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REVIEW

Somatic embryogenesis: cell biological aspects

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INTRODUCTION

Somatic embryogenesis is the development from somatic cells, through an orderly series of characteristic morphological stages, of structures that resemble zygotic embryos. Structures of somatic origin resembling embryos may form spontaneously on, for instance, leaf tips of *Malaxis* (Taylor 1967; for review see Vasil & Vasil 1980). Under experimental conditions somatic cells in tissue culture may enter a developmental pathway resembling that of zygotic embryos in seeds. The mature zygotic embryo typically contains the basic organs of the vegetative plant and one or two storage organs, the cotyledons. Normally it enters a period of metabolic quiescence and developmental arrest before germination, growth and the production of additional tissues and organs of the plant body take place.

Walbot (1968) described zygotic embryo development as five consecutive developmental processes.

(1) Cell division with little growth, and differentiation of all major tissues and organs: embryo specification.
(2) Rapid cell expansion and division: embryo growth.
(3) Little or no cell division and expansion, synthesis of storage molecules: embryo maturation.

Abbreviations: PEM, pro-embryogenic mass; 2,4-D, 2,4-dichlorophenoxy acetic acid; PPB, preprophase band of microtubules; ABA, abscisic acid.

(4) Developmental arrest: embryo dormancy.
(5) Renewed cell expansion and division: embryo germination.

In this review the first three steps of the development of somatic, asexual embryos will be described, up to the cotyledon stage, with emphasis on cell biological aspects of the cytoskeleton, the cell wall, the acquirement of polarity and the development of cotyledons. Somatic embryogenesis in *Daucus carota* L., the dicotyledonous model system, and *Zea mays* L., a monocotyledonous crop plant will be highlighted. The characteristic developmental stages of dicot embryogenesis are the globular, heart and torpedo (=cotyledon) stages. These stages for maize embryogenesis include globular and club-shaped or ovoid stages followed by development of a bipolar embryo axis attached to the scutellum, the embryo's storage organ, the cotyledon-stage.

Somatic embryos can develop directly on an explant from organized tissue, from isolated protoplasts or from microspores, or indirectly from callus aggregates or suspension cells (Williams & Maheswaran 1986). Somatic embryos can develop as single structures in suspension culture, or attached to each other via callus tissue in liquid culture or on agar-solidified medium. In this paper somatic embryogenesis is defined as the development from somatic cells of structures that follow a histodifferentiation pattern which leads to a body pattern resembling that of zygotic embryos. This definition includes the formation of patterns of lethal and precociously germinating zygotic mutants (maize: Clark & Sheridan 1991; Sheridan & Clark 1993). It does not include organogenesis: the development of shoots and (or) roots from callus aggregates with callus tissue between shoot and root meristems and without the formation of an embryo storage organ (see for discussion: Van der Valk *et al.* 1989; Emons & De Does 1993). Reviews on somatic embryogenesis include: Ammirato (1983), Williams & Maheswaran (1986), Komamine *et al.* (1990), and De Jong *et al.* (1993). The present review compares a monocotyledonous and a dicotyledonous system and includes the maturation phase, which for many crop plants is essential for successful germination.

The somatic cells that give rise to embryos are called embryogenic cells. Only a limited number of cells of explants, protoplasts, microspores, callus aggregates and suspension cells in fact eventually give structures that exhibit embryo development. Those cells are commonly called competent (to embryogenesis) cells. Whether these competent cells will indeed express their embryogenic character, depends on the tissue culture environment, such as hormone balance, osmotic condition, sucrose, amino acid and salts concentrations (Armstrong & Green 1985; Rhodes *et al.* 1986). The percentage of cells that actually gives rise to somatic embryos in the successful carrot suspension culture system is not more than 2% (De Jong *et al.* 1993). The methods of finding the right conditions for somatic embryogenesis are as yet for the greater part based on trial and error experiments. Nevertheless, successive developmental steps appear to require some general conditions: (i) dedifferentiation of cells in the explant, in most instances, can be achieved by the application of high auxin concentrations; (ii) initiation of embryo development from a globular stage onwards can be achieved by a drastic reduction of the auxin concentration; (iii) embryo maturation in species with well-developed embryo storage organs can be achieved by the application of a maturation medium with high sucrose concentration; (iv) prevention of germination before embryo maturation can be achieved by using, among others, media with high osmolality and special hormone conditions including abscisic acid (ABA).

