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Transition of somatic plant cells to an embryogenic state

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Abstract

Under appropriate *in vivo* or *in vitro* conditions, certain somatic plant cells have the capability to initiate embryogenic development (somatic embryogenesis). Somatic embryogenesis provides an unique experimental model to understand the molecular and cellular bases of developmental plasticity in plants. In the last few years, the application of modern experimental techniques, as well as the characterization of *Arabidopsis* embryogenesis mutants, have resulted in the accumulation of novel data about the acquisition of embryogenic capabilities by somatic plant cells. In this review, we summarize relevant experimental observations that can contribute to the description and definition of a transitional state of somatic cells induced to form totipotent, embryogenic cells. During this somatic-to-embryogenic transition, cells have to dedifferentiate, activate their cell division cycle and reorganize their physiology, metabolism and gene expression patterns. The roles of stress, endogenous growth regulators and chromatin remodelling in the coordinated reorganization of the cellular state are especially emphasized.

Introduction

The life strategies of multicellular plant and animal species are strikingly different. While active movement is characteristic of animal organisms, plants are sessile. This basic difference is reflected in the evolution of different approaches to short-term environmental adaptation by individuals. Animals respond to environmental signals or conditions by changes in their behaviour, while plants accommodate environmental effects by altering metabolism and/or development. In the absence of the possibility for developmental cell migration and due to the need for continuous organogenesis during their postembryogenic development, plants maintain organ-forming cell files, the meristems. The formation and activity of meristems are highly dependent on environmental, as well as developmental (hormonal), factors. Thus, the ontogenic program of plants is highly flexible and this is linked to the reversibility of the differentiation state of somatic plant cells. Under extreme conditions, these cells have to change their fate: either they have to die ('apoptosis') or dedifferentiate and divide, depending on the needs of the organism. In general, it can be stated that the developmental program of plants is much more open to alternative pathways compared to that of animals and this is manifested at the level of cellular differentiation (for reviews, Walbot, 1996; Sultan, 2000).

One of the most extreme examples of flexibility in plant development is the capability of several cell types, in addition to the zygote, to initiate embryogenic development. In flowering plants, sexual reproduction involves double fertilization that generates the embryo and the endosperm simultaneously. Meiosis precedes the formation of gametes and fertilization restores the somatic chromosome number. However, in many vascular plants, sexual reproduc-

tion is regularly combined with or replaced by different kinds of asexual reproduction (reviewed, e.g., by Sharma and Thorpe, 1995). In addition to vivipary or vegetative reproduction, in seed plants, embryos and seeds can be generated without fertilization through various pathways, which are collectively referred to as apomixis (for reviews, Koltunow, 1993; Koltunow et al., 1995). During gametophytic apomixis, the female gametophyte is formed from an unreduced embryo-sac initial (diplospory) or from somatic cells in the nucellus or chalaza (apospory). In the latter case, which is also called adventitious embryony, one or more embryos are formed directly from the somatic cells in the ovule as nucellar or integumental outgrowths. In most cases, pollination and fertilization are prerequisites for the initiation of adventitious embryos, although in some situations pollination is sufficient. Adventitious and zygotic embryos in the embryo sac compete with each other until one (or a few) of them dominates and gives rise to the mature embryo(s). How and why somatic cells of the ovule change their developmental fate and gain embryogenic potency is not known. In adventitious embryony, although embryogenesis starts from somatic (in most cases, nucellar) cells, and thus falls into the category of somatic embryogenesis, the developing embryos occupy the embryo sac, use the endosperm and 'normal' seeds are the end product. In other examples, as is the case of *Bryophyllum* (Yarbrough, 1932) and *Malaxis* (Taylor, 1967) species, somatic embryos can arise as vegetative propagules from cells at leaf margins.

In addition to these natural, *in vivo*, forms of embryogenesis, there exist at least three ways to induce embryo development from *in vitro* cultured plant cells (for a comprehensive review see Mordhorst et al., 1997): *in vitro* fertilization (for review, Kranz, 1999), from microspores (for review, Reynolds, 1997) and *in vitro* somatic embryogenesis (for review, Dudits et al., 1995). *In vitro* embryogenesis, in addition to its usefulness for cloning and vegetative propagation of a given plant individual, can serve as a model system to study the molecular, cytological, physiological and developmental events underlying plant embryogenesis (Dodeman et al., 1997). The main advantage of these *in vitro* experimental systems is that embryogenic cells are accessible for manipulation by most cellular and molecular techniques, in contrast to gametic cells and zygotes, which develop embedded in maternal tissues.

