Embryo forming cells in carrot suspension cultures

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Stellingen

1. Cellen die zich ontwikkelen tot somatische embryos kunnen morfologisch niet geïdentificeerd worden.

2. Tot nu toe is SERK de enige marker voor vroege stadia van somatische embryogenese.
   Schmidt et al. Development, in press.

3. Embryogene cel clusters zijn alleen embryogeen als ze zich ook tot een embryo ontwikkelen.

4. Embryogenese is een reactie op de drang der omstandigheden.
   Touraev et al. (1996) Planta **200,** 144-152

5. Gezien de grote variatie in biologische effecten van verschillende arabinogalactan eiwitten is het noodzakelijk deze eiwitten te classificeren op grond van hun biologische activiteit.
   Baldwin et al. (1993) Plant Physiol. **103,** 115-123; Komalavis et al. (1991) J. Biol. Chem. **266,** 15956-15965; Li et al.(1996) Plant Mol. Biol. **32,** 641-652; Lind et al. (1994) Plant J. **6,** 491-502; Pogson and Davis (1995) Plant Mol. Biol. **28,** 347-352

6. Expressiepatronen van de CaMV 35S promoter zijn altijd weer verrassend.
   Clapham et al. J. Exp. Bot. **46,** 655-662; Mascarenhas and Hamilton, Plant J. **2,** 405-408

7. Het feit dat automobilisten ondanks de vele files de auto prefereren boven het openbaar vervoer geeft aan dat de kwaliteit van het openbaar vervoer nog steeds te wensen overlaat.

8. De wens is de vader van het resultaat.

Stellingen behorende bij het proefschrift:
'Embryo forming cells in carrot suspension cultures'
door Marcel Toonen, te verdedigen op 11 april 1997

voor mijn ouders

Contents

Scope

Somatic cells of many plant species can be cultured *in vitro* and induced to form embryos that are able to develop into mature plants. This process, termed somatic embryogenesis, was originally described in carrot (*Daucus carota* L.). Somatic embryos develop through the same characteristic morphological stages, i.e. the globular-, heart- and torpedo-stage respectively, as their zygotic counterparts. Due to the different cellular origin of somatic embryos, it is less clear to what extent the earlier pre-globular stages of somatic embryos resemble corresponding stages in zygotic embryo development. In part, this is due to a lack of a precise morphological description of this less defined stage of somatic embryo development. The current stage of these and other, more general aspects of early somatic and zygotic embryo development, are discussed in chapter 1.

While the single cell origin of some carrot somatic embryos has been reported, a more elaborate morphological description of a representative number of such single embryo-forming cells and their early development has been lacking, so far. To describe cells that are in the process of becoming embryogenic, yet still require an externally applied stimulus, the term competent cell has been introduced. Embryogenic cells can develop into somatic embryos in the absence of an externally applied stimulus. In chapter 2 experiments are presented that show the development of many individual single competent carrot suspension cells into somatic embryos employing a cell tracking system. The capability to develop into somatic embryos appeared not to be restricted to a particular cell type distinguishable on cell morphology. In general, oval and elongated cells developed via asymmetrically shaped cell clusters while spherical cells developed via symmetrically shaped cell clusters into somatic embryos. Cells initially more variable in form developed into somatic embryos via aberrantly shaped cell clusters. These results show that the initial form of the cell and subsequent division patterns can be widely variable and yet lead to complete somatic embryos capable of developing into plants.

Based upon previous findings that the monoclonal antibody JIM8 recognises a particular type of single cells only present in embryogenic carrot cell cultures, it was postulated that the JIM8 epitope could be used as a marker for competent and embryogenic cells. The cell tracking system was adapted to study the development of cells labelled with JIM8 in order to determine the reliability of this marker. In chapter 3 it is shown that only few of the single cells developing into somatic embryos reacted with the JIM8 antibody, while most of the embryos developed from cells not labeled with the JIM8 antibody. It was therefore con-

cluded that the JIM8 cell wall epitope reflects embryogenic competence in a cell population rather than competence of individual cells.

Stimulation of somatic embryogenesis in carrot and other species, by the addition of arabinogalactan proteins (AGPs) to the culture medium has been reported previously. In chapter 4 experiments are presented that show that carrot-seed AGP fractions purified by affinity chromatography with ZUM18 monoclonal antibodies do not increase the number of somatic embryos developing in embryogenic cell cultures. An AGP fraction purified with the JIM8 antibody even decreased the number of somatic embryos. Low-embryogenic carrot suspension cultures treated with carrot-seed AGPs did show an increased frequency of embryo development after removal of vacuolated cells and enrichment for cell clusters. These results suggest that complex cell cell interactions, mediated in part by AGPs, occur in embryogenic cultures.

The cell tracking system was also adapted to allow detection of the expression of bioluminescent reporter genes. In chapter 5 the expression of the firefly luciferase coding sequence under control of the *Arabidopsis thaliana* lipid transfer protein 1 (*AtLTP1*) promoter during carrot somatic embryo development is described. The carrot lipid transfer protein EP2 is expressed during protoderm formation and has been used as a molecular marker for embryogenic competence and somatic embryos. The cell tracking experiments on *AtLTP1* luciferase transformed cultures showed that *AtLTP1* expression is correlated with somatic embryo formation, but that not all clusters that express *AtLTP1* developed into somatic embryos. *AtLTP1* expression therefore is a good marker for embryogenic cell clusters, but it is not completely specific.

In chapter 6 a technical description is given of the cell tracking system and the several detection systems connected to it, as applied in the preceding chapters. In chapter 7 the relevance of cell tracking to study somatic embryo development and the implications of the described results on future research are discussed.

1

General introduction

Plant embryogenesis is an unique process in the sense that it can be started not only from the fertilized egg cell but can also be initiated from other cells of the reproductive apparatus and even from somatic cells. One of the challenges of this field is therefore to unravel the molecular mechanisms that lead to the formation of a cell destined to form an embryo. A second important area of research is to determine the molecular basis of pattern formation in the embryo, a process that results in a stereotyped organisation of a seedling. The pattern formation process in plant embryos has to cope with two seemingly paradoxical requirements. On the one hand precisely arranged tissue organisation has to be established and on the other hand sufficient flexibility in adult tissues must be maintained to allow continuous formation of new meristems in an ordered fashion.

In this chapter recent work that employs a variety of experimental systems that range from genetic dissection of pattern formation in the zygotic embryo, androgenesis and *in vitro* fertilisation to somatic embryogenesis will be summarised. While each of these systems highlights a different aspect of embryogenesis, they can be mutually beneficial in helping to understand the making of the plant embryo.

Andreas P. Mordhorst, Marcel A.J. Toonen, Sacco C. de Vries
Plant Embryogenesis, Crit. Rev. Plant Sci., in press

Introduction

The plant embryo is characterised by a stereotyped structure thought to be arranged in a number of elements along an apical-basal or longitudinal axis and along a radial axis. From bottom to top the body pattern elements of a dicot embryo consist of the embryonic root including the root cap and the root meristem, hypocotyl, cotyledons and the shoot apical meristem. In radial fashion, from the outside to the inside, the epidermis, ground tissue and central vascular system are the main tissue types (Jürgens, 1995). Plant zygotic embryogenesis spans the period of plant development that ranges from the fertilised egg cell, the zygote, to the mature desiccated embryo present in a protective seed. While zygotic embryogenesis, by definition, is dependent on fertilisation, the zygote is not the only constituent of the embryo sac or female gametophyte that has this property. Evidence for embryo development *in vivo* without fertilisation comes from studies showing so-called apomictic embryos in certain plant species. These apomictic embryos can have a variable origin ranging from the female gametophyte itself and including the unfertilised egg cell (parthenogenesis) to adventitious embryogenesis initiated from the surrounding maternal tissue (Koltunow, 1993). Even cells of the mature plant body, not in direct contact with the female gametophyte can spontaneously form embryos (Yarbrough, 1932; Taylor, 1967).

Under *in vitro* conditions plant embryos can develop from microspores (androgenesis) after a variety of inducing treatments depending on the species (Ferrie et al., 1995b) while it is now also possible to produce embryos from *in vitro* fertilised egg cells (Kranz and Dresselhaus, 1996). Finally, tissue cultured cells, first shown in carrot (Reinert, 1959) and later in many different species can be induced to form so-called somatic embryos. An important question concerns the molecular basis of the formation of the single cell destined to produce the embryo. While the zygote is destined to develop into an embryo and could therefore be defined as an 'embryogenic' cell, it is less clear in other forms of embryogenesis what changes a cell must undergo in order to become an embryogenic cell, capable of forming an embryo. Therefore, in the apparent absence of a single universally applicable signal that renders cells embryogenic, the unravelling of the molecular mechanisms that underlie the process of embryogenic cell formation is a prime area of interest in plant embryogenesis and one that is so far the exclusive domain of the *in vitro* forms of embryogenesis. In all forms of embryogenesis the same stages are seen as in zygotic embryogenesis. Once an embryo is established as such, it appears therefore safe to assume that the mechanisms of pattern formation that lead to the zygotic embryo is used in all other forms of embryogenesis as well. The genetic dissection of this process, so far mainly in *Arabidopsis*, maize

(*Zea mays*) and in rice (*Oriza sativa*) is therefore likely to yield genes that are also employed under *in vitro* conditions. Advances in particular areas of plant embryogenesis have been reviewed recently (Lindsey and Topping, 1993; Zimmerman, 1993; Goldberg et al., 1994; Ferrie et al., 1995b; Jürgens, 1995; Meinke, 1995; Thorpe, 1995; Kranz and Dresselhaus, 1996). This chapter will focus on the presentation of the different systems of embryogenesis and discuss recent advances. Clearly, one of the challenges of the future will be to combine and integrate these areas in order to gain a much better understanding of plant embryogenesis.

Embryogenesis *in vivo*

The entire sporophyte is produced by two apical meristems, the shoot apical meristem and the root meristem which are formed during embryogenesis as part of the apical-basal pattern. Also the other apical-basal as well as radial body pattern elements are generated during embryogenesis. Therefore, the basic plant body pattern is laid down during embryo formation as superimposition of the apical-basal pattern (order of embryonic organs) and the radial pattern (order of embryonic tissue layers; Jürgens, 1995). In this section a summary of the extensive morphological descriptions during the development of both the dicot and monocot zygotic embryo will be presented to provide a reference for the various experimental approaches aimed to study embryogenesis in plants. In particular the fact that the model of early *Arabidopsis* embryogenesis is now widely known, it is important to bear in mind that in other plant species variations in early cell division patterns exist, yet embryos with correctly organised apical-basal and radial axes develop.

Zygotic embryogenesis: descriptive studies in Dicotyledonae

The unfertilised egg cell as well as the zygote exhibit polarity along the micropylar-chalazal axis of the embryo sac. This is demonstrated by the unequal distribution of cytoplasm and vacuoles (Schulz and Jensen, 1968; Mogensen and Suthar, 1979). The double fertilisation event in flowering plants generates the diploid zygote and the triploid endosperm nucleus, the latter by fusion of the two polar nuclei of the central cell with the second sperm nucleus. The endosperm undergoes a complex series of developmental events and eventually will provide nutrients for the developing embryo and/or for the germinating seedling (for review see Lopes and Larkins, 1993).

Before the first division, the zygote elongates in most angiosperms in the micropylar-chalazal axis that correlates with the apical-basal axis of the future embryo. This elongation coincides which a re-orientation of microtubules to transverse cortical arrays (Webb and Gunning, 1991). In the majority of cases the first division is an unequal transversal division, resulting in two cells of different developmental fates. In general, the smaller apical cell, oriented towards the chalazal end of the embryo sac, will give rise to the embryo proper, while the larger basal cell, oriented towards the micropylar end, will develop into the extra embryonic suspensor. Nevertheless, considerable differences concerning the contribution of derivatives of both, apical and basal cell, to the embryo proper and suspensor, respectively, and the division patterns of the apical cell have been observed (figure 1.1). The apical cell can divide in either one of two perpendicular planes: transversal or longitudinal. Derivatives of the basal cell can contribute not only to the suspensor but in part to the embryo proper as well. On the other hand, derivatives of the apical cell can develop into the embryo proper and also form almost the complete suspensor (reviewed by Johansen, 1950; Maheshwari, 1950; Wardlaw, 1955). The plasticity of apparent cell lineages during plant embryogenesis led to the classification of 5 different embryonic types (Schnarf, 1929; Johansen, 1945; Raghavan and Sharma, 1995). All of these show a transversal division of the zygote (figure 1.1). A 6th embryonic type classifies species exhibiting an uncommon longitudinal or oblique division of the zygote rather than the transversal one (Maheshwari, 1950). Despite the mentioned differences embryos of all embryonic types develop through the same stereotyped stages of globular, heart and torpedo. In cotton (*Gossypium hirsutum*) this 'early plasticity' between various embryonic types is combined in one species. Even in the earliest stages of embryo development no regular division pattern could be determined (Pollock and Jensen, 1964). The 'early plasticity' may be of importance when interpreting some of the mutant phenotypes now observed in *Arabidopsis* embryogenesis (see: molecular genetic analysis).

The Onagrad (or Crucifer) type (figure 1.1) has become the classical example of dicot embryogenesis due to the work in *Capsella bursa-pastoris* (shepherd's purse; Hanstein, 1870; Souèges, 1914; Schulz and Jensen, 1968). The cell division pattern during the formation of the embryo body pattern has already been observed correctly more than 125 years ago, because "es musste endlich ermittelt werden, durch welche Zellgestaltungen überhaupt die ersten Differenzen zwischen Wurzel, Stamm und Blättern zu Stande kommen." ('it had finally to be determined through which cell organisation the first differences between root, stem and leaves are actually achieved'; Hanstein, 1870).

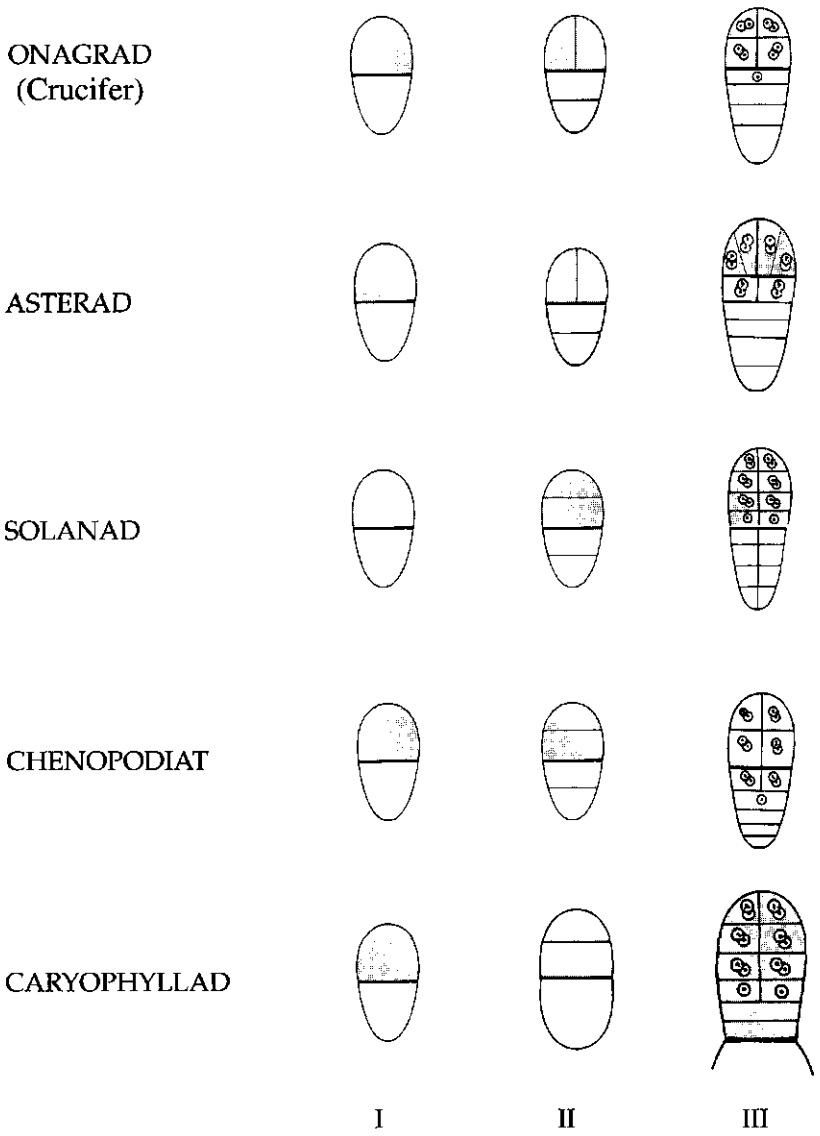

figure 1.1. Schematic overview over the 5 different embryonic types displaying a transversal division of the zygote. Representations demonstrate the zygote after the first (I), and the second division (II), and the early proembryo before periclinal divisions give rise to protoderm formation (III). While grey coloured cells represent derivatives of the apical cell, white coloured cells represent derivatives from the basal cell. Cells containing drawn nuclei (III) will contribute to the embryo, while cells not containing drawn nuclei will contribute to the suspensor. The presence of two nuclei in one cell indicate that one cell lays above and one beneath the drawing plane. Embryonic types are based on the classification of Schnarf (1929) and Johansen (1945). Figure adapted from Natesh and Rau (1984).

Arabidopsis, as member of the *Brassicacea*, follows similarly to *Capsella* the Crucifer embryonic type. The basal cell of the two-celled embryo divides by a series of transversal divisions and gives rise to a filamentous suspensor consisting of 7 - 9 highly vacuolated cells (figure 1.2d; Mansfield and Briarty, 1991). The uppermost lens-shaped cell, called hypophysis (figure 1.2f) this term was introduced by Hanstein (1870), contributes to the embryo by forming part of the root, the collumella root cap and the quiescent centre (Scheres et al., 1994). Development of the suspensor is complete at the globular stage. Subsequently suspensor cells undergo programmed cell death and are hardly visible at maturity. Because of the simple organisation of the suspensor the Crucifer embryonic type is considered to be a rather primitive one (reviewed by Wardlaw, 1955). In other species suspensors develop into haustoria-like organs, demonstrating their role for the uptake of nutrients (Yeung and Meinke, 1993).

The apical cell of the two-celled embryo undergoes two longitudinal divisions at right angles (figure 1.2c), followed by one transversal division (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). The latter plane of division or O' boundary divides the eight cell embryo (octant stage) into an upper an a lower tier (figure 1.2d). From the upper tier the shoot apical meristem and the main parts of the cotyledons are formed, while the lower tier contributes to the cotyledon shoulder, hypocotyl and part of the radicle (Scheres et al., 1994). Until the octant stage, there is a remarkable decrease in relative cell size (Mansfield and Briarty, 1991), bearing analogy with the cleavage divisions characteristic of the early mammalian embryo. Periclinal divisions of all cells of the octant stage embryo lead to the dermatogen stage (figure 1.2e; Jürgens and Mayer, 1994). The formation of each 8 cells of an outer cell layer (protoderm) and of an inner cell group are the first visible signs of radial pattern formation. The protoderm will then be formed by continued anticlinal divisions and develop into the epidermis of the entire embryo (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). The central cells divide again in longitudinal and transversal directions and contribute to the innermost procambium tissue and the parenchymal ground tissue. Together with the protoderm, three concentric tissue layers are thus established that make up the three radial pattern elements of the embryo. The radial pattern is established in a preliminary form when the embryo reaches the mid-globular stage (approximately 64 cells). At the following triangular stage (Jürgens and Mayer, 1994), during the globular-heart transition, the embryo shifts from a radial to a bilateral symmetry as observed by the formation of juxtaposed cotyledon primordia at the apical side of the embryo. At heart stage also the hypocotyl region becomes visible due to cell elongation (figure 1.2g). At the same stage, the root meristem initials are defined. The root meristem performs a few cycles of divisions, similar to the division pattern seen in the seedling (Scheres et al., 1996).

With the completion of the apical-basal pattern in the form of cotyledons, hypocotyl and radicle the body plan of the seedling is essentially finished in the heart shaped embryo (Jürgens and Mayer, 1994). The subsequent torpedo shaped embryo (figure 1.2h) is a result of cell elongation and expansion rather then continued division. Accumulation of starch and other storage products are characteristic of this phase in embryo development. Cells belonging to the shoot apical meristem can now for the first time be distinguished from surrounding cells due to the lack of starch accumulation. Histologically, the shoot apical meristem therefore does not appear before the root meristem is nearly fully formed and functional (Barton and Poethig, 1993). At maturity the shoot apical meristem is relatively undeveloped because leaf primordia are not yet visible. The cotyledons expand further and are finally folded backwards (cotyledonary stage; figure 1.2i). Metabolic activity decreases and the whole seed, including the embryo, undergoes desiccation and finally becomes dormant. After germination, post-embryonic development ensues and the embryo develops into a seedling with two active apical meristems.

While one of the themes in this chapter will be the analogy that may exist between embryos of different origin, it is of interest to discuss zygotic embryogenesis in carrot (*Daucus carota*), the model plant for somatic embryogenesis. Carrot follows the Solanad embryonic type (figure 1.1; Borthwick, 1931) and shows a different pattern in the first divisions. After elongation of the zygote the first division is asymmetric as in Crucifers (figure 1.2j). In contrast to *Arabidopsis*, the apical cell undergoes two transversal rather than longitudinal divisions resulting in a 4-celled 'filamentous' embryo proper (figure 1.2l; Borthwick, 1931; Lackie and Yeung, 1996). The orientation of the subsequent division planes is less regular than in *Arabidopsis*. The 4 filamentous embryo proper cells divide twice longitudinally, and form a 16-celled embryo proper with 4 cell tiers (figure 1.2m). The protoderm is then generated by periclinal divisions (figure 1.2n; Borthwick, 1931; Lackie and Yeung, 1996) one division cycle later compared with *Arabidopsis*. While the number of cells in the carrot embryo is larger than in the comparable stage of *Arabidopsis* embryos, after this stage, the development is very similar to that described for the Crucifer type (figure 1.2o-r).

Zygotic embryogenesis: descriptive studies in *Monocotyledonae*

A high degree of variation in the formation of the embryo is also found in monocots. Members of the *Orchidacea* produce spherical or club-shaped embryos without any visible signs of organ or tissue differentiation (Johansen, 1945). On the other hand, the most advanced type of embryonic development in plants is found in the *Poacea*, characterised by the development of special structures such as the

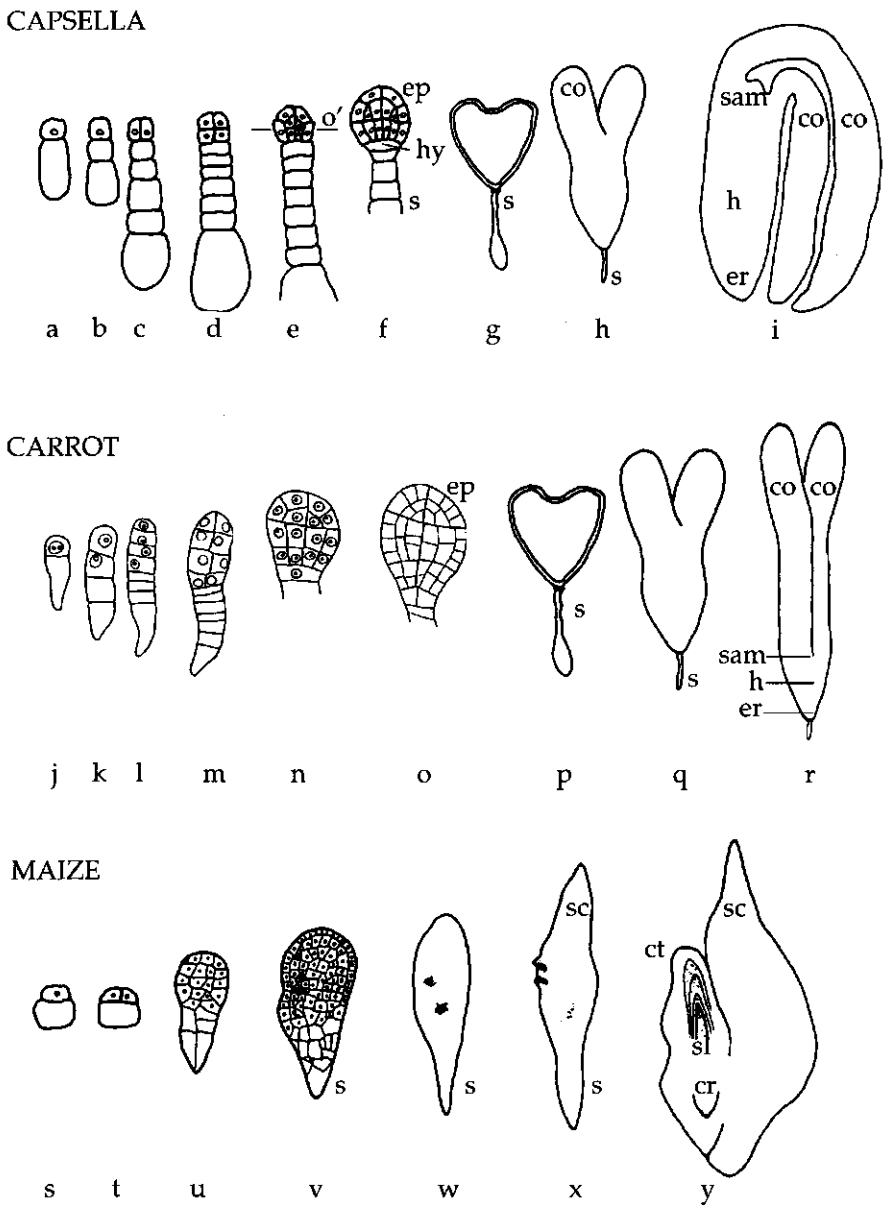

figure 1.2a-y. Schematic representation of: **a-i.** dicot zygotic embryo development of the Onagrad (Crucifer) type in *Capsella bursa-pastoris*, **j-r.** Solanad type in carrot, **s-y.** monocot zygotic embryo development in maize. Cells containing drawn nuclei at early stages will contribute to the embryo, while the cells without drawn nuclei will contribute to the suspensor. Abbreviations: co, cotyledon; ct coleoptile; cr, coleorhiza including embryonic root; er, embryonic root; ep, embryo proper; h, hypocotyl; hy, hypophysis; s, suspensor; sam, shoot apical meristem; sc, scutellum; sl, shoot apical meristem plus leaf primordia. Figure adapted from Borthwick (1931) and Lindsey and Topping (1993).

scutellum (homologous to a single cotyledon), coleoptile, coleorhiza and the presence of several leaf primordia at maturity. Early *monocotyledonae* embryo development will be described based upon studies in *Poa annua* (Souèges, 1924), maize (Randolph, 1936; Van Lammeren, 1986) and barley (*Hordeum vulgare*; Norstog, 1972; Engell, 1989).

As in dicots the monocot egg cell and zygote have a polarised organisation (Faure et al., 1993; Kranz et al., 1995). Also the first division of the monocot zygote is an asymmetric transversal division, giving rise to a small apical and a larger basal cell (figure 1.2s). This division plane stands perpendicular to the longitudinal axis of the future proembryo. In maize the apical cell divides first with a longitudinal division (figure 1.2t), creating a 3-celled embryo. While the subsequent divisions are irregular in maize and clear clonal relationships of cells have not been established (Randolph, 1936), early cell divisions seem to be more regular in barley (Norstog, 1972; Engell, 1989). In the resulting club-shaped embryo of the transition stage a characteristic gradation of cell size with small and cytoplasm-rich apical cells and large and vacuolated basal cells is visible (Randolph, 1936). The 'embryo proper region' is marked by the presence and the 'suspensor region' by the absence of a protodermal cell layer (figure 1.2v). In maize, six to seven days after fertilisation, cells of the subdistal region begin to divide actively on the side facing away from the endosperm (Van Lammeren, 1986). The peripheral shoot meristem becomes visible as an initially inconspicuous indentation (figure 1.2w). Around the shoot meristem the coleoptilar ring is being formed (figure 1.2x). At the same time the formation of the root meristem begins internally. In contrast to dicots, both meristems are laid down in lateral fashion rather than distally. As a result, the axis of the mature embryo does therefore not correspond to the axis of the proembryo. The distal region above the shoot meristem greatly expands to form the scutellum adjacent to the endosperm (figure 1.2x-y). Prior to embryo maturity the shoot meristem has developed three to five leaf primordia apart from the coleoptile, demonstrating a more advance developmental stage at maturity when compared to dicotyledoneous embryos. As in dicots the last steps of embryogenesis are a decrease in metabolic activity followed by desiccation.

Zygotic embryogenesis: molecular-genetic analysis

The genetic dissection of zygotic embryogenesis follows similar approaches that have proven successful in the isolation of genes that control flower development in *Arabidopsis* and *Anthyrrinum* (Yanofsky, 1995). Mutant screens have been performed after chemical and x-ray mutagenesis, as well as by insertion mutagenesis employing T-DNA from *Agrobacterium* or transposable elements (Ac-Ds, En-I/

Spm) from maize; the latter tags facilitate the cloning of the gene. But also positional cloning of an EMS-mutant in *Arabidopsis* is increasingly more efficient with the availability of YAC and cosmid contig libraries. An elegant further development of transposon mutagenesis is the incorporation of enhancer, promoter or gene traps and visible markers into the insertion element (Topping et al., 1994; Topping and Lindsey, 1995; Sundaresan, 1996). In addition to being efficient mutant screens, valuable cell and tissue-specific marker lines have been generated by this way. One example is the cloning of the *PROLIFERA* gene that was identified by gene trap mutagenesis (Springer et al., 1995). In this section, the different screening strategies employed, some of the mutant phenotypes obtained and finally the function of several recently cloned genes involved in embryogenesis will be discussed. While most screens have been done in *Arabidopsis*, also maize, rice and *Petunia* have produced series of embryo mutants.

Immature *Arabidopsis* siliques on selfed M1 plants were screened for the presence of 25 % defective seeds (earlier designated as aborted seeds or embryo lethals; Meinke and Sussex, 1979). Such screens yielded many classes of mutant embryos arrested at different stages of embryo development. Further phenotypes recovered show distorted or fused cotyledons, abnormal suspensors, different size or colour of the embryo or seed or other abnormalities (Meinke, 1985; 1995). A genetic map of more then 100 embryo defective (emb) mutations has been presented (Franzmann et al., 1995). Many of the early arrested embryo mutants are likely to be affected in genes coding for general functions (Meinke, 1995). One such example is the *bio1* mutant (Shellhammer and Meinke, 1990) that can be rescued by culturing in the presence of biotin or biotin precursors (Schneider et al., 1989). The *bio1* mutation could also be complemented with an *Escherichia coli* biotin biosynthetic gene (Patton et al., 1996). Other mutants in this collection are likely to be involved in regulatory functions such as *suspensor* (*sus*; Schwartz *et al.*, 1994), *fusca* (*fus*; Castle and Meinke, 1994; Miséra et al., 1994) and *leafy cotyledon* (*lec*; Meinke et al., 1994) mutants. In mutant *twin* embryos, viable secondary embryos are occasionally produced from the suspensor of the primary embryo (Vernon and Meinke, 1994). Mutant *suspensor* embryos are arrested at the globular stage, while extranumeral divisions in the suspensor lead to a globular structure that is also arrested later on (Schwartz et al., 1994). A similar phenotype was observed in the *raspberry* mutant (Yadegari et al., 1994). These three mutants have been interpreted in the context of signals originating from the embryo proper and that normally suppress the developmental potential of suspensor cells. Both, *suspensor* and *raspberry* embryos, are arrested at the globular stage, yet they do exhibit cellular differentiation in the embryo proper and also in the modified mutant suspensors as judged by the accumulation of markers for maturation stage embryos such as lipid bodies and storage proteins (Schwartz et al., 1994; Yadegari et al., 1994). This

indicates that the expression of certain 'late' embryo genes is not dependent on the corresponding embryo morphology. The *SUS2* gene encodes a spliceosome assembly factor (Meinke, 1995), and this appears to be part of a more general function required not only in embryogenesis.

In maize, *defective kernel* (*dek*) mutants were obtained after pollen mutagenesis or from outcrosses with active *Mutator* plants (Neuffer and Sheridan, 1980; Clark and Sheridan, 1991; Scanlon et al., 1994). The mutants are grouped in several types: mutants that affect both the embryo and the endosperm, resulting in (i) a non-viable embryo or (ii) a viable embryo producing a mutant seedling, (iii) mutants affecting only the endosperm or (iv) only the embryo (Neuffer and Sheridan, 1980). The last class is also described as embryo lethal mutants, blocked at different developmental stages (Sheridan and Neuffer, 1980; Clark and Sheridan, 1991; Sheridan and Clark, 1993). One of the endosperm defective mutants has been shown to lack invertase activity that appeared to be important for normal development of not only the endosperm but also the surrounding maternal tissue (Miller and Chourey, 1992). At present it is not known how many of the *dek* genes code for regulatory genes essential for embryo development.

In *Arabidopsis*, screens were also performed at the seedling level to obtain viable mutants with changes in the apical-basal or radial body pattern (Jürgens et al., 1991; Mayer et al., 1991; Barton and Poethig, 1993; McConnell and Barton, 1995; Scheres et al., 1995). Genes identified in such screens were suggested to contribute to the formation of the body pattern during embryogenesis (Jürgens et al., 1991). A considerable number of mutations concerning the apical-basal pattern resulted in the deletion of one or more pattern element(s). The shoot apical meristem is absent in *shoot meristemless* (*stm*; Barton and Poethig, 1993), *pinhead* (*pnh*; McConnell and Barton, 1995), and *zwille* (*zll*) seedlings (Jürgens et al., 1994). Mutant *wuschel* (*wus*) seedlings display a similar phenotype as observed in *shoot meristemless*, *pinhead* and *zwille* (no direct formation of leaf primordia following germination) but in contrast to them few abnormal cells were present at the corresponding position of the shoot apical meristem forming a flat apex (Laux et al., 1996). Therefore meristem organisation rather then initiation seemed to be affected by the *WUSCHEL* gene (Laux et al., 1996). In *laterne* mutants cotyledons are precisely deleted (Mayer et al., 1991) and concomitant effects on the shoot apical meristem have been observed (Mayer et al., 1993a). Mutations in the *GURKE* (*gk*) gene resulted in a strong reduction or an elimination of the cotyledons (Mayer et al., 1991; Torres-Ruiz et al., 1996b). In strong *gurke* alleles the whole apex and sometimes also parts of the hypocotyl is deleted, while the root part appears not to be effected by the mutation (Torres-Ruiz et al. 1996b). The hypocotyl is deleted in *fackel* seedlings (Mayer et al., 1991), and mutant *monopteros* seedlings lack both,

hypocotyl and root, which are replaced by a basal peg attached to the cotyledons (Berleth and Jürgens, 1993).

Mutants affecting the formation of the embryonic root are *rootless* (Barton and Poethig, 1993), *hobbit, bombadil, gremlin* and *orc* (the 'hypophyseal cell group' mutants; Scheres et al., 1996). In *gnom* the formation of the apical as well as the basal parts is disrupted (sometimes fused cotyledons appear) resulting in a cone or ball shaped embryo (Mayer et al., 1991).

Apart from deletion also addition and replacement of pattern elements has been described. One, three or four cotyledons are found in the *altered meristem program* (*amp*) mutants (Chaudhury et al., 1993), *häuptling* mutant (Jürgens et al., 1991) and in the *monopteros* mutant (Berleth and Jürgens, 1993). The cotyledon number is variable in *fackel* and *fass* mutants (Mayer et al., 1991;Torres Ruiz and Jürgens, 1994). Transformation of cotyledons into shoots or leaves is seen in *toro* (Jürgens et al., 1991) and in *leafy coteledon* mutants (Meinke et al., 1994), respectively.

Phenotypic differences in several of these mutants have been traced back to the earliest visible deviation from wild-type during embryogenesis. *shoot meristemless* mutant embryos are at first distinguishable from wild type at the cotyledonary stage by the lack of the shoot apical meristem (Barton and Poethig, 1993). The effect of *fackel* mutants was visible as early as the heart stage by a broader embryo than wild type (Mayer et al., 1991). *gurke* mutant embryos could be first distinguished from wild type embryos at the triangular/early heart stage of embryogenesis. The apical part of developing *gurke* embryos does not properly widen caused by absent, perturbed or delayed divisions that initiate normally the cotyledon primordia (Torres-Ruiz et al., 1996b). *monopteros* mutants corresponding to the octant stage consist of 4 rather than 2 cell tiers (Berleth and Jürgens, 1993). A mutation in the *GNOM* gene results in a disturbed first zygote cleavage which is more symmetric rather than asymmetric (Mayer et al., 1993a). It is of interest to note that some of these mutants show division patterns found normally in other then the Crucifer embryonic type. For instance the irregular first division as well as the presence of 4 rather then 2 cell tiers at a stage comparable to the octant embryo are both characteristic for wild-type carrot zygotic embryos. In mutant *fass* embryos the initial embryonic divisions are aberrant, yet all pattern elements are developed (Torres Ruiz and Jürgens, 1994) which may point to mechanisms of pattern formation at a later, multicellular embryo stage. Torres Ruiz and Jürgens (1994) and Traas et al. (1995) have suggested that pattern formation does not require directed cell expansion and division plane alignment and is uncoupled from morphogenesis. In *Arabidopsis* mutant *ton/fass* plants the interface microtubules of roots are randomly oriented rather than in transverse arrays and preprophase bands are absent in root meristem and shoot apical meristem (Traas et al., 1995).

Therefore cell expansion is irregular and cell planes could not be aligned in specific orientations.

In order to analyse the morphogenic capacity of embryo defective mutants and to recover homozygous mutant plants, *in vitro* embryo rescue experiments were performed in *Arabidopsis* (Baus et al., 1986; Franzmann et al., 1989) and maize (Sheridan and Neuffer, 1980). These studies can also be employed to select for auxothrophic mutants, such as *bio1* (Schneider et al., 1989), or to try and answer the question whether the function of a particular mutated gene is embryo specific and can be circumvented by *in vitro* organogenesis. Franzmann et al. (1989) showed that embryos arrested at early stages of development seem to have more fundamental defects in morphogenesis than embryos arrested at later stages. From some defected embryos at later stages it was possible to regenerate flowering plants with 100 % defective seeds. Only the function of one gene (*EMB24*) seemed to be embryo specific (Franzmann et al., 1989). Similar experiments show that *gnom* seedlings are unable to produce shoots or even roots in culture but are able to proliferate as callus (Mayer et al., 1993a) or as fast growing cell suspension (Mordhorst et al., submitted). Root segments of *Arabidopsis* are able to regenerate shoots *in vitro* by organogenesis (Valvekens et al., 1988). Mutant *shoot meristemless* roots which are unaffected by the mutation, fail to regenerate adventitious shoots in culture, and only produce abnormal leaves (Barton and Poethig, 1993). Similar results were obtained with mutants belonging to the 'hypophyseal cell group', in which the formation of the embryonic root is disturbed. The mutant seedlings were not able to form a functional root *in vitro* (Scheres et al., 1996). These experiments reveal that these gene functions are required for both embryonic and non-embryonic shoot and root formation and seem therefore not to be embryo-specific. In mutant *pinhead* seedlings normal adventitious shoots can be regenerated *in vitro* from roots as well as from the cotyledonary axis, suggesting that the *PINHEAD* gene product is specifically required for embryonic shoot apical meristem initiation and not for post-embryonic meristem maintenance (McConnell and Barton, 1995). The same conclusions have been drawn for the *ZWILLE* gene. Mutant *zwille* seedlings originally lacking the shoot apical meristem are able to form secondary shoots (Jürgens et al., 1994). *monopteros* mutant seedlings are also able to regenerate adventitious roots *in vitro* revealing that *MONOPTEROS* gene function is not essential for root development in general, but for the embryonic organisation of the basal region of the embryo (Berleth and Jürgens, 1993). A similar argument holds for the *radicleless* mutants in rice, which are able to grow after germination because of the formation of adventitious roots *in vivo* (Hong et al., 1995).

