Plant Molecular Biology **22**: 367–377, 1993.

Update section

Mini review

Early events in higher-plant embryogenesis

Anke J. de Jong, Ed D. L. Schmidt and Sacco C. de Vries*
Agricultural University Wageningen, Department of Molecular Biology, Dreijenlaan 3, 6703 HA Wageningen, Netherlands (author for correspondence)*

Received and accepted 25 February 1993

Introduction

In this review several recent findings in plant embryogenesis will be described. The emphasis will be on a number of selected studies that deal with events in the first and crucial steps of the development of the zygotic embryo and with events in the transition of somatic cells into embryogenic cells. In the first section, early zygotic embryo mutants of *Arabidopsis* will be highlighted. In the second section, essential steps in the formation of embryogenic cells and somatic embryos will be discussed. Based on these studies, the question will be raised which cellular mechanisms control early zygotic embryogenesis and whether analogous mechanisms are involved in the formation of embryogenic cells in tissue culture.

Zygotic embryogenesis

The detailed description of both gametogenesis and zygotic embryo development has been the subject of recent studies [10, 37, 46–49, 85] and will only be recapitulated briefly. During the complex process of plant sexual reproduction, the male gametophytes or pollen grains are formed in the anther. The female gametophyte or embryo sac, is formed in the pistil and consists of seven cells: the egg cell, two synergids, the central cell and three antipodal cells. The polarized egg cell and synergids are positioned at the micropylar pole of the embryo sac. Polarity of the egg cell is evident from the position of the nucleus and most of the cytoplasm at the chalazal side of the cell, while the micropylar part is highly vacuolated. Dual fertilization of the diploid central cell and the haploid egg cell results in the endosperm and the zygote respectively. The first zygotic division is asymmetrical and yields a small apical cell and a large basal cell. The basal cell remains positioned at the micropylar pole of the embryo sac, so the polarity of the unfertilized egg cell appears to predict the future longitudinal axis of the embryo. Development of the *Arabidopsis* embryo from fertilization, through the octant, globular, triangular, heart, torpedo and bent-cotyledon stages, to the mature desiccated embryo has been subdivided into a sequence of 20 different stages [37]. The various classes of genes expressed during plant embryogenesis have been reviewed elsewhere [28, 73].

Generation of the embryo body pattern

In order to ultimately identify genes that direct the formation of the zygotic embryo, a large collection of embryo mutants is required. This has been established for *Arabidopsis* [20, 38, 55] and for *Zea* [6].

The morphology and ultrastructure of a number of embryo lethal mutants has been described [60]. Classification, description of the morphological aberrations, establishment of complementation groups, as well as mapping and cloning of

the mutated genes is currently in progress (D.W. Meinke, pers. comm.).

On the basis of a, most likely saturating, genetic screen for embryo mutations, it was estimated that about 3500 different genes are necessary to complete embryo development [38]. Of these, ca. 40 genes may direct the formation of all body pattern elements in the *Arabidopsis* embryo [50]. Because this number is not very much lower than the number of genes essential for embryo pattern formation in *Drosophila* [69], it may indicate that the apparent morphological simplicity of the plant embryo, when compared to animal embryos, is deceptive.

Jürgens *et al.* [38] have selected for *Arabidopsis* embryo mutants in which germination and seedling development was still possible, with the aim to obtain mutants that were affected in pattern formation rather than mutants affected in more common cellular mechanisms. Based on the mutant phenotypes obtained, a division of the young embryo along the longitudinal axis into an apical, central and basal region was made [38, 50]. A second, radial pattern, superimposed on the apical-basal pattern and consisting of the vascular, ground and epidermal tissues, was proposed. Because in the mutants disturbed in the apical-basal pattern, the three tissue types that make up the radial pattern were all formed, the apical-basal pattern and the radial pattern appear to be established independently. In a separate class of mutants, that exhibited an altered seedling shape rather than a deletion of pattern elements, all pattern elements of the wild-type seedling were still present. The existence of these mutants, in which the shape and spacing of cells in the embryo are abnormal, clearly indicates that changes in cell shape are not essential for the generation of the main pattern elements of the plant embryo.

A very detailed description of the *Arabidopsis* embryo mutant *gnom* was recently completed by Mayer *et al.* [51]. *Gnom* is a terminal pattern mutant [50] and exhibits a highly variable phenotype, that ranges from a ball-shaped seedling with a total lack of root and cotyledon development to a cone-shaped seedling with a clear apical-basal pattern. In all 24 *gnom* mutant alleles investigated, the entire range of phenotypes was found. By tracing the *gnom* phenotype back to the zygote, it was found that the first cell division of the *gnom* zygote is abnormal. Instead of an asymmetric division, resulting in a small apical cell and an elongated basal cell, a nearly symmetric division occurred in the *gnom* zygote. Not only the position, but also the plane of this division appeared to be abnormal, being prone to a variable degree of deviation from the plane of division in wild-type zygotes, which is always perpendicular to the longitudinal axis. Individuals of the same *gnom* mutant allele exhibited variation, both in the position and plane of the first zygotic division, and this most likely accounts for the phenotypic variation observed for each *gnom* mutant allele at seedling stage. The fact that in none of the *gnom* mutant phenotypes a normal root meristem is formed, may be the result of the observed failure in *gnom* to form the hypophysal cell, the direct progenitor of part of the root meristem initial. Whether this points to a continued requirement of the *gnom* gene in all asymmetric divisions in the early embryo, or is due to the previous failure to perform the first asymmetric division of the zygote, is not clear. In several of the *gnom* mutant alleles, it was observed that the first visible event after fertilization of the egg cell, expansion of the zygote in the direction of the future longitudinal axis of the embryo, was suppressed. Consequently, partitioning of the zygote into a cytoplasm-rich apical part with the nucleus and a vacuolated basal part might not have taken place. The resulting aberrant first zygotic division in *gnom* could therefore also be the result of a failure of correct directional cell expansion. Thus, it appears that the two most important determinants of plant morphogenesis, the correct position of the plane of cell division and the controlled directional cell expansion [43, 44], are directly affected by the *gnom* gene.