DEDIFFERENTIATION OF EXPLANT CELLS

Dedifferentiation of plant cells is defined as reinitiation of cell division. Starting from an intact plant part, the explant, a high concentration of an auxin-like substance, such as 2,4-dichlorophenoxy acetic acid (2,4-D), is generally needed to reinitiate cell divisions. Depending on the time during which this synthetic growth regulator is applied, the somatic embryogenesis process is direct (short time of auxin application, no subculturing), or indirect (long time of auxin application, subculturing of calli on fresh media or pro-embryogenic masses (PEMs) in liquid media). In the latter process cells continually proliferate: they successively divide and grow, but do not differentiate into the organs of the plant body; they form secondary pro-embryos. Calli may be held in this condition for years by repeated subculturing, though their embryogenic potential generally decreases in time. These proliferating calli or suspensions have often been described as 'undifferentiated', which is, as De Jong *et al.* (1993) correctly point out a confusing sort of terminology, because in embryogenic callus and suspension cultures subpopulations of morphologically (Emons *et al.* 1992) and biochemically (Pennell *et al.* 1992) different cells occur. De Jong and co-workers (1993) introduced the term 'unorganized'. However, in embryogenic calli and PEMs in suspension cultures there are always several types of 'organized' cells (see next paragraph). In fact the very young pre-globular somatic embryo is already present in those cultures, both in dicots and in monocots (Emons *et al.* 1992).

Auxins stimulate cell division, but also disrupt formerly adhering cells from each other by loosening hemicelluloses from the cellulose microfibrils (Hayashi 1991), and promote cell elongation by vacuole expansion and formation of microtubules transverse to the elongation direction (Shibaoka 1991). As long as, in spite of auxin treatment, highly cytoplasmic cells remain present and their daughter cells adhere to each other, there are cells which are competent to divide and to form multicellular structures, two prerequisites for embryo development. If the auxin concentration is too high or subculturing is done too often, the small and highly cytoplasmic, 'embryogenic', cells disappear from the culture, because further disruption and elongation of cells takes place. Arabinogalactan proteins are able to promote the formation of PEMs in previously non-embryogenic cell lines of carrot (Kreuger & Van Holst 1993). These are the type of proteins that are developmentally regulated and might play a role in cell-to-cell adhesion (Knox *et al.* 1991). Also, a range of molecular links such as exhibited by several pectins in the middle lamella of cell walls serve a role in plant cell adhesion (Knox 1992).

Auxins are not the only substances able to reinitiate cell division and thus mediate the transition from somatic cells to cells able to form embryo-like structures. Molecules totally different from conventional plant growth regulators can also direct this transition. In carrot, somatic embryogenesis was achieved without hormones, e.g. by pH changes (Smith & Krikorian 1990); in citrus a change in carbon source was sufficient to induce somatic embryos (Gavish *et al.* 1991); microspores need a temperature shock to induce somatic embryogenesis (Pechan & Keller 1988) and the developmental stage of the microspores at the time of treatment is highly involved in determining the frequency of embryo formation (Telmer *et al.* 1992).

Because reinitiation of cell division is the essence of the plant cell dedifferentiation process, key steps involved concern the cell cycle and the cytoskeleton. One of the important events in the induction of the embryogenic pathway in tobacco pollen

