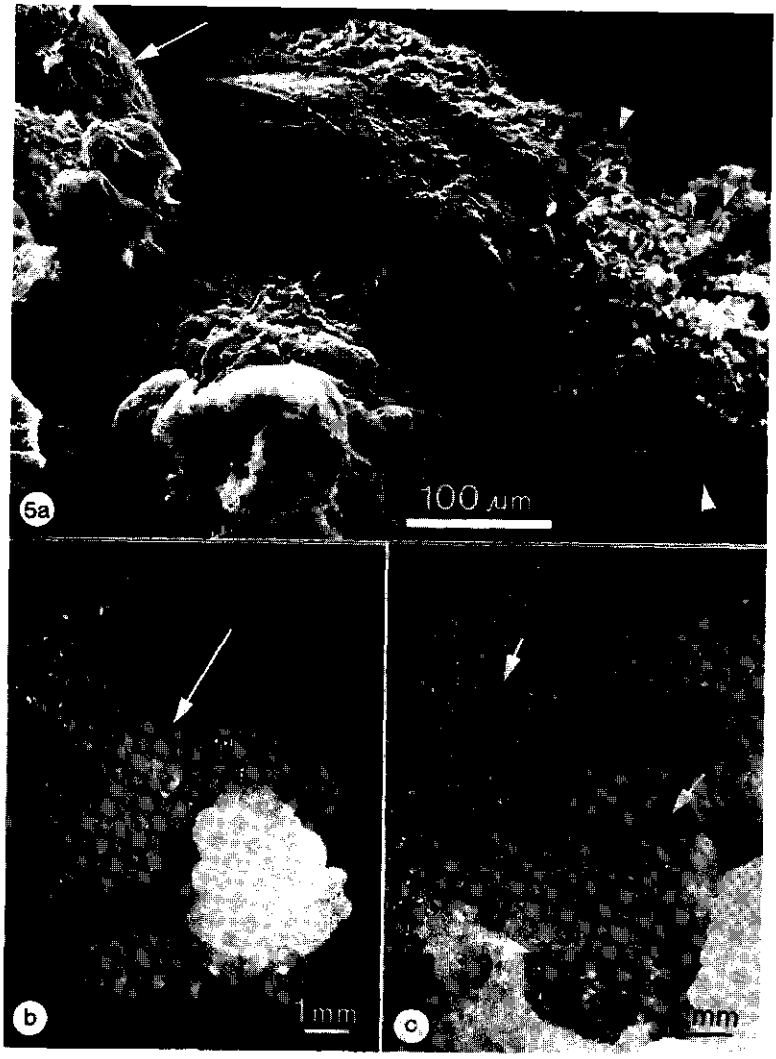

Fig. 5 Morphology of callus formation on maize embryos cultured in vitro on GP-2 medium and observed with SEM and LM. a. After 25 days in culture (DIC) rootlets were formed (arrow at the upper left). In the central area the scutellum epidermis (SCE) is still intact but at the right it is protruded by friable callus (arrow-heads) . b-c. Callus formation on the roots of maize embryos cultured in vitro on GP-2 medium. The highly proliferated callus structures are homologous with lateral roots (b,arrow) and can have a green appearance because of the differentiation of chloroplasts (c,arrows).

beginning of culture, the A188 cells showed cytoplasmic streaming in less than 5% but lissamine green - FDA tests, such as shown in Fig. 7c,d, gave percentages up to 50% viability after 8 weeks. Subculturing of that suspension in GP-1 medium and a subsequent incubation of 8 days resulted in an increased viability that exceeded 90% . A remarkable increase in cell number was noticed concomitantly but cell divisions were not observed in the suspended

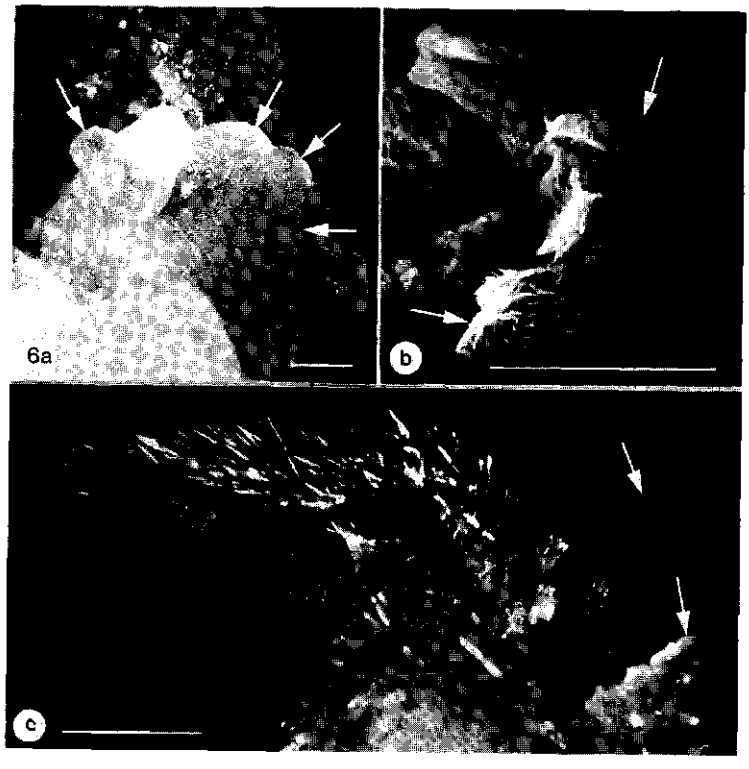

Fig. 6 Regeneration on immature maize embryos (strain A188) on GP-2 medium. a.Formation of adventitious shoots (arrows) at 25 DIC. b-c. Differentiating leaflets of adventitious shoots at 38 DIC. The adventitious roots (c, arrows) dc not originate from the adventitious shoots. The bars represent 1 mm.

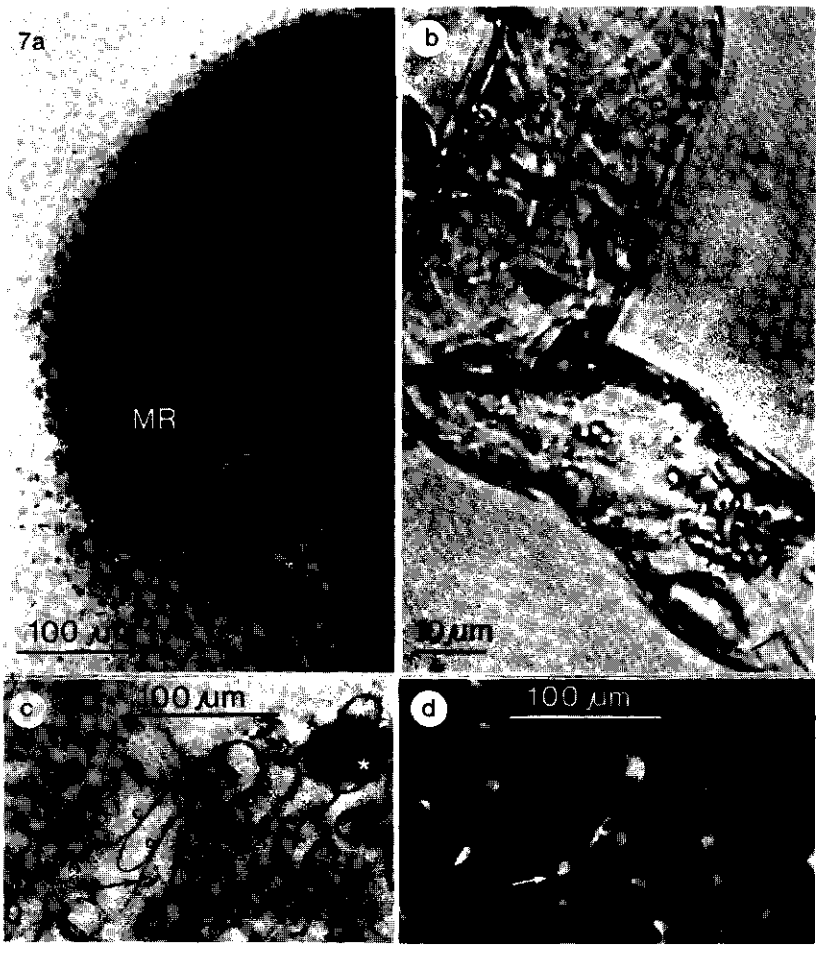

Fig. 7 Culture of immature maize embryos in liquid GP-2 medium resulted in the development of a "cell suspension" containing cell aggregates (a) with meristematic regions (MR). Cell production was dependent on their presence in both strains. b-d. Determination of viability in " cell suspensions" derived from immature maize embryos of strain A188. The viability was either determined by observing cytoplasmic streaming and the integrity of the cytoplasm (b) or by staining with lissamine green (c) and fluorescein diacetate (d). In the phase contrast view (c) some desintegrated and lissamine green accumulating cells are marked (*). The dark-field micrograph (d) of

cells. They were observed in cell aggregates of 1.5 to 3.0 mm size which were not in the inoculum initially. Figure 7a shows such a cell aggregate which has a root-like appearance. The increase in cell number was only observed when the cell aggregates were present.

Subculturing of suspended cells often caused the disruption of cells and the outflow of their cytoplasm. Mannitol was added in 2% steps up to 12%(w/v) to prevent cell lysis, but in all subcultures lysed cells were detected. Only a gradual, 20% dilution of the culture medium was successful to maintain cell stability. Cell aggregates were detected in subcultures with both high (12%) and low (0.8%) concentrations of mannitol. Only in these particular subcultures the number of suspended cells increased.

4. DISCUSSION

4.1. Pollination and caryopsis development in vitro

The in vitro culture of maize embryos offers the opportunity to improve the control of nutritional and environmental conditions in order to study and influence embryo development, callus formation and somaclonal propagation (see Sheridan 1982, Vasil 1984).In vitro embryogenesis ab ovo was realized by in vitro pollination. The technique shortens the progamic phase and might hence synchronize subsequent embryo development (see amongst others Gengenbach 1977, Dhaliwal & King 1978, Raman et al. 1980). It was found, however, that the in vitro pollinated pistils did not develop uniformly. This could either be caused by unsuccessful fertilization, by a failure of endosperm development or by a lack of interaction between the ovary and the nutrient medium. Gengenbach (1977) already stressed the importance of in vitro pollination with maize. He achieved only 5% kernel maturation and suggested that this was caused by deficient endosperm development because fertilization in his experiments ranged up to 84% depending upon the genotypes of the parents. In the

the same cells shows exclusively the "living", FDA-positive cells. The black and white arrows in pictures c and d point towards the same nucleus. Abbreviations: CP = strand of cytoplasm, MR = meristematic region, N - nucleus, P = plastid.

present experiments the highest percentage of kernel development was achieved when silks were pollinated inside instead of outside the Petri dish. Because the site of pollen attachment in that case was closer to the ovaries, more pollen tubes might reach an embryo sac. In the present experiment a limited amount of pollen was used to prevent contamination. Therefore the percentage of kernel development might further be increased by administering the optimal amount of pollen as was demonstrated by Raman et al. (1980).

Although the percentage of kernel development was notably improved upon and the method proved to be successful in comparison to the results of Gengenbach (1977), it was preferred to pollinate pistils in vivo to ensure fertilization for further in vitro culture experiments.

4.2. Pollination in vivo followed by embryo development in vitro

When pistils were pollinated in vivo, then excised at 1 DAP and cultured in vitro, the embryo and endosperm developed (Schel & Kieft 1986). When pistils,however, were pollinated in vivo and sagittal sections of the ovaries containing the fertilized embryo sacs were set in culture at 1 DAP, that culture resulted in deficient embryo development. A callus-like structure formed and germination did not occur. This result indicates that injury strongly influences embryo development. In vivo, the cells of the placento-chalazal region, and the synergids function in the transport of metabolites and inorganic compounds towards the nucellus and the embryo respectively (Van Lammeren 1986a). After the nucellus is replaced by the endosperm, the endosperm cells of the placento-chalazal region and those near the embryo suspensor do function similarly (see Schel et al. 1984). When, however, an ovary is sectioned sagittally and the section side is placed onto the nutrient medium, the nutrients will be taken up through the section surface rather than through the spikelet axis. Consequently, the composition of the nutrient flow will be changed because the selective transport from the somatic tissues of the ovary towards the endosperm and embryo is disturbed.

Proembryos which were set in culture at 3 DAP, generated roots with a length up to 2 cm but shoot development failed. Considering the in vivo stage of development at 3 DAP it was demonstrated before

(Van Lammeren 1986b) that root meristems were not present yet. Thus embryo development in vitro not only means activation of existing meristems but the formation of such meristems as well. So an excised proembryo can transform into an embryo proper stage. When older embryos are set in culture, while they are surrounded by someendosperm, deficient shoot and scutellum formation can also be observed. Whith the endosperm surrounding the embryo in vivo, an intensive interaction between embryo and endosperm can be deduced from ultrastructural evidence (Schel et al. 1984). In vitro, the endosperm influences the embryo growth by its influence on the nutritional environment. In addition there is a wound effect by which there is no selective transferrence of metabolites in the placento-chalazal region any more. Therefore, the composition of the nutrient flow from the endosperm towards the embryo is also changed and influences embryogenesis.

When embryos were excised and not embedded in endosperm, germination only occurred when the scutellum was in contact with the nutrient medium. This suggests the importance of the direction of the nutrient flow. As compared with the in vivo development where most nutrients are taken up through the abaxial epidermis of the scutellum (Van Lammeren 1986b) a similar pathway for nutrients is evident here.

The germination of immature embryos indicates that the technique offers an opportunity to study the morphological differentiation continuously and to simulate germination. Several authors cultured immature maize embryos to achieve complete plant development. Embryos, excised from 15 DAP onwards, germinated and developed normally in the experiments of Mock & Dahmen (1973) who used a solid agar-sucrose composite high-nutrient medium. Sheridan et al. (1978) reported the successful development of excised embryos of 11 DAP. From very young excised embryos (3 up to 7 DAP) only the oldest showed limited growth in the early experiments of Uttaman (1949). In the present experiments, using the medium as described by Graebe & Novelli (1966), the development of plantlets of immature excised embryos was achieved already from 9 DAP onwards. With respect to the data available this means a shortening of the dependence of the embryo upon the endosperm. As compared with the in vivo embryo-

genesis (see Van Lammeren 1986b) a decreased scutellum development is obvious and an activation of meristematic activity and cell elongation results in the early root and shoot formation. The premature activation of the apical meristems is probably initiated by the fall-off of the endosperm suppression. Embryos younger than 9 DAP exhibited growth as well, but deficiencies occurred.

4.3. Callus formation and plant regeneration

Embryo cultures in fluid media were set up to form cell suspensions which could give rise to somatic embryos. The aim was to analyze somatic embryos from the single cell stage onwards to compare their development with the in vivo embryogenesis (see Van Lammeren 1986a). The single cell stage gives the particular opportunity to study the development of structural polarity such as induced in the egg cell-zygote stages in vivo. The culture in media, supplemented with 2.4D resulted in the production of suspended cells in high densities. Cell divisions, however, were not observed in these cells. Therefore the culture is not a real cell suspension but merely the result of cell production in the meristematic, root-like cell clusters. The increase of viability after subculturing was also caused by cell production in the root-like aggregates in the suspension. The tests based on FDA staining gave the best quantification of viability. Cytoplasmic streaming proved not to be a reliable parameter and observing the cytoplasmic integrity is not reliable and laborious.

The regeneration of plantlets from callus cultures of Zea mays L. on solid nutrient media has been acchieved from various tissues such as stem tissue from seedlings (Harms et al. 1976) and from excised immature embryos (see, amongst others, Green & Phillips 1975, Lu et al. 1983, Novák & Dolezelová 1983, Vasil et al. 1984, Duncan et al. 1985, Kamo et al. 1985).

In the present experiments the culture of immature embryos of 16-17 DAP on solidified nutrient media resulted in callus formation at various sites of the embryo. On the scutellum it was mainly restricted to the coleorhizal end which may be related to the high levels of plant growth regulators presumed to be present at this site (Vasil & Vasil 1982). The induced bipolarity existing within the scutellum, which is regarded as a leaf (Van Lammeren 1986b), was

also assigned by the transversal sectioning of the scutellum; most sections exhibited callus formation then. Anatomically, callus formation was found in the epidermal and sub-epidermal zones (see also Springer et al. 1979, Vasil et al. 1985). Callus formation is only one growth response to in vitro culture. The swelling of the scutellum was mainly caused by cell enlargement, by the formation of many large intercellular spaces and, to a lower extent, by the formation of chlorenchyma, collenchyma and vascular tissues. Halperin (1969) and Springer et al. (1979) mentioned a random distribution of vascular tissue in which phloem elements were not detected. Only the compact scutellar callus which was formed at the coleorhizal end showed regenerative capacity. Vasil et al. (1983) also reported embryoid formation in the nodal region of the scutellum but therefore the scutellar side of the embryo was in contact with the nutrient medium. The areas with cells dividing frequently also bore the bipolar, proembryo-like structures. These structures might be of unicellular origin as was also shown for Pennisetum embryoids (Vasil & Vasil 1982, Botti & Vasil 1984). In our experiments the development of leafy structures and adventitious shoots was observed (cf Green & Phillips 1975 and Springer et al. 1979) rather than the formation of somatic embryos as reported by, amongst others, Gordon et al. (1977), Lu et al. (1982, 1983), Novák & Dolezelová (1983), Vasil et al. (1985) and Fransz (pers. comm.).The limited development may be caused by the hormonal level utilized in this study.

5. ACKNOWLEDGEMENTS

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CHAPTER 5

Interactions between embryo and endosperm during early developmental stages of maize caryopses (*Zea mays*)

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Interactions between embryo and endosperm during early developmental stages of maize caryopses (*Zea mays*)

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The early developmental stages of maize caryopses were studied at a fine structural level. Emphasis was placed on the interactions between the developing embryo and the surrounding endosperm. It was found that the placentochalazal region of the endosperm contains cells showing ultrastructural features of transfer cells, including wall ingrowths. This indicates an important function of these cells in the transport of nutrients supplying the developing embryo. Near the basal region of the embryo, densely cytoplasmic endosperm cells occurred, without wall ingrowths but with a mass of highly ordered rough endoplasmic reticulum indicating a synthesis function of this part of the endosperm. The products, probably membranes and proteins, are most likely taken up by the suspensor of the embryo. At about 7 days after pollination, endosperm cells degenerate near the embryo axis and the scutellum. The endosperm remnants might then serve as a food supply for the embryo as well.

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Nous avons étudié les premiers stades de développement des caryopses de maïs au niveau de l'ultrastructure et avons mis l'emphase sur les interactions entre l'embryon qui se développe et l'albumen qui l'entoure. La région chalazo-placentaire de l'albumen inclut des cellules dont l'ultrastructure montre des caractéristiques des cellules de transfert, replis pariétaux inclus. Cela indique que ces cellules jouent un rôle important dans le transport de nutriments à l'embryon en développement. Près de la région basale de l'embryon se retrouvent des cellules d'albumen à cytoplasme dense, sans replis pariétaux mais ayant une masse de réticulum endoplasmique granulaire hautement ordonnée indiquant une fonction de synthèse de cette région de l'endosperme. Les produits, probablement des membranes et des protéines, sont probablement absorbés par le suspenseur de l'embryon. Environ sept jours après la pollinisation, les cellules de l'endosperme dégénèrent près de l'axe embryonnaire et du scutellum. Les restes de l'albumen peuvent donc servir de source de nourriture pour l'embryon aussi.

[Traduit par le journal]

Introduction

The process of embryo formation in grasses is well documented, although mainly by descriptions based on light microscopy (for a survey, see Rost and Lersten 1973). As pointed out by Smart and O'Brien (1983), the only recent studies on the embryo region of grass caryopses at a fine structural cellular level are those of Norstog (1972, 1974) on barley. These authors also emphasized the lack of studies dealing with embryo development in relation to the neighbouring tissues. They therefore investigated in detail and at a fine structural level the process of embryo development in wheat, paying particular attention to structure–function relationships between the developing embryo and the surrounding endosperm.

