CYTODIFFERENTIATION DURING CALLUS INITIATION AND SOMATIC EMBRYOGENESIS IN ZEA MAYS L.

CYTODIFFERENTIATIE TIJDENS CALLUSINITIATIE EN SOMATISCHE EMBRYOGENESE IN ZEA MAYS L.

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Cytodifferentiation during callus initiation and somatic embryogenesis in Zea mays L.

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
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STELLINGEN

I Het toekennen van regeneratievermogen aan de maislijn A632 op basis van slechts visuele waarneming van compact callus is niet verantwoord.

Duncan et al. (1985), Planta 165, 322-332.

II Het optreden van een shock-fase en een groei-fase tijdens de callusinitiatie in het scutellum van onrijpe maisembryo's toont aan, dat de dedifferentiatie direkt plaatsvindt bij het in kweek brengen van dit meristematische orgaan.

Dit proefschrift.

III De term "niet-embryogeen callus", zoals gehanteerd door Heyser et al., dient in te houden, dat plantregeneratie bij dit callustype niet mogelijk is.

Heyser et al. (1985), Z. Pflanzenzuchtg. 94, 218-233.

IV De vergelijking van vochtig en zacht callus met hard callus op basis van hun versgewicht maakt de uitkomst van een analyse van metabolieten discutabel.

Songstad et al. (1988), Plant Cell Reports 7, 262-265.

V Door onderscheid te maken tussen een "embryonic region" en een suspensor in somatische embryos van Picea glauca wordt ten onrechte gesuggereerd, dat de suspensor geen deel uitmaakt van het embryo.

Hakman et al. (1987), Protoplasma 140, 100-109.

VI De voordelen van paraffine versus plastics bij inbeddingen worden te weinig onderkend.

VII Gezien de verwarring die het gebruik van zowel voor- als achternaam bij wetenschappelijke publicaties kan veroorzaken, dient uitsluitend de achternaam voluit geschreven te worden slechts voorzien van voorletters.

Olga E. (1975), cited by S.C. Tiwari (1983) Ann. Bot. 51, 17-26.
Hungtu Ma, Minghong Gu and G.H. Lian (1987). Theor. Appl. Genet. 73, 389-394.

VIII In de controverse rond de verdunningsexperimenten van Benveniste zouden zowel voor- als tegenstanders wat water bij de wijn moeten doen, opdat na een oneindige verdunning de vertroebelde sfeer enigszins opheldert.

Davenas et al. (1988), Nature 333, 816-818.
Maddox et al. (1988), Nature 334, 287-291.
Coles (1988), Nature 334, 372.

IX De doctorsbul is tevens een garantiebewijs voor psychische stressbestendigheid.

X In tegenstelling tot de Iraanse en Iraakse beweringen betreffende successen in de Golfoorlog, zijn het de wapenfabrikanten, die als enige overwinnaars uit de strijd zullen komen.

XI Het verlagen van de subsidies voor de kinderopvang heeft een negatief effect op de positieve discriminatie ten aanzien van vrouwelijke sollicitanten.

Stellingen behorende bij het proefschrift: "Cytodifferentiation during callus initiation and somatic embryogenesis in Zea mays L." door P.F. Fransz.

Wageningen, 7 oktober 1988.

aan Agaath en Casper

VOORWOORD

Dit proefschrift is door mij geschreven, maar had nooit gepubliceerd kunnen zijn zonder de medewerking en de inzet van anderen. Daarom wil ik op deze plaats de mensen bedanken, die mij hebben geholpen bij het tot stand komen van dit proefschrift.

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Agaath heeft mij bijgestaan vanaf het begin van mijn studie. Zij steunde mij wanneer het studeren moeizamer verliep en moest menige eenzame uren doormaken. Vooral tijdens het schrijven van dit proefschrift heeft zij mij over vele "dode punten" heen geholpen. Dank je wel.

ABBREVIATIONS

2,4D	- 2,4 dichlorophenoxyacetic acid
ACP	- acid phosphatase
DAPI	- 4,6 diamidino-2-phenylindole dihydrochloride
dicamba	- 3,6 dichloro-o-anisic acid
EDC	- embryogenically determined cells
FLN	- fraction of labeled nuclei
GDH	- glutamate dehydrogenase
GP 0	- Green and Phillips (1975) nutrient medium without 2,4D
GP 2	- Green and Phillips (1975) nutrient medium with 2 mg 2,4D per liter
MI	- mitotic index
MPG	- multicellular pollen grain
MS	- Murashige and Skoog (1962) nutrient medium
N6	- Nutrient medium according to Chu et al. (1975)
OsO_4	- osmium tetroxide
PAS	- periodic acid-Schiff's reagent
PRX	- peroxidase

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Historical view

The first report on plant tissue culture was published in 1902 by Haberlandt, who cultured isolated vegetative cells of higher plants. Haberlandt did not succeed to obtain cell divisions, due to the use of highly differentiated palisade cells and the simple composition of the medium. Despite this failure it formed no obstacle for later researchers to elaborate his view on the totipotency of plant cells: 'Without permitting myself to pose further questions, I believe in conclusion that I am not making too bold a prediction if I point to the possibility that in this way one could succesfully cultivate artificial embryos from vegetative cells' (Haberlandt, 1902, translated and provided with comments by Krikorian and Berquam in 1969). The demonstration of totipotency, considered as the regenerative capacity of plant cells, became a challenge to botanical scientists working in the field of tissue culture.

Tissue culture experiments were quite unsuccessful for the next thirty years and only poor growth was obtained from excised roots by Kotte (1922) and Robbins (1922). The use of vitamins, meristematic tissue and, most important, the discovery of auxins at last resulted in the first real tissue culture. At the end of the thirties Gautheret (1939) and Nobecourt (1939), both working with Daucus, and White (1939), with Nicotiana, independently published their results on unlimited culture of plant tissue. This breakthrough led to an increased interest in this experimental field in science. The callus cultures from Daucus and Nicotiana were soon followed by cultures of other plants. Increased knowledge of auxins contributed to this success, while another growth regulator, kinetin, was discovered by Miller et al. (1955), who examined the use of coconut milk as a nutrient in tissue culture.

Muir et al. (1954) obtained growing tissue cultures from single tobacco cells by culturing them on a filter paper that was placed on a tobacco callus colony. Although this result was no full proof for the totipotent character of a plant cell it brought the theory of Haberlandt nearer to practice. In 1958, Steward demonstrated the embryogenic ability of a carrot cell suspension. It was unclear, however, whether regeneration was originated from a single cell or from cell clumps. Some years later Vasil and Hildebrandt (1965) were able to obtain plantlets from completely isolated

cells of a tobacco hybrid using a medium with coconut milk and naphtylene-acetic acid. About the same time Guha and Maheshwari (1964) discovered the totipotency of pollen grains from Datura inoxia by culturing anthers in a mineral salt medium supplemented with casein hydrolysate, indole-acetic acid and kinetin. They observed the development of normal embryos and plantlets; their haploidy was confirmed later and by that their origin from male gametes. In conclusion, it took over sixty years after Haberlandts publication to establish the regeneration of a complete plant from a single cell.

During the last two decades the number of publications on plant regeneration has increased enormously (see Flick et al., 1983; Ammirato, 1983; George and Sherrington, 1984 and Williams and Maheswaran, 1986). An increasing number of publications is also observed for tissue culture with respect to biotechnological application. Improvement of biochemical, molecular and cell biological techniques resulted in advanced methods to manipulate plant tissue, e.g. by gene transfer, micro-injection and protoplast fusion, or to isolate specific metabolic products in cell suspensions (see Evans et al., 1983; Vasil, 1984).

Monocotyledonous tissue culture

The in vitro culture of angiosperm explants has long been restricted to plant species of the dicotyledonous class. Daucus, Datura, Nicotiana, Helianthus, Petunia and Lycopersicum proved to be excellent model systems to improve culture techniques or to study fundamental principles of tissue culture. On the contrary, members of the monocotyledons were poorly involved in the development of plant tissue culture. Despite the economic importance of cereals, the efforts appeared unsufficient for a long time to obtain regeneration. Only in 1947, La Rue reported regeneration in cultured maize endosperm. However, it is quite likely that the endosperm tissue was not free from the young embryo and that this resulted in the observed callus culture. More definite reports on morphogenesis in cereal tissue cultures were not established until the late sixties with Avena (Carter et al., 1967), Oryza (Nishi et al., 1968) and Triticum (Shimida et al., 1969). The induction of haploid rice plants was also reported in the same period (Niizeki and Oono, 1968). The relatively unresponsive behaviour of cereals and grasses in tissue culture earned for this group the label 'recalci-

trant'.

The breakthrough in tissue culture of monocotyledons started in the seventies when it became obvious that plant regeneration in this class could be achieved from immature explants, while mature tissue generally produced nonregenerable callus. The use of strong growth regulators, such as 2,4 dichlorophenoxy-acetic-acid (2,4D) or dicamba, contributed to the success in monocotyledonous tissue culture. Extensive lists of publications on regeneration in Gramineae are filed by Flick et al. (1983), Ammirato (1983) and George and Sherrington (1984).

Concerning regeneration through tissue culture of Zea mays we find the first publication by Green and Phillips (1975) who cultured immature embryos on a modified Murashige and Skoog (1962) solid medium with 2 mg per liter 2,4D. Shoot formation in callus cultures was subsequently reported by Freeling et al. (1976), Harms et al. (1976) and Novak et al. (1979). However, success was restricted to a few genotypes. Improvement of culture conditions, mainly obtained by increased efforts of scientists active in the emerging field of biotechnology, finally resolved the growing pains of tissue culture in maize and other cereals. Regeneration is now possible in many genotypes. For example, Duncan et al. (1985) obtained embryogenic callus in 199 out of 218 inbred lines and germplasm stocks using an accurately composed medium with dicamba as a growth regulator. Various immature explants have been succesfully tested (see later in this chapter).

Recently it has been reported that regeneration has been accomplished from maize protoplasts (Vasil and Vasil, 1987; Kamo et al., 1987; Rhodes et al., 1988). Embryoid and plantlet formation, and also mature plant development from protoplasts were already reported in other Gramineae (for reviews, see Vasil, 1987; Lörz et al., 1988). With these experiments, totipotency finally has been demonstrated in single cells of monocotyledons eighty years after Haberlandts prophecy.

Plant regeneration in cereal tissue culture

Factors affecting the culture response

In general, plant propagation through tissue culture is achieved when new plantlets are formed on the cultured explant and develop to maturity. Es-

pecially in monocotyledonous species the establishment of regeneration is strongly affected by important factors, such as explant type, culture conditions and genotype.

Explant type. The use of immature explants with undifferentiated tissue like young leaves, young inflorescences or immature embryos is essential for a successful culture response. A detailed list of immature maize explants which were successfully used is given in Table 1. The detailed specification by the authors of age, developmental stage, and orientation on the medium shows that the conditions in cereal tissue culture are even more strict than just the immature state of the explant. Moreover, even explants with similar developmental stages differ in culture response during various years (Santos and Torne, 1986), or if isolated from mother plants cultivated in different ways (Lu et al., 1983; Tomes, 1985a).

Culture conditions. The media of Murashige and Skoog (MS, 1962) and Chu et al. (N6, 1975) are often used in combination with a strong growth regulator, e.g. 2,4D or dicamba. Other types of culture media are generally modified variants (see also Flick et al., 1983; Ammirato, 1983). Sucrose concentration or the presence of organic components like proline or casein hydrolysate are varied to raise the efficiency of embryogenic callus production and plant regeneration (Lu et al., 1983; Kamo et al., 1985; Armstrong and Green, 1985).

Genotype. It is evident that in monocotyledons there is a strong genotypic influence on the culture response. For example, the maize inbred lines A188 and A619 are very succesfull for tissue culture whereas A632 and B73 are difficult to regenerate (Green and Phillips, 1975; Green and Rhodes, 1982; Tomes, 1985a; but see Duncan et al., 1985). It has been demonstrated by Tomes (1985b), Hodges et al. (1985) and Rhodes et al. (1986) that partially dominant nuclear genes control the formation of embryogenic callus and plant regeneration through tissue culture.

Pathways of regeneration

Plant regeneration in tissue culture may follow different pathways. At first, one might distinguish between a direct and an indirect way. The former implies the de novo development of meristems on the explant from which new explants originate. In the case of indirect regeneration the

Table 1. List of most relevant literature on immature explants used for plant regeneration through tissue culture in maize

explant	reference
immature embryo	Green and Phillips (1975), Springer et al. (1979), Lu et al. (1982, 1983), Vasil et al. (1983, 1984, 1985), Armstrong and Green (1985), Kamo et al. (1985)
germinated immature embryo	
-coleoptile, leaflet	Chang (1983)
-mesocotyl	Torne et al. (1980)
germinated mature embryo	
-mesocotyl	Harms et al. (1976)
-leaf segments	Conger et al. (1987)
-plumule	Wang (1987)
-seedling segments	Santos et al. (1984)
immature tassels	Rhodes et al. (1986)
anther-free glumes	Suprasanna et al. (1986)
anthers	Ku et al. (1978), Miao et al. (1978), Ting et al. (1981), Genovesi and Collins (1982), Nitsch et al. (1982), Petolino and Jones (1986), Tsay et al. (1986)

development of new plantlets from the cultured explant is interrupted by an intervening callus phase. This callus is undifferentiated or slightly differentiated tissue with unorganized growth. Embryogenic callus is regarded as callus tissue with regenerative capacity. On the contrary, non-embryogenic callus does not produce new plantlets, but merely roots or root-like structures.

A second distinction in the regeneration proces is made between organogenesis, also called shoot morphogenesis, and somatic embryogenesis. Organogenesis is the development of shoots from the explant culture. The shoots are formed upon the explant (directly) or on callus tissue (indirectly). After induction of root formation small plantlets develop and may grow to maturity. Somatic embryogenesis is the formation and development of somatic embryos or embryoids and resembles the zygotic embryogenesis during seed development. The embryoids can grow to maturity and subsequently germinate and develop into plantlets. Similar to organogenesis a direct and an indirect development are distinguished depending on the absence or presence of an intervening callus phase. Figure 1 shows the different morphogenetic ways of regeneration as observed in embryo cultures of Zea mays. All studies used immature embryos with a scutellum length of 1.5-2.0 mm, which proved to be a sufficient specification of the required developmental stage. The morphogenetic way of regeneration is genetically controlled (Tomes, 1985b; Hodges et al., 1985). However, the culture conditions strongly affect the pattern of development. This can be deduced from the fact that embryo cultures of the same inbred line, A188, showed various pathways of regeneration on different media (Green and Phillips, 1975; Springer et al., 1979; Armstrong and Green, 1985; McCain and Hodges, 1986).

Callus initiation and development

Indirect regeneration involves the initiation and development of undifferentiated callus, with regenerative potency. In dicotyledonous cultures from mature explants callus is formed after a process of dedifferentiation, by which in general highly vacuolated cells undergo a sequence of changes that ultimately lead to cell division (for a review, see Yeoman and Street, 1977). The differentiated tissue turns into callus and the meristematic regions are extended by continued cell proliferation.

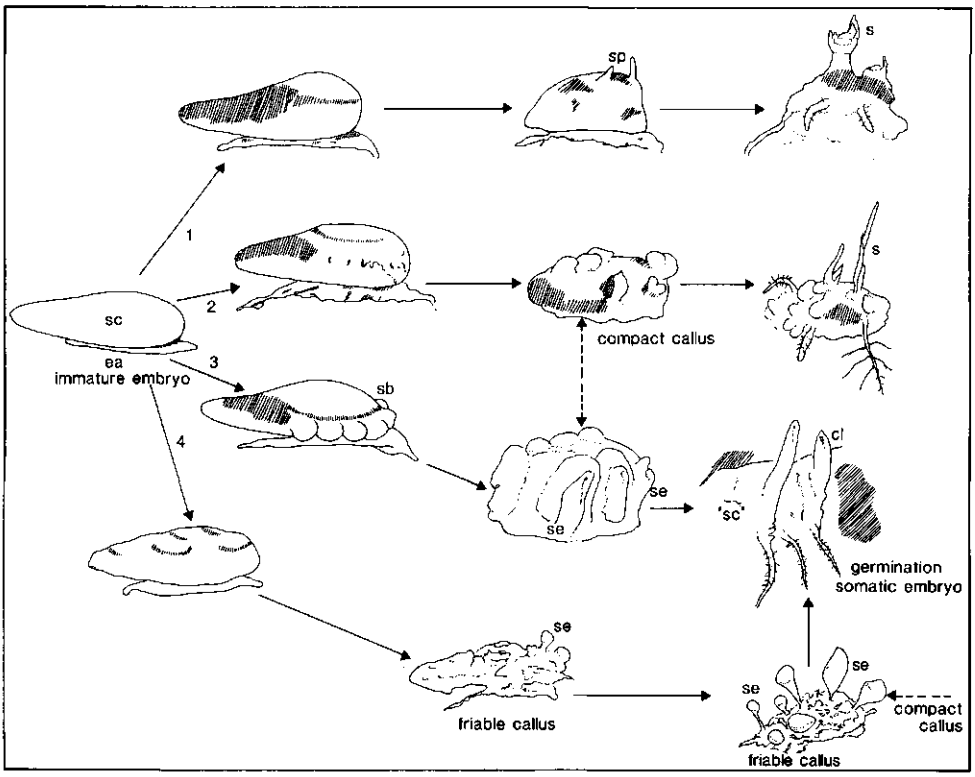

Fig. 1 Schematic representation of the 4 different pathways of regeneration in cultured maize embryos. (1) direct organogenesis, (2) indirect organogenesis, (3) direct somatic embryogenesis, (4) indirect somatic embryogenesis. The developmental pathway can be changed into another pathway, which is indicated by dashes. cl, coleoptile; ea, embryo axis; s, shoot; sb, scutellar body; sc, scutellum; 'sc', scutellum of somatic embryo; se, somatic embryo; sp, shoot primordium.

Role of growth regulators. Using undifferentiated tissue the unability of differentiated cereal tissue to dedifferentiate is circumvented by the establishment of embryogenic callus. Strong growth regulators are needed to achieve this. The synthetic auxin, 2,4 dichlorophenoxyacetic acid (2,4D), is most widely applied for callus induction. The concentration ranges in general from 1 to 5 mg/l. In some cases high doses, up to 15 mg/l (Harms et al., 1976) or 30 mg/l (Wernicke and Milkovits, 1984), were used. Another herbicide, 3,6-dichloro-o-anisic acid (dicamba) also proved to be able to

induce embryogenic callus (Longer et al., 1983; 1987; Duncan et al., 1985; Papenfuss and Carman, 1987). Sanchez de Jimenez et al. (1981) tested the effects of several 2,4D analogues on induction and maintenance of maize callus and concluded that the analogues with the alkyl substitution on the carbon chain of the acids were more effective than 2,4D. Unfortunately, they did not report any regeneration from the calli. It is still unclear by what mechanism 2,4D urges the explant tissue to transform into unorganized callus. Zwar and Brown (1968) reported the accumulation of ^{14}C labelled 2,4D in nucleoli of dividing cells in cultured explants of the Jeruzalem artichoke tuber. This suggests some interaction between the auxin and nuclear regions.

Callus origin sites. As young leaves and immature embryos were most succesfull in cereal tissue culture, callus initiation was mainly studied with these explants. In cultured embryos the scutellum cells proliferate rapidly and embryogenic callus was formed within a few days after the onset of culture (Green and Phillips, 1975; Vasil and Vasil, 1981; Lu et al., 1982, 1983; Novak et al., 1983). A detailed analysis showed that scutellar callus originated (i) from epidermal and subepidermal cells or (ii) around the procambial strand near the scutellar node (Ozias-Akins and Vasil, 1982; Vasil and Vasil, 1982; Vasil et al., 1985). Sometimes the nodal region or the plumule of the embryo axis gave rise to embryogenic callus (Vasil et al., 1983; Heyser et al., 1985).

Callus initiation in young leaves was extensively studied by Wernicke and coworkers in Sorghum and wheat. They cultured immature leaf segments of young plantlets and discovered that the ability to regenerate was rapidly lost during leaf maturation (Wernicke and Brettell, 1980, 1982). It was further suggested by Wernicke and Milkovits (1984) that callus growth was initiated only from cells which had apparently left the mitotic cell cycle and were in the process of differentiation. Cytological examination revealed that proliferation was initiated in mesophyll and epidermal cells close to vascular bundles (Wernicke et al., 1982). Ho and Vasil (1983) observed cell divisions, mainly in mesophyll cells of the abaxial half of culture leaf segments of sugarcane, but also from vascular parenchyma cells. In cultured immature inflorescences embryogenic callus arose from rachis segments or from basal regions of florets (Ozias-Akins and Vasil, 1982; Rhodes et al., 1986).

Cereal callus types. Callus tissue can be subdivided in two types, embryogenic and non-embryogenic, depending on its potentiality to regenerate plants. The description of callus in the literature has often led to confusion or ambiguous terminology. In general, non-embryogenic callus is yellow to translucent, wet, friable, loose and soft. It is composed mainly of large elongated cells, often with meristematic centres producing small cells which enlarge as they move towards the periphery. Non-embryogenic callus often gives rise to roots. Microscopical examination has shown that non-embryogenic callus growth in maize mostly is aberrant growth of roots (Mott and Cure, 1978).

Cereal embryogenic callus can be distinguished in compact callus, or type I, and friable callus, or type II (Green et al., 1983). Type I is most frequently obtained and grows slowly. It is white, pale white or yellowish and compact in appearance. The nodular structures betray the presence of organized tissue. They are termed 'scutellar bodies' and are often accompanied by leafy structures or leafy scutella and trichomes (Ozias-Akins and Vasil, 1982; Novak et al., 1983; Ho and Vasil, 1983). Shoots, roots and embryoids develop from the scutellar bodies (Green and Phillips, 1975; Freeling et al., 1976; Lu et al., 1982; Vasil et al., 1985). During somatic embryo development the embryoids may fuse and form hard and white callus or give rise to secondary somatic embryos (Dunstan et al., 1978; Vasil and Vasil, 1982).

Type II callus was first reported in maize by Green and Rhodes (1982). It grows rapidly and is described as soft, friable, white or pale-yellow and somewhat transparent. It differs from non-embryogenic callus by the presence of numerous smooth and globular embryoids. These embryoids contain a suspensor-like structure, by which they are connected with the callus. After transfer to auxin-free medium they develop into well organized somatic embryos (Green, 1983; Vasil et al., 1984; Kamo et al., 1985). Because of its friability and high regenerative capacity type II callus is very suitable for cell suspensions or protoplast cultures. Complete or partial regeneration from such cultures has been reported for many cereals (for a review, see Lörz et al., 1988).

Microscopical studies on embryogenic callus have been carried out almost exclusively on type I callus (McDaniel et al., 1982; Ozias-Akins and Vasil, 1982; Wernicke et al., 1982; Vasil and Vasil, 1982; Ho and Vasil,

1983; Vasil et al., 1985). The reports confirm the compact appearance by histological evidence. In some reports the presence of a distinct shoot-root axis during somatic embryo dvelopment is clearly demonstrated. On the other hand, type II callus is scarcely studied by light microscopy. Vasil et al. (1985) examined friable embryogenic callus in cultured embryos of maize. They focussed, however, on the somatic embryo development and paid less attention to the morphogenesis of the friable callus.

Ultrastructural studies on embryogenic cultures of Gramineae were made only by Kott et al. (1985) on barley, by Karlsson and Vasil (1986) on guinea and napier grass and by Huang (1986) on androgenesis of barley and wheat. Microscopical research on dicotyledonous tissue cultures is far ahead on research in cereals and intensive research is necessary to elucidate processes like dedifferentiation and callus initiation in the monocotyledons.

Somatic embryogenesis

Williams and Maheswaran (1986) have stated that somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. This includes apomixis or spontaneous somatic embryogenesis in vivo (for a review, see Tisserat et al., 1979) as well as the induced somatic embryo development as known from in vitro tissue culture.

Somatic embryos in tissue culture are recognized by their shape and the presence of a shoot-root axis, a closed vascular system and sometimes a suspensor-like structure. Controversy exists on the point of embryoid origin: single cell or multicellular origin. Somatic embryos have been derived from single cells (Botti and Vasil, 1983) as well as from groups of cells (Vasil et al., 1985; McCain and Hodges, 1986). Except in the case of direct embryogenesis from microspores the reports of a single cell origin are not always convincing and even the close examination of few-celled aggregates in suspension culture did not unequivocally give evidence for a unicellular or multicellular origin (Karlsson and Vasil, 1986). Haccius (1978) generalized all plant embryos, zygotic as well as somatic, as originating from a single cell. Even embryoids which seemed to be derived from a broad meristematic region or embryogenic clumps (Halperin and Jensen, 1967) would fall under this definition. According to Haccius the multicellular

proembryonal complex is formed from a segmenting single cell. However, Williams and Maheswaran (1986) summarize a number of papers that are in conflict with the definition of Haccius. The discussion is still going on but detailed analysis of compact and friable embryogenic callus may provide new information about the process of somatic embryogenesis.

Outline of this thesis

The use of immature plant tissue has opened the way towards cereal tissue culture. This offers prospects for further biotechnological research in this economically important plant group. However, it also uncovers a fundamental difference between dicotyledonous and many monocotyledonous culture systems with respect to dedifferentiation and totipotency. Little is known of the ultrastructural changes occurring around the period of callus initiation and further cytological data concerning this event are also lacking. In fact, all information on this subject comes from research with dicotyledonous cultures (Yeoman et al., 1965, 1966, 1968; Israel and Steward, 1966; Halperin and Jensen, 1967; Yeoman and Evans, 1967; Fowke and Setterfield, 1968; Mitchell, 1968; Bagshaw et al., 1969; Yeoman and Mitchell, 1970; Vasil, 1973; Yeoman and Street, 1977; Macleod et al., 1979; Howarth et al., 1981, 1983).

The lack of information in monocotyledonous cultures is probably due to the late breakthrough and the recalcitrant in vitro behaviour of this group. On the other hand, the process of regeneration from cereal tissue culture is well documented with histological and scanning electron microscopical studies (Springer et al., 1979; Dunstan et al., 1978, 1979; Ozias-Akins and Vasil, 1982; Vasil and Vasil, 1982; Wernicke and Brettel, 1982; Botti and Vasil, 1983; Novak et al., 1983; Vasil et al., 1983; McCain and Hodges, 1986). These reports all show the formation of shoots or somatic embryos from compact callus, which was most frequently obtained. Soft and friable embryogenic callus of Zea mays L. with embryogenic potency has been obtained rather recently and has been examined only by light microscopy (Vasil et al., 1985).

This thesis encloses fundamental research in maize tissue culture. Attention is focussed on cytodifferentiation during callus initiation, callus

development and somatic embryogenesis. The research was carried out on Zea mays L. for the following reasons:

- Maize is, together with wheat and rice, one of the three economically most important crops of the world.
- Maize is one of the most widely used objects in biological research (see Sheridan, 1982) and there is a large amount of information available from many different disciplines. For example, embryogenesis in maize has been documented extensively (Randolph, 1936; Abbe and Stein, 1954; Schel et al., 1984, 1986; Van Lammeren, 1987).
- The presence of many ovaries on one ear and the relatively large embryos facilitate tissue culture.
- Embryo culture and callus induction is easily achieved with a high efficiency (Lu et al., 1983).

The various chapters describe light and electron microscopical changes during early callus formation and during embryogenesis from somatic tissue and microspores. By autoradiography, cell cycle events were studied, while electrophoresis and enzyme histochemistry were used to detect biochemical changes at early embryogeny. Aim of the study has been to provide more insight into the processes leading to cytodifferentiation in cereal embryogenic systems by use of a cytological approach.

CHAPTER 2

AN ULTRASTRUCTURAL STUDY ON EARLY CALLUS DEVELOPMENT FROM IMMATURE EMBRYOS OF THE MAIZE STRAINS A188 AND A632

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Summary

Immature embryos of two maize inbred lines (A188 and A632) have been examined during the first three days of in vitro culture using light and electron microscopy. The first day of culture is characterized by an increase in the amount of organelles, changes in vacuolation and in nucleolar morphology. These ultrastructural events take place in both inbred lines and are not influenced by the presence of 2,4D. After one day of culture variation in developmental patterns occurs, which is dependent on genotype and culture conditions. It is found, that in the scutellum of A188 embryos proliferation is activated strongly within two days of culture, followed by the appearance of a broad scutellar meristematic zone. A second meristematic region is observed around the coleorhiza. In A632 embryos only a coleorhizal meristematic zone developed, while in the scutellum proliferation was hardly observed.

Therefore we have subdivided the first period of maize embryo culture into two phases. The first phase is called the shock response and takes about one day, while the second phase starts after one day and is called growth response.

In concurrent cultures of A188, which were prolonged for several weeks, white compact callus developed accompanied by somatic embryos, while in the A632 cultures non-embryogenic callus was produced.

Introduction

Plant regeneration with maize has been obtained most commonly from immature embryos (Green and Phillips, 1975; for recent reviews, see e.g. Green, 1983; Vasil, 1983; Tomes, 1985). In most cases the scutellum was the origin of embryogenic callus formation; histological studies (Springer et al., 1979; Vasil et al., 1985) revealed that the initiation starts in the epidermal and the subepidermal region towards the coleorhizal end of the scutellum. In both studies morphological changes were observed after the third day of in vitro culture. No cytological study, however, has been done of the first days of maize embryo culture. Electron microscopical investigations about this early period have been carried out almost exclusively on dicotyledonous systems (Yeoman and Street, 1977; see also Howarth et al., 1981, 1983). With

respect to the monocotyledons, Kott et al. (1985) investigated the callus origin sites in haploid barley embryos and described some ultrastructural changes of the epidermal and subepidermal scutellar region.

It was reported earlier that the maize strain A188 produces embryogenic callus with a high regeneration efficiency (Green and Phillips, 1975). On the contrary, in cultures from strain A632 only non-embryogenic callus developed without regeneration capacity (Green and Phillips, 1975; Green and Rhodes, 1982; but see also Duncan et al., 1985). Aim of the present study was to investigate the events occurring during the early (0-3 days) stages of culture of these different genotypes using cytological techniques. Some observations were made on cultures with modified conditions, especially with modified levels of 2,4D and sucrose (see Lu et al. 1983).

Materials and methods

In vitro culture

Maize inbred lines A188 and A632 (kindly supplied by Dr. C.E. Green, Minnesota) were grown in the greenhouse. Pollination and harvesting took place in the late summer of 1985. Within two weeks after pollination whole cobs were broken into three or four parts and surface sterilized with 70% ethanol (30 sec), followed by commercial bleach diluted to 1.5% hypochlorite to which a drop of Tween 80 was added (5-10 min). After three rinses with sterile water immature embryos (1-2 mm) were excised and placed on an agar nutrient medium with the embryo axis in contact with the medium. The medium was composed of the inorganic components of Murashige and Skoog (1962) and the organic compounds as described by Green and Phillips (1975). Sucrose was added in two different concentrations (2% and 6%); in most cases 2,4D (2,4 dichlorophenoxyacetic acid) was present (2 mg/l, abbreviated as GP2; medium without 2,4D: GP0). The embryos and resulting callus cultures were incubated at 28-30 ^{o}C with a 16/8 hours light/dark regime. Subculturing took place after two to three weeks.