embryogenesis is the derepression of G_1 arrest in the cell cycle of the vegetative cell, which is caused by starvation (Zarsky *et al.* 1992). MAP (mitogen activated protein) kinases, a family of protein kinases, are involved in re-entry in the cell cycle at G0/G1 and in entry into mitosis. Animal cells, yeast, but also plant cells (Jonak *et al.* 1993) contain members of this gene family. Protein kinases are characterized by their ability to phosphorylate microtubule-associated protein 2 (MAP2). In *Medicago sativa* cells, MAP-kinases are expressed in a cell-cycle-dependent and organ-specific manner (Jonak *et al.* 1993). MAP kinases belong to the superfamily of *cdc*2 kinases, genes involved in cell division. A central component in the regulation mechanism of cell division in eukaryotic cells is a 34-kD protein encoded by the *cdc*2 gene. This protein mediates cell division and DNA replication (review: Nurse 1989), such as nuclear envelope breakdown, chromosome condensation and spindle formation (review: Lewin 1990). In animal mitotic cells most of the $p34^{cdc2}$ kinase is found associated with the spindle poles (Alfa *et al.* 1990). Higher plant cells also contain a functional $p34^{cdc2}$ kinase (*Zea,* Colasanti *et al.* 1991; *Arabidopsis*, Ferreira *et al.* 1991; *Medicago*, Hirt *et al.* 1991). Colasanti *et al.* (1993) were able to localize the functional $p34^{cdc2}$ homolog of *Zea mays* in root tips and cells of the stomatal complex. At interphase the protein was found in the nucleus, and at early prophase it co-localized with the preprophase band of microtubules. $P34^{cdc2}$ is hypothesized to be involved in setting up the mitotic spindle by the phosphorylation of as yet unknown substrates (animal cells: Verde *et al.* 1990). Traas *et al.* (1992) studied cell-cycle related proteins in partially synchronized cell suspension cultures of tobacco and root meristem cells of maize with an antibody called MPM-2. This antibody recognized a mitosis-specific phosphorylated epitope. In interphase, immunofluorescence was seen in the nucleus and the amount of labelling was low. It increased up to metaphase and was again low at telophase. The MPM-2 reactive protein has not yet been characterized, but there are indications that it is a cytoskeletal protein (Traas *et al.* 1992). There are many control steps in the cell division cycle and research in this field has, for plant cells, just started. The recalcitrance of many of the cereal crops to re-enter the cell cycle from a differentiated cell state as mature leaf cells, would be better understood if the control steps were known. In most systems tested, DNA synthesis starts (Wang *et al.* 1991) and often cells divide (Hahne *et al.* 1989), but then cell proliferation stops. These cells do not seem to be blocked in re-entering the cell cycle but in progression through successive cycles. Jung & Wernicke (1993a) studied the potential of cereal mesophyll protoplasts to enter the cell cycle and found that this potential varied in species and was dependent on maturity of the tissue from which the protoplasts were derived. Abnormal cell cycles coincided with abnormal cytoskeleton configurations (Jung & Wernicke 1993b).

The cytoskeleton, indeed, plays an important role in reinitiation of cell division. The formation of a phragmosome (Venverloo *et al.* 1980; Venverloo & Libbenga 1987; review: Derksen *et al.* 1990), is the first visible phenomenon occurring when cells are brought under such culture conditions that reinitiation of cell division takes place. The phragmosome is constituted of a layer of dense cytoplasm to the centre of which the acentrally localized nucleus moves and which is reinforced with microtubules radiating from that nucleus (Meijer & Simmonds 1988). Phragmosome formation is followed by a cell division in the direction determined by the preprophase band, which lies at the site where the phragmosome touches the cell wall (Cleary *et al.* 1992; Hepler *et al.* 1993).

EMBRYOGENIC CELLS

From the preceding paragraph it is clear that auxin is not primarily the trigger for embryogenesis, but for dedifferentiation. Embryogenic cells are those cells in suspension culture that are still capable of dividing and of adhering to each other, but have escaped the influence of prolonged auxin treatment, which disrupts cells from each other and causes them to elongate excessively. The non-embryogenic cells do not divide; they elongate and only loosely attach to each other or become single cells in the medium.

Embryogenic suspension cultures of carrot contain a number of cell types: at least two types in the PEMs; at least three types of single cells; and loosely adhering clusters of single cells. PEMs are cell aggregates consisting of small cytoplasm-rich dividing cells that are embryogenic, remain attached to each other and form a protoderm in medium without auxin. These groups of cells are comparable to pre-globular zygotic embryos and lie at the outside of cell aggregates. They are held together by the second type of cells within the PEM, the larger, hardly dividing non-embryogenic cells with large vacuoles, which derive from the small cells in the auxin containing medium, and eventually are sloughed off from the cell aggregates and become elongated cells floating free in the medium (Emons *et al.* 1992). In medium without auxin these cells only elongate further but do not divide (for maize see Emons & Kieft 1993). They are also called banana-shaped cells or corkscrew-shaped cells and they may form loose clusters with little cell-to-cell adherence and specific wall characteristics (van Engelen *et al.* 1991). The embryogenic carrot culture contains two other types of single cells. Small densely cytoplasmic embryogenic cells are present (Komamine *et al.* 1990). These may divide, their daughter cells remaining attached to each other, and they may develop into new PEMs. But most PEMs derive from PEMs themselves (Halperin 1966; de Vries *et al.* 1988; Emons *et al.* 1992). The culture also contains small elongated cells that may undergo an asymmetric division, after which the smaller daughter cell will ultimately develop into an embryo (Komamine *et al.* 1990).