For maize, the process of embryo formation has been described extensively, both by light microscopy (Randolph 1936; Kiesselbach 1949; Wolf et al. 1952; Van Lammeren and Schel 1983) and by electron microscopy (Diboll 1968; Van Lammeren 1981; Van Lammeren and Kieft 1983). Endosperm development in maize has also been studied in great detail (Weatherwax 1930; Lampe 1931; Brink and Cooper 1947; Khoo and Wolf 1970; Kyle and Styles 1977). However, an integrated study to elucidate the functional relationships between the developing embryo and endosperm from a fine structural point of view has not yet been carried out.

We report here on the fine structure of early developmental stages from maize caryopses. Attention will be given to a functional interpretation of the structural changes of embryo and endosperm. The results will be discussed in relation to data on embryo formation in other plants. It is felt that a detailed knowledge of the *in vivo* embryogenesis of a cereal such as maize is required, especially now that *in vitro* culture methods using immature embryos from this agronomically important crop are becoming increasingly important (e.g., see Green 1982; Sheridan 1982; Vasil 1982).

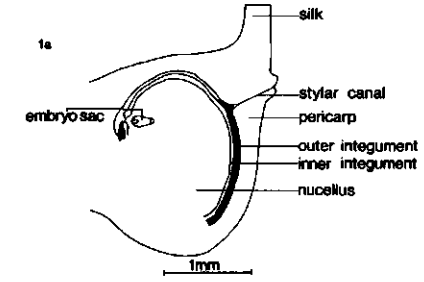
Materials and methods

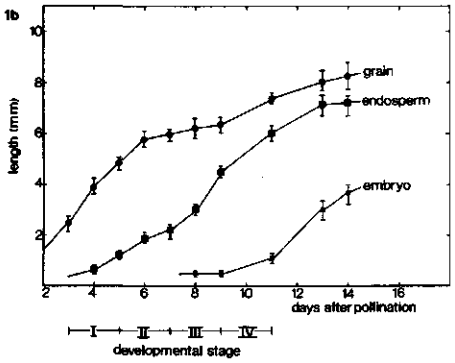
Strain A-188 of *Zea mays* L. (kindly supplied by Dr. C. E. Green, Minnesota) was used. Seeds were grown to maturity under greenhouse conditions. After emergence of the silks, the plants were cross-pollinated by hand. At defined intervals, ranging from 4 to 11 days after pollination (DAP) developing caryopses were removed from the middle of the ears. Small slices, about 2 mm thick, were prepared using razor blades. They were made in such a way that the whole embryo axis and a large part of its surrounding tissues were excised.

The slices were immediately fixed in 5% glutaraldehyde buffered with 0.1 *M* sodium cacodylate buffer (pH 7.0) to which 7 m*M* $CaCl_2$ was added. Fixation took place for 2 h at room temperature. After rinsing, the samples were postfixed for 2 h with 1% OsO_4 and dehydrated in a graded ethanol series, ranging from 30 to 100%, followed by propylene oxide. The pieces of tissue were then transferred to propylene oxide – Epon 812 mixtures and finally to fresh resin. Polymerization occurred at about 600 mbar (1 bar = 100 kPa) for 16 h at 20°C, 8 h at 45°C, and 24 h at 60°C. Semithin (2 μm) and thin sections were prepared on a LKB Ultratome V. The thin sections were poststained with uranyl acetate and lead citrate. They were examined in a Philips EM 301 operating at 60 kV.

Results

The orientation of ovary, ovule, and embryo sac in a median section is represented in Fig. 1*a*, which shows the unfertilized condition. The cob on which the kernels are located is situated at the left. Most micrographs presented here show this type of sagittal section and orientation. The developmental stages fol-





ABBREVIATIONS: *al*, aleuron layer; *ap*, apex primordium; *cp*, coleoptile primordium; *cu*, cuticula; Cw, cell wall; Di, dictyosome; Em, embryo; *End*, endosperm; End ad, adaxial region of the endosperm; End ab, abaxial region of the endosperm; ER, endoplasmic reticulum; *int*, integumentary layer; Li, lipid droplet; Mi, mitochondrium; N, nucleus; Nu, nucellus; *nu*, nucellar layers; *p*, plastid; *pc*, pericarp; Pd, plasmodesma; Rer, rough endoplasmic reticulum; *sa*, shoot apex; Sc, scutellum; Sp, suspensor; *sy*, synergid; *v*, vacuole; *ve*, vesicle.

FIG. 1. (*a*) Schematic representation of a sagittal section through an unfertilized caryopsis of maize. The cob axis is situated at the left (adapted from Randolph 1936). (*b*) Correlation between grain, embryo, and endosperm length and the number of days after pollination. The developmental stages are numbered I to IV. FIG. 2. A nearly sagittal semithin section showing embryo and endosperm development at stage I (3–5 DAP). A degenerated synergid (*sy*) is visible. The embryo contains large vacuolated basal cells and cytoplasm-rich top cells. The cellular endosperm consists of highly vacuolated cells except in the region bordering the embryo at the adaxial side. In that region, the endosperm cells are small and densely cytoplasmic (arrows).

lowing fertilization which are described in this paper have been numbered I to IV. This classification is based on the measurements of the length of excised embryos and endosperm in relation to the number of days after pollination (Fig. 1*b*). Kernels in which the number of DAP and embryo and endosperm lengths did not correspond with Fig. 1*b* were not used for further examination.

The sagittal semithin section in Fig. 2 shows the development of the embryo and endosperm at stage I (3–5 DAP). The embryo is club shaped with a few large, highly vacuolated basal cells and several small cytoplasm-rich top cells. It is surrounded by the endosperm which at this stage is already cellular and consists of large, highly vacuolated cells. However, both near the base and near the side of the embryo which is oriented most to the cob axis (the "adaxial side"), a region of small, densely cytoplasmic endosperm cells is visible (arrows).

As seen at a higher magnification (Fig. 3), the embryo already has a polar orientation showing a gradient in vacuolation from the top to the basal cells. The endosperm cells surrounding the embryo at the abaxial side (End ab) are large, with big vacuoles. The adaxial side of the embryo is covered by endosperm cells (End ad) which are thin walled and contain numerous small vacuoles. A detail of the cytoplasm from such endosperm cells is given in Fig. 4*a*. The cells contain mitochondria which are preferentially located near the crushed nucellus layers. A large amount of rough endoplasmic reticulum (Rer) is present, often with big intracisternal spaces (arrows). These endosperm cells are interconnected by several plasmodesmata (Pd).

Cross sections made perpendicular to the length axis of the embryo at the level of the basal cells reveal that the region of the densely cytoplasmic endosperm is cup shaped and does not surround the embryo completely (Fig. 4*b*, arrows). Higher magnifications show dictyosomes which are highly active judging by the many vesicles (*ve*) emerging at the distal sides (Fig. 4*c*). At the boundary between endosperm and embryo, invaginations of the plasma membrane of the endosperm cells are often found. They often contain dark osmiophilic material (Fig. 4*d*, arrows). When cross sections were made near the embryo tip, these membrane pockets were also found, sometimes with dark granules (Fig. 4*e*, arrow), but also without

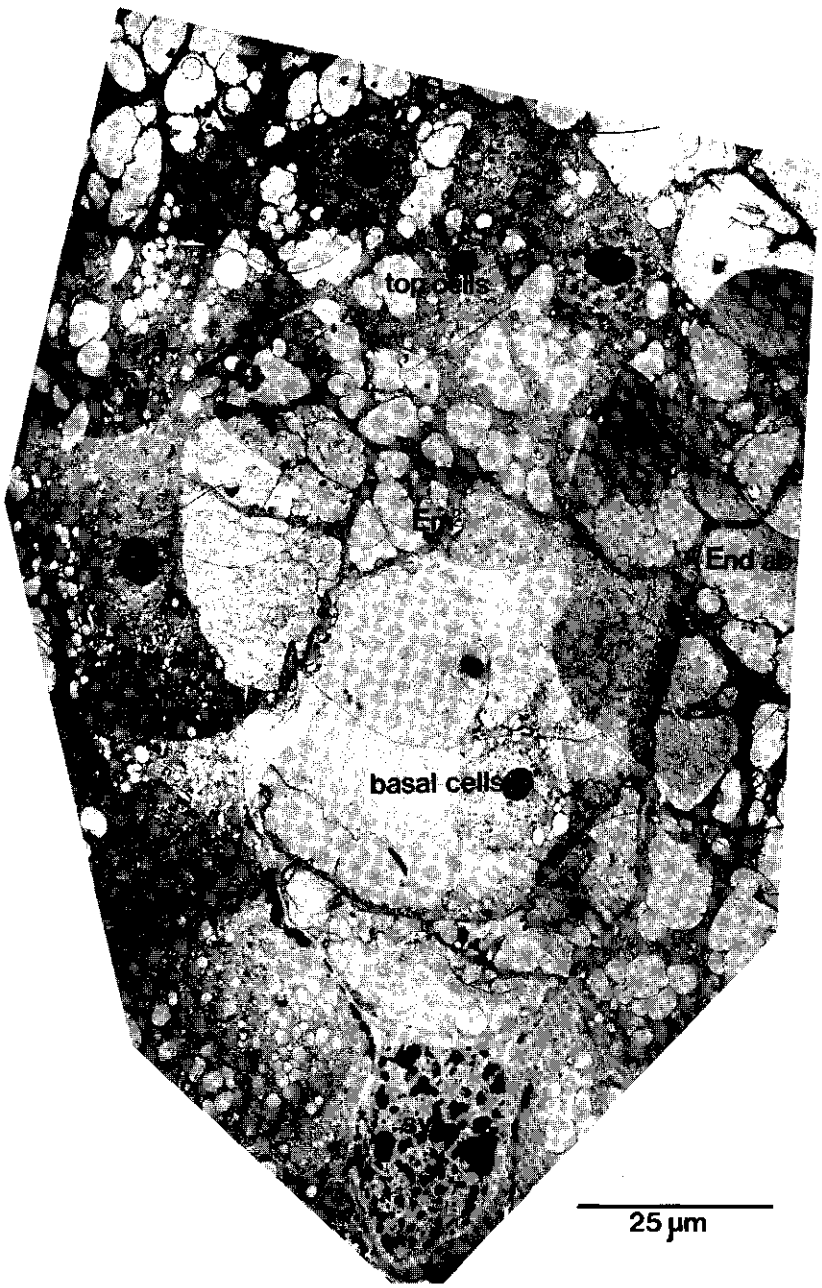


FIG. 3. Electron micrograph of the embryo–endosperm region at stage I. The degenerated synergid (*sy*) is filled with electron-dense material. The polar orientation of the embryo is visible by the change of vacuolation from top to basal cells. The endosperm cells at the abaxial side (End ab) are large and highly vacuolated; the adaxial endosperm (End ad) shows densely cytoplasmic cells with numerous small vacuoles.

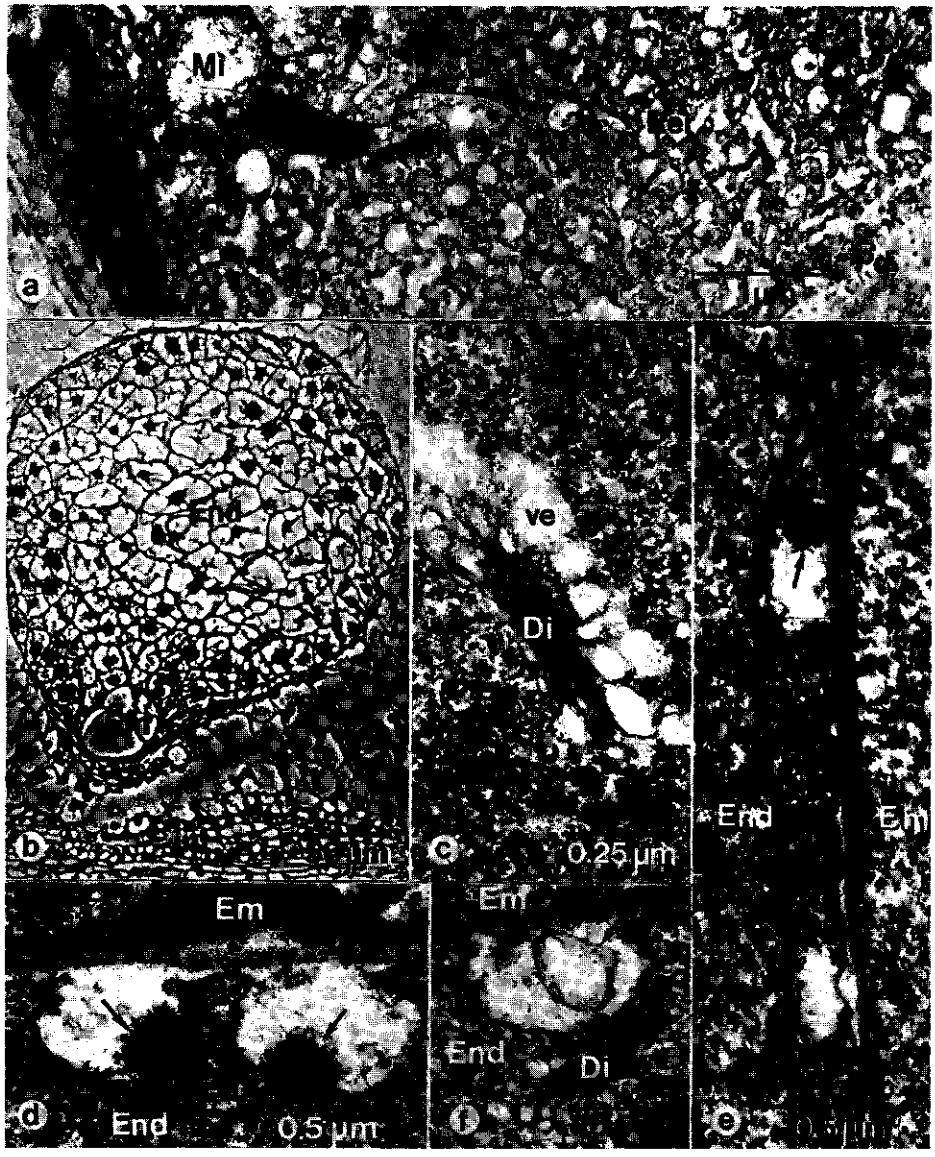


FIG. 4. (*a*) Detail of the adaxial endosperm at stage I. Note the presence of big intracisternal spaces (arrows) occurring in the endoplasmic reticulum. (*b*) Cross section perpendicular to the length axis of the embryo at the level of the basal cells. The cytoplasm-rich endosperm does not surround the embryo completely but is cup shaped (arrows). (*c*) Detail of the cytoplasm of the adaxial endosperm showing a dictyosome pinching off many vesicles (*ve*). (*d*) Boundary region between embryo and endosperm at the level of the basal cells. The plasma membrane of the endosperm shows invaginations, often containing dark osmiophilic material (arrows). (*e*) Embryo–endosperm interface near the embryo tip. Note again the presence of dark granules (arrows). (*f*) Invagination of the endosperm plasma membrane with a membranelike inclusion. The plasmalemma infolds possibly are caused by enhanced dictyosome activity.

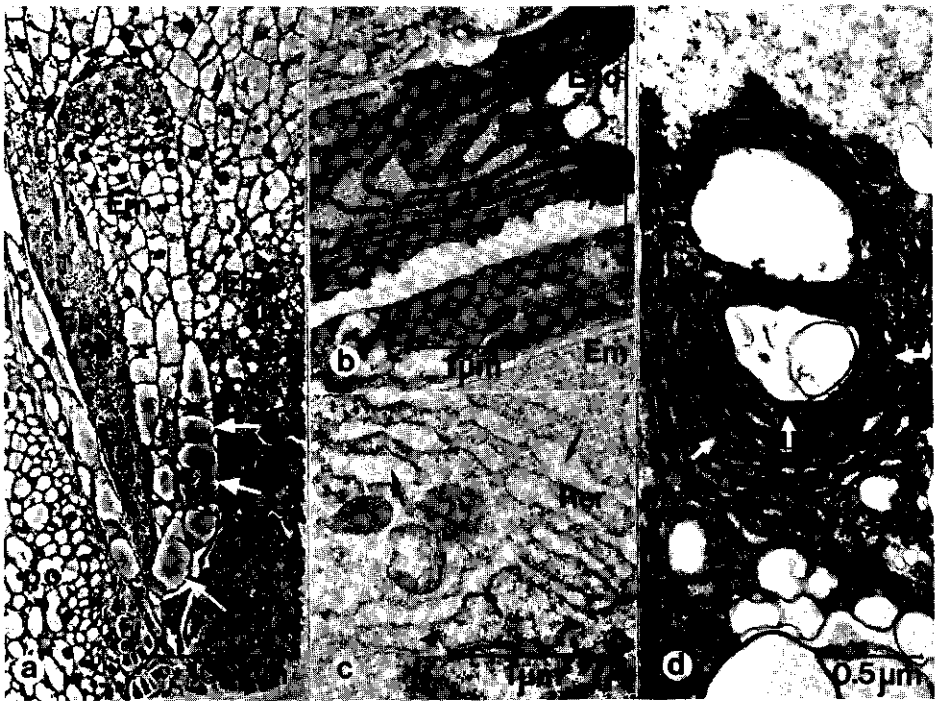


FIG. 5. (*a*) Light micrograph of a sagital section through the embryo sac at stage II (5–7 DAP). The embryo has increased considerably in length; the basal cells are large and highly vacuolated (arrows). In the region near the embryo tip, the endosperm cells are strongly compresssed (arrowheads). (*b*) Detail of the compressed endosperm cells. No intact cytoplasm can be observed. Osmiophilic droplets are visible (arrows). (*c*) Detail of the endosperm near the base of the embryo. Note the presence of the large intracisternal spaces in the rough endoplasmic reticulum (arrows) and the blebbing of the outer nucler membrane (arrowheads). (*d*) Concentrically arranged ER with many "fenestrae" (arrows) giving the appearance of annulate lamellae.

inclusions (Fig. 4*e*) or with membranous inclusions (Fig. 4*f*, arrow). Enlargement of the plasma membrane can also be seen in some of the endosperm cells in the placental region near the chalaza. Before endosperm formation, cell wall protrusions already are synthesized from the lower part of the central cell. At the end of stage I more ingrowths appear in the outer cell walls of the endosperm bordering the placentochalazal region. They are directed towards the cytoplasm and cause the cell membrane to undulate. Most cells have large vacuoles and the cytoplasm is densely stained by the presence of ribosomes. Dictyosomes and endoplasmic reticulum (ER) are not observed in major amounts.

At stage II (5–7 DAP), the embryo has increased considerably in length (Fig. 5*a*). It shows a regular array of cells with increasing vacuolar size ranking from the top. The basal cells are large and highly vacuolated (arrows). The endosperm is strongly compressed in the region near the embryo tip (arrowheads). Details of these compressed cells are given in Fig. 5*b*. The cells are crushed and no longer intact, as indicated by the presence of osmiophilic droplets which are most likely lipids or phospholipids (Fig. 5*b*, arrows). On the contrary, the endosperm lining the base of the developing embryo is highly active. There is a huge amount of ER present, in which large intracisternal spaces occur (Fig. 5*c*, arrows). The outer nuclear membrane in these cells was blebbing very frequently (Fig. 5*c*, arrowheads). In some cases, the ER was arranged concentrically (Fig. 5*d*) and showed the appearance of annulate lamellae (Maul 1977).

At stage III (7–9 DAP), the embryo has developed a long suspensor (Fig. 6*a*). The sites of initiation of the embryo axis (Ea) and the scutellum (Sc) ar visible. The endosperm surrounding the base of the suspensor is densely cytoplasmic, especially at the adaxial side (End ad). The other regions of the endosperm surrounding the embryo consist of large vacuolated cells, sometimes broken down, particularly in the tip and scutellar region of the embryo (arrows).