The capacity of *monopteros* seedlings to form adventitious roots and to develop mutant plants was used to study post-embryonic effects of the *MONOPTEROS* gene (Przemeck et al., 1996). While the MONOPTEROS gene function is

not required for the formation of all major organs in the adult plants, post-embryonic MONOPTEROS functions are revealed by the presence of abnormal flowers, reduced or absent veins in leaf laminae, and not oriented, improperly aligned or isolated vessel elements in mutant *monopteros* plants. Furthermore, the polar auxin transport in inflorescence axis was reduced (Przemeck et al., 1996). This defects are discussed in the sense that the MONOPTEROS gene product is involved in axialisation in plant development possibly mediated by a canalised shoot-to-root signal flux in which polar auxin flux might play a role (Przemeck et al., 1996). The post-embryonic developmental potential of *gurke* seedlings was also analysed in culture. While strong alleles failed to develop further or only produced leaf-like structures, weak alleles developed abnormal leaves and stems and eventually abnormal flowers (Torres-Ruiz et al., 1996b). These observations suggest that apart from organising the apical region during embryogenesis the *GURKE* gene may also be involved in post-embryonic development (Torres-Ruiz et al., 1996b).

Two genes, *EMB30/GNOM* and *SHOOT MERISTEMLESS* have been cloned now that appear to be involved in the apical-basal pattern. The *gnom* mutant turned out to be allelic to the embryo defective mutant *emb30* (Mayer et al., 1993a; Shevell et al., 1994). The *EMB30/GNOM* gene has been cloned from a T-DNA tagged mutant line (Shevell et al., 1994) as well as by positional cloning (Busch et al., 1996). A region of the encoded protein has similarity to the Sec7 domain of yeast. Sec7 is a cytosolic protein linked to the Golgi apparatus and involved in secretory pathways (Shevell et al., 1994). *EMB30/GNOM* is expressed throughout the entire plant and proposed to be involved in cell division, elongation and cell adhesion during the whole life cycle of the plant. Surprisingly the gametophytic generation is not affected as inferred from the presence of 25 % mutants seeds from heterozygous plants (Shevell et al., 1994). The data suggest that the *GNOM* gene seems to be not only involved in asymmetric divisions (Mayer et al., 1993a) but in divisions in general with the first visible effect in the mutant at the first zygote cleavage. Busch et al. (1996) identified another yeast coding sequence, *YEC2*, which product shows a higher similarity to the GNOM protein than Sec7. The deletion of the *YEC2* gene in haploid yeast cells by homologous recombination did not affect cell viability, like the mutation in the *GNOM* gene (Busch et al., 1996). The precise role of the GNOM gene product in apical-basal pattern polarity remains to be determined. Judged by the expression of the *AtLTP1* marker gene, *gnom* embryos can exhibit normal or no apical-basal polarity, and in a number of cases even an inverted polarity (Vroemen et al., 1996). The radial body pattern in *gnom* embryos remains unchanged (Mayer et al., 1991; Vroemen et al., 1996) supporting the hypothesis that independent mechanisms lead to the formation of the apical-basal and radial axes of polarity.

The *SHOOT MERISTEMLESS* gene encodes a class 1 KNOTTED-like protein (Long et al., 1996). The *KNOTTED* class of genes encode homeodomain-containing proteins that have a function in the shoot meristem. *In situ* hybridisation showed expression of *SHOOT MERISTEMLESS* as early as the mid-globular stage in a few cells at a position predicted to form the embryonic shoot apical meristem, yet long before the visible presence of the shoot apical meristem at the torpedo stage. The *SHOOT MERISTEMLESS* gene remains expressed during meristem formation and also post-embryonically for as long as the shoot apical meristem is active (Long et al., 1996). The results support the observation from the *in vitro* culture experiments that suggested SHOOT MERISTEMLESS function is required for initiation as well as maintenance of the shoot apical meristem (Long et al., 1996). Corresponding data have been presented for the expression of the homologue *KNOTTED1* gene in maize. *KNOTTED1* expression is temporally and spatially coincident with first histologically visible signs of shoot meristem formation during embryogenesis and the expression is continuated throughout post-embryonic shoot meristem development (Smith et al., 1995).

Genes involved in the establishment of the radial pattern have been described as the *KNOLLE* and *KEULE* genes of *Arabidopsis*. *knolle* seedlings lack a well formed epidermis and are also characterised internally by enlarged cells and incomplete cell walls (Mayer et al., 1991; Lukowitz et al., 1996). *knolle* embryos are unable to perform periclinal divisions at the octant stage so that the formation of the protoderm fails. In contrast to the wild type development, divisions in *knolle* also appear more randomly (Lukowitz et al., 1996). The initial lack of the radial pattern is revealed by a uniform expression of the *AtLTP1* gene, normally restricted in its expression to the protoderm (Vroemen et al., 1996). Cloning of the *KNOLLE* gene revealed similarity of the predicted KNOLLE protein to syntaxins, a group of proteins involved in vesicular trafficking (Lukowitz et al., 1996). *In situ* hybridisation revealed that *KNOLLE* mRNA accumulates in single cells or small groups in a 'patchy' pattern of cells throughout the wild-type embryo from the octant stage onwards. The *KNOLLE* gene product is likely to be involved in cytokinesis (Lukowitz et al., 1996), its disruption leads to incomplete cytokinesis with groups of interconnected cells, resulting in the failure to specify internal cells with a different cell fate from the outer cells of the octant embryo. Most likely, the KNOLLE protein does not convey specific information for radial patterning. Additional, KNOLLE independent mechanisms are involved in radial patterning as well, because provascular tissue is differentiated in *knolle* embryos (Mayer et al., 1991) and *LTP* mRNA is also excluded from central regions of *knolle* embryos at later stages (Vroemen et al., 1996).

In *keule* seedlings the morphology of the outermost cell layer is affected and consists of bloated and irregular arranged cells, while ground tissue and vas-

cular strands appear to be normal (Mayer et al., 1991; Vroemen et al., 1996). Mutant embryos have large multinuclear cells characterised by interrupted cell walls as well as wall stubs (Assaad et al., 1996). Cell division seemed to be slower compared to wild type and the plane of division is often disorientated. The detailed analysis of the *keule* mutant embryos suggests that this gene is also involved in cytokinesis (Assaad et al., 1996). From both, *knolle* and *keule* mutant seedlings, slowly growing callus could by obtained in tissue culture experiments, but shoot or root regeneration was not possible (Assaad et al., 1996).

Another group of genes (*WOODEN LEG, GOLLUM, PINOCCHIO, SCARECROW,* and *SHORTROOT*) affect the radial pattern organisation of the embryonic axis and the resulting primary root (defects in pericycle, vascular tissue, endodermis, cortex; Scheres et al., 1995). The gene activity in all these cases is not restricted to the embryo or the primary root, also secondary roots and roots regenerated via callus display the same phenotype. It is suggested that the formation of the radial pattern during embryogenesis and during meristematic activity of the root meristem is controlled to a large extent by the same genetic information (Scheres et al., 1995).

Another class of mutants has been described as shape mutants in which the seedling shape is altered in a particular way, yet a complete body pattern is formed (Mayer et al., 1991). Four of such mutants were grouped in this class, *knopf, mickey, fass* and *enano* (Mayer et al., 1991; Torres Ruiz and Jürgens, 1994). The alteration of the seedling shape is reflected in alterations of the cell shape (Torres-Ruiz et al. 1996a). Seedling phenotypes range from growth retardation (*mickey, enano*) to an extreme compression along the axes of the body organs (*fass, knopf*; Mayer et al., 1991; Torres-Ruiz and Jürgens, 1994; Torres-Ruiz et al., 1996a).

Embryo pattern mutation have also been described in other species. The *no apical meristem* (*nam*) mutant seedlings in *Petunia* lacks a shoot apical meristem and resembles in this aspect the *stm* mutation in *Arabidopsis* (Souer et al., 1996). Nevertheless, because *NAM* encodes a different type of protein and displays a different expression pattern than *SHOOT MERISTEMLESS*, meristem formation must be affected by different mechanisms. The *cytokinesis-defective* (*cyd*) mutant in pea (*Pisum sativum*) shares features with the *keule* mutant in *Arabidopsis*, namely multinucleate cells with cell wall stubs in the cotyledons (Liu et al., 1995). Like in the *keule* mutant (Assaad et al., 1996) the cytological phenotype could be mimicked in wild-type cells with caffeine treatments. The *CYD* gene is therefore supposed to be also involved in cytokinesis (Liu et al., 1995). In rice, Nagato et al. (1989) and Hong et al. (1995) described embryo mutants that show deletion of certain pattern elements. Disruption of at least 4 different loci (*shootless 1* to *4*) cause a deletion of the shoot primordium and disruption of 1 locus causes a deletion of the radicle (*radicleless*; Hong et al., 1995). In the mutant *variable embryo*

phenotype 3 a multiplication of radicles by the deletion of the apical regions has been observed similarly to *doppelwurzel*. Another group of mutants show modified positions of organs including the most remarkable mutant *various embryo phenotype 2* with a reversed (rotated by 180°) apical-basal pattern (Hong et al., 1995). Apart from the reversal of marker gene expression in *gnom* mutant embryos, mutants affecting the spatial order of apical-basal pattern elements in this way have not been described in *Arabidopsis*.

In conclusion, it appears that most of the genes that result in embryo phenotypes and that have been cloned cause rather severe pleiotrophic phenotypes with considerable alterations at the cellular level. It is not clear in most cases how the observed cellular changes relate to the morphology of the embryo or seedling (Mayer et al., 1991, 1993b; Schwartz et al., 1994; Shevell et al., 1994; Yadegari et al., 1994; Lukowitz et al., 1996). It does seem to be clear that the assumption that the most severe embryo or seedling phenotypes are the result of very early acting regulatory genes (Mayer et al., 1993b) is not borne out by the presumed function of the genes identified so far.

Apomixis

Gametophytic development starts with the formation of nucellus tissue from the ovule primordium (figure 1.3). One sub-epidermal cell of the nucellus then differentiates into a megaspore mother cell which undergoes meiosis I and II to form four reduced megaspores during the polygonum type of embryo sac development. The functional megaspore, closest to the chalaza enlarges and the three microspores at the micropylar end degenerate. During megagametogenesis the functional megaspore undergoes three mitotic divisions, resulting in the coenocytic megagametophyte. Cell wall formation, nuclear migration and cell differentiation lead to the formation of an eight celled embryo sac which contains three antipodal cells at the chalazal pole, two synergids and one egg cell at the micropylar pole and two polar nuclei in the centre (figure 1.4; reviewed by Reiser and Fischer, 1993). Double fertilisation of the egg cell and central cell are the first processes leading the development of the embryo and the endosperm.

However, in a substantial number of species embryo development in the ovule occurs without fertilisation of the egg cell. Three different forms of this process, diplospory, apospory and adventitious embryogenesis are collectively designated as apomixis (figure 1.3; reviewed by Koltunow, 1993; Sharma and Thorpe, 1995). Apomictic embryos develop from unreduced embryo sac, nucellus or inner integument cells and have the same genetic constitution as the mother plant. Apomictic processes can be initiated at several points during gametophytic de-

velopment. During the two forms of diplospory an unreduced embryo sac is formed from the megaspore mother cell. In the case of meiotic diplospory the megaspore mother cell differentiates from the nucellus and begins meiosis. Meiosis is inhibited at a particular stage by unknown mechanisms and the nucleus is restored to undergo mitosis (Bergman, 1950). During mitotic diplospory the megaspore mother cell does not enter meiosis at all and only undergoes mitotic divisions (Bergman, 1951; Leblanc et al., 1995b). In both forms of diplospory a functional embryo sac is formed consisting of unreduced cells. The unreduced egg cell can then develop into an asexual or apomictic embryo. It is so far not known which molecular events cause the arrest in meiosis in the gamethophytic development nor is it known which processes initiate embryo development from the unreduced egg cell. In diplospory a functional endosperm usually develops autonomously and does not require fertilisation of the central cell either.

In apospory additional embryo sacs that originate from nucellar cells are formed in the ovule. These cells, called aposporous initial cells differentiate via the three mitotic divisions characteristic for the development of the megaspore mother cell. As in diplospory the resulting embryo sacs consist of unreduced cells and the egg cells develop into an embryo without fertilisation. The embryo sac closest to the micropylar pole of the ovule is usually the one entered by the pollen tube and endosperm is formed after fusion of the second sperm cell with the central cell. In case of an aposporic embryo sac the sperm nucleus will fuse with only one of the unreduced nuclei giving rise to the triploid endosperm.

Adventitious embryogenesis starts from somatic tissues of the mature ovule, the nucellus and inner integument. Nucellar cells that are competent to develop into embryos are dense in cytoplasm and contain large nuclei. These cells morphologically resemble the developing megaspore mother cell and apospory initial cells but develop directly into an embryo (Esen and Soost, 1977; Naumova and Willemse, 1982). Normal fertilisation of the sexual embryo sac gives rise to a zygote and endosperm, leading to the formation of sexual and apomictic embryos in the same ovule which compete with each other.

Little is known about the initiation of apomixis. The frequency of the facultatively occurring apospory can be influenced by environmental conditions such as the photo period (Brown and Empry, 1958), temperature and other factors such as inorganic salts and nutrients. Timing of apomixis might also influence the occurrence of apomictic embryo development. Since the apomictic pathway leading to an embryo sac is generally faster than the sexual pathway apomictic embryos may have a head start compared to their sexual counterparts. In the case of diplospory meiosis of the megaspore mother cell is disturbed and would result in sterile plants, so apomixis may be used as an escape to allow viable seed development. In the case of apospory and adventitious embryogenesis embryos are formed

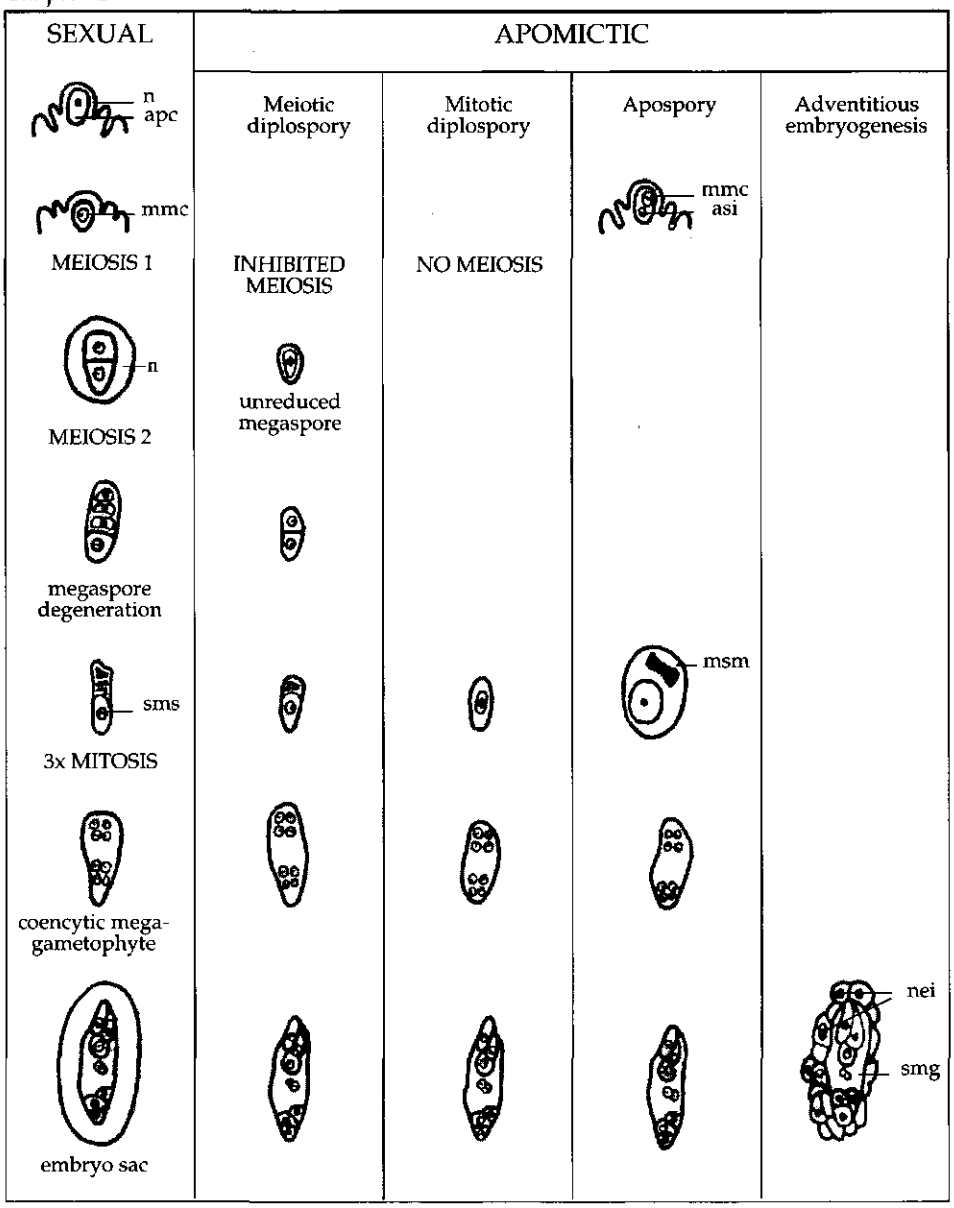

figure 1.3. Schematic representation of embryo sac development during sexual gametogenesis and the three apomictic forms: diplospory, apospory and adventitious embryogenesis. Abbreviations: apc, archesporial cell; asi, aposporous initial cell; mmc, megaspore mother cell; msm, megaspore mother cell or surviving megaspore; n, nucellus; nei, nucellar embryo initial cell; smg, sexual megagametophyte; sms, surviving megaspore. Figure addapted from Koltunow (1993).

from cells that normally do not form the embryo sac. During sexual ovule development one nucellar cell develops into the megaspore mother cell. The formation of additional cells resembling the megaspore mother cell in apospory may reflect a disturbance in the pathway that normally prevents gametophytic development in other cells of the nucellus. Elucidating this mechanism may also help to understand the sexual process better.

At the moment several strategies are applied to identify genes involved in the pathways leading to apomixis. One of these aims to obtain mutants in *Arabidopsis*. The screen employed is to mutagenise male sterile plants (such as *apetala* and *pistillata* mutants) and to select for viable seeds, that may be derived from a non-sexual reproduction event (Koltunow et al., 1995). Preliminary results show the identification of at least three of these *fertilisation independent seed* (*fis*) mutants (Chaudhury et al., 1996). Using a similar strategy a group of *fertilisation independent endosperm* (*fie*) mutants have been isolated. However it is not known whether the autonomous endosperm development in *fie* mutants is the same as in certain apomictic species (Ohad et al., 1996).

In the apomictic model system *Hieracium* comparative studies between mRNA populations derived from sexual and asexual siblings is expected to lead to the isolation of genes involved in the apomictic pathway (Koltunow et al., 1995). Analysis of the progeny of crosses between apomictic and sexual modes of reproduction have shown that the apomictic process is controlled by one single dominant locus (Parlevliet and Cameron, 1959). Based upon RFLP analysis a number of markers have been isolated to distinguish between sexual and apomictic derived embryos (Lubbers et al., 1994; Mazzucato et al., 1995). In *Tripsacum* three RFLP markers co-segregating with diplospory have been mapped to the same locus (Leblanc et al., 1995a).

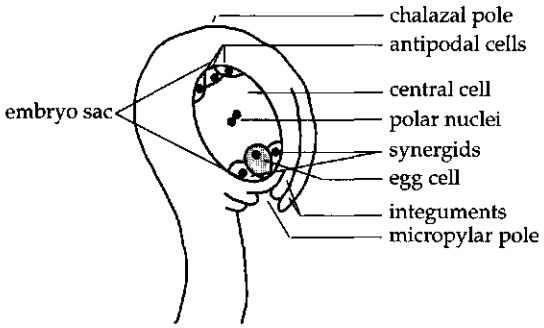

figure 1.4. Schematic representation of the ovule.

In plants that exhibit parthenogenesis, the reduced egg cell starts to divide spontaneously. In the absence of endosperm formation, embryo development is usually aborted at early stages of embryo development. If the same parthenogenetic process is induced by auxin treatment of the parthenogenetic plant, embryo development can proceed to the state of organ differentiation even in

the absence of endosperm development (San and Gelebart, 1986; Matzk, 1991; Ferrant and Bouharmont, 1994). The salmon system of wheat (*Triticum aestivum*) consists of three completely isogenic and homozygous plant lines. One of the lines can propagate sexual while the two other lines have a parthenogenic capacity of about 90 % (Matzk, 1991; 1995). Using 2D protein patterns a water soluble protein with a molecular mass of 50 to 60 kDa was identified that was specifically expressed in the ovaries of the parthenogenic lines (Matzk et al., 1995).

Embryogenesis *in vitro*

Besides the naturally occurring processes of embryogenesis described above, artificial experimental *in vitro* systems provide the opportunity to studying various additional aspects of embryogenesis, which will be highlighted in this section. It has been demonstrated that transfer of the fertilisation process and subsequent embryogenesis *in vitro* is possible in plants (*in vitro fertilisation*). Also gametophytic and somatic cells can be induced to undergo embryogenic development (androgenesis and somatic embryogenesis). The aim of this section is to outline recent progress in these areas of research, that so far have been quite removed from the molecular-genetic approaches as used to dissect zygotic embryogenesis.

In vitro fertilisation of single isolated gametes

Recently an experimental technique has been introduced in maize that allows to study the first events of embryogenesis without the surrounding maternal tissues in an experimental *in vitro fertilisation* system (reviewed by Kranz and Dresselhaus, 1996). Zygotes, created by *in vitro fertilisation* of single isolated gamete protoplasts divided initially (Kranz et al., 1991; Breton et al., 1995b) and were capable of development into seedlings and normal fertile plants (Kranz and Lörz, 1993). Cell divisions resulting in multicellular structures have also been obtained in wheat using a similar system (Kovács et al., 1995). Holm et al. (1994) and Mòl et al. (1995) were able to regenerate fertile plants of barley, wheat and maize, respectively, by isolating and culturing *in planta* fertilised zygotes. Egg cell protoplasts for *in vitro* experiments were isolated from embryo sacs containing slices of unfertilised female flowers by a combination of incubation with cell wall-degrading enzymes and a manual microdissection (Kranz et al., 1991; Kranz and Lörz, 1993; Faure et al., 1994), while sperm cells were released from pollen grains (Kranz et al., 1991; Faure et al., 1994). After alignment of gametes (figure 1.5a) electrofusion (Kranz et al., 1991; Kranz and Lörz, 1993) or calcium mediated fusion (Faure et al., 1994;

Kranz and Lörz, 1994) and karyogamy (figure 1.5b) have been demonstrated (Faure et al., 1993). Zygotes were cultured in phytohormone containing media and required co-cultivation with feeder cells for sustained development (Kranz and Lörz, 1993; Holm et al., 1994) which may replace nutritive functions of the endosperm. In maize the first cleavage of the *in vitro* created zygote is asymmetric as in the *in planta* fertilised egg cells (figure 1.5c). The resulting multicellular structure develops into a transition stage embryo and finally the two meristematic regions, the scutellum and the coleoptile are formed (figure 1.5d-f). After transfer to hormone free media phenotypically normal and fertile plants have been obtained of which the hybrid nature has been demonstrated genetically (Kranz and Lörz, 1993). The similarity to developmental stages of embryo development *in planta* suggests that plant regeneration from *in vitro* fertilised zygotes took indeed place via embryogenesis rather then organogenesis. The development of *in vitro* techniques provides the opportunity to study mechanisms of fertilisation such as adhesion, gamete recognition and fusion, karyogamy and inhibition of polyspermy, which may not be very accessible for genetic approaches (Dumas and Mogensen, 1993; Faure et al., 1994; Kranz et al., 1995). cDNA libraries from a small number of egg cells and zygotes as source for isolation of egg cell or zygotes specific genes (Dresselhaus et al., 1994) as well as from later stages (Breton et al., 1995a) have been produced. As an example of a differential screen of these libraries a cDNA clone encoding calreticulin was isolated (Dresselhaus et al., 1996). Calreticulin is more abundantly expressed in zygotes than in unfertilised egg cells an its expression was further correlated with dividing tissue (Dresselhaus et al., 1996). Using RT-PCR techniques it is possible to detect gene expression on the single cell level (Richert et al., 1996). The system of *in vitro fertilisation* also allows to investigate the role of already known genes, e.g. involved in the cell cycle, or to analyse changes of the cytoskeleton during the fertilisation process and early embryogenesis.

Androgenesis

After certain experimental *in vitro* manipulations the haploid male gametophytic cells are able to switch from a gametophytic into a sporophytic development. Instead of developing into mature pollen, microspores at the uni-cellular stage or immature pollen grains at the early bi-cellular stage can be directed towards formation of so-called androgenic (also known as haploid or pollen) embryos. Both susceptible microspore stages will be referred to as 'microspores' in the following section. Androgenic embryos have first been obtained in *Datura innoxia* by Guha and Maheshwari (1964). Androgenesis was mostly studied in the model plants rape seed (*Brassica napus*; Lichter, 1982; Swanson et al., 1987; Pechan and Keller,

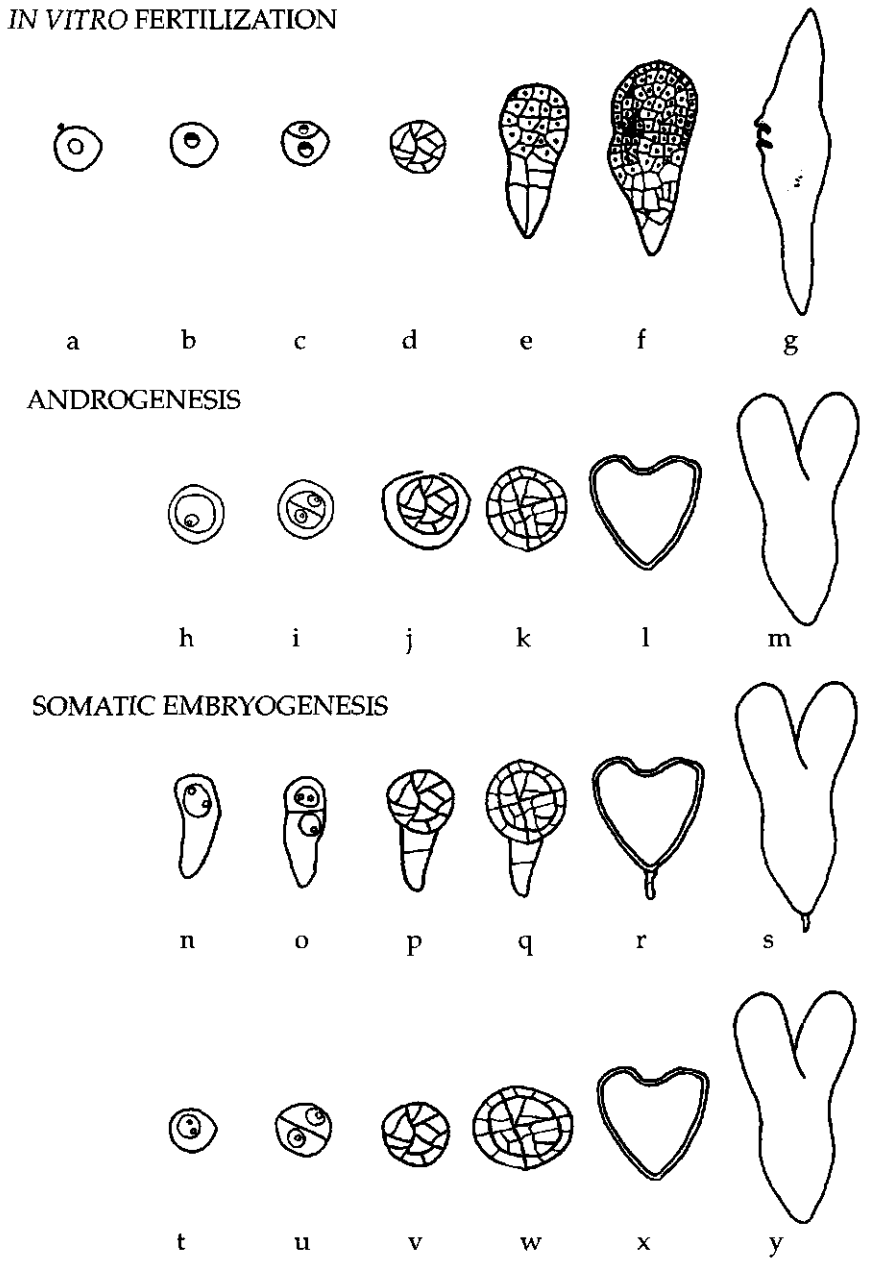

figure 1.5a-y. Schematic representation of *in vitro* forms of embryogenesis. **a-g.** *In vitro fertilisation*: **a.** After alignment of the sperm cell with the egg cell protoplast **b.** fusion and karyogamy takes place. **c.** The first asymmetrical division and **d-f.** subsequent divisions lead to the formation of an embryo (**g**). **h-m.** Androgenesis: **h.** The microspore in the exine **i.** divides symmetrically and develops into a cell

1988), tobacco (*Nicotiana tabacum*; Sunderland and Roberts, 1977; Kyo and Harada, 1985; Heberle-Bors, 1989) and barley (Wei et al., 1986; Olsen, 1991; Hoekstra et al., 1993). Microspores can be cultured inside the anther on solidified medium (anther culture; Hunter, 1988; Siebel and Pauls, 1989), as a shed culture floating on liquid medium (Sunderland and Roberts, 1977) or as isolated-microspore culture (Lichter, 1982; Olsen, 1991). In order to switch developmental fate a species-specific stress pre-treatment of anthers or microspores is necessary. This treatment can be a heat shock (Pechan and Keller, 1988), cold treatment (Huang and Sunderland, 1982), starvation from carbohydrates (Benito Moreno et al., 1988), incubation in a mannitol solution (Roberts-Oelschlager and Dunwell, 1990) or other treatments (reviewed by Ferrie et al., 1995b). A combination of heat shock and carbohydrate starvation have additive effects in tobacco microspore cultures (Touraev et al., 1996). For successful and reproducible microspore culture, donor plants have to be cultivated under controlled environmental conditions (reviewed by Dunwell, 1978; Ferrie et al., 1995b; Jähne and Lörz, 1995). In barley, both growth conditions of donor plants and pre-treatment of microspores are of more importance for the induction of initial divisions than the culture medium used, while the medium composition is essential for further development (Mordhorst and Lörz, 1993). Species specific requirements for the composition of the culture media have been reviewed recently (Ferrie et al., 1995b). The ability of microspores to form androgenic embryos is also genotype dependent (Petolino and Thompson, 1987; Vergne et al., 1993; Murigneux et al., 1994; Ferrie et al., 1995a), suggesting a genetic basis for the ability to develop microspore embryos.
Besides the regeneration of haploid plants, a variable percentage of plants is dihaploid and therefore fertile because of spontaneous auto-reduplication of the genome (Siebel and Pauls, 1989; reviewed by Jähne and Lörz, 1995) so that these plants can be used directly for breeding purposes (Bajaj, 1990).

The analysis of biochemical and molecular changes during the acquisition of embryogenic competence have been a central point of research to elucidate underlying mechanisms (reviewed by Cordewener et al., 1995a). Both tobacco and rape seed microspores can be directed *in vitro* to embryogenesis as well as to pollen maturation (Kyo and Harado, 1986; Custers et al., 1994), giving rise to a non-induced, but nevertheless developing control microspore population. During the starvation period specific changes in the pattern of polypeptide phospho-

colony that is released into the culture medium (**j**). **k-m.** Subsequent divisions lead to the development of the androgenic embryo. **n-y** Somatic embryogenesis: n,t. Single suspension cultured carrot cells can either divide asymmetrically (**n**) or symmetrically (**u**) to develop into a somatic embryo. After an asymmetrical division a suspensor like structure may be formed (**p-s**) which is absent during the symmetrical form of somatic embryo development (**v-y**).

rylation (Kyo and Harada, 1990) and protein kinase activity (Garrido et al., 1993) have been determined, suggesting that protein phosphorylation cascades might accompany the establishment of embryogenic competence. Zársky et al. (1992) showed that derepression of the cell cycle of the vegetative nucleus is involved in the induction of embryogenesis. Changes of gene expression at mRNA and protein level could also be correlated with the stress induced induction of embryogenesis (Pechan et al., 1991; Garrido et al., 1993; Vergne et al., 1993; Boutilier et al., 1994; Cordewener et al., 1994; Ríhová et al., 1996). Furthermore, heat shock proteins are thought to be involved in this developmental switch (Cordewener et al., 1995b; Zársky et al., 1995).

The first microspore divisions take place inside the exine (figure 1.5h-j). Cellular and ultrasturctural changes, e.g. fragmentation of the vacuole, movement of the nucleus to a central position and formation of starchy cytoplasm, dedifferentiation of plastids and loss of nuclear pores in the vegetative nucleus are observed before the first division of embryogenic microspores (Zaki and Dickinson, 1990; Garrido et al., 1995). This division is symmetric (figure 1.5i) in contrast to asymmetric division of the gametophytic pathway (Zaki and Dickinson, 1990; Telmer et al., 1995; Yeung et al., 1996). Besides the stress pretreatment, asymmetric division of microspores could be prevented by depolymerisation of microtubules using colchicin, leading to embryogenic development as well (Zaki and Dickinson, 1991; Zhao et al., 1996). This finding suggests that embryogenesis may occur as a default mechanism (Zhao et al., 1996). The multicellular structure, still developing inside the exine, exhibits an equal distribution of starch granules which might demonstrate the absence of polarity (Hause et al., 1994). The local rupture of the exine and the release of the cell colony into the culture medium (figure 1.5j) is followed by a change of starch distribution, namely a disappearance at the broken side (the future apical pole) and persistence at the opposite side (the future root pole; Hause et al., 1994; Yeung et al., 1996). The polarisation in starch distribution is also found in zygotic embryos, so the side of exine rupture is considered to play an important role in the determination of the apical-basal polarity of microspore derived embryos (Hause et al., 1994).

As member of the *Brassicacea*, zygotic embryogenesis in rape seed follows the strict cell division pattern of the Crucifer type described for *Arabidopsis*, in particular with respect to the first cell divisions (Tykarska, 1976; Yeung et al., 1996). In contrast to zygotic development, early divisions in embryogenic microspores appear to be random rather than regular (Telmer et al., 1995; Yeung et al., 1996). The multicellular structure released from the exine is subsequently 'self-organising' into a globular embryo (figure 1.5k), as evidenced by the formation of a protoderm by periclinal divisions of the outermost cell layer. In the two cell stage of

microspore embryogenesis a cell comparable to the larger basal cell of the two-celled zygotic embryo is absent, as is a suspensor and therefore also a hypohyseal cell in a later embryo stage (Yeung et al., 1996). While the hypophyseal cell is considered to play a central role in the formation of the root meristem (Scheres et al., 1995), other cells in androgenic embryos apparently take over the function of the hypophyseal cell (Yeung et al., 1996). This reinforces the idea that positional information rather than cell lineage is important in plant embryogenesis (Yeung et al., 1996) as has also been demonstrated in *Arabidopsis* root development (Van den Berg et al., 1995). After the 'self-organisation' of the globular embryo, subsequent development follows the stereotyped principles of dicot and monocot development respectively. The resulting androgenic embryos of course contain all embryonic pattern elements as found in zygotic embryos (Engell, 1991; Yeung et al., 1996). In barley the initial divisions of microspores, further proliferation, androgenesis and thereby embryo formation, and finally plant recovery could be manipulated independently by altering the nitrogen composition of culture media (Mordhorst and Lörz, 1993). For instance formation of the secondary embryo axis including scutellum, shoot and root primordia was inhibited in media containing only glutamine as nitrogen source. This inhibition was correlated with the accumulation to a very high level of two embryo-specific transcripts, normally restricted to developmental stages after differentiation of scutellum and secondary embryo axis (Mordhorst et al., 1995; Stirn et al., 1995). As observed in the *raspberry* mutant in *Arabidopsis*, these results show that an arrest in embryo development caused by either a mutation or, as in this case, by manipulation of the culture medium, leads to expression of certain genes in the wrong morphological context of an arrested embryo.

Somatic embryogenesis

The formation of plant embryos discussed so far all start from the zygote or from cells of male or female reproductive tissues. Embryos can also develop from somatic plant cells, a process that can occur naturally on leaf margins in a number of species such as *Bryophyllum* (Yarbrough, 1932) and *Malaxis* (Taylor, 1967). *In vitro* somatic embryo development was first observed in suspension cultured carrot cells (Reinert, 1959). In this section, recent work on the induction phase of somatic embryogenesis is discussed in relation to the early phases of zygotic and androgenic embryogenesis in order to compare the various processes with each other. The term 'embryogenic cell' will be restricted to those cells that have completed the transition from a somatic state to one in which no further externally applied stimuli are necessary to produce the somatic embryo (De Jong et al., 1993b). The

cells that are in this transitional state and have started to become embryogenic, but still require externally applied stimuli, are defined as competent cells (Komamine et al., 1990; Chapter 2).

After appropriate culture manipulations, usually involving synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), either alone or in combination with cytokinins, somatic embryos can develop from almost any part of the plant body. However, immature zygotic embryos are a frequently used source of explant material due to the high rate of success in obtaining embryogenic cell cultures. Several aspects of somatic embryogenesis have been reviewed (De Jong et al., 1993b; Zimmerman, 1993; Schmidt et al., 1994; Yeung, 1995). By virtue of systems that employ cell cultures, somatic embryogenesis has long been considered to be an ideal system to study the early events in embryo formation. In carrot, where it has been demonstrated that single, suspension cultured cells in suitable culture conditions can develop into an embryo (figure 1.5n-y), the emphasis has shifted from relatively late events to those that have to occur during the formation of the first cells that are embryogenic. Analysis employing the recording of many thousands of individual cells has shown that competent single cells to become embryogenic, are of many different morphologically different types. In addition, these studies revealed that these cells require 2,4-D to initiate embryo development (Chapter 2), confirming earlier work on manually isolated single competent cells (Komamine et al., 1990).

An analysis of the events that occur during treatment of hypocotyl explants with 2,4-D has shed light on where the initial population of competent cells originates. Proliferation of provascular cells resulted in a mass of small, rapidly proliferating cells (Guzzo et al., 1994). However, these cells are not yet competent to form embryogenic cells (Schmidt et al., 1997). Some of the proliferating cells elongate, and a limited number of a particular oval to triangular intermediate cell type has acquired competence to become embryogenic and produce a somatic embryo, again demonstrated by cell tracking (Schmidt et al., 1997). None of the other cell types, including the small isodiametric rapidly dividing cells released from the explant developed into somatic embryos. At least one gene is now available, of which the expression pattern coincides with competent cell formation, thus enabling a more detailed analysis of this process (Schmidt et al., 1997). So far the study of the transition of somatic cells into competent and embryogenic cells was hampered by the inability to prove that particular early markers for competent and embryogenic cells were indeed absolutely specific for those few cells that are capable of embryogenic cell formation.

Several systems have been described in which it is possible to induce somatic embryos directly from explant cells, without an intervening callus stage. One of the most advanced systems is based on leaf explants of the *Cichorium* hy-

brid "474" (Dubois et al., 1991). In this system, somatic embryos develop after treatment with growth regulators or by incubation at 35 °C (Decout et al., 1994). The first divisions during embryo development can be synchronised by the addition of glycerol to the incubation medium (Robatche Claive et al., 1992). The nucleus increases in size and is displaced towards the centre of the activated mesophyl cells. The vacuole becomes fragmented and callose is deposited in the cell wall of the embryogenic cell (Dubois et al., 1991; Blervacq et al., 1995). The first division of the embryogenic cell is symmetrical and anticlinal in respect to the orientation of the vascular elements (Blervacq et al., 1995). Subsequent anticlinal divisions lead to the formation of an embryogenic structure that develops into a somatic embryo (Dubois et al., 1991; Blervacq et al., 1995). In other systems somatic embryos develop spontaneously like on megagametophyte tissue of *Pinus taeda* (Becwar et al., 1990) or after thawing of cryopreserved somatic embryos of *Picea sitchensisi* (Kristensen et al., 1994).

The establishment of somatic embryogenesis in *Arabidopsis* allows to combine the molecular genetic approaches used in zygotic embryos (Mayer et al., 1991; Meinke, 1995) with cellular (Van den Berg et al., 1995) and biochemical approaches (De Jong et al., 1992; 1993b). While immediately useful for e.g. mutant embryo rescue and phenotypical analysis, other advantages are the availability of sufficient quantities of developing staged embryos and eventually the establishment of screens for mutants with an altered ability to form somatic embryos. Embryogenic callus cultures of *Arabidopsis* have been obtained by culturing immature zygotic embryos of the ecotype Columbia (Pillon et al., 1996). Embryogenic cell suspensions of a high number of different ecotypes and cell lines with even higher embryogenic capacity were obtained from a mutant, *primordia timing-1* (*pt-1*), allelic to *altered meristem program1* characterised by a higher then normal ability to regenerate via organogenesis (Chaudhury et al., 1993). The embryogenic capacity of these cell suspension cultures was so far stable for more than one year (Mordhorst and De Vries, to be published).