In a mutant of the class of basal pattern mutants, *monopteros* [50], the entire seedling root and hypocotyl is deleted but, in contrast to *gnom*, the cotyledons are formed normally. Based on the *gnom-monopteros* double mutant phenotype, it appears that *gnom* is epistatic to *monopteros* [51]. Thus, without the prior activity of the product of

the *gnom* gene, which apparently has to be active in the unicellular zygote, where it may control correct cell elongation and plane of division, the *monopteros* gene is not able to give rise to the basal part of the seedling. In an experiment similar to that performed by Schiavone and Racusen [69], who have shown that the apical part of transected *Daucus* somatic embryos were able to regenerate the entire missing root part, cut *gnom* seedlings did not regenerate a root [51]. This result suggests an important role for the ability to perform asymmetric cell divisions in (root) regeneration, and it also indicates that the function of the *gnom* gene is not restricted to the embryo.

Cellular mechanisms in zygotic embryogenesis

Maternally acting genes and zygotic genes

The role of the *gnom* gene in the establishment of apical-basal polarity in the *Arabidopsis* embryo marks it as one of the earliest-acting genes so far described. Genetic analysis has indicated that the *gnom* gene is a zygotically acting gene [50, 51]. Cytological observations clearly indicate that the unfertilized egg cell is highly polarized [10], suggesting that maternally expressed genes are involved. Although Meinke [54] found evidence for an overlap between male gametogenesis and a lethal embryo phenotype in some mutants, to date no typical maternal effect mutations that affect pattern formation in the zygotic plant embryo have been reported. Two female-sterile ovule mutants in *Arabidopsis*, *bell* and *sin1*, have recently been described [68]. In these mutants, the formation of the integuments is aberrant. Although megasporogenesis was not affected, a normal mature embryo sac did not develop in these mutants, most likely as a result of the aberrant formation of the integuments. In the *sin1* mutant the defect appeared to be the result of a failure of the integument cells to properly expand after division. This appeared to be a more general effect, in view of reduced internode length observed in the mutant plants. Therefore, putative maternally acting genes that affect oogenesis or direct pattern formation in the early embryo have not been reported in plants. This appears in contrast with the generation of pattern during animal embryogenesis, where, except for mammals, at least one axis and, as in *Drosophila*, two axes of the future embryo are established in the unfertilized egg cell [29, 75]. Whether this reflects a fundamental difference between plant and animal embryo pattern formation or is due to the technical difficulties in isolating such mutants in plants, is at present unclear. The conventional argument that the possibility of somatic embryogenesis precludes an important role for maternal effect genes in plant embryogenesis, seems to be of limited use in view of the fact that very little is known about the molecular mechanisms that underly the transition of a somatic cell into an embryo-forming cell (see next section).

Embryonic induction and asymmetric cell division

Two mechanisms appear to be universally used in animal embryogenesis to initiate cell differentiation. These are the interaction between an inducing cell or tissue and a responding cell or tissue, and asymmetric cell division [29].

No direct evidence is available that cell inductive processes are of importance in the formation of plant gametophytes. A sequential and transient expression of an arabinogalactan-protein (AGP) epitope, recognized by the monoclonal antibody JIM8, was observed in the plasma membranes of diverse parts of both male and female reproductive tissues in *Brassica*. This included sperm cells and the egg cell, the embryo up to early globular stage, and the suspensor and hypophysal derivatives of later embryo stages [62]. AGPs are proteoglycans with poly- and oligosaccharide units covalently attached to a central protein core [82]. They are found in plasma membranes, cell walls and in the intercellular spaces of plant tissues [21]. In the absence of any clear correlation between a particular differentiation event and the expression of the JIM8 plasma membrane epitope, Pennell *et al.* [62] speculated that this epitope might actually be a marker for a cell-inductive process in plants.

Asymmetric cell division occurs frequently in plants, and the analysis of the *Arabidopsis gnom* mutant clearly shows that this mechanism is indeed of crucial importance in plant embryogenesis. In animal cells the plane of cell division is controlled by the positioning of the mitotic spindle. This is in turn mediated by the positioning and anchoring of the centrosome by means of microtubules to a specific cortical site. The 'default' plane of division in animal cells is 90° to the previous plane. This is explained by division and subsequent movement of the daughter centrosomes to opposite sides of the nucleus in the case of the 90° default orientation and alternative or additional movements in the case of deviations of this rule [76]. The asymmetric first division of the *Caenorhabditis* zygote is essential to form daughter cells that differ in their cytoplasmic determinants, such as maternally produced mRNA [34, 76]. As a consequence, these two cells follow different developmental fates. Whether the first asymmetric division of the plant zygote serves the same purpose seems quite reasonable. It is not difficult to envisage that variability in the first zygotic division [51] automatically leads to a variability in the amount of cytoplasmic determinants in each of the resulting daughter cells. However, the nature of these determinants remains to be established.