Some details of the densely cytoplasmic endosperm are given in Fig. 6*b*. There is a large amount of Golgi activity (black arrows), while the ER (mostly Rer) is arranged randomly (arrowheads). Towards the suspensor (Sp) region, however, the arrangement of the ER becomes more ordered, giving rise to parallel arrays of ER cisternae lying close together (white arrows). In Fig. 6*c* the ordered arrangement of ER, vesicles, and mitochondria within the endosperm cells is very striking. There are stacks of ER, while many mitochondria are located near the plasma membrane of the endosperm lining the

FIG. 6. *(a) Light micrograph of a sagittal section through the embryo sac at stage III (7–9 DAP).* The initiation sites of the embryo axis (Ea) and the scutellum (Sc) are visible. The densely cytoplasmic endosperm near the embryo suspensor is prominent, especially at the adaxial side. Near the scutellar region of the embryo some degeneration of endosperm cells occurs (arrows). *(b)* Electron micrograph of the adaxial endosperm. In these cells, a high Golgi activity is present (black arrows). The ER is arranged capriciously (arrowheads) or ordered in parallel stacks (white arrows). *(c)* Detail of the parallel arrays of ER in the active endosperm. Many small vacuoles (*v*) are visible, mostly oriented towards the embryo suspensor. Also, the localization of the mitochondria (Mi) appears to be highly ordered. The plasma membranes contain many osmiophilic droplets.

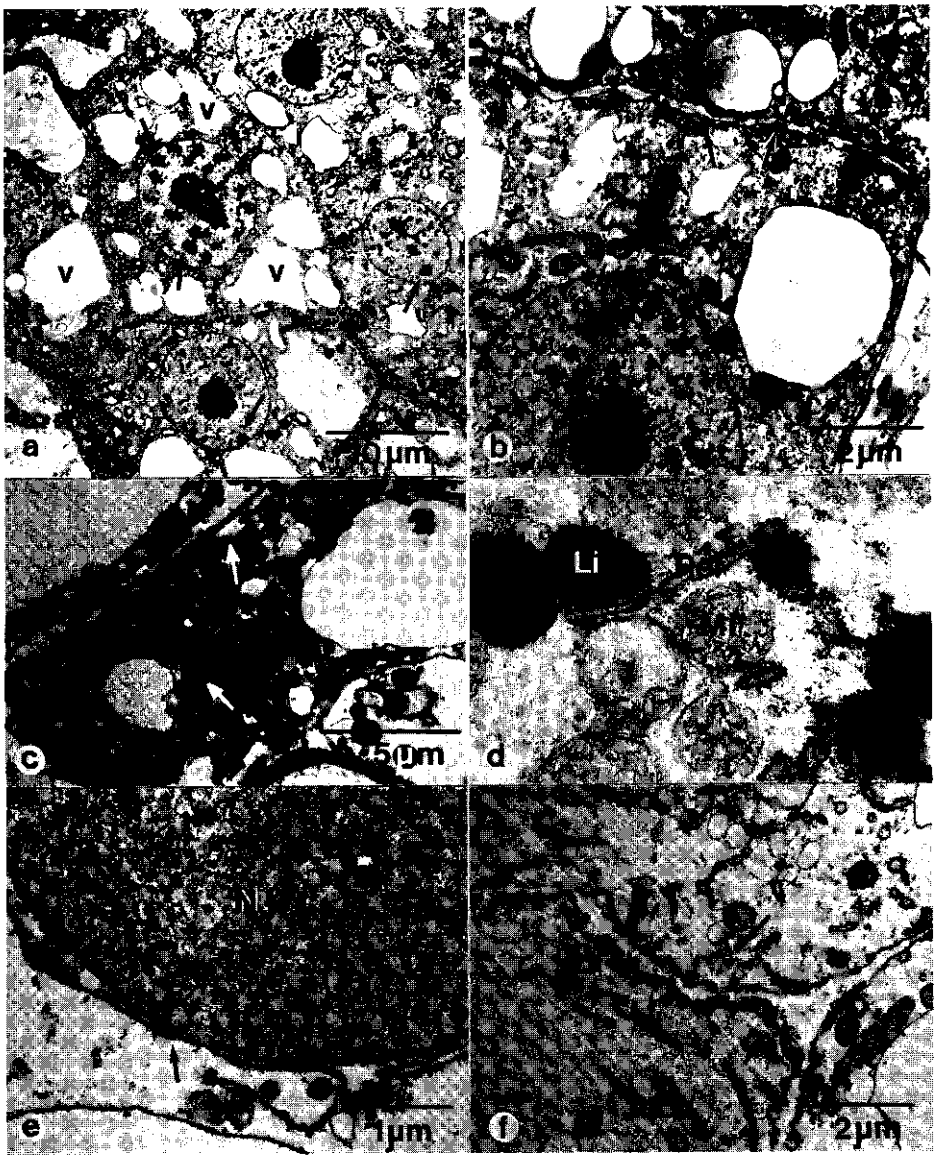


FIG. 7. Some details of embryo and endosperm cells in stage III (7–9 DAP). (*a*) Detail of an embryo cell from the embryo axis region. There are several small vacuoles, while the nuclei often contain many domains of heterochromatin (arrows). (*b*) In the cells of the embryo many osmiophilic droplets occur (arrows), possibly taken up from the surrounding degenerating endosperm. (*c*) Detail of the degenerating endosperm near the embryo axis region. In the cytoplasm, many osmiophilic droplets are present. (*d*) Detail of the cytoplasm remnant showing many lipid droplets (Li). (*e*) Degeneration is also visible by membrane breakdown, e.g., nuclear membrane (arrow). (*f*) Accumulation of osmiophilic droplets (arrows) near the walls of the degenerating endosperm cells.

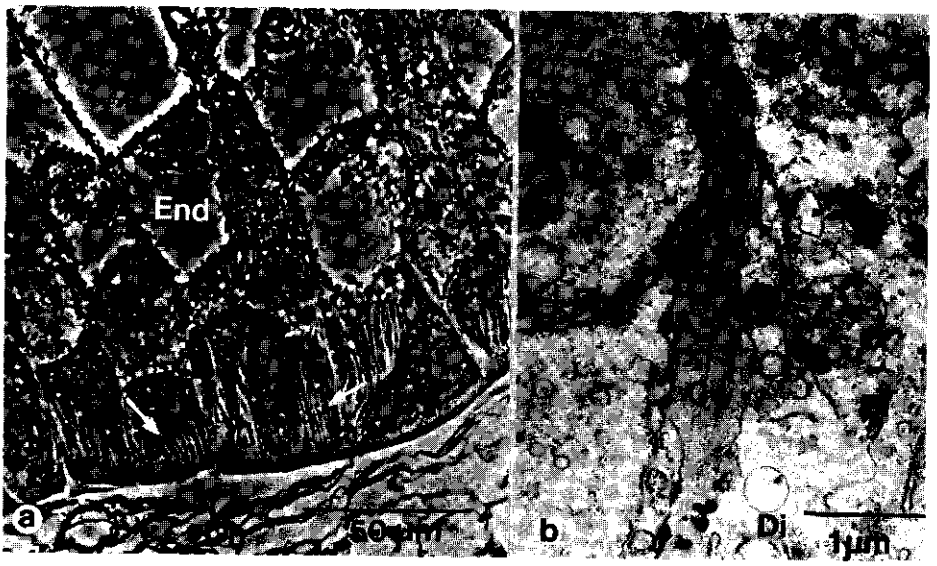


FIG. 8. (*a*) Semithin section of the endosperm bordering the placentochalazal region (Ch) at stage III. The wall ingrowths are very pronounced (arrows). (*b*) Higher magnification of the endosperm cells at the end of stage III. The cytoplasm contains extensive profiles of ER and many polysomes and dictyosomes. Both symplastic transport (by plasmodesmata, see arrows) as well as transmembranal transport of osmiophilic material (arrowheads) takes place.

basal suspensor cells. Moreover, all plasma membranes contain many osmiophilic droplets.

The cells of the embryo in the axis region are small and have several small vacuoles (Fig. 7*a*). The nuclei often contain prominent domains of heterochromatin (arrows). The cells have many osmiophilic droplets (Fig. 7*b*, arrows). The endosperm in this region has degenerated fully at this stage (Fig. 7*c*–7*f*). On the contrary, in the placentochalazal region the wall ingrowths of the endosperm cells have become very pronounced during this stage. They occur initially in the outer layer of the endosperm directly bordering the chalaza (Fig. 8*a*), causing the cell membrane to undulate intensively. Like in the endosperm cells surrounding the base of the suspensor (see Fig. 6*c*), the accumulation of osmiophilic droplets is obvious here as well. Compared with the earlier stages the cells are now densely filled with cytoplasm in which mitochondria, dictyosomes, and profiles of Rer are most prominent. These features are still present at the end of stage III (Fig. 8*b*). Moreover, many plasmodesmata and large ingrowths of periclinal and anticlinal cell walls have appeared.

Figure 9*a* shows a sagittal section through the embryo–endosperm region of a stage IV maize caryopsis (9–11 DAP). The embryo is located very close to the pericarp. The scutellum (Sc) has developed further while the coleptile (*cp*) and shoot apex (*sa*) primordia are clearly visible. The endosperm surrounding the basal suspensor cells has the same appearance as shown in stage III. The endosperm near the scutellum, embryo tip, and embryo axis has totally disintegrated, partially by mechanical stress.

In Fig. 9*b*, the boundary region between endosperm and scutellum is visible. The endosperm has lost its original structure; what remains is a lot of membrane fragments, osmiophilic droplets, and loosened cell wall material (arrows). These osmiophilic droplets are present also in the walls between the degenerating endosperm and the embryonal cells (arrowheads). At this stage, the outer layer of the endosperm near the seed coat contains small vacuoles, protein bodies and other substances (Fig. 9*c*). This layer will develop eventually as the aleuron layer.

Osmiophilic droplets also occur in the embryo axis region (Fig. 9*d*), both near the degenerated endosperm cells and inside the intact embryo cells (arrows), in which the development of starch-containing plastids was also registered around this time (Fig. 9*e*).

Discussion

Plant embryogenesis has for a long time been studied mainly at a descriptive level. However, during the last decade increased attention has been paid to experimental analysis, placing considerable emphasis on functional relationships (for a survey, see Raghavan 1976). In the descriptions of embryo and endosperm the latter has in most cases been described as the nutritive tissue for the developing embryo (Brink and Cooper 1947; for a recent review, see Bhatnagar and Sawhney 1981). Indeed, the role of endosperm extracts on growth and morphogenesis of the embryo has been firmly established (Ziebur and Brink 1951; Pieczur 1952; Williams and De Lautour 1980; Mapes and Zaerr 1981). Not only the nutrient contents, but also the maintenance of a suitable osmotic environment has been proven to be an important function of the endosperm, as has been pointed out by several authors (Raghavan and Torrey 1963; Ryczkowski 1969; Monnier 1976).

Singh and Mogensen (1976) investigated the embryo–

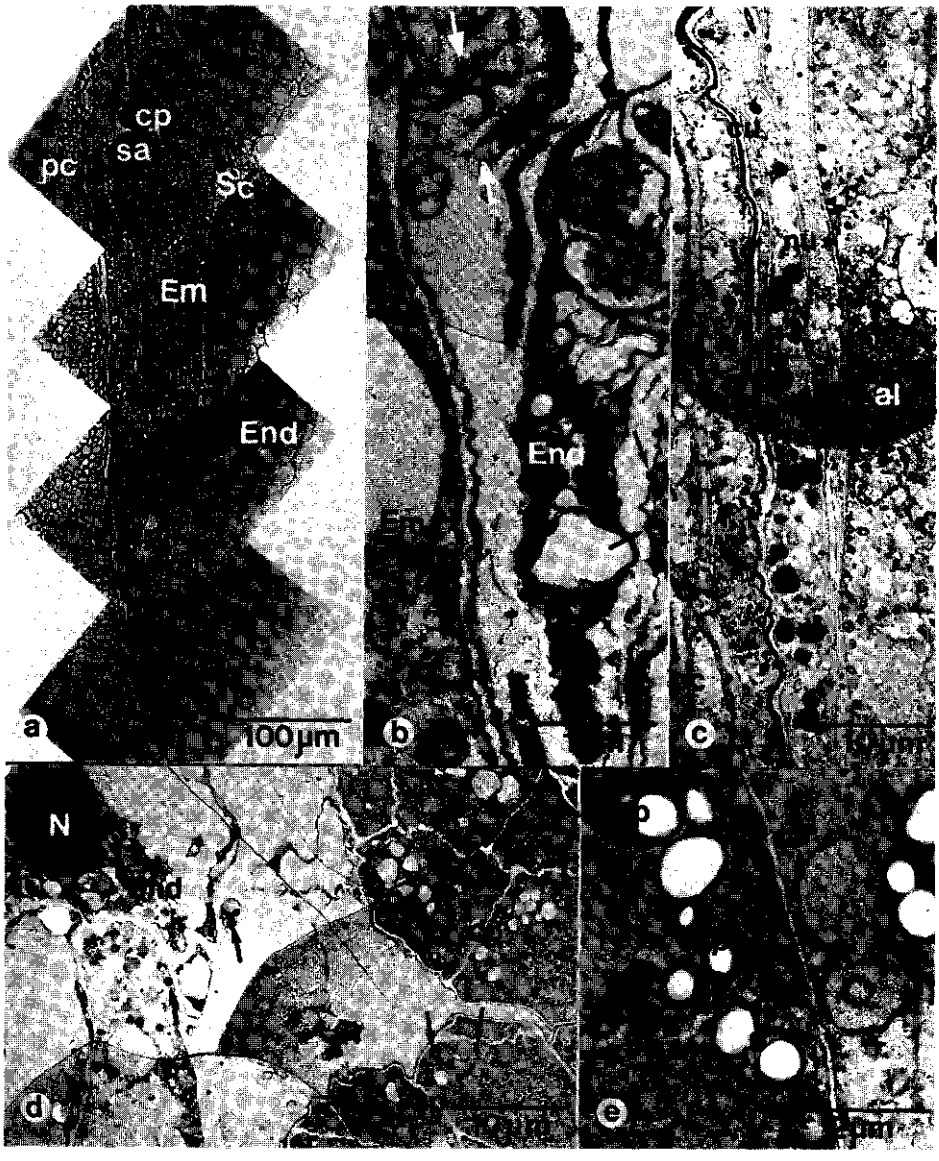


FIG. 9. (*a*) Light micrograph of a sagittal section through the embryo sac at stage IV (9–11 DAP). The embryo is located very close to the pericarp (*pc*). Coleoptile primordium (*cp*) and shoot apex (*sa*) are clearly visible. Near the scutellum, embryo tip, and embryo axis the endosperm is no longer intact. (*b*) Detail of endosperm cell remnants near the scutellum of the embryo. Only membrane fragments and loosened cell wall material remain (white arrows). Osmiophilic droplets are present (black arrows) and might migrate into the scutellum of the embryo (arrowheads). (*c*) Near the pericarp the integumentary layer (*int*) and nucellar layers (*nu*) are compressed. The outer endosperm cell layer develops into the aleuron layer (*al*). (*d*) The embryo axis cells might also take up lipids (arrows) from the degenerated endosperm. (*e*) Detail of an embryo cell containing plastids with starch.

endosperm relationships in *Quercus gambelii*. Because of the small amount of starch and lipid found in the endosperm, they questioned if the endosperm of this species is indeed functioning as a food source. They argued that the food supply might be present in a soluble form, not easily detected, or that the endosperm functions merely as a medium for the transport of food, a suggestion also made by Marinos (1970) for *Pisum sativum*. Yeung and Clutter (1978), in their work on the embryogeny of *Phaseolus coccineus*, have also noted that the contribution of the endosperm to the growth of the early embryo might not be very significant because in this plant the endosperm is still developing at the heart stage of the embryo.

The results, presented in this report on maize embryogenesis, support the emphasis placed on the absorption and transport function of the endosperm during early embryo formation rather than on the nutritive function. We suggest that both the endosperm cells surrounding the basal region of the embryo and those near the placentochalazal region serve this purpose. These cells most likely function as transfer cells in the sense first used by Gunning and Pate (1969), although the very characteristic wall ingrowths were present only in the placentochalazal region, as was observed earlier (Kiesselbach and Walker 1952).

Transport of nutrients from the placentochalazal region into the endosperm was investigated by Shannon (1972), who proved that sucrose is used by developing kernels, provided it is hydrolysed to glucose and fructose by invertase prior to uptake in the symplast of the endosperm. In a later microautoradiographic study, Felker and Shannon (1980) revealed that incoming sugars are not confined to the apoplast but rather are present in the cytoplasm and vacuoles of the endosperm cells. Concerning the transport of amino acids in the placentochalazal region, it was recently shown by Lyznik et al. (1982) that translocation takes place against the concentration gradient present in the developing maize kernel. This implies the involvement of energy in the translocation process. Moreover, significant differences in composition of the amino acid pool were found between the placentochalazal region and the endosperm.

Our results further indicate that besides the absorption and transport function of the endosperm, a high degree of synthesis, at least of membranes, also takes place. This was deduced from the huge amount of endoplasmic reticulum occurring both in the placentochalazal region and in the region bordering the base of the embryo. Because most of the highly ordered ER (Fig. 6*c*) in the endosperm surrounding the base of the embryo proved to be Rer, protein synthesis and subsequent transport in the pinched off vesicles is very likely. This mainly occurs during stages III and IV, in which the embryo has developed a long suspensor. This observation supports the active role of the suspensor in the monocotyledon maize as is the case also in dicotyledons as was shown by Yeung (1980).

The view for maize emerging at a fine structural cytological level closely resembles the situation in wheat as reported by Smart and O'Brien (1983). These authors, however, do not mention the presence of wall ingrowths in the endosperm at all, but only in the nucellar epidermis cells at later developmental stages. Therefore, the term "modified endosperm" has been used by them to describe the highly active endosperm cells lining the embryo, differentiating these cells from transfer cells which are defined by, among other criteria, wall ingrowths. In the modified endosperm of wheat, large quantities of Rer were also found, often with swollen cisternae filled with amorphous content. For maize, we observed no filling of the ER cisternae. Moreover, we found that, unlike in wheat, dictyosomes were very often clearly resolved (see Fig. 6*b*).

The dilated appearance of ER cisternae was also observed by Mares et al. (1976) studying differentiation of the aleuron layer in developing wheat kernels. They further noted a rapid and massive proliferation of ER and demonstrated that the outer nuclear membrane is involved in the process of ER formation. The authors suggested that this mechanism might be a specific response of plant cells to a sudden and massive requirement for membrane and synthetic machinery. They further cited Wischnitzer (1974), who ascribed a similar role to the annulate lamellae in oocytes of various organisms (see also Kessel 1968; Maul 1977). Our results, as shown in Figs. 5*c* and 5*d*, support these views. At least part of the newly synthesized ER membranes might originate from the nuclear envelope.

Smart and O'Brien (1983) conclude that in wheat the developing embryo is initially supplied by hydrolysis of nucellar parenchyma and endosperm cells. Hydrolysis of endosperm cells also occurs in maize, although in this case it starts somewhat later, during stage II (about 5 DAP). Breakdown is partially caused by mechanical stress, as can be deduced from the Figs. 5*a* and 5*b*. Autolysis of the endosperm or digestion by hydrolytic enzymes excreted by the embryo must be an important factor too. The breakdown of the endosperm cells near the scutellum in stage IV (Figs. 9*a* and 9*b*) is mainly an autolytic process caused by internal programmed hydrolytic activity. This is evident from the fact that no significant excretion activity is registered in the neighbouring embryo cells as measured by the absence of high Golgi activity. We also did not observe embryo cells with wall crenulations as reported by Olson (1981) for *Papaver nudicaule* and tentatively suggested by him to be indicative for an embryonic influence on endosperm breakdown. Programmed autolysis of the endosperm has been described before (e.g., see Bhatnagar and Sawhney 1981); *in vitro* studies using isolated embryo and endosperm tissues support this view (Segers et al. 1981). The products, coming free from the degenerating endosperm near the scutellum, can be absorbed by this part of the embryo. The maize scutellum is known as a fat-storing tissue, as indicated by the presence of glyoxysomes (Longo and Longo 1970). Therefore, the osmiophilic droplets as shown in Figs. 9*b* and 9*c* might be markers for such a process. We cannot, however, rule out the possibility that the embryo cells themself have synthesized these droplets.