Comparing early stages of zygotic and somatic embryo development showed similar developmental patterns in a number of species. In rice, as in most other species, the zygote elongates after and then divides unequally and transversal whereafter the terminal cell divides in a variable fashion (Jones and Rost, 1989b). This is similar to the first divisions of epithelial sculellum cells that develop into somatic embryos (Jones and Rost, 1989a). Also somatic embryos of *Vitus* (Altamura et al., 1992) and *Ranunculus* (Konar et al., 1972) follow the same embryonic type of development as their zygotic counterparts. However, early divisions in carrot somatic embryogenesis follow variable division patterns resembling e.g. the Crucifer type (figure 1.5n-s; McWilliam et al., 1974) but also symmetrical divisions have been observed (figure 1.5t-y; Chapter 2). Similar results were obtained during

somatic embryogenesis in alfalfa (*Medicago sativa*) where initial divisions where less precise when compared to the zygotic divisions (Dos Santos et al., 1983). Somatic embryos often lack a suspensor (Xu and Bewley, 1992; Chapter 2) and can develop via morphologically distinct cell clusters (Chapter 2) showing that somatic embryo development may be more flexible than the zygotic development. During zygotic and somatic embryo development in carrot a number of genes is expressed in the globular embryo. Expression patterns of embryogenesis related mRNAs (Franz et al., 1989; Sterk et al., 1991; Wurtele et al., 1993) and the embryogenic ECP40 protein (Kiyosue et al., 1993) are similar during somatic and embryogenic embryo development. However, later in development expression patterns of storage proteins of somatic embryos of alfalfa deviate from the zygotic ones. The transcription of storage protein mRNA is comparable in zygotic and somatic embryos but due to physiological conditions protein translation is altered in somatic embryos (Pramanik et al., 1992; Krochko et al., 1994).

A point of discussion that has surfaced in somatic embryogenesis is whether the cells that are in the transition between the somatic state and the embryogenic state progress in a cell-autonomous way under the influence of exogenous growth regulators or whether they receive specific signals from neighbouring cells. Evidence for the importance of cell to cell communication during embryogenesis comes from the beneficial effect of e.g. suspension cell cultures (Kranz et al., 1991) or embryogenic microspore cultures (Holm et al., 1994) on *in vitro* zygote culture. Similar effects have been observed in somatic embryogenesis by addition of conditioned medium (De Vries et al., 1988). Whether this points to the existence of classes of specific molecules or whether there is a more general effect on cell division, as demonstrated in single cell cultures (Spangenberg et al., 1985) is not known yet. The carrot EP3 endochitinase genes (Kragh et al., 1996), encode secreted proteins able to rescue somatic embryo development in the temperature sensitive mutant *ts11* (De Jong et al., 1992; 1995). The EP3 genes are not expressed in somatic embryos, but in other cell types present in the suspension culture (Van Hengel et al., submitted), pointing to the existence of cell to cell communication events involved in somatic embryogenesis. Another example of such communication in cell culture may be found in certain arabinogalactan proteins (AGPs). Recently it has been shown that AGPs can promote embryogenic cell formation in suspension cultures of carrot (Kreuger and Van Holst, 1993; 1995) and *Picea* (Egertsdotter and Von Arnold, 1995). AGPs are proteoglycans with poly- and oligosaccharide units covalently attached to a central protein core. AGPs react with the ß-glycosyl Yariv reagent (Fincher et al., 1983; Kreuger and Van Holst, 1996). Binding of ß-D glycosyl Yariv reagent to cell wall AGPs of rose (*Rosa* sp.) suspension cells inhibited growth in a reversible fashion, probably due to suppression of the cell cycle

eventually in combination with prevention of cell expansion (Serpe and Nothnagel, 1994). In *Arabidopsis* seedlings and in carrot suspension cultures a similar effect of ß-D glycosyl, but not of AGP-unreactive α-galactosyl Yariv reagents was observed (Willats and Knox, 1996). It has been proposed that carrot suspension cells decorated with the JIM8 AGP cell wall epitope are in a transition between competent and embryogenic cell state (Pennell et al., 1992). This was based on the labelling of a subpopulation of single cells with the JIM8 antibody and also on the occurrence of labelled cells in embryogenic cultures only. Cell tracking of JIM8 labelled cell populations however failed to demonstrate a causal relationship between JIM8 labelling and embryo formation (Chapter 3). Given the demonstrated promotive effects of AGPs, it is possible that the JIM8 decorated cells perform some accessory function in embryogenesis. Such a role for AGPs seems in line with their expression *in planta*, where e.g. JIM4 reactive AGP epitopes mark emerging anatomical patterns in developing carrot somatic embryos (Stacey et al., 1990). Based upon expression of JIM4 and JIM13 epitopes in the carrot root apex a function for AGPs in determination of the cell fate has been postulated (Knox et al., 1989; 1991). Maize coleoptiles that are committed to undergo programmed cell death express a specific set of AGP epitopes (Schindler et al., 1995). These examples suggest that AGPs may play a role in determination of cell fate and cell differentiation (Chasan, 1994; Knox, 1995).

If components of the conditioned medium of plant cell cultures such as chitinases and AGPs have a beneficial effect on several stages of somatic embryo formation, it is of interest to determine where such molecules are found during zygotic embryogenesis. Indeed, developing seeds have shown to be a rich source of AGPs able to promote embryogenic cell formation in tissue culture (Kreuger and Van Holst, 1993), while the carrot chitinase *EP3* genes appear to be expressed in the integuments and in the endosperm (Van Hengel et al., submitted). This seems to point to a quite intimate role between the embryo and the surrounding sporophytic and gametophytic tissues, a topic that can now also be addressed through *Arabidopsis* mutants that show endosperm development without (Ohad et al., 1996). The existence of these *fertilisation independent endosperm* (*fie*) mutants suggest that endosperm and embryo development are genetically divergent. The *fie* mutation also has a female gametophytic embryo lethal phenotype, and while the reasons for this are unclear, it may support the notion that early embryogenesis is dependent on endosperm formation. This idea was exploited by the use of coconut milk in tissue culture experiments and which eventually led to the identification of plant cytokinins.

While the cellular origin of somatic embryos is quite different from zygotic ones, both the radial and the apical-basal pattern formation takes place in correct fashion. Whether apical-basal pattern formation in somatic embryos depends on

least in established carrot cultures such a correlation was not evident (Chapter 2). However, in the absence of experimental data on the nature of the mechanisms that set up and fix polarity in the zygote or in single competent cells, it remains to be established whether apical-basal axis formation in plant embryos always requires such polar cells or whether alternative mechanisms exist. The radial pattern in somatic embryos most likely proceeds by specification of inside and outside cells, as it occurs in zygotic embryos. In this respect it is of interest to note that at least one marker of embryogenic cells in culture systems encodes a protein associated with epidermal cells (Sterk et al., 1991). However, as in zygotic embryogenesis (Vroemen et al., 1996), the molecular mechanisms by which the radial pattern in somatic embryos is established are unknown.

In several systems the question whether auxin gradients are instrumental in apical-basal pattern formation have been addressed. Inhibition of polar auxin transport by application of 2,3,5-triiodobenzoic acid (TIBA) or 9-hydroxyfluorene 9-carboxylic acid (HFCA) in *in vitro* cultured zygotic globular embryos of Indian mustard (*Brassica juncea*) led to the formation of fused cotyledons. The treated embryos did however form shoot and root meristems (Liu et al., 1993), suggesting that apical-basal pattern was complete before the globular stage, or is not dependent on polar auxin transport. Globular and oblong carrot somatic embryos treated with *N*-(-1naphthyl)phthalmic acid (NPA) or TIBA did not develop roots or shoots, but only increased in size (Schiavone and Cooke, 1987) suggesting that in somatic embryos other mechanisms of apical-basal pattern formation operate or that the time at which this is established is later then in zygotic embryos. In *in vitro* cultured globular wheat zygotic embryos the addition of TIBA influenced the position and development of the shoot apical meristem while no root meristem was formed (Fisher and Neuhaus, 1996). Note, that the *monopteros* mutant phenotype, in which also no root meristem is formed, is discussed to be the result of an altered polar signal, eventually auxin flux (Przemeck et al., 1996). Dramatic effects of exogenous auxin were also reported, perhaps through disturbance of the endogenous auxin gradients in the globular embryo (Michalczuk et al., 1992; Cooke et al., 1993). While suggestive of an important role of polar auxin transport in some aspects of pattern formation, it is not clear at all whether the proposed auxin gradients necessary are indeed established already in the pre-globular embryo. While intracellular levels of the synthetic auxin 2,4-D and the endogenous indole-3-acetic acid (IAA) have been measured in whole clusters and embryos (Michalczuk et al., 1992; Ivanova et al., 1994) no auxin gradients have been determined so far.

Cell polarity and asymmetric division

While fundamental problems concerning the mechanisms of initiation and fixation of axes of polarity and embryo pattern formation are being studied now in plants using several different approaches, it is of interest to discuss some aspects of these processes as they have been established in other experimental systems.

In *Fucus* polarity of the zygote can be induced by unilateral light. The zygote polarity can be undone by removal of the cell wall or disruption of microfilament assemblies, and reinduced by the application of light from another direction. Attachment of F-actin or other cytoplasmic compounds to the cell wall may fix the axis of polarity (Kropf et al., 1989). In maize egg cell protoplasts however, polarity may remain fixed even in the absence of the cell wall (Kranz et al., 1995), suggesting that there are different mechanisms for the establishment of polarity. After irreversible determination of the polar axis in the *Fucus* zygote, sulphated fucan polysaccharides and a vitronectin-like protein are specifically localised in the cell wall at the future rhizoid side. It is believed that these molecules are transported along the actin microfilaments. In the two celled embryo an unidentified rhizoid-specific cell wall component determines the developmental fate of the rhizoid cell (Berger et al., 1994). Just after fertilisation mRNA is symmetrically distributed in the apolar *Fucus* zygotes. Soon after fixation of the polar axis mRNA becomes localised in the thallus area of the polar zygote and is often distributed in a gradient with the highest concentration at the thallus side. After division the majority of the mRNA is localised in the thallus cell (Bouget et al., 1995). Actin mRNA deviates from this pattern in that it becomes localised near the plane of division at the time the division plate becomes pronounced. Colocalisation of actin mRNA and the division plane only occurs during the first two transverse divisions of the thallus cell (Bouget et al., 1996).

Mechanisms responsible for cytoplasmic localisation of developmentally important mRNAs have been revealed in *Drosophila* (St. Johnston and Nüsslein-Volhard, 1992). The main anterior-posterior axis is established in the form of a gradient of maternal mRNAs in the fertilised egg. One of these, BICOID, is localised at the anterior part of the egg, where its translation results in a high concentration of the BICOID transcription factor, in turn resulting in a gradient towards the posterior part of the embryo due to diffusion of the protein. At the posterior end *NANOS* and *OSCAR* mRNAs are localized (St. Johnston and Nüsslein-Volhard, 1992). Localisation of these mRNAs in the cytoplasm is mediated by specific sequences in the 3' untranslated regions (3' UTRs). Localisation of *BICOID* mRNA requires the EXUPERANTIA (EXU) protein, proposed to specifically recognise the conserved secondary structure of the 3' UTR of *BICOID* mRNA, and an intact microtubular skeleton. The EXU/BICOID complex is then actively transported

towards the minus ends of the microtubules (Wang and Hazelrigg, 1994).

While an asymmetric zygotic division in plants is usually interpreted as a clear indication of formation of an axis of polarity, in several plant species the first division of the zygote can be symmetrical, oblique or longitudinal (Sivaramakrishna, 1978). Whether there is asymmetric distribution of e.g. mRNAs and/or cell wall components in these zygotes is not established, but it cautions towards placing to much emphasis on the occurrence of asymmetric divisions as an indicator of polarity. Variability in early division patterns is also observed in somatic embryogenesis, where both asymmetric as well as symmetric division of suspension cells resulted in the formation of embryos. Also here, no cellular asymmetry in the distribution of mRNAs was investigated (Chapter 2). However, protoplasts of *Medicago* divide asymmetrically and form a suspensor like structure connected to an embryogenic cell cluster that subsequently will develop into a somatic embryo (Dijak and Simmons, 1988; Song et al., 1990).

In contrast to these studies, androgenesis does appear to depend on a change from asymmetric to symmetric division. In tobacco pollen, an asymmetrical division generates the small generative and the large vegetative cell. When the asymmetrical division is prevented the pollen cells develop a vegetative fate as shown by the expression of the LAT52 gene specific for vegetative cells. Changing the asymmetrical division to a symmetrical division results in LAT52 expression in both daughter cells, indicating that an asymmetrical division is required for differentiation of the generative cell. It has been proposed that an, unidentified, microspore derived cytoplasmic factor controls gene expression before and after this division (Eady et al., 1995). Embryo induction of late uninucleate *Brassica* microspores by heat treatment is followed by a change of the normal asymmetrical division to a symmetrical division. Changes in the microtubular cytoskeleton either lead to a 90° rotation in the division plane or to migration of the nucleus to the centre of the cell. In both cases this leads to a symmetrical division generating two morphologically identical daughter cells that develop into a globular androgenic embryo. No suspensor like structures are observed in this type of embryo development (Zaki and Dickinson, 1990; Hause et al., 1993). One of the challenges in this area now is to identify the molecules that are decisive in the generation of polarity in plant cells and to decide whether these molecules are important for the formation of the apical-basal pattern of the embryo.

Concluding remarks

In the preceding sections, plant embryogenesis has been discussed at various levels. While it seems clear that well-established mechanisms to generate pattern in

animal embryos, such as localized distribution of cytoplasmic determinants in the form of mRNAs will also be functional in plants, clear evidence of this remains to be established. In order to gain a better insight in the mechanisms of plant embryogenesis it will be indispensable to combine in the future various disciplines and the different experimental systems available for research on embryogenesis. The understanding of the mechanisms underlying the transition from a somatic or a gametophytic cell into an embryogenic cell can be expected to be beneficial to develop more efficient procedures for plant regeneration. It is likely that this better understanding will also help to unravel processes of early zygotic embryogenesis. Concerning the mechanisms that plant embryos use to build up the embryo body pattern, cell-cell interactions or positional information are the most promising mode (Van den Berg et al., 1995). However, the demonstrated plasticity in terms of cell fate and determination in plant cell development cannot rule out the existence of so far unknown mechanisms. Examples of this are the considerable degree of plasticity in initial division patterns in both zygotic and somatic embryos and the variable contribution of apical and basal cells of two-celled embryo to the final embryo pattern elements. In this context is is noteworthy that a complete embryo body pattern can be generated in *Arabidopsis* mutants with an aberrant early division pattern. In addition, the formation of a complete embryonic root in somatic and androgenic embryos without the involvement of a hypophyseal cell emphasises the importance of positional information in pattern formation. It may be that there is not a single model applicable to all plant species for generating the plant embryo. The existing developmental plasticity during embryogenesis is further emphasised by the development of androgenic and somatic embryos through different initial stages compared to the zygotic counterparts. While all of these types of embryos result in a stereotyped sequence of embryonic developmental stages, their initial development is vastly different. Finally, if the concept of cell-cell interactions and positional information is the main mechanism by which pattern and cell differentiation is effectuated in plant embryogenesis, the nature of the signals involved and the way they are transduced is essential to understand plant embryogenesis. Whether it involves only the classical growth regulators or a completely different set of regulatory molecules or a combination of both remain to be established.

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2

Description of somatic-embryo-forming single cells in carrot suspension cultures employing cell tracking

A cell tracking system was established to determine the capability of individual single suspension cells of carrot (*Daucus carota L.*) to develop into somatic embryos. Immobilised in phytagel, 127 out of 30 318 single suspension cells smaller then 22 μm in diameter developed a somatic embryo. Single cells present at the start of the experiment were classified on the basis of their morphology into five groups: small spherical vacuolated cells; small spherical cytoplasm-rich cells; oval vacuolated cells; elongated vacuolated cells and cells that could not be classified into either one of these groups. Single cells of all morphologically distinguishable single cell types developed into somatic embryos with a frequency that varied between 19 and 100 somatic embryos per 10 000 cells. This suggests that the capability of individual single cells to form somatic embryos is not restricted to a particular cell type distinguishable on the basis of its morphology. During development three major pathways were observed. Oval and elongated cells developed into somatic embryos via an asymmetrical cell cluster. Spherical cells developed via a symmetrical cell cluster into somatic embryos. Before formation of a somatic embryo, cells of a more variable initial morphology first developed aberrantly shaped cell clusters. This suggests that the developmental pathway leading to a somatic embryo can be predicted by the initial single cell morphology.

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Introduction

Despite the wide use of carrot as a model system for somatic embryogenesis, fairly little is known about the actual origin of somatic embryos in cultures. Under the most commonly employed culture regimes somatic embryos develop from multicellular clusters of embryogenic cells termed proembryogenic masses. Based upon detailed morphological observations, Halperin (1966) postulated that pro-embryogenic masses in fact represent arrested early globular embryos. This hypothesis finds support in the observation that several genes are expressed both in globular embryos and in pro-embryogenic masses (Wilde et al., 1988; Kiyosue et al., 1991; Sterk et al., 1991). It implies that somatic embryogenesis starts during the suspension culture phase in the presence of 2,4-D; dilution of proembryogenic masses into basal medium merely relieves the developmental arrest imposed by the high cell density and the presence of 2,4-D. Backs-Hüsemann and Reinert (1970) were the first to observe that somatic embryos can also have a single cell origin. Employing time-lapse photography they described a relative large, elongated and vacuolated cell type. After a first division in the presence of 2,4-D, one of the daughter cells developed into a callus from which a somatic embryo developed after removal of 2,4-D. Somatic embryogenesis without an intermediate callus stage has been reported by Nomura and Komamine (1985) who obtained, after density centrifugation, two cell types developing somatic embryos. One of these, a small round cell type reported to be cytoplasm-rich (Komamine et al., 1990), developed somatic embryos with a high frequency. The second cell type consisted of small oval cells and developed somatic embryos with a lower frequency (Nomura and Komamine 1985). Histological observations in other plant systems support the hypothesis that somatic embryos in general originate from small cytoplasm-rich cells (Konar et al., 1972; Dos Santos et al., 1983; Ho and Vasil 1983; Botti and Vasil 1984; Vieitez et al., 1992).

A model for the development of the small cytoplasm-rich single cells into somatic embryos was presented by Komamine et al. (1990). In the development of somatic embryos three states were recognised. The first (state 0) consists of single cells that require auxin to form small state 0 cell clusters. After removal of auxin these are designated as state 1 cell clusters that develop slowly into state 2 cell clusters. Rapid cell divisions in certain parts of the state 2 cell cluster then lead to the development of a globular somatic embryo which in turn develops into the later embryo stages such as heart and torpedo. If, as suggested above, the developmental pathways that lead to globular embryos and proembryogenic masses are the same, the single embryo-forming cells that have been described so far would be essential intermediates in the acquisition of embryogenic competence in cell cultures.

Suspension cultures initiated from cut carrot hypocotyl segments initially mainly contained large vacuolated cells (De Vries et al., 1988), with few small cells present. After removal of the explants, proembryogenic masses were first detected in the culture after three weeks (De Vries et al., 1988). Two pathways for the formation of embryogenic cells in the newly initiated cultures have been suggested:

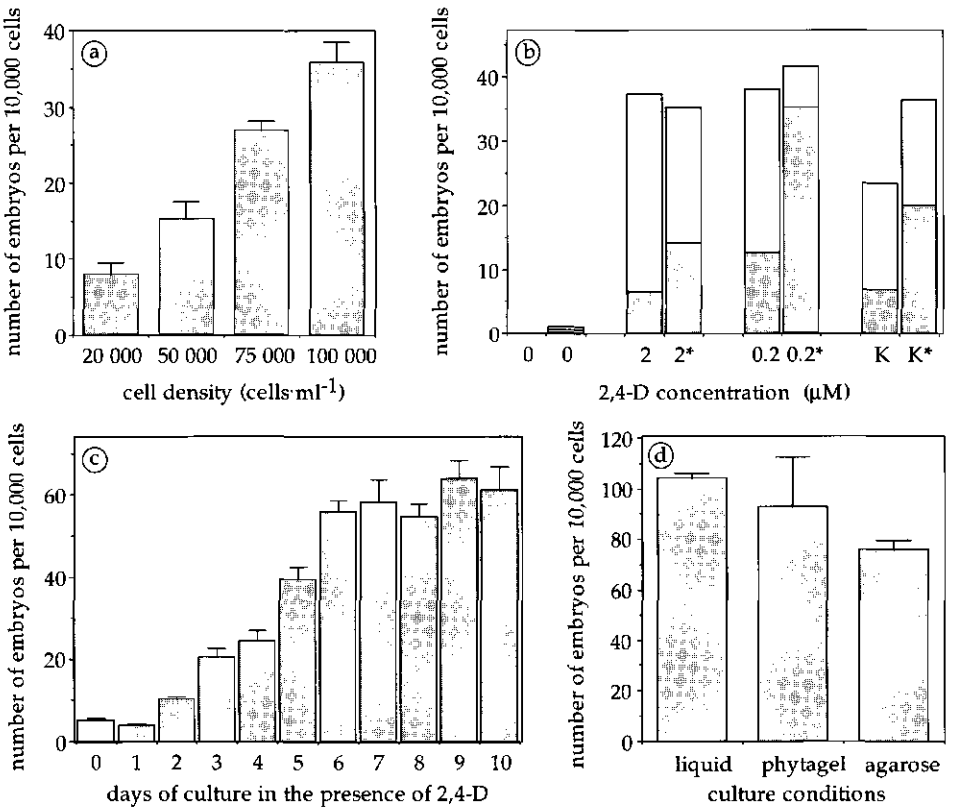

figure 2.1a-d. a. Effect of cell density on the percentage of somatic embryos formed from a < 22 µm cell fraction. After one week of culture in B5 medium in the presence of 0.2 µM 2,4-D, cells were transferred to B5-0 medium. **b.** Effect of 2,4-D concentration on the number of embryos and cell clusters formed in a < 22 µm cell fraction. Single cells were cultured in B5 medium containing 2 or 0.2 µM 2,4-D or B5 medium containing $5 \cdot 10^{-8}$ M 2,4-D, 1 µM zeatin and 0.2 M mannitol in B5 medium (Nomura and Komamine 1985). After one week of culture, cells were either transferred to B5-0 medium (indicated with an asterisk) or transferred to medium containing the same growth regulators. **c.** Induction of embryogenesis from a < 22 µm cell fraction. Cells were incubated for 0 to 10 days in the presence of 0.2 µm 2,4-D and transferred to B5-0 medium. **d.** Effect of immobilisation of the < 22 µm cell fraction in agarose or phytagel. Cells were cultured in B5 medium supplemented with 0.2 µM 2,4-D in liquid cultures, 0.5 % agarose cultures or 0.1 % phytagel cultures. After six days 2,4-D was removed by washing with B5-0 medium. Bars represent the standard deviation (±SD; n=4)

First, these cells can arise from a cell type, susceptible to 2,4-D, which is already present in the explant. This cell type could then develop into a self-perpetuating subpopulation of the embryogenic suspension culture and remain as long as the culture is embryogenic. *Second*, these cells are formed *de novo* from previously non-embryogenic single cells or cell clusters present in the suspension culture (De Vries et al., 1988). In this second model, the population of embryogenic cells is continuously supplemented by other, non-embryogenic, cell types. It is not known whether the first, or second, or both pathways together are used. Neither is it known whether the cell types described by Back-Hüsemann and Reinert (1970) and Nomura and Komamine (1985) are the only single cells present in a cell suspension culture, that can develop into somatic embryos.

This paper describes the identification of 127 single carrot suspension cells and their development into somatic embryos, using a system for semi-automatic cell tracking. The results show that there is a considerable morphological heterogeneity in somatic embryo-forming single suspension cells, and that three different types of somatic embryo development can be observed.

Results

Optimising culture conditions for somatic embryo development

Optimising cell density

To identify embryogenic single cells a cell population containing single cells developing somatic embryos with a reasonable frequency had to be obtained. After sieving an one-week old suspension culture through a 22 μm nylon sieve, the resulting cell population consisted of over 97 % single cells, with the remainder present in small clusters of 2 to 6 cells each. After one week of culture in the presence of 0.2 μM 2,4-D, followed by one week in B5-0 the number of embryos formed per 10 000 cells increased with increasing cell density (figure 2.1a). This effect was seen in three independent cell lines and confirmed observations made by Hari (1980), that somatic embryogenesis is stimulated at higher cell densities. At the highest cell density used (100 000 cells·ml^{-1}), 36 somatic embryos developed from 10 000 cells. Although a further increase in cell density might have increased the frequency of somatic embryogenesis even further, this was not practical because it interfered with the identification of individual single cells during cell tracking experiments.

Concentration and incubation time of 2,4-D

Media with two 2,4-D concentrations were tested and compared to the medium described by Nomura and Komamine (1985). After one week small cell clusters were observed. Upon further incubation in the presence of 2 µM 2,4-D most cell clusters developed into proembryogenic masses while a minority (17 %) of the small cell clusters directly developed somatic embryos (figure 2.1b). Removal of 2,4-D after one week increased the percentage of somatic embryos formed from the small, one week old, cell clusters to 40 %. Small cell clusters present in the culture with an initial 2,4-D concentration of 0.2 µM developed into somatic embryos with a frequency of 84 % when transferred to B5-0 medium after one week. The medium described by Nomura and Komamine (1985), that includes 1 µM zeatin and 0.2 M mannitol, gave results comparable to those obtained at the low 2,4-D concentration (figure 2.1b), indicating that zeatin and mannitol are not critical in somatic embryo formation from single cells under our conditions.

To optimise the duration of culturing in the presence of 2,4-D, samples were taken daily from a culture containing 0.2 µM 2,4-D. Samples transferred to hormone free medium at day 0 contained almost no somatic embryos after 14 days of culture in B5-0 medium (figure 2.1c). These somatic embryos probably arose from the small cell clusters present in the initial < 22 µm cell fraction. Single cells cultured for 2 to 6 days in the presence of 2,4-D showed an increase in the number of somatic embryos formed 14 days after transfer to hormone-free medium. Cultivation for more then 7 days in the presence of 2,4-D did not increase the total number

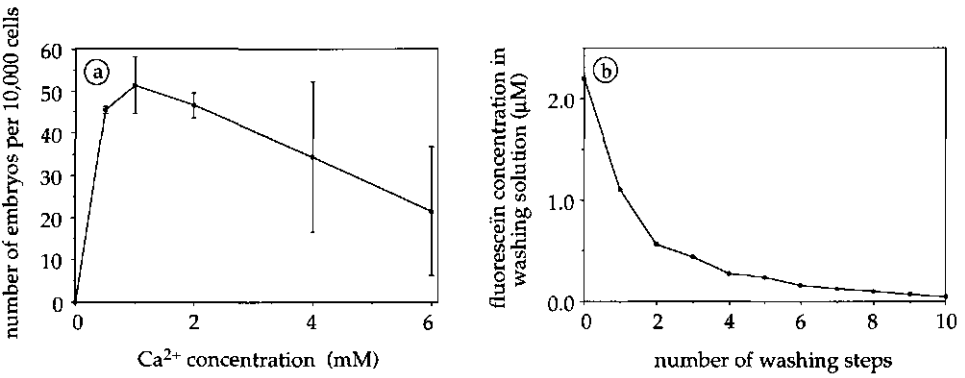

figure 2.2a-b. a. Effect of Ca^{2+} concentration on the number of somatic embryos formed from < 22 µm cell fraction. Single cells were cultured in B5-0.2 medium containing no Ca^{2+}, 0.5, 1.0, 2.0, 4.0 or 6.0 mM Ca^{2+}. After seven days cells were transferred to B5-0 medium containing the same Ca^{2+} concentrations. **b.** Effect of washing to remove fluoresceïn from phytagel. The phytagel layer was washed 10 times with B5-0 medium and the residual amounts of fluoresceïn in the washing solution after each wash step were measured spectrophotometrically. Ninety-nine percent of the total amount of fluoresceïn is removed in ten washing steps. Bars represent ±SD (n=4).

of somatic embryos (figure 1c) but more polyembryos were formed (results not shown).

In conclusion, optimal culture conditions for tracking somatic embryo development from single cells were established in 2 ml cultures of 100 000 cells ml^{-1} B5 medium supplemented with 0.2 µM 2,4-D, followed by transfer to B5-0 medium after 6 days. Under these conditions, somatic embryos could be obtained from single cell populations with a frequency between 36 and 105 somatic embryos per 10 000 cells, depending on the cell lines used. For comparison, the frequency of somatic embryogenesis from an unfractionated and established embryogenic culture, is between 100 and 140 somatic embryos per 10 000 cells (De Vries et al., 1988).

Immobilisation system

To monitor embryo development from individual single cells, these cells had to be immobilised. For this purpose, embedding in phytagel proved to be a satisfactory system. The total amount of Ca^{2+} added to the phytagel culture system equals a Ca^{2+} concentration of 2.4 mM. Because Ca^{2+} may bind to phytagel during polymerisation the free Ca^{2+} concentration is probably below 2.4 mM. Since the Ca^{2+} concentration in normal B5 medium is 1 mM, the effect of Ca^{2+} on somatic embryogenesis from single cells was tested. Figure 2.2a shows that at Ca^{2+} concentrations between 0.5 and 2.4 mM the frequency of somatic embryo development is hardly affected.

To allow embryo development beyond the early globular stage 2,4-D had to be completely removed from the culture system. Ten washing steps of 15 minutes each of the phytagel cell cultures with B5-0 were sufficient to allow embryo development beyond the torpedo stage. To confirm that small aromatic compounds such as 2,4-D could be removed from the phytagel culture system this way, fluoresceïn was used to test diffusion in the phytagel culture system. In 10 washing steps 98 % of the amount of fluoresceïn present in the phytagel culture could be removed (figure 2.2b).

Finally, a comparison of somatic embryogenesis from single cells was made between liquid culture, phytagel embedded and agarose embedded cell cultures. The frequency of somatic embryogenesis in phytagel cultures and agarose embedded cultures was the same when compared to liquid cultures (figure 2.1d). No adverse effect was seen on the morphology of somatic embryo development from embedded cells, but somatic embryos developed in agarose were delayed in their development (results not shown).

Taken together, it was concluded that embryo development from single cells in the phytagel culture system faithfully reflects somatic embryogenesis in liquid cultures.

Cell tracking

Single cells that developed into somatic embryos were identified by studying the video images recorded at day 15 for the presence of somatic embryos and tracing back the corresponding images from previous days. These images were copied in sequence onto a second video tape. In this way the entire developmental history of the somatic embryo down to the single cell from which it originated, could be monitored. This allowed the identification and morphological classification of single embryo-forming cells. In two independent experiments, in the recorded areas of the culture dishes a total of 30 318 single cells and 857 cell clusters of 2 to 6 cells each were observed at the start of the experiment (day 0). From these cells and cell clusters a total of 155 somatic embryos developed (table 2.1).

Single cells present at day 0 could be classified into the following groups, based on their morphology: small spherical vacuolated cells (figure 2.3a), small spherical cytoplasm-rich cells (figure 2.3b), oval vacuolated cells (figure 2.3c) and elongated vacuolated cells (figure 2.3d). Cells that did not fit either of the described types were classified as irregular-shaped cells (e.g. figure 2.3e). To this last group belong single cells such as cubical cells. These cells were either cytoplasm-rich or vacuolated. Cell clusters consisting of 2 to 6 cells and cells that already had divided at the start of the culture were classified as multicellular (figure 2.3f).

table 2.1. Cells present in the scanned area of two culture dishes at day 0 grouped according to their morphology and size. The number of somatic embryos formed from each group after fourteen days of culture is given and is also expressed as number of embryos per 10 000 cells. Single cell sizes are given as an average of 10 single cells. The multicellular type size is the size of the cell cluster.

group	average cell size or cell cluster size (μm x μm)	Total number of cells or cell clusters	total number of embryos formed	number of embryos per 10 000 cells
small sperical vacuolated cells	20 x20	4 247	8	19
small sperical cytoplasm-rich cells	20 x 20	1 203	12	100
oval vacuolated cells	33 x 14	4 205	38	90
elongated vacuolated cells	60 x 15	1 503	9	60
irregular-shaped cells	44 x 25	18 977	60	32
multicellular	26 x 18	857	28	327
total		31 175	1554	50

Over 99 % of the single cells present did not divide, but after one week 70 % of the cells were still viable as determined by fluoresceïn diacetate staining. From the non-dividing single cells the spherical cells did not show any morphological changes, while many oval and elongated cells had expanded. About 50 % of the dividing cells developed somatic embryos, the remaining dividing single cells developed into clusters of vacuolated cells. The contribution of the cell types to the total population as well as the frequency with which these cell types developed into somatic embryos are listed in table 2.1.

From the results presented in table 2.1 three important conclusions can be drawn: *First*, under the conditions employed here, all single cell types distinguishable in a population of single cells smaller then 22 µm can form somatic embryos. *Second*, only a small minority of the cells in each class actually develops into somatic embryos. *Third*, although the highest frequency of embryogenesis was observed for the small spherical cytoplasm-rich cells and the oval vacuolated cells, together these classes contribute only about 40 % of the total number of somatic embryos developed.

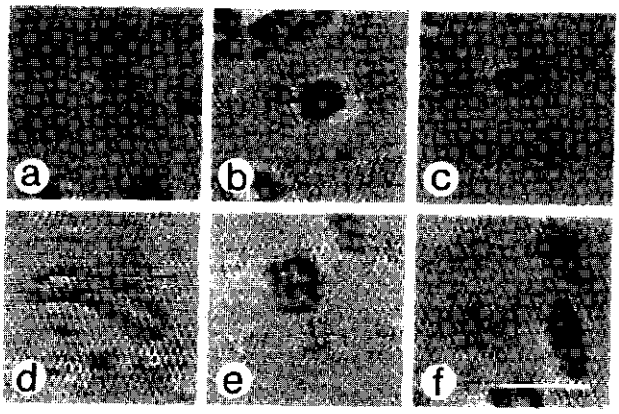

figure 2.3a-f. Examples of cell morphology of the different cell type categories present at day 0 in the embedded < 22 µm cell fraction **a.** small spherical vacuolated cell **b.** small spherical cytoplasm-rich cell **c.** oval vacuolated cell **d.** elongated vacuolated cell **e.** irregular-shaped cell **f.** multicellular cell cluster. Bar = 50 µm.

The first division of cells developing into a somatic embryo usually occurred at day 1 or 2, but in some cases did not occur before day 6. No correlation was found between the cell type and the moment at which the first division was observed. After three to four days most of the individual single cells capable of forming a somatic embryo had progressed into small clusters of cytoplasm-rich cells. According to Komamine et al. (1990) these cell clusters can be regarded as state 0 cell clusters. These cell clusters enlarged and after transfer to B5-0 medium they are referred to as state 1 and state 2 cell clusters (Komamine et al., 1990). Three major types of development from single cell to somatic embryo were observed. The first type (figure 2.4a) was characterised by an asymmetrical morphology of the state 0 as well as the state 1 and state 2 cell clusters. In general, this

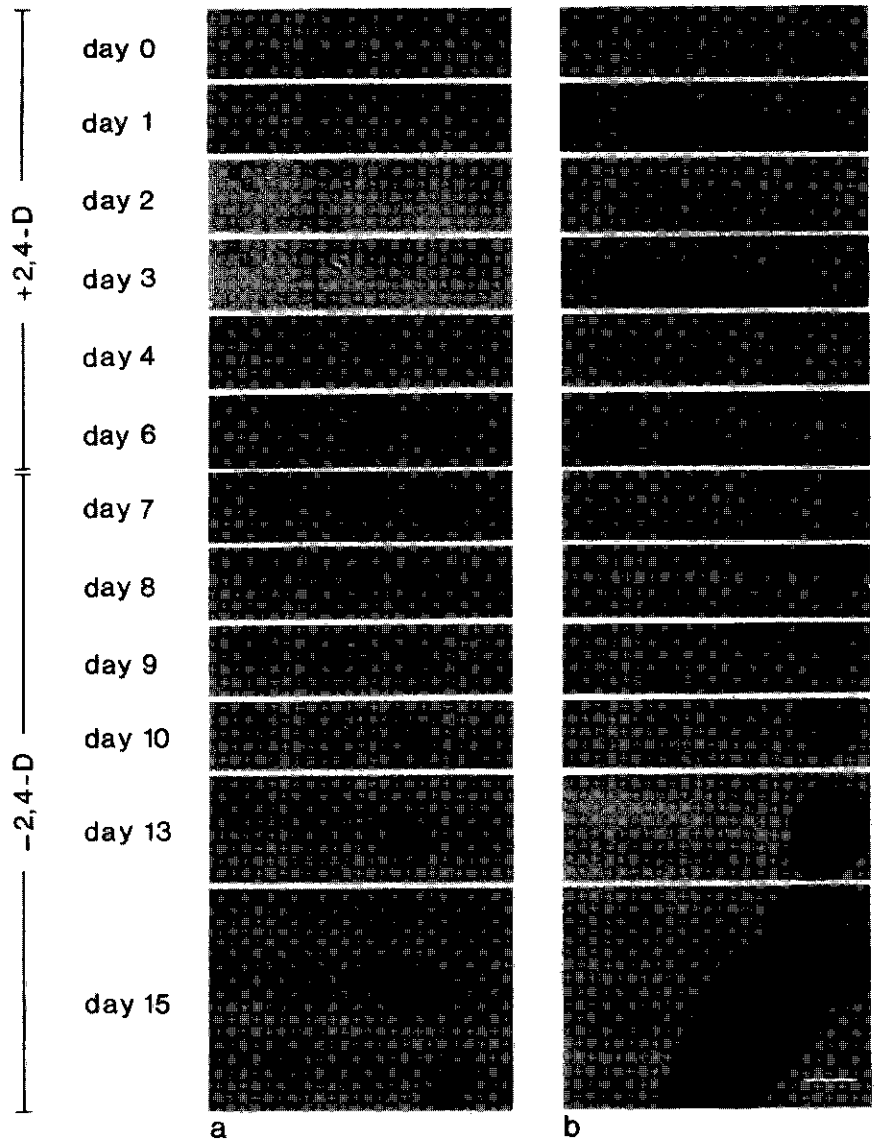

figure 2.4a-b. Development of somatic embryos from single cells. Single cells were embedded in phytagel and cultured in B5-0.2. After six days 2,4-D was removed by 10 washing steps with B5-0. **a.** Somatic embryo developed from a single oval vacuolated cell with asymmetrically developing state 0, state 1 and state 2 cell clusters. **b.** Somatic embryo developed from a single spherical cytoplasm-rich cell with spherically developing state 0, state 1 and state 2 cell clusters. Bar = 100 μm.

table 2.2. Development of somatic embryos from each group of cells via symmetrical, asymmetrical and aberrant state 0 and state 1 cell clusters.

group	number of embryos	symmetrical development	asymmetrical development	aberrant development
small sperical vacuolated cells	8	7	0	1
small sperical cytoplasm-rich cells	12	12	0	0
oval vacuolated cells	38	1	22	15
elongated vacuolated cells	9	0	4	5
irregular-shaped cells	60	11	13	36
multicellular	28	4	5	19
total	155	35	44	76

was preceded by an asymmetrical first division of single oval and elongated vacuolated cell types (table 2.2). One of the daughter cells developed into a cluster of cytoplasm-rich cells, while the other daughter cell expanded to give rise to a large vacuolated cell. Sometimes cell divisions occurred in the vacuolated cell giving rise to more large vacuolated cells. After several days a cell cluster, that was still connected to the large vacuolated cell(s), was formed from the second daughter cell. Via formation of a state 1 and state 2 cluster (figure 2.5a,b) a globular embryo developed, still having one or more large vacuolated cells attached to it. The second type of development (figure 2.4b) was characterised by state 0, state 1 and state 2 cell clusters with a symmetrical morphology (figure 2.5c,d) and was mainly observed in the development of spherical vacuolated and cytoplasm-rich cell types (table 2.2). The remaining cells showed a third developmental pathway with aberrantly formed state 0, state 1 and state 2 cell clusters (figure 2.5e-h) that nevertheless still formed morphologically normal somatic embryos later on (results not shown).