It is of interest to note that there is a certain analogy between the early phenotype of the *Arabidopsis gnom* mutant and the *Caenorhabditis par* mutants. *Par* mutants show defects in spindle orientation that result in aberrant partitioning of cytoplasmic components during the first few divisions. If this analogy is valid, the apical cell of the plant zygote would be equivalent to the AB cell, and the basal cell equivalent to the P1 cell of the two-celled nematode zygote. Phenocopies of *par* mutants could be obtained after treatment with microfilament inhibitors [33].

Control of cell expansion

It is clear that, during early zygotic embryogenesis, cell expansion is rigorously controlled. After the unidirectional expansion of the zygote and the first asymmetric division, the resulting apical cell does not increase in size. Instead, three cleavage-like divisions occur, and in these, but also in the following tangential divisions that form the protodermal precursor cells, no or hardly any increase in the size of the apical part of the embryo occurs. At least one *Arabidopsis* embryo mutant, *emb 101-1*, has been described where cell expansion in the embryo is totally out of control, resulting in giant cells that fill the entire seed (D. W. Meinke, personal communication).

Cell lineage

Fate maps from egg to embryo have been constructed by direct observation of the cell lineage in *Caenorhabditis* [77]. Laser ablation and cell transplantation experiments have shown the presence of groups of cells with a similar competence [34]. From these studies, it appears that embryogenesis continues according to a rigidly fixed programme, initially dependent on the regional location of cytoplasmatic determinants by asymmetric cell division, but also including cell-inductive processes, in the determination of the fate of each individual cell. In plants, no evidence has been found for the existence of such a rigid cell lineage in, for instance, the functioning of the shoot apical meristem. Instead, cell position rather than previous developmental history is considered to be essential for the formation of the somatic tissues [11, 64]. Although the fate of cells in the shoot apical meristem of *Arabidopsis* is predictable to a certain degree [24], Irish and Sussex [35] suggested the term 'probability map' rather than 'fate map' to emphasize the absence of a rigid cell lineage. Studies aimed to determine cell lineage in the generation of the embryo body plan in *Zea* and *Gossypium*, also demonstrated a general but not an absolute predictability in the final position of cells in the embryo [5, 64].

The systematic genetic dissection of plant zygotic embryogenesis has only recently been initiated on a large scale. It is therefore not yet pos-

sible to predict whether these studies will reveal cellular mechanisms analogous to those found for animal model systems such as *Drosophila* or *Caenorhabditis*. It is clear from the description of the *Arabidopsis gnom* mutant, that asymmetric cell division is a key process in plant embryogenesis. It is also evident that this is only one of the cellular processes employed. Whether maternally acting genes, cell-inductive processes and cell lineages are also important in plant embryogenesis, remains to be determined. The role of the cell wall in the controlled directional expansion of cells and the formation of the endosperm may represent aspects of plant embryogenesis that do not have a clear counterpart in animal cells.

Somatic embryogenesis

Somatic or asexual embryogenesis is the process by which somatic cells develop into plants through characteristic morphological stages. For dicots these are the globular, heart and torpedo stages. This process occurs naturally in several species such as *Malaxis*, where somatic embryos form spontaneously on the leaf tips [79], but it can also be induced by experimental manipulation. Under *in vitro* conditions somatic embryos can either form directly on the surface of an organized tissue such as a leaf or stem segment, from protoplasts or from microspores, or indirectly via an intermediary step of callus or suspension culture [87].

Here the focus will be on what is not only the most important, but also the least understood part of somatic embryogenesis, the transition of somatic cells into cells, referred to as embryogenic cells, that are capable of forming an embryo. In animals, the ability to form embryos is restricted to a specific set of stem cells, the germ cells. Germ cells are separated from somatic cells at a very early stage of embryogenesis. *Drosophila* eggs contain a class of maternally provided gene products, like oskar [19], that function in the formation of germ cells. In plants, where the ability to form embryos is not restricted to the germ cells, somatic embryos are used extensively as convenient alternatives for zygotic embryos in many biochemical and molecular studies.

Description of embryogenic cells

Since its first demonstration [68], somatic embryogenesis has been most widely studied in suspension cultures of *Daucus* [3, 30, 41, 53, 80] and *Medicago* [18]. Because a certain amount of confusion exists in the literature on terminology, it may be useful at this point to explain the terms that will subsequently be used in this review. Although suspension cultures are often described as 'undifferentiated', a better term is probably 'unorganized', because in many cultures subpopulations of cells exist that retain characters found to be associated with specific differentiated cell types *in planta* [81]. Also, use of the term 'embryogenic cell or cells' will be limited to describe only those cells that have completed the transition from a somatic cell or cells to a state where no further externally applied stimuli are necessary to produce the somatic embryo. Following from this, a culture or tissue with a variable number of cells in it that have responded to external stimuli will be called 'embryogenic culture or tissue'. Depending on the experimental conditions, the ratio of embryogenic to total cells under these conditions can vary between zero and the theoretical maximum of 1. One of the advantages of this terminology is that the difference between direct and indirect somatic embryogenesis is no longer of importance. Direct embryogenesis on explants, or indirect embryogenesis on callus or clusters of embryogenic cells in suspension cultures probably represent different sides of the same coin [87].