The ordered arrangement of mitochondria just below the plasma membrane of the endosperm cells next to the integumentary remnants or suspensor cells (see Fig. 6*c*) might focus on the highly active transport processes taking place in those areas and requiring considerable energy. As proposed by Oparka et al. (1981), in developing rice caryopses these regularly aligned mitochondria, present along the upper periclinal aleuron cell walls, may function as part of a solute transport system requiring localized ATP production. The plasmalemma foldings, which were frequently observed at the embryo–endosperm interface (Figs. 4*d*, 4*e*, and 4*f*), might also be indicative for the transport processes taking place, e.g., by setting up the standing gradient osmotic flow recently discussed by Harris (1981) for plasmalemma invaginations in cotyledons of germinating mung beans.

In conclusion, we observe a close interrelationship between both embryo and endosperm in the developing maize kernel. During early embryogeny (stage I and II, until about 7 DAP),

the basal endosperm is especially highly active, supplying the embryo all along the suspensor with nutrients, which are transferred directly from the placentochalazal region by a small area of endospermic transfer cells or are newly synthesized in the modified endosperm region lining the embryo. From this study, no conclusions could be drawn as to the chemical composition of the nutrients. It is probable, however, that the main constituents are dissolved salts, peptides, and amino acids. From stage III, starting about 7 DAP, the nutritive function of the highly vacuolated and degenerating upper endosperm might become increasingly important, carbohydrates and (phospho)-lipids most likely being the main food resources.

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CHAPTER 6

Cell differentiation in the pericarp and endosperm of developing maize kernels (*Zea mays* L.) with special reference to the microtubular cytoskeleton

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CELL DIFFERENTIATION IN THE PERICARP AND ENDOSPERM OF DEVELOPING MAIZE KERNELS (*ZEA MAYS* L.) WITH SPECIAL REFERENCE TO THE MICROTUBULAR CYTOSKELETON

ABSTRACT

Maize kernels were investigated by light and electron microscopy to determine and quantify cytological changes in fruit wall and seed at various developmental stages.

Pericarp, integument and nucellus cells differentiated and subsequently degenerated within a period of three weeks. Most prominent feature in the pericarp development was the differentiation of three zones, each with cells that are shaped and arranged differently. Cell elongation and wall thickening in exocarp and endocarp cells lead to protecting layers. The early formation of a spongy mesocarp facilitated compression of the fruit wall in later stages. Integuments and nucellus degenerated soon; the endosperm persisted and enlarged.

Endosperm development is characterized by the formation of the aleurone layer, some sub-aleurone layers and inner endosperm from 5 days after pollination onwards. The cell layers differ from each other by the shapes of the cells, the diverging development of vacuoles and plastids, the rate of starch accumulation, the increase of cytoplasm, and by the formation of various types of spherosomes and protein bodies. The cellular distribution of the mitochondria in aleurone and sub-aleurone layers point to an active transport through cell membranes towards or from the nucellus.

Microtubules were visualized immunocytochemically in semi-thin sections (2-3 µm) to determine their three-dimensional distribution in the cytoplasm. Microtubules were observed in three major configurations: spindle tubules, cytoplasmic microtubules which run throughout the cytoplasm, and cortical microtubules which appear to form various configurations in the periphere cytoplasm. The cortical microtubules were investigated in particular. They formed various configurations from parallel to criss-cross arrangements. From the

development of cell shapes and the observed concomitant configurations of microtubules it is concluded that these microtubules mediate in the cytomorphogenesis and likely by influencing the mode of cell wall synthesis.

1. INTRODUCTION

The structural aspects of caryopsis development have been studied for important cereals as wheat (Buttrose 1963b, Campbell et al. 1981, Cochrane 1983, Rogers & Quatrano 1983, Smart & O'Brien 1983), barley (Olsen & Krekling 1980, Felker et al. 1984, Kvaale & Olsen 1986), rye (Pomeranz 1973) and rice (Bechtel & Pomeranz 1977, Bechtel & Juliano 1980, Miflin et al. 1981, Oparka & Harris 1982). A general survey related to seed development is given by Bhatnagar & Sawhney (1981). Developmental studies of maize go back to the last century; many early workers analyzed spikelet formation, fertilization, and the development of fruits and seeds from a structural point of view (see, amongst others, Guérin 1899, Miller 1919, Weatherwax 1919, Lampe 1931, Randolph 1936, Kiesselbach 1949). More recently, electron microscopy stimulated the sub-cellular analysis of fertilization (Diboll 1964, Van Lammeren 1986a), the development of the proembryo (Van Lammeren & Schel 1983, Schel et al. 1984), the embryo proper (Van Lammeren 1986b), the endosperm (Khoo & Wolf 1970, Kyle & Styles 1977) and of the interaction between embryo and endosperm (Schel et al. 1984).

The endosperm is considered as an important nutritional factor for embryo development and germination. Especially the outermost cell layer, the aleurone layer which can be distinguished by the shapes of its cells and the organelles within the cells, accumulates storage products during seed development and releases hydrolytic enzymes during germination (Lampe 1931, Bhatnagar & Sawhney 1981). The ultrastructural development of the aleurone layer and the sub-aleurone layers have been studied before (Khoo & Wolf 1970, Kyle & Styles 1977); however, the development of the cell shapes, the interaction

between the various tissues of the caryopsis and the concomitant manifestation of (ultra)structural phenomena have as yet not been observed.

The present study focusses on the cellular and sub-cellular changes in carpels, integument, nucellus and part of the endosperm from the moment of cellularization of the endosperm up to and including the senescence of the pericarp cells. In all tissues of the caryopsis cell differentiation resulted not only in a diverse composition of the cytoplasm but in varying cell shapes as well. In order to elucidate the function of the microtubular cytoskeleton in this process of cytomorphogenesis (Lloyd 1982, Lang Selker & Green 1984, Van Lammeren 1985, Van Lammeren et al. 1985, Traas 1986) special attention was paid to the structure of the cytoskeleton by the application of immunofluorescence microscopy.

2. MATERIALS AND METHODS

Maize plants of strain A188 were grown and pollinated as described previously (Van Lammeren 1986b). From 3 up to 22 days after pollination (DAP) thick (1mm) median sections were cut from the ovaries and small, 2 mm^3 blocks were excised from a defined region (see Fig. 1). The blocks contained the pericarp, the aleurone and sub-aleurone layers and some inner endosperm.

For phase contrast and electron microscopy the tissue blocks were fixed with 2.5% glutaraldehyde in 0.02 M phosphate buffer, pH 6.8 with 2 mM EGTA for 2 hrs at 20°C. After rinsing, the samples were post-fixed in 2% OsO_4 in buffer and dehydrated through a graded series of acetone and embedded in Epon. Semithin (2 µm) sections were cut with glass knives and ultrathin sections were prepared with a diamond knife on an LKB Ultrotome V. The latter sections were post-stained in an LKB Ultrostainer with uranyl acetate for 15-30 min. at 40°C and with lead citrate for 30-60 sec. at 20°C before investigation in a Philips EM 301 operating at 60 kV.

The fixation, embedding and sectioning of plant tissues preceding the immunocytochemical labelling of microtubules were described

previously (Van Lammeren et al. 1985) but small adaptations were performed. Blocks were fixed in 3% para-formaldehyde and 0.13% glutaraldehyde in phosphate buffered saline (PBS) pH 7.2 for 1 hr at 20°C. After rinsing and dehydration the samples were transferred into a mixture of ethanol and two types of polyethylene glycol (PEG 1500/4000, BDH Chemicals Ltd., Poole); ethanol:PEG= 2:1 (w/w) for 1hr, 1:1 for 1 hr, and 100% PEG for 1 hr, all at 55°C. Finally the samples were embedded in pure PEG 1500/4000 and cooled down. Sections of 2-3 μm were picked up with hanging drops of 40% (w/w) PEG 6000 in PBS using a wire loop and mounted on poly-lysine coated slides as described before (Van Lammeren et al. 1985). After that, the PEG was washed away with PBS; the aldehydes in the embedment-free sections were quenched with 15 mM $NaBH_4$ and 0.1 M NH_4Cl for 5 min. each and treated with 2% bovine serum albumin (BSA, essentially globulin free, Sigma, St Louis) in PBS for 30 min. To detect microtubules, the sections were then incubated with the IgG fraction of a polyclonal antitubulin (dilution 1:25 in PBS) raised in rabbit against rat brain tubulin (see Van Lammeren et al. 1985). After 45 min. incubation at 20°C the sections were rinsed in PBS for 45 min. and incubated again with a second, FITC-conjugated antibody which was raised in goat against rabbit (GAR/FITC, Nordic, Tilburg; dilution 1:25 in PBS). After that, the sections were rinsed again and embedded in a mixture of 20% Mowiol 5-88 (Hoechst, Frankfurt am Main) in Citifluor (Citifluor Ltd, London).

3. RESULTS

3.1. Development of somatic tissues in the caryopsis

The development, differentiation and degeneration of the carpels, the integument and the nucellus were traced by means of light and electron microscopy within a restricted zone at the germinal face of the caryopsis (Fig. 1) during a period of three weeks after pollination. In the pericarp, three zones were distinguished at three days after pollination (3 DAP): an endocarp zone near the ovule consisting

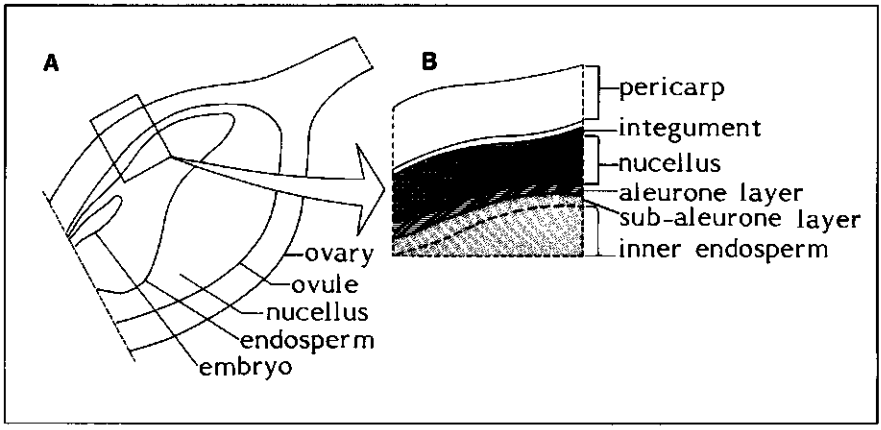


Fig. 1 Schematic representation of the median section of a young maize caryopsis with the sampling area enlined (A) and the anatomy of the sampling block (B).

of about three cell layers of small, thin-walled fusiform cells which were often isodiametric in section and elongated out of the plane of sectioning (Fig. 2a); a mesocarp zone with larger, loosely arranged cells which are isodiametric to irregular in shape (Fig. 2a) and an exocarp zone containing cylindrical cells which bordered each other leaving only little intercellular space. All pericarp cells had large central vacuoles and only little cytoplasm in which amyloplasts were observed (Fig. 3a, arrow). As development proceeded, however, the growth of the developing seed forced the pericarp to enclose a larger area. At 8 DAP the exocarp cells had elongated. In the mesocarp region a spongy mesophyll developed with spherical, slightly elongated or curled cells (Fig. 2b). The endocarp cells did not increase much in diameter but they separated, thus enlarging the intercellular space (Fig. 2b, arrow). In cross-sections the cells were often found parallel to each other forming fibrous sheaths covering the developing seed. The length axes of the various sheaths were not fixed. At 17 DAP the pericarp cells were still intact yet at an end phase of development (Fig. 2c). The diameter of the endocarp cells had not enlarged much. The exocarp cells had reached their maximal length

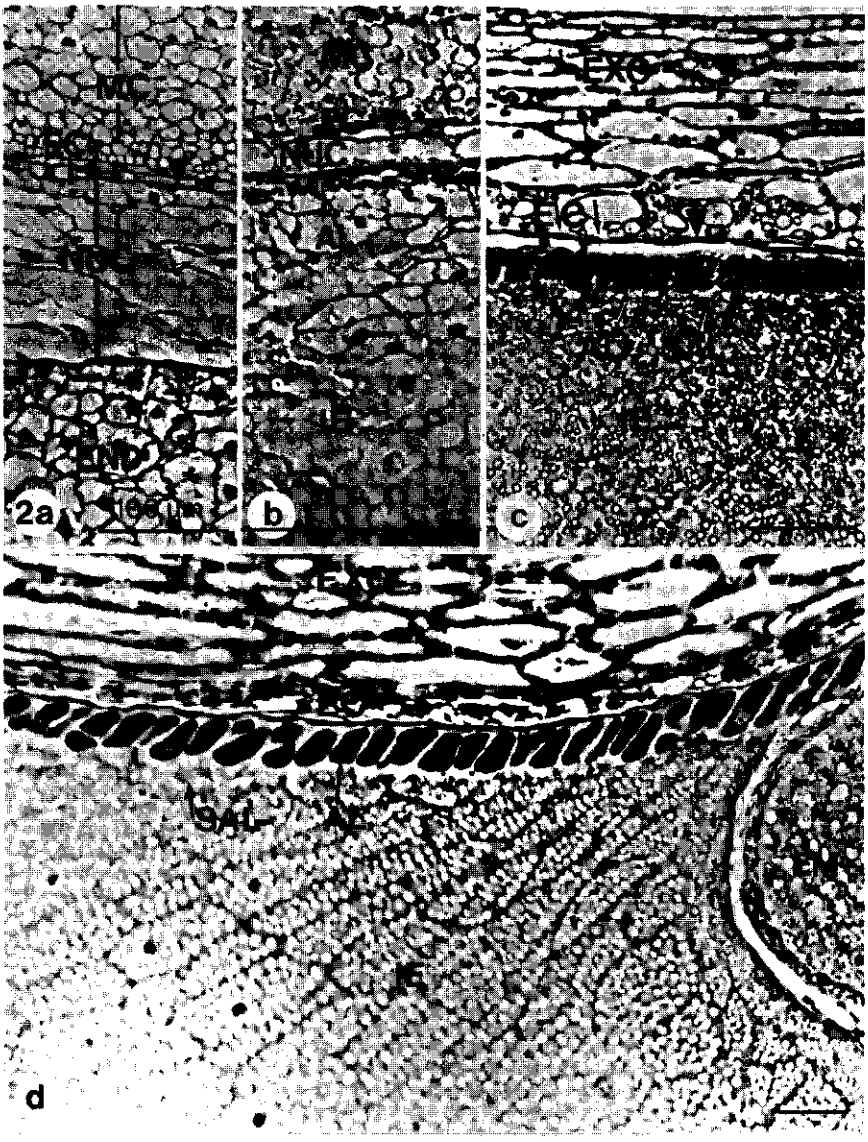


Fig. 2 Low magnification light micrographs of 2 µm Epon sections from the sampling area of the maize caryopsis at various developmental stages. Note the enlargement, differentiation and subsequent degeneration of the endocarp, mesocarp and exocarp cells. The

thereby leaving only little intercellular space. Their cell walls had thickened giving solidity to the fruit wall. The spongy mesocarp cells were compressed and so the thickness of the whole pericarp decreased. The cytoplasm of the remaining cells degenerated at about 20 DAP thus leaving a multi-layered dead fruit wall mainly consisting of cell walls. At 22 DAP the small endocarp cells were often crushed whereas the shape of the dead exocarp cells was still preserved (Fig. 2d).

Integuments covered the nucellus and the endosperm during the early developmental stages. Because of the retarded growth of the outer integument during ovule development, only an inner integument covered the nucellus at the sampling area. It consisted of two cell layers and a thin cuticle. The integumentary cells were cylindrical at 3 DAP (Fig. 2a, arrow). Sometimes they were only seen at this stage but occasionally the intact cytoplasm was still observed at 8 DAP. Hereafter cells were crushed between the pericarp and the expanding endosperm and their thin cell walls and the cuticles disappeared completely.

The nucellus is an ephemeral tissue, too. Except for the epidermis and its cuticle, the nucellus degenerated fast. At 3 DAP a multi-layered nucellus still existed (Fig. 2a) but at 5 DAP only 1 or 2 layers of nucellus cells remained (Fig. 3a) in the sampling area.

integument and sub-epidermal nucellus cells degenerated soon (a-b). The endosperm developed into the aleurone, sub aleurone and inner endosperm, all accumulating proteins and starch. a= 3 DAP; b= 8 DAP; c= 17 DAP; d= 20 DAP. The bars represent 100 μm.

Abbreviations to the figures: AB= aleurone body; AL=aleurone layer; DAP=days after pollination; EC=endocarp; EM=embryo; END= endosperm; EXC=exocarp; EPB=endosperm protein body; I=integument; IE=inner endosperm; IS=intercellular space; MC=mesocarp; NUC=nucellus; PC= pericarp; S=starch; SAL=sub-aleurone layer; S1=spherosome type 1 present in aleurone and sub-aleurone layer; S2=spherosome type 2 present in the aleurone layer from 8 DAP onwards.

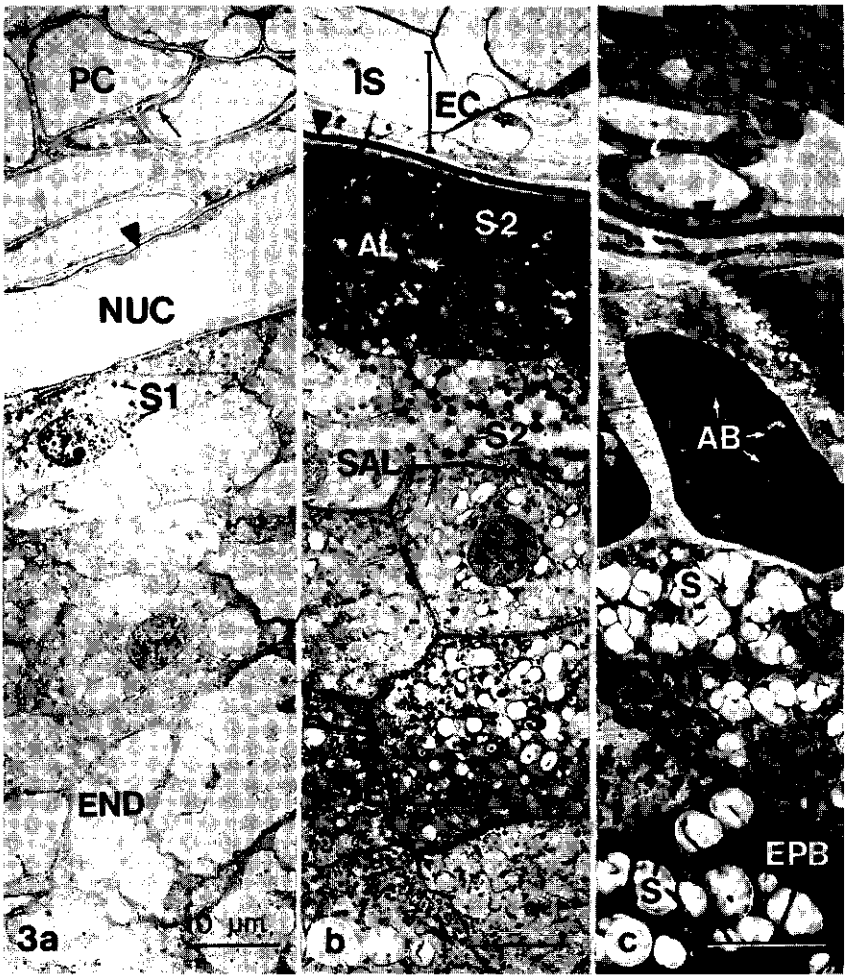


Fig. 3 Electron micrographs of the developing maize caryopsis in the sampling area at various developmental stages. Conspicuous changes in the aleurone and sub-aleurone layers include the differentiation of an epidermal-like tissue (a-b) and the accumulation of starch (S, b-c) and storage products in two types of spherosomes (S1, S2), aleurone bodies (AB) and endosperm protein bodies (EPB).For abbreviations see Fig. 2. a=5 DAP; b=12 DAP; c=22 DAP. The bars represent 10 µm.