The results presented in table 2.2, figure 2.4 and figure 2.5 show that, apparently dependent on the initial cell morphology, three different developmental pathways by which a single cell develops into a somatic embryo could be identified in carrot cultures.

Discussion

This paper describes the identification of single carrot suspension cells capable of somatic embryo formation, by recording the development of individual single cells on video. The single cells observed at day 0 were categorised into five groups, in part based on previous descriptions (Backs-Hüsemann and Reinert 1970; Nomura and Komamine 1985): 1, small spherical vacuolated cells, 2, small spherical cytoplasm-rich cells, 3, oval vacuolated cells, 4, elongated vacuolated cells and 5, irregular-shaped cells. Somatic embryos were seen to develop from all five cell types, suggesting that the ability to develop somatic embryos is not restricted to one morphologically distinct cell type. Although they are somewhat smaller, the elongated vacuolated cells described here resemble the cells previously described by Backs-Hüsemann and Reinert (1970). The spherical vacuolated cell type morphologically resembles the type 1 cells identified by Nomura and Komamine (1985). However, Komamine et al. (1990) have described these type 1 cells as cytoplasm-rich. The observations presented here clearly show that both cytoplasm-rich and vacuolated spherical cells occur and can develop somatic embryos, albeit with a different frequency.

In the development from single cell to somatic embryo three characteristic pathways were observed. In the first pathway, asymmetrical cell clusters developed after the first division of oval or elongated single cells. One of the daughter cells developed a somatic embryo while the other daughter cell expanded and in some cases divided. This development morphologically resembles the development of the unicellular zygote. The second pathway was characterised by the formation of symmetrical cell clusters, developed from spherical vacuolated or spherical cytoplasm-rich cells. This pathway resembles most closely the development of somatic embryos as described by Komamine et al. (1990). However, in our work globular somatic embryos were seen to develop from the entire state 2 cell cluster whereas Komamine et al. (1990) have stated specifically that globular somatic embryos were formed by cell divisions in only certain parts of the state 2 cell cluster. In our analysis, this last type of development was observed to occur more frequently in the third developmental pathway. In this pathway aberrantly formed cell clusters developed, mainly from the irregular-shaped cell types. This indicates that aberrant initial development of cell clusters can be corrected at a later stage in somatic embryo development.

The different pathways as observed here, can be interpreted in two ways. *First*, it is possible that the spherical cells originate from oval or elongated cells by separation of the two daughter cells formed after the first asymmetrical division. However, because cell separation, if it occurs, will only take place in liquid cul-

tures and not in immobilised cultures this will be difficult to demonstrate. *Second*, according to Pennell et al. (1992) the origin of spherical cells is a larger cell cluster and the oval and elongated cells develop from spherical cells by auxin-induced elongation. In this scheme, the cytoplasm-rich spherical cells are the first population of single embryogenic cells to develop, while formation of, first, vacuolated spherical cells and subsequently oval and elongated cells represent progressive loss of embryogenic competence, finally leading to large vacuolated cells incapable of further development. In both cases, the question whether the initial population of small cells arises from the explant directly or is produced continuously from non-embryogenic suspension cells, remains to be answered. At present, it is not yet possible to draw a conclusive model for the origin of the small spherical cells and their subsequent developmental fate.

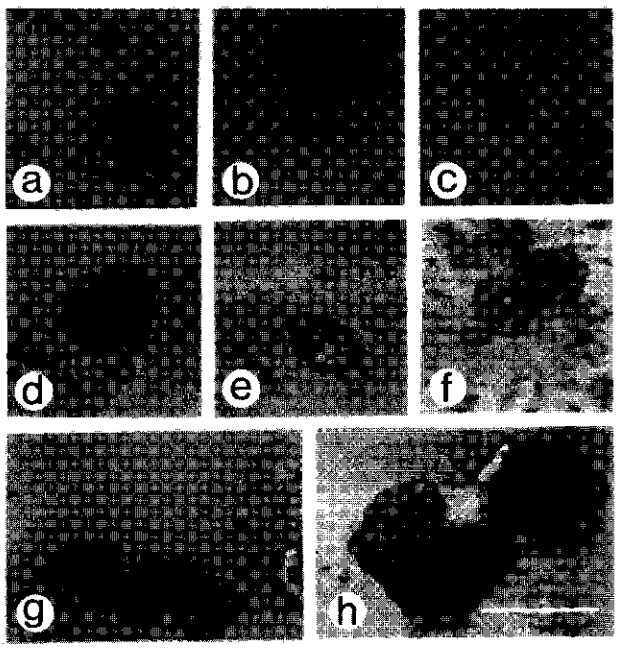

figure 2.5a-h. Examples of cell morphology of type 1 and type 2 cell clusters observed during somatic embryo development. **a.** 8 day asymmetrical cell cluster developed from an oval vacuolated cell. **b.** 8 day asymmetrical cell cluster developed from a multicellular cell cluster. **c.** 8 day symmetrical cell cluster developed from a oval vacuolated cell. **d.** 8 day symmetrical cell cluster developed from a small spherical cytoplasm rich cell. **e.** 7 day cell cluster with aberrant morphology developed from a multicellular cell cluster. **f.** 9 day cell cluster with aberrant morphology developed from an irregular-shaped cell. **g.** 9 day cell cluster with aberrant morphology at day 9 developed from an irregular-shaped cell. **h.** 10 day cell cluster with aberrant morphology developed from a multicellular cell cluster. Bar = 100 μm

In discussing the development of somatic embryos from single cells the need arises to precisely define the onset of embryo formation. In zygotic embryogenesis this point can be defined by fertilisation of the egg cell. In *in vitro* cultures externally applied auxin is the most frequently used inducer of embryogenic cells (Dudits et al., 1991), although, also other methods such as a pH shift (Smith and Krikorian 1990) have been employed. In the presence of auxin single cells can undergo a cell division eventually leading to somatic embryos. At the point in this process where auxin is no longer required to complete embryo formation, the cell or cell cluster is called embryogenic (De Jong et al., 1993b). In this definition state 1 cell clusters (Komamine et al., 1990) are embryogenic whereas single cells that still require externally applied auxin to form somatic embryos are not. The exact time point at which the embryogenic state is obtained may be anywhere between the moment the cell is exposed to auxin and the moment it has undergone the first cell division, or even at a later stage e.g. after the first cell division. Since it is not possible to define this transition with any precision, the term competent single cell has been introduced by Komamine et al. (1990) to describe single cells that form embryogenic cell clusters in the presence of auxin. Apart from auxins, other plant-produced compounds have been implicated in promoting the formation of embryogenic cells. Addition of conditioned medium to newly initiated suspension cultures of carrot stimulated the formation of embryogenic cells (De Vries et al., 1988), and addition of carrot-seed arabinogalactan proteins to a non-embryogenic cell line reinduced the embryogenic potential in this cell line (Kreuger and Van Holst 1993). In individual cytoplasm-rich *Brassica* protoplasts it was shown that the frequency of microcallus formation could only be stimulated by coculturing with a specific vacuolated protoplast type (Spangenberg et al., 1985). All these results hint at a further level of complexity, where particular non-embryogenic cell types may produce compounds that stimulate other cells to become competent. In a situation where pure cell populations are isolated these putative support cells may be lacking, resulting in a lower frequency of competent cells from such a pure cell population when compared to the frequency of somatic embryo development from the same cell type in the original heterogeneous cell suspension culture. Based on the observation that polyploid cells do not develop somatic embryos (Coutos-Thevenot et al., 1990), and that certain carrot somatic embryos were haploid, it has been proposed that chromosome reduction mechanisms play a role in the formation of embryogenic cells (Nuti Ronchi et al., 1992a, 1992b).

The observations presented here on the identification of competent single cells appear in general agreement with the model presented by Pennell et al. (1992). In this model a transitional single cell state (state B), characterised by the presence of an cell wall epitope recognised by the monoclonal antibody JIM8, was proposed to develop from a state A non-embryogenic cell devoid of this epitope.

Cells in this state B might either elongate and die or form a state C cell defined as the somatic embryo initial cell. The JIM 8-reactive state B cells, cells in the transition from state B to state C or both state B and C cells then would correlate with the competent cell state. According to the model of Pennell et al. (1992) JIM8 might act as a marker for the competent, but not the embryogenic cell state. With the use of the described cell tracking technique experiments are currently in progress to further explore the role of the JIM8 cell wall epitope in the pathway from somatic to embryogenic cell.

At present very little is known about the molecular details that are involved in the pathway that leads from somatic cells via competent cells to embryogenic cells (for review see De Jong et al., 1993b). It is however clear that the formation of somatic embryos is a process in which various cell types appear in a sequential fashion. The methodology presented in this work to follow the fate of individual cells or clusters of cells will be of great use to help define further the events that are taking place in individual cells in the pathway of somatic cell to somatic embryo.

Materials and methods

Plant material and cell culture

Embryogenic carrot (*Daucus carota L.*) suspension cultures designated cell lines "10" were maintained as described previously (De Vries et al., 1988). One-week old high-density (10^6 - 10^7 cells·ml^{-1}) cell suspensions were sieved through nylon sieves with successive 300, 125, 50 and 22 µm pore sizes. Single cells were obtained from the < 22 µm fraction. These single cells were washed twice with B5 medium (Gamborg et al., 1968) supplemented with 0.2 µM 2,4-D (B5-0.2 medium) and resuspended in the same medium. Unless stated otherwise single cells were incubated in 2 ml liquid cultures in 35-mm-diameter Petri dishes (Greiner, Frickenhausen, Germany) containing 100 000 cells·ml^{-1} in B5-0.2 medium. After seven days in B5-0.2 the culture was centrifuged for 5 minutes at 300 g and the cells were resuspended in 2 ml hormone-free B5 medium (B5-0 medium). Fourteen days after the start of the experiments the number of cell clusters and somatic embryos was determined and expressed according to De Vries et al. (1988) as the number of somatic embryos developed from 10 000 cells present at day 0 in the culture.

Cells cultured for different periods of time in the presence of 2,4-D were obtained by daily withdrawal of 2 ml samples from a suspension culture containing 100 000 cells·ml^{-1} in B5-0.2 medium. This culture was grown on a rotary shaker

at 50 rpm. Samples were washed once with B5-0 medium and subcultured in 35-mm-diameter Petri dishes. The number of cell clusters and somatic embryos was determined fourteen days after transfer to B5-0 medium. All cultures were incubated at a 16 hour light/8 hour darkness regime at 25 ± 0.5 °C. Different cell lines "10" used developed under these conditions between 36 and 105 somatic embryos from 10 000 cells. Cell viability was determent by fluoresceïn diacetate (F 7378; Sigma, St. Louis, Mo., USA) staining as described by Widholm (1972) .

Cell immobilisation

The temperature at which phytagel (P 8196; Sigma) solidifies is dependent on the Ca^{2+} concentration. In the absence of Ca^{2+} phytagel hardly solidifies at room temperature. In medium containing 1 mM Ca^{2+} (as in B5 medium) phytagel solidifies at approximately 30 °C and in medium supplemented with 5 mM Ca^{2+} at approximately 55 °C.

Petriperm dishes (Hereaus, Hanau, Germany), used for cell tracking experiments, were prepared by pouring a bottom layer of 1 ml 0.2 % phytagel in B5-0.2 medium containing 5 mM Ca^{2+} into the dish at 60 °C, and allowing the phytagel to solidify at room temperature for 5 minutes. The single cell fraction (< 22 µm) was washed twice with B5-0.2 medium without Ca^{2+}. This fraction was resuspended in the same medium at a cell density of 800 000 cells·ml^{-1} and mixed with the same volume of 0.2 % phytagel in B5-0.2 medium without Ca^{2+}. Of this mixture 0.5 ml was poured at room temperature onto the bottom phytagel layer. Within two to three hours, cells were immobilised by phytagel polymerisation due to diffusion of Ca^{2+} from the bottom layer. One ml of B5-0.2 medium was added on top of the cell-containing phytagel layer to prevent dehydration of the phytagel. After six days of culture 2,4-D was removed by replacing the liquid B5-0.2 medium with B5-0 medium. After 15 minutes incubation the B5-0 medium was replaced by fresh B5-0 and this was repeated 9 times. The effectiveness of 2,4-D removal was tested by using fluoresceïn (13200; Gurr, High Wycombe Bucks, UK). Removal of fluoresceïn from the phytagel culture system was performed as described for 2,4-D. Fluoresceïn was added at ± 60 °C to the autoclaved phytagel to a final concentration of 2 µM. Fluoresceïn diffused into the washing solutions was measured using a Perkin Elmer LS 5 luminescence spectrometer (λ_{ex} 491 nm., λ_{em} 515 nm.)

Low melting agarose embedding was performed by mixing 1 ml 1.0 % low melting agarose (Seakem; FMC Corporation, Rockland, Me., USA) in B5-0.2 medium with 1 ml of the < 22 µm cell fraction at a cell density of 200 000 cells·ml^{-1} in B5-0.2 medium at 39 °C. This mixture was poured into an 60-mm-diameter Petri dish (Greiner).

Cell tracking

Cell tracking was performed using a Zeiss inverted microscope (C. Zeiss, Oberkochen, Germany) equipped with a motorised microscope stage (Märzhäuser, Wetzlar, Germany). The microscope stage was linked to control electronics and operated by a personal computer. Following calibration of the culture dish 960 microscopic images (covering 18 % of the dish) were recorded on a VHS video recorder using the scanning program STEPS (available at CPRO-DLO). Microscopic images were recorded in replicate dishes at day 0, 1, 2, 3, 4 and 6. At day 6 2,4-D was removed and subsequent recordings of the microscopic images were made at day 7, 8, 9, 10, 13 and 15.

Acknowlegdements

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3

Expression of the JIM8 cell wall epitope in carrot somatic embryogenesis

Certain single cells in carrot (*Daucus carota* L.) suspension cultures react with the monoclonal antibody JIM8, and it has been proposed that these cells represent a transitional stage in somatic embryo formation. Shortly after isolation of the single cells by sieving, up to 80% of the cells react with JIM8. Within 4 days JIM8 labelling becomes restricted to 1% of the single cells. To obtain evidence for the proposed correlation between expression of the JIM8 cell wall epitope and somatic embryo formation the developmental fate of carrot single cells labelled with JIM8 was determined by cell tracking. The results, obtained by recording 43 000 cells, show that only a few JIM8-labelled cells give rise to embryos, and most somatic embryos develop from cells devoid of the JIM8 cell wall epitope. We therefore conclude that the presence of the JIM8 cell wall epitope does not coincide with the ability of single suspension cells to form embryos.

Marcel A.J. Toonen, Ed D.L. Schmidt, Theo Hendriks, Harrie A. Verhoeven, Ab van Kammen, Sacco C. de Vries (1996) Planta **200**, 167-173

Introduction

Embryogenic suspension cultures of carrot can be obtained by incubation of hypocotyls in culture medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). After five to seven days of treatment, cells from the provascular tissue show a reduced vacuolar volume, and have started to divide in both longitudinal and transverse planes to form small isodiametric cells. Cells on the surface of the resulting provascular mass enlarge and detach to give rise to the embryogenic suspension culture. Three days of treatment with 2,4-D is needed to generate an embryogenic cell culture, whereas a shorter treatment leads to organogenesis (Guzzo et al., 1994, 1995).

In an established suspension culture, somatic embryos can develop from embryogenic cell clusters (De Vries et al., 1988) and single cells (Backs-Hüsemann and Reinert 1970; Nomura and Komamine 1985; Chapter 2). Initiation of somatic embryogenesis from single cells requires 2,4-D and re-activation of the cell cycle (reviewed by Dudits et al., 1995). Cells that develop into embryos in the presence of 2,4-D are defined as competent cells. The first morphologically visible sign of embryo development is division of a single cell that subsequently develops into a cluster of small cytoplasm rich cells. Via successive globular-, heart- and torpedo-stage embryos these cells develop into a plantlet. The small cell clusters do not require externally applied 2,4-D for embryo development and are defined as embryogenic (De Jong et al., 1993b; Chapter 2).

Only a very limited number of cells in a hypocotyl explant or in a single cell population respond to treatment with 2,4-D by becoming competent to develop into somatic embryos. To study the initial processes in somatic embryogenesis it is essential to identify competent cells as early as possible during development. Using cell tracking it has been shown that morphologically distinct single cells can develop into somatic embryos and that cell morphology cannot therefore be used to identify competent cells (Chapter 2).

In oilseed rape the monoclonal antibody JIM8 has been shown to react with arabinogalactan protein (AGP) epitopes in sexual organs, the eight-celled embryo and a very limited number of other cell types (Pennell et al., 1991). In carrot suspension cultures the JIM8 epitope has been localised on three different cell-membrane AGPs (Pennell et al., 1991) and on AGPs secreted into the suspension culture (Knox et al., 1991). The cell wall epitope, present in spherical, oblate and oval carrot suspension cells (Pennell et al., 1992) has recently been identified as an AGP (R.I. Pennell personal communication). Spherical and oblong cells showed an uniform distribution of the cell wall epitope while the epitope was present at one or both cell poles of more-elongated cells. Large single cells and

proembryogenic masses did not react with the JIM8 antibody (Pennell et al., 1992). It is unclear whether the JIM8 cell wall AGP epitope is related to the membrane AGPs or to secreted AGPs.

It has been postulated that the JIM8 cell wall epitope marks a transitional state in the formation of embryogenic cells. Pennell et al. (1992) presented a model in which a somatic cell (state A) can obtain the JIM8 cell wall epitope (state B). This cell can either elongate and form a non-embryogenic state-D cell or divide to form a state-C cell devoid of the JIM8 epitope. This state-C cell will develop into the embryo.

According to this model, expression of the JIM8 epitope coincides with the transition from somatic cell into competent cell, which probably represents the first and maybe most important stage of somatic embryogenesis. Therefore, JIM8 might be used to identify competent single cells, facilitating further research into the early stages of somatic embryo development. Here we report on the developmental fate of JIM8-labelled cells, determined by semi-automatic cell tracking. The results show that most somatic embryos develop from single cells that are devoid of the JIM8 epitope, indicating that the JIM8 cell wall epitope is not a marker for competent single cells. A role for the JIM8 cell wall epitope as a marker for competency or embryogenic capacity of a cell culture rather than for a competent cell state is discussed.

Results

Optimising conditions for in vivo JIM8 labelling

Labelling with JIM8 requires high Ca^{2+}

To allow normal development of single cells labelled with JIM8, labelling conditions have to mimic standard culture conditions used for somatic embryo development. Therefore, labelling of carrot suspension cells in Gamborg B5 medium was compared with the labelling in phosphate buffered saline (PBS) described by Pennell et al. (1992). In their original immersion immunofluorescence experiments where JIM8 labelling was visualised with a second goat anti-rat IgG FITC conjugate, Pennell et al. (1992) showed that in PBS single cells, loosely attached groups of cells and cells at the surface of cell clusters reacted with JIM8. Our experiments gave similar results in PBS (figure 3.1a). In parallel, a control labelling experiment with only the goat anti-rat FITC conjugate did not show any specific bright green FITC immunofluorescence signal (figure 3.1b). However, a green/yellow and a red fluorescent background signal was observed. This signal was also visible in the JIM8 labelling under the different conditions applied. The green/yellow sig-

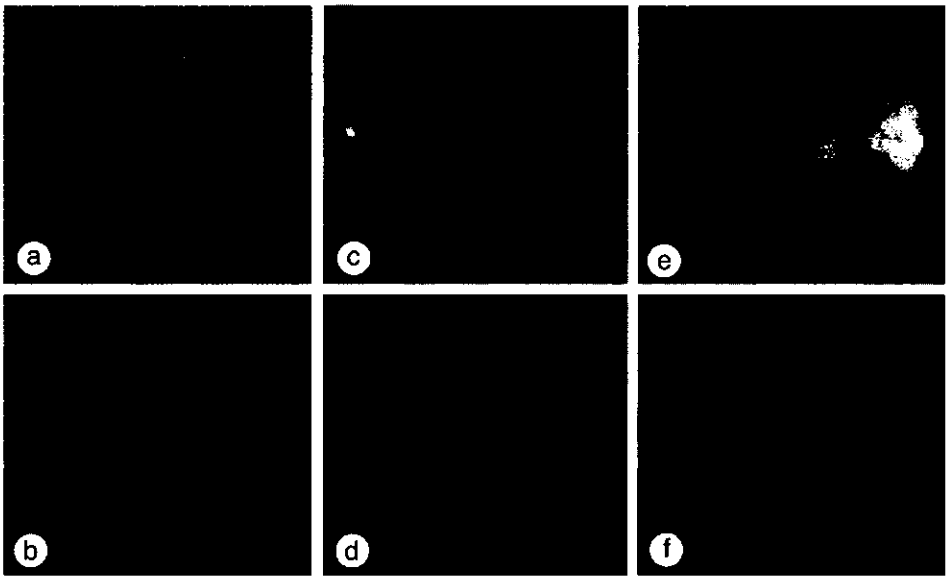


figure 3.1a-f. Expression patterns of the JIM8 epitope in carrot suspension cultures. Labelling with JIM8 was performed in PBS medium, B5 medium and B5 medium supplemented with 20 mM Ca^{2+} to test the effect of high salt concentrations on labelling patterns. One-week-old cell cultures were incubated in calf serum to block aspecific binding sites and thereafter either treated with JIM8 hybridoma supernatant followed by the FITC-conjugated second antibody or only with the FITC conjugated second antibody. **a.** Labelling in PBS medium gives a bright green FITC signal, showing expression of the JIM8 epitope; **b.** control labelling in PBS of cells only treated with the goat anti-rat FITC conjugate without JIM8 gives only faint green auto-fluorescence signal; **c.** and **d.** Labelling with JIM8 in B5 medium does not give a specific signal of expression of the JIM8 epitope but some red auto fluorescence of chloroplasts is observed; **e.** and **f.** JIM8 labelling in B5 medium supplemented with 20 mM Ca^{2+} restores the specific JIM8 signal as observed for JIM8 labelling in PBS medium. Bar = 20 µm

nal, caused by phenolic compounds present in the cell walls, was mainly detected in large cell clusters (figure 3.1b,e). Red autofluorescence caused by chloroplasts was observed in a small number of single cells and cell clusters (figure 3.1d,e). The labelling pattern of JIM8 in B5 medium (figure 3.1c,d) resembled the non-specific signals observed in the control FITC conjugate labelling rather than the specific JIM8 labelling obtained in PBS. The JIM8 labelling pattern as observed in PBS could be restored by addition of 100 mM NaCl (data not shown) or 20 mM $CaCl_2$ to the B5 medium (figure 3.1e,f).

Previously, we have shown that increased Ca^{2+} concentrations in the culture medium has an inhibitory effect on somatic embryo development from single cells. Culturing single cells for two weeks in the presence of 5 mM Ca^{2+} resulted in a 50 % decrease in the number of somatic embryos formed (Chapter 2). Culturing single cells for 4 hours in 20 mM Ca^{2+}, to allow JIM8 labelling and re-

cording of single cells, and subsequent removal of excess Ca^{2+} did not affect the frequency or development of somatic embryos from single cells (figure 3.2a). The control experiments presented in figure 3.2a showed that there is no effect on the number of somatic embryos after incubation in the JIM8 conjugate, Ca^{2+}, calf serum or with calf serum, Ca^{2+} and JIM8 conjugate (the entire labelling procedure). Morphologically, the embryos derived from the treated single cells were identical to embryos derived from the non-treated control cells.

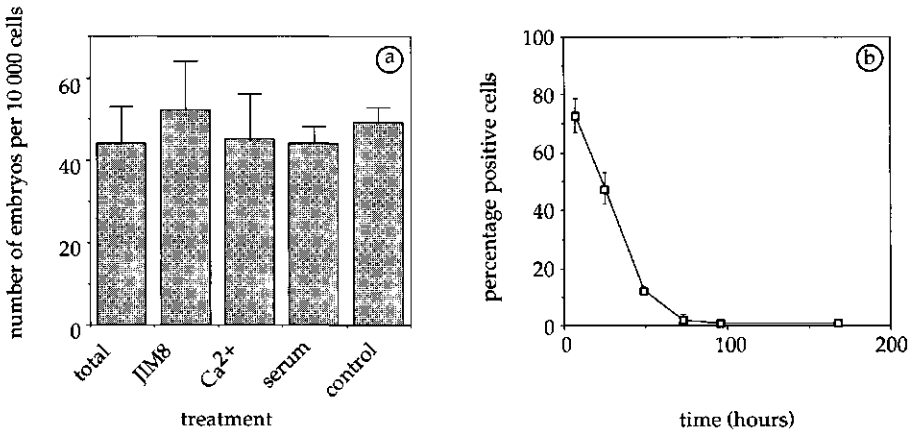


figure 3.2a-b. Effects of the different treatments required for *in-vivo* JIM8 labelling on the number of somatic embryos formed from 10 000 cells. **a.** Single cells smaller than 22 µm were incubated in B5-0.2 medium supplemented with 1.25% calf serum and 20 mM Ca^{2+} for 30 minutes. Subsequently, JIM8-FITC conjugate was added to a final concentration of 5% (total). In addition, single cells were cultured in B5-0.2 medium supplemented with either 5% JIM8-FITC conjugate, 20 mM Ca^{2+}, 1.25% calf serum or in basal B5 medium (control). After 4 hours incubation, all cultures were washed with basal B5-0.2 medium to remove the supplemented compounds and cells were cultured to allow somatic embryo development. The number of embryos scored after two weeks is indicated per 10 000 cells present at day 1 of the experiment; **b.** Single cells smaller than 22 µm were isolated by sieving through a 22 µm polyester sieve, concentrated by centrifugation and cultured in 2 ml cultures in B5 medium. After the indicated times, samples were labelled with JIM8 and a viability stain was performed. The percentage of JIM8 positive cells (open squares) and percentage of viable cells (closed squares) at the time after isolation are shown. Bars represent the standard deviation (± SD; n=3)

Induction of the JIM8 epitope

In unfractionated carrot suspension cultures, 0.5 to 5 % of the single cells smaller than 22 µm reacted with the JIM8 antibody. However, isolation of these cells by sieving the suspension culture induced the presence of the JIM8 cell wall epitope and to our surprise, 70 to 80 % of the single cells then became labelled with the

JIM8 antibody. Approximately 7 % of the single cells present at the start of the experiment were dead, as determined by viability staining. Large numbers of cells lost the JIM8 epitope with time as shown by the decrease in the percentage of JIM8-reactive cells (figure 3.2b). Twenty hours after JIM8 labelling the number of JIM8 reactive cells varied between 5 and 50 % in different experiments, and after 40 to 90 hours the number of JIM8-reactive cells reached a steady state level of about 1 %. These results show that there is a large variation in the speed with which the JIM8 cell wall epitope becomes undetectable by the antibody. During the first week embryogenic cell clusters developed from single cells. The number of remaining single cells stayed constant but the number of viable cells decreased from 93 % to 85 % (figure 3.2b) indicating that an additional 7 % of the cells died during the first week of culture.

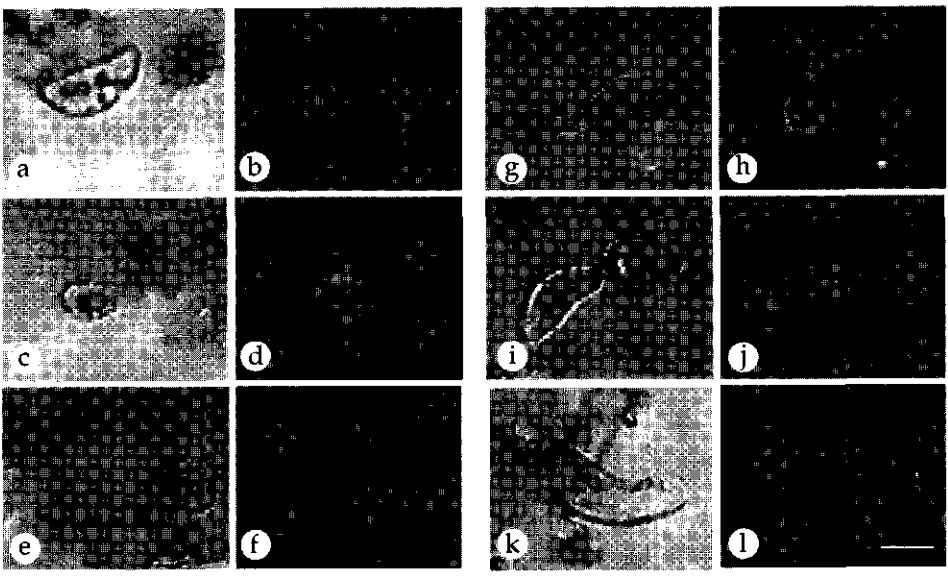


figure 3.3a-l. Expression patterns of the JIM8 epitope of embedded single carrot cells labelled with JIM8-FITC conjugate on day 1 of the cell tracking experiment. First and third columns from the left show visible light images. Second and fourth columns show the JIM8-FITC signal as a white colour. Different types of expression patterns are shown e.g.: **a.** an oval cell; **b.** with no expression of the JIM8 epitope; **c.** a dead cell; **d.** showing strong JIM8 expression on the cell wall; **e.** a spherical cell; **f.** expressing the JIM8 epitope at the cell wall around the cell; **g** an elongated cell; **h.** expressing the JIM8 epitope at the cell wa.ll around the cell; **i** two oval cells; **j.** of which only one shows expression of the JIM8 epitope along one side; **k.** an elongated cell; **l.** showing expression of the JIM8 epitope at one pole of the cell. Bar = 50 µm

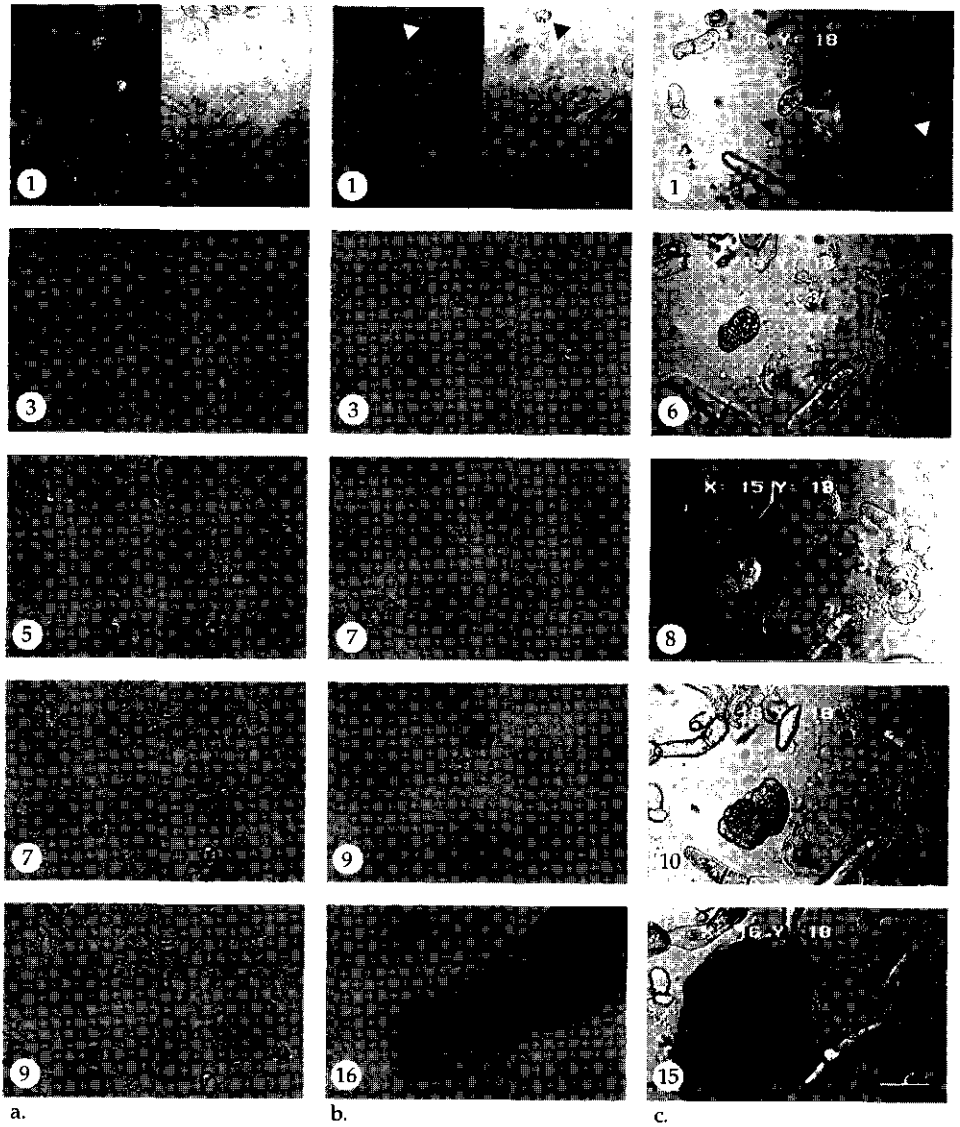
Immobilisation system

Immediately after isolation of the single-cell fraction by sieving, the cells were embedded in phytagel in B5-0.2 medium (day 0). Embedding in phytagel does not influence somatic embryo development or the frequency of embryo development from single cells (Chapter 2). After 16 to 20 hours of incubation, non-specific cell wall epitopes were blocked by calf serum added to the embedded cells in liquid B5-0.2 medium supplemented with $CaCl_2$. Diffusion of these compounds into the phytagel layer gave a final concentration of 1.25 % calf serum and 20 mM Ca^{2+} in the single-cell culture. After 30 minutes the liquid layer was replaced by a 10 % JIM8-FITC conjugate in B5-0.2 medium supplemented with 20 mM $CaCl_2$ and 1.25 % calf serum. The JIM8-FITC conjugate was chosen because it precluded a second labelling step with a second FITC-conjugated antibody. Labelling patterns obtained with the JIM8-FITC conjugate were similar to the patterns obtained with JIM8 detected by the goat anti-rat FITC conjugate as described above (data not shown). After one hour unbound JIM8 antibodies were removed by extensive washing of the embedded single cells. At this time the percentage of JIM8 reactive cells varied between 5 and 10 % in the three different cell tracking experiments described. Visible light images and fluorescent images of a defined area of the culture dish were recorded using the cell tracking equipment coupled to the confocal scanning laser microsope (CSLM). After scanning the culture dish, the phytagel layer was washed with basal B5-0.2 medium to remove excess Ca^{2+}, calf serum and JIM8-FITC conjugate. After washing, the Ca^{2+} concentration was reduced to the standard 1 mM. Fluorescent signal from the JIM8-FITC conjugate was mainly observed on cell wall remnants. Single cells showed only traces of JIM8-FITC labelling. At day 6, 2,4-D was removed from the culture to allow embryo development beyond the globular stage.

Cell tracking

The development of JIM8-labelled single cells into somatic embryos was followed by employing semi-automatic cell tracking. This system allowed the recording of the development of many thousands of individual cells. The cell tracking system was coupled to the CSLM to detect the JIM8-FITC signal. Damage to the cells due to UV light was prevented by operating the CSLM at low light intensity. After JIM8 labelling the fluorescent and visible light images of several thousand cells present in the scanning area of one culture dish could be recorded in 75 minutes. In three independent experiments, each experiment consisting of two to four culture dishes, the development of single cells was monitored by recording images every second day for over two weeks.

Of the embedded single cells 6.9 % reacted with the JIM8 monoclonal anti-



a. b. c.

figure 3.4a-c. Development of embedded, JIM8 labelled carrot cells into somatic embryos as observed by cell tracking. On day 1, both the visible light image and the JIM8-FITC conjugate fluorescent signal are shown. The fluorescent signal is visualised as white signal. subsequent development of the embryo, indicated by the day numbers, is shown in the lower panels; **a.** Development of a partially reactive JIM8 labelled cell into an early globular embryo. The JIM8 signal is visible on the partly reactive cell *a* that develops into the early globular embryo. Negative cells are characterised by the absence of fluorescent signal (cell *b*); **b.** Development of a JIM8 unreactive single cell into a torpedo stage somatic embryo; **c** Development of an JIM8 unreactive divided cell into a heart stage somatic embryo. The numbers on the pictures in c are coordinates of the cell tracking system used to identify the image position. Bar = 20 μm

body. This included all morphologically distinct cell types, spherical, oval, elongated and irregular-shaped cells, present in the cell culture. Two types of labelling with the JIM8 monoclonal antibody were observed. (i) 3.7 % of the cells showed JIM8 labelling on patches of the cell wall or at the poles (figure 3.3i,j and k,l). This distribution was observed mainly in oval and elongated cells. (ii) The other 3.2 % JIM8-reactive single cells consisted of all cell types and showed an equal distribution of the JIM8 epitope over the entire cell wall (figure 3.3e,f and g,h). The cell wall of dead plasmolysed cells (figure 3.3c, d) and cell wall debris from damaged cells (figure 3.4b1) reacted strongly with the JIM8-FITC conjugate. The majority of the embedded single cells (93 %) did not react with the JIM8 antibody (figure 3.3a,b).

In total, 45 somatic embryos were seen to develop from a collection of 43 000 single cells. This frequency of somatic embryo development is correlated with the low embryogenic capacity of the cell lines used and is not due to JIM8 labelling (figure 3.2a). Of the 45 embryo-forming cells, 42 cells could be identified. The remaining three cells were not in the focal plane at day 1 and therefore could not be classified. No somatic embryos developed from JIM8-reactive single cells, two developed from partially JIM8-positive cells and 38 developed from JIM8-negative single cells. In two cases a somatic embryo developed from a two-celled cluster, morphologically resembling a divided zygote (figure 3.4b). Both cells of this cluster were JIM8 negative.

Discussion

This paper describes the development of carrot suspension cells labelled with the JIM8 antibody. Directly after isolation of a single cell population by sieving, about 70-80 % of the isolated cells were JIM8 reactive. This percentage slowly decreased to about 1 % after 40 to 90 hours. In a typical cell-tracking experiment about 7% of the cells in the total single cell population and 5 % of the cells that formed embryos were labelled by the JIM8 antibody. This indicates that there is no direct relation between the presence of the JIM8 epitope in the cell wall and the ability of that cell to develop into a somatic embryo. It can therefore be concluded that the JIM8 monoclonal antibody is not a marker for competent single cells. However, the JIM8 antibody might be used as a marker for embryogenic competence of a total cell population.

A surprising observation was the increase in the number of JIM8-reactive cells after sieving a cell population. In established embryogenic suspension cultures the number of JIM8-reactive single cells smaller than 22 μm is between 0.5 and 5 %. In different experiments this percentage increased to 70-80 % after isola-

tion of the single cells by sieving and centrifugation. This increase in the number of JIM8-reactive single cells resembles a stress response due to treatment of the cells. The basis of this response is not clear. Expression of the JIM8 epitope could have been caused by *de-novo* synthesis of new JIM8-containing cell wall AGPs that were subsequently transported to the cell wall. Another possibility is that the JIM8 epitope was present in the cell wall but was masked by other cell wall components which were removed by mechanical damage during sieving and centrifugation, thus exposing the JIM8 epitope. Cell wall remnants also reacted strongly with the JIM8 antibody, indicating that the JIM8 epitope was already present in the cell walls before the cells burst and that no *de-novo* synthesis of the epitope is required. Removal of the JIM8 epitope from the cell walls proceeded with variable speed in the different experiments performed. It took 40 to 90 hours to reduce the number of JIM8-labelled cells to the basic level of about 1 %. It is possible that the JIM8 epitope threfore, becomes masked again as a result either of cell wall synthesis or rearrangement of cell wall proteins. Another possibility is that the JIM8 epitope is released into culture medium, either as total AGP, or as polysaccharides released after the action of degrading enzymes.

Addition of 20 mM Ca^{2+} to the B5 medium was required to obtain a JIM8 expression pattern in B5 medium that resembled the JIM8 expression pattern in PBS as described by Pennell et al. (1992). A similar effect could be observed in the presence of 100 mM Na^{+}. Without additional Ca^{2+}, JIM8 labelling in B5 medium gave patterns comparable to background signals. High salt concentrations might be required to induce a change of conformation in sugar groups present on JIM8-reactive AGPs. Only after this conformational change might the epitope be recognised by the JIM8 antibody. Another possibility is that high salt masks electrical charges in either the AGP or JIM8 molecule.