In *Daucus*, the usual strategy to start an embryogenic suspension culture is to expose explants to a high concentration of auxin. After reinitiation of cell division and a period of proliferation of the released explant cells in the presence of auxin, embryogenic cells appear in the culture [13]. These are usually in the form of clusters of small cytoplasmic cells, referred to as proembryogenic masses [31]. It is of importance to note that in almost all embryogenic *Daucus* cultures, the per-

centage of cells that actually are embryogenic is fairly low, and never amounts to more than about 1–2% [13]. The remainder of the suspension cells are not directly capable of forming somatic embryos.

By using time-lapse photography, Backs-Hüseman and Reinert [3] have described an elongated single vacuolated suspension cell able to develop into a somatic embryo. Using cell purification techniques, Nomura and Komamine [57] described a much smaller, almost spherical and cytoplasmic suspension cell, designated a type 1 cell, as being able to develop into a somatic embryo. Because both require preculturing in auxin, neither of these cell types can be called embryogenic under our definition. In the case of the type 1 cell, the derived state 1 cell cluster [41] would be the first to contain embryogenic cells in the pathway leading to somatic embryos from single cells.

Identification of embryogenic cells

Several molecular markers have been reported that are able to distinguish between embryogenic and non-embryogenic cell cultures [63, 73]. One of these is the *Daucus* EP2 gene [74]. Employing *in situ* mRNA localization, the EP2 gene was found to be exclusively expressed in peripheral cells of proembryogenic masses and in the protoderm of somatic embryos. In *Daucus* zygotic embryos, EP2 expression was detected in a protoderm-specific fashion as early as a 60-celled globular embryo. The EP2 gene encodes a secreted lipid transfer protein, postulated to function in cutin synthesis [74; E. Meijer and T. Hendriks, manuscript submitted].

Another marker for embryogenic cultures consists of a cell wall epitope in *Daucus* suspension cells, that is recognized by the monoclonal antibody JIM8 [63]. The JIM8 epitope has been localized on three different plasma membrane AGPs [62], on secreted AGPs in *Daucus* suspension cultures [40] and on an unidentified cell wall molecule present in a sub-population of *Daucus* suspension cells. It is unclear whether the molecule that bears the JIM8 cell wall epitope is related to the plasma membrane AGP epitope described previously [62] or the epitope present on secreted *Daucus* AGPs [40]. The presence of the cell wall JIM8 epitope in *Daucus* suspension cell cultures is highly correlated with the presence of embryogenic cells. Surprisingly, immersion immunofluorescence showed that several morphologically different cells react with the JIM8 antibody, but not the proembryogenic masses [63]. Instead, mainly small single cells, including cells morphologically similar to the type 1 cells, were recognized. The hypothesis put forward by Pennell *et al.* [63] is therefore that the JIM8 cell wall epitope marks a transitional state in the formation of embryogenic cells. Because the number of JIM8 reactive single cells exceeds by far the number of single cells that are able to develop into an embryo, apparently only few cells in this transitional state are actually able to reach the status of the embryogenic state 1 cell cluster. Although the JIM8 plasma membrane epitope, as observed in *Brassica* flowers [62], is most likely present on a molecule different from the JIM8 cell wall epitope observed in *Daucus* suspension cultures, the observation that both visualize a transient developmental process, not restricted to a particular set of morphologically recognizable cells, represents an intriguing parallel.

Formation of embryogenic cells

It has often been observed that the developmental stage of the explant is of prime importance for the transition of somatic cells into embryogenic cells [2, 7, 86]. However, it is not clear whether these observations reflect genetic differences in the ability of somatic cells to become embryogenic, or whether they are due to the frequency of a particular responsive cell type in these tissues.

The fact that almost all cells of mature organs in plants, including *Arabidopsis* [25], are polyploid has led to the question whether polyploidy is negatively correlated with the ability to regenerate. However, in *Zea*, no evidence was found that this is indeed the case [17]. In *Daucus* suspension cultures a correlation was found between the tet-

raploid state and the inability to produce somatic embryos [9, 71]. Based on the occurrence of meiotic-like cell division configurations and the presence of a limited number of haploid nuclei in newly initiated cultures of *Daucus*, Nuti-Ronchi *et al.* [58, 59] postulated a requirement for DNA reducing mechanisms in the formation of embryogenic cells. Support for this hypothesis is the observation that, after chemical mutagenesis of embryogenic *Daucus* suspension cultures, an unexpectedly high number of recessive mutants were recovered [27]. Definite evidence for the occurrence of reductional divisions in tissue culture awaits segregation analysis in the regenerants.

Although auxins are the best studied inducers for obtaining embryogenic cells [1, 30, 36, 56, 72, 84, 86], they are certainly not unique in the ability to mediate the transition of somatic cells into embryogenic cells. For example in *Citrus* suspension cultures, a change in carbon source is sufficient [26] and for *Brassica* microspores a temperature shock is employed to render cells embryogenic [61]. In *Medicago*, the ability of cells to become embryogenic appeared to depend on their sensitivity to auxin, as illustrated by the totally different response to 2,4-D of leaf protoplasts derived from a genotype that readily forms embryogenic cells *in vitro* and one that does not [4].

Recent evidence suggests that particular purified AGPs, isolated from the culture medium of embryogenic *Daucus* lines and from dry *Daucus* seeds were able to promote the formation of proembryogenic masses, even in previously non-embryogenic *Daucus* cell lines, when added in nanomolar concentrations. Other AGPs, isolated from the medium of a non-embryogenic line, acted negatively on the formation of proembryogenic masses [42]. These results show that specific members of the family of AGPs are involved in the formation of embryogenic clusters. Although the underlying mechanisms are unclear, these observations, together with earlier ones employing unfractionated conditioned medium [14], suggest that molecules totally different from conventional plant growth regulators are able to direct the transition of somatic cells into embryogenic cells. Since cell-surface AGPs turn over very rapidly [85], and their expression is clearly developmentally regulated [39], they are likely candidates for molecules able to mediate developmental processes in plants, perhaps by a cell-inductive mechanism [62].