Light and electron microscopy

After various periods of in vitro culture embryos were sampled and fixed for microscopical examination.

Light microscopy (LM):
Specimens were fixed in 4% glutardialdehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 hr at room temperature. After dehydration through an ethanol series (50-70-90-100-100%) the specimens were embedded in 2-hydroxyethyl-methacrylat (Technovit 7100, from Kulzer, Wehrheim, Germany). Sections (5 µm) were stained with toluidine blue.

Transmission electron microscopy (TEM):
Specimens were fixed in 2% glutardialdehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 hrs at room temperature. Postfixation, sectioning and staining procedures used were as described by Schel et al. (1984).

Scanning electron microscopy (SEM):
Fixation and postfixation took place as for TEM. The specimens were further processed for SEM using the OTO-method, according to Postek and Tucker (1977). After the last rinsing period the specimens were dehydrated and critical point dried. The dried samples were examined with a Jeol ISM-35C scanning electron microscope.

Results

General morphology

Immature embryos from the A188 and the A632 lines had a scutellum length of 1-2 mm at the onset of culture and showed a well defined shoot-root axis. We have subdivided the scutellum into a top, middle and basal region (Fig. 1).

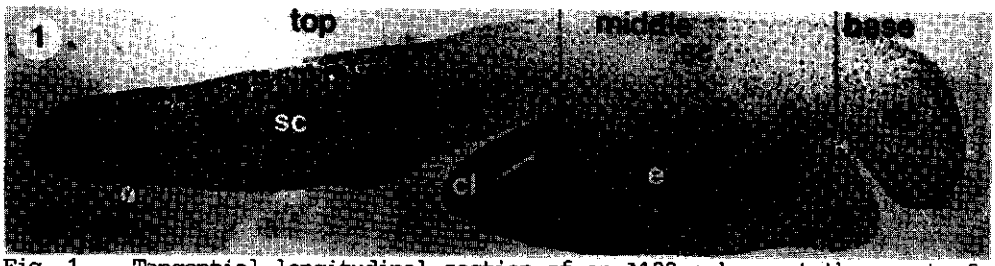

Fig. 1 Tangential longitudinal section of an A188 embryo at the onset of culture (t=0). The scutellum is subdivided into three regions: top, middle and base. e, embryo axis; cl, coleoptile; cr, coleorhiza; sc, scutellum; s, suspensor.

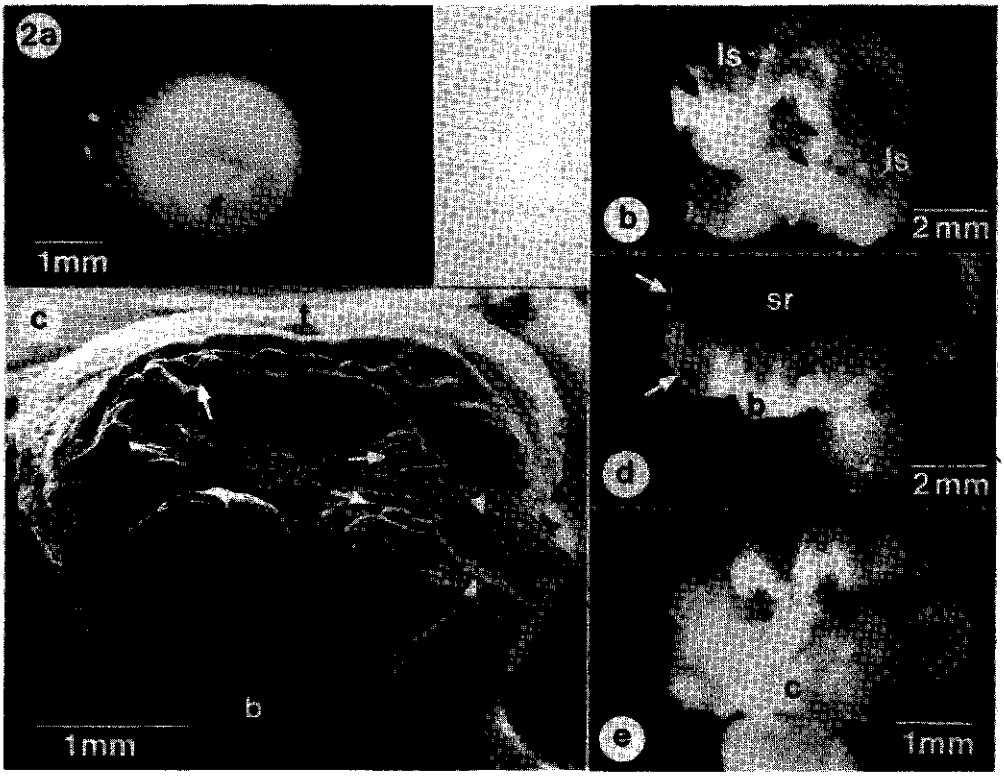

Fig. 2a. Three days cultured A188 embryo on GP2 with 6% sucrose showing the scutellar roof. Note the beginning of disruption (arrow).
b. The same embryo after 10 days of culture. The scutellar roof is completely disrupted. White compact scutellar bodies (arrows) arise at the base of the green leafy structures (ls).
c. Scanning electron micrograph of a five days cultured A188 embryo. Note the presence of scutellar bodies (arrows). Trichomas (arrowheads) develop on the leafy structures. b, scutellar base; t, scutellar top.
d. Ten days cultured A188 embryo on GP2 with 6% sucrose. Note the appearance of trumpet-like structures (arrows). sr, scutellar roof; b, scutellar base; e, embryo axis.
e. After three weeks the scutellar-like bodies have developed into somatic embryoids (arrows). c, compact callus.

Embryos of both strains, cultured on GPO medium, germinated within a few days and the swollen scutellum became green. In the presence of 2,4D embryos from the A188 line developed protuberances within one week and a green spot appeared at the scutellar top. Subsequently, leafy structures were recognized in the middle and basal region. Opaque scutellar-like bodies

appeared at the base of these structures, while friable non-embryogenic callus developed near the coleorhiza. Elongation of the coleoptile often took place. Young leaves emerged from the scutellar-like bodies after three weeks and developed into plantlets. In general, no embryoids could be observed in these cultures. However, in some cases soft and friable embryogenic callus was formed with typical globular embryoids.

When these A188 embryos were cultured with a raised sucrose level (GP2-6% medium) the roof of the swollen scutellum broke open after 3 to 4 days at the centre or the edges of the basal and middle part, but never at the top (Fig. 2a). A green spot appeared at the top and within 10 days a completely disrupted scutellum was present (Figs. 2b and c). When the scutellum roof remained intact, "trumpet-like" structures with knurled edges appeared (Fig. 2d). White compact embryogenic callus developed at the base of the these structures or from under the disrupted scutellum roof. After several weeks shoots and roots and somatic embryos (Fig. 2e) were observed. Subculturing was then needed to prevent excessive root formation.

If A632 embryos were put on GP2-medium with 6% sucrose the events as described above were not observed. These embryos generally became yellow to brown; friable non-embryogenic callus was produced starting near the coleorhiza from under the scutellum. This callus mainly produced roots; in one case, a white scutellar-like body was observed, but regeneration could not be obtained.

Cytomorphology

In previous studies by Springer et al. (1979) and Vasil et al. (1985) it was reported that scutellar embryogenic callus develops in the (sub)epidermal region near the base of the scutellum. Therefore we focussed in this study on the middle and basal scutellar region.

Observations on both strains at the onset of culture (t = 0).

As shown in Fig. 3a, cells of the line A188 contained a round nucleus, whereas the nucleus of A632 cells was more or less lobed (Fig. 3). Several mitochondria and plastids were present. Ribosomes were observed free as well as bound to the endoplasmic reticulum (Fig. 3c). Cells of strain A632 also contained many polysomes (Fig. 3d, arrows). The cell wall in both strains

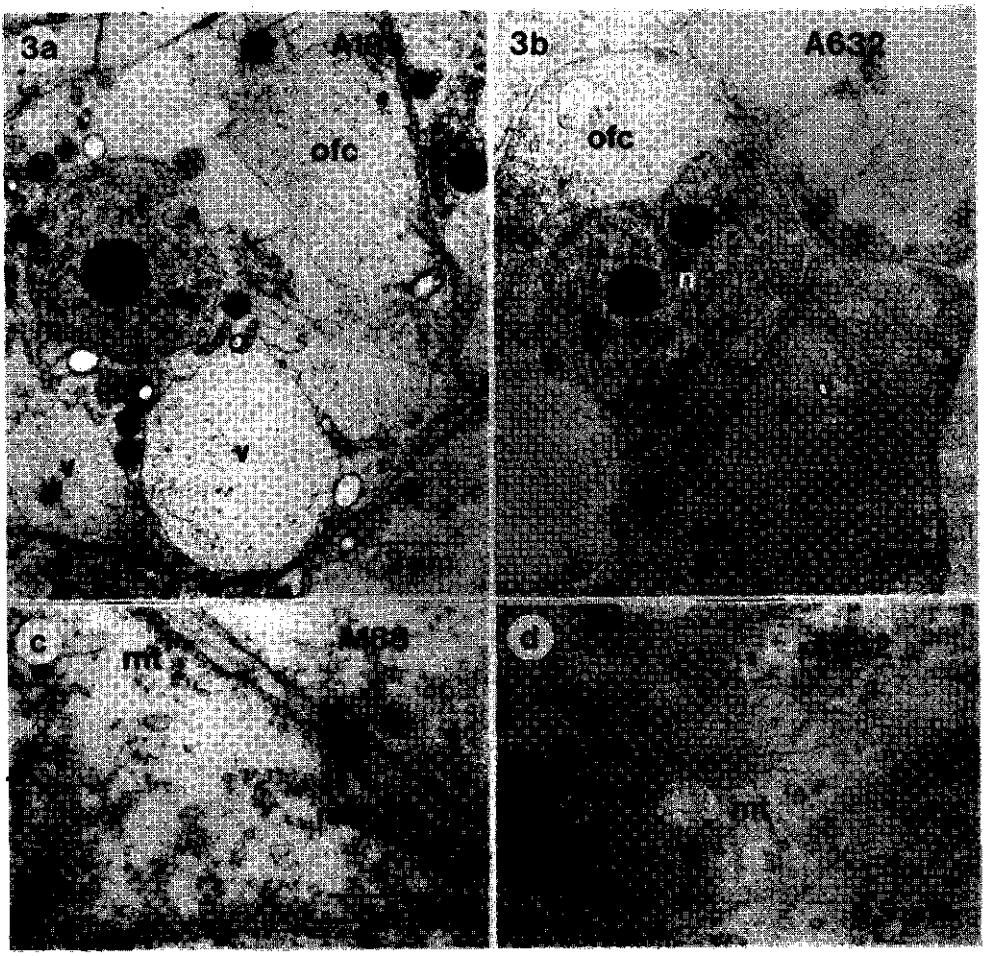

Fig. 3a. Electron micrograph of a scutellum cell at the basal region from A188. n, nucleus; v, vacuole; m, mitochondrion;p, plastid; ofc, organelle-free cytoplasm.

b. As in Fig. 3c, but now from A632.

c. Detail near the cell membrane of an A188 scutellum cell. Many ribosomes are present. rer, rough endoplasmic reticulum; d, dictyosome; mt, microtubules.

d. Detail near the cell membrane of an A632 scutellum cell. Note the microtubules (mt) and the helical polysomes (arrows).

had a firm appearance except for newly formed cell walls which were slightly irregular. Plastids sometimes contained starch granules. The nuclear chromatin was diffuse with patches of condensed chromatin. The nucleolus was characterized by a peripheral granular region and a central fibrillar region. The vacuoles were small or medium-sized and cytoplasmic areas were observed in which no organelles other than some ribosomes were found (Figs 3a,b). This organelle-free cytoplasm was encircled by vacuoles, small vesicles and endoplasmic reticulum, but not by a continuous membrane. Cortical microtubules were abundant (Figs. 3, d).

Cytological changes during the first days of culture on GP2 medium containing 6% sucrose.

The cytoplasm of A188 embryos, studied by light microscopy, was no longer uniformly stained after 30 min of culture. This was mainly due to the vacuolation, which was more evident. Mitotic activity was strongly reduced. After one day of culture vacuolation had decreased, while proliferating cells were present in the whole scutellum. Day 2 embryos showed an increased mitotic activity in the whole scutellum, especially in the middle and basal region. At day 3 a broad meristematic zone was present in the middle part of the scutellum and also around the coleorhiza (Fig. 4a). Tracheary elements developed in the top of the scutellum.

The A632 scutellum cells showed an identical pattern as A188 after 30 min of culture: an increased vacuolation accompanied by a reduced mitotic activity. After one day vacuolation decreased while mitotic figures reappeared in the scutellum, especially in the vascular region. At day 3 the embryos showed a meristematic zone around the coleorhiza (Fig. 4b). Little meristematic activity is seen in the scutellum.

The ultrastructural observations confirm the increase in vacuolation of the A188 scutellum cells after 30 min of culture (see Table 1). Many polysomes with helical configurations appeared in the cytoplasm. Organelle-free cytoplasmic regions were still present. In the nucleolus the fibrillar region was surrounded by the granular part, although in some cells these regions were intermingled. Dictyosomes were frequently observed while microtubules could not be found.

After one day of culture the scutellum cells showed a reduced vacuolation (Table 1). The small vacuoles contained electron-dense material and

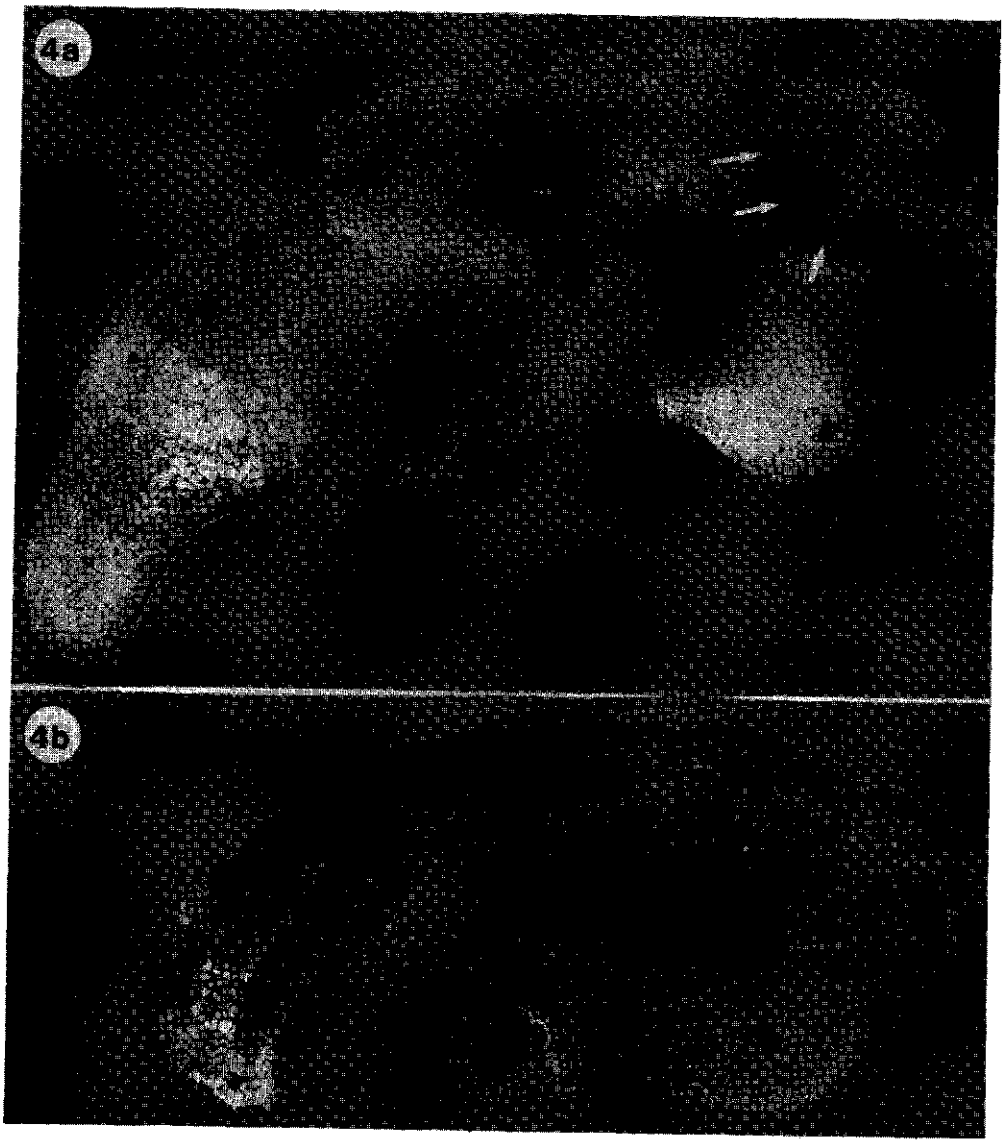

Fig. 4. Tangential longitudinal section of an A188 embryo (a) and an A632 embryo (b) after three days of culture on a GP2 medium with 6% sucrose. Note the meristematic zones (white arrows) in the scutellum (sc) and around the coleorhiza (cr, arrowhead) from A188 and around the coleorhiza (black arrows) only in the A632 embryo. Also note the protuberance (pr) at the basal end of the A188 embryo.

sometimes osmiophilic droplets. Areas of organelle-free cytoplasm were no longer detected. The nucleolus showed intermingled granular and fibrillar regions, often with a so-called nucleolar vacuole (Jordan, 1984). The cytoplasm was filled with high concentrations of polyribosomes, numerous mitochondria, dictyosomes and plastids, some of which contained starch (Fig. 5a). Microtubules were observed near the cell wall which is more or less irregular.

Table 1 Surface density* of vacuoles in scutellum cells during the first three days of culture. Data expressed as means ± standard error.

time in culture	A188 GP2 6%	A188 GPO	A188 GP2	A632 GP2 6%
0	0.203 ± 0.055			0.180 ± 0.091
30 min	0.391 ± 0.133	0.831 ± 0.053		0.794 ± 0.065
1 d	0.085 ± 0.031	0.501 ± 0.178	0.294 ± 0.078	0.114 ± 0.048
2 d	0.058 ± 0.026[a] 0.232[b]		0.439 ± 0.139	
3 d	0.138 ± 0.067	0.374 ± 0.050	0.348 ± 0.115	0.023 ± 0.012

* Surface density (s^{vac}/s^{cell}), calculated from several cells, is expressed in units of $\mu m^2/\mu m^2$.

a Surface density at the basal region.

b Surface density in a representative cell at the middle region.

Scutellum cells of day 2 embryos showed meristematic characteristics: relatively small cells, with a central nucleus and a high cytoplasmic density. The vacuoles contained electron-dense material. In the nucleolus the fibrillar and granular regions were segregated or intermingled. One large or several small nucleolar vacuoles were often observed. In the middle part of the scutellum, amyloplasts were numerous, and shape of the cell wall was very irregular. Intercellular spaces were prominent (Fig. 5b). At the scutellar base the cell wall was thin and wrinkled. Here no intercellular spaces were observed (Fig. 5c). After three days of culture vacuolation

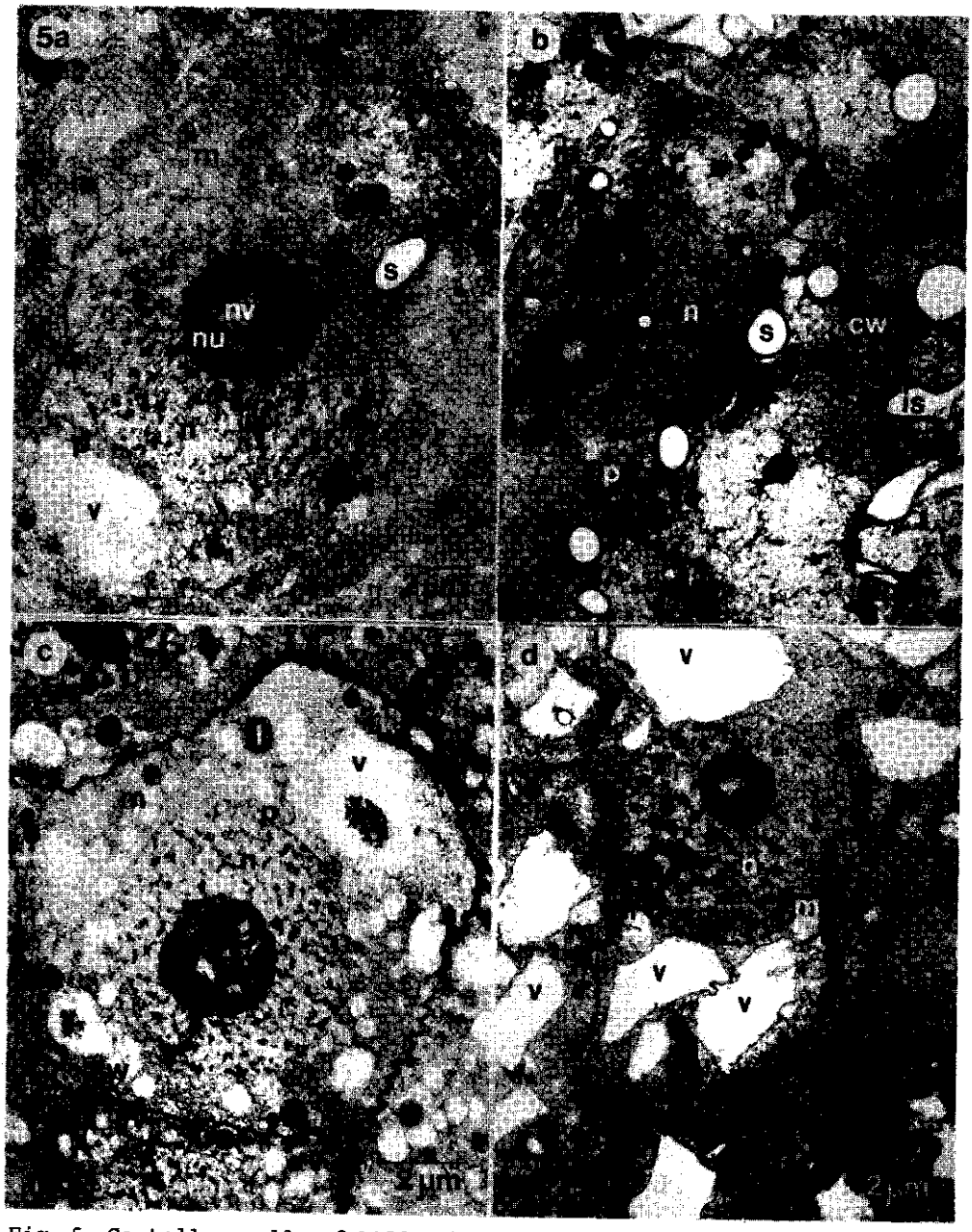

Fig. 5. Scutellum cells of A188 embryos cultured on a GP2 medium with 6% sucrose. a, one day culture; b, two days culture at the middle region; c, two days culture at basal region; d, three days culture middle/base. n, nucleus; nu, nucleolus; nv, nucleolar vacuole; v, vacuole; m, mitochondrion; p, plastid; s, starch; l, lipid droplet; cw, cell wall; is, intercellular space.

increased in the basal scutellum but reduced in the middle region (Table 1). The vacuoles contained no more electron-dense material (Fig. 5d). The amount of starch granules was decreased.

In the A632 scutellum cells the vacuoles were significantly enlarged after 30 min of culture (Table 1). No change in nucleolar morphology was observed. The high concentrations of ribosomes caused a high cytoplasmic density. After one day of culture the same characteristics as in the A188 cells were observed. Vacuolation was decreased and vacuoles contained electron-dense material. Many helical polysomes, dictyosomes and mitochondria were present and plastids sometimes contained starch. The nucleolus, however, still had the segregated arrangement of granular and fibrillar regions. The cell wall was irregularly formed.

After three days the cells of the meristematic zone around the root apex (see Fig. 4b) had penetrated into the scutellum and showed a high cytoplasmic density. They did not differ in morphology from the subepidermal cells of the middle and basal scutellum. In contrast to the A188 cells the cells of the A632 strain contained very small vacuoles (Fig. 6a). In the nucleolus the fibrillar and granular regions were generally intermingled although incidently segregation occurred. Nucleolar vacuoles were infrequently observed. Plastids contained starch.

Cytomorphogenesis of A188 embryos cultured on GPO and GP2 medium with 2% sucrose.

At the light microscopical level A188 embryos cultured on 2 medium esembled the GP2-6% cultured embryos during the first days of culture, although the degree of vacuolation differed. Embryos cultured on GPO medium at first showed no differences as compared with the GP2 cultures. However, after two days no extensive increase in mitotic activity, as observed in the presence of 2,4D, took place, while vacuolation increased in the basal part of the scutellum. After three days no meristematic zone was observed.

At the EM level embryos of all cultures showed enlarged vacuoles after 30 min of incubation (Table 1). During the following period GP2 embryos cultured at the low sucrose concentration differed from those at the high sucrose concentration. For example, vacuoles were completely filled with electron-dense material and cell walls were very irregular (Fig. 6b). Embryos cultured on GPO medium, also contained darkly stained vacuoles. After

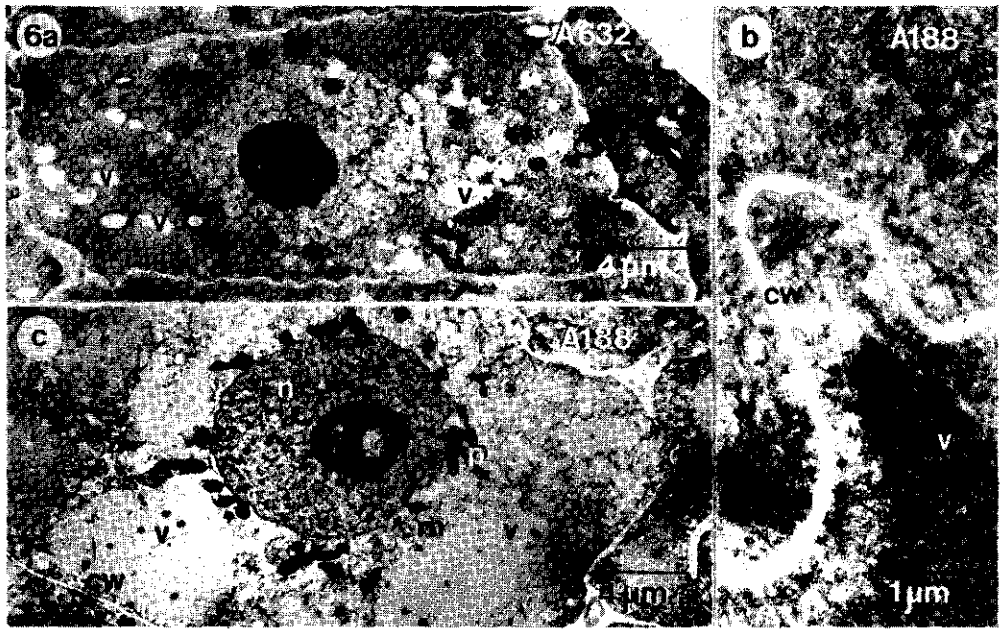

Fig. 6a. Embryo of the A632 line cultured on a GP2 medium with 6% sucrose for three days. Scutellum cell in middle region. Note the presence of many small vacuoles (v).
b. Embryo of the A188 line cultured on GP2 medium with 2% sucrose for one day. Detail of the vacuoles (v) of two adjacent cells, containing electron-dense material. Also note the irregularly shaped cell wall (cw).
c. Embryo of the A188 line cultured on a GP0 medium with 2% sucrose for three days. n, nucleus; v, vacuole;, m, mitochondrion; p, plastid; cw, cell wall.

three days of culture they tended to the t=0 situation: a lower cytoplasmic density, medium-sized vacuoles and a decrease of the amount of mitochondria and plastids. The plastids showed a darker groundplasm (Fig. 6c).

Discussion

White and compact callus tissue is an important stage in maize regeneration through tissue culture. Green and Phillips (1975) and Freeling

et al. (1976) regenerated maize plants from immature embryo cultures by organogenesis, while Lu et al. (1982, 1983) and Novak et al. (1983) obtained somatic embryogenesis. This callus, also called type I callus (Armstrong and Green, 1985), may be transformed into soft embryogenic callus which can be maintained for longer periods (type II callus; see also Vasil et al., 1984).

Our histological observations are in agreement with the work of Springer et al. (1979) and Vasil et al. (1985). The embryogenic callus originated in the scutellar region towards the coleorhiza. However, the pattern of development, leading to plant regeneration, may differ. Springer et al. (1979) obtained regeneration by organogenesis, which started in the epidermal and subepidermal layers. A cambium produced typical radial seriation. It is unlikely that this development results into white and compact callus, because prominent shoot apices originate directly from this cambial zone. Vasil et al. (1985) reported the establishment of somatic embryogenesis through the white compact callus. Proliferation started in cells near the procambial strands. We conclude from these and our data that different types of callus initiation patterns give rise to different types of callus. It is likely, that both embryogenic and non-embryogenic callus is capable to develop in the immature embryo and may do so simultaneously from different origins. However, several factors, e.g. genotype, culture conditions and physiology of the explant tissue may influence this development. This suggests that 'difficult' maize lines are also capable in producing regenerable callus, which is confirmed by Duncan et al. (1985), who reported regenerable callus "in appearance" from 199 maize inbred lines. However, for strain A632 the authors were unable to show regeneration in their 1983 experiments, while their claim for regeneration capacity in the 1984 experiments was only based on visual appearance of the calli. Regeneration from these calli was not further attempted.

Our ultrastructural observations indicate that the period of callus initiation can be subdivided into two phases. The first phase takes about one day, while the second starts after one day of culture. During the first phase changes occur in nucleolar morphology, degree of vacuolation and cell wall shape, while several organelles increase in number. This accumulation of organelles is a general feature in callus initiation (Israel and Steward, 1966; Halperin and Jensen, 1967; Fowke and Setterfield, 1968; Yeoman and Street, 1977; Kott et al., 1985). The second phase starts with the recovery

of mitotic activity. During this phase ultrastructural variation becomes visible between the different ways of culture. These differences comprise the degree in vacuolation, cytoplasmic density and organelle structure.