While re-activation of the cell cycle (Dudits et al., 1995).and chromosome-reducing mechanisms (Nuti Ronchi et al., 1992b; Giorgetti et al., 1995) have been implicated as important mechanisms in competent cell formation, no specific markers are presently available to identify these cells. According to the model presented by Pennell et al. (1992), expression of the JIM8 epitope coincides with the competent cell stage and thus can be used as a molecular marker. However, the experiments described here show that there is no direct correlation between expression of the JIM8 epitope on the cell wall of a cell and the competent cell state. The JIM8 cell wall epitope has been identified as an AGP (R.I. Pennell, personal communication). It has been shown that this type of proteins plays a role during somatic embryo development (Kreuger and Van Holst 1993; Egertsdotter and Von Arnold 1995). When added in nanomolar concentrations AGPs purified from embryogenic *Daucus* suspension cultures and from dry *Daucus* seed were able to promote the formation of embryogenic cell clusters and somatic embryos

in embryogenic and even previously non-embryogenic carrot suspension cultures (Kreuger and Van Holst 1993; 1995). AGPs isolated from dry Norway spruce-seeds allowed the transition from a less developed embryo type (group B embryos) into a more mature embryo type (Egertsdotter and Von Arnold, 1995). Binding of ß-D glucosyl Yariv reagent to cell wall AGPs of rose suspension cells inhibited growth in a reversible fashion, probably due to eventual suppression of the cell cycle in combination with prevention of cell expansion (Serpe and Nothnagel 1994). These observations hint that AGPs have a function in cell growth and division. Culture-medium AGPs carrying the JIM8 epitope might be developmentally related to the cell wall epitope and produced by specific 'nursing' cells in the culture. Such nursing effects have been described for individual *Brassica* protoplasts, where the frequency of microcallus formation from cytoplasm-rich protoplasts was increased by co-cultivation with a more vacuolated protoplast type (Spangenberg et al., 1985). In established carrot suspension cultures there is a good correlation between the level of JIM8-reactive cells and the number of somatic embryos formed (Pennell et al., 1992). However, since most somatic embryos develop from JIM8-negative cells this may reflect the embryogenic competence of the entire culture rather than mark individual competent cells. This suggests that acquisition of embryogenic competence involves cells or the product of cells that are themselves not able to develop into somatic embryos.

Materials and methods

Plant material and cell culture

Embryogenic carrot (*Daucus carota* L. cv. Trophy; S&G seeds, Enkhuizen, The Netherlands) suspension cultures were maintained as described previously (De Vries et al., 1988). Single cells smaller then 22 µm were obtained by sieving through polyester sieves (Monodur-PES; Verseidag Techfab, Walbeck, Germany; Chapter 2). All cultures were incubated in a 16 h light/8 h darkness regime at 25 ± 0.5°C. Embryogenic competence is expressed according to De Vries et al. (1988) as the number of somatic embryos developed per 10 000 cells present at the initiation of the culture. Cell viability was determined with fluoresceïn diacetate (F7378; Sigma, St. Louis, Mo., USA) as described by Widholm (1972).

Monoclonal antibodies

JIM8 antibodies (Pennell et al., 1991) were isolated from the JIM8 hybridoma culture supernatant by binding to Bakerbond 40 µm prepscale ABx (7269-02; J.T. Baker

Inc., Phillipsburg, N.J., USA) as described by the manufacturer. A column with JIM8-bound ABx was prepared and antibodies were eluted using 20 mM NaAc, 500 mM $(NH_4)_2SO_4$, pH 6.8. The JIM8 antibody fraction was dialysed against 0.1 M sodium bicarbonate buffer (pH 9.0) and conjugated to fluorescein isothiocyanate (FITC) using the QuickTag FITC conjugation kit (1248 618; Boehringer, Mannheim, Germany) according to the manufacturer's protocol.

Immersion immunofluorescence

Labelling of the JIM8 cell wall epitopes was performed on one week old suspension cultures or < 22 µm cell fractions. One millilitre of the suspension cultures was transferred into 10 ml PBS (150 mM NaCl, 10 mM Na_2PO_4, pH 7.2), Gamborg's B5 medium (B5 medium) or B5 medium supplemented with 20 mM Ca^{2+}. All solutions contained 2 % calf serum (56010-010; GibcoBRL, Breda, The Netherlands). After washing, cells were resuspended in 2 ml of the same solution containing 5 % JIM8 hybridoma culture supernatant or 2 % FITC-JIM8 conjugate. After 1 h excess unbound JIM8 antibody was removed by washing twice with the washing solution. The JIM8 antibody was localised by resuspending the cells in 0.1 % goat anti-rat IgG FITC conjugate (F6258; Sigma) followed by washing to remove excess FITC conjugate. Control experiments were performed using the goat anti-rat IgG FITC conjugate as antibody. Immunofluorescence was observed with a Nikon Optiphot with epifluorescence-optics using a B1a filterset (excitation 470-490 nm, dichromatic mirror 510 nm, barrier filter 520 nm) and a low level of phase contrast (Nikon, Tokyo, Japan). Colour micrographs were taken using Kodak Ektachrome 1600.

Cell immobilisation and in-situ immersion immunofluorescence

Single cells were immobilised in 0.1 % phytagel (P8196; Sigma) in B5-0.2 medium without Ca^{2+}. This suspension was poured onto a 0.2 % phytagel-layer in B5-0.2 medium containing 5 mM Ca^{2+} in Petriperm dishes (Hereaus, Hanau, Germany) as described in chapter 2. After 16 h incubation, non-specific binding was blocked by adding onto the phytagel layer an equal volume of 2.5 % calf serum in B5-0.2 medium containing 40 mM Ca^{2+}. After 30 minutes the blocking solution was replaced with 10 % JIM8 FITC conjugate in B5-0.2 medium containing 20 mM Ca^{2+}. One hour later, unbound JIM8 was removed by eight washing steps of 10 minutes each with B5-0.2 medium supplemented with 20 mM Ca^{2+}. After recording (see below), excess Ca^{2+} was removed from the immobilised cell culture by washing ten times with basal B5-0.2 medium. Due to this treatment the JIM8 signal was

also removed. Subsequent visible light images were recorded for two weeks on alternate days to follow development of the single cells. At day 6, 2,4-D was removed to allow embryogenesis to proceed beyond the globular stage (Chapter 2).

Cell tracking

Cell tracking was performed using a Nikon Diaphot microspcope equipped with a BioRad MRC-600 confocal scanning laser system (BioRad, Cambridge, MA, USA) and a Märzhäuser EM 32 IM cross-table (Märzhäuser, Wetzlar, Germany). The cross-table was controlled by the program MicroScan 1.0 (Agricultural University) running on a Tulip 486 personal computer (Tulip, 's Hertogenbosch, The Netherlands) via a MultiControl 2000-3 unit (Märzhäuser). This set-up allowed scanning of the dishes in the X and Y directions as well as in the Z direction and the recording of the fluorescent and visible signals simultaneously. FITC was detected using the FITC filterset (excitation 488 nm, emission 515 nm). The images obtained by the BioRad system were recorded on a VHS video recorder (Hitachi, Tokyo, Japan). Initial experiments were performed using a similar set-up using a Zeiss Axiovert inverted microscope (Zeiss, Oberkochen, Germany).

Video images were transferred to and digitised by a Macintosh 7100 AV PowerPC computer (Apple, Cupertino, Ca, USA) using the VideoMonitor image-grabbing program (Apple). Contrast and brightness of the images was optimised in Photoshop 2.5.1 (Adobe Systems Inc., Mountain View, Ca, USA).

Acknowledgements

We thank Marijke Hartog (dept. of Molecular Biology, Agricultural University Wageningen, The Netherlands) for maintenance of the cell cultures. E.D.L.S. is supported by the Technology Foundation (STW, Utrecht, The Netherlands) and T.H. was supported by the European Commission Biotechnology Program PTP-BIOTECH.

4

Promotive and inhibitory effects of arabinogalactan proteins on *Daucus carota* somatic embryogenesis

Extracellular secreted proteins play an important role in the development of carrot (*Daucus carota* L.) somatic embryos. Secreted arabinogalactan proteins (AGPs) isolated on the basis of specific epitopes have been reported to be able to either promote or inhibit somatic embryo development. Isolation of AGPs containing these epitopes has resulted in an AGP fraction able to stimulate somatic embryo development (Kreuger and Van Holst (1995) Planta **197**, 135-141). Addition of this AGP fraction to different size-fractionated cell populations isolated from carrot suspension cultures did not show any significant effect on the frequency nor on the cellular morphology of the somatic embryos produced. An AGP fraction containing the JIM8 epitope showed an inhibitory effect on the frequency of somatic embryo development from single cells. Addition of carrot-seed AGPs to non-embryogenic cell suspensions did not directly promote embryogenic competence in the suspension culture. Only after enrichment for cell clusters and removal of most of the single cells an increase in embryogenic competence was observed.

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Sacco C. de Vries (1997) Planta, in press

Introduction

During carrot somatic embryogenesis several proteins are secreted into the culture medium. Some of these extracellular proteins appear to play an important role in the development of somatic embryos. The arrest during somatic embryo development of the carrot *ts11* variant could be rescued by addition of the secreted endochitinase EP3 (De Jong et al., 1992; 1995) while a secreted peroxidase (EP5) could reverse the inhibitory effects of tunicamycin on embryo development (Cordewener et al., 1991). Arabinogalactan proteins (AGPs) also play a role in somatic embryo development in carrot and *Picea*. When added to embryogenic carrot cell populations AGPs increased the number of cells that morphologically resemble embryogenic cells. In non-embryogenic carrot suspension cultures AGPs re-initiated embryogenic potential (Kreuger and Van Holst 1993). In *Picea* AGPs promoted the transition to a more mature embryo type (Egertsdotter and Von Arnold 1995).

AGPs are present on cell membranes, in cell walls, in the intercellular spaces of tissues and are secreted into the medium in cell cultures (for review see Fincher et al., 1983; Kreuger and Van Holst 1996). AGPs are proteoglycans with poly- and oligosaccharide units covalently attached to their protein moiety (Van Holst and Klis 1981) that form a complex with a specific synthetic ß-glucosyl Yariv reagent (Yariv et al., 1962). The carbohydrate part is rich in 1-3 linked ß-D-galactopyranose residues branched with 1-6 linked ß-D-galactopyranose which in turn carry arabinofuranose and other less abundant monosaccharides like L-rhamnose, glucoronic acid and D-xylose (Fincher et al., 1983). The polypeptide part constitutes between 2 and 10 % of the molecule and the total molecular weight is over 100 000 daltons. Some cDNAs encoding the protein moiety have been isolated (Chen et al., 1994; Du et al., 1994; Mau et al., 1995). No sequence homology exists between the different protein backbones, but all contain domains rich in alanine, hydroxyproline, serine and threonine (Mau et al., 1995). Also AGP protein backbones are known without hydroxyproline (Baldwin et al., 1993; Mollard and Joseleau 1994).

In plants a wide variety of AGPs is expressed. Membrane bound AGPs in root and floral meristems have been shown to display developmentally regulated patterns of expression. They are not tissue specific markers but rather seem to predict emerging tissue patterns (Pennell and Roberts 1990; Stacey et al., 1990; Knox 1993; Smallwood et al., 1994) or developmental fate of cells such as cell death (Schindler et al., 1995). For the monoclonal antibody JIM8 it has been postulated that it recognises AGP epitopes (R.I. Pennell, personal communication) on the surface of cells in the transition from somatic to embryogenic cells (Pennell et al., 1992). Recently, it was shown that there is no correlation between the expres-

sion of the JIM8 cell wall epitope and the ability to develop into a somatic embryo (Chapter 3).

Different types of AGPs are secreted into the culture medium of cell suspension cultures from various plant species (Van Holst et al., 1981; Knox et al., 1991; Komalavilas et al., 1991; Smallwood et al., 1996). Treatment of *Rosa* sp. cell cultures with ß-glucosyl Yariv reagent led to suppression of cell division, while cell elongation was not affected (Serpe and Nothnagel 1994). In carrot suspension cells however, Yariv reagent inhibited cell elongation (Willats and Knox 1996) suggesting that different, possible AGP-mediated processes can be affected depending on the cell line and culture conditions employed. Different AGP populations were able to increase or decrease embryogenic potential in carrot suspension cultures. In old cell lines that lost the ability to develop somatic embryos, embryo formation could be re-initiated by addition of carrot-seed AGPs (Kreuger and Van Holst 1993). Addition of Norway spruce-seed AGPs to an immature type B spruce somatic embryo culture resulted in the formation of more developed embryos. But to complete development into the mature type A somatic embryos, addition of total seed extract was required, indicating that beside AGPs other components are required as well (Egertsdotter and Von Arnold 1995).

Different AGP fractions were obtained by column affinity chromatography with monoclonal antibodies raised against carrot-seed AGPs (Kreuger and Van Holst 1995). The AGP fraction isolated with the monoclonal antibody ZUM18 (ZUM18AGPs) was shown to increase the percentage of embryogenic cells in carrot suspension cultures about two-fold. An about two-fold decrease on the percentage of embryogenic cells was shown with the AGP fraction isolated with the ZUM15 antibody (ZUM15AGPs). The effect of ZUM15AGPs was comparable to AGPs isolated from non-embryogenic suspension cultures. ZUM18AGPs as well as ZUM15AGPs are present in seeds and in suspension cultures. It was concluded that the ratio of different AGPs in suspension cultures will determine the embryogenic potential (Kreuger and Van Holst 1995).

Here we report on the effect of unfractionated and monoclonal antibody-purified AGP fractions on both non-embryogenic and size fractionated populations of embryogenic suspension cultures. While both unfractionated carrot-seed AGPs and the ZUM18 AGP fraction were not effective in any size fractionated population of our embryogenic cell lines, JIM8 isolated AGPs inhibited embryogenesis in a defined population of suspension cells. Unfractionated carrot-seed AGPs could increase the number of somatic embryos formed in cell populations enriched for clusters of small cytoplasm-rich cells isolated from low-embryogenic suspension cultures.

Results

Addition of AGPs to embryogenic cell lines

Carrot single cells were isolated by sieving an one week old suspension culture through a 22 µm mesh sieve (< 22 µm cell population). The thus isolated cells were cultured in Gamborgs B5 medium at 100 000 cells·ml^{-1} in the presence or absence of 0.2 µM 2,4-D. ZUM18AGPs were added to a maximum of 0.8 µg·ml^{-1} and after seven days cultures were washed to remove 2,4-D and AGPs. Fourteen days after the start of the experiment the number of somatic embryos was determined. In figure 4.1a a representative example of the results of one of these experiments is shown. Initiation of somatic embryogenesis is increased by a factor of 10 due to the presence of 2,4-D, while no significant effect of ZUM18AGPs is observed. This experiment was repeated 4 times with 3 independent "10" cell lines. The frequency of embryo development in the ZUM18AGP treated cultures varied between 70 to 130 % of the untreated controls. To compare the results of all 5 individual experiments, statistical analyses based upon a generalised linear model were performed (Aitkin et al., 1991). The overall effect of ZUM18AGPs on single cell populations was assessed by means of a F test and P values over 0.0005 were obtained (table 4.1) indicating that the ZUM18AGP fraction neither significantly increased or decreased the number of somatic embryos formed from single cells. To test whether ZUM18AGPs affect cell clusters rather then single cells, similar experiments were performed on 22-50 µm populations containing single cells and

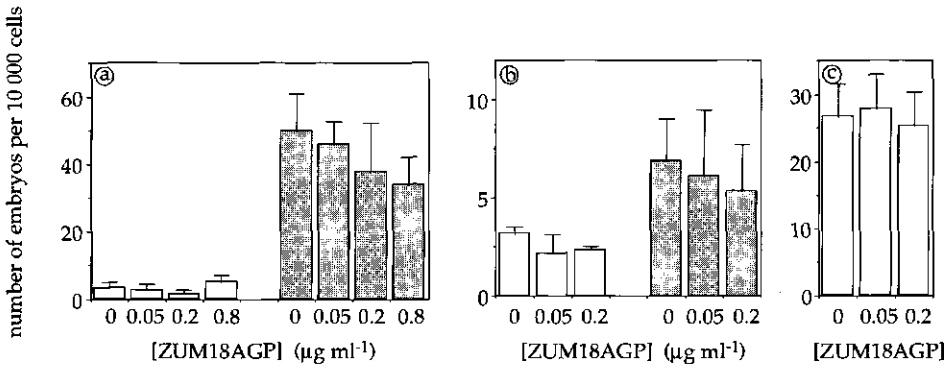

figure 4.1a-c. Effect of isolated ZUM18AGP fraction on the number of somatic embryos formed. Each graph shows one representative example from a series of experiments. Such a representative experiment consists of size-fractionated populations in the absence (*open boxes*) or presence (*grey boxes*) of 0.2 µM 2,4-D treated with various concentrations of ZUM18AGPs in triplicate. **a.** < 22 µm cell population treated with ZUM18AGPs; **b.** 22-50 µm population treated with ZUM18AGPs; **c.** 50-125 µm population treated with ZUM18AGPs. Bars represent the standard deviation in this one experiment (± SD; n=3)

table 4.1. P values for the effect of ZUM18AGPs on the frequency of somatic embryo development in a number of independent experiments with size-fractionated populations isolated from suspension cultures in the absence or presence of 0.2 µM 2,4-D. P values are calculated according to the generalised statistical model (Aitkin et al. 1991) and are calculated based upon data of several independent experiments of which one example is shown in figure 4.1. $P \leq 0.0005$ indicate that treatment with ZUM18AGPs in the range given, significantly affects the frequency of somatic embryogenesis.

size fractionated population	cell density (cells·ml^{-1})	[2,4-D] (µM)	ZUM18AGP range (µg·ml^{-1})	P
< 22 µm	100 000	0	0 - 0.8	0.7245
< 22 µm	100 000	0.2	0 - 0.8	0.4256
22 - 50 µm	100 000	0	0 - 0.2	0.5258
22 - 50 µm	100 000	0.2	0 - 0.2	0.1127
50 - 125 µm	20 000	0	0 - 0.2	0.3799

cell clusters of up to 50 cells and on 50-125 µm populations containing larger cell clusters and pro-embryogenic masses. Also in these experiments no significant effect of ZUM18AGPs in concentrations ranging from 0 to 0.2 µg·ml^{-1} on the frequency of embryo development was observed (see figure 4.1b-c for representative examples of the results of the experiments and table 4.1).

JIM8AGPs were isolated from carrot-seed AGPs by affinity column chromatography with the JIM8 antibody and tested on <22 µm cell populations and 22-50 µm populations. Since optimal AGP concentrations vary for different AGP fractions (Kreuger and Van Holst 1995) the effects of JIM8AGPs were tested in a concentration range of 0 to 1.2 µg·ml^{-1}. To our surprise the frequency of somatic

table 4.2. P values for the effect of JIM8AGPs on the frequency of somatic embryo development in a number of independent experiments with size-fractionated populations isolated from suspension cultures in the absence or presence of 0.2 µM 2,4-D. P values are calculated according to the generalised statistical model (Aitkin et al. 1991) and are calculated based upon data of several independent experiments of which one example is shown in figure 4.2. $P \leq 0.0005$ indicate that treatment with JIM8AGPs in the range given, significantly affects the frequency of somatic embryogenesis.

size fractionated population	cell density (cells·ml^{-1})	[2,4-D] (µM)	JIM8AGP range (µg·ml^{-1})	P
< 22 µm	100 000	0	0 - 1.2	0.0001
< 22 µm	100 000	0.2	0 - 1.2	0.0001
22 - 50 µm	100 000	0	0 - 0.6	0.0426
22 - 50 µm	100 000	0.2	0 - 0.6	0.5874

embryo development from single cells decreased significantly with increasing JIM8AGP concentrations up to 1.2 $\mu g \cdot ml^{-1}$ both in the presence or absence of 2,4-D (figure 4.2a and table 4.2). At a JIM8AGP concentration of about 0.6 $\mu g \cdot ml^{-1}$ the frequency of embryo development was inhibited 50 %. In comparison, the concentration at which ZUM15AGPs showed 50 % inhibition on the formation of morphologically defined embryogenic cells was 2.0 $\mu g \cdot ml^{-1}$ (Kreuger and Van Holst 1995). The effect of JIM8AGPs on 22-50 µm populations in the absence of 2,4-D was less severe, but still statistically significant. In the presence of 0.2 $\mu g \cdot ml^{-1}$ JIM8AGPs the number of embryos formed decreased with 25 % while in the presence of 0.2 µM in 2,4-D in the same population, no effects of JIM8AGPs were observed (figure 4.2b and table 4.2).

The results obtained by Kreuger and Van Holst (1995) were obtained in liquid cultures on a rotary shaker, while in our experiments stationary cultures where employed. To test the influence of culture conditions on the frequency of somatic embryogenesis 22-50 µm populations were cultured under standard conditions (stationary at 25 ± 0.5 ℃ with 16 h light/8 h dark regime), in the dark (stationary at 25 ± 0.5 ℃) and on a rotary shaker at 100 rpm (25 ± 1.0 ℃ with 16 h light/8 h dark regime). No significant differences in the frequency of somatic embryo development were observed between the different culture conditions applied (data not shown) indicating that different culture conditions cannot be responsible for the differences between our results and those reported by Kreuger and van Holst (1995).

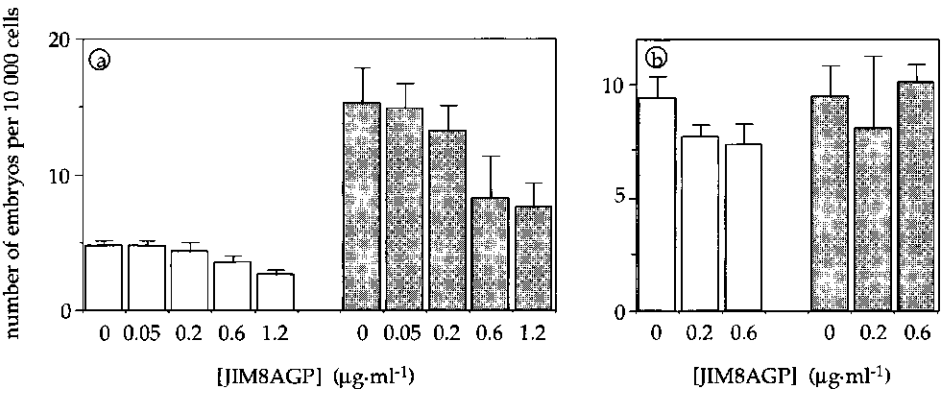

figure 4.2a-b. Effect of isolated JIM8AGP fraction on the number of somatic embryos formed. Each graph shows one representative example from a series of experiments. Such a representative experiment consists of size-fractionated populations in the absence (*open boxes*) or presence (*grey boxes*) of 0.2 µM 2,4-D treated with various concentrations of JIM8AGPs in triplicate. **a.** < 22 µm cell population treated with JIM8AGPs; **b.** 22-50 µm population treated with JIM8AGPs. Bars represent the standard deviation in this one experiment (± SD; n=3)

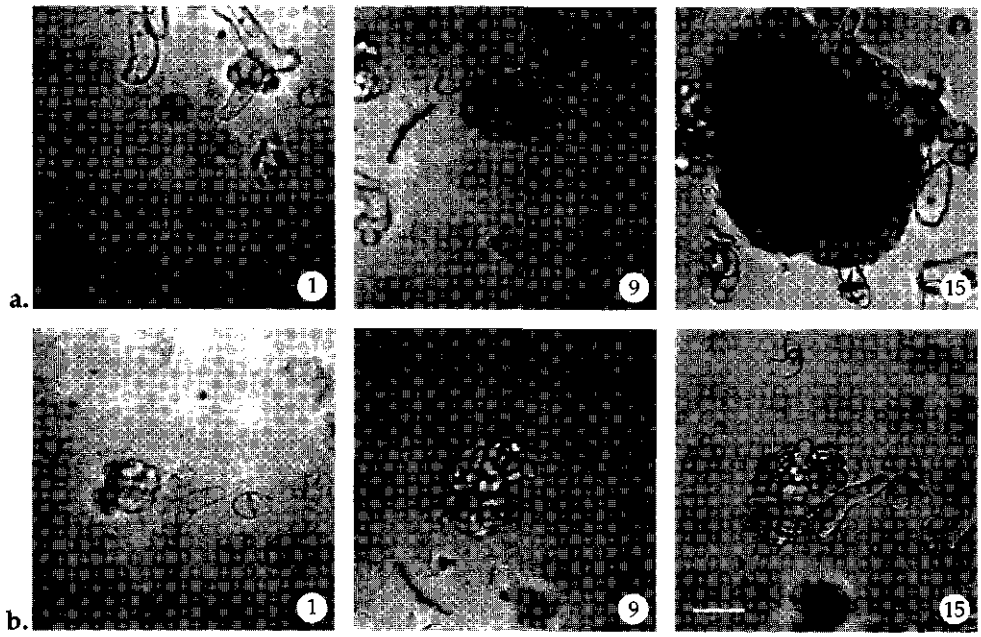

figure 4.3a-b. Development of cytoplasm-rich cell clusters present in a size-fractionated <50 µm population isolated from suspenion cultures, which morphologically resemble embryogenic cell clusters. **a.** An embryogenic cytoplasm-rich cell cluster as determined by cell tracking. **b.** A morphologlcal similar cell cluster, yet non-embryogenic. Indicated in the images are the days after isolation of the < 50 µm population. Bar = 50 µm

In previous studies, it was concluded that AGPs affect mainly the number of morphologically characterised 'embryogenic cells' in cytoplasm-rich cell clusters (Kreuger and Van Holst 1993; 1995) further referred to as 'embryogenic cell clusters'. To determine the correlation between the number of 'embryogenic cell clusters' and the number of somatic embryos, 22-50 µm populations were cultured for seven days and based upon morphology the number of 'embryogenic cell clusters' was determined. The number of 'embryogenic cell clusters' obtained by different researchers varied two to three fold due to different interpretation of cell cluster morphology. Furthermore, no direct correlation was obtained between the number of cell clusters counted in different samples and the number of somatic embryos formed after two weeks of incubation. By cell tracking, development of large series of cell clusters in <50 µm and 50-125 µm populations was followed during two weeks. It was shown that only two thirds of the cell clusters developed into somatic embryos (Chapter 5). Based upon morphology at day 1, clusters like the ones shown in figure 4.3a-b would both be described as 'embryogenic cell cluster'. However, only one of these clusters developed into a somatic embryo (figure 4.3a) while the other cluster formed a group of loosely attached,

vacuolated cells after 15 days of culture (figure 4.3b). Based on these results, it can be concluded that there is no direct relation between morphology of a cell cluster and the capability of that cluster to develop into a somatic embryo. Therefore, morphology is not a reliable criterion to predict the embryogenic competence of cell clusters.

Addition of AGPs to low- and non-embryogenic cell lines

The effect of carrot-seed AGPs was tested on three independent low- and non-embryogenic cell lines. All three lines were over one year old and had lost their embryogenic capacity. Grown under standard conditions these lines developed 0 to 0.3 somatic embryos per 10 000 cells while embryogenic cell lines develop 20 to 120 embryos per 10 000 cells (De Vries et al., 1988). To diminish the effect of endogenous AGPs cell cultures have to be cultured at low cell densities (M. Kreuger, personal communication). Therefore the three non-embryogenic cell lines were sub-cultured at 10 000 or 20 000 cells·ml^{-1} in the presence of 0, 1.0 or 10.0 µg·ml^{-1} of carrot-seed AGPs. After three subcultures the embryogenic competence of low cell density suspension cultures was tested. Cultures derived from one cell line (Af 2) did not develop any somatic embryos while the cultures derived from the two other cell lines developed in duplicate experiments on average 2.1 and 4.3

table 4.3. Effect of carrot-seed AGPs on the development of somatic embryos from low- and non-embryogenic cell lines. Indicated in the table are the number of somatic embryos per 10 000 cells, formed after two weeks of culture. Three cell lines were cultured in duplicate at low cell density (10 000 or 20 000 cells·ml^{-1}) in the absence or presence of 1.0 or 10.0 µg·ml^{-1} carrot-seed AGPs. After three subcultures of the cell lines (Af 10)was enriched for cell clusters and subcultured under the same conditions as before the cluster enrichment. Control cell lines were not treated in any way and subcultured at normal cell density (10^5 cells·ml^{-1}). nt = not tested

[AGP] (µg·ml^{-1})	Ai 10	Af 2	Af 10	Af 10 after cluster enrichment
0	2.6	0	2.3	0.3
0	1.5	0	6.3	0.1
1.0	0	0	3.9	4.1
1.0	3.8	0	2.4	4.3
10.0	n.t.	0	.01	2.3
10.0	n.t.	0	0.9	15
control	0.3	0	0.3	

somatic embryos per 10 000 cells in the absence of AGPs (table 4.3). In the presence of carrot-seed AGPs the number of embryos formed did not increase in the low density cultures (table 4.3). The Af 10 derived suspension cultures grown at low cell density in the absence or presence of 1.0 or 10.0 $\mu g \cdot ml^{-1}$ seed AGPs were enriched for cytoplasm-rich cell clusters as described by Kreuger and Van Holst (1993). By slowly rotating a large Petri-dish cell clusters accumulated in the centre of the dish while other cell types such as large vacuolated cells accumulated at the rim. Cell clusters in the centre of the dish were collected and cultured for another two weeks at the same cell density and AGP concentration as before cluster enrichment. Cultures grown in the absence of seed AGPs developed on average only 0.2 somatic embryos per 10 000 cells, which is comparable to the low-embryogenic control line grown at normal cell density (table 4.3). However, low cell density Af 10 suspension cultures grown in the presence of seed AGPs now developed more embryos as the cultures grown in the absence of AGPs. Also when compared to the situation before cluster enrichment more somatic embryos were formed in the AGP treated cell lines. The frequency of embryo development was on average 4.2 and 8.7 somatic embryos per 10 000 cells. During the experimental period control cell lines subcultured at normal cell density (10^5 cells$\cdot ml^{-1}$) developed somatic embryos with frequencies of 0 and 0.3 embryos per 10 000 cells (table 4.3).

In summary, low-embryogenic cell lines grown at high cell densities can be re-initiated to develop somatic embryos by culturing them at low cell densities. Addition of seed AGPs to these suspension cultures does not increase the frequency of somatic embryo development. After enrichment for cytoplasm-rich cell clusters, the embryogenic capacity in the cultures is lost and can be restored by addition of carrot-seed AGPs confirming the results described by Kreuger and Van Holst (1993). The frequency of somatic embryo development is even higher after cluster enrichment in comparison to the situation before enrichment. The cell line Af 2 was completely non-embryogenic and could also not be re-initiated to form somatic embryos by either dilution or carrot-seed AGP treatment.

Discussion

This paper describes diverse effects of arabinogalactan proteins on carrot somatic embryogenesis. Isolated ZUM18AGP fractions did not significantly increase the number of somatic embryos formed from single cells (< 22 µm cell population), single cells and small cell clusters (22-50 µm population) or large cell clusters and pro-embryogenic masses (50-125 µm populations). The results on the 50-125 µm population are not in agreement with results described by Kreuger and Van Holst

(1995). They described an increase in the percentage of embryogenic cells in a 50-150 µm population from about 40 % up to 80 % due to treatment with 0.2 $\mu g \cdot ml^{-1}$ ZUM18AGPs. Cell populations sieved between 50 and 125 and between 50 and 150 µm are fully comparable in composition and in embryogenic potential, except for a slightly higher frequency of pro-embryogenic masses. It is unlikely that this accounts for the differences in response to AGPs between this work and the results of Kreuger and van Holst (1995). The difference in the results obtained are probably caused by analysing and counting different events. While in this paper the number of somatic embryos was determined after AGP treatment, Kreuger and Van Holst (1995) described changes in cell morphology. Cell tracking experiments have shown that only about two third of the 'embryogenic cell clusters' develop into somatic embryos (Chapter 5) showing that it is difficult to define an 'embryogenic cell cluster' based upon morphology. Thus, several experimental uncertainties may result in errors of the same magnitude of the AGP effects reported, so it is difficult to confirm the previous results by Kreuger and Van Holst (1995).

Culturing low-embryogenic cell lines at low cell densities increased the embryogenic potential in these lines. Adding carrot-seed AGPs to these cultures did not increase the embryogenic potential. This increase in embryogenic potential in the low-embryogenic lines could be a result of the dilution of factors that inhibit embryo-development. This could either occur directly by dilution of the factors or indirectly by changing the growth conditions for the cells that produce these factors.

Extracellular glycoproteins produced by *Citrus* pro-embryogenic masses have been reported to inhibit somatic embryo development (Gavish et al., 1992) and certain classes of carrot AGPs might inhibit the formation of embryogenic cells (Kreuger and Van Holst 1995). Another option is that growth conditions for 'nursing' cells that produce embryogenesis-promoting factors are improved in low cell density cultures. In this way more promoting factors are present in the culture. This idea is supported by the observation that embryogenic potential is decreased after enrichment for cell clusters in cultures of the Af 10 cell line, grown in the absence of AGPs. Due to enrichment of cytoplasm-rich cell clusters certain other cell types, like 'nursing' cells that are necessary for maintaining embryogenic potential, might be removed from the culture. Embryogenic potential is only restored when cultures are grown in the presence of carrot-seed AGPs. The action of these AGPs on somatic embryo development can either be direct on embryo development but can also be indirect by promoting development of 'nursing' cells that produce the embryogenesis-promoting factors. The latter might be favoured since no direct effect of carrot-seed AGPs on embryo development is observed in cultures grown at low cell density. The results of the low-embryogenic cell lines

described here match perfectly with the results of Kreuger and Van Holst (1993) on the effect of carrot-seed AGPs on one non-embryogenic cell line.

AGPs isolated from carrot seeds carrying the JIM8 epitope, influenced the frequency of embryo development. Surprisingly, JIM8AGPs showed an inhibitory effect on the frequency of embryo development from single cells. The number of somatic embryos formed is reduced by 50 % at about 0.6 $\mu g \cdot ml^{-1}$. For comparison, ZUM15AGPs give a 50 % reduction in the number of 'embryogenic cells' in 50-150 µm populations at 2 $\mu g \cdot ml^{-1}$ (Kreuger and Van Holst 1995). A slight decrease in the number of somatic embryos was observed in the 22-50 µm population in the absence of 2,4-D. The JIM8 arabinogalactan epitope is expressed in plasma membranes of cells of the zygotic *Brassica* embryo (Pennell et al., 1991). In carrot suspension cultures the JIM8 cell wall epitope can be regarded as a marker for embryogenic competence of a cell line (Chapter 4). In contrast soluble JIM8AGPs isolated from carrot-seed AGPs inhibit somatic embryo formation from single cells. It seems contradictory that on one hand the cell wall epitope is related to embryogenic competence and that on the other hand the soluble JIM8AGP fraction inhibits somatic embryo development. The JIM8AGPs might be formed due to increase of embryogenic potential of a culture and for example may be involved in the limitation of embryogenic potential of a culture once it is established. An other possibility is that soluble JIM8AGPs are not related to the JIM8 cell wall epitope and that the JIM8AGP fraction isolated from carrot seeds also carry many active groups that are inhibitory to embryo development.

Several studies suggest that particular classes of AGPs are involved in complex interactions between different cell types in suspension cultures and act indirectly on somatic embryo development. The nature of these interactions is far from clear but apparently results in changes in cell morphology. Addition of ß-glucosyl Yariv reagent to cultured carrot cells prevented elongation (Willats and Knox 1996) and promoted formation of cell clusters that morphologically resemble the 'embryogenic cell clusters' described by Kreuger and Van Holst (1993). Willats and Knox (1996) therefore proposed a function for carrot cell wall AGPs in the regulation of cell wall extension. When bound with ß-glucosyl Yariv reagent cells are not able to elongate anymore and consequently would form cytoplasm-rich cell clusters. In contrast, Kreuger and Van Holst (1993, 1995) showed that addition of carrot-seed AGPs or ZUM18AGPs leads to the formation of cytoplasm-rich cell clusters. Since from the work presented here, it is evident that different carrot suspension cultures show different responses to mixtures of particular AGPs, it is difficult to compare the effects of addition of such AGP preparations and the addition of ß-glucosyl Yariv reagents directly. It seems clear that several different types of AGPs occur in unfractionated suspension cultures and that some of these promote while others inhibit cell elongation. Our results show that seed AGPs

can completely restore the inhibitory effect that removal of most of the single cells present, has on somatic embryogenesis. This points to the existence of a population of 'nursing' cells, that could produce a class of AGPs necessary for somatic embryo development. Whether these 'nursing' effects are specific or whether they primarily influence cell elongation in general remains to be determined.

From our results, it is clear that in certain carrot cell lines there is a promotive effect on somatic embryogenesis upon addition of unfractionated carrot-seed AGPs. This effect is only seen after removal of certain single cell types in these cultures, suggesting that these cells normally produce the promotive AGPs under tissue culture conditions. Manipulation of somatic embryogenesis in suspension cultures now requires that both promotive and inhibitory AGPs are purified and their precise cellular origin is determined. In this way, manipulation of the cell type composition of embryogenic cultures could be carried out with greater precision and more control over the outcome.

Materials and methods

Plant material and cell culture

Embryogenic carrot (*Daucus carota* L. cv. Tropy; S&G Seeds, Enkhuizen, The Netherlands) suspension cultures designated cell lines "10" were maintained as described previously (De Vries et al., 1988). One-week old high-density (10^6 - 10^7 cells·ml^{-1}) suspension cultures were sieved through nylon sieves with successive 300, 125, 50 and 22 µm pore sizes (Monodur-PES; Verseidag Techfab, Walbeck, Germany). The population containing large single cells, cell clusters and pro-embryogenic masses (50-125 µm population) retained on the 50 µm sieve. The cells and cell clusters passing the 50 µm sieve were transferred to the 22 µm sieve. Single cells and cell clusters retained on this sieve where isolated as 22-50 µm population while single cells (< 22 µm cell population) were collected after passage through this sieve. Size-fractionated populations were washed twice with B5 medium (Gamborg et al., 1968) without 2,4-D (B5-0 medium) or B5 medium supplemented with 0.2 µM 2,4-D (B5-0.2 medium) and resuspended in B5-0 or B5-0.2 medium. Single cell populations (< 22 µm) and 22-50 µm populations were cultured in 2 ml cultures at a cell density of 100 000 cells·ml^{-1} and 50-125 µm populations at 20 000 cells·ml^{-1}. After seven days, cultures were centrifuged for 5 minutes at 300 *g* and the cells were resuspended in 2 ml hormone-free B5 medium without AGPs. Fourteen days after the start of the experiments the number of cell clusters and somatic embryos was determined and expressed according to De Vries et al. (1988) as the number of somatic embryos developed from 10 000 cells present at

day 0 in the culture. Unless stated otherwise cultures were incubated in 2 ml liquid cultures in 35-mm-diameter Petri-dishes (Greiner, Frickenhausen, Germany) at a 16 hour light/8 hour darkness regime at 25 ± 0.5 °C. The different cell lines "10" used developed between 36 and 105 somatic embryos from 10 000 cells under these conditions (Chapter 2).

Cell tracking experiments were performed on < 50 µm and 50-125 µm populations as described in chapter 5.

Isolation of AGPs from seeds

AGPs were isolated from carrot seeds (S&G Seeds) by precipitation with ß-glucosyl Yariv reagent (Yariv et al., 1962) according to the methods described by Kreuger and Van Holst (1993, 1995). ZUM18AGPs were either kindly provided by S&G Seeds or isolated from carrot seed AGPs using the monoclonal antibodies ZUM18 bound to Bakerbond ABx (J.T. Baker Inc. Phillipsburg, NJ, USA) as described by Kreuger and Van Holst (1995). The same protocol was used to isolate JIM8AGPs using the JIM8 monoclonal antibody (Pennell et al., 1991). The AGP concentration was determined by radial gel diffusion method as described by Van Holst and Clarke (1985).

AGP complementation assays

Carrot seed AGPs, ZUM18AGPs or JIM8AGPs were filter sterilised (0.2 µm disposable filter, Schleicher & Schüll, Dassel, Germany). AGPs were added in concentrations between 0.05 and 1.2 $\mu g \cdot ml^{-1}$ to 2 ml cultures of < 22 µm, 22-50 µm or 50-125 µm populations in the presence of 0 or 0.2 µM 2,4-D. After one week cultures were washed and resuspended in fresh B5-0 medium without AGPs. In each experiment the same total amount of buffer was added to each sample and control dish.