In vitro zygote development, initiated by the artificial fusion of isolated gametes, probably follows the pathway of *in vivo* events most closely (Kranz, 1999). Despite some technical limitations, this experimental approach has provided basic, novel information about early molecular events following fertilization (Kranz, 1999; Faure and Dumas, 2001). A mass of cells with acquired embryogenic competence can be studied in specific cultures of microspores or somatic cells (Dudits et al., 1991; Reynolds, 1997; Osuga et al., 1999). There is debate, however, as to the suitability of these embryogenic systems as models for zygotic embryogenesis (Zimmerman, 1993).

Events of zygotic embryogenesis, mainly based on studies in *Arabidopsis*, can be subdivided into different stages (Jurgens et al., 1991; Jurgens, 1992; Mayer and Jurgens, 1998) such as the first asymmetric division, and formation of the globular, oblong, heart, torpedo, cotyledonary and finally the mature dehydrated embryo. Although somatic (and microspore) embryogenesis may serve as a model for most of these stages, there are several obvious differences:

- the lack of endosperm differentiation,
- the missing or retarded suspensor development,
- the lack of embryo desiccation and dormancy during somatic embryogenesis, and
- the acquisition phase of embryogenic competence by a somatic cell prior to the initiation of embryo development (for review, Dodeman et al., 1997).

While the zygote, formed as a consequence of egg cell fertilization, is clearly determined to follow the embryogenic cell fate, in other forms of plant embryogenesis, including apomixis and somatic embryogenesis, there is a transition phase during which competent and embryogenic cell types are formed. This transition phase is very difficult to define, but an understanding of the underlying mechanisms can provide insight into the developmental strategy of plants.

In this review, we aim to summarize selected, recent information on the induction phase of somatic embryogenesis up to the formation of globular embryos. Early cellular and molecular events of somatic embryogenesis will be described in relation to characteristic, although overlapping, phases of dedifferentiation, cell division, acquisition of competence, induction and determination of the embryogenic cell fate.

Induction of somatic embryogenesis

Stress: the force to switch

As shown by several experimental observations, the

differentiated fate of plant cells, dependent on positional information and developmental signals, can be easily altered under *in vitro* conditions. Drastic changes in the cellular environment, such as exposing wounded cells or tissues to sub-optimal nutrient or hormone supply (e.g., *in vitro* culture conditions), generate significant stress effects. The response to stress conditions depends on two main parameters: the level of stress and the physiological state of the cells. If the stress level exceeds cellular tolerance, the cells die. In contrast, lower levels of stress enhance metabolism and induce adaptation mechanisms (Lichtenthaler, 1998). Adaptations include the reprogramming of gene expression, as well as changes in the physiology and metabolism of the cells. Stress alters source/sink regulation by switching on genes of sink-specific enzymes in parallel with stress defence genes (Roitsch, 1999). This transient cell state induced by stress conditions can be characterized by extensive cellular reorganization and allows for a developmental switch, if appropriate signals are perceived.

In vitro tissue culture conditions expose the explants to significant stresses, as they are removed from their original tissue environment and placed on synthetic media containing non-physiological concentrations of growth regulators, salts and organic components. Wounding itself is a significant signal in the induction of dedifferentiation: most of the genes expressed in freshly isolated leaf protoplasts, not only those involved in the stress response, are already induced as a result of wounding (Grosset et al., 1990). In alfalfa leaf protoplasts, oxidative stress-inducing compounds increased the endogenous auxin level of the cells and promoted dedifferentiation, as indicated by faster acidification of the medium and earlier cell division at a smaller size (Pasternak et al., 2002). Dedifferentiation, in many cases, can be clearly correlated with stress and/or auxin responses of cells.