Cell polarity and asymmetrical cell division

Several observations support the hypothesis that plant growth regulators employed to form embryogenic cells, do this by alteration of cell polarity and promotion of subsequent asymmetric divisions. When immature zygotic embryos of *Trifolium* were cultured in the presence of cytokinin, somatic embryos are produced directly from the hypocotyl epidermis. The first sign of the induction of embryogenic cells was a shift from the normal anticlinal division pattern in the epidermis, to irregular periclinal and oblique divisions [45]. The effect of the cytokinin was not entry into mitosis per se, but rather an alteration of the division planes, because regular anticlinal divisions persisted for some time in the absence of cytokinin. As pH gradients and electrical fields can change cell polarity [67], the positive effect on embryo development of pH shifts [70] and electrical fields [16] may be due to their effect on cell polarity. It is plausible, but unproven, that exogenously applied plant growth regulators directly modify cell polarity, by interference with pH gradients or the electrical field around cells. Following stimulation by auxin, asymmetric cell divisions were frequently observed in leaf protoplast cultures derived from an embryogenic *Medicago* cultivar, while in protoplast cultures from a non-embryogenic cultivar cells divided symmetrically [4, 18]. The different types of cell division in *Medicago* leaf protoplast cultures appeared to be correlated with differences in microtubule organization [15]. In *Daucus*, the first division of single suspension cells capable of forming embryogenic cells is also asymmetric [3, 41], and only the smaller daughter cell will ultimately develop into an embryo. As the future root pole of the somatic embryo is always oriented towards

the larger cell, the polarity of the entire somatic embryo is already determined prior to the first division of an embryogenic cell.

Brassica microspores are highly polarized during normal development into pollen, *in vivo* as well as under *in vitro* conditions. Depending on the developmental stage at the time of isolation, after heat shock induction to induce the formation of microspore derived embryos, the first visible change is either a 90° shift in the orientation of the mitotic spindle, or a migration of the nucleus from an acentric to a central position [32]. In both cases the result is a 90° shift of the division plane and replacement of an asymmetric cell division with a symmetric cell division. Artificially increasing the number of symmetric cell divisions by colchicine resulted in a larger number of microspores proceeding towards embryogenesis [89]. These results suggest that alteration of division symmetry is required to switch from the gametophytic to the sporophytic developmental pathway. Although many variations have been observed, the replacement of the normal asymmetric cell division with a symmetric one appears to be a general phenomenon in microspore embryogenesis [88].

With the exception of microspore embryogenesis, the ability to perform an asymmetric cell division, based on a change in cell polarity, seems to be an important and perhaps universal mechanism in the formation of embryogenic plant cells from somatic cells. This change in cell polarity can apparently be initiated by a variety of inducers, among which plant growth regulators. As in zygotic embryogenesis (see previous section), the nature of the cytoplasmic determinants that are partitioned by asymmetrical cell divisions, remains to be identified. The fact that in microspore embryogenesis a symmetric first cell division is the first one in the sporophytic pathway may be a consequence of the previous highly specialized developmental history of the microspores.

Control of cell expansion

A second mechanism that is of importance in the formation of embryogenic cells *in vitro* is the ability to restrict cell expansion under hypotonic conditions [23, 78, 80, 81]. The ability to control cell expansion is generally accepted to reside in the cell wall, and is probably mediated by specific sets of cell wall proteins and enzymes [22]. These enzymes may act by breaking and reforming bonds in cell wall polymers by for instance the action of glucanases, cellulases and peroxidases [81], and other not yet identified proteins [52]. In *Daucus*, the glycosylation inhibitor tunicamycin arrests somatic embryogenesis, perhaps by the gradual disruption of proembryogenic mass due to expansion of its outer cell layer. This effect could be counteracted by addition of a single protein, exhibiting peroxidase activity, purified from medium conditioned by a somatic embryo culture [9]. A mechanism that limits cell expansion may also be required at later stages of somatic embryo development, as indicated by the rescue of arrested globular embryos of the temperature-sensitive *Daucus* mutant *ts11* with a single secreted acidic endochitinase [12]. Addition of the endochitinase appeared to prevent the formation of an aberrant, irregular protodermal layer, consisting of enlarged, vacuolated cells. A positive effect was also seen on the formation of proembryogenic masses and globular embryos from *ts11* suspension cells, which implies that more than one stage in the development of embryos is affected in *ts11*.

The mechanisms by which secreted proteins influence somatic embryogenesis are unknown, but it is reasonable to postulate that their function can be explained in terms of an effect on particular cell wall polymers [83].

Concluding remarks

In the preceding sections, several recent approaches used to understand the molecular and cellular basis of zygotic and somatic embryogenesis in plants have been dealt with. It appeared from several studies that in the *in vitro* formation of embryogenic plant cells both asymmetric cell division and control of cell expansion are important mechanisms. There is some evidence that cell

polarity and a postulated subsequent partitioning of cytoplasmic determinants can be influenced by a variety of factors among which plant growth regulators. Other molecules that profoundly influence, for instance, the formation of embryogenic cells and the restriction of cell expansion characteristic of these cells have been found with biological assays based on *in vitro* systems. Analysis of *Arabidopsis* mutants, such as *emb 101-1* and *gnom*, that are affected in early stages of zygotic embryogenesis, have also pointed to control of cell expansion and asymmetric cell division as important mechanisms. The results obtained so far suggest that, although their starting points are quite different, the same basic cellular mechanisms are used in somatic as well as in zygotic plant embryogenesis.