Three low- and non-embryogenic "10" cell lines were cultured at a cell density of 10 000 or 20 000 $cells \cdot ml^{-1}$ in 10 ml B5-2 medium in 50 ml Erlenmeyer flasks. Carrot-seed AGPs were added to the low cell density suspensions at a concentration of 0, 1.0 or 10.0 $\mu g \cdot ml^{-1}$ in duplicate. A control was sub-cultured at normal cell density (10^5 $cells \cdot ml^{-1}$). Cell suspensions were subcultured every two weeks and embryo formation was tested after three subcultures. One cell line was enriched for cell clusters by slowly rotating a large Petri-dish to accumulate cell clusters in the centre of the dish. These cell clusters were collected and subcultured (Kreuger and Van Holst 1993).

To relate the number of somatic embryos obtained to a particular treatment in series of individual experiments the SAS System based upon a generalised linear model (Aitkin et al. 1991) was used. The overall effect of treatment was assessed by means of F tests and significant differences expressed with P values $\leq$ 0.0005.

Acknowledgements

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5

AtLTP1 expression during carrot somatic embryogenesis

The carrot (*Daucus carota* L.) *EP2* gene encodes a Lipid Transfer Protein (LTP) which is expressed during protoderm formation in developing embryos. To develop a vital reporter system for gene expression during somatic embryo development a 1.1 kB fragment of the *Arabidopsis thaliana LTP1* promoter was fused to the firefly luciferase (LUC) coding sequence. The *AtLTP1* luciferase expression pattern in transformed carrot suspension cultures was identical to the expression pattern of the endogenous carrot *EP2* gene. Cell tracking experiments revealed that all somatic embryos were derived from *AtLTP1* luciferase expressing cell clusters, which substantiates the predicted correlation between *EP2* gene expression and somatic embryogenesis. However, not all cell clusters that expressed the *AtLTP1* luciferase reporter gene developed into a somatic embryo, suggesting that initiation of an embryogenic pathway in tissue culture does not always lead to development of a somatic embryo.

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Introduction

Apart from the zygote, many other types of plant cells have the remarkable capability to form embryos. Experimental systems such as microspore embryogenesis and somatic embryogenesis have the advantage that large numbers of staged embryos can be obtained. The different systems for plant embryogenesis have been compared in chapter 1. Many studies have been devoted to cloning genes differentially expressed during induction and early stages of embryogenesis. The glycine-rich protein CEM6 for example, is expressed in early globular embryos and may be localised within the cell wall (Sato et al., 1995). Somewhat later in development the *EMB1* gene is expressed in globular embryos (Wurtele et al., 1993). The translational elongation factor 1α encoding gene *CEM1* (Kawahara et al., 1992), the homeobox gene *CHB2* (Kawahara et al., 1995), the LEA gene *DC3* (Wilde et al., 1988) and the abscisic acid-inducible gene *ECP40* (Kiyosue et al., 1993) are all expressed in developing somatic embryos starting from the globular embryo stage. The *EMB1* gene is one of the few genes analysed to date, that is specifically expressed during early stages of embryogenesis. All other genes described are also expressed in other plant organs. Induction of microspore embryogenesis is accompanied by specific changes in mRNA and protein synthesis in the total population of triggered microspores (Cordewener et al., 1994; Pechan et al., 1991; Ríhová et al., 1996). Initiation of sporophytic development of *Brassica* microspores by heat shock was accompanied by increased synthesis of heat shock proteins (Cordewener et al., 1995) and levels of napin gene expression (Boutilier et al., 1994) in the total microspore population.

It has proven to be quite difficult to obtain genes or proteins that are specifically expressed in cells that will develop into embryos, the so-called embryogenic cells. Pro-embryogenic masses have often been defined as the initial stage in somatic embryo development (Aleith and Richter, 1990; Wilde et al., 1988). Screening for genes expressed in this stage of embryo development resulted in genes expressed in globular and later staged somatic embryos. This is not suprising since pro-embryogenic masses are in fact early globular embryos arrested by the presence of 2,4-D and the high cell density (Halperin, 1966). Isolation of markers for more early stages of embryo development that were based upon cell morphology, were obscured by the fact that cell morphology is not a reliable criterion to predict embryo-forming capacity. This problem became evident with the JIM8 cell wall epitope, first reported to be a marker for single cells designated to develop into somatic embryos (Pennell et al., 1992). Later, cell tracking experiments showed that the presence of the JIM8 cell wall epitope does not coincide with the ability of individual single cells to form somatic embryos (Chapter 3).

Since it is difficult to predict embryo-forming single cells or cell clusters solely by morphology, cell destructive staining methods such as *in situ* hybridisation or promoter-GUS analysis do not prove a direct correlation between gene expression and the embryogenic capacity of a cell or cell cluster. Such a correlation can only be obtained by following the developmental fate of cells expressing a particular gene by means of a viable marker system. The two systems presently available for this purpose are based on the green fluorescent protein (GFP) and luciferase (LUC). GFP has the advantage that it does not require a substrate but only needs blue light for excitation. The GFP protein, however, has a half life of three days, which does not allow to detect rapid changes in gene expression. Furthermore, GFP can form aggregates that are toxic to plant cells (Haselhoff and Amos, 1995). The firefly luciferase reporter system has been shown to work very well in plant systems (Chang and Geider, 1995; Charng and Pfitzner, 1994; Chia et al., 1994; Kost et al., 1995; Millar et al., 1992b, 1995a, 1995b; Ow et al., 1986; Quandt et al., 1992; Schneider et al., 1990). In the absence of its substrate luciferin, luciferase is very stable. In the presence of luciferin however, luciferase has a half life of 3 hours which makes it suitable as a reporter to monitor changes that occur within hours in the expression of temporally regulated genes (Millar et al., 1992a, 1995a, 1995b).

The luciferase enzyme catalyses an ATP-dependent oxidative decarboxylation of D-luciferin in the presence of magnesium. This reaction produces yellow-green light with an emission peak at 560 nm. The firefly luciferase acts as a monomer with a high quantum yield (one photon is emitted in 88 % of the catalytic cycles) and is encoded by a single gene. Plants do not contain background luciferase activity (Abeles, 1986) and the cellular concentrations of ATP, magnesium and oxygen are generally sufficient for maximum activity of the enzyme (Aflalo, 1991). The substrate luciferin is a weak acid that is easily taken up by cells but concentrations above 400 μM are toxic to suspension cultured tobacco cells (Ow et al., 1986).

Here we describe the results of a study using the promoter of the *Arabidopsis thaliana* lipid transfer protein 1 (*AtLTP1*) gene (Thoma et al., 1994). The gene is homologous to the carrot lipid transfer protein *EP2* gene, expressed during zygotic and somatic embryogenesis. The EP2 protein was originally found in embryogenic but not in non-embryogenic carrot suspension cultures. The encoding gene is also expressed in the shoot apex of seedlings, developing flowers and maturing seeds (Sterk et al., 1991). In developing embryos expression is restricted to the protoderm. *In vitro* the LTP protein has binding capacity for oleic acid (Meijer et al., 1993) and a role of the protein in transport of cutin monomers has been proposed (Sterk et al., 1991). In developing *Arabidopsis* embryos *AtLTP1* mRNA is

distributed in a similar fashion as *EP2* mRNA in carrot embryos (Thoma et al., 1994). These data were confirmed using the ß-glucoronidase gene under influence of the *AtLTP1* promoter (Thoma et al., 1994; Vroemen et al., 1996). Using the *AtLTP1* promoter fused to the luciferase coding sequence *AtLTP1* promoter activity was monitored *in vivo* in developing carrot cell clusters. Development of these clusters into somatic embryos was subsequently determined by cell tracking.

Results

Transformation of *Daucus carota* and establishing cell lines from single transformed cells

The firefly luciferase coding sequence under control of the duplicated CaMV 35S promoter or the *AtLTP1* promoter (figure 5.1a) was introduced into carrot by *Agrobacterium tumefaciens* mediated transformation of hypocotyl segments. Less than one transformed callus per 100 hypocotyl segments was obtained from the *Daucus carota* cultivar 'Trophy' while the cultivar 'Amsterdamse bak' produced up to one transformed callus per hypocotyl segment. Therefore the latter cultivar was used in transformation experiments.

One week after transfer to kanamycin selection medium, hypocotyl segments were sprayed with luciferin to test whether luciferase expression could be detected in transformed callus shortly after transformation. A large number of hypocotyl segments showed luciferase activity at the cut edges, but did not develop calli. Instead, growth of bacteria occurred, suggesting that the luciferase

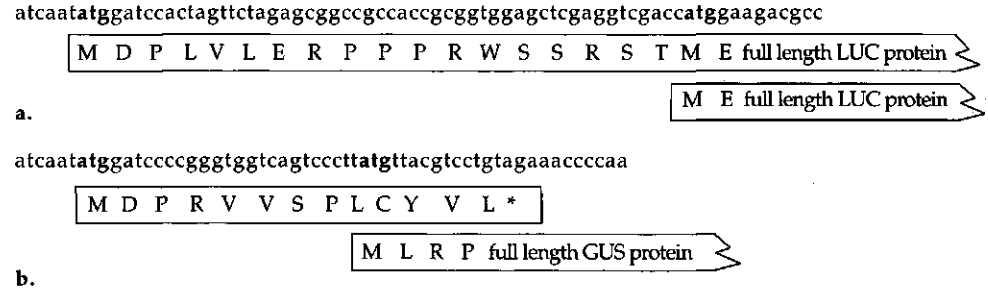


figure 5.1a-b. The nucleotide and deduced amino acid sequences of *AtLTP1*-LUC and *AtLTP1*-GUS fusion constructs. **a.** The *AtLTP1*-LUC fusion contains two ATG start codons. Usage of the first (LTP1) codon results in a N-terminal fusion of 18 amino acids to the LUC protein while start at the second (LUC) start codon results in the normal LUC protein. **b.** Usage of the first (LTP1) codon in the *AtLTP1*-GUS fusion results in a 13 amino acid fusion protein while start at the second (GUS) start codon results in the normal GUS protein.

activity was of bacterial origin. Six to ten weeks after transformation, calli where obtained that showed luciferase activity in variable amounts, while no bacterial growth could be observed anymore. After 12 weeks, calli measuring 5 to 10 mm in diameter were used to start suspension cultures. At this time no bacterial contamination was observed. Luciferase expression under influence of the duplicated CaMV 35S promoter was observed in single cells and cell clusters in the suspension culture (figure 5.2a) demonstrating that the luciferase protein is active in *Daucus carota* suspension cultured cells.

To demonstrate a causal relationship between *AtLTP1* promoter activity and embryo formation, the use of chimaeric cell lines containing a mixture of transformed and non-transformed cells has to be excluded. For this reason, secondary cell lines were initiated, starting from a single somatic embryo, that was in turn derived from either a single cell or a single protoplast under continuous selection for transformed cells. A series of the thus established secondary cell lines was used for further analysis and shown to behave like non-transformed cell lines started from seed-grown seedlings. Most cell lines were slightly more embryogenic then the non-transformed cell lines.

AtLTP1 luciferase expression in suspension cultures

AtLTP1 luciferase expression was tested in several individual secondary cell lines. Single cells and cell clusters of up to 20 cells were obtained by sieving a suspension culture through a 30 µm mesh sieve (< 30 µm population). In addition to these cells, populations sieved through 50 µm mesh sieves (< 50 µm population) also contained larger cell clusters. The population size fractionated between 50 and 125 µm (50 - 125 µm population) consisted mainly of large cell clusters and large vacuolated single cells. About 65 % of the cell clusters obtained in the < 30 µm and < 50 µm populations expressed luciferase. The luciferase appeared to be distributed throughout these cell clusters (figure 5.2b). A similar expression pattern was observed for the endogenous carrot *EP2* gene as shown by whole mount *in situ* hybridisation experiments with an *EP2* antisense RNA probe (figure 5.2c). In globular and heart shaped somatic embryos *AtLTP1* luciferase expression was detectable throughout the embryo (figure 5.2d) while in torpedo staged embryos expression decreased in the primary root. *AtLTP1* luciferase expression was also observed in a very low percentage of single cells and very small cell clusters of up to 5 cells (figure 5.2e). In the original analysis of the carrot *EP2* gene expression this was not observed (Sterk et al., 1991), probably due to the technique of *in situ* hybridisation on sections of fixed and embedded suspension cultured cells employed in that work. Whole mount *in situ* experiments with a carrot *EP2* antisense RNA probe on the same cell population of the transformed cell line used for the

AtLTP1 luciferase expression studies, revealed the presence of *EP2* mRNA in 0.1 to 0.2 % of the single cells in this population (figure 5.2f). *EP2* mRNA could also be detected in single cells in regular embryogenic carrot suspension cultures (results not shown). These results demonstrate that the *AtLTP1* luciferase expression in single cells is not an artefact of the luciferase system, but reflects endogenous *EP2* gene expression.

In the size-fractionated 50-125 µm populations about 80 % of the cell clusters showed detectable amounts of luciferase expression. These large cell clusters, commonly designated as pro-embryogenic masses, consisted of morphologically different cell types. More centrally located cells in the pro-embryogenic masses apparent to be dead as revealed by fluoresceïn diacetate staining (figure 5.2g). The observed localised expression of *AtLTP1* luciferase probably reflects expression in those cells within the pro-embryogenic mass that were still alive (figure 5.2h). Similar expression patterns were observed for the endogenous *EP2* gene in this transformed cell line (figure 5.2i) and in regular carrot suspension cultures (results not shown).

Based on the correspondence between the *AtLTP1* luciferase expression pattern and the expression pattern of the endogenous carrot *EP2* gene, we conclude that the *AtLTP1* promoter faithfully reflects *EP2* expression in the cell line used. Therefore this cell line was used for cell tracking experiments.

Luciferin inhibits somatic embryo development

To monitor *AtLTP1* promoter driven luciferase expression during embryo development, single suspension cells and cell clusters as present in a size-fractionated < 50 µm population, were embedded in phytagel and luciferin was added to a final concentration of 1.0 mM. Using the iCCD camera attached to the inverted microscope several cell clusters showed expression of luciferase after 10 minutes of photon counting. Even after seven days, luminescent images could still be obtained without addition of fresh luciferin. Since luciferase activity in transgenic plants is destabilised in the presence of luciferin (Millar et al., 1992b; Quandt et al., 1992), this indicates that luciferin itself is highly stable. However, only a small percentage of cell clusters had developed into somatic embryos in these cultures in comparison to control cultures without luciferin. Most of the embryos that were formed, did not proceed beyond the late globular stage and only expanded in radial fashion. The inhibiting effect of luciferin on embryo development was confirmed in non-transformed < 22 µm cell populations. Addition of luciferin in concentrations ranging from 0.02 to 1.0 mM led to a decrease up to 80 % in the number

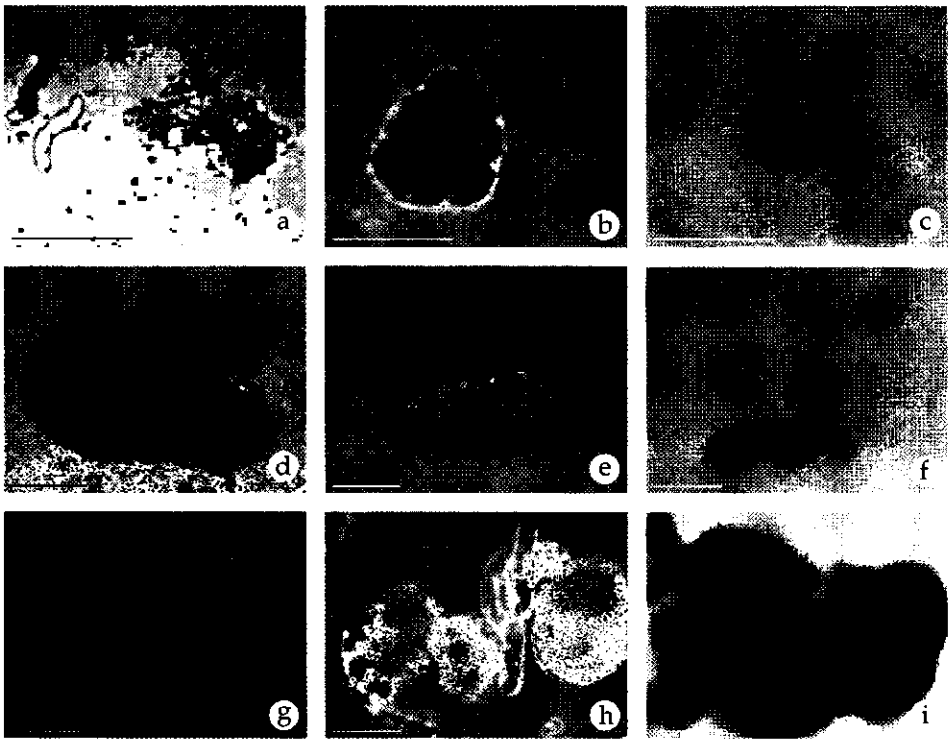


figure 5.2a-i. a. CaMV35S luciferase expression in transformed carrot single cells present in a size fractionated < 30 µm population. Signals were obtained after 30 minutes photon counting in slice mode and shown as superimposed pseudocolors. Low signals are indicated by blue and range via red to white to indicate high signals. **b.** *AtLTP1* luciferase expression in a carrot < 50 µm population. Signals obtained after 10 minutes photon counting in gravity mode are indicated as blue dots, each dot representing one luciferase released photon. Shown is a cell cluster expressing *AtLTP1* luciferase. **c.** Whole mount in situ hybridisation on a cell cluster from the same population as in panel b with an antisense *EP2* RNA probe. *EP2* mRNA localisation is visualised as a dark purple colour in the cell cluster. **d.** *AtLTP1* driven luciferase expression in a late heart shaped somatic embryo. Signals obtained after 10 minutes photon counting in gravity mode **e.** *AtLTP1* luciferase expression in an elongated cell cluster consisting of five cells present in the < 50 µm population. **f.** Whole mount in situ hybridisation on the same population as in panel e with an antisense *EP2* RNA probe. *EP2* mRNA is located in the elongated single cell. **g.** Viability staining of cells in a pro-embryogenic mass indicating that a number of cells in the cluster are metabolic inactive. **h.** *AtLTP1* luciferase expression in a 50-125 µm population. Parts of the pro-embryogenic mass show *AtLTP1* luciferase expression while other parts do not show expression. **i.** Whole mount in situ hybridisation on the same population as in panel h with an antisense *EP2* RNA probe. *EP2* mRNA is located in specific parts within the cell cluster. Bar = 50 µm.

of embryos formed (figure 5.3), and also to an arrest at the globular stage at higher luciferin concentrations (results not shown). As the intensity of the luminescent signal at 0.02 mM luciferin was comparable to the signal at higher luciferin concentrations, and hardly affected the number and correct development of the somatic embryos, this luciferin concentration was used for subsequent experiments.

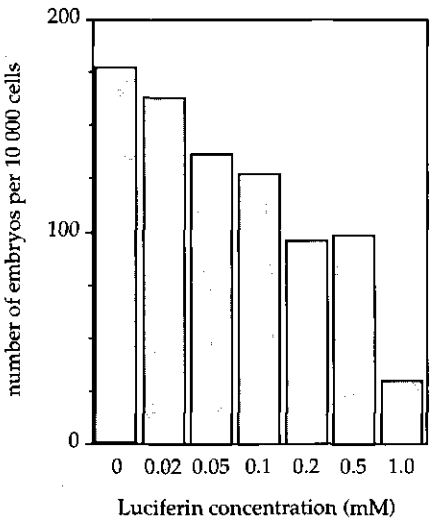


figure 5.3. Effect of luciferin on the frequency of somatic embryo development from non-transformed single cells. Indicated are the number of somatic embryos formed per 10 000 cells at luciferin concentrations from 0 to 1.0 mM.

AtLTP1 expression during somatic embryo development

To be able to follow *in vivo* gene expression during development of somatic embryos it was essential to be able to merge the images obtained from the intensified CCD (iCCD) camera used to detect luciferase expression with those obtained from the automatic cell tracking system used to record development of individual cells and cell clusters. For this purpose the existing iCCD camera was fitted to a microscope equipped with a computer controlled cross-table allowing recording of luciferase expression and of development with time in a cell specific manner. A detailed description of the system is given in chapter 6.

To determine the development of AtLTP1 luciferase expressing cells and cell clusters, size fractionated populations < 30 μm and < 50 μm were embedded in phytagel at a cell density of 100 000 cells·ml^{-1}. During the first week bioluminescent images of *AtLTP1* luciferase expression were obtained by 10 minutes of photon counting at alternate days. Development of the cell clusters was followed during two weeks. At day 1, 85 out of 142 of small cell clusters of the < 30 μm population that were present in the scanned area showed *AtLTP1* luciferase

expression (table 5.1). In most of the clusters that showed luciferase activity, it decreased with time during the first week. These clusters did not develop into somatic embryos. Cell tracking showed that only 20 of the *AtLTP1* luciferase expressing clusters developed into a somatic embryo. In these clusters, expression was maintained during the first seven days of development. Embryos were also seen to develop from cell clusters that did not express *AtLTP1* luciferase at day 1. However, in these cases *AtLTP1* luciferase expression became visible at a later stage during the experiment. This resulted in a total of 69 cell clusters in the < 30 µm population that expressed *AtLTP1* luciferase at day 7 of which 41 developed into a somatic embryo (table 5.1). *AtLTP1* luciferase expression determined at day 7 was therefore better correlated to the embryogenic competence of cell clusters. Examples of described patterns of *AtLTP1* luciferase expression during embryo development are shown in figure 5.4a. While three out of the six cell clusters visible in this image expressed *AtLTP1* luciferase at day 1 only one developed into a heart shaped somatic embryo (figure 5.4a1). Two other cell clusters (figure 5.4a2, a3) first expanded and luciferase activity was seen for the first time at day 5 or 7. After 15 days one of these clusters had developed into a globular shaped embryo (figure 5.4a3) while the other cluster did not (figure 5.4a2). It appears therefore that there is variation in the level and in the temporal regulation of *AtLTP1* expression. As noted previously (Chapter 2 and unpublished results) there is also quite some variation in the precise time point at which somatic embryogenesis is initiated leading to unsynchronised embryo development. In this respect *AtLTP1* luciferase expression appears to coincide with embryo development. It also shows that although embryo formation is always associated with *AtLTP1* expression, not all clusters that express the *AtLTP1* promoter indeed develop into somatic embryos.

table 5.1. Percentages of cell clusters expressing luciferase under control of the *AtLTP1* promoter at day 1 (or day 7) and percentage of cell clusters that subsequently develop into somatic embryos.
* percentages determined at day 7.

cell population	total number of cell clusters analysed	number of cell clusters expressing luciferase	number of expressing clusters developing into an embryo	number of embryos derived from luciferase expressing cell clusters
< 30 µm	142	85 (60 %)	20 (24 %)	20 (100 %)
< 30 µm*	132	69 (52 %)	41 (59 %)	41 (100 %)
< 50 µm	64	47 (73 %)	41 (87 %)	41 (100 %)
50 - 125 µm	41	34 (83 %)	25 (74 %)	25 (100 %)

Of the 64 cell clusters in the < 50 µm population analysed, 47 showed *AtLTP1* luciferase expression. Out of these, 41 *AtLTP1* expressing clusters developed into somatic embryos (figure 5.4c) and could morphologically be identified as early globular embryos around day 7. The remaining 6 cell clusters with high *AtLTP1* luciferase expression were morphologically indistinguishable from the ones that developed into somatic embryos. These clusters however, only expanded and failed to develop into a somatic embryo (figure 5.4b). In two cases this was accompanied by decreased *AtLTP1* luciferase expression levels. As was observed for the < 30 µm populations somatic embryo development of cell clusters in the < 50 µm populations was accompanied by variable levels of *AtLTP1* luciferase expression.

In 50-125 µm populations 34 out of 41 cell clusters showed a detectable amount of *AtLTP1* luciferase expression (table 5.1) and 25 of these clusters developed into a somatic embryo (figure 5.4d). While in other populations cell clusters merely expanded, in this 50-125 µm population cell clusters expanded and large aberrant clusters where formed (results not shown).

It is evident that the correlation of *AtLTP1* luciferase expression with somatic embryo development improves with increasing cell cluster size. In populations < 30 µm only 24 % of the clusters that expressed *AtLTP1* luciferase at day 1 indeed developed into somatic embryos. When *AtLTP1* luciferase expression was again recorded at day 7, when many of the clusters have increased in size, the percentage of *AtLTP1* luciferase expressing cell clusters that develop into somatic embryos increased to 59 %. This percentage approaches to the 87 % *AtLTP1* expressing clusters in the <50 µm population that develop into embryos. While part of this improved correlation is due to the lack of synchronisation in initiation of somatic embryo development, another part is due to the fact that at day 7 clusters in the < 30 µm population have reached a size comparable to those in the < 50 µm population. In none of the cell populations investigated, there seemed to be a direct correlation between the intensity of the luciferase expression signal and the ability to develop into somatic embryos.

Discussion

In this work the expression of the *AtLTP1* promoter-luciferase gene construct during development of live somatic embryos is described. The use of luciferase allowed to monitor *AtLTP1* promoter acitivity at two day intervals. One of the problems encountered was the toxic effect at 50 µM luciferin or higher to somatic embryo development. Toxicity was observed in transformed and non-transformed cell lines and is therefore due to luciferin itself and not to an interaction between luciferin and luciferase or a product of this reaction. The luciferin concentration

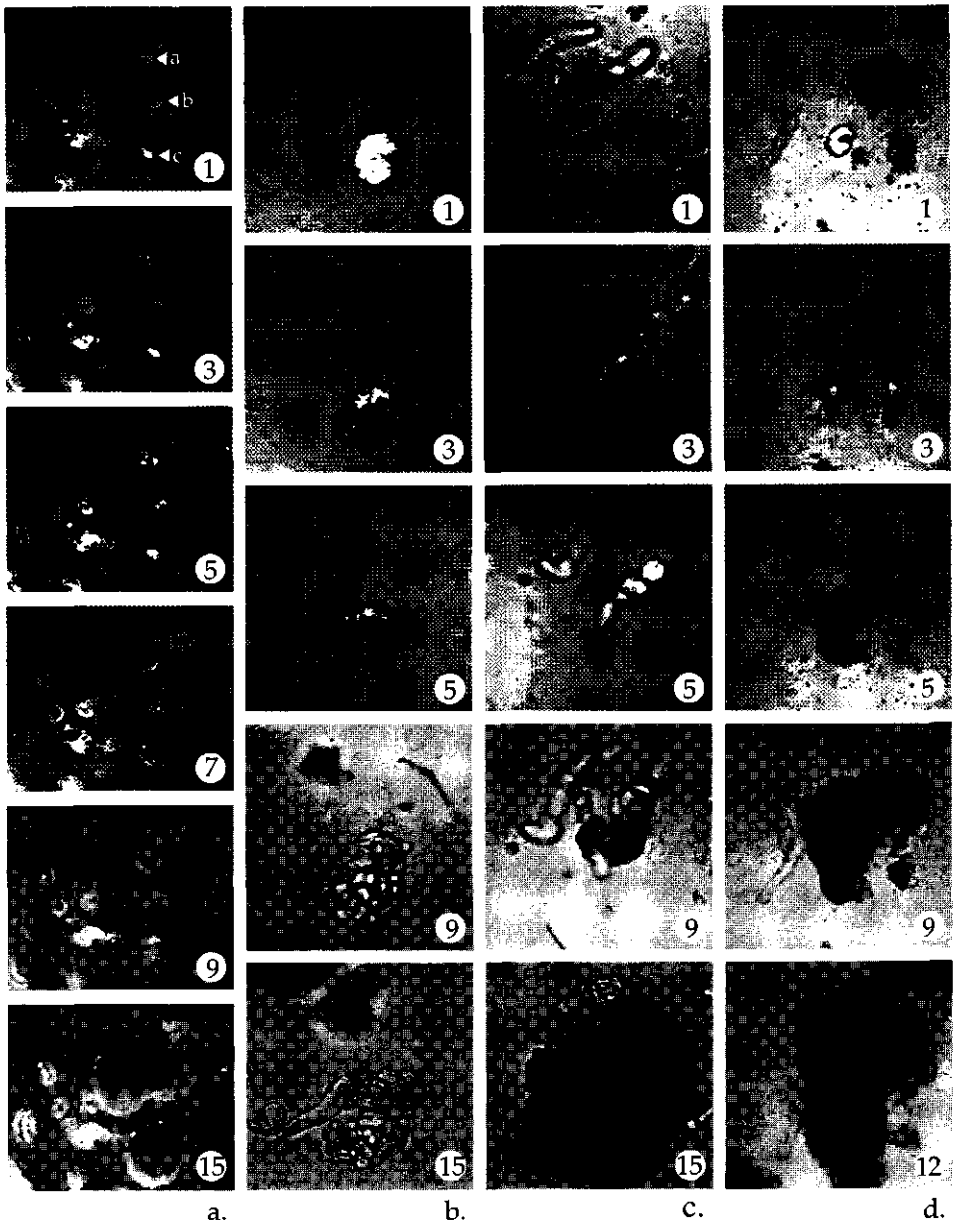


figure 5.4a-d. Luciferase expression under control of the *AtLTP1* promoter during somatic embryo development of cells present in size fractionated <30 µm, <50 µm or 50-125 µm populations. At days 1, 3, 5 and 7 *AtLTP1* luciferase expression, visible in pseudocolors, was obtained after 10 minutes photon counting in slice mode. Low signals are indicated by blue and range via red to white to indicate high signal intensity. **a.** Development of two somatic embryos from cell clusters present in a < 30 µm

could be lowered to 20 µM without affecting the strenght of the luciferase signal. It can probably be recommended to lower the luciferin concentration in tissue culture experiments.

The iCCD camera in conjunction with cell tracking provided a direct correlation between expression of the *AtLTP1* promoter in a cell cluster and the formation of a somatic embryo. The importance of this kind of studies has been shown for the monoclonal antibody JIM8 which reacted only with cells in embryogenic carrot suspension cultures. Based upon immersion immuno-fluorescence studies it was proposed that the JIM8 antibody reacted with a cell wall epitope of embryo-forming single cells present in these cultures (Pennell et al., 1992). Cell tracking revealed however, that most of the somatic embryos actually derived from unreactive cells, indicating that the JIM8 cell wall epitope cannot be used as a marker for embryo-forming cells, but rather reflects embryogenic competence of the culture as a whole (Chapter 3).

Analysis of the *AtLTP1* luciferase expression pattern showed that the 1.1 kB *Arabidopsis thaliana LTP1* promoter faithfully reflects expression of the endogenous carrot *EP2* gene under tissue culture conditions. The *AtLTP1* luciferase expression pattern was similar in independent transformants. The expression pattern of the *AtLTP1* promoter in transformed carrot somatic embryos was similar to the expression pattern of this promoter in *Arabidopsis* zygotic embryos (Thoma et al., 1994). Cell tracking experiments revealed that all somatic embryos developed from *AtLTP1* luciferase expressing cell clusters. This clearly shows the correlation between *AtLTP1* luciferase expression and somatic embryogenesis. However, not all clusters that expressed *AtLTP1* luciferase developed into somatic embryos. Lipid transfer proteins have been proposed to be involved in protoderm formation (Sterk et al., 1991; Thoma et al., 1994) and may also have a role in plant pathogenesis (Canevascini et al., 1996; Molina and Garía-Olmendo, 1993). One reason for 'false positives' might be that protoderm formation can occur independently from somatic embryo development. Alternatively, these *AtLTP1* luciferase expressing cell clusters, though designated to develop into somatic embryos, are disturbed in other processes such as the initiation of apical meristems or other elements of pattern formation. Evidence for this can be found in the *Arabidopsis* zygotic embryo mutant *gnom*, in which protoderm formation is normal and evident by the expression of the *AtLTP1* gene, yet no apical-basal axis of

population. **b.** Cell cluster present in the < 50 µm population with a high At*LTP1* luciferase expression does not develop into a somatic embryo. **c.** Cell cluster present in the < 50 µm population with A*tLTP1* luciferase expression developing into two somatic embryos. **d.** Development of a somatic embryo from a cell cluster in the 50-125 µm population expressing A*tLTP1* luciferase. Two clusters with low luciferase expression do not develop into an embryo.

polarity develops (Vroemen et al., 1996). This phenomenon may in general cause uncertainties in establishing the correlation between molecular markers and somatic embryo formation.

The correlation of *AtLTP1* promoter activity with somatic embryo development improves with increasing cell cluster size. *AtLTP1* luciferase expression in single cells was not correlated at all with embryogenesis while about 87 % of the *AtLTP1* luciferase expressing cell clusters < 50 µm developed into somatic embryos. Later stages were not analysed statistically in this study, but it can be predicted that the correlation is absolute for morphologically recognisable somatic embryos. This described correlation of *AtLTP1* luciferase expression patterns and cell cluster size seems in part to be correlated with protoderm development in the developing embryos. Cell clusters in the < 50 µm population consist of up to 50 cells. In carrot zygotic embryos the protoderm is formed at the 32 cell stage (Borthwick, 1931; Lackie and Yeung, 1996) at which time the carrot EP2 gene is first expressed (Sterk et al., 1991).

A number of other genes expressed during early stages of somatic embryogenesis (Kawahara et al., 1995; Sato et al., 1995; Wilde et al., 1988; Wurtele et al., 1993) might be useful markers for embryogenic cells. Expression patterns of these genes have been established in embryogenic suspension cultures and developing somatic embryos. However, due to the experimental conditions applied, expression of these genes has not been determined during the very early stages of somatic embryo development. Some of these genes might like the *AtLTP1* gene, be used as marker for embryogenic cell clusters. However, cell tracking experiments are required to determine the potential role of these genes. Genes expressed more early during induction of embryogenesis have been isolated from microspore populations induced to develop into embryos (Cordewener et al., 1994; Kyo and Harada, 1990; Ríhová et al., 1996; Vergne et al., 1993). Since these induced populations also consist of non-embryo-forming microspores, it is not known whether the isolated genes are indeed expressed only in the microspores that develop into embryos. Alternatively they might also be expressed in microspores not directly involved in the process of embryogenesis.

From the results presented in this paper it becomes evident that the ability to causally relate gene expression with the very early stages of somatic embryo development, it is essential to determine expression of such genes in living developing cells.

Materials and Methods

Construction of plasmids

The binary vector pMT500 containing the firefly luciferase gene downstream of a polylinker containing 5 unique restriction sites was created by uni-directional ligation of the firefly luciferase coding region followed by the polyadenylation sequence from the pea *rbcS*::E9 gene (Millar et al., 1992a) in the *Hin*dIII-*Xba*I site of the binary vector pMOG800 (kindly provided by Mogen N.V., Leiden, The Netherlands). The binary vector pMOG800 is based upon pBIN19 (Bevan, 1984) but while in pBIN19 the polylinker is flanked by the left border and the neomycin phosphotransferase (NPT II) expression cassette, the polylinker in pMOG800 is flanked by the right border and the NPT II expression cassette. The CaMV 35S promoter enhanced by duplication of the -343 to -90 region (Kay et al., 1987) was isolated from the pMON999 vector by digestion with *Hin*dIII and *Sst*I and cloned into the pBluescript SK$^-$ vector (pMT120). The CaMV 35S promoter was cloned upstream of the luciferase gene in the binary vector pMT 500 as a *Kpn*I-*Sst*I fragment to give the 35S::LUC::E9 binary vector pMT510. The *Arabidopsis thaliana LTP1* promoter fragment was obtained from the binary plasmid pUH1000 (Thoma et al., 1994) by digestion with *Bam*H1 and *Hin*dIII, cloned into pBluescript SK$^-$ (pMT121) and subcloned into the pMT500 vector as a *Kpn*I-*Sst*I fragment to give the *AtLTP1*::LUC::E9 binary vector pMT520.

Additional sequencing of genomic *AtLTP1* DNA revealed that the *AtLTP1* promoter fragment used contains the methionine translational start codon of the *AtLTP1* gene (C. Somerville, unpublished; see GenBank accession number M80567 for the modified sequence). When translation starts with this codon, an N-terminal fusion protein of 18 amino acids of the *AtLTP1* with the luciferase protein is produced. When the luciferase start codon is used, the complete luciferase protein is synthesised (figure 5.1a). When the *AtLTP1* promoter fragment is fused to the ß-glucoronidase (GUS) coding sequence translation from the first (*AtLTP1*) start codon results in a fusion protein of 13 amino acids. A functional full length ß-glucoronidase protein is produced when translation starts at the GUS translational start codon (figure 5.1b). This ß-glucoronidase protein could be detected in transformed *Arabidopsis* plants although in some cases GUS expression patterns were not consistent with At*LTP* mRNA localisation (Thoma et al., 1994). However, in developing zygotic *Arabidopsis* embryos, the GUS expression pattern was identical to the *AtLTP1* mRNA localisation pattern (Thoma et al., 1994; Vroemen et al., 1996) indicating that the *AtLTP1* promoter faithfully reflects endogenous *AtLTP1* gene expression in developing embryos.

The binary vectors pMT510 and pMT520 were transformed into *Agrobacterium tumefaciences* strains MOG101 and MOG301 (Hood *et al.*, 1993) by electroporation as described by the manufacturer (CellJect; EquiBio, Angleur, Belgium) and selected for resistance to kanamycin (100 $mg \cdot l^{-1}$; Duchefa, Haarlem, The Netherlands).

Plant material, gene transfer and cell culture

Transformation of *Daucus carota* cv. 'Amsterdamse bak' was performed as described by Thomas et al. (1989). One week old dark grown seedlings were sliced into segments of 10 to 20 mm and incubated for 20 minutes in a freshly prepared 10 fold diluted overnight culture of *Agrobacterium*. The segments were dried and transferred to a modified Gamborgs B5 medium (P1 medium; S&G seeds, Enkhuizen, The Netherlands) supplemented with 2 µM 2,4-D (P1-2) and solidified with agar (Difco, Detroit, Mi, USA). After two days of culture in the dark at 25 ± 0.5 °C, segments were transferred to solidified P1-2 medium supplemented with kanamycin (100 $mg \cdot l^{-1}$), carbenicillin (500 $mg \cdot l^{-1}$; Duchefa) and vancomycin (100 $mg \cdot l^{-1}$; Duchefa). After three weeks segments were transferred to fresh plates and transformed calli were selected after an additional three weeks. Transformed calli were grown on P1-2 plates with antibiotics for 3 weeks at a 16 hour light/8 hour darkness regime.

Primary transformed embryogenic suspension cultures were initiated as described by (De Vries et al., 1988) by transferring 0.2 g callus to 10 ml liquid P1-2 medium supplemented with 200 $mg \cdot l^{-1}$ kanamycin, 250 $mg \cdot l^{-1}$ carbenicillin and 50 $mg \cdot l^{-1}$ vancomycin. During the first weeks 1 to 3 volumes of fresh medium were added to the culture at weekly intervals. After 5 to 7 weeks cultures were subcultured to a packed cell volume of 2 ml per 50 ml medium every two weeks and incubated at a 16 hour light/8 hour darkness regime at 25 ± 0.5 °C.

Protoplasts were isolated by overnight incubation of 5 ml suspension culture in 50 ml enzyme solution (2 % cellulase "Onzaka R10" (Yakult Biochemicals, Japan), 1 % macerozyme R10 (Yakult), 0.4 M mannitol, 50 mM sodium citrate pH 4.8) in the dark. Protoplasts were sieved through a 50 µm mesh sieve to remove undigested plant material, washed twice with washing solution (100 mM $CaCl_2$, 0.3 M mannitol) and transferred to P1 medium supplemented with 0.2 µM 2,4-D and 0.3 M mannitol. Protoplasts are grown at 100 000 cells $\cdot ml^{-1}$ and after one week the medium was replaced by fresh basal P1 medium without growht regulators, mannitol and additional $CaCl_2$. After two weeks cell clusters derived from single protoplasts developed into somatic embryos. Alternatively, somatic embryos were derived from transformed single suspension cells obtained after sieving through

a 22 µm mesh sieve. Individual torpedo stage embryos or small plantlets were isolated, chopped into pieces and either transferred to P1-2 plates or liquid P1-2 medium. Secondary transformed suspension cultures were maintained as described above. One-week old high-density (10^6 - 10^7 cells·ml^{-1}) secondary suspension cultures were sieved through nylon sieves with successive 300, 125, 50 and 22 µm pore sizes (Monodur-PES; Verseidag Techfab, Walbeck, Germany). The cell population containing mainly cell clusters (50-125 µm population) was retained on the 50 µm sieve. The cell population passing through the 50 µm sieve was either isolated as < 50 µm population or transferred to the 30 µm sieve. Single cells and cell clusters passing this last sieve are designated as < 30 µm populations (Chapter 2).