The importance of nucleolar morphology in growth-induced cultures has been shown by Zwar and Brown (1968), who reported the accumulation of ^{14}C labeled 2,4D in nucleoli of dividing cells in cultured explants from the Jeruzalem artichoke tuber. Nucleolar modifications also occur during activation of quiescent root cells from Zea mays (Deltour and Bronchart, 1971; De Barsy et al., 1974; Deltour et al., 1979) and in Allium cepa (Risueno and Moreno-Diaz de la Espina, 1979). In cells with low activity the fibrillar and granular regions are compact and segregated. In contrast, nucleoli of proliferating cells contain less compact and intermingled regions (Nagl, 1976; Yeoman and Street, 1977). The term "nucleolar vacuole" is used to describe the nucleolar areas with a generally nucleoplasmic appearance, containing loosely dispersed pre-ribosomal-like particles and fibrils (Jordan, 1984). Deltour and De Barsy (1985) suggest that the nucleolar vacuoles, observed during early seed germination of maize, participate in the increase in the nucleolus-nucleoplasm exchange interface and are established when the nucleolar volume decreases, while the output of ribonucleoproteins increases rapidly. Taking into account these data, nucleolar activity and probably RNA synthesis in embryo cultures of maize start between 30 min and one day of incubation. Because the polysomes accumulate within 30 min of culture, they are either formed from pre-existing cytoplasmic ribosomes and/or from pre-existing ribosomal subunits stored in the nucleolus. Similar observations on nucleolar modifications have been reported in cultured Jeruzalem artichoke tuber tissue (Fowke and Setterfield, 1968; Yeoman and Street, 1977). Vasil (1973) also observed a vacuolated nucleolus in callus cell nuclei of the hypocotyl of an in vitro germinated pea seedling in the presence of 2,4D. Fibrillar and granular regions, however, became segregated in the presence of 2,4D.

Vacuolation and cell wall morphology are parameters indicative for the changes in water potential in the plant tissue. Cell expansion and turgor depend on cell wall elasticity, while the vacuole serves as an osmotic system that develops turgor pressure (see monograph of Kramer, 1983). Before culture the vacuoles of the scutellum cells are in an early stage of development as can be noticed from the areas of organelle-free cytoplasm (Amelun-

xen and Heinze, 1984; Hilling and Amelunxen, 1985). After 30 min of culture an inward diffusion of water leads to an increase in vacuolation and turgor pressure, especially in embryos cultured on medium with a low sucrose concentration. The walls of the young scutellum cells are very elastic and, as a result, the cells expand as the turgor pressure increases. However, some doubt exists weather the primary cause of cell enlargement is turgor pressure or the difference in water potential (Ray et al., 1972; Kramer, 1983).

The observed ultrastructural changes are in good agreement with the cytological process of wound-healing in injured plants tissue, which is known from early studies (Barckhausen, 1978). Two distinct stages have also been described by Macleod et al. (1979) who studied the response of Jeruzalem artichoke tissue when cultured on medium containing 2,4D. The first stage was called the wound response and occurred in the presence or absence of 2,4D. The second stage was called the growth response and was initiated by 2,4D. The wound response was hardly influenced by seasonal variation, while the growth response showed seasonal dependency. Our results confirm Macleods two-component hypothesis with electron microscopical evidence; however, in the case of maize embryo culture there is hardly any wounding. The embryo can be excised very easily from the caryopsis without being disrupted, because it is not grown together to the endosperm. At most some deletion of the suspensor may take place, but in embryos of 13 DAP the functional role of this tissue seems to be less important (Schel et al., 1984). Although we cannot really speak of a wound response, the scutellar cells show similar ultrastructural characteristics. We prefer to use the term shock response instead of wound response.

The proces of callus initiation is an important step of plant regeneration through somatic embryogenesis as well as through organogenesis. We conclude that the pattern of development leading to any callus type is influenced by several main factors: genotype, culture conditions and physiology of the explant. This pattern is effected during the second phase of callus initiation, after a first period of culture. No distinct ultrastructural parameters were observed which might be characteristic for embryogenic callus.

Acknowledgements

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C H A P T E R 3

CELL CYCLE EVENTS DURING THE EARLY PERIOD OF CALLUS INITIATION IN THE SCUTELLUM OF CULTURED MAIZE EMBRYOS. AN AUTORADIOGRAPHIC STUDY

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Submitted for publication

Summary

Cell cycle events were examined in scutellar regions of immature maize embryos at the onset of culture. The embryos were cultured on a modified MS agar medium containing 1 mg per liter 2,4D and 6% sucrose. From the onset of culture embryos were transferred after constant periods of eight hours to the same medium supplemented with 5 µCi per ml ^{3}H-thymidine and incubated for eight hours to examine the fraction of DNA replicating nuclei. During the first day, similar changes in the fraction of labeled nuclei and the mitotic index were observed in all regions. After the first day the scutellar regions showed differences with respect to the cell cycle. The areas from which callus originates remained mitotically active and showed high fractions of labeled nuclei, whereas in the other regions these activities declined. A difference is also observed in the timing of the cell cycle changes between the adaxial and the abaxial regions, which suggests a shift in the cell cycle from the scutellar node towards the epidermis.

It is concluded, that a shock response and a growth response are manifested in the sequence of cell cycle events. These events are discussed with respect to callus induction and callus development in the scutellum tissue.

Introduction

Cereal tissue culture has become more successful during the last decade, due to the use of immature explants (Torne et al., 1980; Lu et al., 1983; Chang, 1983; Rhodes et al., 1986; Suprassanna et al., 1986; Tsay et al., 1986). This breakthrough has opened new ways for plant breeders and other scientists dealing with important crops such as rice, wheat and maize. Still, however, the cereals are regarded being difficult for in vitro propagation as compared to the dicotyledons. For example, the genetic constitution appears to be an important factor for a successful in vitro response (Green and Phillips, 1975; King et al., 1978; Maddock, 1985) and culture studies on reciprocal F1 genotypes suggest that partially dominant genes control the culture response, at least in maize (Rhodes et al., 1986; Hodges et al., 1986; Tomes, 1987). However, extensive research on genotype and culture medium has provided evidence that improvement of the culture medium may overcome this genetic blockade (Duncan et al., 1985).

Plant regeneration from cereal explants mostly takes place through an embryogenic callus phase. From this callus shoots (Green and Phillips, 1975) and somatic embryos (Kamo et al., 1985; Armstrong and Green, 1985) develop, which can grow further into complete plants. Embryogenic callus also plays an important role in the production of cell suspension and protoplast cultures with regeneration capacity (Green et al., 1983; Vasil and Vasil, 1986; Kamo, 1987). Induction of this callus type is therefore of utmost importance for e.g. plant regeneration, genetic manipulation or somaclonal variations. Many articles deal with how to obtain embryogenic callus from cereals efficiently, using different genotypes (Duncan et al., 1985) and immature explant sources (see above), while others report on the morphogenetic processes of callus initiation with histological techniques (Springer et al., 1979; Vasil and Vasil, 1982; Wernicke et al., 1982; Vasil et al., 1985). On the contrary, only a few studies have been published on the ultrastructural level of early callus initiation in cereals (Kott et al., 1985; Fransz and Schel, 1987), while cytological data about this early period are lacking. Most reports on the cell division cycle come from the work with dicotyledons and the Jeruzalem artichoke in particular (Yeoman et al., 1965, 1966). The late breakthrough in cereal plant regeneration may be the reason for this gap of information.

Using an in vitro culture system with immature embryos from the maize inbred line A188, we can obtain embryogenic callus in more than 90% of the cultured embryos. This high percentage gives the opportunity to examine the early period of culture, during which no macroscopical changes are visible that would indicate callus induction. The objective of this study was to find a possible relationship between cell cycle events and callus initiation, and to examine the different culture responses in the scutellar subregions. These data may provide additional information to the recalcitrant behaviour of cereal tissue culture.

Materials and methods

Culture

Immature embryos (approx. 1.5 mm) of the maize strains A188 and A632 were cultured as described before (Fransz and Schel, 1987). The solid medium was

composed of the inorganic constituents of Murashige and Skoog (1962) and the organic compounds as described by Green and Phillips (1975). The medium further contained 6% sucrose and 1 mg per liter 2,4 dichloro-phenoxy-acetic acid (2,4D).

Labeling of the embryos with ^{3}H-thymidine and autoradiography.

From the onset of culture (t = 0) embryos were transferred every 8 hrs to the same medium supplemented with 5 µCi per ml ^{3}H-thymidine (sp. act. 5.0 Ci per mmol, Amersham). After an incubation time of 8 hrs on labeled medium the embryos were sampled and fixed in 4% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, for one hour at room temperature. After several rinses in the same buffer for a period of 2 days the specimens were dehydrated and embedded in 2-hydroxy-ethyl-methacrylat (Technovit 7100, Kulzer, Wehrheim, Germany). Sections (3 µm) were cut on a Reichert-Jung Supercut 2050.

Selected sections were mounted on cleaned slides, dipped in Ilford K_2 photographic emulsion diluted with aqua dest (1:2), dried overnight at room temperature in open boxes and afterwards stored in closed boxes at 4°C. After two weeks exposure time the autoradiographic slides were developed in Kodak Microdol X for 4 min., rinsed in aqua dest. for 30 sec., fixed in Ilford Ilfospeed 200 fixer for 8 min, and rinsed in aqua dest. for 5 min. Sections were stained with 1% toluidine blue O in 1% boric acid.

Quantifications

The autoradiographic sections were examined with a light microscope at a magnification of 200x. From median sections of the scutella drawings were made using a drawing-prism. Per stage two embryos were taken. Parenchyma nuclei of the scutellum were used for quantification. All labeled and unlabeled nuclei, interphases as well as mitoses were noted. Only nuclei that were distinctly covered by silver grains were counted as labeled nuclei.

The scutellum was subdivided into several subregions as follows. The insertion points A and B (Fig. 1) are the attachment points of the coleoptile resp. coleorhiza to the scutellum. The line AB connects these points

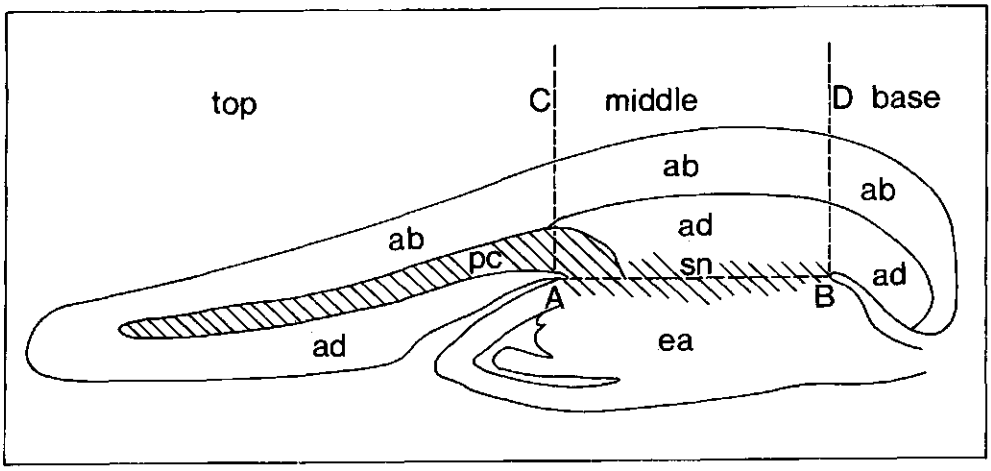


Fig. 1. Schematic representation of an immature embryo, the scutellum of which is subdivided into several subregions. AB, base line; AC and BD, perpendiculars in A resp. B; ab, abaxial; ad, adaxial; ea, embryo axis; pc, procambium; sn, scutellar node.

and forms the base line in all embryos. The lines AC and BD are perpendiculars of AB in A resp. B and divide the scutellum into a top, a middle and a basal region. The top region is divided into an abaxial and an adaxial region by the procambium strand which is recognized by elongated cells. The region which is located apical from the procambium strand in the top is assigned to the adaxial top region. The middle abaxial and adaxial regions are separated by the line that is located between the scutellar node and the epidermis at equal distances. The scutellar node is positioned between the embryo axis and the scutellum.

The same approach of division is used for the basal abaxial and adaxial region. In the most basal part (a region of about 100 µm) no discrimination is made between abaxial and adaxial. Because the labeling index and also the histological appearance closely resembled that of the abaxial basal region it is assigned to the abaxial basis. The areas of the scutellar subregions were measured using a Kontron MOP-30 image analyzer.

DAPI densitometry.

Selected sections of embryos at t=0 and t=8 were mounted on cleaned slides and stained with DAPI (4,6 diamidino-2-phenylindole dihydrochloride). Fluorescence intensities of nuclei sections were recorded in a microspectrophotometer using a mercury high pressure lamp (100W) and a UV filter combination with an excitation wavelength of 365 nm and a barrier filter at 410 nm. Only nuclei containing nucleoli and nuclei of comparable size were selected.

Results

The subdivision into a top, middle and basal region, each consisting of an adaxial and abaxial subregion (Fig. 1) allowed to study the fraction of labeled nuclei (FLN) and the mitotic index (MI) in more detail. Although by this approach the borders are more or less arbitrary, it provides an impression of the various responses that take place in the scutellum of a cultured embryo during callus initiation. Because of the relatively complex structure of the scutellum and the specific location of embryogenic callus origin (Springer et al., 1979; Vasil et al., 1985; Fransz and Schel, 1987) we used autoradiography on median sections of cultured embryos to study the cell cycle.

When embryos are placed on a nutrient medium they swell and the scutellum length increases from 1.8 mm to 2.5-3.0 mm during the first three days. Figure 2 shows the increase of the area of the scutellar regions and it is evident that especially the adaxial top region strongly enlarges from the onset of culture. There is an increase in the number of nuclei per area in all regions after 32 hrs of culture (Fig. 2). In the abaxial middle and basis the increase is significant after 48 hrs, while in the top there is a decrease in the number of observed nuclei, which is due to cell elongation.

Figure 3 shows the fraction of nuclei that had incorporated ^{3}H-thymidine during an incubation of 8 hrs. This fraction of labeled nuclei (FLN) represents the percent of nuclei that passed the S-phase during that period.

In the histograms similar changes in the FLN can be observed in all subregions during the first 32 hrs of culture. A low FLN in the initial 8 hrs is followed by a rise in FLN to values around 60%. In general the adaxial subregions have a lower FLN than the abaxial subregions. The same

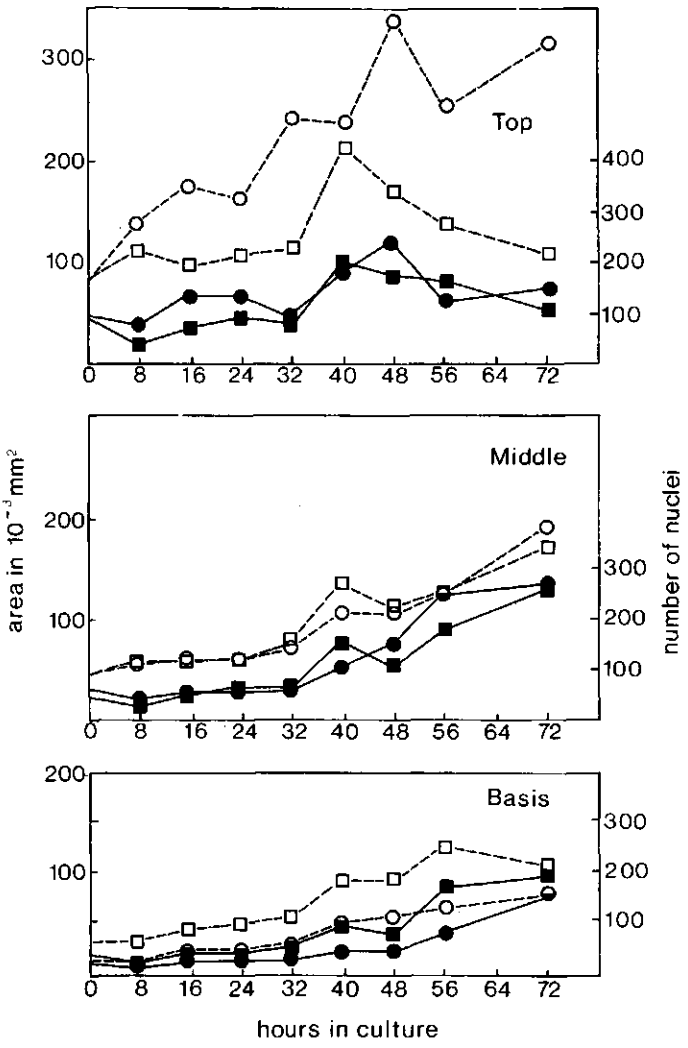


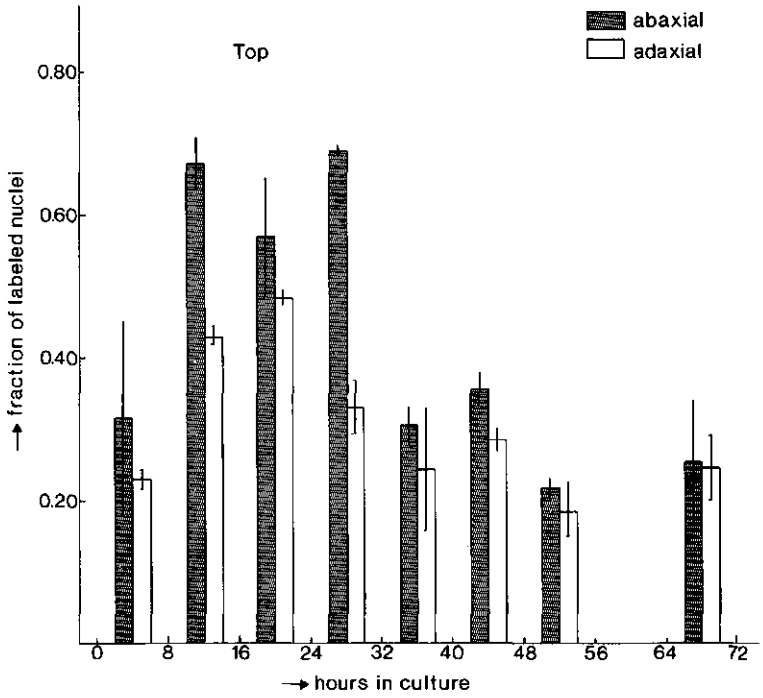
Fig. 2. Comparison of the areas of the subregions and the number of observed nuclei in the subregions during culture. abaxial area (open box); adaxial area (open circle); number of abaxial nuclei (closed box); number of adaxial nuclei (closed circle).

observations have been done on embryos of the maize line A632, that does not produce embryogenic callus (Table 1). It is suggested that this change in FLN during the first day is part of a general response of the explant cells to the culture.

After 32 hrs variation took place between the scutellar subregions with respect to the FLN. In the top regions and the adaxial basis the FLN declined to values around 25%, while in the abaxial basis and especially the middle region the FLN fluctuates with maximum values in the periods 40-56 hrs (adaxial middle), 48-56 hrs (abaxial middle and basis) and 64-72 hrs (abaxial basis). At two places in the histograms the adaxial subregions show a change in FLN 8 hrs before their abaxial counterparts. In the period round 24 hrs the adaxial FLN declined in the top and the middle, while the diminution of the abaxial FLN took place eight hours later. A similar situation is applicable for the basis at 32 hrs and 40 hrs of culture. An increase of FLN in the adaxial middle is observed in the period 40-48 hrs, which is 8 hrs sooner than the abaxial subregion. This indicates a gradient in cell cycle activity from the adaxial region near the embryo axis towards the abaxial regions in the subepidermis of the scutellum.

Figure 4a shows the percent of observed mitoses (MI); a difference is made between labeled and unlabeled mitoses. Before culture the scutellum tissue was mitotically active in all regions, with a mean MI of 5%. At 8 hrs of culture the MI was completely reduced. A recovery in mitotic activity was observed after 16 hrs and 24 hrs, by which the middle and basal regions showed only labeled mitoses, whereas in the top unlabeled mitotic figures were also detected. In preliminary studies we examined the relatively DNA content by DAPI densitometry on 3 μm sections. Although we have not determined the relative 2C or 4C value, the results indicate a shift in DNA content from staggered values at t=0 to relatively lower values (Fig. 4b). This suggests, that nuclei of proliferating cells are distributed randomly in the cell cycle at t=0 and become arrested in G1 in the period 0-8 hrs.

After 24 hrs of culture the MI in the top region and the adaxial basis declined and the few mitotic figures were usually observed close to the middle region. This reduction in MI and the low FLN suggest that the cells in these regions become arrested in either G1 or G2. At this stage the top cells were elongated and the cytoplasmic density was low (Fig. 5).


Top
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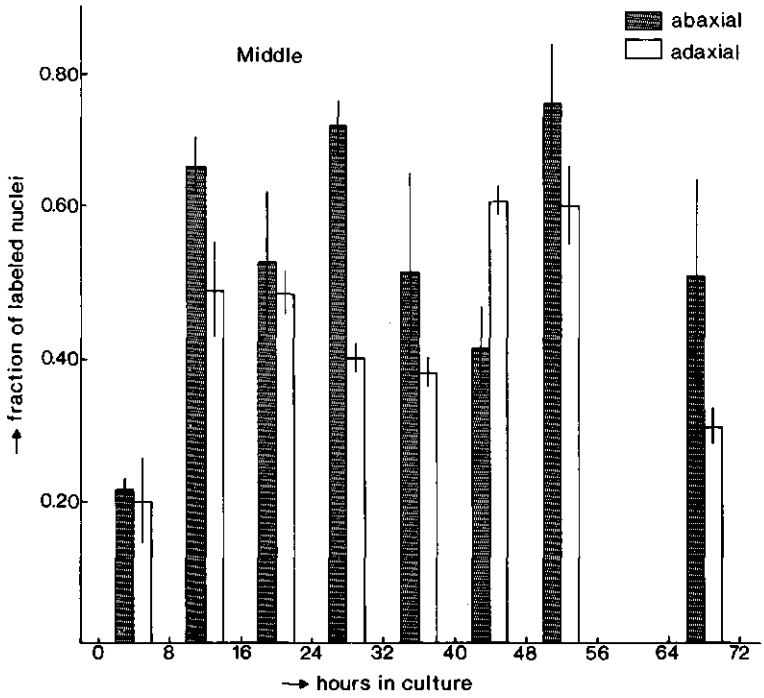
hours in culture


Middle
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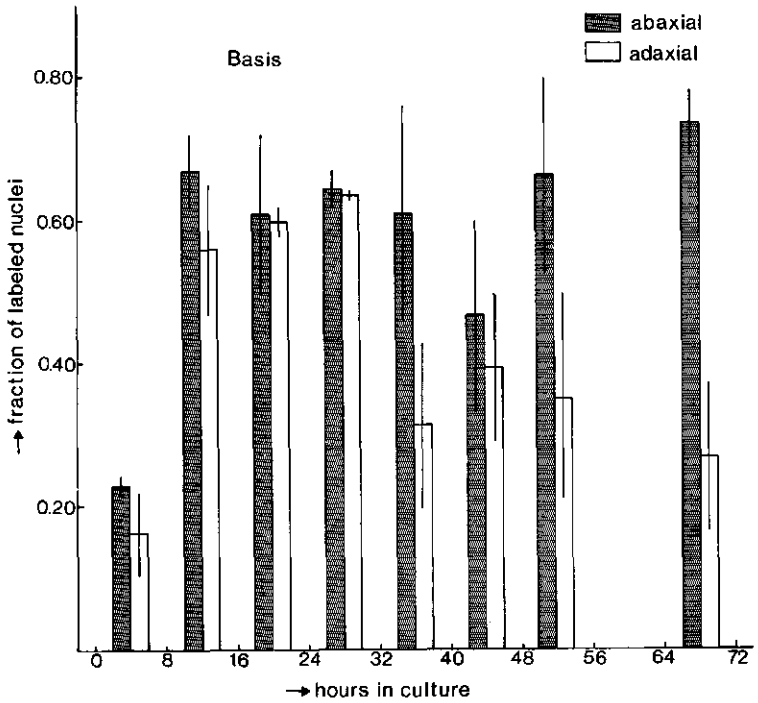


Fig. 3. Comparison of the fraction of labeled nuclei in the top, the middle and the basis. The position of the bars corresponds with the eight hours labeling period preceding the fixation. The left bar (gray) represents the abaxial region, while the right bar (white) represents the adaxial region.

Table 1: A comparison of FLN in A188 and A632 in the period 0 to 19 hours of culture.

hours in culture	Top		Middle		Base	
	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial
A188 (0-8)	0.23	0.17	0.22	0.20	0.32	0.23
A632 (0-8)	0.24	0.09	0.29	0.09	0.12	0.08
A188 (8-16)	0.67	0.56	0.67	0.50	0.68	0.43
A632 (11-19)	0.64	0.81	0.81	0.58	0.57	0.43

In the middle and abaxial basis the MI appeared constant or showed some fluctuations. A maximum in cell division activity was noticed at 56 hrs, suggesting that a certain degree of synchronization has taken place. At this time a region of meristematic cells, characterized by small cytoplasmic cells, was observed in the middle and abaxial basal scutellum (Fig. 6a). All divisions took place randomly in the subepidermal middle and basal region and most division planes were periclinal (Fig. 6b). After three days a clear protuberance could be observed in the scutellar basis of some embryos (Fig. 7), which will develop into a scutellar body and later into a somatic embryo (Vasil et al., 1985; Fransz and Schel, 1987).

The cells of the embryo axis showed no disturbance of the cell cycle during the first 8 hrs of culture. Labeled nuclei as well as cell divisions were observed, indicating that the culture response of these cells with respect to the cell cycle differs from the parenchyma cells of the scutellum. The procambium, which can be recognized by small elongated cells, also showed continuous DNA replication and mitotic activity at the start of culture. In later stages the FLN was comparable with values found in the surrounding parenchyma tissue in the top. High rates of labeled nuclei in vascular cells of the top are mainly due to those cells that are located close to the scutellar node. During the early period of callus initiation, vascular cells differentiate and tracheal elements are clearly observed (Fig. 5, see also Fransz and Schel, 1987). Epidermal cells also showed DNA replication activity in all regions, but the FLN is in general lower than in the parenchyma tissue. In the adaxial epidermis of the scutellum top the mitotic activity before culture has disappeared completely as soon as culture is started and during culture no mitotic figures have been observed anymore. This indicates that proliferating epidermal cells in the adaxial top become arrested in G2 during culture.

Although it was not intended to examine the duration of the cell cycle, some remarks can be made. During the 8 hrs of incubation with ^{3}H-thymidine, cells that passed the S-phase replicated DNA and became labeled. In the total experiment labeled as well as unlabeled mitoses were detected in all regions (Fig. 4a). However, in the middle and basal regions the fraction of labeled mitoses was generally higher than in the top region. When embryos were incubated for only 4 hrs with ^{3}H-thymidine, no labeled mitoses were found. In procambium and coleorhiza cells, however, labeled as well as

unlabeled mitoses were found (Fig. 8). Therefore, it is concluded that the period between S-phase and mitosis lasts about 8 hrs in parenchyma cells, whereas in cells of the procambium and the coleorhiza it takes about 4 hrs.

Discussion

During the initiation of callus in cultured carrot slices Komamine and Shimizu (1975) discovered two stages on account of changes in enzyme activities and respiration. Examination of RNA metabolism in cultured artichoke tuber tissue by Macleod et al. (1979) indicated the existence of a wound response and a growth response at the onset of culture. Fransz and Schel (1987) ascertained a shock response and a growth response in cultured maize embryos by electron microscopy. This study presents a sequence of cell cycle events which confirms the concept of a two-phasic process during callus initiation in maize embryos.

The shock response, which occurs during the first day of culture, comprises two events; (i) a decrease in mitotic activity and probably also in DNA replication, and (ii) a recovery of both activities. It is suggested, that the shock response takes place in the whole scutellum regardless the further development of the tissue, e.g. into embryogenic callus, non-embryogenic callus, or no callus development at all. During the growth response only regions that produce callus remain mitotically active and replicate DNA, whereas in the other regions these activities slow down, and cells starts to differentiate. The increase in the number of nuclei in the adaxial basis after 48 hrs, despite a decline in mitotic and DNA synthesizing activity, might be explained by the arbitrary fixing of the basal subregions. Meristematic cells in the abaxial basis divide and produce cells towards the adaxial side that elongate and do not proliferate further.

Extensive studies on callus initiation have been carried out by Yeoman and coworkers, using tuber explants of the Jeruzalem artichoke. The originally resting tuber cells show a more or less synchronous first cell division in the superficial layers in the period from one to two days of culture, followed by an abrupt increase in the number of cells (Yeoman et al., 1966; Yeoman and Evans, 1967; Mitchell, 1968). Prior to this synchronous cell division DNA is replicated only in cells that are induced to divide and subsequently produce callus (Yeoman and Evans, 1967; Yeoman and Mitchell,

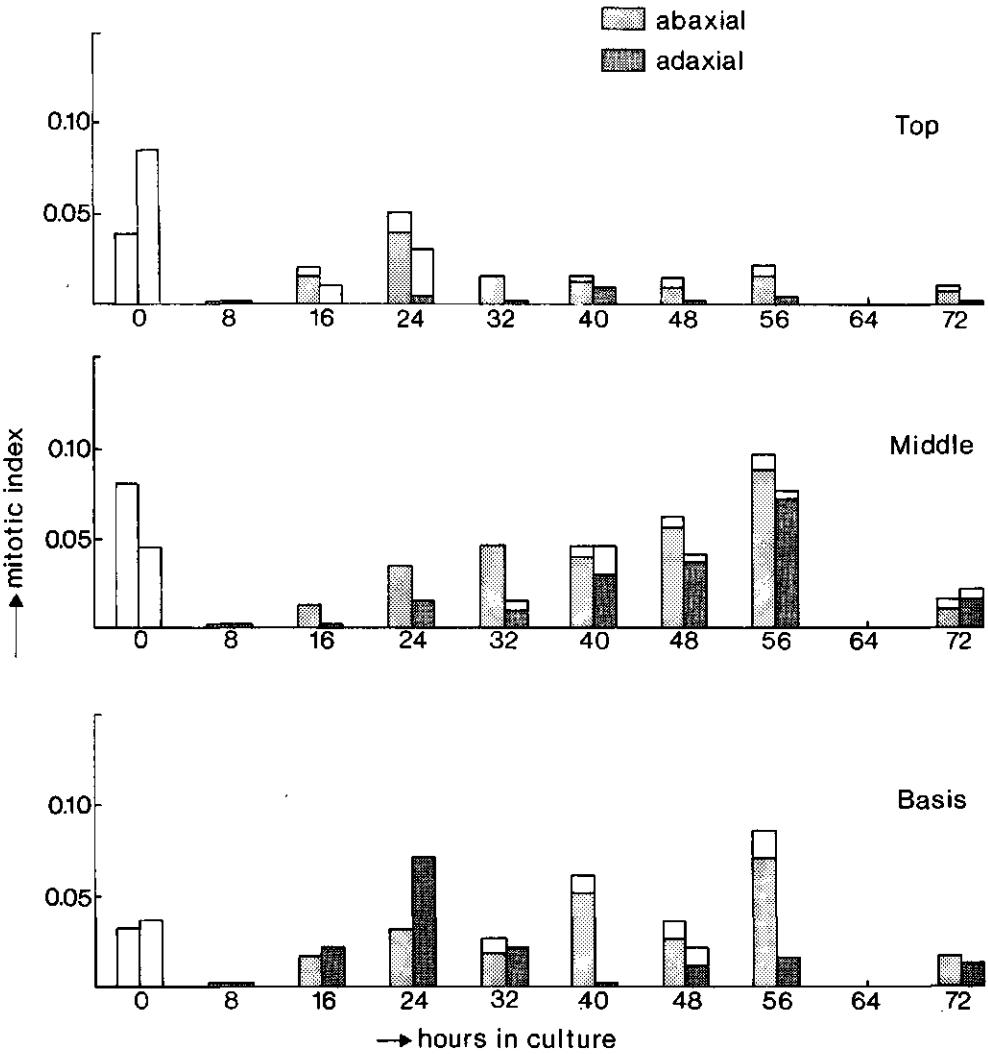


Fig. 4a. Comparison of the fractions of mitoses in the top, the middle and the basis at various times after the onset of culture. The left bar represents the abaxial region, while the right bar represents the adaxial region. The shaded areas mark the labeled fraction.