Tautorus *et al.* (1992) found interesting microtubule organizations after asymmetric divisions in the latter type of embryogenic cells of black spruce cultures: at the two-celled stage the small isodiametric, embryogenic cell had a net of cortical microtubules, indicating limited expansion in all directions; the long, vacuolated, non-embryogenic cell had transversely aligned microtubules, indicative of elongation in the direction of the long axis of the cell.

In maize embryogenic calli, the embryogenic cell clusters develop from cells at the outside of the callus aggregate (Emons *et al.* 1993b). Internal meristems produce only elongated non-embryogenic cells and the callus in which they reside is rhizogenic, i.e. only gives rise to roots when subsequently grown in auxin-free medium (Emons *et al.* 1993a).

Freeze-fracture studies of embryogenic and non-embryogenic cells of carrot PEMs showed a number of differences (Emons *et al.* 1992). Embryogenic cells were small (400–800 μm^3), angled, had contact at all wall facets with many plasmodesmata (2–4 per μm^2), had small vacuoles (30% of cell volume), many starch grains (5–25 per fractured cell), many exocytosis configurations on the plasma membrane (0·6 per μm^2) and polylamellate walls (lamellae with two alternating directions and an angle of 20–40° between lamellae) at all wall facets. The non-embryogenic cells of a PEM were larger (1000–3000 μm^3), rounded, had intercellular spaces, fewer plasmodesmata (0·1–1 per μm^2), larger vacuoles (80% of cell volume), fewer starch grains (1–2 per fractured cell),

sometimes fewer exocytosis configurations (0·3–0·7 per μm^2), and the side walls had polylamellate texture with alternating microfibril orientations transverse to the long axis. The elongated cells showed these tendencies even more: their volumes were 10 000–15 000 μm^3, plasmodesmata frequency was 0–0·1 per μm^2, vacuole percentage was 95, the number of starch grains was 0–1 per fractured cell, there were many exocytosis configurations during the first days after subculture and almost none later in the batch cycle, and walls had, apart from a more or less polylamellate texture in the older wall areas, random microfibril textures in the wall part deposited after cell expansion. Similar differences have been found for maize embryogenic and non-embryogenic suspension cells (Emons & Kieft 1990). From these data it may be concluded that an embryogenic cell is small, densely cytoplasmic, has food reserves and that daughter cells tightly adhere and have intercellular communication and the texture of their cell walls is indicative for a suppressed and controlled process of cell elongation.

Gorst *et al.* (1986) suggested that a high PPB index, i.e. the number of cells with preprophase bands of microtubules in relation to the number of cells with phragmoplasts (microtubule configuration at telophase), would be correlated with potential for organized growth. They reported a PPB index of 2·0 for embryogenic suspension cultures of carrot and 0·16 for non-regenerable tobacco suspensions. The results of Traas *et al.* (1990) with *Petunia hybrida* callus derived from protoplasts agree with this hypothesis. Polarized development, leaf regeneration, occurred if the PPB index was high. Tautorus *et al.* (1992) studied the correlation between PPB index and somatic embryogenesis by comparing these indices for embryogenic black spruce cultures and non-embryogenic jack pine cultures and found that the PPB index was significantly higher in the somatic embryos. However, high numbers of PPBs are not always indicative of organized growth (Wang *et al.* 1989).

SOMATIC EMBRYO DEVELOPMENT

First cell division plane

Zygotic embryo development starts with fertilization of the polarized egg cell in which the nucleus and most of the cytoplasm are positioned at the chalazal side of the cell, while the micropylar part contains a large vacuole. The first division of the zygote is asymmetrical and gives rise to a small apical cell, the future embryo proper, and a large basal cell, which becomes the suspensor, the tissue that connects the embryo to the mother plant (Cresti *et al.* 1992). Real suspensor-like cells are often not present in somatic embryogenesis (Ho & Vasil 1983; Emons & De Does 1993), but in PEMs (*Daucus*: Emons *et al.* 1992) and in embryogenic callus aggregates (*Zea*: Emons & De Does 1993) the small cytoplasm-rich embryogenic cells are connected with the large vacuolated callus cells, which have a suspensor-like function in so far as they provide nutrients and hormones to the non-photosynthesizing embryo. Suspensor-like structures have, however, been reported for somatic embryos of white (Fowke *et al.* 1990) and black spruce (Tautorus *et al.* 1992).