Control experiments with untransformed cells were performed with *Daucus carota* cv. 'Trophy' (S&G seeds) suspension cultures grown in P1-2 medium. The embryogenic competence is expressed according to De Vries *et al.* (1988) as the number of somatic embryos developed from 10 000 cells present at day zero in the culture.

Cell immobilisation

Size fractionated populations smaller then 30 µm, 50 µm or between 50 and 125 µm were immobilised in phytagel (P8196; Sigma, St Louis, Mo, USA) in petriperm dishes (Heraeus, Hanau, Germany). The bottom layer consisted of 1 ml P1-0.2 medium with 5 mM Ca^{2+} and 0.2 % phytagel. Two hundred thousand cells (< 30 µm and < 50 µm populations) in B5-0.2 medium without Ca^{2+} supplemented with 0.1 % phytagel were poured on top of the bottom layer. For this layer B5 was applied since, at room temperature, phytagel solidified in P1 medium without Ca^{2+}. After 2 hours of solidification an additional P1-0.2 layer with 0.2 % phytagel was poured onto the cell layer preventing the B5 layer to move. To prevent dehydration of the phytagel layers and to supply luciferin to the cells, 0.5 ml P1-0.2 medium containing 0.05 mM luciferin (Promega, Madison, Wi, USA) was added after solidification. The final luciferin concentration in the culture was 0.02 mM. After 7 days of culture 2,4-D and luciferin was removed from the cultures by washing as described in chapter 2. The 50-125 population was embedded in a similar way at 40 000 cells per dish in P1/B5 medium without 2,4-D.

***In-vivo* imaging of luciferase bioluminescence**

Transformed calli were sprayed with filter sterile 0.1 mM luciferin in water. Luciferase expression was observed with a Hamamatsu CCD camera fitted with

an intensifier (C2400-47; Hamamatsu, Hamamatsu City, Japan) and a 55 mm f1.1 lens (Nikon, Tokyo, Japan) in a dark box. Images were obtained using the ARGUS 50 photon counting image processor. For 35S::luciferase and LTP1::luciferase constructs images were obtained by 10 minutes of photon counting.

The cell tracking equipment (Chapter 6) consisted of the iCCD camera connected to the 100% light camera port of a Zeiss Axiovert 135-TV inverted microscope (Zeiss, Oberkochen, Germany) equipped with a Märzhäuser cross table (Märzhäuser, Wetzlar, Germany). The cross table is driven by the MultiControl 2000 unit (Märzhäuser) under control of the MicroScan 6 program (Agricultural University Wageningen, The Netherlands). MicroScan allowed to follow the developmental fate of single cells and cell clusters for over two weeks. Luciferase derived photons were detected and bioluminescent images were obtained by digital accumulation of images by photon counting for 2 to 10 minutes from cell clusters and somatic embryos. Images of AtLTP1 luciferase expression shown were obtained by 10 minutes counting either in slice mode or gravity mode. Signals obtained in slice mode represent the group of photons detected by the iCCD camera. This method gives high signals when compared to gravity mode. Signals obtained in gravity mode represent individual photons released by luciferase. Images were stored as TIFF file on a 1 Gbyte server disk. Adobe Photoshop 3.0 (Adobe Systems Inc. Mountain View, Ca, USA) was used to adjust grey levels and to prepare superimposed images of bioluminescent and visable light images. Ilustrations where prepared with Adobe Pagemaker 6.0.

Viability staining and whole mount *in situ* hybridisation

Viability staining was performed with fluorescein diacetate as described by Widholm (1972). Whole mount *in situ* hybridisation on <50 µm and 50-125 µm populations using the EP2c riboprobe derived from the EP2c cDNA (Sterk et al., 1991) was performed as described elsewhere (Engler et al., 1994; Schmidt et al., 1997)

DNA isolation and PCR

To confirm the presence of luciferase constructs in plants 0.5 ml transformed suspension culture was centrifuged and DNA was extracted from the pellet with a 1:1 mixture of phenol and extraction buffer (0.2 M NaCl, 0.006 M $MgCl_2$, 1 % SDS, 0.01 M Tris/HCl pH 7.4). After several phenol-chloroform extractions, DNA was precipitated by ethanol in 0.3 M NaAc. RNA was removed from the pellet by RNase A treatment (a modification of the method described by Frankis and

Mascarenhas (1980)). PCR was used to amplify sequences upstream of the luciferase coding sequence using AmpliTaq polymerase (Boehringer, Mannheim, Germany) and two primers flanking the upstream cloning site. This resulted in a 100 bp fragment for plants containing pMT500 DNA without promoter. For constructs containing the CaMV 35S or *AtLTP1* promoter, fragments of respectively 800 and 1250 bp were obtained.

To determine the copy number of the inserted luciferase construct genomic DNA was isolated according to (Rogers and Bendich, 1985). Samples of 10 µg genomic DNA were digested with restriction enzymes, separated on an agarose gel and transferred to Nytran Plus membrane. Hybridisation was performed as described previously (Sterk et al., 1991) using the luciferase cDNA fragment as probe.

Acknowledgements

The pUH1000 plasmid was kindly provided by Dr. Chris Somerville (Dept. of Plant Biology, Carnegie Institution of Washington, Stanford, Ca, USA) and the pMON999 was provided by Dr. Cynthia Hemenway (Monsanto Co., St. Louis, Mi, USA). *Agrobacterium* strains MOG101 and MOG301 and the binary vector pMOG800 were kindly provided by Mogen N.V. (Leiden, The Netherlands). We are grateful to Dr. Sander van der Krol (Dept. of Plant Physiology, AUW) for introducing the 2D luminometer and providing the luciferase::E9 construct and Marijke Hartog (Dept. of Molecular Biology, AUW) for technical assistance.

6

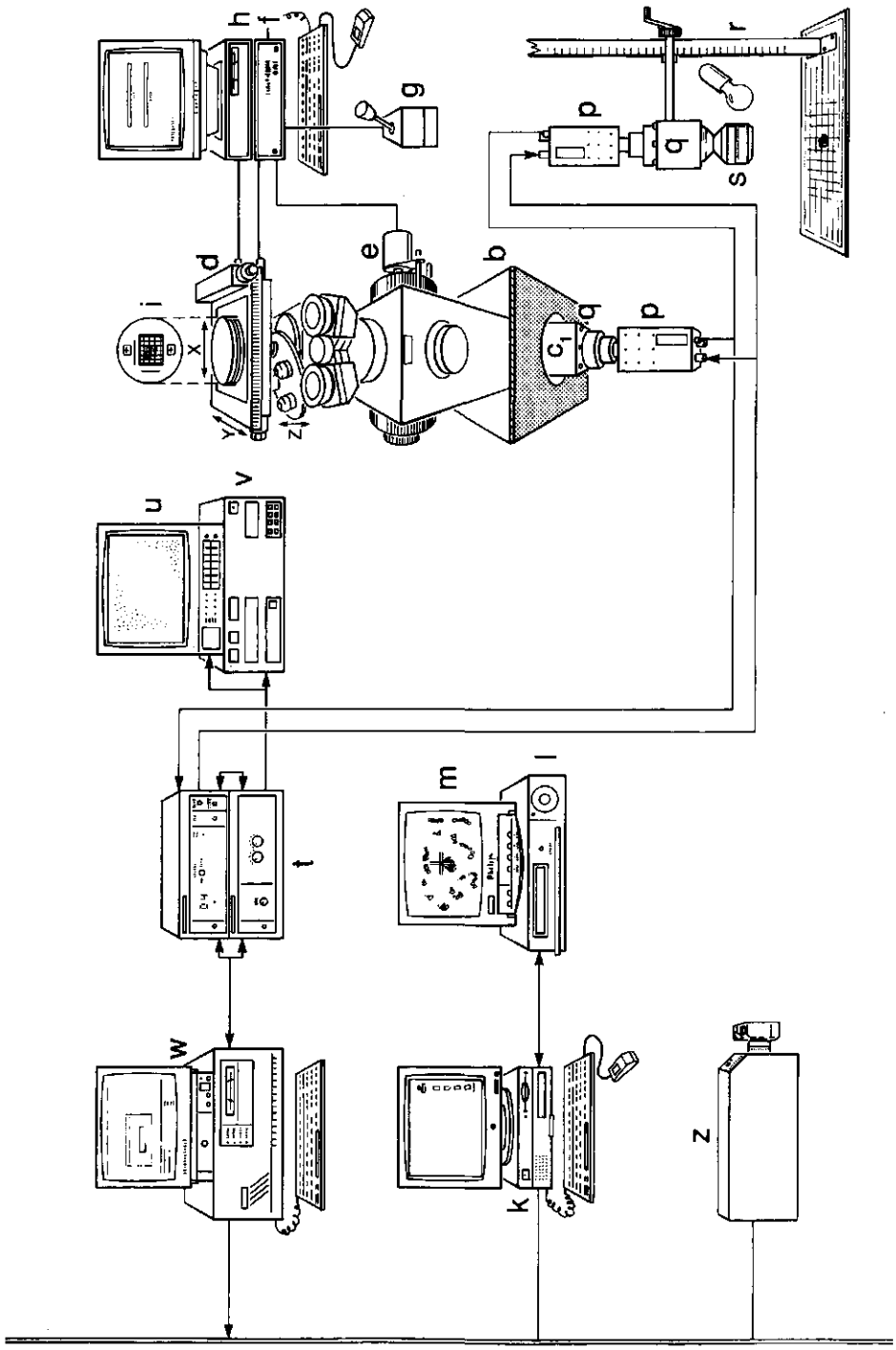
Use of video cell tracking to identify embryogenic cultured cells

Marcel A.J. Toonen & Sacco C. de Vries (1997)
adapted from: Plant tissue culture manual, pp H 1-45, Lindsey K. ed.
Kluwer Academic Publishers, The Netherlands

Introduction

Plant somatic embryos develop from embryogenic cells, that in turn originate from somatic cells. Embryogenic cell formation usually commences when explants or suspension culture cells are exposed to exogenous plant growth regulators. A large number of cells in the explant or culture generally responds to the inducing treatment, while only a limited number of cells actually becomes embryogenic. There is a considerable gap in our knowledge of the events that take place between the moment the inducing treatment is applied and the first morphologically visible signs of somatic embryo development occur. This is due in part to the fact that changes observed after particular treatments with, for example growth regulators at the culture level are often interpreted based on effects in very few cells. It is therefore important to be able to identify the few responding cells in culture and follow their development into somatic embryos. For this purpose a cell tracking system was established based upon the system previously developed by Dr. H.A. Verhoeven (CPRO-DLO, Wageningen, The Netherlands). The cell tracking system allows recordings of protoplasts, cells or cell clusters over periods of several weeks. In this way development of individual cells into somatic embryos can be monitored. This cell tracking system has been used to identify single carrot suspension cells that are in the process of acquiring embryogenic capacity (Chapter 2). It has also been used to identify guard cells as the cell type that develops microcalli in the recalcitrant crop species sugar beet (Hall et al., 1995).

A second useful procedure combines cell tracking with Confocal Scanning Laser Microscopy (CSLM) as a method to test potential markers for their role in embryogenic cell formation. This was used to follow development of cells labelled with the monoclonal antibody JIM8, reported to be a marker for embryogenic cells (Pennell et al., 1992). However, cell tracking experiments showed no correlation between expression of the JIM8 cell wall epitope and the ability of that cell to develop into a somatic embryo (Chapter 3). A third application uses cell tracking in combination with a 2D luminometer system to detect *in vivo* gene expression during embryo development. For this purpose carrot suspension cultures were obtained from cells transformed with the embryo-expressed *AtLTP1* promoter fused to the firefly luciferase coding sequence. The 2D luminometer system is able to detect photons released by the luciferase and in this way enables the detection of gene expression in live, developing somatic embryos. Here we will describe the equipment needed and procedures used to allow cell tracking in combination with the different detection systems.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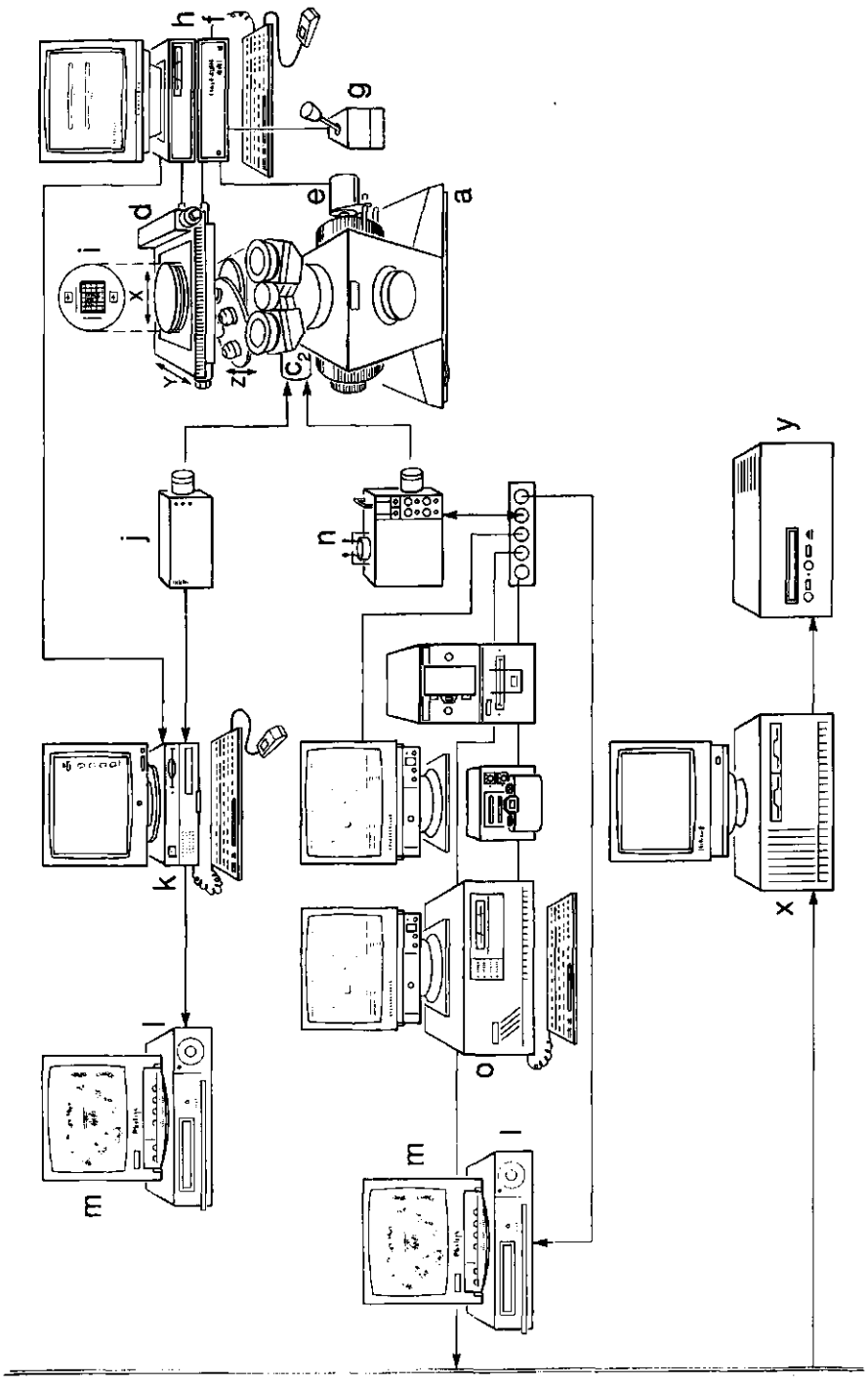

figure 6.1. Equipment employed for cell tracking and the detection of light images and images of fluorescent and bioluminescent signals. Each part is displayed in detail in the text.

Instrumental set-up

Figure 1 shows a schematic overview of the cell tracking equipment and the various signal detection systems. The system is based upon a step motor driven cross table attached to an inverted microscope equipped with a step motor-controlled focus knob. MicroScan 6 cell tracking software has been developed to allow control of the plane of focus and movement of the cross table. The combination of the cross table with the MicroScan software allows recording of the same positions in culture dishes over periods of several weeks. The area of the culture dish at such a position that is visible on the video screen is defined as 'frame'. For detection of visible light images a video camera can be connected to the microscope. For detection of fluorescent signals a CSLM system can be used while the 2D luminometer system is used for bioluminescent signals. In all cases data can be stored either as video images or as digital images. In case of large numbers of images video recording is the preferred option, since a 3 hour video tape can store over 20 000 images in colour, which is beyond the capability of digital storage media commonly used at this moment. In case of bioluminescent signals less images are obtained and they can be stored digitally on optical disks or CD-ROMs.
Features of each individual part of the equipment and software are described below.

Microscope and cross table

The cell tracking equipment is based upon the Märzhäuser EM-32 IM cross table (figure 6.1d; Märzhäuser, Wetzlar, Germany). The cross table itself is universal but it is built in such a way that it can only be attached to one specific type of microscope. Different holders can be placed into the cross table to fit culture dishes of various sizes. To perform cell tracking a microscope containing a video connector is required. We used a Nikon Diaphot inverted microscope (figure 6.1a; Nikon, Tokyo, Japan) or a Zeiss Axiovert 135-TV inverted microscope (figure 6.1b; Zeiss, Oberkochen, Germany) both equipped with their specific EM-32 IM cross table. Each type of microscope is equipped with a specific light path. In the Zeiss microscope we used, all light derived from the specimen is either directed to the eyepiece for visual observation or alternatively, through the video connection (100 %) at the bottom of the microscope (figure 6.1c1). The advantage of the video connection at the bottom of the microscope is that the distance between specimen and camera is as short as possible and little signal is lost in the optical path. In the Nikon Diaphot we also used, 20 % of the light derived from the specimen is directed to the eyepiece and the remaining 80 % is directed through the video connection (figure 6.1c2). For detection of low intensity signals such as produced in

bioluminescence experiments, a 100 % light video connection, as in the Zeiss microscope, is preferred. In addition, the microscope optics must be of first quality to avoid loss of signal strength in the optical path. Objectives ranging from 2.5x to 20x can be used, but routinely 4x or 10x objectives are applied.

The Märzhäuser EM-32 IM cross table (figure 6.1d) is equipped with two step motors with a 0.4 millimetre pitch enabling movements of the cross table in the X- and Y-direction with steps of 10 nanometres. To control the Z-movement of the objective both microscopes are equipped with a step motor (figure 6.1e; Märzhäuser) attached to the focus knob. All three motors are controlled by the MultiControl 2000 unit (figure 6.1f; Märzhäuser). This unit contains a RISC-processor controlled by the operating software VENUS-1 (Märzhäuser). Connected to the MultiControl unit is a Tulip DC486 sx PC (figure 6.1h; Tulip, 's Hertogenbosch, the Netherlands). The computer acts as terminal to control the RISC-processor by the VENUS-1 communication software under MS Windows. By using VENUS-1 commands, settings of the MultiControl 2000 unit can be adjusted to individual preferences. Movement of the cross table and focus motor can be controlled using a joystick (figure 6.1g) or entering coordinates. The step motors are driven by sequential pulses, each pulse allowing the cross table to make one step of 10 nanometres. After calibration of the cross table every position of the table is exactly defined and can be retrieved using the VENUS-1 software or MicroScan 6 software (Agricultural University, Wageningen, The Netherlands).

MicroScan 6

MicroScan 6 allows scanning of immobilised cells or cell clusters at defined positions. The program uses VENUS-1 commands to control the MultiControl unit. Either single positions or a matrix of adjacent positions can be scanned. For convenience the area of the culture dish at one position that is visible on the video screen is defined as 'frame' (figure 6.2a). Each frame position can also be scanned in the Z-direction in a number of steps. The position of the culture dish relative to the cross table does not have to be fixed, because this is determined by the two mark-points present in each dish (figure 6.2b). Based upon these two points a new set of mathematically defined axes is calculated by the MicroScan program. All positions in the culture dish are determined relative to these axes. This feature allows to retrieve exactly the same position in the culture dish at any desired time point. In addition, it makes it possible to exchange dishes between the different microscopes equipped with a Märzhäuser EM-32 IM cross table and to retrieve particular single frame positions in a given dish. Individual frame positions can be programmed manually or a matrix of adjacent frames can be calculated by the MicroScan program. The programmed positions or the set of frame information

calculated, are stored to the computers hard disk by the program. To follow development into somatic embryos of large numbers of cells distributed in a petri dish the matrix of adjacent frames is used. Frame images are recorded on video tapes during embryo development. Placing the images of the same frame into sequence then gives an overview of the development of all individual cells visible in that frame.

Besides the MicroScan 6 software two data files are present: profiles.dat and msconfig.dat. The msconfig.dat file contains standard settings such as the cross table movement speed and settings to define the communication port (COM port) used for connection with the MultiControl unit (figure 6.1f), the Macintosh computer (figure 6.1k) and the computer mouse. The profiles.dat file contains pre-defined settings used for scanning a matrix. These settings are the size of a frame visible on the monitor screen, the number of frames to be scanned in X-, Y- and Z- direction and the time to stop at each frame position. For video microscopy the cross table stops 350 milliseconds at each position to allow recording of one image. For CSLM it takes 1.1 second to obtain an image of the fluorescent signal and this is the minimal time to stop at each frame position. Both data files can be changed using the MS-DOS edit command.

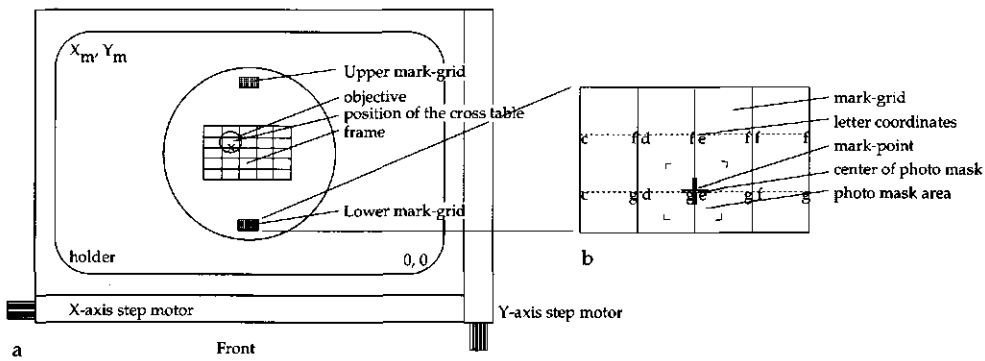

figure 6.2a-b. a. Schematic drawing of a top view on the cross table. The table consists of two step motors to control movement in X and Y direction. The axes defined by the VENUS software. The basis of the set of axes is the (0, 0) position at the right bottom of the cross table. The maximal XY position (X_m, Y_m) is located at the outer right top. The culture dish fitted with two mark-grids is placed in the holder is . The calculated matrix of adjacent frames is in the centre of the culture dish. One frame is the area that is visible on the monitor with a given objective. The position of the cross table with respect to the objective is defined as the position of the cross table (x). **b.** The mark-points are located on the mark-grid. The grid consists of a number of lines in horizontal and vertical direction. The distance between the two lines is 400 nm. On crossings of the lines letter coordinates are given. One letter coordinate is selected as mark-point and placed in the centre of the photo mask (represented as a set of black lines in the form of a cross on the Nikon and Zeiss microscopes used).

Individual frames to be scanned can be programmed using Point Scan. A matrix of frames for cell tracking can be calculated by Matrix Scan. For both type of scans a frame is selected by the user where it is possible to define a low and a high plane of focus, between which the program will scan in the Z-direction. Point scan and Matrix scan are used to scan the programmed frames. Before cell tracking the cell tracking system has to be calibrated with a full calibration. Hereby the effective area of the cross table that can be scanned is determined. First the cross table is sent to the 0, 0 position (figure 6.2a). Thereafter the table moves to its maximal X, Y position (figure 6.2a; X_m, Y_m). In this way the exact dimensions to be scanned are known and the basic set of axes are defined by the MicroScan program. Half calibration will send the cross table to the 0,0 position. After calibration of a culture dish a new set of axes is defined by the MicroScan program. If a new culture dish is placed on the cross table the set of axes has to be recalibrated. This is done by the MicroScan software itself and does not require additional calibration.

TitleGen 1.1

The TitleGen 1.1 program (ID Systems, Vaassen, The Netherlands) has been developed to record the position number on the corresponding frame image. This feature considerably speeds up the retrieval of recorded images of the same frame after the cell tracking experiment has been performed. The program uses standard Macintosh software modules and requires a Macintosh 7100 AV Power-PC (figure 6.1k; Apple, Cupertino, Ca, USA). Using additional video settings such as brightness and contrast the video image can be adjusted. The video signal is obtained from the video camera by the Macintosh video-card-in port and the position numbers are obtained from the MicroScan 6 program via the serial port of the Macintosh connected to the RS232 port of the Tulip cell tracking computer (figure 6.1h). Position numbers are visualised by a text generator, merged into the video signal and send to the video recorder (figure 6.1l; Hitachi F780E) via the Macintosh video-card-out port.

Video recording

To record frame images a video camera (figure 6.1j; KP-C503, Hitachi, Tokyo, Japan) is attached to the video connection (figure 6.1c2) of the Nikon Diaphot microscope (figure 6.1a). This microscope is equipped with a 50 W halogen lamp. Eighty percent of the light from the frame image is available for the camera. Due to the low sensitivity of the Hitachi KP-C503 camera visible light images can be obtained with up to a 20x objective. With the TitleGen video settings, image qual-

ity can be improved and a 40x objective can be used. Alternatively, more sensitive CCD cameras can be used for low light conditions.

Video images are stored on video tape by continuous recording of the images on a VHS video recorder (figure 6.1l) and displayed on a monitor (figure 6.1m; CM11342, Philips, Eindhoven, The Netherlands). Better image quality can be obtained using a Super-VHS recorder. This type of machine has an improved recording and replay system. Normally they have also additional options which facilitate for example mounting the tapes. This type of video recorder has a good performance/price ratio especially when compared to professional video recorders.

An average cell tracking experiment consists of a set of 2 to 6 culture dishes. In each dish 1 000 frame positions are scanned in XY direction and each frame position is scanned in 5 Z-steps. Such a scan of 5 000 images requires about 45 minutes scanning time. Frame images of four culture dishes can be recorded on one tape. Scanning at two-daily intervals for two weeks thus will generate seven video tapes for every four dishes. When the cell tracking experiment is completed, the video tape of the last recorded day is examined for the presence of frame images containing somatic embryos. Images of the corresponding frame positions of previous days are collected and then sequentially copied onto a tape in a second video recorder. Alternatively, all frame images from one tape where somatic embryos have developed are transferred to the Macintosh Power PC and digitised by the VideoMonitor program (Apple). Corresponding frame images recorded on previous days are also transferred and digitised, omitting the need of a second video recorder. Placing the corresponding images in their correct temporal sequence will give the desired developmental history of a particular embryo down to the original cell or cluster of cells that produced it.

Confocal Scanning Laser Microscopy

To detect fluorescent signals from single cells labelled with specific antibody-FITC conjugates the Nikon Diaphot inverted microscope with the Märzhäuser cross table was connected to the BioRad MRC-600 Confocal Scanning Laser Microscopy system (figure 6.1n; BioRad, Cambridge, MA, USA). The advantage of CSLM over a UV source is that it generates single wavelength light and it is possible to adjust the light intensity. In this way damage to the cells due to strong UV-light can be prevented. The argon laser light passes an excitation filter and the 488 nm light excites FITC. The emitted 515 nm light passes the 500-650 nm transmission filter and is detected by a photo multiplier. Visible light is detected by a second multiplier. The entire equipment is controlled by the MRC600 control software running on a 486 PC (figure 6.1o). The image of the fluorescent signal as well as the visible

light image are displayed on a monitor. Brightness and contrast of the image can be adjusted using the software. The latest version of the control software allows images to be stored in program specific PIC-format as well as the MS-DOS Tagged Interchange File Format (TIFF). For cell tracking a video recorder (figure 6.1l) can be attached to the video-out connection of the BioRad MRC 600. The time (1.1 sec) the cross table stops at each frame position is set to the time the BioRad system requires to build one image in the Fast 1 (F1) scanning mode. Images from the fluorescent signal and visible light of each frame are continuously recorded onto the video recorder.

As an alternative for the CSLM system, Silicon Intensified Tube (SIT) camera systems can be used. For this type of camera a UV source attached to the microscope is required. SIT camera systems have about the same sensitivity as the human eye, a high resolution, are easy to use and are less expensive when compared to CSLM systems.

2D Luminometer

Transgenic plant cells containing promotor::firefly luciferase constructs have been used to detect *in vivo* gene expression (Millar et al., 1992a; 1995a; 1995b). After application of D-luciferin (Promega, Madison, Wi, USA) to the cells, the luciferase enzyme converts this substrate into oxyluciferin. Photons released in this reaction are visualised with the Hamamatsu ARGUS 50 system (Hamamatsu, Hamamatsu City, Japan) based on an intensified CCD camera (C2400-47; Hamamatsu). Photons detected by the CCD camera are displayed as spots on a monitor. By digitally accumulating the images of these spots over time an image of the bioluminescent signal can be obtained which is displayed on the monitor using false colours. Due to the intensifier the resolution of this system is fairly low (300 lines).

The ARGUS luminometer system consists of a CCD camera (figure 6.1p) coupled to an intensifier (figure 6.1q). To obtain images from calli ($\geq$ 1 millimetre in diameter) or whole plants ($\leq$1 metre) the camera is attached to a support (figure 6.1r) in a dark box and connected to a 55 mm f1.1 Nikon lens (figure 6.1s), optionally fitted with extension tubes for the smaller objects. To obtain microscopic images, the intensifier and CCD camera can be connected to the 100 % light video connection at the bottom of the Zeiss Axiovert 135 TV inverted microscope (figure 6.1c1) placed in a dark box (Hansa, Tokyo, Japan). Both CCD camera and intensifier are controlled by the ARGUS 50 control unit (figure 6.1t; Hamamatsu). All functions of the control unit can be controlled by the ARGUS V3.3 software, except for the sensitivity of the intensifier which is set manually. Images of the bioluminescent signals are obtained by photon counting, either in slice or gravity mode. Photon relased by luciferase acitivity are multiplied in the intensifier and

detected by the CCD camera. The multiplication is dependent of the set sensitivity of the intensifier. In 'slice mode' the group of photons detected by the camera are displayed on the Trinitron monitor (figure 6.1u; PVM1444QM, Sony, Tokyo, Japan) as a group of pixels representing the number of photons detected by the camera. In 'gravity mode' the group of pixels is replaced by one single pixel, representing the original photon that generated the signal, giving a more accurate representation of the number of photons released by the luciferase. To allow 'gravity mode' operation the PC (figure 6.1w; DeskPro 66M, Compaq, Houston, Tx, USA) running the ARGUS software is equipped with a gravity memory board (Hamamatsu). During or after photon counting intensity levels of obtained images can be converted in various ways to allow correct interpretation. Obtained images can either be printed with a photo printer (figure 6.1v; CP100E, Mitsubishi, Tokyo, Japan) or stored digitally by the ARGUS software as ARGUS software specific IMA files or as interchangeable TIFF files. Use of IMA files is restricted to the ARGUS software but these files contain additional information when compared to the TIFF files. TIFF files however can be used by most image processing software. Both type of files can contain up to 0.25 Mbyte of data per image and are stored on a 1 Gbyte network server disk (figure 6.1x). A CD-ROM writer (figure 6.1y) is connected to this server for long time storage of the image files. A 2D-luminometer based upon an Astromed cooled CCD camera has recently been described by Kost et al. (Kost et al., 1995).

Macintosh 7100 AV Power PC

This computer is equipped with a Power PC 601-chip, 16 Mbyte ROM memory, 2 Mbyte video memory, ethernet network connection and an Audio/Video card. With the VideoMonitor program (Apple) images from the video recorder or camera can be digitised and stored as Macintosh interchangeable PICT file. Using the local ethernet, TIFF files stored by the ARGUS 50 luminometer system can be accessed. Quality of these images can be improved in Photoshop 3.0 (Adobe Systems Inc. Mountain View, Ca, USA) by adjusting brightness and contrast or by expanding grey levels and colour levels. Subsequently, images can be combined in Pagemaker 6.0 (Adobe Systems Inc.) to make compositions for publication. These can be transferred to either photo or slide film using a film recorder (figure 6.1z).

Using the TitleGen program the computer is used as title generator, merging position numbers of the cell tracking into the video signal.

Embedding of cells

One of the main requirements for cell tracking is the ability to immobilise cells in the culture dish. In our experiments we use the phytagel system. Phytagel (Sigma, St. Louis, Mo, USA) gives a strong and completely transparent matrix. The pore size allows diffusion of molecules as large as an IgM molecule. This facilitates the addition or removal of plant growth regulators at certain timepoints during the culture period. The temperature at which phytagel solidifies depends on the concentration of free calcium ions. When cooled down phytagel hardly solidifies at room temperature in the absence of Ca^{2+}. With 1 mM Ca^{2+} phytagel solidifies at approximately 30 °C and medium with 5 mM Ca^{2+} solidifies at approximately 55 °C. In case of Gamborgs B5 medium the Ca^{2+} concentration is 1 mM (Gamborg, 1962). Immobilisation of cells depends on two layers of phytagel. The bottom layer consists of 0.2 % phytagel in B5 medium with Ca^{2+} added to a final concentration of 5 mM. On top of this layer a layer containing 0.1 % phytagel in B5 medium without Ca^{2+} is poured at room temperature. Ca^{2+} diffuses from the bottom layer to the cell layer allowing the phytagel to solidify. After two hours the phytagel matrix containing the cells has been solidified. On top of this layer of phytagel a layer of liquid B5 medium is placed to prevent dehydration of the phytagel (figure 6.3). The final Ca^{2+} concentration is below 2.4 mM and does not affect somatic embryo development (Chapter 2).

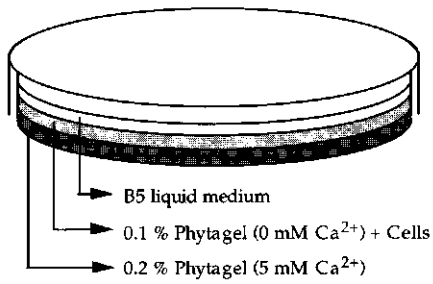

figure 3. Schematic drawing of a culture dish containing a bottom layer of 0.2 % phytagel with 5 mM Ca^{2+} and a cell containing layer without Ca^{2+}. Ca^{2+} diffuses from the bottom layer to the cell layer and solidifies the phytagel. The liquid top layer prevents dehydration of the phytagel layers.

Protocols

1. Isolation of cell populations

1). Sieve one to two litres of a 7 day old carrot suspension culture (De Vries et al., 1988) first through a 200 and then through an 125 µm polyester sieve (Monodur-PES, Verseidag Industrietextilien, Kempen, Germany).
2). Transfer half of the filtrate to 50 ml tubes (Sarstedt, Nümbrecht, Germany) and centrifuge for 5 minutes at 200 g.
3). Discard the supernatant and sieve the pelleted cells together with the remaining filtrate through a 50 µm polyester sieve (Monodur-PES).
4). Collect the cell population remaining on the sieve and transfer them to a tube. This is the 50-125 µm cell population.
5). Store one hundred ml of the filtrate obtained by the 50 µm sieve step and centrifuge the remaining filtrate at 200 g for 5 minutes.
6). Discard the supernatant and sieve the pelleted cells together with the remaining 100 ml of the filtrate gently through the 22 µm sieve (Monodur-PES).
7). Collect the cell population remaining on the sieve and transfer them to a tube. This is the 22-50 µm cell population. Cells passing the 22 µm sieve are the < 22 µm cell population.
8). Wash all cell populations twice with B5 medium without Ca^{2+} in 50 ml tubes. Pellet them for 5 minutes at 200 g.
9). Determine the cell density in each population using a haemacytometer and dilute the cell populations to the desired cell density.

2. Preparation of phytagel embedded cultures

1). Pour one ml of B5 medium containing 5 mM Ca^{2+} and 0.2 % phytagel (P 8196; Sigma, St. Louis, Mo, USA) in a Petriperm culture dish (Hereaus, Haneu, Germany) at 60 °C and allow the phytagel to solidify for 5 minutes at room temperature.
2). Prepare a cell population in B5 medium without Ca^{2+}. The cell density should be 10 times as high as the desired cell density in the embedded culture. This is 1 000 000 cells·ml^{-1} for the < 22 µm and 22-50 µm carrot cell populations and 200 000 cells·ml^{-1} for the 50-125 µm carrot cell populations.
3). Mix the diluted cell population with an equal amount of 0.2 % phytagel in B5 medium without Ca^{2+}, and pour at room temperature 0.5 ml onto the phytagel bottom layer using a 5 ml wide-mouthed disposable pipette (Sarstedt).

4). After two to three hours the phytagel is polymerised. Add 1 ml liquid B5 medium on top of the cell containing phytagel layer to prevent dehydration of the phytagel.

5). The final cell density of the embedded cells is 100 000 cells·ml^{-1} for the < 22 µm and 22-50 µm cell populations and 20 000 cells·ml^{-1} for 50-125 µm cell populations.

Notes

1. For other types of media the concentrations of Ca^{2+} might be adapted to the Ca^{2+} concentration used in the medium. If media contain Ca^{2+} concentrations of 2.5 mM or higher, cells can be embedded as described above. The desired Ca^{2+} concentration of the medium can be reached by adding additional Ca^{2+} to the liquid medium added to the phytagel layers.
2. In most cases plant growth regulators are added to the media. For 2,4-D a 0.2 µM concentration is used for single cell embryogenesis. This amount is sufficient to induce embryo development and is easily removed from the cultures after one week. It is added before autoclaving to all media. Heat labile compounds can either be added to all media after cooling or in a 2.5 times excess to the liquid B5 medium that is added to the culture dish after solidification. The compounds then diffuse into the phytagel layer.
3. Using diffusion also other components can be transferred into the phytagel medium. In our experiments we added 2.5 times excess of the IgM class JIM8 monoclonal antibody conjugated to FITC or D-luciferin to the B5 medium. After 30 minutes the presence of all of these compounds in the cell containing layer was shown by labelling of cell wall epitopes or release of photons due to luciferase activity respectively (see section: cell tracking combined with immersion immunofluorescence).
4. The cell containing phytagel layer is 0.25 mm thick. Large cell clusters or embryos stick out of this layer during their development. If this area becomes to large they might be released from the phytagel and move through the culture dish. To prevent this an additional 0.1 % phytagel layer in B5 medium can be poured on top of the cell containing layer at 30 to 35 °C.
5. This protocol has also been applied for video cell tracking of protoplasts. To adjust the osmolarity of the culture medium 0.3 M mannitol has to be added to all media.

3. Washing the phytagel cultures

1). Remove the liquid medium on top of the phytagel with a 1 ml disposable pipette (Sarstedt).
2). Add carefully 2 ml fresh medium with a 5 ml wide-mouthed pipette and incubate for 10 minutes to allow diffusion of molecules from the phytagel layer into the liquid medium.
3). Remove the liquid medium on top of the phytagel with a 1 ml disposable pipette (Sarstedt).
4). Repeat steps 2 and 3 for 9 times.
5). Add 1 ml fresh liquid medium on top of the cell containing phytagel layer.

Note
Using this procedure 98 % of small molecules like Ca^{2+} or 2,4-D are removed from the phytagel medium. For 2,4-D this is sufficient to allow carrot embryo development beyond the globular stage (Chapter 2).

4. Video cell tracking

4.1 Preparation of the culture dishes

1). Stick a piece of double sided Sellotape (Sellotape GB LTD, Dunstable, UK) on a Leica S54 cell finder grid (Leica, Wetzlar, Germany) and cut the grid in square pieces of about 5 to 5 mm.
2). Mount two pieces on the bottom side of a Petriperm culture dish (Hereaus) as indicated in figure 6.2a. These pieces of finder grid consist of a number of lines with coordinates consisting of two letters (figure 6.2b). From both pieces one letter combination is selected as mark-point for the dish. These letter combinations are referred to as Lower and Upper mark-point.
3). Place the culture dish in the holder of the cross table.

4.2 Initiation of a matrix video cell tracking experiment

1). Start the MicroScan program by typing *micscan*. The Main menu will appear. To calibrate the cross table select full calibration by choosing *1*. A full calibration is only required when the MicroScan program is initiated.
2). After calibration the main meu will apear again. Select the Matrix menu by choosing *5*. The Matrix Scan menu will apear.