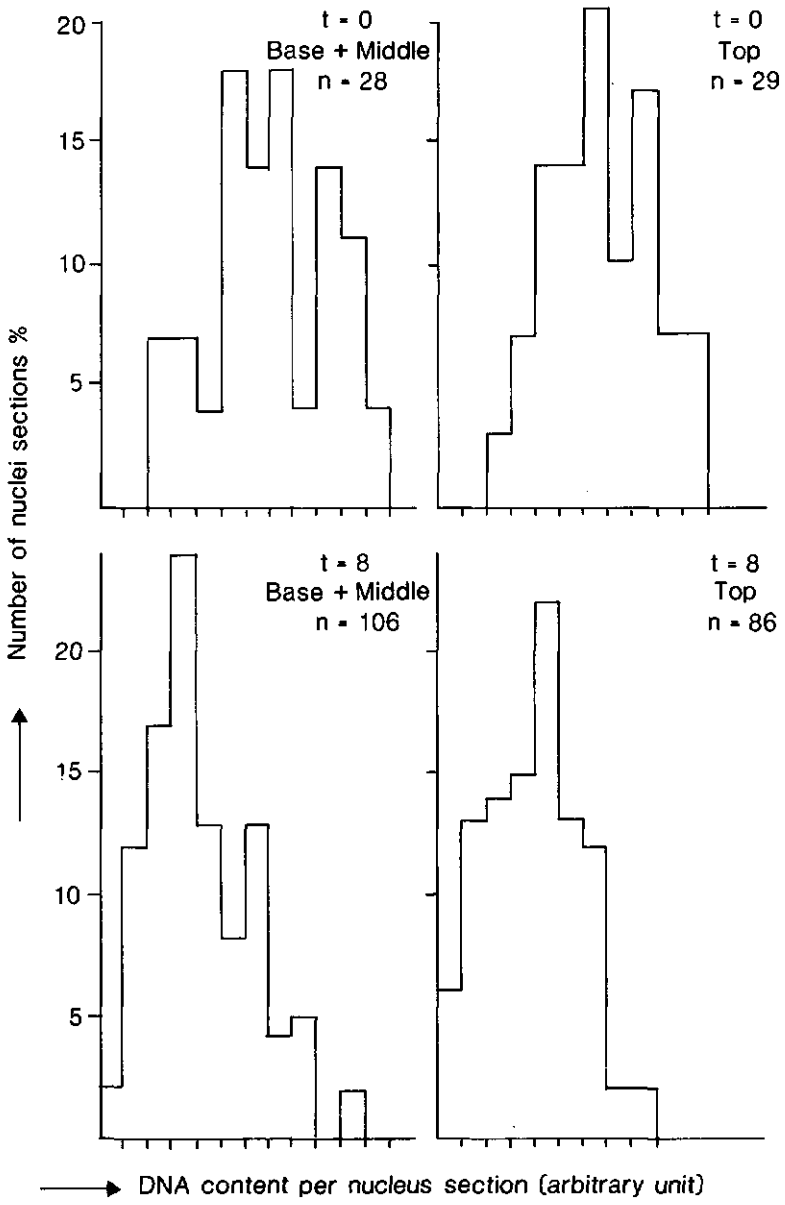


Fig. 4b. Distribution of DAPI values in nuclei sections of two different scutellum regions just before culture (t=0) and 8 hrs after the start of culture (t=8).

1970). Here we find a discrepancy between the tuber slice and the maize embryo. In the cultured embryo there is no synchronous first cell division in the scutellum and DNA is also replicated in cells that do not produce callus. We must consider that the maize embryo has a mitotically active scutellum already before excision and therefore dedifferentiation does not account for this tissue. Recovery of mitotic activity during the shock response is not affected by the presence of 2,4D (Fransz and Schel, 1987). On the contrary, the growth response is completely dependent on the induction by 2,4D. In the tuber explant the synchronous first DNA synthesis and cell division are induced only in the presence of 2,4D (Yeoman and Evans, 1967; Yeoman and Mitchell, 1970). We therefore conclude that the shock response in maize immature embryos and probably more meristematic explants, differs from the shock or wound response in the tuber slices.

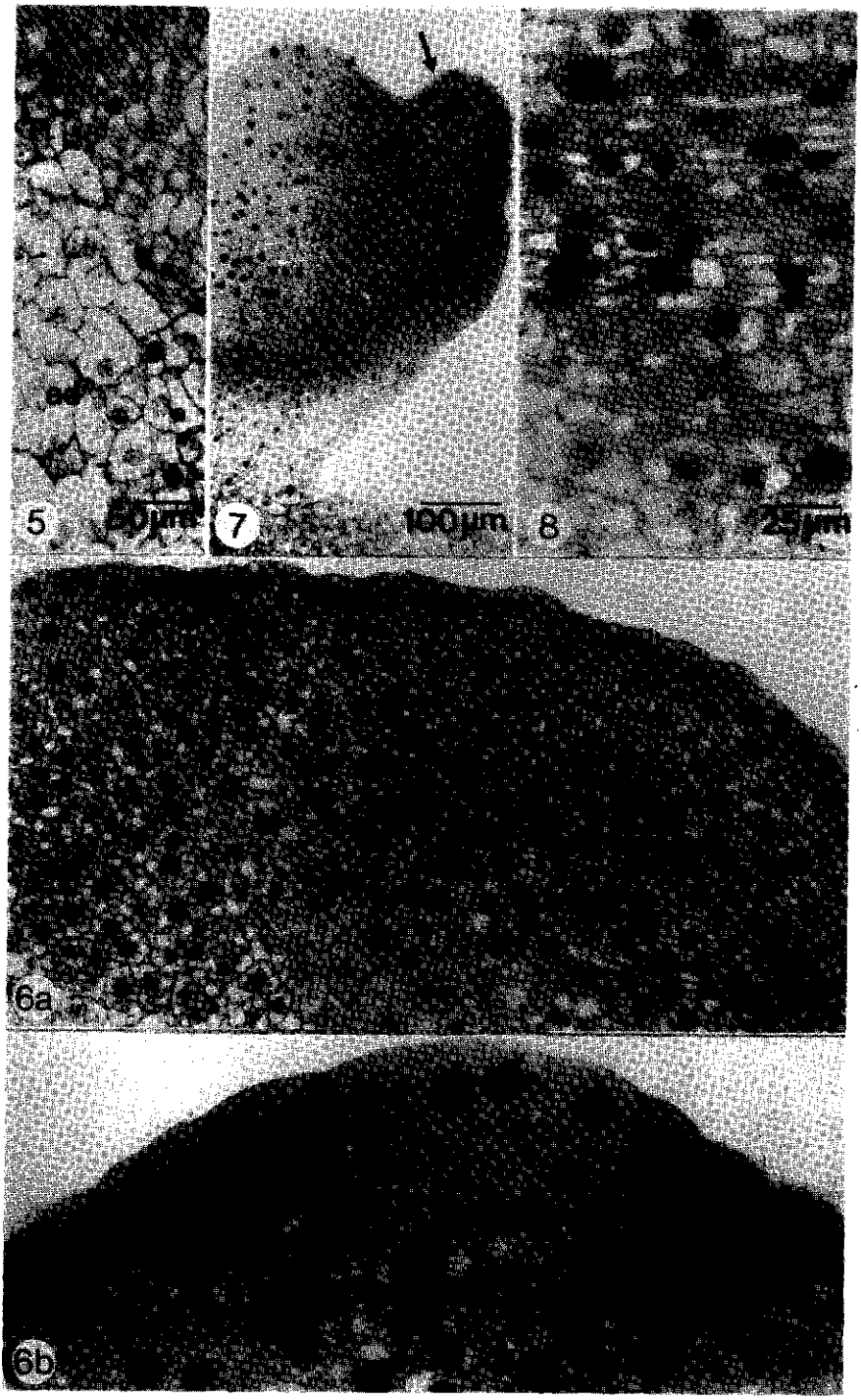
The results show that the shock response starts with a deregulation of the cell cycle in cells that are actively proliferating. Springer et al. (1979) already reported that the scutellum of the maize embryos before culture is mitotically very active. It is further ascertained that immature embryos which differed in age, 11-14 DAP, and in length, 1-3 mm, all showed a high mitotic activity randomly distributed in the scutellum, indicating asynchrony. Therefore it is concluded that the scutellum cells become arrested in either G1 or G2 immediately at the onset of culture. This cell cycle arrest is probably due to energy-consuming processes, that accompany the shock response. Fransz and Schel (1987) have shown, that within 30 min. of culture the scutellum cells undergo several ultrastructural changes, a.o.

Fig. 5. Detail of the adaxial region in the top at 56 hrs, showing vascular cells (v) and enlarged adaxial parenchyma cells. ad, adaxial. x280.

Fig. 6a. Light microscopical autoradiograph of the abaxial middle and basal region, showing a zone of meristematic cells with labeled nuclei and mitoses (arrows) after 56 hrs of culture. x280.
b. Detail of the basal region. Note the periclinal division planes (arrows) of the labeled mitoses. x540.

Fig. 7. Light micrograph of the basal region at 72 hrs, showing a protuberance (arrow). x140.

Fig. 8. Light microscopical autoradiograph of procambium cells at 20 hrs after a four hours incubation with ^{3}H thymidine. Note the labeled mitosis (arrow). x540.


5
50 µm
7
100 µm
8
25 µm
6a
6b

accumulation of polyribosomes, indicating active protein synthesis. The appearance of high molecular weight proteins (Fransz and Boersma, 1988) confirms the synthesis of polypeptides, which might cause the deregulation of the cell cycle. This explanation is in agreement with the principal control point hypothesis (Van 't Hof and Kovacs, 1972) which implies that cell division is regulated by factors that operate during G1 and G2. Under conditions where cell division ceases, these factors become limiting, causing cell cycle arrest in G1 or G2.

Rost (1977) summarizes the response of the plant cell cycle to stress conditions and concludes that G1 and G2 are sensitive to environmental stress. Furthermore, there is evidence that the arrest in either G1 or G2 is genetically controlled and not affected by the cause of arrest (Evans and Van 't Hof, 1974). Most reports on cell cycle arrest in the maize embryo concern the shoot and root of the embryo axis during seed maturation. The majority of these cells become arrested in G1, although some controversial observations exist (for a review, see Deltour, 1985). Our results suggest an arrest of the scutellum cells by preference in G1 during the period 0-8, because in the following eight hour period the majority of the scutellar nuclei pass the S-phase.

The presumed arrest in G1 during the shock response makes it interesting to know if there is any relationship between a cell cycle phase and callus induction by 2,4D. Is some kind of arrest necessary for callus initiation in cells that are competent for 2,4D? There are no concrete data available concerning these specific correlation. It is not inconceivable if we consider that callus growth in immature wheat leaves could be initiated in cells that were just arrested in G1 after the last mitosis (Wernicke & Milkovits, 1984). Moreover, several cereal anther cultures are most successful when the microspores are in the early or mid-uninucleate stage, which corresponds with G1 (rice: Genovesi & Magill, 1982; barley and wheat: Wheatley et al., 1986, Huang, 1986). Also in the case of dicotyledons evidence is presented that the differentiated cells are arrested in G1 and in this state are induced by 2,4D to rapid cell division (Yeoman et al., 1966). In all these cells there may be some state of susceptibility for the transformation from normal tissue to callus by induction of some external factor (e.g. 2,4D). It is found that an excellent correlation exists between the stage of wound response and the rate of tumor growth (Braun, 1975). Host cells of

Kalanchoe stems reached a maximum of competence for transformation in the crown gall disease between the second and third day after a wound was made. A certain conditioning is therefore necessary for optimal competence.

Substantial evidence concerning the relationship between 2,4D starvation and G1 arrested cells comes from Nishi et al. (1977). They transferred carrot cells, that grew exponentially in the presence of 2,4D, to a medium without the growth regulator. Timing of DNA synthesis and of mitosis indicated that the cells had been arrested at G1 phase. Everett et al. (1981) repeated these experiments on cultured sycamore cells and concluded that 2,4D starved cells do not arrest at a specific cell cycle control point. The authors suggest that 2,4D does not regulate cell division by interacting with specific cell cycle controls. This, however, does not exclude the phase-specific sensitivity of certain plant cells to the action of 2,4D as discussed above.

The phase difference between the abaxial and adaxial part of the scutellum possibly reflects the flow of nutrients and 2,4D from the embryo axis towards the subepidermal layers of the scutellum. This indicates a polarity, which is later recognized during organogenesis and direct somatic embryogenesis. It is known, that in cultured maize embryos shoot apices develop in the epidermal layer of the scutellum (Springer et al., 1979), whereas root primordia originate in the adaxial scutellum (Vasil et al., 1985). Moreover, during somatic embryogenesis the position of the embryoid axis is such, that the root primordium is located adaxial, towards the zygotic embryo axis, whereas the shoot apex develops at the opposite site (Vasil et al., 1985). It is suggested that embryo development is possible by the presence of a polarity, which is considered as a morphogenetic factor (Van Lammeren, 1987). Brawley et al. (1984) suggest, that embryo polarity in somatic embryos of Daucus carota is established and maintained by ionic currents, which are affected by hormones. Electrical current enters the exposed surface of incipient globular embryos and leaves the region near the presumptive radicle. A correlation between embryo polarity and cell cycle events is found during seed maturation in Triticum durum, where the accumulation in G1 starts at the shoot apex and subsequently in the root (Avanzi et al., 1969). The different timing of the cell cycle changes in the abaxial and adaxial subregions may thus reflect the polarity in the scutellum and thereby contribute to somatic embryo development.

The study of cell cycle events in the early period of cultured immature explants provides valuable information about the role of the cell cycle in callus initiation, cell differentiation and shock response with or without wounding. A system represented by the immature maize embryo might be a valuable tool in these studies. We are quite aware that the problem of recalcitrant monocotyledons cannot be tackled just by cytological observations. We feel, however, that this study provides additional information on the extreme culture conditions in Zea mays and possibly cereals in general.

Acknowledgements

We thank: Dr. R. Deltour (Universite de Liège, Liège, Belgium) for his advices in autoradiography and critical reading of the manuscript; Prof. M.T.M. Willemse for valuable comments; S. Massalt, P. van Snippenburg and A. Haasdijk for preparing the photographs and the drawings; J. Cobben-Molenaar and T. van de Hoef-van Espelo for the preparation of the typescript.

CHAPTER 4

CYTOMORPHOGENESIS OF FRIABLE EMBRYOGENIC CALLUS OF *ZEA MAYS* L.

Summary

Immature embryos of Zea mays L. were cultured on N6 and MS medium to obtain friable resp. compact embryogenic callus. Cultured tissue fragments with various developmental stages of friable callus were sampled and prepared for light microscopy and transmission electron microscopy. Pieces of compact callus were only prepared for light microscopy in order to compare the structural organization of both callus types.

Friable callus develops from a thin layer of abaxial scutellum cells including the epidermis. During further development callus cells dissociate due to the breakdown of the middle lamellae, while older vacuolated cells degenerate. This results into long cell aggregates separated by large intercellular spaces, giving the callus its friable appearance. The microscopical sections showed a striking difference between friable and compact callus, not only in structure but also in organization. Vascular elements were not found in friable callus. On the contrary, vascular bundles were prominent in compact callus.

The embryogenic potential of friable callus is situated in embryogenic units. These are independent aggregates of small isodiametric cells containing a central nucleus, an electron-dense cytoplasm and many organelles. Proliferation is only observed in these cells, which are therefore presumed to generate new embryogenic units, somatic embryos and vacuolated callus cells. The results indicate, that discrete masses of embryogenic cells, possibly early embryoids, have a unicellular origin.

Introduction

Plant regeneration in cereal tissue culture can be obtained from embryogenic callus, which is white and compact in appearance. On the contrary, friable callus in cereal cultures has long been associated with non-embryogenic callus generating only roots (Freeling et al., 1976; King et al., 1978; Mott and Cure, 1978; Ozias-Akins and Vasil, 1982). Only occasionally embryogenic callus has been derived from this tissue (Botti and Vasil, 1983; Haydu and Vasil, 1981). Since the discovery by Green and Rhodes (1982) of friable callus with high embryogenic potency from embryo cultures of maize this new embryogenic callus type has become of more interest because of its highly

regenerative capacity on long terms. Moreover, friable embryogenic callus has proven to be a valuable source for the production of maize cell suspensions and protoplasts with regeneration capacity (Green et al., 1983; Vasil and Vasil, 1986; Kamo et al., 1987). Thus, in cereal tissue culture we can distinguish a non-embryogenic and two embryogenic callus types. The embryogenic types are termed compact, or type I, callus and friable, or type II, callus (Green et al., 1983).

Studies of embryogenic callus in cereals mostly concern the compact callus development and somatic embryogenesis using light microscopy or scanning electron microscopy (Dunstan et al., 1978; Wernicke et al., 1982, Vasil and Vasil, 1982; Lu et al., 1982, 1983; McCain and Hodges, 1986; Fransz and Schel, 1987). On the contrary, only a few light microscopical studies on friable callus have been published (Vasil et al., 1984, 1985). These studies deal with the sites of callus origin and the ontogeny of somatic embryos, while cytomorphogenesis of friable embryogenic callus has hardly come up for discussion. It is not clear how this friable callus with its fragile appearance comes about and is organized. Therefore, we examined the development and morphology of friable embryogenic callus in Zea mays by light and electron microscopy. The anatomy of friable and compact callus is compared.

Materials and methods

Maize plants of the inbred line A188 were grown in the greenhouse. Compact callus was obtained from immature embryos cultured on GP-medium (for details, see Fransz and Schel, 1987).

The GP-medium was composed of the inorganic components of Murashige and Skoog (1962) and the organic components as described by Green and Phillips (1975), with 6% sucrose and 1 mg/l 2,4D (2,4 dichlorophenoxy-acetic acid). Friable callus was established from immature embryos (1.0-1.5 mm long), cultured on N6 medium (Chu et al., 1975) supplemented with 2% sucrose, 1 mg/l 2,4 D, 100 mg/l casamino acids and 6 mM L-proline. All cultures were incubated at 28-30 °C with a 16/24 h light/dark regime. Subculturing occurred every two weeks (Malmberg et al., 1985).

Friable callus and compact callus of about 1 month were sampled and fixed for light microscopy in 4% glutardialdehyde in 0.1 M sodium cacodylate

buffer, pH 7.2 for 1 h at room temperature. After dehydration the specimens were embedded in 2-hydroxy-ethyl-methacrylate (Technovit 7100). Sections (5 um) were stained with toluidine blue. Some sections were stained with PAS according to the method as described by O'Brien and McCully (1981). Embryos cultured for 3 d and 10 d on N6 medium and friable callus of about 1 month were sampled and fixed for transmission electron microscopy in 2% glutardialdehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 h at room temperature. Post-fixation in 1% OsO_4, sectioning and staining procedures were as described by Schel et al. (1984).

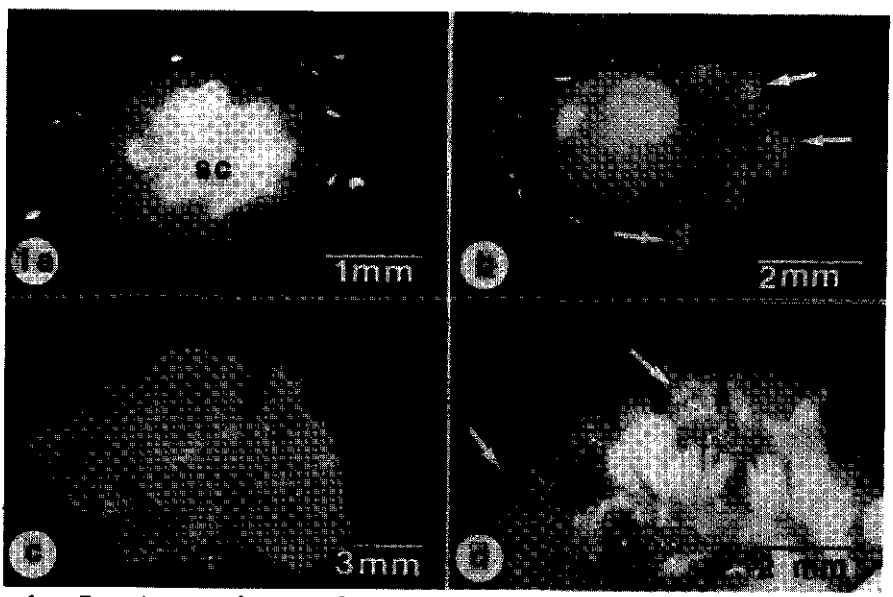


Fig. 1a. Immature embryo cultured on N6 medium for 6 days. The exposed scutellum (sc) has a soft and translucent appearance.
b. Immature embryo cultured on N6 medium for 13 days. The scutellum has partly become friable. Globular embryoids are present on its surface (arrows).
c. Friable embryogenic callus obtained from proliferating scutellum tissue with friable appearance after subculture on N6 medium.
d. White compact embryogenic callus obtained from the scutellum of embryos, that were cultured on GP-medium with 6% sucrose. Note the presence of somatic embryos (arrows).

Results

General morphology

After about 6 days of culture on N6 medium the embryo became soft and almost translucent (Fig. 1a). The embryo axis elongated poorly and often degenerated and within two weeks the scutellum surface became irregular and transformed into a friable callus tissue (Fig. 1b). The callus is characterized by a soft, white or yellow-white, friable appearance and is embedded in a mucilaginous substance. Numerous globular somatic embryoids developed on its surface (Figs. 1b and c). When it is transferred into liquid medium it falls to small fragments. The difference in appearance between friable callus and compact callus which is cultured on GP-medium with 6% sucrose, is very clear (Figs. 1c and 1d).

Friable callus could be maintained for long terms when subcultured every two weeks. This frequent subculturing was necessary, not only to refresh the culture medium and prevent necrosis, but also because of the vigorous callus growth. These results are in agreement with those of Green et al. (1983).

Histological and ultrastructural examination.

Early development of friable callus (0-10 days in culture).

The scutellum of three days cultured embryos contained a broad subepidermal region with meristematic cells (Fig. 2a). From the epidermis towards the scutellar node the cytoplasmic density declined, while cell volume and vacuolation increased, which indicates a gradient in cell differentiation in the scutellum (Fig. 2b). Near the coleorhiza an intercellular space was observed surrounded by degenerating cells (Figs. 2b and 2c). Tracheary elements were hardly observed.

After ten days of culture the adaxial scutellum contained enlarged vacuolated and degenerating cells and large intercellular spaces (Fig. 3a). The epidermal and subepidermal region showed many small cells, which divided rapidly. These cytoplasm-rich cells are most likely the embryogenic cells of the friable callus. At this time a globular embryoid could already be observed. At several places in the abaxial scutellum we found clusters of very

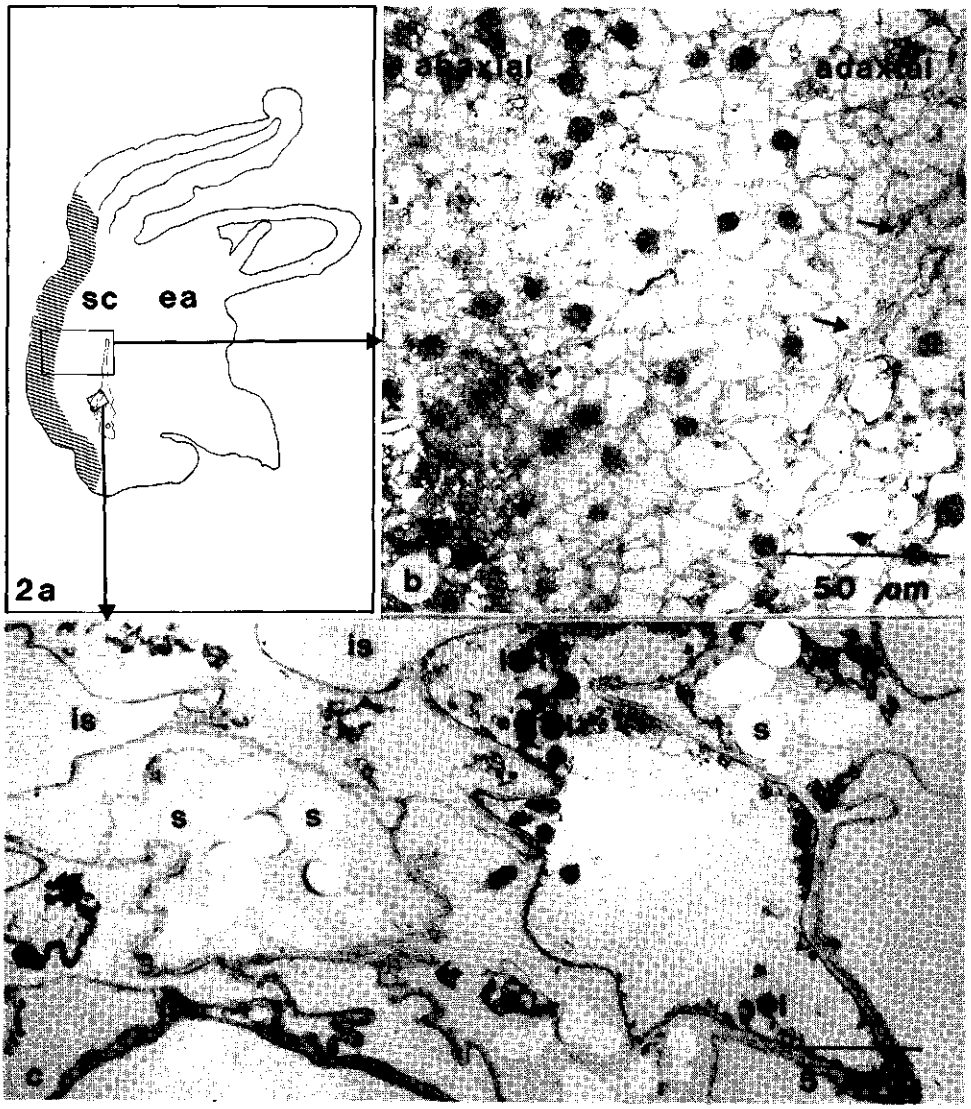


Fig. 2a. Schematic representation of an immature embryo after three days on N6 medium. The shaded area denotes the meristematic region of the scutellum (sc). ea, embryo axis.
b. Detail of the middle scutellum showing small cytoplasm-dense cells on the abaxial side, whereas towards the adaxial side the cells are larger and more vacuolated. Note the degeneration of cells close to the scutellar node (arrows).
c. Electron micrograph of the scutellum near the scutellar node, where cells are degenerating. is, intercelluar space; l, lipid droplet; s, starch.

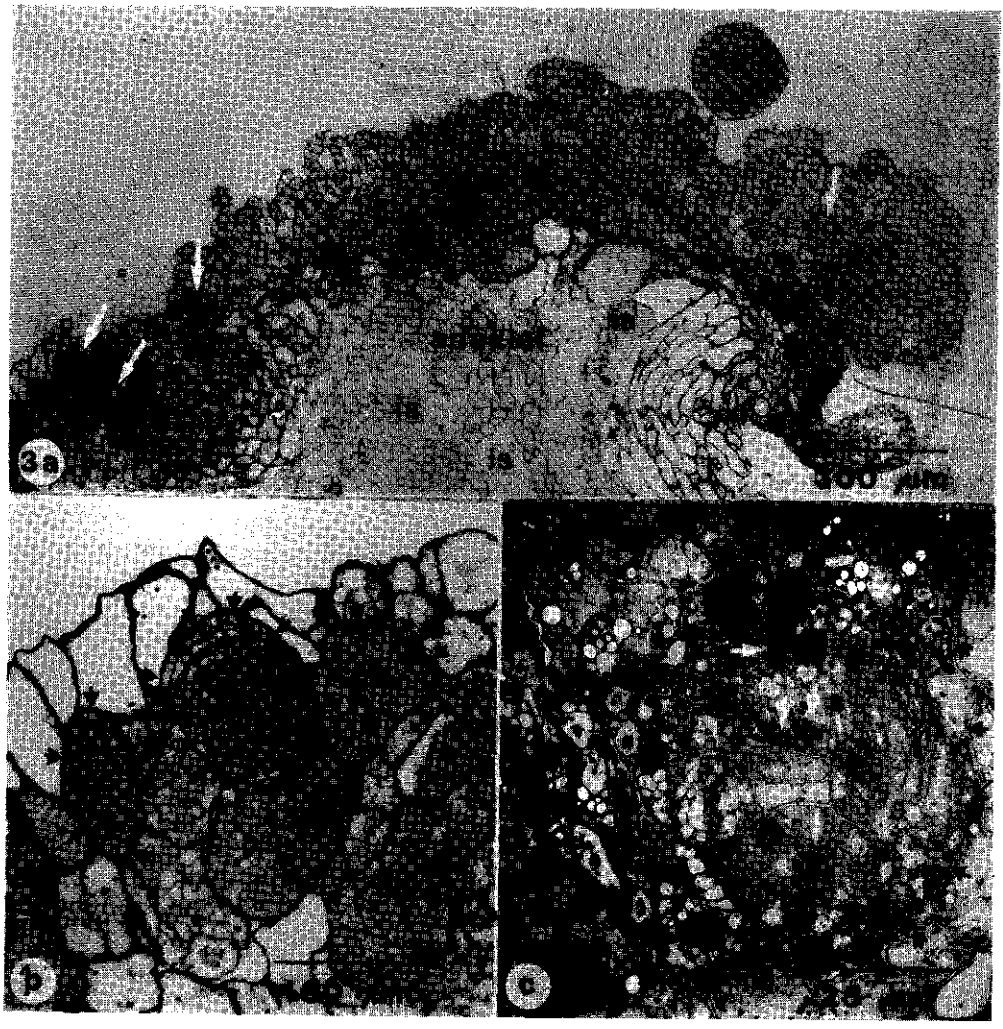


Fig. 3a. Longitudinal section of the scutellum of an embryo cultured for ten days on N6 medium, showing meristematic activity in the epidermal and subepidermal region. The adaxial region shows large intercellular spaces (is). Note the presence of discrete cell clusters with dense cytoplasm (arrows). se, somatic embryo.
b. Detail of Fig. 3a, showing some clusters of very small cells with a high cytoplasmic content. The contours of some clusters are indicated by arrow heads. cc, cell cluster.
c. Electron micrograph of a cell cluster. Note the electron density, the small cells and nuclei (white arrows) as compared to the surrounding tissue (black arrow). The contour of the cell cluster is indicated by arrowheads.

small cells with a very high cytoplasmic content (Fig. 3b). These were probably derived from a single cell after several divisions during which the total volume of the cell cluster apparently does not change. This can be deduced from the small cells and the size of the nuclei as compared to the cells and nuclei of the surrounding tissue (Figs. 3b and c). Contact between individual clusters or between clusters and the surrounding tissue by plasmodesmata has not been observed. This physiological isolation suggests a separate development from the other cells in the scutellum tissue.