Because plant cells do not move and change position in the plant body, the two most important determinants of plant morphogenesis, apart from the order of cell divisions in time, are the correct positioning of the plane of cell division and the determination of the direction and degree of cell expansion. In plant cells the exact plane of cell division is controlled by the preprophase band, the spindle, and the phragmoplast (Cleary *et al.*

1992; Gunning 1992; Hepler *et al.* 1993). These tubulin and actin cytoskeletal systems act through a number of as yet unknown associated proteins, and it could well be that the *gnom* phenotype of *Arabidopsis*, in which the first division of the zygote is abnormal, i.e. not asymmetric (Mayer *et al.* 1993), is deficient in one of the cytoskeleton-associated proteins. The direction of cell expansion appears to be determined by, or at least related to, the orientation of the cortical microtubules (Williamson 1991), and the degree of cell expansion is determined by the properties of the cell wall (Taiz 1984; Fry 1986; Fry 1993). Plant growth regulators act on both the microtubular cortical cytoskeleton (Shibaoka 1991) and on cell wall properties (Hayashi 1991). For the role of the cytoskeleton in cell morphogenesis and differentiation see Derksen *et al.* (1990).

One of the questions is: 'Is an asymmetric division a prerequisite for somatic embryogenesis?' The first sign of the induction of embryogenic cells in the *direct embryogenesis* process in plant tissue is a shift from the normal division pattern to division planes in atypical directions, which are not necessarily asymmetric (Maheswaran & Williams 1985). This is not different from what happens in induced organogenesis in tissue culture (Venverloo *et al.* 1980; Wilms & Derksen 1988; review: Derksen *et al.* 1990).

In *indirect somatic embryogenesis* (from PEMs and embryogenic calli) the first prerequisite for embryo development is a cell that is able to divide and produces daughter cells which remain attached to each other. This is the case for the small cytoplasm-rich cells in an embryogenic suspension and for the small cytoplasm-rich cells at the outside of embryogenic calli. These cells themselves may have arisen from a symmetric or an asymmetric division.

In *somatic embryo formation from microspores* the first division resulting in embryo development is symmetrical (Zaki & Dickinson 1991), in contrast to the normal asymmetrical division which gives rise to pollen development. As expected, this symmetrical division is preceded by a different microtubule organization (Hause *et al.* 1993).

In cultures of embryogenic *protoplasts* of *Larix*, embryogenic differentiation was often associated with the formation of Y-shaped phragmoplasts by which large parts of cells were eliminated (Staxen *et al.* 1990). After electrical treatment of protoplasts of *Medicago sativa* the asymmetrically divided protoplasts further developed into somatic embryos, whereas the non-electrically treated protoplasts developed ordered arrays of microtubules, divided symmetrically and formed calli (Dijak & Simmonds 1988). For review on microtubules in protoplasts see Simmonds (1991).

One should bear in mind that the asymmetric division of the zygotic embryo produces a vacuolated cell which gives rise to the suspensor through which the embryo is fed, and a densely cytoplasmic small cell, the embryo proper, which develops into a globular stage embryo by means of equal divisions. Whether somatic embryos develop directly on the plant tissue, indirectly from calli, from protoplasts or from microspores, the requirement for an embryogenic pathway from a somatic cell is not an unequal division, but the presence of a densely cytoplasmic cell which, through a number of cell divisions that are not followed by cell growth, forms a globular multicellular structure. And this densely cytoplasmic cell often is the product of an unequal division.

Globular stage somatic embryo

The direction of cell expansion in plant cells is under control of cortical microtubules: cell elongation occurs in the direction perpendicular to the cortical microtubules

(Williamson 1991). All known plant hormones act on the orientation of the cortical microtubules and exert their influence on growth via this cytoskeletal system: gibberellins and auxins promote an orientation of the cortical microtubules perpendicular to the cell axis and therefore promote growth in length (Bergfield *et al.* 1988; Akjashi & Shibaoka 1987; Shibaoka 1991; Ishida & Katsumi 1991); ethylene, cytokinins and ABA promote an orientation of the cortical microtubules in the direction of the long axis of the cell and consequently diminish growth in cell length (Steen & Chadwick 1982; Lang *et al.* 1982; Roberts *et al.* 1985; Shibaoka 1991; Ishida & Katsumi 1992). Both for ABA and gibberellins it is known that this change in orientation of microtubules is not preceded by changes in the rate of cell elongation (Sakiyama-Sogo & Shibaoka 1993). Microtubules may be the causal agents through which hormones modify plant cell growth characteristics. The signal transduction pathway leading from phytohormones to remodelling of the cytoskeleton is poorly understood and work on tubulin-associated (Schellenbaum *et al.* 1993) and actin-binding proteins such as spectrin (De Ruijter & Emons 1993) has only recently begun.