Cell degeneration occurred near the outermost cell layer of the endosperm. First the cells exhibited cytoplasmic degeneration. Then they collapsed and eventually they disappeared. The nucellus epidermis was observed as a living tissue at 8 DAP. It consisted of flattened cells covered with a thick, conspicuous cuticle which was still found in the late developmental stages (Figs. 2 and 3,arrow-heads). There was a large central vacuole and only little cytoplasm. From 12 DAP onwards nucellar epidermis cells collapsed (Figs. 3b, 7-IV). The remnants were found up to 22 DAP because the cuticle, the outer cell walls and the partly thickened anticlinal cell walls persisted (Fig. 3c, arrow-heads).

The course of cell development of the various somatic tissues described above is summarized schematically in Table 1 with respect to cell shape development, cell wall formation and viability.

3.2. Development of the endosperm

The development of aleurone, sub-aleurone and inner endosperm involved the multiplication, enlargement and differentiation of cells and the accumulation of storage products (see Fig. 1b for the topography of the sampling area and the Figs. 7 I-V for 'camera lucida' drawings). The development of the aleurone layer became prominent from 5 DAP onwards (Fig. 3a-c). Initially the outer endosperm cells, which developed into the aleurone cells, were large (one cell of the nucellus epidermis covered 2-3 pre-aleurone cells, Fig. 7-I) and divided in various directions but at about 7 DAP they divided simultaneously and in only one direction. Many nuclei exhibited prophase configurations at 5-6 DAP and at 8 DAP cytokinesis had resulted in the formation of an epidermal-like cell layer in which mainly anticlinal cell walls had formed (Fig. 7-II). The newly formed cells were much smaller; up to 8 epidermal cells were now covered by one nucellus epidermal cell which had not increased in size. After that, the epidermal cells became rectangular, the amount of cytoplasm and organelles still increased, and the accumulation of storage products started (Fig. 3b, 12 DAP). From 8 to 12 DAP the cells of the outer-

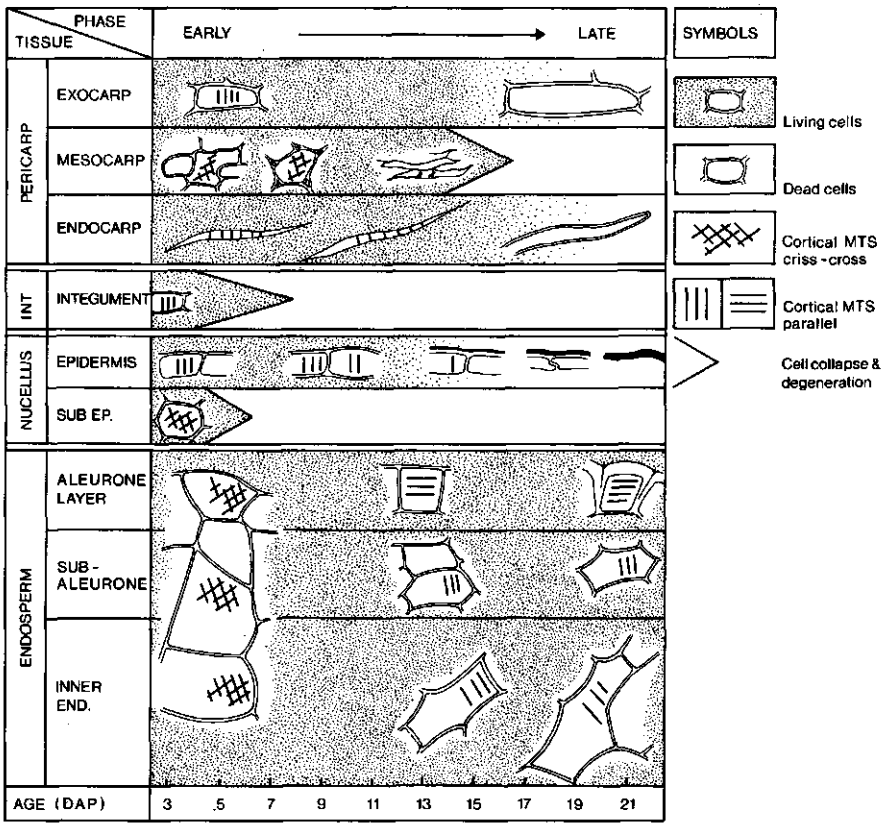


Table 1 Schematic representation of cell differentiation and concomitant configurations of microtubular cytoskeletons in various tissues of the developing caryopsis of maize.

most cell layer of the endosperm differentiated into aleurone cells. They enlarged in periclinal direction and up to 5 aleurone cells were covered by one nucellus cell at 12 DAP. Sub-aleurone cells were often found in line with the aleurone cells indicating that periclinal cell wall formation took place repeatedly as endosperm development proceeded. These cells continued to divide, sometimes in a meristem-like fashion forming rows of cells with new periclinal cell walls. The newly formed, isodiametric cells were often smaller than the

bordering aleurone cells (Fig. 3b). The inner endosperm cells, however, enlarged as shown in Fig. 3b. During further seed development the aleurone cells enlarged in periclinal and anticlinal direction (Up to 4 aleurone cells were covered by one cell of the nucellus epidermis at 17 DAP, Fig. 7-III). In the ensuing development, from 17 to 22 DAP, only slight changes in the sizes and shapes of the cells were to be seen. The anticlinal and outer cell walls increased in thickness from 17 DAP onwards (Fig. 3c). Sub-aleurone and inner endosperm cells, however, still increased in size (cf Figs. 2c and 2d). These cytomorphological data of endosperm formation are summarized schematically in Table 1 together with the somatic tissues of the caryopsis.

The submicroscopical aspects of endosperm differentiation are summarized for the developing aleurone layer and the sub-aleurone endosperm in Table 2 and 3 respectively. Nuclei, being the largest organelles, were about spherical to ellipsoidal and had undulating membranes. They did not differ in size and shape in aleurone and sub-aleurone cells, nor did their internal structure. At 3 and 5 DAP the nuclei of the outermost endosperm layer were often observed near the outer cell wall. Plastids were found in all endosperm cells. In the aleurone layer they were smallest and accumulated starch from 12 DAP onwards. At 22 DAP these amyloplasts occupied up to 12% of the cell volume. In the sub-aleurone cells the plastids accumulated starch from 8 DAP onwards and up to 46% of the cell volume was filled with these second largest organelles at 22 DAP. Thylakoids were found in all stages but grana were never detected. Mitochondria were never abundant in the endosperm. At 3 DAP they were not equally spread over the cytoplasm in the outer cell layers but preferentially found in the cytoplasm adjacent to the outer periclinal cell walls (Fig. 3a, thin arrow). This phenomenon could be observed at later stages, too, and also in the sub-aleurone layer (Fig. 3b, thin arrows).

Vacuoles occupied about 40% of the cell volume of young aleurone and sub-aleurone endosperm cells (Fig. 2a, 3 DAP). At 22 DAP only few, small vacuoles were observed in the sub-aleurone cells but in the aleurone layer about 10% of the cell volume was occupied by

Table 2 Schematic representation of organelle development in differentiating aleurone cells of Zea mays L. from 3 to 22 days after pollination (DAP).

Table 3 Schematic representation of organelle development in differentiating sub-aleurone endosperm cells of Zea mays L. from 3 to 22 days after pollination (DAP).

vacuoles. Only in the aleurone cells they functioned as aleurone vacuoles by the accumulation of storage proteins, a phenomenon which is depicted in Fig. 4 and which was observed from 5 DAP onwards. An osmiophilic, flocculent precipitate was formed in the centre of the spherical vacuoles (Fig. 4a, arrows) and at 12 DAP a dense precipitate was often found near the tonoplast (Fig. 4b, asterisk). The

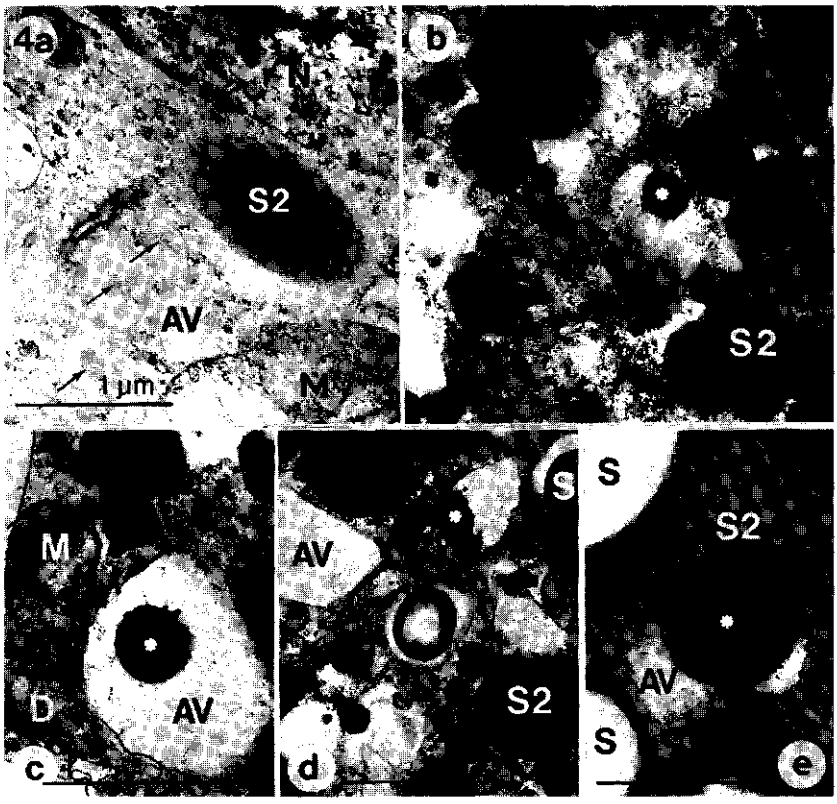


Fig. 4 High magnification electron micrographs of the aleurone cells of Zea mays L. endosperm at various stages of development. Note the accumulation of spherosomes (S1) and the development of the aleurone bodies (AB) from the aleurone vacuoles (AV). a=8 DAP, b=12 DAP, c-d=19 DAP, e=22 DAP. For further abbreviations see legend Fig. 2. The bars represent 1 µm.

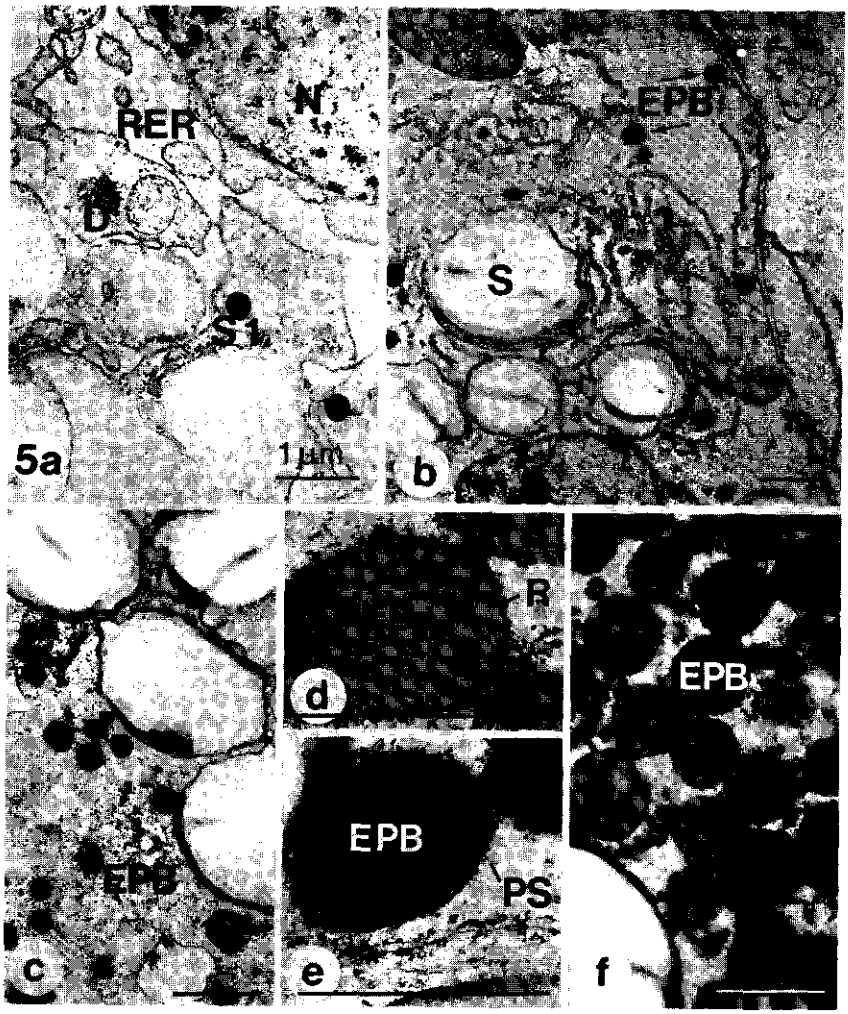


Fig. 5 High magnification electron micrographs of the cytoplasm of sub-aleurone and inner endosperm cells of Zea mays L. at various developmental stages. Note the changes in ER configuration, the development and accumulation of endosperm protein bodies (EPB) from the ER and the appearance of amyloplasts. a=5 DAP, b=8 DAP, c=12 DAP, d=17 DAP, e=19 DAP, f=22 DAP. For abbreviations see legend Fig. 2. The bars represent 1 µm.

amount of storage proteins clearly increased and at 22 DAP they almost filled the vacuoles, which are then called aleurone bodies (Figs. 4c-e, asterisks).

The endoplasmic reticulum proved to be a conspicuous organelle system, especially in the sub-aleurone cells which ultrastructural development is shown in Fig. 5. Initially, at 3 DAP, the ER occupied but a minor part of the cell volume. At 5 DAP there was, however, a significant increase of the cisternal space because of the swelling of the ER by which up to 15% of the cell volume was occupied (Fig. 5a). In the sub-aleurone and inner endosperm the ER, to which many ribosomes were attached, gave rise to electron-dense globular structures which are called endosperm protein bodies (EPBs, Figs. 5b-e). These EPBs which were surrounded by a ribosome carrying ER membrane (Figs. 5d,e) were formed from 8 DAP onwards and had an average diameter of 0.14 $\pm$ 0.04 μm at 8 DAP and 0.44 $\pm$ 0.09 μm at 22 DAP. Their presence was restricted to the sub-aleurone and inner endosperm where they eventually occupied up to 70% of the cell volume (see Figs. 3c and 5f) at 22 DAP. The heavily stained ribosomes occurred singly or as short coils of polysomes. Dictyosomes were not seen frequently and in the aleurone layer their presence could not be established at 22 DAP because of the densely stained cytoplasm.

Concerning other storage products, two types of spherosomes were discerned in the developing endosperm. The first type (Type S1) was mainly seen in the outer cell layers of the young endosperm up to an age of 8 DAP (Figs. 3a and 5a). Their number and total amount decreased in the aleurone layer when the first spherosomes of a second type (Type S2) were discerned. This second type predominantly occurred in the aleurone layer (Figs. 3b and 4). Some of these spherosomes were seen in the adjacent cell layer but none in the inner endosperm. Tye S2 spherosomes were largest (1.26 + 0.68 μm at 17 DAP) and sometimes filled the aleurone cell cytoplasm for over 90% at 22 DAP (Figs. 3c,4e).

3.3 Differentiation of the microtubular cytoskeleton

Microtubules were present in all developmental stages. The shape of the cytoskeleton and the arrangement of the microtubules in the cells were related to the cell shape and to the developmental stage of the tissue rather than to the position of the organelles. Three major configurations of the microtubular cytoskeleton were distinguished. In dividing cells spindle microtubules were observed and in interphase cells two more configurations of cytoskeletal microtubules were found. In one configuration microtubules ran throughout the cytoplasm and are known as cytoplasmic microtubules and in the other the microtubules were found in a thin layer of cytoplasm within the first 100 nm adjacent to the cell membrane and are known as cortical microtubules (Fig. 6).

At 3 DAP microtubules were found in the various tissues of the sampling area i.e. in the pericarp, the integument, the nucellus and the endosperm (Fig. 7a,b). During cell division microtubules were only found in the spindles. In interphase cells most microtubules were cortical microtubules. Their orientation varied at this stage of development. In isodiametric mesocarp cells most cortical microtubules were randomly oriented. When, however, pericarp cells were cylindrical, the microtubules were oriented normally or obliquely to the length axis of the cells (Fig. 7a). The same phenomenon was observed in the long integumentary cells and in the nucellus epidermis cells (Fig. 7b). The sub-epidermal and inner cells of the nucellus were isodiametric. They exhibited a clear criss-cross arrangement of cortical microtubules. This arrangement was also found throughout the young endosperm.

At 5 DAP the orientation of microtubules in the carpels had not changed. When the integumentary cells were still intact they exhibited the original distribution of microtubules. Within the highly vacuolated endosperm cells most microtubules were found adjacent to the cell membranes but near the nucleus cytoplasmic microtubules were observed as well. When anti-clinal rows of endosperm cells were formed the cortical microtubules were arranged periclinally against those anti-clinal cell walls. In isodiametric endosperm cells still a

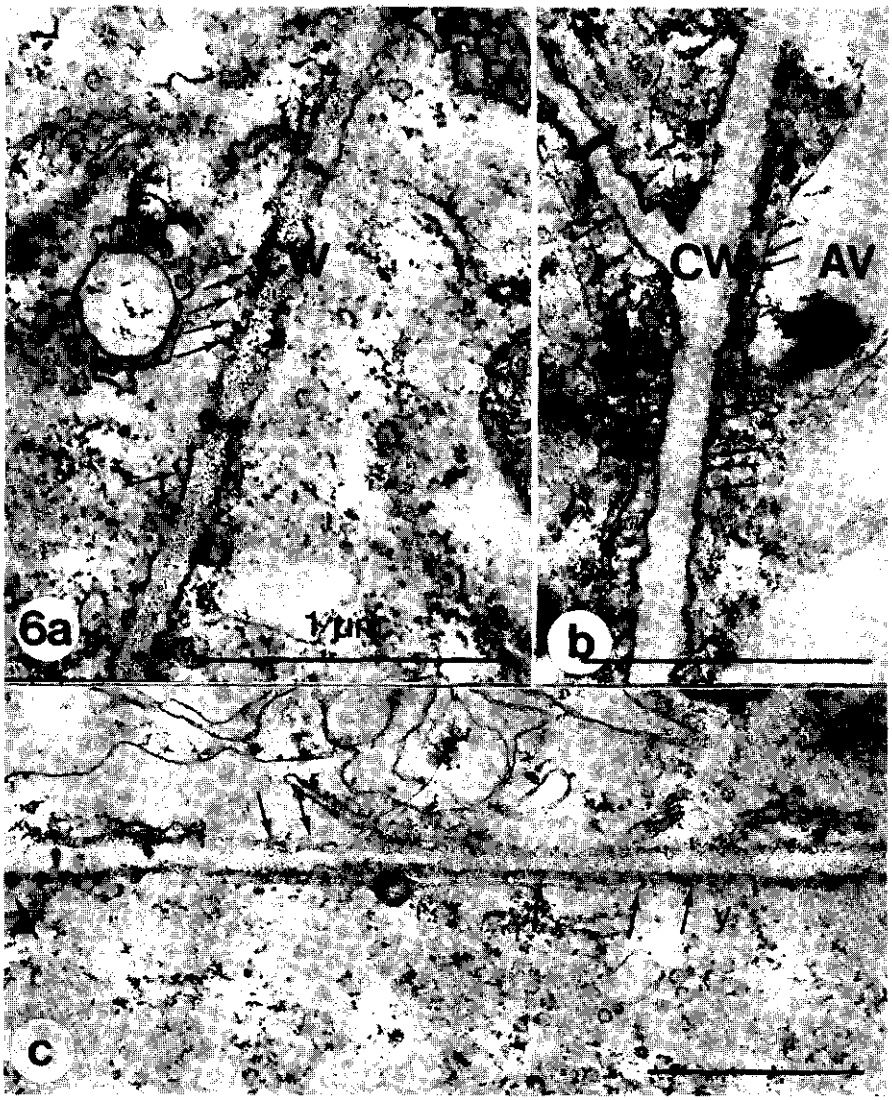


Fig. 6 Electron micrographs of cytoplasm and cortical microtubules (arrows) in ultrathin Epon sections of the endosperm of Zea mays L. Glutaraldehyde- OsO_4 fixation and Pb-citrate and Ur-acetate post staining. a.Inner endosperm at 8 DAP; b. Aleurone cells at 8 DAP; c. Aleurone cells at 17 DAP. The bars represent 1 µm. For abbreviations see legend Fig. 2.

criss-cross pattern of cortical microtubules was observed both in the outermost layer as well as in the adjacent cell layers.