Later stages of friable callus.

After prolonged subculturing, a spongy and poorly organized tissue could be observed (Fig. 4a). It consisted of two types of cells: large, elongated and highly vacuolated cells and small isodiametric cells with densely stained cytoplasm. The highly vacuolated cells occupied the major part of the callus tissue, mainly because of their voluminous appearance. They did not show proliferating activity and were probably not totipotent. We did not found vascular elements in this callus.

The isodiametric cells were grouped together as narrow strands or small clumps. These cell aggregates showed mitotic activity and are termed embryogenic units. They have intercellular spaces and apparently are not bordered by specially differentiated cells (Figs. 4a and 5a). In callus colonies which were not subcultured for longer periods embryogenic units were not detected. Strands of isodiametric cells were often connected to strands of vacuolated cells. The transition from one to the other seems to be gradual; however, it appeared to be abrupt at higher magnification (Fig. 5b).

Fig. 4b shows a section through the compact callus. The difference in organization with friable callus is striking. Scutellar bodies and broad meristematic regions developed on the surface of the callus, whereas in the center vacuolated parenchyma cells were found. Vascular bundles surround this center and sieve elements as well as vessel elements were observed between the central parenchyma cells.

At the ultrastructural level the embryogenic cell is characterized by a relatively large nucleus, the presence of small vacuoles and an electron-dense cytoplasm (Fig. 5a), caused by the presence of numerous free ribosomes. The cytoplasm contained many mitochondria and plastids, sometimes containing starch. Dictyosomes and microbodies were also frequently obser-


eu
is
vc
eu
is
vc
eu
vc
is
is
4a
150 µm
pc
b

ved. We found many plasmodesmata in the thin cell walls of adjacent cells (Fig. 5b). The vacuolated cells are less electron dense and Fig. 5c clearly shows this difference. There is also a difference in electron-density of the cell wall between the two cell types, which indicates a difference in composition. In vacuolated cells which are located next to or near embryogenic units prominent stacks of RER with spirally arranged polysomes were often observed (Fig. 5d).

Dictyosomes are frequently observed with vesicles that have an electron-dense content. These dark vesicles are possibly excreted on the plasma membrane and the content is present between the plasma membrane and the cell wall (Figs. 6a and 6b). Callus cells, located at distance from the embryogenic unit are very large and probably older. These cells are characterized by a single large vacuole and mitochondria with an electron-dense matrix and well developed cristae (Fig. 6c). We did not observe plasmodesmata between the vacuolated cells and close contact is impeded by thick cell walls that sometimes contained small vesicles (Fig. 6d). Adjacent cells were often separated by a narrow space, filled with electron-dense material, probably remnants of degenerated cells (Fig. 6a). We regularly found long strands of fibrillar material not only between the cell walls of the vacuolated cells, but also between isodiametric cells or between the two types (Fig. 6e). The fibrillar strands often ran into the large intercellular spaces (Figs. 4a and 6f), and appeared PAS positive, indicating a polysaccharide component.

Fig. 4a. Light micrograph of friable callus tissue showing embryogenic units (eu) and strands of vacuolated callus cells (vc). Note the enormous intercellular spaces (is).
b. Light micrograph of compact callus tissue showing regions with meristematic cells (mc) and parenchyma cells (pc). Note the presence of vascular bundles (arrows) and vessel elements (ve).


ou
vc
vc
ip
5a
20 µm
b
c
d

Discussion

The results confirm the report of Vasil et al. (1985), that a small zone of epidermal and subepidermal cells proliferates to form friable callus, while the adaxial scutellum cells enlarge and degenerate. Furthermore, we present extended information on the development and morphology of friable callus in maize tissue culture.

The main characteristic of friable embryogenic callus is its friability, which makes it easy for fragmentation in order to subculture it and to obtain cell suspensions. This report clearly shows the striking difference in morphology between the compact type I and the friable type II callus (Figs. 4a and b). The compact appearance of type I callus is due to the presence of broad meristematic regions and regions with vacuolated parenchyma cells and tracheary elements (Fig. 4b, see also Vasil and Vasil, 1982; Wernicke et al., 1982; Ozias-Akins and Vasil, 1982; Vasil et al., 1985). Compact callus originates from a broad scutellar area (Vasil et al., 1985; Fransz and Schel, 1987). As opposed to the situation in friable callus no gradient in vacuolation and degeneration was observed during early development of compact callus. This suggests that the morphogenetic differences between friable and compact callus are visible already after three days of culture.

Several studies have been reported on the factors that affect the formation of either compact or friable callus. Armstrong and Green (1985) demonstrated the important role of L-proline in the N6 medium in obtaining friable callus and suggested that L-proline may function at least partially as a protectant against a stress or stresses associated with in vitro culture. This enhancing effect of L-proline on type II formation is confirmed

Fig. 5a. Detail of friable callus showing an embryogenic unit (eu) next to vacuolated callus cells (vc). is, intercellular space.
b. Electron micrograph of an embryogenic unit cell, showing mitochondria (m), microbodies (mb), and numerous ribosomes. pd, plasmodesma.
c. Detail of an embryogenic unit cell (eu) neighbouring a vacuolated callus cell (cc). Note the difference in electron density of the cytoplasm and of the cell wall (cw).
d. Detail of the cytoplasm of a vacuolated callus cell near an embryogenic unit. Note the prominent stacks of rough endoplasmic reticulum (rer) and the spirally arranged polysomes (arrows).

by Tomes (1985). On the contrary, L-proline had no effect on callus formation of MS cultures (Armstrong and Green, 1985).

Another factor, which influences the callus type is the sucrose concentration (Lu et al., 1983; Vasil et al., 1984, 1985; Tomes, 1985). Low concentrations (2%) stimulate type II production, whereas at higher concentrations (6%) type I develops. It is also possible to generate type II callus from type I cultures by reducing the sucrose concentration (Vasil et al., 1984). A similar transformation from compact to friable callus without changing the culture conditions was observed in callus cultures of Aesculus hippocastanum (Profumo et al., 1987). According to these authors this transformation is accompanied by the appearance of starch in the friable embryogenic callus, which is due to an enhanced ability to absorb sugar from the substrate. We therefore propose, that the ability to obtain friable callus from compact callus indicates, that embryogenic units are present in potential in compact callus.

The genetic constitution appears to be an important factor controlling the formation of type I or type II callus. Tomes (1985) found, that the callus type response differs considerably between different genotypes, which are all capable of plant regeneration. It is suggested, that type I and type II culture responses are conditioned by separate nuclear genetic determinants. Hodges et al. (1985) confirmed the genotypic influence. Moreover, they found that the inheritance of somatic embryogenesis and plant regeneration in maize involved primarily two genes. These data suggest that before transformation of type I into type II, certain nuclear genes are switched off, while others are turned on, either after lowering the sucrose level or as a 'spontaneous' event.

Figs 6a-e. Electron micrographs of vacuolated callus cells.
a and b. Many dictyosomes (d) are present often in the vicinity of dark vesicles (arrow). Note the presence of electron-dense material in the intercellular space (asterisks). p, plastid.
c. Callus cell at distance from the embryogenic unit. Note the mitochondria (m) with well developed cristae.
d. Cell walls containing small vesicles (arrows). d, dictyosome.
e. Electron-dense fibrillar material running from one to the other cell.
f. Light micrograph of isodiametric cells stained with PAS. PAS positive fibrillar strands (arrows) run from the cells into the intercellular space.


a
b
c
d
e
f
20 µm

During friable callus development cell clusters proliferate and dissociate resulting in long aggregates separated by large intercellular spaces. A similar process of fragmentation is also observed in callus cultures of Ranunculus sceleratus (Thomas et al., 1972) and Aesculus hippocastanum (Profumo et al., 1987) and in cell cultures of Daucus carota (Halperin and Jensen, 1967; McWilliam et al., 1974). According to Thomas et al. (1972) the prominent air spaces in friable callus are due to differentiation and ultimate death of the vacuolated central cells in the cell aggregates. McWilliam et al. (1974) also considered the large vacuolated cells to be involved in fragmentation. The presence of fibrillar strands between isodiametric cells in type II callus (Fig. 6f) indicates that cell separation also takes place in the embryogenic unit, which thereby contributes to fragmentation of the aggregates. Button et al. (1974) and Profumo et al. (1987) observed vesicles deposited in the cell wall, the contents of which cause digestion of the middle lamellae. This report also shows, that vesicles appear in the cell walls of the vacuolated cells, and these might be responsible for the breakdown of the middle lamellae. These changes in the cell walls are presumed to be the result of auxins (Halperin and Jensen, 1967). However, auxins are possibly not the only necessary factor to induce tissue fragmentation, since compact callus tissue cultured in the presence of 2,4D did not show cell wall degradation. Israel and Steward (1966) conclude, that cell separation in cultured carrot explants is due to pronounced cell expansion. After cell separation a fine fibrillar network remains in the intercellular space. An excessive growth of fibrillar wall material extending into the liquid culture has also been found by Halperin and Jensen (1967).We suggest that in the case of maize tissue culture, external factors enclosed in the culture medium, like 2,4D or sucrose, induce biochemical processes which are responsible for cell dissociation in friable callus or differentiation of certain cells into vascular elements in compact callus.

The embryogenic component of type II callus is represented by embryogenic units. These aggregates of small cytoplasm-rich cells closely resemble in form and structure the superficial cells of embryogenic masses (Halperin and Jensen, 1967) or embryogenic clumps (McWilliam et al., 1974) in carrot suspensions and Ranunculus sceleratus (Thomas et al., 1972). There is a difference in composition between the cell aggregates of maize and the embryogenic clumps in carrot. Unlike the clump-like composition in carrot

and Ranunculus sceleratus, where superficial embryogenic cells encircle central vacuolated cells, the embryogenic units in maize cultures are arranged in long strands of aggregates together with non-embryogenic cells. Clump-like compositions have been observed in maize callus, but these were described as intermediate stages between the embryogenic unit and the early somatic embryo (see Chapter 4). It is suggested, that cells of the embryogenic unit (i) are involved in production of new isodiametric cells, (ii) give rise to somatic embryos, and (iii) turn into vacuolated non-embryogenic callus cells.

The apparent lack of vascular elements in type II callus makes us consider alternatives for transport of nutrients and metabolites. Symplastic transport is excluded because of the absence of plasmodesmata in the highly vacuolated cells. This implicates two alternatives of apoplastic transport, (i) diffusion from cell to cell, and (ii) diffusion via the large intercellular spaces presumed that they are occupied by some liquid substance. This is not inconceivable if we consider, that soft and friable callus but also other callus types are often embedded in some mucilaginous substance (Wernicke et al., 1982; Everett et al., 1985; Karlsson and Vasil, 1986). It is reported that polysaccharide slime is transported through the cisternae and vesicles of dictyosomes (Morre et al., 1967; Paul and Jones, 1976), an organelle, which is observed very frequently in callus cells (Halperin and Jensen, 1967). The observed vesicles with dark content (Figs. 6a and b) may be involved in this process. Based on this evidence, we suggest, that callus cells excrete mucilaginous slime, which fill the large spaces between the cell aggregates, thereby facilitating the transport of nutrients and metabolites by diffusion.

Although a study on somatic embryogenesis is reported in more detail in Chapter 5, some observations concerning the origin of embryoids are also reported here. Somatic embryos may be of multicellular or unicellular origin. The matter is discussed in many papers (for a review, see Williams and Maheswaran , 1986). A consideration is that a multicellular origin produces embryoids fused with the parent tissue over a broad area of the root pole or axis region, whereas a unicellular origin is more likely to give embryos attached by a narrower suspensor-like organ. Both types have been reported in cultures of cereals and grasses. For example, a multicellular origin is proposed in Sorghum bicolor (Dunstan et al., 1978; Wernicke et al., 1982)

and Zea mays (Vasil et al. 1985; McCain and Hodges, 1986; Fransz and Schel, 1987). A unicellular origin is suggested in Pennisetum americanum (Vasil and Vasil, 1982; Botti and Vasil, 1983). Except for haploid embryoids, which are derived directly from microspores, there is no direct evidence for unicellular origin. Most data are based on microscopical observations of discrete groups (proembryoids) of two or few cells within a common thick wall. In the present study, we have not only observed discrete and physiologically isolated masses of embryogenic cells, but we also found indications that these cells are derived from one single cell (Fig. 3c). Although their further development is not examined, we suggest, that they may develop directly into embryoids. Because we have observed such cell clusters only at the early development of type II callus, when scutellar tissue is still recognized, they may be developed directly from scutellum cells (see also Vasil et al., 1985). The presence of a globular embryoid at this early stage of friable callus development supports this argument.

CHAPTER 5

AN ULTRASTRUCTURAL STUDY ON THE EARLY DEVELOPMENT OF *ZEA MAYS* L. SOMATIC EMBRYOS.

Summary

Friable embryogenic callus, obtained from immature embryos of Zea mays L. was cultured on N6 medium supplemented with 1 mg per liter 2,4D, 6 mM proline and 2% sucrose. Cultured tissue fragments containing several globular embryoids were excised and examined by light and electron microscopy in order to follow the early development of maize embryoids. The somatic embryo consists of an apical region and a suspensor region. Cells of the apical region are small, cytoplasm-rich, and mitotically active. They contain much starch and numerous bundles of microtubules. Suspensor cells are larger and more vacuolated. A high metabolic activity in both cell types is indicated by the presence of many organelles, coated vesicles, membranous invaginations and multivesicular bodies.

We propose a transition unit which forms an intermediate stage between the embryogenic callus cells and the somatic embryo. The transition unit is a group of embryogenic cells and shows an apical and a basal region. The unit has many intercellular spaces, while within the cells organelle-free cytosol is frequently observed. We suggest, that the acquisition of polarity and the appearance of numerous microtubules mark the initiation of somatic embryogenesis.

Introduction

Somatic embryogenesis can be described as the process by which haploid or diploid somatic cells develop into different plants through characteristic embryological stages without fusion of gametes (Williams and Maheswaran, 1986). The phenomenon has been observed in tissue cultures of numerous plant species since the first publication by Steward (1958) for carrot suspension cultures. In gramineous cultures organized structures with a single cotyledon developing at the callus surface were recognized as somatic embryos (for a review, see Ammirato, 1983). However, it has often appeared difficult to detect a shoot-root axis which is regarded as the criterium to distinguish somatic embryogenesis from organogenesis with independent shoot and root formation. The organization of compact callus, which is most frequently obtained, hampers the determination of a somatic embryo. Only few studies on cereal tissue culture make notice of a pronounced shoot-root axis (Vasil and

Vasil, 1981, 1982; Wernicke et al., 1982; Botti and Vasil, 1983; Vasil et al., 1985).

A second point of controversy concerns the initiation and establishment of somatic embryogenesis. Many reports deal with the origin of somatic embryos: single cellular or multicellular. Both concepts are supported by microscopical evidence (Vasil and Vasil, 1982; Botti and Vasil, 1983; Ho and Vasil, 1983; Vasil et al., 1985; McCain and Hodges, 1986). Unfortunately, the reports do not explain why certain single cells or groups of cells become, or perhaps remain, determined for embryogenic development, while others do not.

We examined friable callus in Zea mays L. which has been found highly embryogenic because of the development of numerous globular embryoids on its surface (Armstrong and Green, 1985). Moreover, embryonal stages were found from globular to mature embryo (Green, 1983) and a typical embryo axis is clearly recognized by light microscopy (Vasil et al., 1984, 1985). Early developmental stages, however, are poorly examined (Vasil et al., 1985). In Chapter 4 we studied the development and structure of friable embryogenic callus and found that aggregates of cytoplasm-rich cells, so-called embryogenic units, form the embryogenic potential of this callus type. The units are assumed to be the sites of somatic embryo origin. In this study we describe the early development of somatic embryos up to the late globular stage. We were especially interested in cytological parameters that accompany the initiation of somatic embryogenesis. Such parameters provide information on the mechanisms that trigger this initiation.

Materials and methods

Maize plants of the inbred line A188 were grown in the greenhouse. Friable embryogenic callus was obtained by culturing immature embryos (approx. 1.5 mm) on N6 medium (Chu et al., 1975) supplemented with 1 mg per liter 2,4D, 6 mM L-proline, 100 mg per liter casamino acids and 2% sucrose (for details, see Malmberg et al., 1985). The cultures were incubated at 28-30^{o}C with a 16/24 h light/dark regime. Subculturing took place every two weeks. Callus fragments containing several somatic embryos were fixed in 4% glutardialdehyde for light microscopy and in 2% glutardialdehyde followed by a 1% OsO_4 fixation for electron microscopy (for details, see Chapter 4).

Some callus fragments were prepared for immunocytochemical analysis. Processing for embedding and indirect immunolabeling occurred using the method essentially as described by Van Lammeren (1988). The samples were fixed in 3% paraformaldehyde and 0.25% glutardialdehyde in 0.1 M phosphate buffered saline at pH 7.2 for 1 h at room temperature, dehydrated and embedded in polyethylene glycol. Sections (3 µm thick) were incubated with a polyclonal antiserum raised against tubulin and microtubule-associated pro-

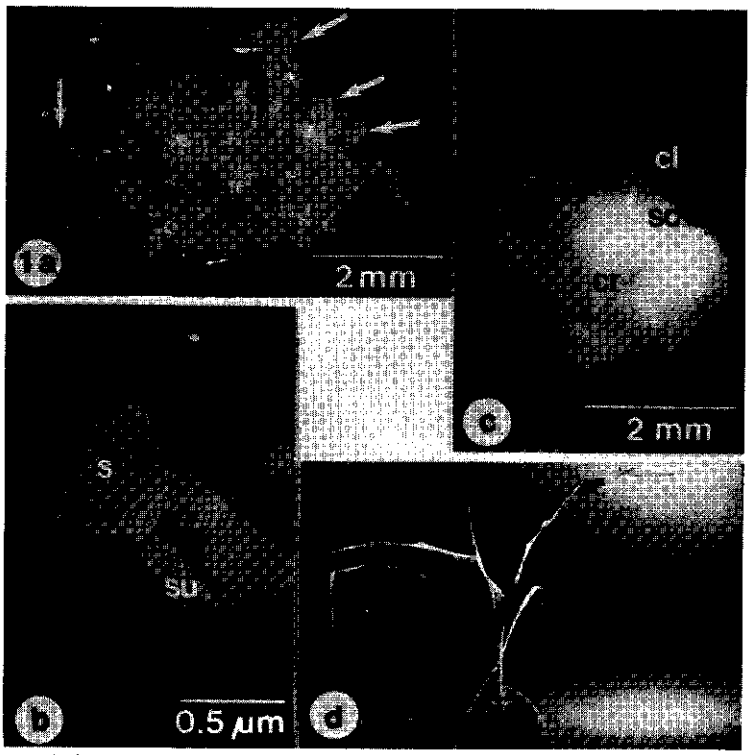


Fig. 1a Friable callus colony on N6 medium with 1 mg per liter 2,4D containing numerous globular somatic embryos (arrows).
b. Detail of a somatic embryo (s) attached to a suspensor-like stalk (su).
c. Mature somatic embryo after transfer to a 2,4D free medium. cl, coleoptile; cr, coleorhiza; sc, scutellum.
d. Young maize plant obtained from a somatic embryo after germination and transfer to soil.

teins for 45 min at room temperature. After rinsing, the sections were incubated with a FITC-conjugated goat-anti-rabbit serum (GAR/FITC, Nordic, Tilburg) for 45 min in the dark at room temperature. The sections were rinsed, mounted and examined under a fluorescence microscope (Nikon, Labophot).

Results

General morphology

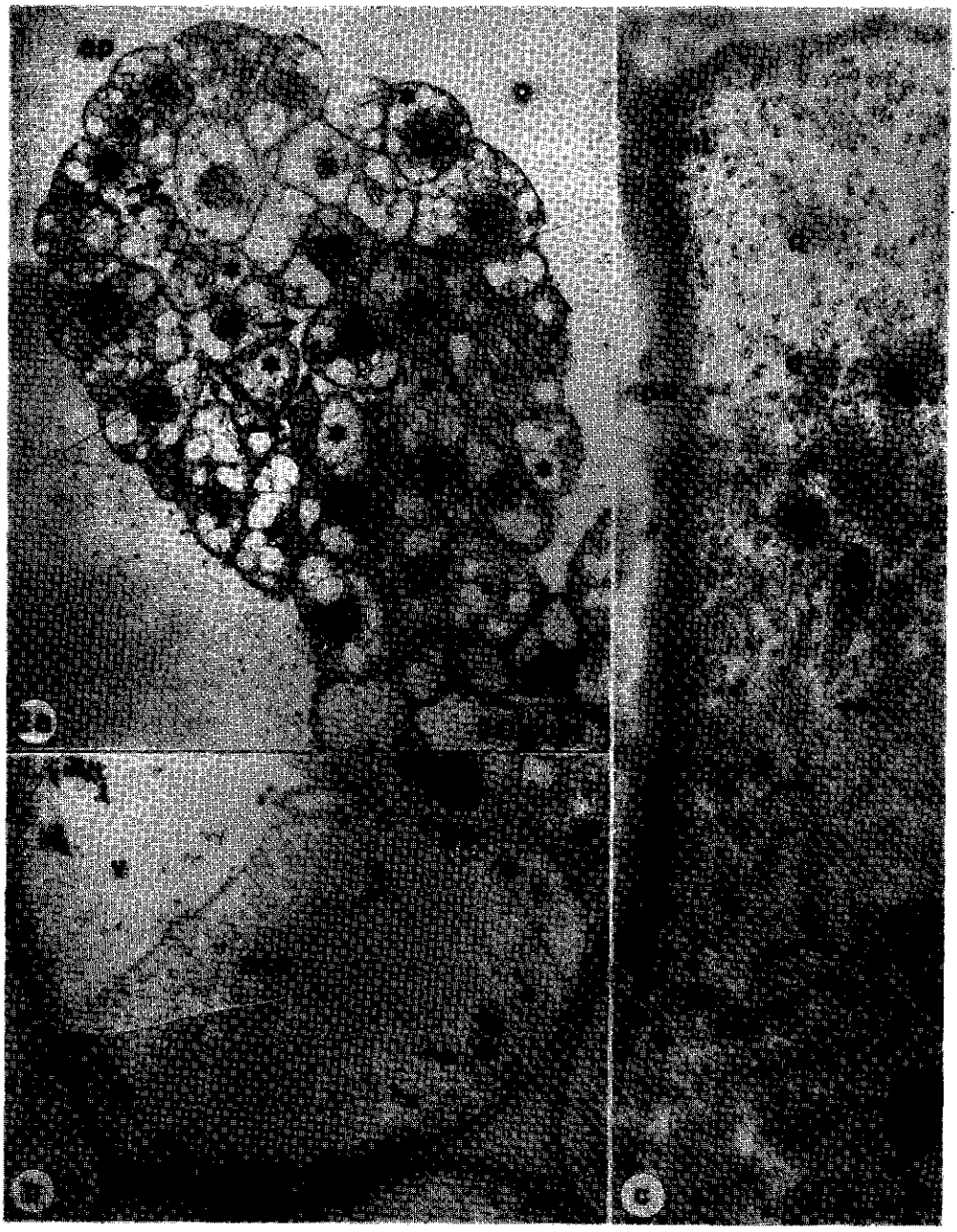
Soft and friable callus was initiated on N6 medium containing 1 mg per l 2,4D and had a high embryogenic potency. Already within ten days globular somatic embryos were observed at the callus surface. Older cultures showed numerous globular embryoids (Fig. 1a), which were often connected with the callus by a stalk (Fig. 1b). This stalk is described as the suspensor of the embryoid (Armstrong and Green, 1985; Vasil et al., 1985). The embryoids did not develop beyond the late globular or coleoptilar stage as long as 2,4D was present; omission of 2,4D resulted in a further growth and development of the embryoid (Fig. 1c). Precocious germination could be suppressed on a medium with a raised sucrose level (4%). In some cases nodular structures were observed, obviously the result of fusion of embryoids sharing the same suspensor. Without subculturing, embryoids may also develop to maturity and germinate, probably as a consequence of exhaustion of 2,4D. Upon transfer to MS medium without hormones small plantlets developed from which complete plants could be obtained (Fig. 1d).

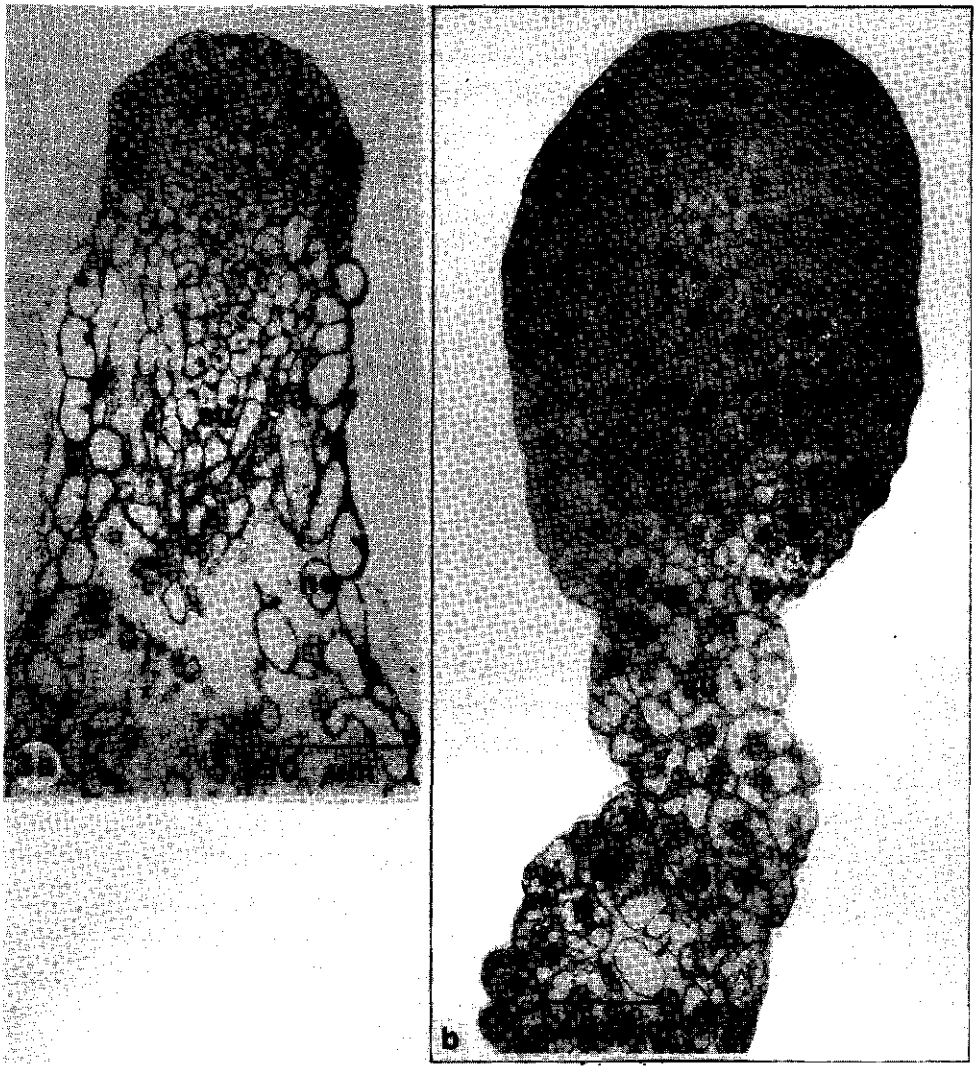
Histological and ultrastructural examination

Transition stage.

Figure 2a shows a globular structure, which was apparently more organized than the embryogenic units as described in Chapter 4. It consisted of an apical protoderm-like region with small cells, while larger more vacuolated cells were observed in the central or basal part towards the vacuolated callus cells. It was further characterized by the presence of intercellular spaces in the central region. We call this structure a transition unit because it probably forms an intermediate stage between the embryogenic unit and the somatic embryo.

Fig. 2a A transition unit showing small cells at the apical side (ap) and larger cells at the basis. Note the presence of intercellular spaces (arrows) and organelle-free cytosol (asterisks).
b. Electron micrograph of organelle-free cytosol (ofc), delimited by vacuoles (v), small vesicles (arrows) and endoplasmic reticulum (er).
c. The cytoplasm of a cell of the transition unit shows many dictyosomes (d) and spirally arranged polysomes (arrows). mt, microtubule; pd, plasmodesma.


ap
a
b
c



Figs. 3a and b. Early stages of somatic embryos consisting of an apical region (ar) and a suspensor region (su). Note the presence of starch (arrows) in the apical region especially near the suspensor. ne, non-embryogenic callus cells.