In the first zygotic divisions leading to a globular stage embryo the cells hardly expand. In carrot PEMs the cells that will form the globular stage somatic embryo become smaller, because their daughter cells do not expand, while the non-embryogenic cells to which they are attached expand (Emons, unpublished results). The ability to restrict cell expansion is of importance in somatic embryogenesis. The degree of plant cell expansion is generally believed to be limited by properties of the cell wall (Taiz 1984). The ability of the wall to control cell expansion may be mediated by cell wall proteins and enzymes (Fry 1986; Fry 1993). Glucanases, cellulases and peroxidases may function in this process by breaking and reforming bonds between cell wall polymers. It has been shown that peroxidase, purified from the medium in which carrot somatic embryos had been growing, can counteract the effect of the glycosylation inhibitor tunicamycin which arrests somatic embryos in this species in the globular stage (Cordewener *et al.* 1991). Peroxidase could limit cell expansion by coupling hemicelluloses together via phenolic acids (Fry 1988). Another wall enzyme with rescue activity for temperature-sensitive somatic embryos of carrot is a 32 kD endochitinase (De Jong *et al.* 1992), the *in vivo* substrate of which may be a non-chitin molecule, and thus the enzyme might function in wall-loosening and reshaping.

When carrot PEMs are depleted of auxin, elongation growth and disruption of cells from each other stops and globular stage somatic embryos develop (de Vries *et al.* 1988). The first differentiation step is the formation of a protoderm at the outside of the globule. In maize, globular stage somatic embryos, and also the later stages, ovoids and polars, develop when still in the 2,4-D containing medium, but the protoderm is only formed after transfer to auxin-free medium (Emons, unpublished results). The globular stage somatic embryo is a ball of small cytoplasm-rich cells, confined by still smaller and even more cytoplasm-rich protodermal cells. In suspension the globule may be free in the medium; in an embryogenic callus it is attached to vacuolated cells of the callus via a more or less broad base. In the protodermal cells of carrot PEMs and globules the EP2 gene is expressed (Sterk *et al.* 1991). The EP2 gene codes for a lipid transfer protein, which is secreted and was hypothesized to have a function in cutin synthesis (Sterk *et al.* 1991). The protoderm cells form an epidermis, by which the globule becomes isolated from its surroundings. In contrast, the cells within the globule are all connected via a high number of plasmodesmata. Already at PEM-stage the embryogenic cells of the

PEM have more plasmodesmata per μ^2 than the non-embryogenic cells (Emons *et al.* 1992). Thus, while isolated from its surroundings, cell-to-cell signalling within the PEM is important. In nucellar polyembryony in *Sarcococca humilis*, closing of plasmodesmata by the apposition of a new wall layer is a prerequisite for the differentiation of proembryos (Naumova & Willemse 1982), and the initial for the aposporic embryo sac cell of *Panicum maximum* Jacq. has a thickened cell wall and few, probably closed, plasmodesmata (Naumova & Willemse 1992).

In maize somatic embryogenesis, the development of the epidermis can be stimulated by ABA application (Emons *et al.* 1993b). Ishida & Katsumi (1992) have shown that ABA treatment of cucumber seedlings results in an increase in the number of epidermal cells with longitudinally oriented microtubules, i.e. microtubules oriented in the elongation direction and thus limiting cell growth.