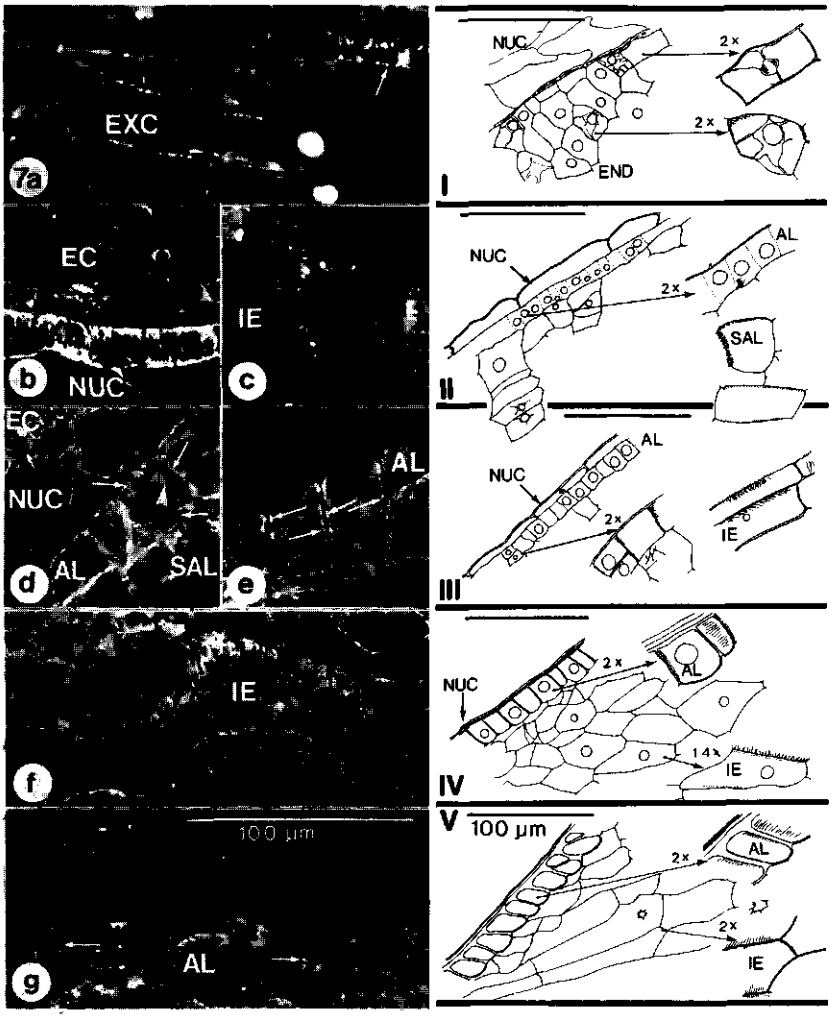
At 8 DAP microtubules were found in all living cells. Pericarp cells exhibited predominantly cortical microtubules in the same orientations as observed before. Additionally the endocarp cells developed parallel arrays of microtubules perpendicular to the length axis of the elongated cells. In the cytoplasmic remains of the compressed integuments microtubules were observed in the original distribution. The outermost one or two layers of nucellus cells exhibited microtubules in an unchanged orientation. The inner endosperm cells still had criss-cross patterns of cortical microtubules (Fig. 7c). After the synchronous division of the outermost cell layer of the endosperm a higher concentration of cortical microtubules was observed in the newly formed cells (Fig. 7d, arrow) but near to the nuclei cytoplasmic microtubules were also found (Fig. 7d, arrowhead).

In the period of 12 to 15 DAP microtubules were still present in the inner cells of the pericarp and in living cells of the nucellus epidermis which covered the endosperm. The aleurone layer exhibited a special arrangement of microtubules from then on. Initially microtubules were observed alongside all cell walls (Fig. 7d, arrows). Hereafter bundles of cortical microtubules were found in parallel arrays along the anticlinal cell walls (Fig. 7e, arrows). Cytoplasmic microtubules were frequently observed around the nuclei. Spindle tubules were seen when anticlinal cell rows were formed.

At 15 to 17 DAP the pericarp still contained living cells with a well detectable microtubular cytoskeleton. In the fusiform endocarp cells microtubules exhibited a parallel arrangement and in the spongy mesocarp cells, if not yet compressed, a random orientation was observed. Microtubules were found in the nucellus epidermis. Sometimes, howevever, the nucellus epidermis had already collapsed and cytoplasm could no longer be observed. In the aleurone layer the cortical microtubules had the same orientation as before. The subaleurone and inner endosperm cells exhibited criss-cross patterns of microtubules when they were isodiametric but they showed an parallel

arrays of microtubules normal to the growth direction when the cells enlarged and elongated (Fig. 7f).

From 19 to 22 DAP the degeneration of the carpel was nearly completed and consequently only few cells still contained a microtubular cytoskeleton (Fig. 7g). The remnants of the nucellus were without any cytoplasm. In the aleurone layer most microtubules were positioned as before. In the sub-epidermal and inner endosperm microtubules were either parallel to each other in the elongated cells or



they exhibited a criss-cross pattern in the isodiametric cells. The data concerning the microtubular configurations as well as the concomitant cell shape formation in the endosperm are summarized in the Figs. 7 I to V and a complete scheme for microtubular configurations in the whole sampling area is given in Table 1 together with the cytomorphological data.

Fig. 7 Distribution of microtubules in the developing caryopsis of Zea mays L. from 3 to 22 DAP. Microtubules in 2 µm sections are labeled indirectly with FITC conjugates after formaldehyde-glutaraldehyde fixation (a-g). a= exocarp at 3 DAP with cortical microtubules often arranged normal to the length axis of the cell. b=endocarp, integument and nucellus at 3 DAP. Note the abundance of parallelly arranged cortical microtubules normal to the length axis of the nucellus epidermal cells. c=Inner endosperm cells at 8 DAP with a criss-cross texture of cortical microtubules. d=Endocarp, nucellus epidermis, aleurone and sub-aleurone cell at 15 DAP. Note the presence of the microtubules in endocarp and aleurone cells (arrows). e=Aleurone and sub-aleurone cells at 17 DAP. Microtubules are predominantly observed along the anticlinal cell walls of the aleurone cells (arrows). f=long inner endosperm cells at 17 DAP show parallel arranged microtubules. g=Aleurone cells at 19 DAP with microtubules along the anticlinal cell walls (arrows). All micrographs have the same magnification. Based upon light micrographs the distribution of microtubules is represented schematically in the drawings I to V. I= 3-4 DAP: criss-cross patterns occur frequent in the endosperm. II=8 DAP: Nucellus epidermis remains; criss-cross and parallel arrangements are present in aleurone and sub-aleurone cells. III=17 DAP: Aleurone layer is well organized and exhibits parallel cortical microtubules. IV=19 DAP : Nucellus epidermis collapsed; parallel arrays of microtubules occur in aleurone and inner endosperm. V=22 DAP : Cell walls of the aleurone cells thicken, inner endosperm cells enlarge and both have parallel arrays of microtubules. The bars represent the magnification of the left-hand drawings; the details are enlarged twofold or 1.4x. For abbreviations see legend Fig. 2.

4. DISCUSSION

4.1. Cell differentiation in the caryopsis.

The pericarp, known for its determined life time (see , amongst others, Randolph 1936), developed various cell layers each with cells of particular shape, wall thickness and corresponding mechanical properties. From 22 DAP onwards the exocarp consisted of rigid cell walls while the cytoplasm of the cells was degenerated. Most dead mesocarp cells were compressed between the exocarp and the seed but the walls of the small fusiform cells of the endocarp were still well recognizable. The pericarp, which initially showed a three-partite structure as is common for the caryopses of many Gramineae, protected the seed. Throughout their life span pericarp cells had a large central vacuole. As in other Gramineae the carpels did not contribute to any food storage. The accumulation of starch was ephemeral. The intimate relation between pericarp and underlying tissues was expressed by the lack of a real inner epidermis. As was also mentioned for wheat and barley the fusiform cells of the endocarp vary in direction between individual caryopses. They appeared as a network of so called "cellules tubulaires" (Guérin 1899) forming the inner face of the pericarp. Actually the nucellar cuticle is the demarcation between fruit wall and seed because the integumentary cells had already degenerated.

The life time of the integuments has been discussed by various authors, amongst others, Randolph (1936), who observed initial stages of the desintegration of the integuments at the germinal face of the caryopsis in samples examined 3 DAP. He reported that the walls of the outer cell layer disappeared first while nuclei and cytoplasm often persisted for some time. In the present study the degeneration was often accomplished before 5 DAP but incidentally at 8 DAP cytoplasm with microtubules was still observed. This asynchrony in development can be attributed to differences in developmental rates in the various caryopses. The inner integument influences the pathway of the pollen tube (Van Lammeren 1986a) rather than the subsequent seed formation.

The nucellus is also an ephemeral tissue and it is likely that it functions in nourishing the developing endosperm when cells are digested near the endosperm epidermis. The nucellus epidermis and its cuticle existed longest and covered the endosperm. They might function as a layer providing mechanical protection because its cells lived until the cell walls of the aleurone layer started to thicken. Eventually the cells died and only the remnants of the anticlinal and outer cell walls and the cuticle of the nucellus persisted. These remnants were called the nucellar membrane (Randolph 1936, Kiesselbach 1949). At this stage that structure is likely to function as a protective covering of the embryo and endosperm because of its semi permeable properties (Randolph 1936).

Endosperm formation in maize is of the nuclear type initially but cellularization started within 3 DAP in the present study. The fine structure of the formation of the first anticlinal cell walls and the subsequent elaboration of periclinal cell walls and rows of endosperm cells has only been described for Triticum aestivum L. (Buttrose 1963a, Morrison et al. 1978, Fineran et al. 1982). For maize the process was studied by light microscopy (Weatherwax 1930, Lampe 1931, Randolph 1936 and Kiesselbach 1949). Kyle and Styles (1977) applied electron microscopy and described the development of the aleurone and sub-aleurone layer of the maize inbred line W-22. The endosperm development in wheat was studied by, amongst others, Mares et al. (1975,1976) and Morrison et al. (1978).

In the caryopses of the maize inbred line A188 the differentiation of the aleurone layer started at about 7 DAP with the synchronous cell divisions by which one layer of columnar cells was formed. In our experiments the phase of differentiation started 3 days earlier than reported by Kyle & Styles (1977). In contrast to e.g. barley, in which the aleurone layer consists of as many as four cell layers, one layer is usual in maize but two cell layers were found incidentally. Aleurone and sub-aleurone endosperm differed from each other in many aspects. From 8 DAP onwards periclinal cell divisions were not observed any longer in the aleurone layer but the cells enlarged and became rectangular. Sub-aleurone cells descended from the aleurone

cells by asynchronous, anticlinal cell divisions.

With respect to the type and location of organelles some similarities were observed in the aleurone and sub-aleurone layers of this maize strain. Both layers had the typically located mitochondria. The alignement along the outer periclinal cell walls points to a high and local demand for energy to permit active transmembranal export and import. This phenemenon was also observed in the synergids of maize before fertilization (Van Lammeren 1986a) and in the modified endosperm bordering the maize embryo suspensor (see Schel et al. 1984). Both cell types showed remarkable proliferation of the ER, though, not as intense as was reported for wheat where evaginations of the nuclear membrane took part of the ER system (Kyle & Styles 1977). Furthermore, an increasing amount of cytoplasm was noticed on all cell types and starch accumulated in all plastids which is in contrast to the observations of Khoo & Wolf (1970) who only found proplastids in the aleurone layer. The difference might be caused by varying developmental rates which are influenced by genotype and environmental conditions. On the other hand the organization of the cytoplasm differed between the epidermal, subepidermal and inner endosperm cells as early as 3 DAP. Differences were found in the accumulation of spherosomes (Type S1) in the various tissues at 3 DAP; The synthesis of endosperm protein bodies from 7 DAP onwards was restricted to the sub-aleurone cells and inner endosperm cells, and the formation of aleurone bodies and spherosomes which started at 9 DAP occured only in the aleurone cells.

The development and structure of protein bodies and spherosomes in seeds is discussed by Lott (1980). Khoo and Wolf (1970) reported about the development of the aleurone vacuoles and protein bodies in the outer cell layer of the maize endosperm. They failed to detect protein accumulation in the vacuoles in 7-10 day kernels but found protein granules by day 12. As compared to e.g. barley and wheat (Jacobsen et al. 1971) maize forms only few aleurone protein bodies (see also Khoo & Wolf 1970, Kyle & Styles 1977) which occupied only a minor part of the aleurone layer but spherosomes were abundant and took up much more space. The origin of the spherosomes was not es-

tablished. The existence of spherosome membranes in maize was reported by Kyle & Styles (1977). Such a membrane was only observed in the present study when spherosomes were pressed to each other. Spherosomes show variations in staining which might be related to the heterogenous composition (Wolf et al. 1969). Aleurone protein bodies have electron dense and electron opaque inclusions. The electron dense parts, which are hard and do not penetrate well with embedded medium, often chatter during sectioning leaving holes in the section. Protein bodies of the sub-aleurone and inner endosperm were found to have polysomes associated with the protein body membrane. These endosperm protein bodies of maize probably accumulate the main storage protein called zein (Burr & Burr 1976). When the polysomes were isolated at a time when storage protein synthesis was active, they were able to synthesize zein.

With respect to the function of the aleurone layer Bhatnagar and Sawhney (1981) reviewed the literature about hydrolytic enzymes such as α-amylase that brings about the breakdown of reserve food materials in the endosperm tissue which are utilized by the embryo during its growth and development. Protein bodies have several functions that were summarized by Matile (1968). First they store certain reserve substances such as proteins, metals and phytin the synthesis of which might be mediated by mitochondria (Morrison et al. 1975). Second, during germination the aleurone vacuoles are transformed to a 'lysosome' and their food reserves are mobilized. Finally they may form a lytic compartment in which other constituents of the cell are broken down.

4.2. The microtubular cytoskeleton.

In the caryopsis of maize microtubules were found in three major configurations: a) spindle tubules, the functions of which are discussed by amongst others Kreis & Birchmeier (1982), b) cytoplasmic microtubules running throughout the cytoplasm and probably functioning in the positioning and moving of organelles and in sustaining the integrity of the cytoplasm (Van Lammeren et al. 1985, Lloyd et

al. 1986) and c) cortical microtubules interacting with cell shape development. The latter exhibited a variety of configurations. Criss-cross patterns and parallel arrangements were the extremes. The patterning is likely to influence the morphogenesis of a growing plant cell (see e.g. Marchant 1979, Lang Selker & Green 1984, Lloyd 1984) although the data are not equivocal (Emons 1986, Traas 1986).

Cortical microtubules in criss-cross arrangements were observed in small and large cells which had no clear length axis. This observation might imply that such a configuration of cortical microtubules restricts or determines cell enlargement and prevents cell elongation. As soon as parallel arrangements were observed, cells were more or less cylindrical or had a length axis. In these cells it was found that the direction of the cortical microtubules was normal to the growth direction which was determined by the increase in length and in width. When cells just elongated without a remarkable lateral expansion, as was observed in the integuments and nucellus epidermis, the microtubular orientation was perpendicular to the length axis. When, however, the elongated cells expanded in lateral direction the orientation of the microtubules was oblique with respect to that direction and the deviation appeared to be related to the intensity of the lateral expansion. From the above data it is concluded that in maize caryopses the cortical microtubules are involved in the cytomorphogenesis in the sense as described by Lang Selker and Green (1984). The way how microtubules interact with microfibril deposition and the direction of the process is not clear up to now although some possible mechanisms have been described (Emons 1983, Heath & Seagull 1982, Hepler 1985). The majority of the microtubules in the pericarp cells was observed in the cortical cytoplasm suggesting a relation with cell wall formation (see also Roberts et al. 1985). In elongated cells we suppose the microtubules to be the inducers of the dominance of longitudinal growth. The cells of the aleurone layer exhibited some deviant phenomena. Parallel arrangements of microtubules were observed along anticlinal cell walls from 12 DAP onwards. Aleurone cells elongated only slightly in a direction normal to the plane of the microtubules. Instead of elongation, cell wall thickening was

notable both on anticlinal and outer periclinal cell walls adjacent to which only few microtubules were observed.

In conclusion, the data of the present investigation point toward a strict sequence of events leading to the maturation of the caryopsis. Pericarp differentiation resulted in a dead protective fruit wall covering the seed. Differentiation and cell wall synthesis in the pericarp lead to thick-walled exo- and endocarp cells and thin-walled mesocarp cells. The differentiation of the endosperm is analyzed cytologically, however, the formation and composition of various organelles in aleurone and sub-aleurone layers will have to be studied in more detail. The configurations of the microtubular cytoskeleton are related to the development of cell shape in growing cells. Table 1 gives a scheme which summarizes the data of microtubular distribution and cell shape development in the various tissues of the caryopsis.

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CHAPTER 7

Some concluding remarks on endogenous and exogenous influences on embryo development in maize

A.A.M. van Lammeren

SOME CONCLUDING REMARKS ON ENDOGENOUS AND EXOGENOUS INFLUENCES ON EMBRYO DEVELOPMENT IN MAIZE AND THE SIGNIFICANCE OF THE STUDY FOR PLANT BREEDING

During caryopsis development the shape and structure of both the embryo and the extra-embryonal tissues of the caryopsis change continuously. This chapter will expand on when and how embryogenesis is influenced. Factors inducing or influencing the development of the shape of the embryo will be postulated. They are called endogenous when their influence manifests from inside the embryo and exogenous when they come through from outside the embryo.

1. Effects of endogenous morphogenetic factors

Although one has to keep in mind that morphogenesis is largely an expression of the genetic constitution of the embryo, some characteristics of embryo development can be analysed. Embryo development is determined or influenced by the initiation and maintenance of polarity. The shape of the embryo is further influenced by the formation and activity of meristems and the directed cell elongation in the various organs.

Induction of polarity becomes manifest during the transition of the egg stage to the zygote stage. The new cell polarity is established when the location of the cytoplasm in the cell changes (Chapter 1). It can be worked out in later developmental stages and is then expressed by the positions of the suspensor and the apical meristems. This polarity determines further proembryo development and is expressed by the positions of the suspensor and the apex. Polarity determines further proembryo development and can be considered as a morphogenetic factor. Chapter 2 highlights the fact that suspensor cells of the proembryo elongate. Elongation occurs after the apical meristem has developed a certain size and can therefore be regarded as an effect of polarity. Finally, the transition of the proembryo stage to the embryo stage should be based upon a regulation which is expressed in the further development of the apical meristems which will develop into the shoot meristem and the root meristem

(Chapter 3). The nature of this regulation is complex but some characteristics will be discussed.