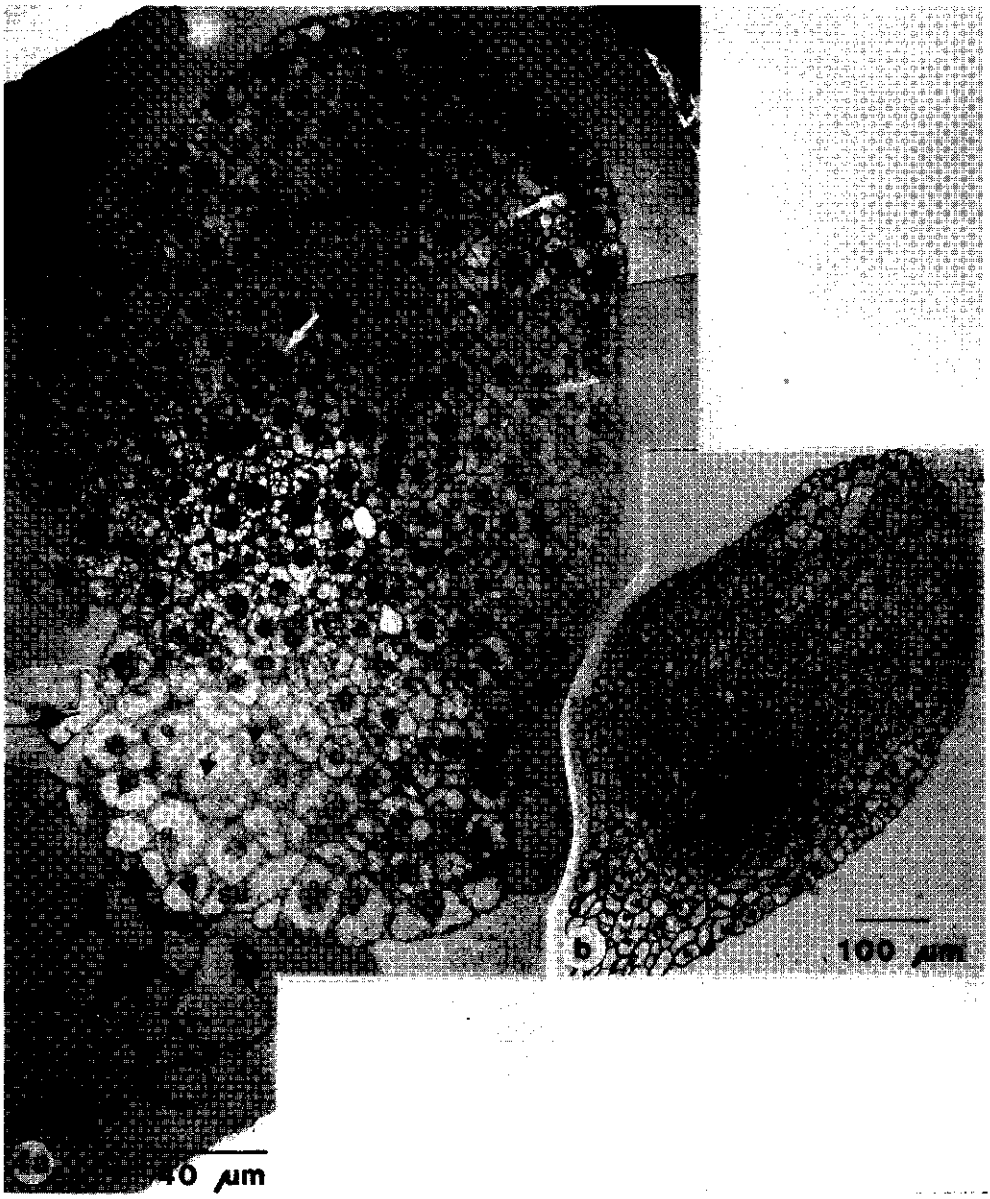


Fig. 4a A typical globular somatic embryo with meristematic cells in the apical region (ar) and more vacuolated cells in the suspensor region (su). Mitotic figures (white arrows) are present in the apical region. Note the presence of starch (black arrows) especially near the suspensor.
b. A late globular embryoid showing a root apex (ra) and the future shoot apex (arrows).

Cytoplasmic areas without organelles were observed frequently (Fig. 2b), especially in the central and basal cells. These areas are encircled by medium-sized vacuoles, small vesicles and endoplasmic reticulum and are termed 'organelle-free cytosol' (Hilling and Amelunxen, 1985). We found mitotic figures only in the apical and subapical region, suggesting that growth of the transition structure is due to cell division in the apical regions and cell elongation in the central and basal parts. The cytoplasm in all cells contained numerous polysomes often in spiral configuration, mitochondria with well developed cristae and dictyosomes pinching off many vesicles (Fig. 2c). These observations denote an active metabolism. We found starch containing plastids in the whole unit, especially in the central cells (Fig. 2a). All cells of the transition unit showed many plasmodesmata unless the cells were separated by an intercellular space (Fig. 2c).

Globular embryoids.

To avoid confusion in terminology we regard the globular embryoid as a club-shaped or globular structure attached to a stalk and without distinct differentiated regions such as a shoot apex or scutellum. The stalk is termed 'suspensor'.

The globular embryoids at the callus surface closely resembled zygotic maize proembryos at 5-7 DAP with respect to shape and structure (Figs. 3a, 3b, 4a and 4b, see also Van Lammeren, 1987). They were characterized by an apical region containing small cells with a high nucleus/cell volume ratio and a suspensor region which consisted of elongated highly vacuolated cells. The cells of the apical region showed mitotic activity and were surrounded by an epidermis or in early stages by a protoderm (Fig. 3a). In contrast with the transition unit intercellular spaces were not observed in the apical regions. Starch granules were generally confined to the embryonic cells, especially in the basal part towards the suspensor (Figs 3b, 4a, 4b). During further development of the somatic embryo, the apical region enlarged as a result of cell division and cell elongation (Fig. 4a). A typical shoot-root axis was lacking at this stage; the basal part, however, often showed characteristics of a root apex. In some cases a margin of small meristematic cells was recognized as the future shoot apex (Fig. 4b), which indicates the early embryo proper stage.

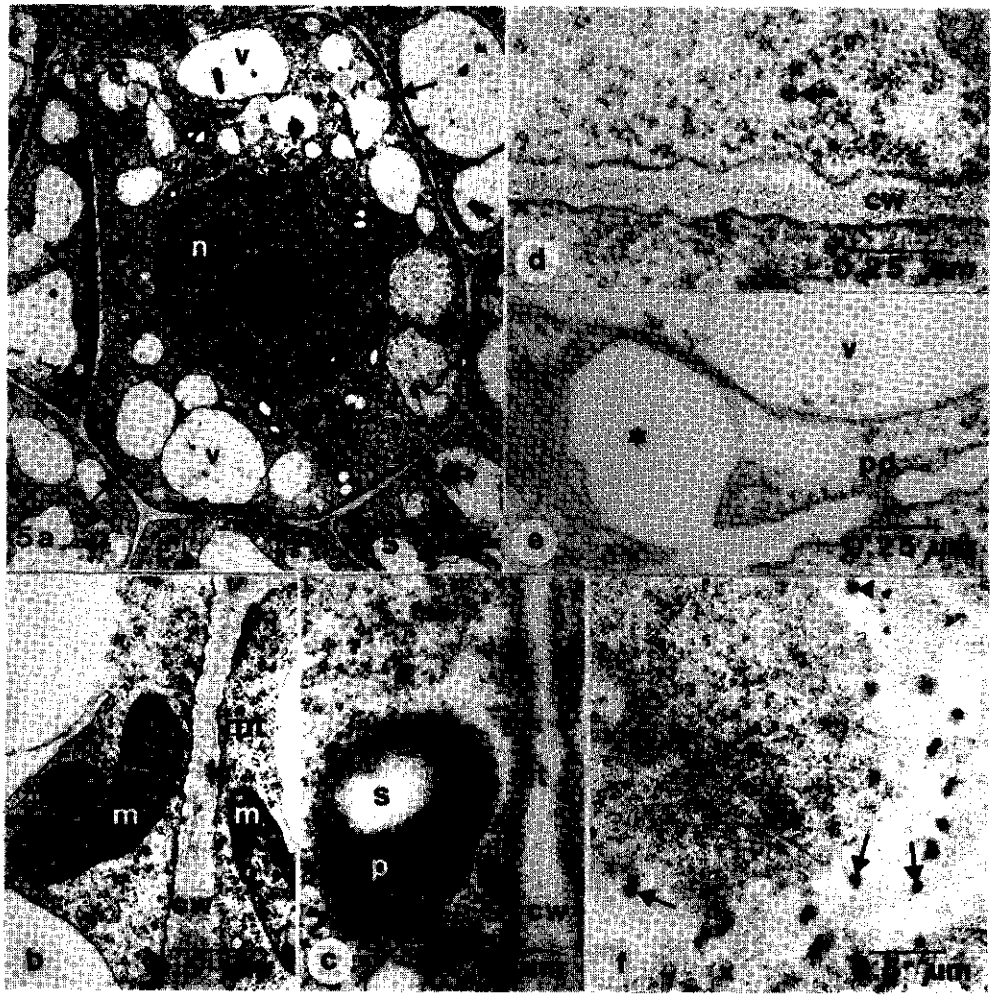


Fig. 5a Electron micrograph of a cell in the apical region. Note the presence of many plasmodesmata (arrows). n, nucleus; v, vacuole.
b. en c. Detail near the cell wall (cw) showing mitochondria (m) and a plastid (p) close to cortical microtubules (mt). s, starch.
d. Detail of the cytoplasm showing numerous free ribosomes and a coated vesicle (arrow). cw, cell wall.
e. Detail near the cell wall (cw) showing a large membraneous invagination (asterisk). v, vacuole.
f. Detail of the cell wall containing many plasmodesmata (arrows). Also note the presence of cortical microtubules (mt).

At the fine structural level we observed in cells of the apical region a central nucleus surrounded by many organelles. The cytoplasm was electron-dense which is most probably due to the presence of numerous free ribosomes (Fig. 5a). In some cells we frequently found mitochondria and plastids close to the plasma membrane near cortical microtubules, suggesting some kind of association with the cytoskeleton (Figs. 5b and c). The mitochondria showed well developed cristae. Plastids, often containing starch, did not show well-developed thylakoid membranes. Besides these characteristics, metabolic activity was also demonstrated by the presence of coated vesicles (Fig. 5d) and membraneous invaginations often appearing in the vicinity of a vacuole (Fig. 5e), where they are enlarged similar to the simultaneous invaginations as described by Zamski (1986). As was the case for the transition unit the apical region showed numerous plasmodesmata which indicates an intensive interaction through the symplast (Fig. 5f).

The suspensor cells resembled those of the apical region with respect to the organelles and their location. They also contained plasmodesmata but these were found less frequently. We observed plasmodesmata contact between apical cells and suspensor cells (Fig. 6a). However, in most cases both regions were separated by a thick cell wall with an electron-dense zone. Numerous dictyosomes were present in the suspensor cells near multivesicular bodies (Fig. 6b). On the outer side of the epidermal cells of the suspensor we frequently observed electron-dense material, stuck to the cell wall whereas on the inside we found dark granules, probably dictyosome vesicles (Fig. 6c).

Special attention was paid to the presence of microtubules in the different cells. Many cortical microtubules were found in the apical region perpendicular to the cell wall but also running parallel with it (Figs. 5f, 7a). In the suspensor cells the microtubules were also present although less frequently. Using immunocytochemistry we observed numerous bundles of microtubules in the apical region (Fig. 7b), whereas the suspensor cells (Fig. 7c) contained less microtubules. The cells of the embryogenic unit (Fig. 7d) and the vacuolated cells (Fig. 7e) remarkably showed a relatively low fluorescence, as compared to the apical cells, suggesting that they contain relatively few microtubules.

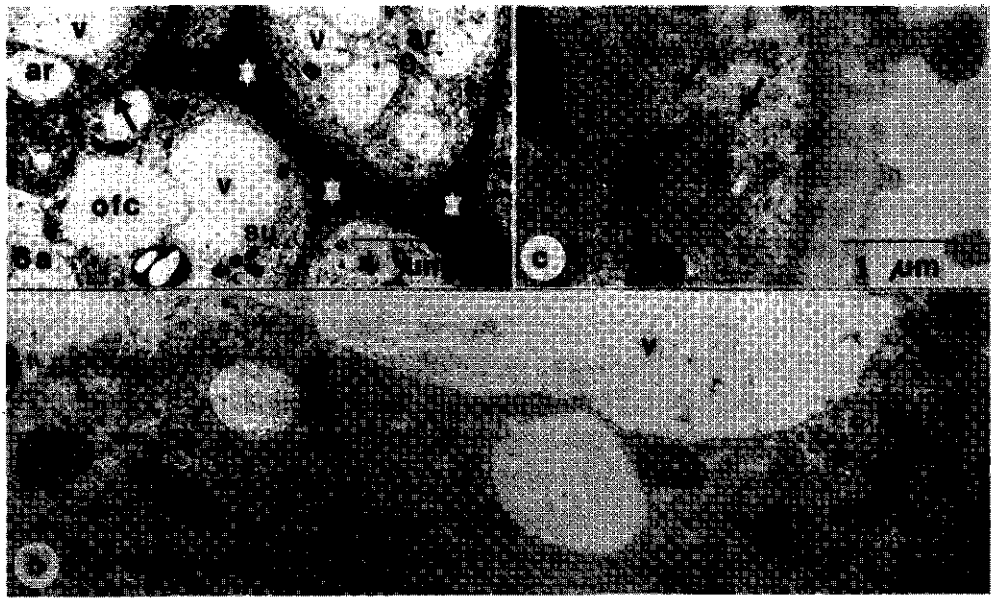


Fig. 6a Electron micrograph of a suspensor cell (su) adjoining cells of the apical region (ar). Note the presence of plasmodesmata (arrows) and an electron-dense thick cell wall (asterisks). ofc, organelle-free cytosol; v, vacuole.
b. Detail of a suspensor cell, showing many dictyosomes (d) and multivesicular bodies (arrows). m, mitochondrion; pd, plasmodesma.
c. Detail of an epidermal cell of the suspensor, showing a dictyosome (d) probably pinching off dark granules (black arrows). Note the presence of electron-dense material on the outside of the cell wall (white arrow).

Discussion

Somatic embryos growing on the surface of maize friable callus resemble zygotic embryos in shape and structure. Green (1983) presents a complete series of embryoids of subsequent stages excised from the callus culture. These stages range from globular to coleoptilar (Abbe and Stein, 1954) on 2,4D containing medium and subsequently to late embryo stages on 2,4D free medium. Our results demonstrate that early developmental stages of somatic embryos, which were cultured in the presence of 2,4D closely resemble the

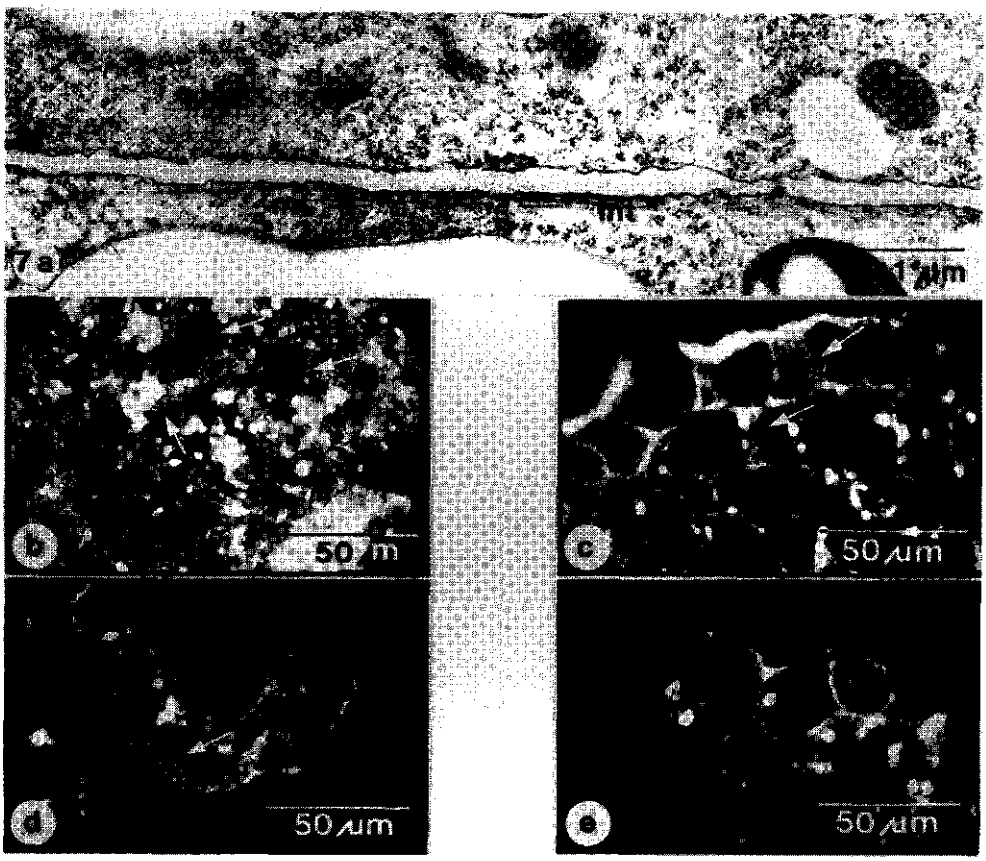


Fig. 7a Electron micrograph of a cell in the apical region showing cortical microtubules (mt) running parallel with the cell wall.
b-e. Fluorescence micrographs of PEG sections immunolabeled with anti tubuline. (b) apical region of embryoid, (c) suspensor region of embryoid, (d) embryogenic unit, (e) vacuolated callus cells. Note the presence or absence of bundles of microtubules (arrows) in the various tissues.

zygotic embryos from 5-7 days after pollination. During this period they developed from globular proembryo to an embryo-proper with a suspensor (Van Lammeren, 1987). In both studies the maize inbred line A188 was used. Although somatic embryos have been reported up to the coleoptilar stage on medium containing 2,4D (Green et al., 1983; Vasil et al., 1984, 1985), in

most cases a distinct shoot-root axis was not demonstrated. The inability to form a shoot-root axis may be explained by the growth-suppressive effect of 2,4D at or in the vicinity of the apical meristems (Wernicke et al., 1986). Pereira and Dale (1982) examined the effect of 2,4D on the development of plumular apices in seedlings of Phaseolus vulgaris and reported that the primordia show a distorted development, especially the most recent primordium (as in other experiments referred). A similar suppressive effect of 2,4D was also suggested for the transition from proembryogenic mass to embryoid in carrot cultures (Halperin and Jensen, 1967; McWilliam et al., 1974). However, Borkird et al. (1986) showed that 2,4D is not the only factor responsible for this developmental blockade. The suppressive effect of 2,4D was only established when coupled with high cell density. Nevertheless, for maize cultures we suggest that the formation of a shoot apex is suppressed by the auxin.

According to Hakman et al. (1987) the suspensor cells in Picea glauca embryoids are derived from the basal embryonic cells. This may also be the case for Zea mays. The role of the suspensor in somatic embryos is not well understood. It is suggested, that it plays a role in the uptake and transport of nutrients. Symplastic transport from suspensor to apical cells would then take place through the plasmodesmata, which are observed between these cells. Typical transfer cells, however, were not detected. On the other hand, dictyosomes and multivesicular bodies, as found in the suspensor cells, may be involved in endocytic as well as exocytic processes (Halperin and Jensen, 1967; Tanchak and Fowke, 1987). Together with the observed dictyosome activity in the epidermal suspensor cells (Fig. 6c) these data are consistent with a transport function of the suspensor.

It is assumed that in zygotic embryos nutrients are transported from the surrounding tissue into the embryo via the suspensor (Young, 1980; Schel et al., 1984). Furthermore, it is suggested that the suspensor is functional at the early stage of embryo development in Phaseolus coccineus L. (Young and Sussex, 1979). In maize, the suspensor is most active in uptake of endosperm products during early embryogeny (Schel et al., 1984). At about seven days after pollination the maize embryo has reached the coleoptilar stage and the nutritive function of the degenerating endosperm near the scutellum becomes more important. Lack of an intact endosperm has been suggested to be the cause of aberrant zygotic embryo development in vitro

(Schel and Kieft, 1986). Somatic embryos growing on the surface of friable callus lack a surrounding endosperm. It might not be a coincidence, that the embryoid does not develop beyond the coleoptilar stage in the presence of 2,4D.

We propose a transition structure between the embryogenic unit and the somatic embryo, which marks the transition from unorganized to organized growth and which reflects the initiation of somatic embryogenesis. In contrast with the embryogenic unit, which contains morphologically identical cells, the transition unit expresses polarity because the apical cells differ in size and degree of vacuolation as compared with the central and basal cells. Nutrient supply to the transition unit is most probably effected by the most basal cells which function as a region of nutrient uptake from the callus cells. On the other hand, the apical cells, which show a higher cytoplasmic density, may be regarded as a sink. Thus we find a flow of nutrients from basal cells to apical cells, emphasizing the polarity of this unit.

At the fine structural level, we reported the appearance of numerous organelle-free cytosol areas in cells of the transition unit (Fig. 2a). This indicates a vigorous vacuole formation from smooth ER vesicles and not from pre-existing vacuoles (Amelunxen and Heinze, 1984; Hilling and Amelunxen, 1985). This formation of new vacuoles in cells, which already contained vacuoles, implies that the lytic compartment (Matile, 1978) is renewed or extended. This suggests a change in metabolic activity in the transition unit. The appearance of numerous spirally arranged or coiled polyribosomes indicates a change in polysome configuration as compared to the free ribosomes, which were observed in cells of the embryogenic unit (Chapter 4). Although a change in aggregation provides no evidence on the rate of protein synthesis, a conversion of monosomes or small polysomes into large polysomes is reported in plant tissue undergoing different stages of development (Davies and Larkins, 1980). These data fortify the idea of an intermediate stage.

The transition from embryogenic unit to somatic embryo is accompanied by the appearance of many bundles of cortical microtubules in the apical part of the early embryoids. Halperin and Jensen (1967) already noticed a striking increase in the number of microtubules in the whole proembryo, after removal of 2,4D. This is confirmed by the work of Wochock (1973) and

Van Lammeren et al. (1987). We suggest that microtubules are being synthesized on a high rate, when somatic embryogenesis is initiated. This initiation is marked by the transition from unorganized to organized growth. It is not surprising, that the embryonic cells of the organized embryoid contain numerous bundles of cortical microtubules, as compared to the embryogenic unit or the vacuolated callus cells. Organized growth of the embryoid depends on a coordinated growth of the individual cells, which in turn is determined by the orientation of the cellulose fibrils within the cell wall. Cortical microtubules participate in regulating the orientation of microfibrils (Lloyd, 1982) and thus play an important role during growth and the establishment of polarity in somatic embryogenesis. Furthermore, microtubules are often associated with cell organelles and it is suggested, that the movement of mitochondria takes place along the microtubular network (Tilney, 1971). The close association of microtubules with mitochondria and plastids, observed in this study, supports this theory.

The results described here do not provide evidence for a unicellular origin of the somatic embryos which were generated by friable callus. On the other hand, the multicellular structure of the embryogenic unit and the transition unit is no evidence for a multicellular origin. According to the definition of an embryo (zygotic or non-zygotic) made by Haccius (1978), the observed somatic embryos arise from single cells. In that case the embryogenic unit would be homologized with the suspensor during normal angiosperm embryogeny.

Regardless the origin of somatic embryos the initiation of the process of embryoid development seems to be the major step in somatic embryogenesis. Once the process is triggered there appears to be no difference between somatic embryogenesis with a single cell origin or from a group of cells (for a review, see Williams and Maheswaran, 1986). The nature of the stimulus, necessary for the initiation of embryoid development, is unclear. Some factors, however, affect this initiation. At first components present or absent in the medium are known to induce somatic embryo development: omission of 2,4D (Halperin and Jensen, 1967), the cell density factor (Borkird et al., 1986) or starvation of sucrose (Heberle-Bors et al., 1988). Secondly, physical and physiological isolation from neighbouring cells was reported to induce somatic embryogenesis (Wetherell, 1984; see also Tisserat et al., 1979). It was suggested, that such an isolation

removes embryogenesis-repressive effects of neighbouring cells. Thirdly, there is substantial evidence that the acquisition of polarity accompanies the initiation of somatic embryogenesis. For example, electrical currents may be involved in determination and maintenance of polarity during somatic embryo development of carrot (Brawley, 1984). Moreover, Dyak et al. (1986) have found that electrical pulses promote embryogenesis in alfalfa protoplasts. Polarity with respect to morphological gradients is observed during early somatic embryogenesis (Halperin and Jensen, 1967; Ho and Vasil, 1983; see also Heberle-Bors, 1985).

Our results indicate that polarization is one of the first events during the initiation of somatic embryos in friable callus of maize. A causal relationship, however, is not demonstrated. There are no indications whether an embryogenic unit either proliferates or becomes a transition unit. We suggest, that the morphogenetic change from embryogenic unit to transition unit may be a result of mechanical forces, caused by the proliferating callus, creating a polarized structure within the embryogenic unit. Once polarity is established, gradients of hormones, nutrients, and possibly ionic currents are build up which fortify the polarity and activate the process of somatic embryogenesis.

CHAPTER 6

ISOZYMES AS BIOCHEMICAL AND CYTOCHEMICAL MARKERS IN EMBRYOGENIC CALLUS CULTURES OF *ZEA MAYS* L.

Summary

Isozyme analyses were carried out on protein extracts of non-embryogenic and embryogenic callus from Zea mays L., using polyacrylamide gelelectrophoresis. We examined the isozyme patterns of glutamate dehydrogenase, peroxidase and acid phosphatase on their utility as biochemical markers of maize embryogenic callus cultures. These isozyme systems were also used to examine possible correlations between isozymes and different stages of regeneration. We found glutamate dehydrogenase and peroxidase useful as markers to distinguish between embryogenic and non-embryogenic callus. Some isozymes appeared to be correlated with the morphological appearance of the tissue while others seem to be involved with the duration of the culture period. Using the same enzyme assays on fresh tissue samples we were able to test the three enzymes as cytochemical markers in embryogenic cultures. Glutamate dehydrogenase proved to be most succesful to discriminate embryogenic from non-embryogenic cells.

Introduction

The enormous progress in cereal tissue culture during the last ten years has led to plant regeneration not only from callus cultures, but also from cell suspensions and protoplast cultures (for reviews, see Flickinger et al., 1983, Ammirato, 1983, Lörz et al., 1988). In the establishment of plant regeneration the screening of an embryogenic culture plays an important role, especially when cultures are derived from numerous cell lines. In many cases the embryogenic potential is identified on morphological characteristics. However, such a visual screening is subjective and often only applicable after prolonged culture periods. An early biochemical identification of embryogenic potency would therefore be a great help for an efficient plant regeneration. In relation to this, reports have been published on biochemical differences between embryogenic and non-embryogenic callus cultures with respect to antigens (Khavkin et al., 1977), polypeptide pattern (Stirn and Jacobson, 1987, Chen and Luthe, 1987), ethylene production (Wann et al., 1987) and the amount of trypsin inhibitor (Carlberg et al., 1987). However, these biochemical systems are either time-consuming or unsufficient specific to identify subsequent stages of development. As an alternative a

simple and rapid identification of embryogenic callus might be established by isozyme analysis. Isozymes are easily detectable and their variation is often associated with genetic differences and developmental stages (Scandalios, 1974). The application of isozymes as markers in embryogenic cultures has therefore been reported in several studies (Wochock and Burleson, 1974, Negrutiu et al., 1979, Everett et al., 1985, Kay and Basile, 1987, Chawla, 1988). Using starch gel electrophoresis Everett et al. (1985) analyzed the zymograms of glutamate dehydrogenase, alcohol dehydrogenase, β-glucoronidase and esterase in callus cultures of maize. They found the esterase and glutamate dehydrogenase zymograms useful to distinguish between embryogenic and shoot forming cultures. However, they did not analyze isozyme differences between embryogenic and non-embryogenic callus.

In this paper we report the application of acid phosphatase, glutamate dehydrogenase and peroxidase as markers in embryogenic cultures of maize. The isozyme patterns of non-embryogenic callus were compared with embryogenic callus on N6 medium. The zymograms of embryogenic callus were also examined after transfer to regeneration medium. In addition, cytochemical assays were performed using the same enzyme systems for an in situ detection of possible embryogenic potential.

Materials and methods

Tissue culture and sampling

Immature embryos of the maize inbred line A188 were cultured on a modified N6 medium (Chu et al., 1975) in order to obtain friable embryogenic callus (see also Chapter 4). Friable callus was subcultured every two weeks. After transfer to N6 medium lacking 2,4D but with 4% sucrose (regeneration medium) the embryoids developed to maturity within four weeks. Non-embryogenic callus was excised from friable callus colonies on N6 medium that produced only roots, and lacking somatic embryos on its surface. Compact embryogenic callus was obtained from immature embryos that were cultured on a modified Murashige and Skoog (1962) medium (for details, see Fransz and Schel, 1987).

Tissue samples of all callus types were taken just before subculturing. In the case of friable callus, samples were also taken 1, 2, 4, 8 and 15 days after transfer to regeneration medium. All samples were frozen in

liquid nitrogen and stored at -70 °C until use.

Protein extraction and electrophoresis.

Frozen tissue samples were ground to a fine powder in liquid nitrogen with an equal volume of buffer (40 mM Tris-HCl, pH 7.2, 1 mM EDTA, 5% glycerol and 0.01 mg/ml bromo phenol blue). The homogenate was incubated at 0 °C. After 1 h the homogenate was centrifuged at 15.000 x g for 10 min. The supernatant was used for isozyme analyses.

Vertical electrophoresis was performed using polyacrylamide gels. The stacking gel contained 4% acrylamide, while in the slab gel a linear gradient of acrylamide was build up from 5 to 16% (w/v). The electrophoresis buffer was composed of 32 mM Tris-HCl (pH 8.3) and 250 mM glycine. The gels were run for 4 h at 7 °C and 120 mA.

Staining methods

The gels were preincubated for 20 min at room temperature in a buffer with the same composition used in the enzyme assay solution.

Glutamate dehydrogenase. (GDH): The enzyme assay solution contained 100 ml 0.1 M Tris-HCl at pH 7.5, 0.2 ml 10 mM $CaCl_2$, 800 mg sodium glutamate, 30 mg nicotinamide adenine dinucleotide (NAD^+), 20 mg nitro-blue-tetrazolium and 4 mg phenazine methosulfate. The gels were incubated overnight in the dark at 30 °C.

Peroxidase. (PRX): The enzyme assay solution contained 140 ml 0.05 M acetate at pH 5.0, 3 ml 0.1 M $CaCl_2$, 75 ul 30% H_2O_2, 75 mg 3-amino-9-ethyl carbazole, 7.5 ml dimethylformamide. The gels were incubated overnight in the dark at 4 °C.

Acid phosphatase. (ACP): The enzyme assay solution contained 150 ml 0.1 M acetate at pH 4.8, 150 mg sodium α-naphtyl acid phosphate, 150 mg fast-garnet GBC. The gels were incubated overnight in the dark at 37 °C.

Cytochemical assay.

Small pieces of friable embryogenic callus were sampled and preincubated in a buffer with the same composition used in the enzyme assay solution. The

composition of the enzyme assay solution for GDH, PRX and ACP were the same as those used for staining the gels. The tissue samples were incubated in the dark for 30 min at 30 °C (GDH), 4 °C (PRX) or 37 °C (ACP). After staining, the specimens were fixed in 3% paraformaldehyde, 0.05% glutardialdehyde in 0.1 M phosphate buffer at pH 7.2, rinsed in buffer and mounted with glycerol under a coverglass.