3). Select Dish with NEW Profile by choosing *1*. When another dish was scanned before the current dish the alignment of the previous dish will be lost. Confirm this by typing *y*.

4). To make a new point profile enter the new profile name for the current experiment. Subsequently select a general profile with the arrow keys and press <*enter*> to select the profile.

chose a general profile (from file profiles.dat)
by using <↑> and <↓> and <*enter*> keys
press <*esc*> to abort

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description :	Nikon MicroScan 10x
Number X, Y, Z steps :	30 / 33 /5
Size X and Y steps :	0.620 / 0.405
Z-step delay (ms) :	350

figure 6.4. Selection screen for the MicroScan 6 matrix scan.

5). Enter your name and plate number or name. Together with the date, these will be displayed on the video tape before scanning starts to facilitate identification of tapes.

6). Use the joystick to move the cross table so that the Lower mark-grid is visible through the eye piece. Pull the microscope photo mask into the view field and move the cross table so that one of the letter coordinates is in the centre of the photo mask. (figure 6.2b). This letter combination is defined as the Lower mark-point.

7). Write down the letter combination of the Lower mark-point. Type <*enter*> to automatically register the XY coordinates of the cross table for the Lower mark-point by MicroScan.

8). Use the joystick to move the cross table so that the Upper mark-grid is visible in the photo mask. Move the cross table so that one of the letter coordinates is in the centre of the photo mask (figure 6.2b). This letter combination is defined as the Upper mark-point.

9). Write down the letter combination of the Upper mark-point. Type <*enter*> to automatically register the XY coordinates of the cross table for the Upper mark-point by MicroScan. MicroScan then measures the distance between the two mark-points and displays it on the monitor.

10). Move the cross table with the joystick to a position in the area of the dish that will be scanned. This position is called focussing point and cells visible

in this frame have to be present both in a low and in a high plane of focus. XY coordinates of the cross table are registered by MicroScan by typing *<enter>*.

11). With the Z-direction of the joystick the lowest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*.

12). With the Z-direction of the joystick the highest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*. During focussing the distance to the lowest focus plane is displayed as focus depth.

13). The MicroScan program stores the data registered on the computer's hard disk. Press *<enter>* to continue.

14). Select Matrix Scan in the Matrix Scan Menu by choosing 4.

15). Activate the TitleGen program by double clicking the program icon on the Macintosh computer and start recording of the video recorder.

16). Select the row you want to start. Normally, this is row 1, but in case of an error during scanning, scanning can be continued at an other row. Start the cell tracking with MicroScan by typing *<enter>*.

17). The video monitor shows the first frame image with in a sequential fashion the Name, Plant Number/Name and the Day of recording. Thereafter cell tracking will start and frame images with the corresponding position numbers will be displayed. Progress of the scan is also indicated by two bars on the monitor of the cell tracking computer.

18). If all programmed frames are scanned by MicroScan, 'Ready!' is shown on the monitor of the cell tracking computer. Stop the recording of the video and remove the culture dish. Press enter to go back to the Matrix Scan Menu. Cell tracking for subsequent dishes can be initiated by choosing *1*, Dish with new profile.

Notes

1. Full calibration is only required when the MicroScan program is initiated or after an error or power failure.
2. Recording has to take place using normal playing speed of the video recorder. Some recorders are equipped with long play options that allow recording twice the data when compared to normal speed. However, when paused long play recordings give low image quality and cause problems with mounting the tapes (see section 4.7).

4.3 Matrix video cell tracking on subsequent days

1). MicroScan is started by typing *micscan* and the main menu will appear.

2). Select the full calibration option by choosing *1*. Select the Matrix menu after calibration by choosing *5*.
3). Choose *2*, Dish with old profile, in the Matrix Scan Module.
4). To open an exisiting profile enter the profile name without extention. The cross table will move to the lower marker point.
5). Turn the culture dish by hand until the letter coordinates of the mark-point are near the centre of the photo mask.
6). Use the joystick to exactly position the Lower mark-point in the centre of the photo mask and register the cross tables XY coordinates by MicroScan by typing *<enter>*.
7). The cross table moves to near the Upper mark-point. Use the joystick to exactly position the Upper mark-point in the centre of the photo mask and register the cross tables XY coordinates by MicroScan by typing *<enter>*. The module measures the distance between the two mark-points and will compare it with the distance at the time of initiation. The difference is expressed as the measurement error and expressed as a percentage. If the error is above 1 % the calibration procedure should be repeated.
8). The cross table moves to the focusing point.
9). With the Z-direction of the joystick the lowest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*.
10). With the Z-direction of the joystick the highest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*. During focussing the distance to the lowest focus plane is displayed as focus depth.
11). Activate the TitleGen program by double clicking the program icon on the Macintosh computer.
12). Start the cell tracking with MicroScan by typing *<enter>*.
13). The video monitor first shows the Name, Plate Number/Name and Day. Thereafter cell tracking starts and the frame images with the corresponding position numbers are displayed. Progress of the scan is also indicated by two bars on the monitor of the cell tracking computer.
14). If all programmed frames are scanned by MicroScan, 'Ready!' is shown on the monitor of the cell tracking computer. Stop the recording of the video and remove the culture dish. Press enter to go to the Matrix Scan Menu for scanning subsequent dishes.

4.4 Initiation of a point scanning profile

1). Start the MicroScan program by typing *micscan*. The Main menu will appear. To calibrate the cross table select full calibration by choosing *1*. A full calibration is only required when the MicroScan program is initiated.

2). After calibration the main meu will apear again. Choose Points menu by choosing *5*. The Point Scan menu will apear.

3). Select Dish with NEW Profile by choosing *1*. When another dish was scanned before the current dish the alignment of the previous dish will be lost. Confirm this by typing *y*.

4). Enter the new profile-name to make a new point profile.

5). Type your Name and the Plate Number/Name. The current date will be displayed. This date will be used to calculate the number of days the experiment is in progress. At the first day it will display 'day 1'. Via the TitleGen program these data will be shown to the video monitor before scanning of the frame images byMicroScan, facilitating identification of the tapes.

6). Type the number of Z-steps to scan (between 1 and 10).

7). Give the number of milliseconds to hold at each Z-step (minimum value 100 ms). For normal video cell tracking at least 350 ms are required.

8). Use the joystick to move the cross table so that the Lower mark-grid is visible through the eye piece. Pull the microscope photo mask into the view field and move the cross table so that one of the letter coordinates is in the centre of the photo mask. (figure 6.2b). This letter combination is defined as the Lower mark-point.

9). Write down the letter combination of the Lower mark-point. Type *<enter>* to automatically register the XY coordinates of the cross table for the Lower mark-point by MicorScan.

10). Use the joystick to move the cross table so that the Upper mark-grid is visible in the photo mask. Move the cross table so that one of the letter coordinates is in the centre of the photo mask. (figure 6.2b). This letter combination is defined as the Upper mark-point.

11). Write down the letter combination of the Upper mark-point. Type *<enter>* to automatically register the XY coordinates of the cross table for the Upper mark-point by MicroScan. MicroScan then measures the distance between the two mark-points and displays it on the monitor.

12). Move the cross table with the joystick to a focusing point. Cells visible in this frame have to be present both in a low and in a high planes of focus. XY coordinates of the cross table are registered by MicroScan by typing *<enter>*.

13). With the Z-direction of the joystick the lowest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*.

14). With the Z-direction of the joystick the highest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*. During focussing the distance to the lowest focus plane is displayed as focus depth.

15). The MicroScan program stores the data registered on the computers' hard disk. Press *<enter>* to continue.

16). Select the EDIT point option in the Points Scan menu to add points to the profile, by choosing *4*.

17). The culture dish is graphically represented on the monitor. The blinking cursor represents the present position of the cross table with respect to the objective (figure 6.2a). The cross table can be moved using the joystick. Points representing frame positions can be stored into the profile by typing the *a*, *<space >*or *<enter>* when the cross table is at the desired position. Points can be deleted by typing *d*. With the *r*, *n* or *p* command the table will respectively move to the nearest, next or previous point of the present position. Maximally 1 000 point can be added per profile.

18). Typing *q* or *<esc>* will end the edit sesion. Data are sored on the computers' hard disk in a new file with the extension .pt. Old data will be stored in an additional file. Press *<enter>* to continue.

19). Select the Point Scan option in the Point Scan Menu by choosing *5*.

20). Select the point you want to start.

21). Select automatic (*a*) or manual (*m*) movement. Manual movement will go to the next point by typing <enter>. Automatic will use the time defined in the program during initialisation.

22). Activate the TitleGen program by double clicking the program icon on the Macintosh computer and start recording of the video recorder.

23). Start the video and thereafter the point tracking with MicroScan by typing *<enter>*.

24). First the Name, Plate name and Date information are send to the video recorder. Hereafter the scanning of the programmed frames will start.

25). The video monitor shows the frame images with the corresponding position numbers. Progress of the scan is also indicated by a bar on the monitor of the cell tracking computer.

26). If all programmed frames are scanned by MicroScan, 'Ready!' is shown on the monitor of the cell tracking computer. Stop the recording of the video recorder and remove the culture dish. Press *<enter>* to continue.

4.5 Point scanning on subsequent days

1). Start the MicroScan program by typing *micscan*. The Main menu will appear. To calibrate the cross table select full calibration by choosing *1*. A full calibration is only required when the MicroScan porgram is initiated.

2). After calibration the main meu will apear again. Choose Points menu by choosing *5*. The Point Scan menu will apear.

3). Select Dish with old profile by choosing 2. When another dish was scanned before the current dish the alignment of the previous dish will be lost. Confirm this by typing *y*.

4). Enter the profile-name to open an existing point profile. the cross table will move to the lower mark point.

5). Turn the culture dish by hand until the letter coordinates of the mark point are near the centre of the photo mask.

6). Use the joystick to exactly position the Lower mark-point in the centre of the photo mask and type *<enter>* to register the XY coordinates.

7). The cross table moves to near the Upper mark-point. Use the joystick to exactly position the Upper mark-point in the centre of the photo mask and register the cross tables XY coordinates by MicroScan by typing *<enter>*. The module measures the distance between the two mark-points and will compare it with the distance at the time of initiation. The difference is expressed as the measurement error and expressed as a percentage. If the error is above 1 % the calibration procedure should be repeated.

8). The cross table moves to the focusing point.

9). With the Z-direction of the joystick the lowest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*.

10). With the Z-direction of the joystick the highest plane of focus is selected and the Z-position is registered by MicroScan by typing *<enter>*. During focussing the distance to the lowest focus plane is displayed as focus depth.

11). Activate the TitleGen program by double clicking the program icon on the Macintosh computer.

12). Start the video and thereafter the point tracking with MicroScan by typing *<enter>*.

13). First the Name, Plate name and Date information are send to the video recorder. Hereafter the scanning of the programmed frames will start.

14). The video monitor shows the frame images with the corresponding position numbers. Progress of the scan is also indicated by a bar on the monitor of the cell tracking computer.

15). If all programmed frames are scanned by MicroScan, 'Ready!' is shown on the monitor of the cell tracking computer. Stop the recording of the video recorder and remove the culture dish. Press *<enter>* to continue.

4.6 Analysing recorded data by video recorder

1). The tape recorded at the last day of cell tracking is played and stopped using pause at the frame in which one or more somatic embryos develop.

2). The corresponding frame images at tapes of previous days are retrieved aided by the position numbers included on the frame images.
3). The video recorder playing the primary tapes, is connected to a second video recorder.
4). The tape containing the selected frame image of day 1 is placed in the playing recorder and the image is recorded on a new tape in the second video recorder for about 6 seconds.
5). These steps are repeated for the corresponding frame images from subsequent days.

4.7 Analysing recorded data by computer

1). The tape recorded at the last cell tracking day is played and stopped using pause at frames images in which somatic embryos develop.
2). Each frame image is stored digitally as PICT file using the 'copy' function of the VideoMonitor program.
3). Frames images of previous days are retrieved from tapes of previous days and also stored digitally.
4). PICT files are transferred to Photoshop 3.0 PICT files and sequentially mounted in Pagemaker 6.0.

4.8 Changing MicroScan 6 profile settings

1). Select the objective you want to use.
2). Determine the size of the frame that is visible on the video monitor. (You can use the Cell Finder mark-grids for this purpose. The distance between two lines on these grids is 400 µm).
3). Type edit profiles.dat to start the MS-DOS edit program.
4). Use the mouse or arrow keys to move the cursor to the end of a line with settings.
5). Start a new row by typing *<enter>*
6). Type the number of frames to be scanned in the X-direction (NX) and Y-direction (NY), the size of the frame in X (dX-mm) and Y (dY-mm) in millimetres, the number of planes of focus to be scanned in Z-direction (NZ) and the time to stop at each plane of focus at each frame (t_Z) in milliseconds. Finally you can a enter a description to describe the profile.
7). Leave the edit-mode by *File Exit*.

NX	NY	dX-mm	dY-mm	NZ	t_Z	description
30	33	1.60	1.12	5	350	Nikon MicroScan 4x
30	33	0.62	0.405	5	350	Nikon MicroScan 10x
30	33	0.31	0.23	5	350	Nikon MicroScan 20x
30	33	0.62	0.405	10	120	Nikon SuperScan 10x
30	33	0.26	0.38	4	1100	Nikon Confocal 10x

figure 6.5. Screen of the MicroScan profiles.dat screen with profile settings.

4.9 Changing MicroScan 6 settings in msconfig.dat

1). Type *edit msconfig.dat* to start the MS-DOS edit program.
2). Use the mouse or arrow keys to move the cursor to the settings.
3). The first number represents the default setting. Replace the settings with the new settings. See the VENUS-1 manual (Märzhäuser) for more information about the settings.
4). Leave the edit-mode by *File Exit.*

```
10    =    speed [ mm/sec ]
10    =    max joystick speed [ mm/sec ]
50    =    acceleration [ mm/sec2 ]
2     =    Com-port for Controller
1     =    Com-port for TitleGen      ( 0 = no computer connected )
0     =    operation  :  0 = normal   1 = test
```

Don't change the sequence of the lines above !

figure 6.6. Screen of the MicroScan msconfig.dat screen with standard settings for the MultiControl unit and MicroScan 6 program.

5. Cell tracking combined with immersion immunofluorescence

5.1 Preparation of cultures

1). Prepare a FITC conjugate of the monoclonal antibody of choice.
2.). Prepare phytagel embedded cultures as described in section 2 without liquid medium.

3.) Prepare a filter sterile solutions of 2.5 % calf serum in B5 medium and an 1.25 % calf serum, 10 % FITC conjugated antibody solution in B5 medium.
4). To block non-specific binding sites add 1 ml B5 medium with 2.5 % calf serum with a 5 ml wide-mouthed pipette to the culture dish.
5). Incubate 30 minutes.
6). Remove the B5 medium with a 1 ml pipette.
7). Add 1 ml B5 medium with 1.25 % calf serum and 10 % FITC conjugated antibody with a 5 ml wide-mouthed pipette.
8). Incubate one hour.
9). Remove excess unbound antibody by washing 10 times with basal B5 medium as described in section 3.

5.2 Cell tracking

10). The cell tracking system is coupled to the CSLM as shown in figure 6.1. The image of the fluorescent and visible signal is optimised by adjusting the intensity of the laser and the gain control of the photo multipliers detecting the fluorescent and visual signals. The scan speed setting Fast 1 (*F1*), will require 1.1 second to build up one image. This has to be defined in the MicroScan profiles.dat file.
11). Perform a matrix cell tracking experiment as described in sections *4.2* and *4.3*. Select a general profile designed for confocal cell tracking (see also section *4.9*).
12). Cell tracking at subsequent days can be performed using the CSLM or a video recorder.

Notes.

1. Antibodies can be isolated using Bakerbond 40 µm prescale ABx (7269-02, JT Baker Inc., Phillipsburg, NJ, USA) as described by the manufacturer. FITC conjugated antibodies can be prepared using the QuickTag FITC Conjugation Kit (1248 618, Boehringer Mannheim, Germany) according to the manufacturers protocol.
2. For JIM8 cell tracking 20 mM Ca^{2+} is required for obtaining a JIM8 signal comparable to control labelling in PBS. For this reason the blocking solution used in step 2 contained an additional 40 mM Ca^{2+}. In the other steps 20 mM Ca^{2+} was added to the B5-0.2 medium. After the cell tracking excess Ca^{2+} was removed by 10 washing steps with basal B5-0.2 medium as described in section 3.

3. A number of methods is available to obtain the image of fluorescent signals. For specific applications we refer to the CSLM manual.

6. Cell tracking combined with bioluminescence

6.1 Preparation of cultures

1). Prepare phytagel embedded cultures of cells containing promotor::luciferase constructs as described in section 3 without liquid medium.
2). Prepare a filter sterile solution of 50 µM D-luciferin (Beetle luciferin E1603, Promega, Madison, Wi, USA) in B5 medium.
3). Add 1 ml B5 medium with 50 µM D-luciferin with a 5 ml wide-mouthed pipette to the culture dish.
4). Incubate 30 minutes to allow diffusion of the D-luciferin. The final D-luciferin concentration in the culture will be approximately 20 µM.

6.2 Cell tracking

5). The cell tracking system is coupled to the 2D-luminometer as described in figure 6.1
6). Initiate a point scan with the as described in section 4.4. Select cells or cell cluster and program their position in the as described in section 4.4.
7). Perform a point-scan with visible light as described in section 4.4. Select the manual *(m)* option. To obtain a visible light image the ARGUS 50 system takes the average of several visible light images. Select the 'rolling average' option of the ARGUS software and save the obtained image to disk as a TIFF file.
8). Go to the next frame position by typing *<enter>* on the cell tracking computer.
9). Repeat step 7 and 8 until all programmed frame positions are recorded.
10). Perform a point-scan with the ARGUS 'photon counting' option. The images obtained from the photon signals are digitally accumulated to generate an images of the bioluminescent signal. The time required to obtain one image depends on the promoter used, the number of positive cells, the microscope optics, ect. Therefore this time has to be determined empirically in a pilot experiment. Save the image to disk as a TIFF file.
11). Go to the next frame position by typing *<enter>* on the cell tracking computer.

12). Repeat step 10 and 11 until all programmed frame positions are recorded.
13). Cell tracking at subsequent days can be performed using the 2D-luminometer or normal video recording.

Notes.

1. Several types of luciferase proteins are available. The bacterial proteins use aldehydes as their substrate. In our opinion this protein is not suited for cell tracking due to the toxic characteristics of its substrate. In our experiments we have used the firefly luciferase coding sequence (De Wet et al., 1987). The substrate of the encoded protein is D-luciferin. At concentrations of 50 µM or higher D-luciferin inhibits embryo development in carrot (Chapter 5). D-luciferin concentrations above 400 µM are toxic to tobacco cells (Ow et al., 1986).
2. The time required to build up one image depends on a large number of factors. In our system it takes about half an hour to obtain a significant signal of CaMV 35S driven luciferase expression of a single cell. Significant signals of large cell clusters and somatic embryos can be obtained in 5 minutes.
3. The number of photons released is dependent on gene expression but also on ATP and O_2 concentration. Culturing cells in liquid medium or embedded in phytagel alters oxygen tension and can therefore influence activity of the luciferase enzym.
4. A number of methods is available to obtain images of bioluminescent signals. For specific application we refer to the luminometer manual.

6.3 Analysing recorded data

1). The server containing the stored files can be accessed via the local ethernet computer network.
2). Images are opened as TIFF files in Photoshop 3.0 on the Macintosh PowerPC.
3). Image contrast, brightness, and colour use can be adjusted.
4). Files can be stored as PICT files.
5). Sequential compositions of PICT files can be made in Pagemaker 6.0.

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7

General discussion

The sporophytic phase of plant development starts with the fertilisation of the egg cell by one of the sperm cells. In dicotelydons the resulting zygote develops via the globular, heart and torpedo stage into a mature embryo. The embryo enters into a dormant state during desiccation of the seed. After germination the embryo develops into a seedling and subsequently into a mature plant. The molecular-genetic analysis of zygotic embryo development is an intensively studied area of research (Jürgens, 1995) and is now yielding insight into the molecular details of for instance the establishment and maintenance of the shoot apical meristem (Long, 1996). As reviewed in more detail in chapter 1, several forms of spontanous in vivo embryogenesis or apomixis have been described that originate from cells of the female gametophyte without prior fusion of gametes (Koltunow, 1995). *In vitro*, embryos can develop from microspores or even from somatic cells in tissue culture. In all of these non-zygotic forms of embryogenesis, single cells are believed to undergo a transition or fate change that renders the cells embryogenic. An important question is whether the molecular mechanisms that lead to such a change in fate are similar in all cases and whether they are comparable with the formation of the zygote.

The subject of the work presented in this thesis is to identify the events that take place during the transition between the somatic and the embryogenic cell state of *in vitro* grown carrot cells. This was initiated by the observations of Komamine and co-workers (Nomura and Komamine, 1985, Komamine et al., 1990) and Pennell et al. (1992). Nomura and Komamine (1985) and Komamine et al. (1990) showed that carrot suspension cultures contained so-called type 1 single cells, that were able to produce somatic embryos at a very high frequency of approximately 90 %. Embryogenic development of type 1 cells required 2,4-D and was suggested to proceed via an asymmetrical first division, reminiscent of the first division of the zygote in many plant species (Komamine et al., 1990). Due to exposure to 2,4-D these cells start to divide to form a cell cluster. Cells in this stage of development are initiated to become embryogenic and are described as competent. After a few divisions the requirement for exogenous 2,4-D is diminished and somatic embryogenesis proceeds in the absence of exogenous growth regulators. At this stage of development the cell cluster is defined as embryogenic (De Jong et al., 1993b).

Pennell et al. (1992) showed that the epitope recognised by the monoclonal antibody JIM8 was present on the surface of cells morphologically resembling type 1 cells. In plants, the JIM8 epitope appeared in several cell types of the reproductive tissue, including the early globular embryo. These observations suggested that the JIM8 epitope marks a transitional stage in the formation of embryogenic cells (Pennell et al., 1992). Since, as shown by the work of Backs-Hüsemann and

Reinert (1970), type 1 cells are not unique in their embryogenic capacity the observations of Nomura and Komamine (1985) had to be validated. For this, the cell tracking system described in this thesis (Chapter 2 and 6), has been developed in collaboration with Dr. Harrie Verhoeven of the CPRO-DLO in Wageningen. It was possible to identify and describe single cells with the proven capability to develop into somatic embryos. Analysing the development of large populations of single cells smaller than 22 µm showed that a small percentage of all cell types could develop into embryos. This development is characterised by a great plasticity in the division patterns. Cell tracking experiments on cell clusters showed that morphologically similar clusters consisting of cytoplasm-rich cells that are routinely described as 'embryogenic' or 'pro-embryogenic mass' can have different developmental fates (Chapter 4 and 5) which indicates that it will be difficult to predict embryogenic capacity beforehand.

In order to answer the question whether the JIM8 antibody could be used as a marker to more precisely identify cells capable of embryo formation, the cell tracking system was adapted to record fluorescent images of JIM8-FITC labelled cells (Chapter 3 and 6). It was shown that the majority of single cells that react with the JIM8 antibody do not develop into somatic embryos and that most of the somatic embryos derived from JIM8 negative cells. While JIM8 labelled cells are only found in embryogenic suspension cultures and the number of labelled cells is variable and dependent on the culture conditions, it is clear that the process visualised by the JIM8 antibody may be necessary for embryogenic competence but that this process is not specific for those cells actually developing into somatic embryos.

Numerous studies have shown that secreted compounds such as extracellular proteins can stimulate the formation of embryogenic cells in e.g. newly started carrot suspension cultures. For low-embryogenic carrot suspension cultures grown at low cell densities it has been shown that removal of most of the vacuolated cells resulted in a decreased frequency of somatic embryo development, which could be restored by addition of carrot seed AGPs (Kreuger and Van Holst, 1993, Chapter 4). The role of AGPs in embryo development is still not clear. AGPs may increase the number of competent cells through prevention of cell enlargement (Willats and Knox, 1996) but can also influence cell division rates (Serpe and Nothnagel, 1994). Alternatively, AGPs may interact with other cell types like 'nursing' or 'feeder' cells and thereby indirectly improving conditions for somatic embryo development. The role of such 'nursing' populations has been suggested based upon expression patterns of the JIM8 cell wall epitope (Chapter 3) and the AGP experiments described in chapter 4. A similar role has been proposed for the carrot endochitinase EP3. This protein is able to rescue the *ts11* embryo mutant

(De Jong et al., 1992) but is not produced by embryogenic cells but by non-embryogenic suspension cells (Van Hengel et al., submitted). In developing seeds the EP3 protein is produced in the endosperm. These results hint at a further level of complexity where particular cell types may produce compounds that influence other cells. This can either be by stimulation of embryogenic development or by formation of competent cells but might also include an inhibitory effect on embryo development such as shown for the JIM8AGPs. One of consequences of this scenario of complex interaction between several cell types is that a population that only consists of competent or embryogenic cells may be theoretically impossible to archive, due to the lack of 'nursing' compounds.

Since it is difficult to predict embryo forming cells and cell clusters based upon morphology, cell destructive staining methods do not allow to correlate gene expression to somatic embryo development. The only way to correlate the expression of genes to competent cells the expression pattern of the gene has to be monitored during in vivo development. The combination of cell tracking with a 2D luminometer allowed detection of firefly luciferase expression during embryo development in transformed *Daucus* cells. This was initially shown by the *Arabidopsis thaliana* lipid transfer protein 1 (*AtLTP1*) gene which is homologous to the carrot *LTP* gene *EP2*, specifically expressed in the protoderm of developing carrot embryos. A*tLTP1* luciferase expression patterns represented expression of the endogenous carrot *EP2* gene. Cell tracking experiments showed that all somatic embryos developed from *AtLTP1* luciferase expressing cell clusters. These results indicate that *EP2* expression is directly correlated to somatic embryogenesis. In contrast to the JIM8 monoclonal antibody, *AtLTP1* is a good marker for embryogenic cells in embryogenic suspension cultures. However, since not all cell clusters that express *AtLTP1* luciferase develop into somatic embryos this construct also gives a low percentage of false positives.

In parallel to the research described in this thesis dr. Ed Schmidt developed a method to isolate genes expressed in single competent cells by several screening approaches. With the use of cold plaque screening a cDNA was isolated with homology to plant and animal receptor kinases. Expression patterns of the SERK gene coincided very well with the presence of single competent cells in both activated explants and in established suspension cultures. During somatic and zygotic embryogenesis the SERK gene was transiently expressed up to the globular embryo stage and was not detectable anymore shortly thereafter and during the entire further life cycle of the plant. Definite proof that the SERK gene indeed marks single competent cells was obtained by following SERK promoter-luciferase expression in single cells in combination with cell tracking (Schmidt et al., 1997). This gene therefore appears to be the first one that fulfils most criteria to be a

specific marker for competent and embryogenic cells. The fact that it encodes a transmembrane receptor kinase with extracellular leucine-rich repeats may indicate that cell-cell interactions also play a role during induction of embryo development. It will now be a further challenge to unravel the signals involved in the formation of embryogenic cells.

In conclusion, the experiments described in this thesis demonstrate that it is essential to follow development of individual cells in order to study embryogenic cell formation. Only in this way it is possible to precisely correlate molecular and cellular events that occur in cells that acquire embryogenic competence. It is likely that cell-cell interactions are crucial to embryogenic cell formation.

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Samenvatting

De ontwikkeling van een plant begint in de bloem met de bevruchting van een eicel. De bevruchte eicel of zygote deelt en ontwikkeld zich via een globulair- en hartvormig stadium naar een torpedo-vormig embryo. Na het kiemen van het zaad groeit het embryo uit tot een plant. De moleculair-genetische analyse van de ontwikkeling van het zygotische embryo is een intensief bestudeerd terrein van onderzoek en heeft inzicht verschaft in bijvoorbeeld de wijze waarop het apicale scheut meristeem wordt aangelegd. Embryogenese is bij veel planten ook langs niet geslachtelijke weg, buiten de plant mogelijk. Hiertoe wordt een stukje weefsel van de plant gesneden en in een kweekmedium gebracht met een hoge concentratie van de groeiregulator auxine. Cellen uit het weefsel gaan vervolgens delen en komen vrij in het kweekmedium. Na enige tijd ontstaat een suspensie met daarin enkele cellen en verschillende soorten celclusters. In de aanwezigheid van auxine kunnen enkele cellen geïnduceerd worden tot het vormen van embryogene cel clusters. Na verdunning in auxine vrij medium kunnen deze celclusters verder ontwikkelen tot embryo's. Deze vorming van embryo's langs niet geslachtelijke weg wordt somatische embryogenese genoemd. Dit proces verloopt efficiënt in *Daucus carota* (Peen) waardoor deze plant geschikt is om het proces van somatische embryogenese te bestuderen.

In celsuspensies van *Daucus* komen kleine cellen voor welke rijk zijn aan cytoplasma maar ook cellen van verschillende afmetingen die grote vacuoles en relatief weinig cytoplasma bevatten. Een aantal van deze cellen bevind zich in celclusters, maar er zijn ook een groot aantal enkele cellen aanwezig. Een klein percentage van deze enkele cellen kan zich in aanwezigheid van auxine ontwikkelen tot een embryogene celcluster. Na verdunning in auxine vrij medium ontwikkelen deze clusters zich tot globulair- hart- en torpedo vormige somatische embryo's. Het moment waarop een cel geinitialiseerd wordt om een somatische embryo te vormen is waarschijnlijk tijdens het enkele cel stadium. Dit type cellen is dan ook bij uitstek geschikt voor onderzoek naar de moleculaire processen die leiden tot de vorming van een embryo. Dit wordt echter bemoeilijkt doordat maar een klein percentage van de enkele cellen competent is om zich te ontwikkelen tot een somatische embryo en het niet duidelijk is welk type enkele cellen dat is. In hoofdstuk 2 wordt het gebruik van een cel volg systeem beschreven om de ontwikkeling van enkele cellen in de tijd te volgen. Met behulp van nylon zeven werden individuele enkele cellen geïsoleerd uit een celsuspensie en vastgelegd in een gel in petrischalen. Met behulp van een video camera en recorder werden elke dag opnamen gemaakt van een bepaald gebied van de petrischaal. De cel-volg apparatuur maakte het mogelijk dat iedere dag exact het

zelfde gebied van het schaaltje opgenomen werd. Door de opnamen van verschillende dagen achter elkaar te plaatsen kon de ontwikkeling van vele enkele cellen gevolgd worden. Na analyse van de resultaten bleek dat zowel cytoplasma rijke als gevacuoliseerde cellen zich tot een embryo ontwikkelden. Het bleek niet mogelijk aan de hand van de vorm (morfologie) van de cel te voorspellen of de cel zich tot een embryo kan ontwikkelen. Wel bleek er een correlatie te bestaan tussen de morfologie van de enkele cellen en de vorm van de embryogene celcluster die ontstaat uit de enkele cellen. Ronde cellen ontwikkelden zich tot symmetrische celclusters terwijl ovale en langgerekte cellen asymmetrische celclusters vormden. De vorm van de laatste groep leek sterk op de vorm van zygotische embryo's. Sommige celclusters hadden een sterk afwijkende vorm maar waren toch in staat zich tot een normaal embryo te ontwikkelen. Omdat embryo-vormende enkele cellen niet geïdentificeerde konden worden op grond van morfologie werd naar andere mogelijkheden gezocht om deze cellen te identificeren.

Eén mogelijkheid om embryo-vormende cellen te identificeren is het gebruik van moleculaire markers. Dit kunnen bijvoorbeeld eiwitten zijn welke gevormd worden tijdens een specifiek stadium van de embryo ontwikkeling. Het monoclonale antilichaam JIM8 reageert met een arabinogalactan eiwit (AGP) in vroege zygotische embryo's van koolzaad planten. Daarnaast reageert JIM8 met een AGP in celwanden van enkele cellen in embryogene *Daucus* suspensie cultures. Dit AGP komt niet voor in niet-embryogene suspensie culturen. Om na te gaan of JIM8 gebruikt kan worden voor het identificeren van embryo-vormende cellen werd het cel-volg systeem aangepast. Door aan het JIM8 antilichaam een fluorescerende groep te koppelen lichten cellen op als JIM8 bindt aan het AGP. Met behulp van een confocale laser scanning microscoop kan de fluorescentie waargenomen worden. In hoofdstuk 3 wordt de ontwikkeling beschreven van enkele cellen in JIM8 behandelde culturen. Het merendeel van de embryo-vormende enkele cellen reageerde echter niet met JIM8, terwijl een groot deel van de cellen welke met JIM8 reageerden geen embryo vormden. Het JIM8 antilichaam kan dus niet gebruikt worden als marker voor competente enkele cellen maar geeft wel het embryogene vermogen weer van een cultuur als geheel.

Eerder onderzoek heeft aangetoond dat cellen in de suspensie culturen eiwitten uitscheiden in het kweekmedium en dat deze eiwitten de embryogenese kunnen beïnvloeden. Met name een gedeelte van de AGP populatie die geïsoleerd kan worden met behulp van het monoclonale antilichaam ZUM18 zou het aantal somatische embryo's dat geproduceerd wordt in een cultuur verdubbelen. In hoofdstuk 4 worden de effecten van verschillende AGP fracties op somatische embryogenese beschreven. In *Daucus* zaden komen grote hoeveelheden AGPs voor. Na extractie van de zaden worden de AGPs met behulp van het Yariv reagens

geïsoleerd, waarna door middel van de moleculaire antilichamen ZUM18 en JIM8 hieruit respectievelijk ZUM18AGP en JIM8AGP populaties geïsoleerd kunnen worden. Toevoegen van ZUM18AGPs aan enkele cellen bleek echter geen effect the hebben op het aantal embryo's dat gevormd werd. Ook het toevoegen van ZUM18AGPs aan populaties van celclusters had echter ook geen effect op de frequentie van somatische embryogenese. Het toevoegen van JIM8AGPs aan enkele cellen heeft geleid tot een afname van het aantal somatische embryo's dat gevormd werd uit enkele cellen. Dit lijkt in tegenspraak met de positieve correlatie die gevonden is tussen de JIM8 celwand epitoop en het embryogene vermogen in een cultuur. Het is mogelijk dat er meerdere typen JIM8AGPs bestaan en dat deze een verschillende functie hebben tijdens de ontwikkeling van somatische embryo's. Eén van die functies zou een soort signalerings-functie kunnen zijn. Naast cellen die in staat zijn zich te ontwikkelen tot somatische embryo's kunnen er andere cellen die factoren produceren die nodig zijn voor het ontwikkelende somatische embryo. Daarnaast produceren andere cellen mogelijk factoren die somatische embryogenese juist remmen. Het is zeer waarschijnlijk dat AGPs bij positieve als negatieve effecten op de embryogenese betrokken zijn. Of soortgelijke interacties ook betrokken zijn bij andere vormen van embryogenese, waaronder zygotische embryogenese is een nog niet beantwoorde vraag.

Parallel aan het hier beschreven onderzoek is door dr. Ed Schmidt gewerkt aan methoden om moleculaire markers te isoleren welke specifiek zijn voor de vroege stadia van somatische embryogenese. Het mogelijk om gericht genen te isoleren welke specifiek tot expressie komen in enkele cellen en kleine celclusters in embryogene celsuspensies. Van één van deze genen, Somatische Embryogene Receptor Kinase (SERK) genoemd is vastgesteld dat het tot expressie komt in enkele cellen en in celclusters, en ook in vroege zygotische embryo's. Om na te gaan of de cellen die het SERK gen tot expressie brengen ook inderdaad de cellen zijn die zich tot somatische embryo's ontwikkelen, is de expressie van het SERK gen gevolgd met behulp van een markersysteem gebaseerd op het luciferase eiwit dat wordt beschreven in hoofdstuk 5.

Vuurvliegjes maken een eiwit dat fotonen produceert die waargenomen worden als geel-groen licht. Het gen dat codeert voor dit eiwit; luciferase, is geïsoleerd uit vuurvliegjes en is door een aantal onderzoeks-groepen gebruikt voor onderzoek naar gen expressie in planten. In hoofdstuk 5 wordt de expressie van het gen dat codeert voor het lipide transport eiwit (LTP) beschreven. Expressie patronen van het *LTP* gen zijn goed gedocumenteerd. Tijdens somatische embryogenese in *Daucus carota* komt het *LTP* gen tot expressie tijdens de ontwikkeling van het protoderm. Door de promotor van het *LTP* gen uit *Arabidopsis thaliana* te plaatsen voor het luciferase gen, zullen getransformeerde *Daucus* cellen

welke *AtLTP* tot expressie brengen zullen ook luciferase gaan produceren en dus licht uitzenden. Dit licht kan waargenomen worden met een luminometer welke bestaat uit een video camera met een lichtversterker. De expressie patronen van het luciferase gen onder invloed van de *AtLTP* promotor kwamen overeen met de patronen zoals beschreven voor het endogene *LTP* gen. De ontwikkeling van getransformeerde celclusters kon gevolgd worden met het cel-volg systeem en het bleek ook mogelijk gen expressie te correleren aan de morfologische ontwikkeling van het embryo. Het merendeel van de clusters die het *AtLTP* gen tot expressie brachten ontwikkelden zich tot somatische embryo's. Deze experimenten lieten zien dat expressie van een gen gevolgd kan worden tijdens somatische embryogenese. Een soortgelijk experiment, met de SERK promotor liet zien dat het SERK gen inderdaad tot expressie komt in enkele cellen die embryo's produceren (Schmidt et al., 1997).

De in dit proefschrift beschreven experimenten laten zien dat het voor de bestudering van vroege stadia van somatische embryogenese essentieel is de ontwikkeling van individuele cellen tot embryo te volgen. Alleen op deze manier kunnen moleculaire processen die plaats vinden tijdens de initiatie en de eerste fase van somatische embryo ontwikkeling bestudeerd worden. Het is duidelijk dat hierbij speelt de interactie met andere cellen in de celsuspensie een belangrijke rol. Het is zeer waarschijnlijk dat extracellulaire eiwitten en AGPs hierbij betrokken zijn.

Nawoord

Aan het einde van dit proefschrift is een woord van dank aan alle mensen die de afgelopen vijf jaren een bijdrage hebben geleverd aan het hier beschreven onderzoek, op zijn plaats. In de eerste plaats Sacco de Vries die mij de mogelijkheid geboden heeft dit promotie onderzoek uit te voeren. Onze gesprekken hebben mij geleerd hoe je je onderzoek opzet en uitvoerd. Onder jou leiding heb ik geleerd hoe je een wetenschappelijk publicatie schrijft die zeer precies weergeeft wat de resultaten zijn en hoe die geinterpreteerd dienen te worden. Daarnaast natuurlijk mijn promotor; Ab van Kammen. Altijd op de achtergrond aanwezig met een kritisch oog voor het werk.

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Ook dank aan de mensen die meegewerkt hebben aan het opzetten van het cell tracking systeem. In de eerste plaats Harrie Verhoeven. Jou manipulaties met microscopen, computers en videorecorders hebben de basis gelegd voor het cell tracking systeem zoals dat hier beschreven is. Ook de hulp van Jan Blaas was onontbeerlijk als er eens iets niet werkte. Sander van der Krol voor je interesse in ons werk en de hulp bij het ontwikkelen en uitvoeren van de luminometer proeven.

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Curriculum vitae

Marcel Toonen werd geboren op 22 augustus 1967 te Wijchen. Nadat hij in 1984 het HAVO diploma behaalde werd begonnen met de chemische laboratoriumopleiding aan de Hogeschool Interstudie te Oss. Het stage- en afstudeervak werd uitgevoerd bij de afdeling Chemische Cytologie van de Katholieke Universiteit Nijmegen onder begeleiding van dr. A. Theuvenet. Na het behalen van het getuigschrift op 24 juni 1988 werd de studie voortgezet aan de Landbouw Universiteit Wageningen. Het afstudeervak Moleculaire Biologie (dr. S. de Vries en prof. A. van Kammen) was de eerste kennismaking met het veld van de somatische embryogenese. Het moleculair-biologisch stagevak werd uitgevoerd bij Mogen N.V. te Leiden onder begeleiding van dr. L. Melchers. Op 23 maart 1992 werd het doctoraalgetuigschrift van de studierichting Moleculaire Wetenschappen behaald. Op 1 december 1991 werd begonnen met het hier beschreven promotie onderzoek naar de moleculaire basis van embryogenese bij hogere planten in dienst van de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO). Sinds 1 december 1996 is hij als post-doc werkzaam aan de Lehrstuhl für Allgemeine Genetik van de Eberhard-Karls Universität in Tübingen.

List of publications

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