Embryo polarity

Somatic embryos at the outside of a callus aggregate have intrinsic polarity, because they are attached to callus cells at one end. This attachment is comparable to the attachment of the zygotic embryo to the mother plant. The future root of the somatic embryos of maize grown attached to callus is always oriented towards the callus centre, as the root of the zygotic embryo is towards the suspensor. In maize, attachment to the callus is very important. Only those somatic embryos that have been attached to the callus until the formation of a proper shoot meristem become complete embryos. Those grown from cell clusters in liquid medium do not form a proper shoot meristem with leaf primordia and a scutellum (Emons & Kieft 1991). In carrot, a species which easily forms free complete somatic embryos in liquid culture, polarity is found in the distribution of the calcium-calmodulin complex at the PEM stage. That is, when the pro-embryos are still attached to non-embryogenic cells. This complex is only present at the future root side, even before morphological polarity becomes evident (Timmers *et al.* 1989). How this polarity is acquired is not known. A steady ionic current directed along the axis of somatic embryos could be involved in the initiation, determination and maintenance of embryo polarity (Brawley *et al.* 1984). Hormones, like cytokinin, act on the localization of membrane associated calcium and the redistribution of plasma membrane ion channels (Saunders & Hepler 1986).

From Ca^{2+} and calmodulin localization studies of somatic, zygotic and germinating embryos, Timmers (1993) concluded that the carrot somatic embryos he used were more comparable with germinating embryos than with zygotic embryos. Calmodulin was found evenly distributed in the zygotic embryos and the polarized distribution of the somatic embryo was observed in the germinating zygotic embryo. In animal cells high calmodulin levels are seen in late G1 and early S phase of the cell cycle (Means *et al.* 1982). The polarized presence of calmodulin in the somatic embryos and germinating zygotic embryos might just be related to a higher frequency of mitotic cells in the root meristem. Somatic embryos of carrot under the conditions as used by Timmers do not show developmental arrest and the root meristem is active at a very young stage.

Embryo maturation

Embryo polarity acquirement ultimately results in the formation of two meristems: a root and a shoot meristem at opposite sites of the structure. In species such as *Daucus*,

regeneration potential, i.e. potential for germination leading to a plantlet, is acquired as soon as root and shoot meristems have been formed. In species such as *Zea*, in which the zygotic embryo has a large storage organ, successful regeneration of somatic embryos depends on the formation of this organ, the scutellum (Emons & Kieft 1993). Such somatic embryos require an embryo maturation phase in a medium with a high sucrose concentration during which the typical storage organ of the species is formed. During this period starch accumulates in the cells of the scutellum and the formation of lignin is suppressed as in the zygotic situation (Emons *et al.* 1993a; Mulder & Emons 1993). Apart from starch, zygotic embryos of maize have large quantities of the protein globulin in their cells (Kriz 1989). The globulins are present in protein bodies from 11 days after pollination onwards and the total amount of protein considerably increases during embryo maturation (Emons & Thijssen 1993). Both embryogenic callus as well as somatic embryos possess only the proglobulin (Emons & Thijssen 1993), the protein which in zygotic embryos is transiently present and modified to globulin (Belanger & Kriz 1989). Thus, the accumulation of globulins is different in zygotic and somatic embryos, as has also been reported for alfalfa (Krochko *et al.* 1992). A difference between somatic embryos and zygotic embryos was also seen in carrot at the ultrastructural level. Timmers (1993) found that somatic embryos and germinating zygotic embryos were rich in vacuoles and large amyloplasts which were not seen in torpedo-stage zygotic embryos. These, however, had many lipid bodies, which were not very abundant in somatic embryos and in germinating zygotic embryos.

CONCLUSIONS

In a number of species, especially those like carrot with only a limited storage organ, the similar morphology and histodifferentiation of somatic and zygotic embryos is striking, though at the ultrastructural level differences may exist (Timmer 1993). Ideally, somatic embryo development should follow species-specific developmental stages, such as bent-cotyledon-stage in many dicotyledons. Histodifferentiation patterns occur in somatic embryos that to a great extent resemble those of zygotic embryos, even in species such as maize which have a large storage organ.

Crucial steps in the developmental pathway of somatic embryogenesis may be summarized as follows. (i) Somatic embryo development starts with a (group of) densely cytoplasmic cell(s), the fate of which has been altered by altering the cell division event. (ii) Through repeated cell divisions without cell expansion this (group of) cells is able to form a globule consisting of small cells, which are connected via plasmodesmata that enable intercellular communication, and is surrounded by a protoderm of even smaller cells, which isolate the globule from its surroundings. (iii) Independent of and supplementary to the requirement to form a globular somatic embryo is the requirement of this meristematic ball of cells to initiate and control bipolar embryo growth by the formation of a shoot and a root meristem at opposite sides. (iv) In many species the first primordia at the shoot side should develop as storage organs.

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