In the above mentioned examples, common structural features are observed. Among these the symplastic isolation, which precedes the morphological changes, is obvious. Symplastic isolation is the consequence of the absence of plasmodesmata between the embryo and its environment. Figure 1 gives a schematic representation of five developmental stages of the maize embryo.

-In Fig. 1a the zygote is shown with plasmodesmata at its micropylar side. Symplastic contact with the endosperm is therefore restricted to that area and a symplastic pathway for nutrients can only be such as indicated by the arrows. The cell membrane at the antipodal side of the zygote forms a so-called symplastic boundary because of the absence of plasmodesmata. In this area most cytoplasm is concentrated. The plasmodesmata and the symplastic boundary are formed when the egg cell develops. It is therefore a property of the zygote which thus determines the direction of symplastic transport. When the direction of symplastic transport influences the induction or maintenance of polarity, this influence is determined by the zygote because of the location of the plasmodesmata and therefore it can be said that this morphogenetic factor has an endogenous character. On one hand the pathway of transport is determined by the structure of the zygote. On the other, however, the nutrients influence the embryo from the outside. This implicates that a morphogenetic factor can have endogenous and exogenous features.

-Figure 1d shows a proembryo such as studied in the Chapters 2 and 5. Although there is no longer symplastic contact between endosperm and proembryo, the endosperm functions in feeding the proembryo via the apoplast, and especially at the base of the suspensor (Chapter 5). Thus the base of the suspensor is a region of nutrient uptake. The apex ingests most of these nutrients and is a sink within the embryo. The flow of nutrients towards the proembryo is indicated by the striated arrows. It should be noted that the protodermal cells of the apex can be regarded as symplastic boundary cells containing much cytoplasm. From these remarks it can be concluded that the embryo

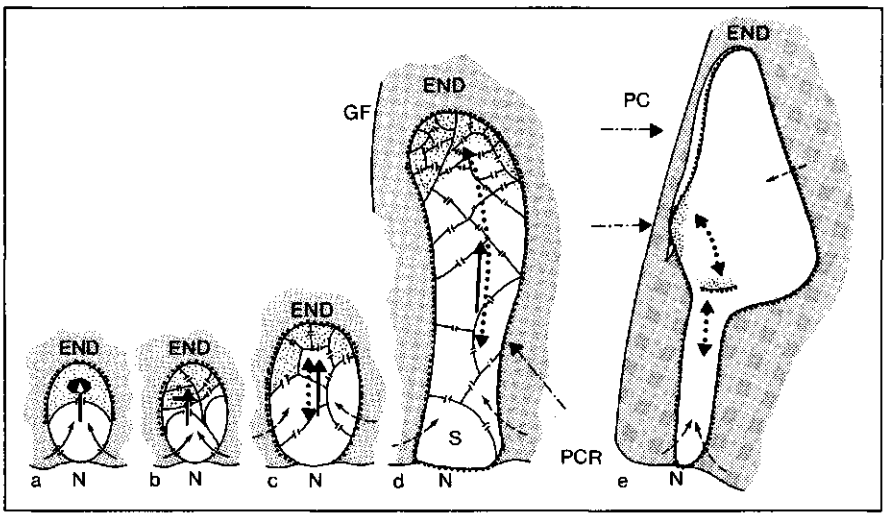

Fig. 1 Schematic representation of endogenous and exogenous factors influencing the embryogenesis in maize at various developmental stages. a. Zygote at 30 hrs after pollination; b. Multi-cellular proembryo at 3 DAP (days after pollination); c. Elongating proembryo at 4 DAP; d. Proembryo with suspensor at 5 DAP; e. Early developmental stage of the embryo proper at 7 DAP.

-Embryo development is influenced by endogenous factors such as the symplastic boundary at the antipodal side of the zygote (▲▲▲▲), the presence of plasmodesmata (⊣⊢) in the globular proembryo at its micropylar side, and the maintenance of zygotic polarity (↑) through subsequent developmental stages which is expressed by the unequal distribution of the cytoplasm as is shown by punctated areas ( ) and stippled arrows (⋮) which also indicate embryo elongation.

-Embryo development is influenced by exogenous factors such as the symplastic pathway for nutrients through plasmodesmata as indicated by arrows ( ) in a and b; the apoplastic uptake of nutrients by the embryo such as visualized by striated arrows ( ) in c-e; and a mechanical factor developed by the growth of the endosperm at the basal region of the suspensor in Fig. d (→). Abbreviations: END = endosperm: GF = germinal face, N = nucellus, PC = pericarp; PCR = placento-chalazal region.

development is not only influenced by the endogenous polarity but by an exogenous factor, in this case the endosperm which supplies the nutrients, as well.

-During the development of the embryo proper (Fig. 1e) the shoot meristem develops from the protoderm cells of the apex of the proembryo. The meristem is therefore formed in symplastic boundary cells. These cells already formed a sink to which the scutellum develops laterally. The initial formation and further activity of the root meristem depends on the developmental stage of the shoot meristem and thus appears to be determined endogenously (Chapter 3). At the onset of the formation of the root meristem, which develops endogenously, a symplastic boundary is created between the area of the root meristem and the calyptrogen (Fig. 1e, arrow-head). Thereafter the meristems are activated. Herewith the necessity of symplastic isolation with respect to meristem formation is demonstrated once again.

In the foregoing examples the sink within the embryo is situated adjacent to existing or newly created symplastic boundaries. The fact that sinks are also found far from regions that function in the uptake of nutrients emphasizes the presence of polarity. In all examples the apical poles can be distinguished from other areas by the accumulation of cytoplasm in relatively small cells. The directed elongation of cells in tissues was frequently observed and is shown in the Figs. 1b,c and e by arrows (↕). It is remarkable that cells do not elongate untill the apical pole of the organism contains relatively large quantaties of cytoplasm. This degree of organization could well be a prerequisite for the induction of cell elongation.

Cell elongation coincides with certain configurations of the microtubular cytoskeleton. Microtubules in the central region of the cytoplasm function in the positioning, motility and movement of organelles but are not likely to influence cell shapes directly. Cortical microtubules, however, influence the mode of cell wall synthesis and determine the development of cell shapes (Chapter 6). Cell elongation can be connected with a rearrangement of the cortical microtubules of the cytoskeleton as is illustrated for the endosperm

and pericarp cells in Chapter 6. The microtubular cytoskeleton can therefore be called a morphogenetic factor. Whether the microtubular cytoskeleton has to be considered as an independent morphogenetic factor or that the changes in the microtubular configurations are the expression of an other cell signal, is not elucidated. The latter possibility is more likely because of the prerequisite of cell elongation as mentioned before.

The genetic constitution of embryos appears to be an important endogenic morphogenetic factor. With in vitro studies in particular there is a striking difference in the ability to regenerate amongst various inbred lines, but it is constant within an inbred line (Fransz, pers. comm.) Such a property is expressed in many structural aspects which are related to e.g. development of callus and somatic embryogenesis (Chapter 5).

2. Effects of exogenous morphogenetic factors

Factors exerting an influence on the embryo from outside can be distinguished in physical, physico-chemical and organic-chemical factors.

An example of physical influences on shape development is the mechanical influence which becomes prominent when the suspensor bends because of the intensive growth of the endosperm at its base by which a lateral pressure is exerted upon the suspensor (Chapter 2). When, at a later stage, the elongated embryo axis is cramped by the suspensor epidermis and the pericarp at its germinal face (Chapter 3) an other example is demonstrated. In this context it is remarkable that in vitro experiments show a sideward outgrowth of the axis when pericarp and endosperm are removed (Chapter 6). From the foregoing arguments it appears that the shape of the embryo is, at least partly influenced by external tissues which develop continuously. Such an influence can be considered as a force or pressure which is caused by growth, cell pressure and tension in the surrounding tissues.

Other physical factors such as light, humidity and temperature strongly influence the whole metabolism of the sporophyte (mother plant) and thus the rate of success and the speed of embryo and

endosperm development. Although both embryo and endosperm are heterotrophic organisms in vivo, illumination in vitro initiates the differentiation of chlorenchyma in very young excised embryos. This experiment demonstrates the sensitivity to this exogenous physical factor but it also puts forward the ability to autotrophy (Chapter 5). The endosperm lacks this ability and other types of differentiation are either not observed or less obvious than in the embryo. It is not known whether in vivo illumination in early developmental stages inhibits or stimulates further development.

The physico-chemical and organic chemical influences are based upon osmolarity, the composition and the direction of the nutrient flow, and the activity of growth regulators amongst others.

It has been demonstrated that growth and premature germination of excised embryos on artificial nutrient media frequently occur. However, these phenomena are reduced significantly or disappear when the osmolarity of the medium is increased. In vivo the premature germination is inhibited and it is likely that it is caused by an increase of the osmolarity in the endosperm because carbohydrates are accumulated from 8 DAP onwards (Chapter 6). The ingestion of sugars in the endosperm is expressed by the synthesis of starch in the plastids at the very moment that the embryo has developed its apical meristems. Starch is osmotic inactive but its accumulation demonstrates the presence of soluble carbohydrates. It is suggested that the osmolarity in the environment of the embryo increases and inhibits germination from the moment that the immature embryo is germinative though premature germination has to be suppressed.

The directedness of the nutrient flow is an exogenous morphogenetic factor at various stages of embryo development. -First, a directional symplastic flow of nutrients towards the zygote can be deduced from the non-random distribution of plasmodesmata in that cell. The endogenous aspect of this factor has already been mentioned.

-Secondly the elongation of the embryo brings the embryo apex in a part of the endosperm which differs from the endosperm surrounding the suspensor. At the apical side highly vacuolized endosperm cells

surround the embryo. At its basal side the embryo suspensor is connected with endosperm cells containing dense cytoplasm. These cells supply nutrients taken up by the growing embryo (Chapter 5) through the apoplast.

-Thirdly the scutellum of the embryo proper is formed in the region where the nutrient supply is abundant. This points to the possibility that the development of a second cotyledon fails to come because of a local deficiency of nutrients. This in its turn means that the directedness of nutrient supply influences morphogenesis. Experiments in which the development of somatic embryos is observed show a comparable type of development of the cotyledons (Fransz, pers. comm.) In these experiments there is, however, no endosperm which supplies nutrients. The embryo protrudes from the callus tissue and is then surrounded by air at its apex. It must therefore be concluded that the absence or presence of the nutrient flow is not a prerequisite any more and that the lack of development of the second cotyledon must point to an acquired local and structural deficiency.

-A forth type of exogenous morphogenetic influence is observed when bilateral symmetry is expressd in the embryo. When the new symmetry becomes prominent there is a bilateral symmetry in the ovary and ovule. This symmetry is thought to be superimposed onto the existing radial symmetry of the proembryo by nutritional or other organic-chemical influences. The directedness of these influences, however, is determined by the anatomy and thus the developmental history of the ovule and ovary. On the other hand it should be noted that in vitro studies demonstrate a change of symmetry within a proembryo in the absence of endosperm (Chapter 5). In this case, the development of shapes appears to be regulated by the embryo and might therefore have become an acquired property.

-Finally, Chapter 6 describes some experiments in which morphogenesis is clearly influenced by the way the nutrients were ingested. Immature maize embryos of 9 DAP exhibit an independent regulation of growth which leads to the development of germlings.

Conclusion.

The morphogenesis of the embryo can be seen as a continuous process in which the shape is influenced or determined by a combined action of exogenous and endogenous factors. The nature of these factors is partly analyzed but experimental embryology in particular will provide more details in future.

3. Significance of the study for plant breeding

This cytomorphological study of the crop plant Zea mays L. provides information of the level of organization and structural complexity of the embryo on subsequent moments of differentiation. It was found that the investigated inbred lines exhibited differences in shape and position of structures such as the micropyle and embryo sac. This kind of study might be of use in the research of intra-specific crossings and the success or failure of fertilization. The structural investigation of inbred lines revealed inbred line-specific differences in developmental rates and in organ and organelle differentiations. Therefore it provides the possibility to get an accurate embryological characterization of lines which can be of use to analyse lines and the intra-specific crosses. The (ultra)structural study of in vivo embryogenesis provides a tool to determine the regular sequence of morphological events and furthermore the influence of external factors on that sequence. Long before the maturity or the degeneration of the caryopsis the expression of the influence of the factors which accelerate or impede development can be registrated.

In vivo studies about cell differentiation and the function of the cytoskeleton such as described in Chapter 6 not only revealed the relation of the arrangement of microtubules with the way of cell enlargement, but they also put forward the advantages of the technique of immunological labeling of tubulin with fluorescein isothiocyanate (FITC) in sections of large tissue blocks which were original embedded in polyethylene glycol (PEG). The investigation of the structural aspects of the initiation of polarity, the development of

meristems and the directed cell enlargement in proembryo and embryo (Chapters 1,2 and 3) attributes to a better knowledge of embryogenesis. The knowledge can be the tool to manipulate specifically during differentiation.

In vitro experiments open ways to interfere and compare with normal embryogenesis. In vitro experiments showed the embryo to become increasingly independent from the endosperm which ultimately can be replaced. The increasing independence can be related to the complexity of structure and organization within the embryo. This procedure offers the opportunity to determine anatomical and cytological parameters to establish the moment to initiate in vitro culture. The differentiation of the apical meristems might be such a moment. Culture of excised immature embryos in the absence of endosperm is of value in crosses with deficient endosperm development. Cytological approaches permit the early registration of structural phenomena induced by physical and organic-chemical factors. For instance, the early recognition of various differentiation forms, such as the development of callus, adventitious shoots and somatic embryos.

In vitro culture makes it possible to manipulate the developing embryos which might lead to acquire properties which might be of advantage in a later phase of plant life. For example, the inhibition of the early germination of somatic embryos might result in a further embryological development which results in arger embryos with larger meristems and perhaps less dominance towards adjacent somatic embryos.

Conclusion.

This morphological study on maize embryogenesis characterizes the various developmental stages of the embryo, the endosperm and the pericarp. Interactions between the tissues can be shown and inbred line specific properties can be visualized. This approach combined with the experimental approach to the in vitro culture of embryos opens ways to breeding.

SUMMARY

In this thesis the embryological development of the maize plant (Zea mays L.) is described. The investigations aim at analysing the development of polarity, the initiation of meristems, the differentiation of tissues within the embryo and the interaction between the embryo and the extra-embryonal tissues of the growing caryopsis. The (ultra)structural aspects of the ontogenesis of both the embryo and endosperm form the main topic.

Caryopses which developed in vivo were used; besides that, tissue culture techniques were applied to pollinate in vitro, to culture excised immature embryos, to regenerate somatic embryos and to initiate callus formation. The structural analysis was mainly based upon transmission electron microscopy, scanning electron microscopy and light microscopy with the belonging (immuno)cytochemical and morphometrical techniques. The gathered information contributes to the knowledge of embryogenesis in vivo and in vitro and to the practical insight in how to influence embryogenesis. The comparison of inbred lines and the comparison of in vivo and in vitro embryo development in particular resulted in a better understanding of the embryogenesis of maize.

Comparable investigations mainly date from the first half of this century and information on the level of organelles lacks for the greater part in those reports. More recently, sub-microscopical investigations on the in vivo embryogenesis of maize have been performed incidentally. The interaction of embryo and endosperm, however, has not been considered intensively. The latter aspect has been stressed in the present investigations. In the various chapters of this thesis one will find subsequently a treatise of the structure of the ovule and embryo sac (megagametophyte) in the progamic phase, of the fertilization, the early embryogenesis in vivo and in vitro, the interaction between the young embryo and the endosperm, and finally the developmental history of the endosperm and the fruit wall (pericarp).

The general introduction reviews the literature, summarizes the aims of the study and of the various chapters, and describes the morphology and anatomy of the inflorescences of maize, its pistillate spikelets and the pistil itself.

Chapter 1 presents the results of an ultrastructural study in which two inbred lines, A188 and BMS, are compared. The distribution of organelles in the cells of the megagametophyte has been determined qualitatively and quantitatively.

Before fertilization the synergids exhibit a conspicuous distribution of organelles such as mitochondria which might be correlated to the transmembranal transport of nutrients in the filiform apparatus. With reference to the location of the cytoplasm in the egg cell, the cytoplasm in the zygote moves towards the antipodal side of the cell. A change in polarity is thus created and it will be maintained in the developing proembryo. In the central cell of the megagametophyte the cytoplasm changes place, too, because of fertilization. The structure and the number of organelles point to a high metabolic capacity which permits the fast synthesis of cytoplasm and the frequent occurrence of cell divisions. Differences between both inbred lines are expressed in, amongst others, the sizes of organelles and sometimes in their functions such as the accumulation of starch in plastids. Concerning the structure of the ovule, the development of the inner integument and the micropylar part of the nucellus appears different in the two lines. In strain BMS the pollen tube penetrates the integument and the nucellus without curving whereas in A188 no straight growth was detected.

Chapter 2 comprises a light microscopical investigation of the morphogenesis of the proembryo from 1 up to 7 days after pollination (DAP). The cellular polarity of the zygote results in an unequal first cell division at about 36 hours after pollination. The two-celled proembryo is spherical and this shape is maintained up to 3 DAP. In this period several small apical cells and somewhat larger basal cells are formed. A pattern in cell division was not observed. Hereafter, the increase in length is mainly caused by cell division and directed elongation in the parts below the apex. Because of the

stretching of the proembryo, its apex, which will form the embryo proper, is directed towards the future germinal face of the caryopsis. This phenomenon can be explained by the bending of the suspensor which is caused by an intensive development of the endosperm near the base of the suspensor. The data mentioned point towards the importance of polarity, to the function of the suspensor which pushes the embryo apex into the endosperm and to the morphogenetic influence of the endosperm.

Chapter 3 gives a compilation of data concerning the induction of bilateral symmetry in the embryo, the development of a single cotyledon, and the formation of the embryo axis. The morphogenesis and cytological differentiation of the young embryo are analysed in order to follow the initiation and development of the apical meristems from the proembryo stage (5 to 6 DAP) up to the moment the shoot and root have been formed (about 13 DAP). The morphology of the embryo is determined by SEM, its histogenesis with LM and its cytological development with TEM. The original radial symmetry of the proembryo changes into a bilateral symmetry. This is caused or influenced by the excentric position of the embryo apex in the endosperm which on its turn influences the embryo as an exogenous factor. The development of the shoot meristem is preceded by the formation and enlargement of the scutellum and is localized in the protoderm at the future germinal face of the embryo. The shoot meristem is oriented asymmetrically because a second cotyledon is not developed. One or two days after the appearance of the shoot meristem, the root meristem is established at the inside of the embryo proper and in line with the suspensor. Initially it is discernable as a group of cells with few vacuoles and much cytoplasm. The new root-shoot axis deviates from the original length axis of the proembryo but during further development a sideward outgrowth of shoot and coleoptile is impeded mechanically by the pericarp and further development of tissues in the root-shoot axis of the embryo.

Chapter 4 presents the investigations on in vitro pollination and the development of embryos which are cultured under various experimental conditions. The pollination of excised pistillate spikelets

resulted in the development of 24% of the ovaries. Next it was determined at which age and developmental stage excised immature embryos were still able to germinate and to grow to maturity on nutrient media without growth regulators. It was found that both apical meristems have to be formed in order to establish an endogenous regulation for further root and shoot development. Embryos should have an age of at least 9 DAP and a size of about 1.5 mm.

Regeneration phenomena have been studied in immature embryos. The presence of 2,4 D appeared a prerequisite for the induction of regeneration. In order to initiate regeneration, the germinal face of the embryo had to be placed onto the nutrient medium. Various tissues were formed such as chlorenchyma, collenchyma, vascular and callus tissues and more complex structures such as adventitious shoots, roots and somatic embryos. Finally attempts have been made to initiate embryogenesis in suspension cultures. Cell suspensions were prepared from callus tissues which were generated by cultured embryos. A suspension of living cells was established. The cells, however, were formed in root meristem-like cell conglomerates and did not divide at all.