Stresses not only promote dedifferentiation, but also can be used to induce somatic embryo formation. For example, embryogenic cells could be formed from alfalfa leaf protoplasts in response to different oxidative stress-inducing compounds in the presence of auxin and cytokinin (Fehér et al., 2001, 2002; Pasternak et al., 2002). Mitogen-activated protein kinase (MAPK) phosphorylation cascades may link oxidative stress responses to auxin signalling and cell cycle regulation (as reviewed by Hirt, 2000). For example, the tobacco MAPK Kinase Kinase (MAPKKK), NPK1, was shown to be involved in oxidative stress response, auxin signalling and cell cycle regulation (Hirt, 2000). Wounding, high salt concentration, heavy metal ions or osmotic stress positively influenced somatic embryo induction in diverse plant species (reviewed by Dudits et al., 1995). These procedures were accompanied by increased expression of diverse stress-related genes, evoking the hypothesis that somatic embryogenesis is an adaptation process of *in vitro* cultured plant cells (Dudits et al., 1995).

Growth regulators: outside and inside

Hormones are the most likely candidates in the regulation of developmental switches. Auxins and cytokinins are the main growth regulators in plants involved in the regulation of cell division and differentiation. The influences of exogenously applied auxins, preferentially 2,4-dichlorophenoxyacetic acid (2,4-D), on the induction of somatic embryogenesis are well documented (for reviews, e.g., Dudits et al., 1991; Yeung, 1995). However, embryo development in somatic tissues has been reported in the absence of growth regulators (e.g., Choi et al., 1998), as well as in the presence of other growth regulators such as cytokinin (e.g., Sagare et al., 2000) or abscisic acid (e.g., Nishiwaki et al., 2000) (for reviews, Nomura and Komamine, 1995; Yeung, 1995, in Leguminosae Lakshmanan; Taji, 2000). Non-hormonal inducers can also be used to promote the somatic/embryogenic transition. Such inducers include high sucrose concentration or osmotic stress (Kamada et al., 1993), heavy metal ions (Kiyosue et al., 1990; Pasternak et al., 2002) and high temperature (Kamada et al., 1989). In most of the cases where no growth regulators were included for embryogenic induction, somatic embryos formed directly on the surfaces of explants without recognisable callus formation.

Based on the wide variation of inducer types, somatic embryogenesis cannot be defined as a specific response to one or more exogenously applied growth regulators. Rather, these observations indicate a critical role of stress as an embryogenic stimulus (see also Stress: the force to switch section). Endogenous hormone levels, however, can be considered as major factors in determining the specificity of cellular responses to these rather general stress stimuli. During the last few years, a large body of experimental observations has accumulated on the central roles of endogenous indoleacetic acid (IAA) and abscisic acid (ABA) levels during the early phases of embryogenesis.

In addition to the absolute requirement of exogenous auxins for sustained growth in *in vitro* cultures,

plant cells may produce substantial amounts of the native auxin, IAA. Higher endogenous IAA concentration has been shown to be associated with increased embryogenic response in various species/explants (Rajasekaran et al., 1987; Ivanova et al., 1994; Michalczuk and Druart, 1999; Jimenez and Bangerth, 2001a, b, c). In carrot cells, exogenous 2,4-D stimulated the accumulation of large amounts of endogenous IAA (Michalczuk et al., 1992a, b). These authors hypothesized that embryogenic competence of carrot cells is closely associated with the several-fold increase in endogenous IAA levels due to the presence of 2,4-D. It was suggested that this synthetic compound acts indirectly by disturbing endogenous auxin metabolism and the direct auxin effect of 2,4-D is less significant.

In alfalfa leaf protoplasts cultured in the presence of 2,4-D, the endogenous IAA levels increased considerably during the first 2–3 days of culture (Pasternak et al., 2002). This increase was transient and comparable under both embryogenic and non-embryogenic conditions; however, the timing of the synthesis peak showed approximately a 1-day delay under non-embryogenic conditions. This peak shift was strongly correlated with the fate of the cells that were manipulated by altering either 2,4-D, iron concentration (the level of stress) or medium pH (Pasternak et al., 2002).

A similar peak of endogenous IAA level has been observed in immature zygotic sunflower embryos induced to form somatic embryos (Charriére et al., 1999). In the sunflower system, cells can be induced to form either adventitious shoots or somatic embryos by simply modifying the sucrose content in the culture medium. The tissues grown under embryogenic conditions showed a 4-fold increase in their IAA content as compared to those tissues that followed the caulogenic pathway. The timing of the peak (at approx. 24 h of culture) correlated well with the time of the irreversible determination of the morphogenetic response. Immuno-cytochemical localization of IAA in the immature zygotic embryos before, during and after the induction of somatic embryo development provided direct evidence that an endogenous auxin pulse may be one of the first signals leading to somatic embryogenesis (Thomas et al., 2002).