Results

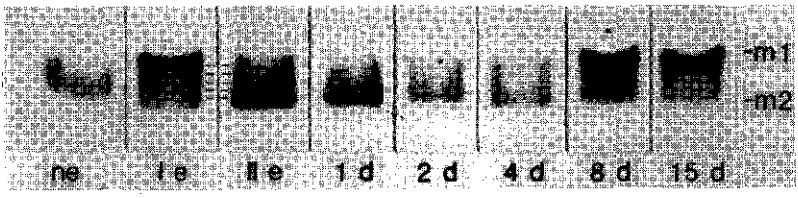
Glutamate dehydrogenase (GDH)

The total number of GDH bands found in the different zymograms consisted of two intensively stained major bands (M1 and M2) and five minor bands which were localized between the positions of the major bands (Fig. 1). The different tissues varied in isozyme pattern. In some cases only a smear of GDH activity was observed. Non-embryogenic callus showed a smear of GDH activity. However, in a second experiment (not shown) the isozyme activity appeared to be clearly localized at major band 2. Both types of embryogenic callus, friable as well as compact, showed one major band and a spectrum of five minor bands, the intensities of which formed a gradient which increased in the direction of the major band. Major band 1 was present in compact callus whereas friable callus showed GDH activity especially at the position of band 2. When friable callus cultures were transferred to regeneration medium with a raised sucrose level and omission of 2,4D, GDH activity shifted from major band 2 immediately after transfer towards major band 1 after 15 days.

Fig. 1. Zymogram of GDH isozymes. (ne) non-embryogenic callus; (Ie) embryogenic compact callus; (IIe) embryogenic friable callus; (1d, 2d, 4d, 8d, 15d) embryogenic friable callus 1, 2, 4, 8 and 15 days after transfer to regeneration medium. m1, major band 1; m2, major band 2.

Fig. 2. Zymogram of PRX isozymes. (ne) non-embryogenic callus; (IIe) embryogenic friable callus; (1d, 2d, 4d, 8d, 15d) embryogenic friable callus 1, 2, 4, 8, and 15 days after transfer to regeneration medium.

Fig. 3. Zymogram of ACP isozymes. (ne) non-embryogenic callus; (IIe) embryogenic friable callus; (se) somatic embryos obtained from embryogenic callus on regeneration medium; (Ie) embryogenic compact callus.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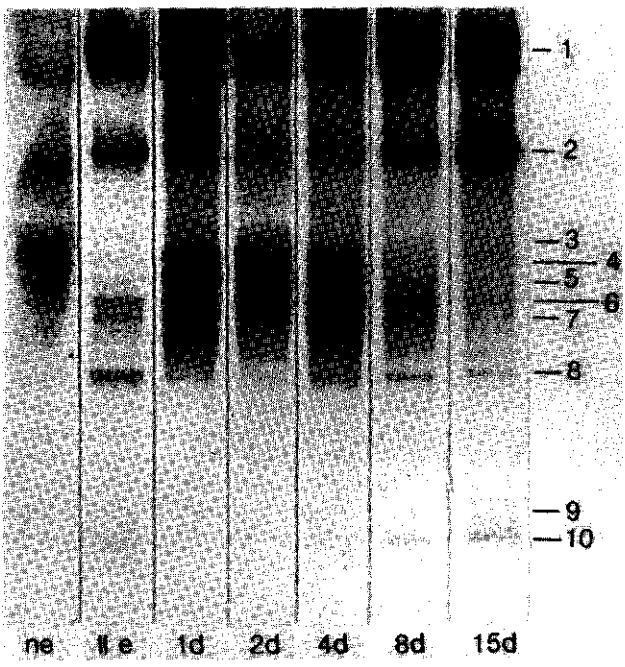
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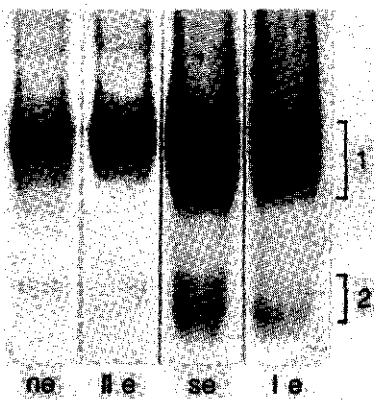
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Peroxidase (PRX)

We found ten different bands in the PRX zymograms of various samples (Fig. 2). Embryogenic and non-embryogenic callus differed in the presence of bands 5 and 7, which were found in embryogenic callus but not in non-embryogenic callus. The other bands differed in intensity; for example, band 1 was prominent in embryogenic callus, whereas the bands 3, 4 and 5 were clearly present in non-embryogenic callus. When embryogenic callus was transferred to regeneration medium without 2,4D, the PRX isozyme pattern changed.

The intensities of bands 1, 2, 8 and 10 at first decreased but increased again after eight days on the regeneration medium. After fifteen days on regeneration medium some of these bands (2 and 10) were even more intense than those of the embryogenic callus before transfer. The bands 3, 4, 5, 6 and 7 showed a reverse pattern: an increase followed by a decrease in intensity. Band 9 was only found in embryogenic callus on regeneration medium and appeared after fifteen days.

Acid phosphatase(ACP)

In the ACP zymograms two zones of enzyme activities were observed, in which no separate bands could be distinguished. All samples showed acid phosphatase activity in zone 1. High intensities were observed in the second zone of somatic embryos and compact callus, whereas friable embryogenic and non-embryogenic callus showed only little activity in this region.

GDH localization

When tissue of friable callus was stained for GDH activity only small isodiametric cells of the embryogenic clumps showed an intense staining, indicating a high GDH activity. On the contrary, non-embryogenic and elongated cells were not or just slightly stained (Fig. 4). Fig. 5 shows a detail of friable callus cells. The difference in GDH activity between the dark stained isodiametric cells and the bean-shaped or elongated callus cells is evident. The reaction for GDH did not stop until the fixative was added and we found an incubation time of 20-30 min sufficient to demonstrate the difference in GDH activity between embryogenic cells and non-embryogenic

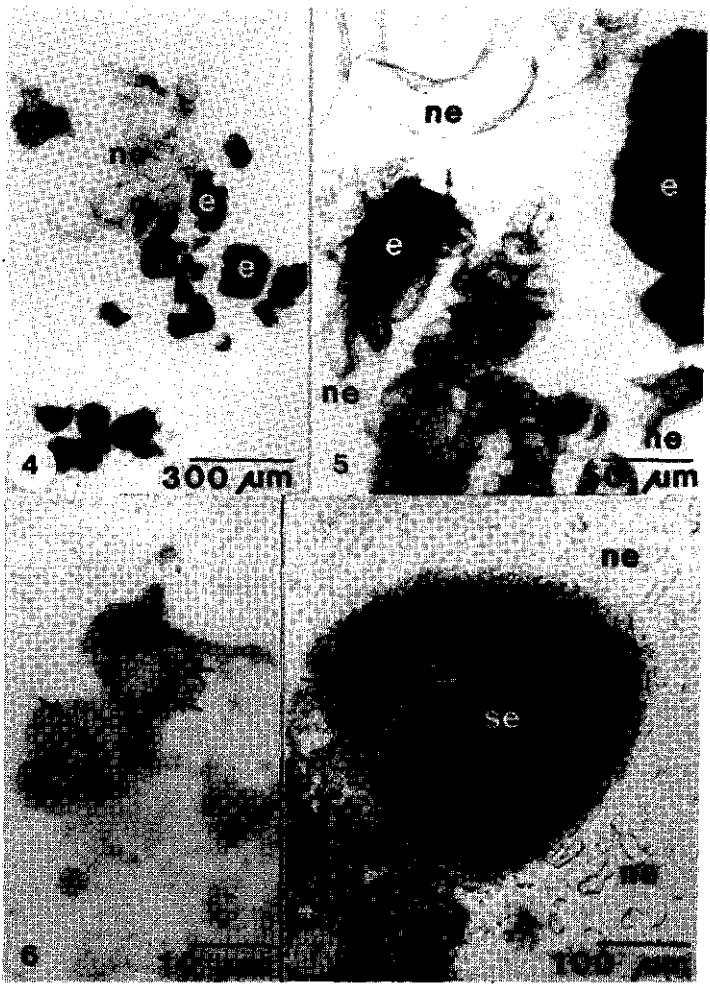

Fig. 4-7. Tissue from friable embryogenic callus stained for GDH activity. (4 and 5) non-embryogenic (ne) and embryogenic (e) cells. Note the difference in GDH activity, (6) high magnification of an embryogenic cell. The GDH activity is located in plastids (arrows) and in the cytoplasm,. (7) an intensively stained somatic embryo (se) surrounded by callus cells (c) and non-embryogenic cells (ne).

cells. The GDH activity is localized in the cytoplasm of the isodiametric cells, and more distinct in plastids (Fig. 6). Fig. 7 clearly shows a darkly stained globular embryoid, surrounded by bean-shaped probably non-embryo-

genic callus cells with very low GDH activity. The callus tissue which is connected with the embryoid also shows some GDH activity. The cytochemical assays for PRX and ACP were positive for both embryogenic and non-embryogenic cells. However, a clear distinction between both cell types could not be made on account of differences in isozyme activity.

Discussion

Everett et al. (1985) found five GDH bands in organogenic callus against seven bands in embryogenic callus of maize. They suggest GDH as a biochemical marker of embryogenesis on account of the similarity in GDH zymograms between embryogenic callus and zygotic embryos and between organogenic callus and shoots. However, they did not compare embryogenic callus with non-embryogenic callus which might have given information about GDH as a marker to distinguish between embryogenic and non-embryogenic callus. Our results confirm the utility to apply GDH as a biochemical marker of embryogenic callus. Not only did we observe differences in isozyme pattern between non-embryogenic and embryogenic callus, but also between compact embryogenic and friable embryogenic callus. Moreover, we found that friable callus after transfer to regeneration medium resembled compact callus with respect to the presence of the slow-migrating GDH isozyme (major band 1). In Chapter 5 we observed that friable callus after transfer became compact in appearance due to the presence of mature somatic embryos. These data suggest a correlation between the slow-migrating GDH isozyme and the compact appearance of the embryogenic callus.

In maize tissue, glutamate dehydrogenase is presumed to be active as a hexamer (see Newton, 1983), the subunits of which are most probably coded by two structural GDH genes (Goodman and Stuber, 1982a, b and c). A seven-banded isozyme pattern would reflect the seven possible combinations between the two types of subunits in a hexamer, i.e. two homohexamers and five heterohexamers. Such a pattern is observed in earlier studies by Sukhorzhevskaya (1978 and 1980) and Everett et al. (1985). Sukhorzhevskaya (1978) suggests, that there is a non-random association of the subunits resulting in a isozyme pattern strongly skewed to either one of the two major bands depending on the tissue. Such a pattern with one darkly stained major band and a spectrum of five bands with decreasing intensity is also present in

both compact and friable embryogenic callus (Fig. 1). This would suggest, that in our results only the homohexamer of the fast migrating GDH isozyme is present in non-embryogenic callus, whereas in embryogenic callus all heterohexamers are synthesized. Compact callus and friable callus apparently differ in the homohexamer-type.

The cytochemical detection of GDH, as presented here, is a useful and fast method to discriminate between embryogenic and non-embryogenic callus cells, because only the small isodiametric cells are intensively stained. It has been suggested, that GDH activity is localized in mitochondria an plastids but it is also observed in the cytosol (Lees and Dennis, 1981; see also Newton, 1983). The presence of GDH activity in plastids and in the cytosol is confirmed by our results. In contrast with the GDH system we consider PRX and ACP not adequate in a cytochemical assay to discriminate between embryogenic and non-embryogenic cells.

In the PRX zymogram we found five bands (1, 2, 8, 9 and 10) that may be useful to discriminate between non-embryogenic and embryogenic callus. Although some of them were also observed in non-embryogenic callus, their intensities were very low. The decline in intensity of some isozyme bands (1 and 8) soon after transfer to regeneration medium followed by an increase after prolonged culture suggests that these isozymes are correlated with the duration of the culture period. The same suggestion applies for bands 3, 4, 5, 6, and 7, which first decreased and subsequently increased in intensity. Peroxidase is proposed to be controlled by auxins (for a review, see Scandalios and Sörensen, 1977). Wochok and Burleson (1974) suggest a regulatory role of 2,4D for PRX activity in carrot cultures. They found a qualitative change in isozyme pattern after omission of 2,4D. In our study some bands (2, 6 and 7) changed in intensity after transfer of embryogenic callus to a 2,4D free medium and subsequent embryoid development. These enzymes are presumably involved with the developmental stage of the tissue, because the callus became more differentiated and organized after transfer to regeneration medium. A similar correlation between specific developmental events, histological changes and isoperoxidase patterns has recently been reported by Kay and Basile (1987) in cultured tobacco explants.

Compact embryogenic callus and non-embryogenic callus varied in ACP isozyme pattern with respect to the intensity of the third zone. These differences are probably correlated with a different morphological appear-

ance, because friable embryogenic callus showed a similar ACP zymogram with non-embryogenic callus, both of which are lower organized as compared to compact callus (Chapter 4). Somatic embryos, which showed ACP activity similar to compact callus, resembled this callus type in organization. Therefore we presume ACP not suitable to discriminate between embryogenic and non-embryogenic callus.

It is impossible to interpret the changes in the zymograms in terms of physiological significance without adequate characterization. The simple method of isozyme detection as presented here does not allow to distinguish different isozymes categories (allelic, non-allelic, conformational, conjugated, etc.). However, our primary interest has been to find an isozyme system as a marker of maize embryogenic callus. On the basis of the data described above, we conclude that GDH and PRX are useful biochemical markers, not only to distinguish between embryogenic and non-embryogenic callus, but also between developmental stages of embryogenic callus cultures. Moreover, we find GDH suitable as a cytochemical marker for embryogenic callus cells.

CHAPTER 7

ULTRASTRUCTURAL STUDIES ON POLLEN EMBRYOGENESIS IN MAIZE (*ZEA MAYS* L.)

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Summary

Maize anthers have been induced on modified N6 medium to produce embryoids. Different stages from the cultures were sampled and prepared for microscopical examination. The microspores at the onset of culture were in an early developmental stage, with the nucleus and numerous organelles centred in the middle, surrounded by many small vacuoles with a lipid content. The binuclear pollen grains contained small vesicles and much starch. The partially condensed vegetative nucleus indicated participation of the vegetative component in the formation of multicellular pollen grains (MPGs). Several MPGs have been observed which differed in morphology. We suggest, on the basis of these ultrastructural observations, that in maize mainly the vegetative cell contributes to the MPG which further develops directly into embryoids.

Introduction

Since the last decade considerable efforts have been made to get haploid plants from anther cultures (for reviews, see e.g. Clapham 1977, Maheswari et al. 1982, Heberle-Bors 1985, Hu and Yang 1986, Raghavan 1986). For maize, results were initially obtained by Chinese groups (401 Research Group 1975, Ku et al. 1978, Kuo 1978, Miao et al., 1978, Ting et al. 1981) but more recently also other laboratories have reported on maize anther culture (Brettell et al. 1981, Genovesi and Collins 1982, Nitsch et al. 1982, Janos 1985, Petolino and Jones 1986, Tsay et al. 1986a).

Because of the strong genotype dependency of the anther response during the in vitro culture of maize it is unfortunately time consuming to find a good embryogenic genotype after testing numerous ones. This might be the reason why much less research was carried out on the ultrastructural aspects of maize pollen embryogenesis, while several publications can be found about the cytochemical and ultrastructural features of the in vitro androgenesis in other species of the Gramineae (Sunderland et al. 1979, Genovesi and Magill 1982, Tsay et al. 1986; for a survey, see Huang 1986). There is obviously a lack of information about maize in this field.

The aim of the present work was to use a relatively highly embryogenic maize genotype (Barnabas et al., in prep.) for ultrastructural studies of pollen embryogenesis and to observe different features of multicellular

pollen grain formation and production of embryoids.

Materials and methods

In vitro anther culture

A field grown maize hybrid (Mv Exp. 2804), supplied by the breeders of the Martonvasar Institute, was used as the experimental material. The plants were raised in the glasshouse during springtime at 20/17 °C for 3 weeks and then at 22/18 °C for the further development of the plants. The anthers containing microspores in the early developmental stage were sampled and cultured at 29 °C in Petri dishes on modified N6 medium with 15% sucrose (Chu 1978) for 30 days in the dark. The medium was supplemented with 25 ppm $Na_2MoO_4.2H_2O$, 25 ppm $CuSo_4.5H_2O$ and 25 ppm $CoCl_2.7H_2O$ (Dr. Jia Xu, personal communication).

Light microscopy and cytochemistry

In general, 10 anthers were randomly chosen from the selected tassels as a control and from the Petri dishes after 6, 8, and 29 days in culture. The anthers were then fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 2 h and postfixed in 1% OsO_4 solution in the same buffer for another 2 h at room temperature. After dehydration they were embedded in Araldite. Semithin and ultrathin sections were then prepared of two anthers selected from each stage, using a LKB Ultramicrotome V. The semithin sections were stained with 0.5% toluidine blue O in 0.1% sodium carbonate. Lipid material was detected with a saturated solution of Sudan Black B in 70% ethanol. Polysaccharides were stained with periodic acid-Schiff's reagent (PAS). At various time intervals 2-3 anthers were stained with acetocarmine and squashed. For all methods, see O'Brien and McCully (1981).

Electron microscopy.

Ultrathin sections were post-stained with uranyl acetate and lead citrate using a LKB Ultrastainer. They were examined with a Philips EM 301 electron microscope at 60 kV.

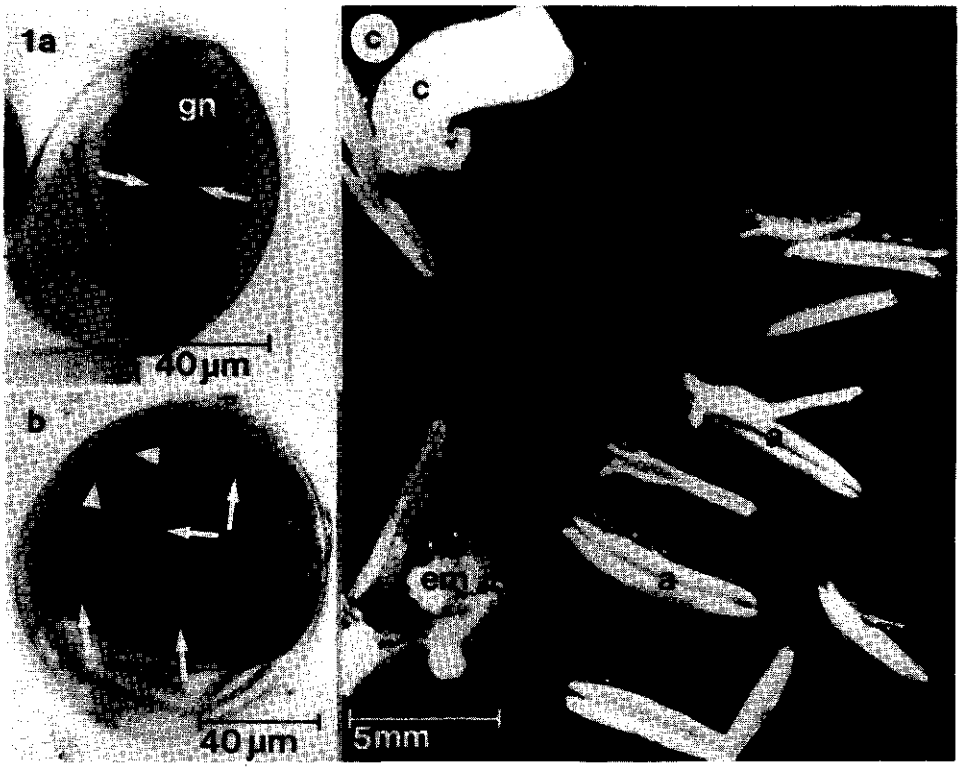


Fig. 1a. Light micrograph of a squash preparation after 6 days in culture, stained with acetocarmine. The generative nucleus is visible (gn); the vegetative nucleus has divided (arrows).
b. The same preparation, but now showing many nuclei (arrows) obviously originated from the vegetative nucleus. The generative nucleus has also divided (arrowheads).
c. Anther (a) cultures after one month showing some callus (c) formation and embryoids (em).

Results

Squash preparations of about 1 week cultures from early microspores showed pollen grains in varying stages of androgenic development. In Fig. 1a a grain is shown in which most likely the vegetative nucleus has divided (arrows) while in Fig. 1b many nuclei are present (arrows) which seem to

have originated from repeated divisions of the vegetative nucleus. The generative nucleus, as deduced from its position, also divided (Fig. 1b, arrowheads). Although, in our opinion, squash preparations are no conclusive evidence because of possible shifts in nuclear positions induced by the mechanical stress, we have used this information to focus the further ultrastructural studies on this stage of culture, i.e. 6-8 days after onset. After one month in culture the anthers gave rise to small clumps of callus and embryoids (Fig. 1c). After prolonged culture small plantlets developed (Barnabas et al., in prep.).

Early microspores

The peak response of pollen embryogenesis in cereals is achieved with microspores before the first mitosis. In general, the so-called mid-uninucleate or early unicellular stage is optimal. This was confirmed for maize by Miao et al. (1978) who found an induction frequency of 7.0% at the mid-uninucleate stage against 4.35% at the late uninucleate stage. We prefer, however, to speak about early microspores because they are, by definition, uninucleate.

An electron micrograph, showing this early stage of a maize microspore is given in Fig. 2a. The nucleus (n) is centred in the middle of the grain. The chromatin is diffuse and often a prominent nucleolus is visible (not shown in Fig. 2a, but see Fig. 2b). Also located in the center, adjacent to the nucleus, is a region with many organelles, a.o. proplastids, mitochondria and dictyosomes (Fig. 2a, arrows). The microspore center is surrounded by many small vacuoles (v) containing electron-dense material. After staining with toluidine blue the vacuoles remained colorless (Fig. 2b) but with Sudan Black they werke darkened (Fig. 2c) indicating the presence of lipid-like components. The exine layer was thick, with channels, while the intine layer was hardly developed.

Bicellular pollen grains

After 8 days in culture some pollen grains were bicellular. The generative cell was situated free in the vegetative cytoplasm which suggests a haploid development. A totally empty collapsed cell wall, however, often indicated

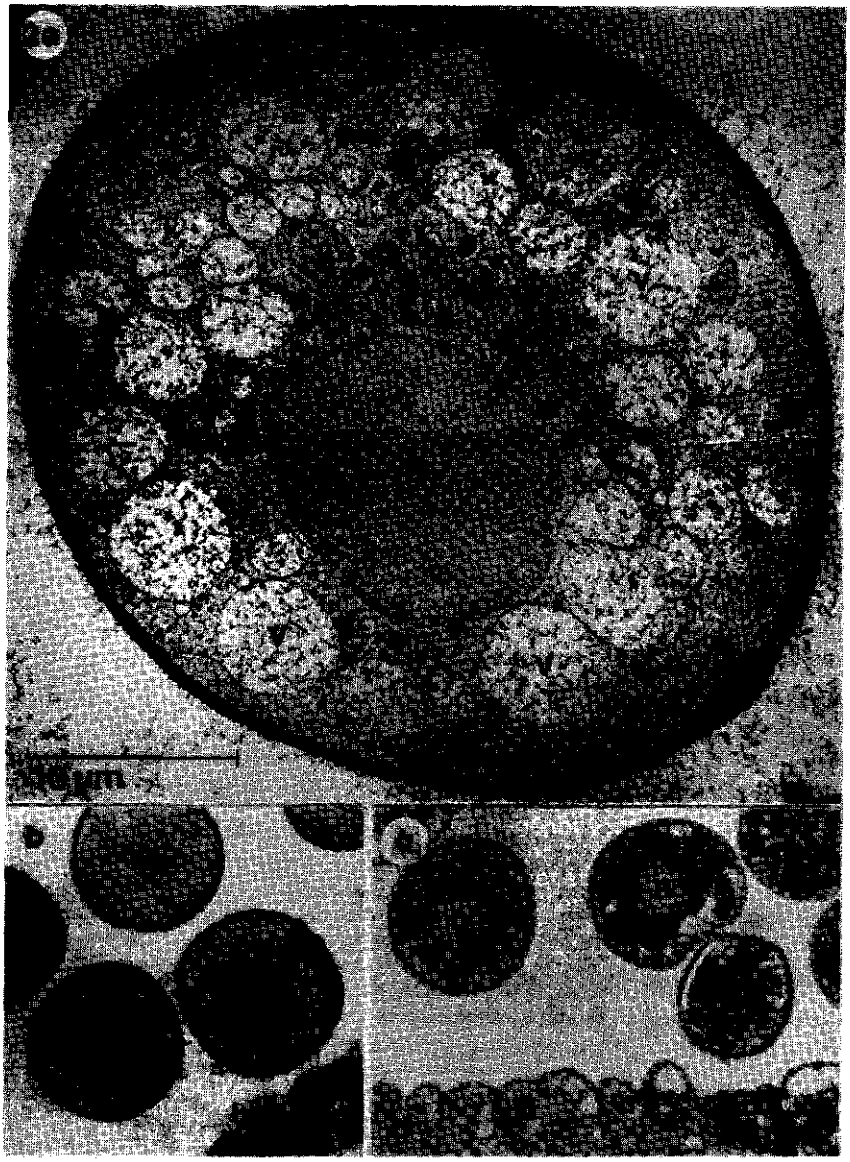


Fig. 2a. Electron micrograph of an early microspore at the onset of culture. Note the central position of the nucleus (n) surrounded by many small vacuoles (v). A region with many organelles is visible (arrows).
b. Light micrograph of an early microspore stained with toluidine blue. Vacuoles are not stained. A distinct nucleolus (arrow) is visible.
c. Same preparation, stained with Sudan Black. Many lipid-like components (li) are visible.

degeneration of these pollen grains. When they remained intact, a situation as shown in Fig. 3a could be observed. The first nuclear division resulted in the formation of a generative (gn) and a vegetative nucleus (vn). The generative nucleus has condensed chromatin with a pronounced nucleolus and is located near the intine (int). The vegetative nucleus is less electron-dense but condensed chromatin is visible indicating that the nucleus is about to divide or just divided. Cytokinesis takes place by invagination of the plasma membrane (arrows). This cell formation is inequal and gives rise to a large vegetative cell and a small generative cell. The vegetative cytoplasm does not differ from the cytoplasm around the generative nucleus. It contains small lipid droplets (li) and many starch grains (st). Mitochondria are hardly observed.

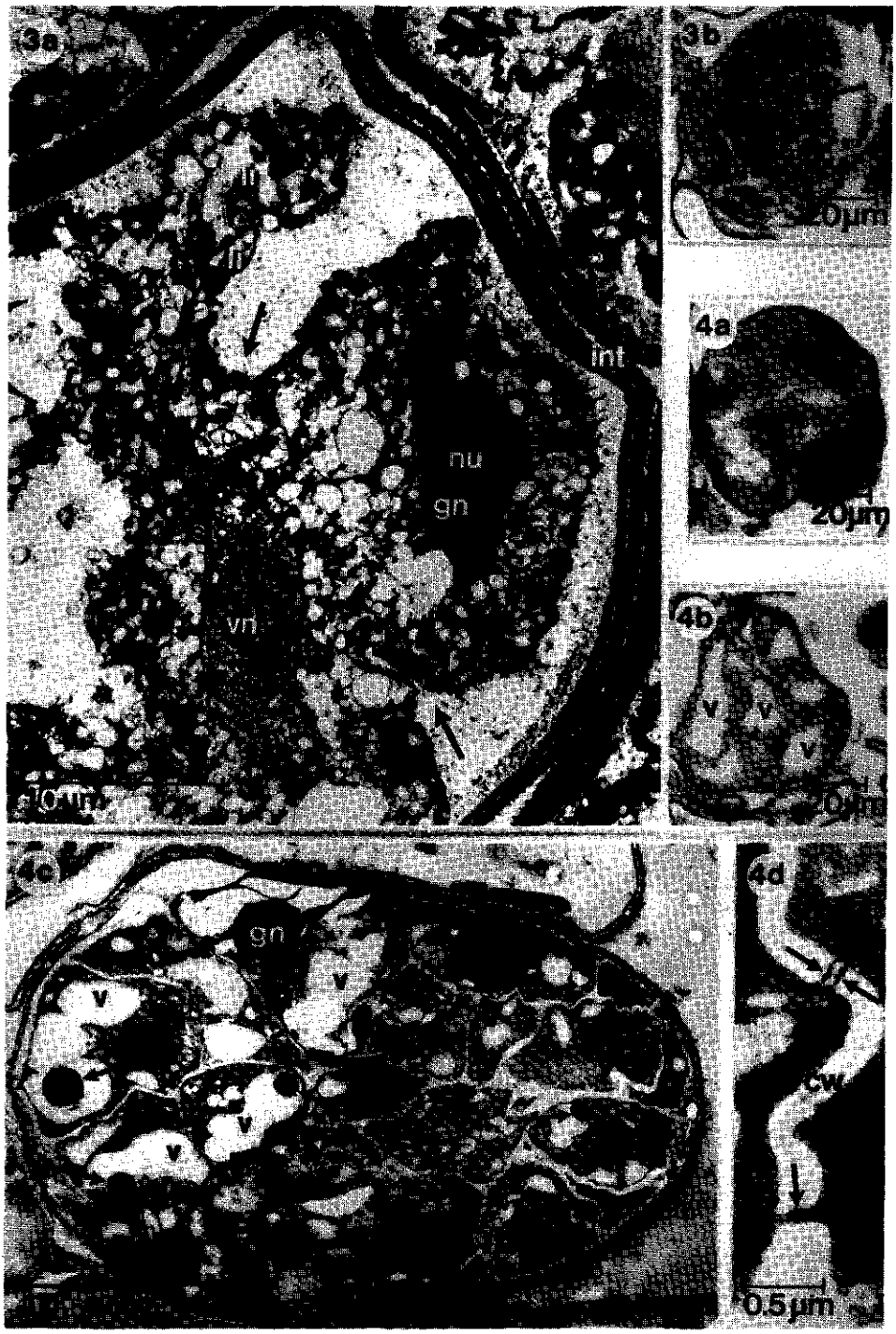
Sections, stained with PAS, showed that 50% of the grains were filled with starch (Fig. 3b, arrows), 38% contained some starch and 12% were starchless. The counted pollen grains were round or nearly round and intact; they formed 37% of the whole population.