Chapter 5 presents an ultrastructural study of the interaction between the embryo and the endosperm from 3 to 11 DAP. Pathways of nutrient flow towards the embryo are characterized structurally by the positions and numbers of various organelles. Nutrients enter the ovule through the placento-chalazal region. They are taken up by the endosperm in a layer of cells with transfer characteristics, and move to various regions in the inner endosperm such as the region of the embryo suspensor. The nutrient flow is characterized structurally by the positions of organelles such as mitochondria, dictyosomes, ER and osmiophilic droplets. A second pathway of nutrient flow, which runs from the degenerating nucellus towards the endosperm, is deduced from the positions of mitochondria in the outer endosperm cells. They were found especially adjacent to the cell membranes bordering the nucellus.

From early developmental stages onwards (3 to 5 DAP) the endosperm cells in the basal region of the suspensor exhibit a large number of

ordered ER profiles. Products, synthesized by the ER, are likely to be ingested by the suspensor of the embryo seeing the accumulation of osmiophilic material in invaginations of the cell membranes bordering the suspensor cells. Energy is supplied locally by mitochondria which are found here in particular. Near to the enlarging embryo proper endosperm cells degenerate continuously and the degraded products are also taken up by the embryo.

The in vivo development of the extra-embryonal parts of the caryopsis is described in Chapter 6. Transmission electron microscopy, LM and immunocytochemical techniques are used to visualize cell differentiation in the pericarp, integument, nucellus and endosperm from 3 to 22 DAP. The arrangements of the microtubular cytoskeleton in these tissues are determined and the accumulation of organelles in the various layers of the endosperm is quantified. Microtubules were observed in three main configurations: a. Spindle tubules which function in the movement of the chromosomes; b. cytoplasmic microtubules which run throughout the cytoplasm and which are likely to have a function in the motility and movement of organelles and in stabilizing the cytoplasm; and c. cytoplasmic microtubules which run in the cortical cytoplasm. The function of the cortical microtubules is related to cytomorphogenesis.

In the pericarp three zones differentiate. The development of cell shapes appears to be related to the organization of the cytoskeleton. The cell differentiation in each zone of the pericarp coincided with a certain arrangement of the cortical microtubules. Mesocarp cells exhibited preferentially criss-cross microtubules. These cells were not cylindrical, had thin cell walls and disappeared almost completely, including the cell walls. Endocarp and exocarp cells elongated and got thick cell walls. The differentiation coincided with parallel arrangements of microtubules. Most cells had degenerated at 22 DAP but their cell walls remained. In the cells of the integument, which is a transient tissue, most microtubules are arranged normal to the length axis of the cylindrical cells. Nucellus cells were mostly isodiametrical but the outer cells were flattened. Depending on the type of the cell shape either criss-cross or paral-

lel arrangements of microtubules were observed. At 17 DAP only cell walls of the epidermis and its cuticle were observed. The endosperm development is characterized by the formation of the aleurone layer, some sub-aleurone layers and the inner endosperm. The cells of the various layers have different shapes and a different patterning of microtubules. Bundles of cortical microtubules were found in parallel arrays along the anticlinal cell walls of the aleurone cells. Criss-cross patterns were found in isodiametrical cells of the sub-aleurone layers and parallel configurations in cells which had a length axis.

From these results it is concluded that when cortical microtubules are found in parallel arrangements in growing cells, elongation is about perpendicular to the microtubules. When cortical microtubules are arranged in criss-cross textures, isodiametrical patterns of growth are found or are to be expected. Although the development of cell shapes can be predicted it has as yet not been found which factor determines the microtubular configuration.

The distribution, the sizes and numbers of organelles within the various cell layers of the endosperm, were determined. Clear differences were found in the three layers with respect to variations in the accumulation of storage products such as starch in the plastids, proteins in the two types of protein bodies and of lipids in the spherosomes.

Finally in Chapter 7 the data of the foregoing chapters are summarized and related to each other. In a model of differentiation endogenous and exogenous factors which influence the development of the embryo shape continuously are postulated. Some developmental stages are mentioned in particular and the influence of transport and the accumulation of nutrients, of polarity, genetical variability and experimental parameters are emphasized.

SAMENVATTING

Dit proefschrift beschrijft de embryonale ontwikkeling van de maïsplant. Het doel van het onderzoek was de ontwikkeling van polariteit, de meristeeminitiatie en de weefseldifferentiatie in het embryo te volgen en de interactie tussen embryo en extra-embryonale weefsels in de groeiende graanvrucht (caryopsis) te analyseren. Daarbij lag het accent op de (ultra)structurele aspecten van de ontogenie van embryo en endosperm. Er is gebruik gemaakt van zich in vivo ontwikkelende maïskorrels. Tevens zijn weefselkweektechnieken toegepast om in vitro bestuiving, kweek van uitgeprepareerde onvolgroeide embryos en vermeerdering in celsuspensies als experimentele condities in te voeren. Bij de structurele analyse is in hoofdzaak uitgegaan van transmissie elektronenmicroscopie, scanning elektronenmicroscopie en lichtmicroscopie met bijbehorende (immuno)cytochemische en morfometrische technieken. De verworven kennis is een bijdrage tot inzicht hoe de maïsplant in vivo en in vitro opgekweekt kan worden.

Vergelijkbaar onderzoek dateert voornamelijk uit de eerste helft van deze eeuw waarbij informatie op organelniveau grotendeels ontbreekt; meer recent is echter slechts incidenteel submicroscopisch onderzoek verricht aan de in vivo embryogenese van maïs. Daarbij is bovendien de interactie tussen embryo en endosperm grotendeels buiten beschouwing gebleven, een aspect dat juist in het hier beschreven onderzoek sterker aandacht heeft gehad. In de verschillende hoofdstukken van dit proefschrift worden dan ook achtereenvolgens de progame fase, de bevruchting, de vroege embryogenese in vivo en in vitro, de interactie van het jonge embryo met het endosperm en de ontwikkeling van het endosperm en de vruchtwand behandeld. Uit deze studie blijkt dat vergelijking van inteeltlijnen onderling en van de in vivo en de in vitro ontwikkelingen bijdragen tot een beter begrip van de embryogenese van maïs.

De algemene inleiding geeft, naast de algemene doelstelling van het onderzoek en de specifieke doelstellingen van de verschillende hoofdstukken een kort literatuuroverzicht en een morfologische be-

schrijving van de bloemgestellen, de stamperbloemen en de stamper van maïs.

In hoofdstuk 1 worden de resultaten van een ultrastructureel onderzoek weergegeven waarbij twee inteeltlijnen, A188 en Black Mexican Sweet, vergeleken zijn. Van de cellen van de megagametofyt (embryozak) is de organelverdeling gelokaliseerd en gekwantificeerd. Vóór de bevruchting is in het cytoplasma van de synergiden bij beide lijnen een uitgesproken verdeling van organellen zoals mitochondrien waargenomen hetgeen in verband lijkt te staan met het stoftransport door de celmembraan van het fadenapparaat. Ten opzichte van de eicel vertonen de zygotes van beide lijnen een verschuiving van het cytoplasma naar de antipodale zijde van de cellen waardoor in de zygote een nieuwe polariteit ontstaat die zich in het pro-embryo blijkt te handhaven. Het cytoplasma van de centrale cel verandert eveneens van plaats als gevolg van de bevruchting. De hoeveelheid organellen in het cytoplasma en de structuur ervan wijzen op een hoge metabolische capaciteit die na bevruchting leidt tot een snelle toename van de hoeveelheid cytoplasma en een frequent optreden van kerndelingen. Verschillen tussen beide lijnen komen met name tot uiting in de verdeling van de grootte van organellen en in b.v. de mate van zetmeelaccumulatie in plastiden. De ontwikkeling van zowel het binnenste integument als van de nucellus blijkt bij de twee lijnen verschillend waardoor de penetratieweg van de pollenbuis bij BMS rechtlijnig is en bij lijn A188 kronkelig verloopt.

Hoofdstuk 2 omvat een lichtmicroscopisch onderzoek naar de morfogenese van het pro-embryo van 1 tot 7 dagen na bestuiving (1-7 DAP=days after pollination). De cellulaire polariteit in de zygote, geeffectueerd in de excentrische ligging van het cytoplasma, resulteert ongeveer 36 uur na bestuiving in een inequale celdeling. Het twee-cellig embryo is bolvormig. Het pro-embryo behoudt deze vorm tot ongeveer 3 DAP in welke periode door deling meerdere kleine apicale en wat grotere basale cellen ontstaan. Een patroon in volgorde van celdeling is niet aangetoond. De hieropvolgende lengtetoename wordt vnl. veroorzaakt door celdeling en celstrekking aan de basis, in het midden- en sub-apicale deel van het pro-embryo. Tijdens

de strekking van het embryo wordt de apex, waaruit zich het eigenlijke embryo zal ontwikkelen, sterk gericht naar de kiemzijde van de caryopsis hetgeen verklaard kan worden doordat de suspensor tijdens de groei een kromming gaat vertonen als gevolg van een sterke lokale endospermontwikkeling aan de basis van de suspensor. Deze gegevens wijzen enerzijds op het belang van polariteit om het eigenlijke embryo, gedragen door de suspensor, goed te positioneren in het endosperm, en anderzijds op de belangrijke vormbepalende invloed van de interactie tussen embryo en endosperm.

In hoofdstuk 3 worden onderzoeksgegevens betreffende de ontwikkeling van de bilaterale symmetrie van het embryo, van het enkele cotyl en van de embryo-as in een differentiatiepatroon samengebracht. De morfologie en de cytologie van zich ontwikkelende jonge maïsembryos is geanalyseerd met SEM, TEM en LM technieken om de initiatie van de apicale meristemen vanaf het einde van het pro-embryo (5-6 DAP) stadium te volgen tot het stadium waarin zowel wortel- als spruitmeristemen aangelegd zijn (13 DAP). De embryo-morfologie is met SEM bepaald, de histogenese met LM en de cytologische ontwikkeling met TEM. De oorspronkelijke radiale symmetrie van het pro-embryo, dat een eigen, endogene polariteit bezit, verandert in een tweezijdige symmetrie hetgeen veroorzaakt is of beinvloed wordt door de excentrische positie van de embryo-apex in het endosperm dat op zijn beurt als een exogene factor op het embryo inwerkt. De ontwikkeling van het spruitmeristeem wordt voorafgegaan door de vorming van het scutellum en vindt plaats in het protoderm aan de toekomstige kiemzijde van het embryo. De asymmetrische orientatie van het spruitmeristeem vindt zijn oorzaak in het achterwege blijven van de ontwikkeling van een tweede zaadlob. Het wortelmeristeem ontwikkelt zich een tot twee dagen later binnen het eigenlijke embryo in het verlengde van de suspensor en wordt daar gezien als een groepje zich delende cellen met weinig vacuolen en veel cytoplasma. De nieuwe wortel-spruit-as wijkt af van de oorspronkelijke lengte-as van het pro-embryo maar tijdens de verdere ontwikkeling wordt een zijdelingse uitgroei verhinderd door de mechanische eigenschappen van het pericarp en de verdere weefselontwikkeling in de spruit-wortel-as van het embryo.

Hoofstuk 4 beschrijft de in vitro ontwikkeling van embryo's onder diverse experimentele omstandigheden. Ten eerste zijn kolfdelen met vruchtbeginsels op kunstmatige voedingsmedia gebracht. In vitro bestuiving had tot resultaat dat 24% van de vruchtbeginsels zich ontwikkelde. Ten tweede is geanalyseerd vanaf welk ontwikkelingsstadium uitgeprepareerde embryo's in staat blijken te zijn zich tot volwassen planten te ontwikkelen op media zonder groeiregulatoren. Het blijkt dat in alle gevallen het pro-embryostadium niet tot die ontwikkeling leidt maar dat beide apicale meristemen aangelegd moeten zijn om onder de kunstmatige omstandigheden spruit- en wortelvorming te reguleren en te realiseren; dat is bij een leeftijd van minimaal 9 DAP en een grootte van 1,5 mm. Ten derde is het regeneratievermogen van onvolgroeide embryo's bestudeerd. Anatomisch en cytologisch blijken een aantal differentiatieprocessen in het embryo op te treden met als resultaat de vorming van chlorenchym, collenchym, vaat- en callusweefsel en de initiatie van adventiefspruiten en somatische embryos. Zowel de aanwezigheid van groeiregulatoren alswel de wijze van nutrienten-opname bleken bij deze experimenten essentieel. De opname van nutrienten wordt beinvloed door de positie van het embryo op het vaste voedingsmedium. Tenslotte is gepoogd de zygotische embryogenese in suspensiecultuur te simuleren door uit te gaan van een celsuspensies die afkomstig waren van in vloeibaar medium gebracht callus weefsel van embryonale oorsprong of van embryos die reeds wortel- en spruitmeristemen gevormd hadden. Er ontstaat een suspensie van levende cellen maar deze cellen blijken afkomstig van celproducerende, wortelmeristeem-achtige celklompjes en vertonen in het geheel geen delingsactiviteit.

In het vijfde hoofdstuk is de interactie tussen embryo en endosperm tijdens de vroege ontwikkelingsfase (3-11 DAP) op ultrastructureel niveau bestudeerd. De nutrientenroute naar het embryo is gekenmerkt door de frekwentie van voorkomen en ligging van organellen en loopt enerzijds van de placento-chalazale regio via een aangepaste buitenste endospermcellaag met transfer-eigenschappen door het endosperm naar o.a. het gebied van de embryo-suspensor. Anderzijds is er een sterke indicatie dat voedingsstoffen opgenomen worden vanuit

degenererend nucellusweefsel hetgeen afgeleid kan worden uit de speciale ligging van de mitochondrien nabij de celmembranen grenzend aan de nucellus. Al in een zeer vroeg stadium van ontwikkeling (3-5 DAP) zijn de endospermcellen in de basale regio van de suspensor rijk aan cytoplasma en bevatten een enorme hoeveelheid sterk geordend ER. De producten, door dat ER gesynthetiseerd, worden waarschijnlijk opgenomen door de suspensor van het embryo, getuige de accumulatie van osmiofiel materiaal in de invaginaties van de celmembranen grenzend aan de suspensorcellen. Ook op deze plaats wordt aan energiebehoeften voldaan door een concentratie van mitochondrien. Nabij het eigenlijke embryo degenereren endospermcellen als het scutellum in grootte toeneemt en de afbraakproducten worden dan eveneens door het embryo opgenomen.

De in vivo ontwikkeling van de extra-embryonale delen van de caryopsis vanaf 3 tot 22 DAP wordt in hoofdstuk 6 beschreven. Hierbij is gebruik gemaakt van TEM, LM en immunocytochemische technieken ter detectie van het microtubulaire cytoskelet. Vanaf het moment van bestuiven ontwikkelen vruchtwand, integument en nucellus zich op verschillende wijzen. De vruchtwand vormt drie lagen en wel een exocarp, een mesocarp en een endocarp . Na een periode van celgroei en -differentiatie waarin met name de ordening van de microtubuli een bepalende rol bij de morfogenese speelt, verdwijnt het mesocarp vrijwel geheel inclusief de celwanden. Tijdens de differentiatie van exo- en endocarp ontwikkelen de celwanden juist sterk en ze zijn aan het einde van de onderzoeksperiode de enige structuren die over blijven. Het integument bestaat kortstondig en van de nucellus resteren op 17 DAP slechts de celwanden en de cuticula van de epidermis.De endospermontwikkeling wordt gekarakteriseerd door de ontwikkeling van de aleuronlaag, enige sub-aleuronlagen en het centraal gelegen endosperm. Kwantificering van organelverdeling, organelgrootte en organelhoeveelheid geeft een duidelijk verschil te zien tussen de diverse weefsellagen. Belangrijke cytologische gebeurtenissen zijn ondermeer de accumulatie van voedingsstoffen zoals zetmeel in plastiden, opslageiwitten in twee typen "protein bodies" en van vetten in sferosomen, ieder met een eigen lokatie in het endo-

sperm. De lokatie hangt samen met de differentiatie van verschillende lagen in het endosperm hetgeen zich ondermeer uit in de ontwikkeling van verschillende celvormen. Die morfogenese van de cellen in de maïscaryopsis is gerelateerd aan de organisatie van het cytoskelet. Drie hoofdvormen van microtubulaire configuraties zijn onderscheiden. Allereerst zijn er de microtubuli in de spoelfiguren. Zij hebben een functie bij de verplaatsing van chromosomen tijdens kerndelingen. Ten tweede zijn er cytoplasmatische microtubuli die door het cytoplasma lopen en waarschijnlijk de beweeglijkheid en de verplaatsing van organellen beinvloeden en een functie hebben in het stabiliseren van de ruimtelijke ordening binnen het cytoplasma . Tenslotte zijn er cytoplasmatische microtubuli die in het perifere ofwel corticale cytoplasma van de cel voorkomen. Deze corticale microtubuli blijken een functie te hebben in de cytomorfogenese. De ordening van de corticale microtubuli in de verschillende weefsellagen bleek als volgt. In de langwerpige cellen van exocarp, endocarp en integument werden microtubuli in parallelle rangschikking gevonden en wel bijna loodrecht op de lengterichting van de cellen. In de isodiametrische en armpallisade-vormige mesocarpcellen werd een kris-kras patroon gezien. In de aleuronlaag liggen microtubuli parallel aan elkaar tegen de anticlinale celwanden. In de isodiametrische cellen van de sub-aleuronlagen worden kris-kras patronen gezien en in langwerpige cellen weer parallelle patronen. Hieruit wordt geconcludeerd dat wanneer parallelle ordening wordt aangetroffen dit aanleiding geeft tot het langwerpig worden of verlengen van cellen in een richting loodrecht op die van de microtubuli. Indien echter corticale microtubuli in een kris-kras configuratie worden gevonden is er ofwel sprake van een isodiametrisch groeipatroon of het is te verwachten. Alhoewel op deze wijze de vormontwikkeling van cellen verklaard kan worden, is met dit onderzoek niet geanalyseerd welke factoren de ordening van de microtubulaire configuraties bepalen.

In het zevende hoofdstuk tenslotte worden de gegevens van de voorgaande zes hoofdstukken met elkaar in verband gebracht en in een breder kader geplaatst met als doel de morfogenese als proces in iedere ontwikkelingsfase herkenbaar te maken en te verbinden met

diverse parameters. Daarbij wordt gelet op de relaties met nutriententransport en -accumulatie, polariteit, genetische variatie en experimentele beinvloeding.

CURRICULUM VITAE

Andreas Antonius Maria van Lammeren werd op 5 december 1950 te Eindhoven geboren. In 1969 behaalde hij het HBS-b diploma aan het Hertog Jan College te Valkenswaard. In hetzelfde jaar begon hij zijn universitaire studie aan de Katholieke Universiteit Nijmegen en behaalde het doctoraalexamen in het hoofdvak Biologie in 1975.

De doctoraalstudie omvatte het hoofdvak Botanie en de bijvakken Medische Biologie en Chemische Cytologie. Het hoofdvak met specialisatie Virologie bestudeerde hij bij de vakgroep Virologie van de toendertijd nog geheten Landbouwhogeschool te Wageningen, de Medische Biologie met specialisatie Endocrinologie aan het Pre-klinisch Instituut van het Academisch Ziekenhuis in Nijmegen en de Chemische Cytologie aan de Faculteit der Wiskunde en Natuurwetenschappen van de Katholieke Universiteit Nijmegen.

Vanaf augustus 1975 tot december 1976 doceerde hij Biologie aan de Rooms Katholieke Havo School Maris Stella te Dongen.

Sedert september 1976 is hij verbonden aan de Landbouwuniversiteit als universitair docent bij de vakgroep Plantencytologie en -morfologie. In die periode verzorgde hij onderwijs en verrichtte onderzoek op het gebied van de geslachtelijke voortplanting bij de hogere plant wat geleid heeft tot het verschijnen van diverse publicaties en de totstandkoming van dit proefschrift.