An auxin surge has been reported to take place after fertilization in carrot zygotic embryos as well (Ribnicky et al., 2001), further emphasizing the significance of temporal changes in endogenous auxin levels in the expression of cellular totipotency. The polar transport of endogenous auxin was found to be an important factor in somatic embryo formation on cotyledon explants of ginseng, which did not require exogenous growth regulator application (Choi et al., 1997). These observations altogether suggest that temporal and spatial changes in endogenous auxin levels are important factors controlling the embryogenic cell fate.

Among the different auxin analogues used to induce somatic embryogenesis, 2,4-D is by far the most efficient and, therefore, this synthetic growth regulator is used in the majority of embryogenic cell and tissue culture systems. It can be suggested that 2,4-D, above a certain concentration, has a dual effect in these cultures, as an auxin (directly or through endogenous IAA metabolism; see above) and as a stressor (Fehér et al., 2001, 2002). 2,4-D is an auxinic herbicide with diverse effects associated with its phytotoxic activity, which cannot be ascribed simply to an auxin overdose. It has been suggested that 2,4-D (or other auxin-type herbicides) affect electrical patterns (Goldsworthy and Mina, 1991), membrane permeability (Schauf et al., 1987), IAA binding to the auxin-binding protein ABP1 (Deshpande and Hall, 2000) and photosynthesis of algae (Matsue et al., 1993; Fargasova, 1994). Recently, auxinic herbicides have been shown to interact with ethylene and ABA synthesis, increasing the cellular levels of these so-called ‘stress’ hormones (Grossmann, 2000; Wei et al., 2000).

In carrot, simple application of abscisic acid to seedlings efficiently induced somatic embryo formation (Nishiwaki et al., 2000). However, this response was dependent on the presence of the shoot apices, the main source of auxin in the seedlings. This finding suggested that, as a stress signal, exogenous ABA was effective only in the presence of an endogenous auxin supply. The level of endogenous IAA was influenced (increased) by the application of ABA to immature zygotic sunflower embryos and resulted in the induction of somatic embryogenesis under sucrose conditions which otherwise allow only caulogenesis to occur (Charriére et al., 1999). Amounts of endogenous ABA were inversely correlated with the embryogenic capacity of alfalfa cultivars (Ivanova et al., 1994). This observation seems to be contradictory to the positive role of exogenous ABA on somatic embryo induction (e.g., Nishiwaki et al., 2000), but it can be explained by the differences in the effects of exogenously applied and endogenous hormones: a relatively higher endogenous ABA level may result in a decreased response to exogenous stress or ABA signals.

Direct experimental evidence of the contribution of endogenous ABA to the induction phase of somatic embryogenesis was provided by Senger et al. (2001). These authors used different approaches to reduce cellular ABA levels in *Nicotiana plumbaginifolia* plants: a homozygous transgenic line was produced which over-expressed an anti-abscisic acid single chain variable fragment (scFv) antibody, wild-type cultures were treated with the ABA synthesis inhibitor flouridone and abscisic acid synthesis mutants (*aba1* and *aba2*) were also used. In all cases of ABA deficiency, disturbed morphogenesis could be observed at pre-globular embryoid formation, which could be reverted by exogenous ABA application.

All of the above-described experimental results and observations (Stress: the force to switch and Growth regulators: outside and inside sections) highlight the importance of the interaction between auxin and stress/ABA signalling. One can hypothesize that the parallel induction of these pathways can lead to the morphological and developmental changes observed during the transition from somatic to embryogenic cell types due to rapid de- and redifferentiation. The simultaneous activation of auxin and stress responses may be a key event in cellular adaptation, causing genetic, metabolic and physiological reprogramming, which results in the embryogenic competence (totipotency) of somatic plant cells.