Acknowledgements

We thank Ab van Kammen, Jacques van Went, Gerd Jürgens and Dénes Dudits for valuable comments on the manuscript. We are grateful to our colleagues for communicating unpublished results. A.J.d.J. was supported by the Foundation for Biological Research, subsidized by the Netherlands Organization for Scientific Research. E.D.L.S. is supported by the Technology Foundation, subsidized by the Netherlands Organization for Scientific Research. Primary research in our laboratory is supported by the EC-BRIDGE programme.

References

1. Abdullah R, Cocking EC, Thompson JA: Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/technology 4: 1087–1090 (1986).
2. Ammirato PV: Embryogenesis. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds), Handbook of Plant Cell Culture, vol. 1; pp. 82–123. MacMillan, New York (1983).
3. Backs-Hüsemann D, Reinert J: Embryobildung durch isolierte Einzelzellen aus Gewebekulturen von *Daucus carota*. Protoplasma 70: 49–60 (1970).
4. Bögre L, Stefanov I, Ábrahám M, Somogyi I, Dudits D: Differences in responses to 2,4-dichlorophenoxyacetic acid (2,4D) treatment between embryogenic lines of alfalfa. In Nijkamp HJJ, van der Plas LHW, van Aartrijk J (eds) Progress in Plant Cellular and Molecular Biology, pp. 427–436. Kluwer Academic Publishers, Dordrecht (1990).
5. Christianson ML: Fate map of the organizing shoot apex in *Gossypium*. Am J Bot 73: 947–958 (1986).
6. Clark JK, Sheridan WF: Isolation and characterization of 51 embryo-specific mutations of maize. Plant Cell 3: 935–951 (1991).
7. Conger BV, Hanning GE, Gray DJ, McDaniel JK: Direct embryogenesis from mesophyll cells of orchardgrass. Science 221: 850–851 (1983).
8. Cordewener J, Booij H, van der Zandt H, van Engelen F, van Kammen A, de Vries SC: Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. Planta 184: 478–486 (1991).
9. Coutos-Thevenot P, Jouanneau JP, Brown S, Petiard V, Guern J: Embryogenic and non-embryogenic cell lines of *Daucus carota* cloned from meristematic cell clusters: relation with cell ploidy determined by flow cytometry. Plant Cell Rep 8: 605–608 (1990).
10. Cresti M, Blackmore S, van Went JL: Atlas of sexual reproduction in flowering plants. Springer-Verlag, Berlin (1992).
11. Dawe RK, Freeling M: Cell lineage and its consequences in higher plants. Plant J 1: 3–8 (1991).
12. de Jong AJ, Cordewener J, Lo Schiavo F, Terzi M, Vandekerckhove J, van Kammen A, de Vries SC: A carrot somatic embryos mutant is rescued by chitinase. Plant Cell 4: 425–433 (1992).
13. de Vries SC, Booij H, Meyerink P, Huisman G, Wilde DH, Thomas TL, van Kammen A: Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta 176: 196–204 (1988).
14. de Vries SC, Booij H, Janssens R, Vogels R, Saris L, Lo Schiavo F, Terzi M, Van Kammen A: Carrot somatic embryogenesis depends on the phytohormone-controlled presence of correctly glycosylated extracellular proteins. Genes Devel 2: 462–476 (1988).
15. Dijak M, Simmonds DH: Microtubule organization during early embryogenesis from mesophyl protoplasts of *Medicago sativa*. Plant Sci 58: 183–191 (1988).
16. Dijak M, Smith DL, Wilson TJ, Brown DCW: Stimulation of direct embryogenesis from mesophyll protoplasts of *Medicago sativa*. Plant Cell Rep 5: 468–470 (1986).
17. Dolezelová M, Dolezel J, Nesticky M: Relationship of embryogenic competence in maize (*Zea mays L.*) leaves to mitotic activity, cell cycle and nuclear DNA content. Plant Cell Tissue Organ Culture 31: 215–221 (1992).
18. Dudits D, Bögre L, Györgyey J: Molecular and cellular approaches to the analysis of plant embryo development from somatic cells in vitro. J Cell Sci 99: 475–484 (1991).
19. Ephrussi A, Lehmann R: Induction of germ cell formation by oskar. Nature 358: 387–392 (1992).
20. Errempalli D, Patton D, Castle L, Mickelson L, Hansen K, Schnall J, Feldmann K, Meinke D: Embryonic lethals and T-DNA insertional mutagenesis in *Arabidopsis*. Plant Cell 3: 149–157 (1991).
21. Fincher GB, Stone BA, Clarke AE: Arabinogalactan proteins; structure, biosynthesis and function. Annu Rev Plant Physiol 34: 47–70 (1983).