Multicellular pollen grains (MPGs)

After about 1 month we observed several kinds of MPGs which all possessed an intact exine layer and complete cell walls. They differed in cell wall morphology, cytoplasmic density and vacuolation. Fig. 4a shows a cross-

Fig. 3a. Electron micrograph of a bicellular pollen grain after 8 days in culture. The chromatin of the generative nucleus (gn) is condensed while the vegetative nucleus shows a pre- or postmitotic configuration. Note the invaginations of the plasma membrane (arrows) and the presence of starch (st) and lipids (li).
b. Light microscopical section, stained with PAS. Many starch grains are present (arrows).

Fig. 4a. en b. Light micrographs showing MPGs with cytoplasmic heterogenecity (Fig. 4a) or with highly vacuolated cells (Fig. 4b). v, vacuole.
c. Electron micrograph of a MPG. Note the polarized orientation of the vacuoles which are sometimes filled with electron-dense deposits (arrows).
d. Higher magnification showing the numerous plasmodesmata (arrows) in the cell walls of the MPG.


3a
li
int
nu
gn
vn
3b
4a
4b
v
4c
gn
v
4d
cw
0.5 µm

section of an MPG. The cells contain thick cell walls and some have darkely stained cytoplasm with many lipids (arrows). The cells are very irregular of shape, sometimes completely pressed together. The MPG in Fig. 4b is characterized by the presence of highly vacuolated cells. It seems to be composed of only one cell type, with few starch and lipid droplets. The electron-density of the cytoplasm is low. Electron microscopy of this type of MPG shows many free ribosomes, few starch and lipid droplets (Fig. 4c). The lobed vacuoles (v) sometimes contain osmiophilic deposits and plasmodesmata are prominent (Fig. 4d, arrows).

Discussion

For maize, four types of pollen plant development from MPGs were described by Miao et al. (1978): 1. MPG mainly derived from the vegetative cell; 2. MPG derived from the reproductive cell; 3. MPG, derived from both the vegetative as well as the reproductive cell; and 4. MPG, derived from the uninucleate pollen without differentiation of the vegetative and reproductive cells. No ultrastructural studies, however, were performed. Our electron microscopical observations support the pathway as described under 1. Differentiation of the vegetative and reproductive cells clearly takes place as was shown in Fig. 3a. After that, the MPG most likely develops from repeated divisions of the vegetative cell, which was indicated already by the vegetative nuclear morphology, showing a pre- or postmitotic configuration. The generative cell seems to have degenerated while the cytoplasm of the multicellular mass is uniform but not heavily stained (Fig. 4c). It cannot be excluded fully, however, that the generative nucleus in Fig. 3a has not yet started mitosis and will contribute later to the formation of the MPG. In that case the microspore follows the embryogenic pathway with partitioned units in which both the vegetative and generative cell participate as observed by light microscopy also for barley by Sunderland et al. (1979).

At the organelle level, the binucleate pollen grain as shown in Fig. 3a conforms to the characteristics of the haploid program as mentioned for barley and wheat by Huang (1986) with regard to the location of the generative and the vegetative cell. Our observations, however, do not support the view that the first sporophytic division (we prefer to call it vegetative

division) takes place in less differentiated cytoplasm with little storage material and a small number of organelles. Particularly much starch was present (Figs. 3a, 3b), which was reported also by Dunwell and Sunderland (1975) for anther cultures of Nicotiana tabacum.

Recently, Tsay et al. (1986b) suggested that in rice starchy microspores might provide a nutritive function during the development of MPGs while the ability of the microspores to synthesize and utilize lipids at the early stages of development might be a decisive factor in switching from gametophytic to vegetative development. Our results support this view (see Fig. 3a) although we think it more likely that lipid accumulation is caused by (programmed) degradation of tapetal or microspore membranes rather than by the synthesis of new lipids.

Membrane degradation and subsequent accumulation of lipids was also observed during the early stages of zygotic embryogenesis in maize as described by Schel et al. (1984). Another feature of normal embryogenesis, the polarity of vacuolation, was also shown in that paper. This polarity of vacuole formation is also distinct during maize pollen embryogenesis as is evident from Fig. 4c. Therefore, it cannot be excluded that also in maize the MPGs develop with a "zygote-like" behaviour as was discussed for barley by Sunderland et al. (1979). The formation of cell walls during early pollen embryogenesis has been reported to occur by centripetal ingrowth of the intine or by cell plate formation (Huang 1986). The latter mechanism can be recognized by the presence of many plasmodesmata. Based on this criterium and considering the numerous plasmodesmata found in the MPG cell walls (Fig. 4d) we think cell plate formation to be the most frequently occurring process in maize.

Acknowledgements

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CHAPTER 8

GENERAL DISCUSSION

Callus initiation

The morphogenetic processes during callus development and early stages of regeneration in cereal tissue culture do not involve drastic cytological changes on balance as compared to the dicotyledonous cultures from mature explants. Callus initiation in cereals takes place in cells which come from an immature tissue (Wernicke and Brettel, 1980). It is, however, likely that some cell determination has already taken place before callus is initiated, because in a too young stage the explant is not able to produce callus (Green and Phillips, 1975, Armstrong and Green, 1985). Probably these young cells are not yet competent to respond to the action of 2,4D. On the other hand, cells in stages that are too old can not produce callus anymore. Older cells are probably no longer sensitive towards 2,4D (Wernicke and Brettell, 1980, 1982). It is demonstrated by Brettell and coworkers, that in grasses only cells that are close to the undifferentiated state are sensitive towards 2,4D. However, the immature state is a primary condition but not useful as a parameter for callus producing capacity (see Chapter 1). The optimum developmental stage has a narrow range and the morphological appearance of the cells during this short period of development does not change much. There are probably no cytological parameters that mark the competence for callus induction.

The scutellum cells of the immature maize embryo have a cytoplasm with a low electron density and relatively few organelles. The scutellum showed mitotic activity in all regions. When the embryos were placed under in vitro culture conditions, all cells became active as noticed by the ultrastructural changes (Chapter 2). However, such changes were not characteristic for cells that are induced to generate callus tissue, because all scutellum cells including those that do not produce callus showed a similar response. The growth regulator 2,4D did not affect these changes. Therefore, we consider this first reaction to the in vitro culture as a shock response, probably induced by the in vitro stress situation.

In cultured explants of dicotyledonous species a first response, or wound response, is described with similar ultrastructural changes as mentioned before (Yeoman and Street, 1977, Barckhausen, 1978). However, these cells are derived from a mature state (e.g. the highly vacuolated parenchyma cells of the artichoke tuber). Thus we find a similarity in response between

the differentiated tuber cells of the dicotyledonous explant and the undifferentiated scutellum cells of the cereal explant. On the other hand, the discrepancy in proliferating activity between both explant types seems to provoke an essential difference in the shock response. In Chapter 3 it is described, that all scutellum cells after a short distortion of the cell cycle recovered mitotic activity within one day of culture while the fraction of DNA sysnthesizing cells increased. The cell division activity and probably also the DNA synthesizing activity at the end of the shock response were not affected by 2,4D. On the contrary, the cells of the tuber explant only become active in proliferation and DNA replication in the presence of 2,4D (Yeoman and Mitchell, 1970). The situation may be explained as follows. As soon as the explant is placed under in vitro culture conditions, a range of mechanisms is set up in order to adapt to the changed environment involving polypeptide synthesis and production of cell organelles. As a consequence other cell processes that are stress-sensitive are disturbed. This is detectable in the scutellum tissue, when after the onset of culture cell division is no longer observed, which implicates a distortion of the cell cycle. In the storage tissue of the artichoke tuber such a clear disturbance of a stress-sensitive cell activity is not yet shown. In the scutellum the cells recover from the in vitro shock within one day and cell divisions are observed. It is this recovery which is not affected by 2,4D. We therefore propose that during the shock response similar processes (e.g. resulting in ultrastructural changes) take place in the undifferentiated scutellum cells and in the differentiated tuber cells, while stress sensitive processes, which may be cell-specific, are disturbed (e.g. those involved in the cell cycle).

A second reaction during callus initiation takes place after the shock response and is called a growth response. This growth response is characterized by mitotic activity in cells that are sensitive towards 2,4D. Proliferation takes place in the presence of 2,4D and results in callus formation.

Callus development

Callus is produced by cells of the middle and basal scutellum. Depending on the origin site embryogenic or non-embryogenic callus develops. Meristematic

cells located in the abaxial scutellum, proliferate and form embryogenic complexes such as a shoot apex, the shoot apical part of a somatic embryo, a globular embryoid or embryogenic callus. Adaxial cells near the nodal region or the coleorhiza produce non-embryogenic callus, roots or root-like structures (Springer et al., 1979, Vasil et al., 1985, this thesis Chapters 2 and 4). Site-specific development is also reported in leaf cultures of Sorghum bicolor (Wernicke and Brettell, 1980). However, in this case the ability to regenerate depended on the distance from the apical meristem which is closely related with the differentiation state. It is suggested, that in maize embryos the site-specific callus development reflects the polarity of the scutellum (Chapter 3). However, the capacity to regenerate is controlled by nuclear genes (Hodges et al., 1985, Tomes, 1985b). This implies that the expression of these genes becomes manifest in the epidermal and subepidermal region of the middle and basal scutellum in 1.5 mm embryos under appropriate culture conditions. This is supported by the fact, that in the inbred line A632, only non-embryogenic callus is produced close to the coleorhiza, while cells of the abaxial scutellum do not proliferate.

Embryogenic callus

Cereal plant regeneration from immature embryos may follow four pathways depending on the culture conditions (see Chapter 1). As regard to the indirect somatic embryogenesis, two embryogenic callus types are distinguished: compact callus, or type 1, and friable callus, or type II. Both originate from the abaxial region of the middle and basal scutellum (Chapters 2 and 4). However, compact callus is derived from a broad meristematic zone having a future shoot apical part with embryogenic cells and a future root apical part. In contrast, friable callus originates only from a narrow superficial meristematic zone consisting of embryogenic cells without any indications of differentiation. It is suggested, that in the broad meristematic region the coordinated development of embryogenic cells and future root cells results in normal somatic embryos or compact callus consisting of deformed or fused somatic embryo structures with an incomplete shoot-root axis. This might explain the often reported observation of normal and abnormal embryoids with only a shoot apex, between which the transition appeared gradual (Maddock, 1985; Wernicke and Milkovits, 1986). In the case of the

narrow superficial meristematic region only the embryogenic cells remain while the adaxial "presumptive root" cells degenerate. The embryogenic cells proliferate to give rise to friable embryogenic callus and somatic embryos.

Somatic embryogenesis

Somatic embryogenesis starts from a single embryogenic cell or from a group of embryogenic cells. During androgenesis the microspore origin is evident after determining the haploidy of the tissue. For early androgenetic stages the presence of an exine layer also clearly indicates a microspore origin (Chapter 7). In contrast, the unicellular origin of embryoids from somatic cells is more difficult to demonstrate. Even in suspension cultures the development of a single cell is not easy to follow (Karlsson and Vasil, 1986). In this thesis we have evidence that somatic embryos arise from a broad pro-embryogenic cell mass in the scutellum (Chapters 2 and 3), from single cells in the scutellum (Chapter 4), from a pro-embryogenic cell aggregate, the embryogenic unit in friable callus (Chapters 4 and 5) and from an early microspore (Chapter 7). The reason why embryoids develop from a single cell or from a group of cells is unclear. It is also unclear why some cells directly develop into embryoids, whereas other cells first undergo an intermediate callus phase. The different pathways of regeneration from the same explant tempt us to generalize the process of somatic embryogenesis and to class the different pathways in one unified scheme.

Figure 1 shows such a hypothetical scheme which in broad outline is adopted from Williams and Maheswaran (1986). In this concept it is assumed, that the regenerative capacity is genetically controlled. The expression of these genes becomes manifest in the EDCs, which need an environmental stimulus (e.g. 2,4D) to divide and to produce the proembryogenic complex of EDCs. Undser conditions that are permissive for regeneration a stimulus for somatic embryogenesis effects the EDCs to develop into somatic embryos. This stimulus is a main event in all pathways of regeneration. The nature of this stimulus is undefined. In Chapter 5 some factors are discussed, that might play a role during the activation and further establishment of somatic embryogenesis. These factors comprise (i) chemical components (ii) physical isolation and (iii) polarity. The hypothesis implies no differences in origin between indirect and direct somatic embryogenesis as suggested by

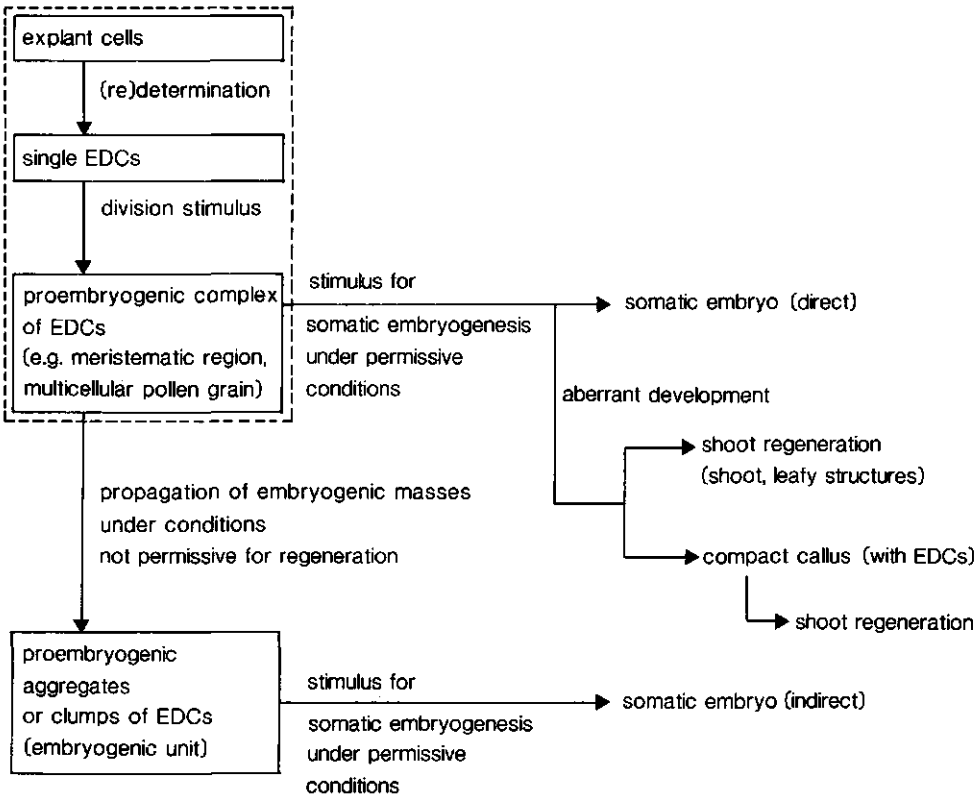

Fig. 1. Schematic synthesis of process to occur in somatic embryogenesis in broad outline adopted from Williams and Maheswaran (1986). The development of embryogenically determined cells (EDC) and subsequent proliferation take place in the explant which is indicated with a dashed line. Further proliferation in the pro-embryogenic complex by the embryogenically determined cells (EDCs) results in somatic embryo development or embryogenic callus.

Sharp et al. (1980). They proposed, that direct somatic embryogenesis proceeds from pre-embryogenic determined cells (PEDC), whereas indirect somatic embryogenesis requires the redetermination of differentiated cells, callus proliferation and differentiation of embryogenic determined cells (IEDC). According to our hypothesis the degree of organization of the pro-embryo-

genic complex or aggregates, to which the stimulus for somatic embryogenesis is given, determines the unicellular or multicellular origin of an embryoid. The decision of an embryogenically determined cell or group of cells to go through a callus phase depends on the time at which a stimulus for somatic embryogenesis is given. During the early period of tissue culture, when the explant is more or less intact, the stimulus will induce direct somatic embryogenesis. When the conditions are not permissive for somatic embryogenesis the embryogenically determined cells (EDCs) proliferate and produce embryogenic callus. After stimulation the EDCs turn from an unorganized to an organized structure with a coordinated growth which results in somatic embryogenesis.

SUMMARY

This thesis deals with cytomorphogenic aspects during various phases of regeneration in tissue cultures of Zea mays L. Regeneration through tissue culture has been shown in an increasing number of plant species and is applied on large scale in agriculture and horticulture. Nevertheless, several fundamental scientific data are lacking, which hampers further development of tissue culture, particularly where it concerns tissue culture of monocotyledonous crops. For example, callus initiation and somatic embryogenesis are important processes during plant regeneration, which can be induced and controlled increasingly with success. However, basic knowledge with regard to these processes is often confined to dicotyledonous crops. In particular, information is lacking about structural changes at the cellular and subcellular level. Research on callus initiation and somatic embryogenesis in maize by structure analysis at the light and electron microscopical level therefore can lead to a better understanding of in vitro cultures, surely for maize, but possibly for monocotyledons in general.

Chapter 1 gives a survey of the present information on callus initiation, callus development and somatic embryogenesis. At the end of this Chapter the aim of the thesis is described.

In Chapter 2 callus initiation is described based on the results obtained from a light and electron microscopical study. A comparison is made between two inbred lines, A188 and A632, which differ in culture resonse. The initiation of callus in immature embryos has come about within three days after the onset of culture. The first day of culture is characterized by an increase in the number of cell organelles, changes in vacuolation and in nucleolar morphology. These ultrastructural changes take place in embryos of both genotypes and are not affected by 2,4D. After the first day of culture changes occur, dependent on genotype and culture conditions. In embryos of the A188 line a broad meristematic zone develops in the middle and basal scutellum under the influence of 2,4D. From this region somatic embryos and embryogenic compact callus arise. A second meristematic region is observed around the coleorhiza. In embryos of the A632 line callus formation occurs only around the coleorhiza. This callus tissue is non-embryogenic and not capable to regenerate. In the period of callus initiation a shock response can be discerned from a growth response. The shock response

takes place in the whole scutellum of both genotypes during the first day of culture and is not affected by 2,4D. On the other hand, the growth response is a local event, after the first day of culture under the influence of 2,4D.

In Chapter 3 the period of callus initiation is studied using autoradiography. Special attention was paid to the fraction of cells with DNA synthesizing activity and to the mitotic index in different scutellum regions. It appears that the shock response and the growth response also become manifest during the cell cycle. Moreover, the variation in culture response between the different regions of the scutellum is also expressed in the course of the cell cycle. We observed, that cells in the top of the scutellum terminate their DNA synthesizing and mitotic activity during the growth response, while cells in the middle and basis clearly retain these activities. The results further suggest that scutellum cells preferentially arrest in the G2 phase of the cell cycle during the first eight hours of culture.

In the Chapters 4 and 5 the development of the embryoids via an embryogenic callus phase is described for the inbred line A188. Compact callus develops from the embryo when cultured on MS medium with 6% sucrose, whereas friable callus arises on N6 medium with 2% sucrose. Both callus types originate from the abaxial region of the scutellum. After a few days on N6 medium the scutellum is transformed into an almost translucent and soft callus tissue with a fragile appearance. This friable callus is composed of long aggregates of large vacuolated cells and embryogenic units, groups of small isodiametric cells. The cell aggregates are surrounded by large intercellular spaces, which gives the callus its fragile appearance. This friability is caused by cell dissociation between embryogenic cells as well as non-embryogenic cells, cell proliferation in the embryogenic unit and elongation and degeneration of the non-embryogenic cells. No vascular tissue is observed in friable callus. Transport of nutrients probably takes place through the intercellular spaces by diffusion. Compact callus is more organized; it contains vascular bundles around a centre of parenchyma cells, while intercellular spaces are lacking.

Somatic embryogenesis in friable callus cultures starts with the transition of an unorganized embryogenic unit to an organized structure of embryogenic cells with a coordinated growth. The transition unit is regarded

as an intermediate stage between the embryogenic unit and the somatic embryo. It is a globular structure with a distinct morphological difference between the apical cells and the basal cells that are connected with the callus. In this transition unit a polarity is established, which possibly leads to a gradient of hormones and nutrients. This polarity contributes to an important extend to the activation of somatic embryogenesis. The appearance of large numbers of microtubules in somatic embryos confirms the transition to a coordinated growth. The early somatic embryogenesis strongly resembles the zygotic embryo development with respect to shape and structure. The inability of the embryoid to develop beyond the late globular stage in the presence of 2,4D is probably due to the repressive effect of the auxin on the growth of the shoot apex.

Chapter 6 shows the results of the study on the utility of isozymes as biochemical and cytochemical markers in tissue cultures of maize. The zymograms of glutamate dehydrogenase (GDH), peroxidase (PRX) and acid phosphatase (ACP) were analysed in different callus cultures. Variation was demonstrated in the isozyme patterns of GDH and PRX between embryogenic and non-embryogenic callus. Some isozyme bands appeared to correlate with the degree of organization, while the activity of other isozymes was dependent on the length of the culture period. When the same enzyme assays were carried out in situ on fresh callus samples, it appeared that only a clear discrimination in enzyme activity between embryogenic and non-embryogenic was possible with the GDH assay. Therefore, GDH is regarded as an useful biochemical and cytochemical marker in embryogenic callus of maize.

In Chapter 7 it is demonstrated, that during pollen embryogenesis in maize, early microspores develop in multicellular pollen grains which consist of cells that differ in vacuolation. During the development repeated divisions especially take place in the vegetative cell. Although participation of the generative cell to the formation of the multicellular pollen grain can not be excluded, it is assumed, that in our experiment the multicellular pollen grain is mainly derived from the vegetative cell.

In Chapter 8 the information, obtained from the Chapters 1 to 7, is summarized in the subjects callus initiation, callus development and somatic embryogenesis and provided with comments. At the end a hypothesis is formed, in which the different ways of regeneration are classed in one scheme. In this concept it is assumed that the regenerative capacity is genetically

controlled and the expression of the genes becomes manifest under permissive conditions.

SAMENVATTING

Dit proefschrift behandelt cytomorfogenetische aspecten tijdens de verschillende fasen van regeneratie vanuit weefselcultures van Zea mays L. Regeneratie via weefselkweek is aangetoond bij een toenemend aantal plantesoorten en wordt in de land- en tuinbouw op grote schaal toegepast. Desondanks ontbreken talrijke gegevens van fundamenteel-wetenschappelijke aard, die een verdere ontwikkeling binnen de weefselkweek belemmeren, met name waar het weefselcultures van monocotyle gewassen betreft. Bijvoorbeeld, callusinitiatie en somatische embryogenese zijn belangrijke processen tijdens de plantregeneratie, welke men in toenemende mate met succes kan induceren en controleren. Echter, fundamentele kennis omtrent deze processen is veelal beperkt tot dicotyle gewassen. Er is met name een schaarste aan informatie over structuurveranderingen op cellulair en subcellulair niveau. Onderzoek naar callusinitiatie en somatische embryogenese bij mais d.m.v. structuuranalyse op licht- en electronenmicroscopisch niveau kan derhalve leiden tot een beter begrip van in vitro cultures, zeker van mais, maar wellicht van monocotylen in het algemeen.

Hoofdstuk 1 geeft een overzicht van de aanwezige informatie over callusinitiatie, callusontwikkeling en somatische embryogenese. Aan het eind van het hoofdstuk wordt de doelstelling van dit proefschrift beschreven.

In Hoofdstuk 2 wordt de callusinitatie beschreven aan de hand van resultaten verkregen uit een licht- en elektronenmicroscopisch onderzoek. Hierbij is een vergelijking gemaakt tussen twee inteeltlijnen, A188 en A632, die een verschillende kweekrespons vertonen. De initiatie van callus bij onrijpe maisembryo's voltrekt zich binnen drie dagen vanaf het begin van de kweek. De eerste kweekdag wordt gekenmerkt door een toename van het aantal celorganellen, veranderingen in vacuolisatie en in morfologie van de nucleolus. Deze ultrastructurele veranderingen vinden plaats in embryo's van beide genotypen en worden niet beinvloed door de aanwezigheid van 2,4D. Na de eerste kweekdag vindt een verdere ontwikkeling plaats, die afhankelijk is van genotype en kweekcondities. In embryo's van de lijn A188 ontwikkelt zich onder invloed van 2,4D na twee dagen een brede meristematische zone in het midden en basale deel van het scutellum. Vanuit deze zone ontstaan somatische embryo's en embryogeen compact callus. Een tweede meristematisch gebied is waargenomen rond de coleorhiza. In embryo's van lijn A632 vindt

callusvorming alleen plaats rond de coleorhiza. Dit callusweefsel is niet embryogeen en derhalve niet in staat tot regeneratie. In de gehele periode van callusinitiatie kan een onderscheid gemaakt worden tussen een zogenaamde "shock"respons en een "groei"respons. De shockrespons vindt plaats in het gehele scutellum van beide genotypen gedurende de eerste kweekdag en wordt niet beinvloed door 2,4D. De groeirespons, daarentegen, treedt lokaal op vanaf de eerste kweekdag onder invloed van 2,4D.

In Hoofdstuk 3 wordt de periode van callusinitiatie onderzocht m.b.v. autoradiografie. Speciale aandacht werd besteed aan het percentage cellen met DNA synthese activiteit en aan de mitotische index in verschillende gebieden van het scutellum. Het blijkt dat de shockrespons en de groeirespons zich ook gedurende de celcyclus manifesteren. Bovendien komt ook de variatie tussen de verschillende gebieden van het scutellum m.b.t. de kweekrespons tot uitdrukking in het verloop van de celcyclus. Zo is waargenomen, dat de meeste cellen in de top van het scutellum hun DNA-synthese- en celdelingsactiviteit beëindigen tijdens de groeirespons, terwijl cellen in het midden en de basis duidelijk deze activiteiten behouden. De resultaten geven verder aan dat de scutellum cellen met name in de G1-fase verkeren tijdens de eerste acht uur van de kweek.

In de Hoofdstukken 4 en 5 wordt de ontwikkeling van embryoiden via een embryogene callusfase beschreven voor inteeltlijn A188. Afhankelijk van het medium vindt vanuit het embryo de vorming van 'compact' callus (op MS medium met 6% sucrose) of 'friable' (lett. brokkelig, broos) callus (op N6 medium met 2% sucrose) plaats. Beide callustypen ontstaan in het abaxiale gebied van het scutellum. Na enkele dagen op N6 medium is het scutellum getransformeerd in een half-doorschijnend en zacht callusweefsel met een broos voorkomen. Dit 'friable' callus is opgebouwd uit lange aggregaten van grote gevacuoliseerde cellen en embryogene eenheden, groepjes van kleine isodiametrische cellen. De celaggregaten zijn omgeven door grote intercellulaire ruimtes, hetgeen dit callus het broos voorkomen geeft. Deze broosheid wordt veroorzaakt door celdissociatie tussen zowel embryogene als niet-embryogene cellen, celdeling in de embryogene eenheden en strekking en degeneratie van de niet-embryogene cellen. In 'friable' callus is geen vaatweefsel waargenomen. Transport van voedingsstoffen vindt waarschijnlijk plaats via de intercellulaire ruimtes middels diffusie. Compact callus is meer georganiseerd; het bevat vaatbundels rond een centrum van parenchymweefsel, terwijl inter-

cellulaire ruimtes ontbreken.

De somatische embryogenese in 'friable' callus cultures begint met de overgang van ongeorganiseerde embryogene eenheid naar een georganiseerde structuur van embryogene cellen met een gecoördineerde groei. De transitie-eenheid wordt beschouwd als een tussenstadium tussen embryogene eenheid en somatisch embryo. Het is een globulaire structuur met een duidelijk morfologisch verschil tussen de apicale cellen en de basale cellen, welke verbonden zijn met het callus. In deze transitie-eenheid komt een polarisatie tot stand, die mogelijk aanleiding geeft tot een gradiënt van hormonen en voedingsstoffen. Deze polariteit draagt in belangrijke mate bij aan de activatie van de somatische embryogenese. Het verschijnen van grote aantallen microtubuli in somatische embryo's bevestigt de overgang naar een gecoördineerde groei. De vroege somatische embryogenese vertoont sterke overeenkomsten met de zygotische embryo-ontwikkeling wat betreft vorm en structuur. Het feit, dat op medium met 2,4D het embryoid zich niet verder ontwikkelt dan het late globulaire stadium is waarschijnlijk te wijten aan de remmende werking van het auxine op de groei van de spruittop.

In Hoofdstuk 6 zijn de resultaten weergegeven van het onderzoek naar de mogelijkheid om isozymen te gebruiken als biochemische en cytochemische markers in weefselcultures van mais. De zymogrammen van glutamaat dehydrogenase (GDH), peroxidase (PRX) en zure fosfatase (ACP) werden geanalyseerd voor verschillende callus cultures. In de isozympatronen van GDH en PRX konden duidelijke verschillen worden aangetoond tussen embryogeen en niet-embryogeen callus. Sommige isozymbandjes bleken te correleren met de organisatiegraad van het callus, terwijl de activiteit van andere isozymen afhankelijk was van de duur van een kweekperiode. Wanneer dezelfde enzymtesten in situ werden uitgevoerd op vers callus materiaal bleek alleen met de GDH-test een duidelijk verschil in enzymactiviteit aantoonbaar tussen embryogene en niet-embryogene cellen. Daarom wordt GDH als een bruikbare biochemische en cytochemische marker beschouwd in embryogeen callus van mais.

In Hoofdstuk 7 wordt aangetoond, dat tijdens de pollen embryogenese in mais, vroege microsporen zich ontwikkelen tot multicellulaire pollenkorrels bestaande uit cellen met een verschillende vacuolisatiegraad. Tijdens deze ontwikkeling vinden herhaalde delingen vooral plaats in de vegetatieve cel. Hoewel deelname van de generatieve kern aan de vorming van de multicellulaire pollenkorrel niet kan worden uitgesloten, wordt aangenomen, dat in ons

experiment de multicellulaire pollenkorrel hoofdzakelijk afkomstig is van de vegetatieve cel.

In Hoofdstuk 8 wordt de informatie, verkregen uit de Hoofdstukken 1 t/m 7, ondergebracht in de thema's callusinitiatie, callusontwikkeling en somatische embryogenese en van commentaar voorzien. Aan het eind is een hypothese opgesteld, waarin de verschillende wegen van planteregeneratie binnen een schema zijn ondergebracht. Daarbij wordt ervan uitgegaan, dat het regeneratievermogen genetisch is vastgelegd en dat deze genen tot expressie komen wanneer de omstandigheden dat toelaten.

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CURRICULUM VITAE

Paul Frederic Fransz werd geboren op 23 mei 1958 te Oldebroek. In 1976 behaalde hij het diploma Atheneum B aan het Carolus Clusius College te Zwolle, waarna hij begon met de studie biologie aan de Landbouwuniversiteit in Wageningen. De doctoraalstudie omvatte de hoofdvakken Celbiologie en Erfelijkheidsleer en het bijvak Moleculaire biologie. De praktijktijd bracht hij door op het Hubrecht Laboratorium te Utrecht. In januari 1985 behaalde hij het ingenieurs-diploma Biologie.

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