22. Fry SC: Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol 37: 165–186 (1986).
23. Fry SC: Roles of the primary cell wall in morphogenesis. In: Nijkamp HJJ, van Der Plas LHW, van Aartrijk J (eds) Progress in Plant Cellular and Molecular Biology, pp. 504–513. Kluwer Academic Publishers, Dordrecht (1990).
24. Furner IJ, Pumfrey JE: Cell fate in the shoot apical meristem of *Arabidopsis thaliana*. Development 115: 755–764 (1992).
25. Galbraith DW, Harkins KR, Knapp S: Systemic endopolyploidy in *Arabidopsis thaliana*. Plant Physiol 96: 985–989 (1991).
26. Gavish H, Vard A, Fluhr R: Extracellular proteins and early embryo development in Citrus nucellar cell cultures. Physiol Plant 82: 606–616 (1991).
27. Giuliano G, Lo Schiavo F, Terzi M: Isolation and developmental characterization of temperature-sensitive carrot cell variants. Theor Appl Genet 67: 179–183 (1984).
28. Goldberg RB, Barker SJ, Perez-Grau L: Regulation of gene expression during plant embryogenesis. Cell 56: 149–160 (1989).
29. Gurdon JB: The generation of diversity and pattern in animal cells. Cell 68: 185–199 (1992).
30. Halperin W: Morphogenetic studies with partially synchronized cultures of carrot embryos. Science 146: 408–410 (1964).
31. Halperin W: Alternative morphogenetic events in cell suspensions. Am J Bot 53: 443–453 (1966).
32. Hause B, Hause G, Pechan P, Van Lammeren AAM: Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol Int Rep 17: 153–168 (1993).
33. Hill DP, Strome S: Brief cytochalasin-induced disruption of microfilaments during a critical interval in 1-cell *C. elegans* embryos alters the partitioning of developmental instructions to the 2-cell embryo. Development 108: 159–172 (1990).
34. Horvitz HR, Herskovitz I: Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. Cell 68: 237–255 (1992).
35. Irish VF, Sussex IM: A fate map of the *Arabidopsis* embryonic shoot apical meristem. Development 115: 745–753 (1992).
36. Jones TJ, Rost TL: The developmental anatomy and ultrastructure of somatic embryos from rice (*Oryza sativa* L.) scutellum epithelial cells. Bot Gaz 150: 41–49 (1989).
37. Jürgens G, Mayer U: *Arabidopsis*. In: Bard J (ed) A colour atlas of developing embryos. Wolfe, London. (1992).
38. Jürgens G, Mayer U, Torres Ruiz RA, Berleth T, Miséra S: Genetic analysis of pattern formation in the *Arabidopsis* embryo. Development (Suppl 91.1): 27–38 (1991).
39. Knox JP, Day S, Roberts K: A set of cell surface glycoproteins forms an early marker of cell position, but not cell type in the root apical meristem of *Daucus carota* L. Development 106: 47–56 (1989).
40. Knox JP, Linstead PJ, Peart J, Cooper C, Roberts K: Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. Plant J 1: 317–326 (1991).
41. Komamine A, Matsumoto M, Tsukahara M, Fujiwara A, Kawahara R, Ito M, Smith J, Nomura K, Fujimura T: Mechanisms of somatic embryogenesis in cell cultures – physiology, biochemistry and molecular biology. In: Nijkamp HJJ, van Der Plas LHW, van Aartrijk (eds) Progress in Plant Cellular and Molecular Biology, pp. 307–313. Kluwer Academic Publishers, Dordrecht (1990).
42. Kreuger M, van Holst GJ: Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta 189: 243–248 (1993).
43. Lloyd CW: How does the cytoskeleton read the laws of geometry in aligning the division plane of plant cells: Development (Suppl 91.1): 55–65 (1991).
44. Lyndon RF: Plant Development. The Cellular Basis. Unwin Hyman, London (1990).
45. Maheswaran G, Williams EG: Origin and development of somatic embryos formed directly on immature embryos of *Trifolium repens* in vitro. Ann Bot 56: 619–630 (1985).
46. Mansfield SG, Briarty LG: Development of the free-nuclear endosperm in *Arabidopsis thaliana* L. *Arabidopsis* Inf Serv 27: 53–64 (1990).
47. Mansfield SG, Briarty LG: Endosperm cellularization in *Arabidopsis thaliana* L. *Arabidopsis* Inf Serv 27: 65–72 (1990b).
48. Mansfield SG, Briarty LG: Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. Can J Bot 69: 461–476 (1991).
49. Mansfield SG, Briarty LG, Erni S: Early embryogenesis in *Arabidopsis thaliana*. I. The mature embryo sac. Can J Bot 69: 447–460 (1991).
50. Mayer U, Torres Ruiz RA, Berleth T, Miséra S, Jürgens G: Mutations affecting body organization in the *Arabidopsis* embryo. Nature 353: 402–407 (1991).
51. Mayer U, Büttner G, Jürgens G: Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene. Development 117: 149–162 (1993).
52. McQueen-Mason S, Durachko DM, Cosgrove DJ: Two endogenous proteins that induce cell wall extension in plants. Plant Cell 4: 1425–1433 (1992).
53. McWilliam AA, Smith SM, Street HE: The origin and development of embryoids in suspension cultures of carrot (*Daucus carota*). Ann Bot 38: 243–250 (1974).
54. Meinke DW: Embryo–lethal mutants of *Arabidopsis thaliana*: evidence for gametophytic expression of the mutant genes. Theor Appl Genet 63: 381–386 (1982).
55. Meinke DW: Embryonic mutants of *Arabidopsis thaliana*. Devel Genet 12: 382–392 (1991).
56. Mórocz DJ, Donn G, Németh J, Dudits D: An improved system to obtain fertile regenerants via maize protoplasts isolated from a highly embryogenic suspension culture. Theor Appl Genet 80: 721–726 (1990).
57. Nomura K, Komamine A: Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol 79: 988–991 (1985).
58. Nuti Ronchi V, Giorgetti L, Tonelli M, Martini G: Ploidy reduction and genome segregation in cultured carrot cell lines. I. Prophase chromosome reduction. Plant Cell Tissue Organ Culture 30: 107–114 (1992).

59. Nuti Ronchi V, Giogetti L, Tonelli M, Martini G: Ploidy reduction and genome segregation in cultured carrot cell lines. II. Somatic meiosis. Plant Cell Tissue Organ Culture 30: 115–119 (1992).
60. Patton DA, Meinke DW: Ultrastructure of arrested embryos from lethal mutants of *Arabidopsis thaliana*. Am J Bot 55: 807–819 (1990).
61. Pechan PM, Keller WA: Identification of potentially embryogenic microspores in *Brassica napus*. Physiol Plant 74: 377–384 (1988).
62. Pennell RI, Janniche L, Kjellbom P, Scofield GN, Peart JM, Roberts K: Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell 3: 1317–1326 (1991).
63. Pennell RI, Janniche L, Scofield GN, Booij H, De Vries SC, Roberts K: Identification of a transitional cell state in the developmental pathway to carrot somatic embryogenesis. J Cell Biol 119: 1371–1380 (1992).
64. Poethig RS: Genetic mosaic and cell lineage analysis in plants. Trends Genet 5: 273–277 (1989).
65. Poethig RS, Coe EH, Johri MM: Cell lineage patterns in maize embryogenesis: a clonal analysis. Devel Biol 117: 392–404 (1986).
66. Quatrano RS: Development of cell polarity. Annu Rev Plant Physiol 29: 487–510 (1978).
67. Reinert J: Über die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebekulturen aus Karotten. Planta 53: 318–333 (1959).
68. Robinson-Beers K, Pruitt RE, Gasser CS: Ovule development in wild-type *Arabidopsis* and two female-sterile mutants. Plant Cell 4: 1237–1249 (1992).
69. Schiavone FM, Racusen RH: Regeneration of the root pole in surgically transected carrot embryos occurs by position-dependent, proximodistal replacement of missing tissues. Development 113: 1305–1313 (1991).
70. Smith DL, Krikorian AD: Somatic proembryo production from excised, wounded zygotic carrot embryos on hormone-free medium: evaluation of the effects of pH, ethylene and activated charcoal. Plant Cell Rep 9: 468–470 (1990).
71. Smith SM, Street HE: The decline of embryogenic potential as callus and suspension cultures of carrot (*Daucus carota* L.) are serially subcultured. Ann Bot 38: 223–241 (1974).
72. Song J, Sorensen EL, Liang GH: Direct embryogenesis from single mesophyll protoplasts in alfalfa (*Medicago sativa* L.). Plant Cell Rep 9: 21–25 (1990).
73. Sterk P, De Vries SC: Molecular markers for plant embryos. In Redenbaugh K (ed), Synseeds: Applications of Synthetic Seeds to Crop Improvement, pp. 115–132. CRC Press, London (1992).
74. Sterk P, Booij H, Schellekens GA, van Kammen A, de Vries SC: Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell 3: 907–921 (1991).
75. St Johnston D, Nusslein-Volhard C: The origin of pattern and polarity in the *Drosophila* embryo. Cell 68: 201–219 (1992).
76. Strome S: Determination of cleavage planes. Cell 72: 3–6 (1993).
77. Sulton JE, Schierenberg E, White JG, Thomson JN: The embryonic development of *Caenorhabditis elegans*. Devel Biol 100: 64–119 (1983).
78. Taiz L: Plant cell expansion: regulation of cell wall mechanical properties. Annu Rev Plant Physiol 35: 585–657 (1984).
79. Taylor RL: The foliar embryos of *Malaxis paludosa*. Can J Bot 45: 1553–1556 (1967).
80. van Engelen FA, de Vries SC: Extracellular proteins in plant embryogenesis. Trends Genet 8: 66–70 (1992)
81. van Engelen FA, de Vries SC: Secreted proteins in plant cell cultures. In Roubelakis-Angelakis KA (ed) Markers of Plant Morphogenesis. NATO-ASI series, Plenum, New York (in press).
82. van Holst GJ, Klis FM: Hydroxyproline glycosides in secretory arabinogalactan proteins of *Phaseolus vulgaris* L. Plant Physiol 68: 979–980 (1981).
83. van Holst GJ, Klis FM, de Wildt PJM, Hazenberg CAM, Buijs J, Stegwee D: Arabinogalactan protein from a crude cell organelle fraction of *Phaseolus vulgaris* L. Plant Physiol 68: 910–913 (1981).
84. Vasil V, Redway F, Vasil IK: Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L. Bio/technology 8: 429–434 (1990).
85. Webb MC, Gunning BES: Embryo sac development in *Arabidopsis thaliana*. I. Megasporogenesis, including the microtubular cytoskeleton. Sex Plant Reprod 3: 244–256 (1990).
86. Wernicke W, Brettell R: Somatic embryogenesis from *Sorghum bicolor* leaves. Nature 287: 138–139 (1980).
87. Williams EG, Maheswaran G: Somatic embryogenesis: Factors influencing coordinate behavior of cells as an embryogenetic group. Ann Bot 57: 443– 462 (1986).
88. Zaki MAM, Dickinson HG: Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma 156: 149–162 (1990).
89. Zaki MAM, Dickinson HG: Microspore-derived embryos in Brassica: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex Plant Reprod 4: 48–55 (1991).