Changing fate: dedifferentiation and the acquisition of cellular totipotency

Acquisition of embryogenic competence largely relies on dedifferentiation, a process whereby existing transcriptional and translational profiles are erased or altered in order to allow cells to set a new developmental program. The activation of cell division is required to maintain the dedifferentiated cell fate, as well as for embryo differentiation. This is not only true for those embryogenic systems where embryogenic callus formation precedes somatic embryo development (‘indirect somatic embryogenesis’), but also for those where somatic embryos develop on primary explants without an intervening callus formation (‘direct embryogenesis’).

Dedifferentiation-specific genes: are there any?

As dedifferentiation of plant cells is a basic phenomenon underlying *in vitro* cell and tissue culture responses, there have been many attempts to isolate potential key genes with altered transcriptional profile during this process. Most available data comes from genes switched on in freshly isolated protoplasts. Leaf protoplasts are ideal experimental objects of studies on cellular dedifferentiation: they are a population of single cells derived from fully differentiated, specialized tissues and they respond to *in vitro* culture conditions uniformly and with reliable synchrony. The synthesis of proteins involved in photosynthesis is completely inhibited, while other proteins, absent from differentiated mesophyll cells, are actively synthesized in protoplasts as soon as they are isolated from leaves (Fleck and Durr, 1980; Vernet et al., 1982). Among those genes responsible for the synthesis of these proteins, many are associated with stress response, primary metabolism, cell wall synthesis or cell division (Grosset et al., 1990; Marty et al., 1993; Yu et al., 1999). During dedifferentiation, all these functions participate in the reorganization of cellular metabolism and the developmental switch. Most of these genes, not only those related to stress responses, have been induced by signals resulting from the mechanical wounding of tissues during protoplast isolation (Grosset et al., 1990; Marty et al., 1993). These data also emphasize the role of stress in cellular reprogramming.

Nagata et al. (1994) reported the isolation of cDNA clones for three auxin-regulated genes, *parA*, *parB* and *parC,* from tobacco protoplasts that had regained their division activity. The protein products of these auxin-induced genes belong to the plant glutathione *S*-transferase (GST) family (for review, Marrs, 1996). Their expression is induced in leaf protoplasts within 20 min of auxin application and fully repressed when the protoplasts start to divide (by 48 h of culture) (Takahashi et al., 1994). GSTs can have diverse roles, including the detoxification of xenobiotics, targeting their substrates for transmembrane transport and protection from oxidative stress. It has also been shown that some plant GSTs can bind, and probably carry and store, natural auxin (IAA) (for review, Marrs, 1996). There is accumulating evidence that the redox status and the glutathione content of cells have important roles in developmental processes and especially in the reactivation of cell division (for review, den Boer and Murray, 2000).

A carrot cDNA (*Dcarg1*), homologous to the *parA* sequence, was isolated as a differentially expressed gene in hypocotyl cells, induced to form somatic embryos by a 2-h treatment with high concentrations of 2,4-D (Kitamiya et al., 2000). The expression of the *Dcarg1* gene was not responsive to stress treatments,

but a close relationship between somatic embryo induction and *Dcarg1* expression could be observed under different hormonal conditions. The expression of this gene was repressed during embryo development (Kitamiya et al., 2000). These observations may indicate that *par*-related gene products (GSTs) are not only involved in the process(es) of dedifferentiation and reactivation of cell division, but may also have roles in developmental reprogramming.

In *Nicotiana sylvestris*, genes expressed in mesophyll protoplasts, before the reinitiation of the DNA replicational activity, were identified through the differential screening of a cDNA library made from 6-h-old protoplasts (Criqui et al., 1992). Three mRNAs were characterized to represent a novel type of trypsin inhibitor, a moderately anionic peroxidase and a protein of unknown function with a nuclear localization signal. The expression of these genes was independent of $HgCl_2$ treatment, heat shock, pathogen infection and tumour formation, and declined in protoplast-derived cells entering cell division. Thus, they were hypothesized to have specific roles during cellular dedifferentiation.

As it can be seen, none of the above approaches resulted in the identification of master genes clearly responsible for the dedifferentiation processes. One can hypothesize, however, that such master genes, if they exist, should be involved in the general reprogramming of gene expression (chromatin remodelling, transcription machinery) and/or protein turnover.

Cellular reorganization: proteins are turned over

Establishment of a new cellular state is not only governed at the level of gene expression, but requires modification and/or removal of unnecessary polypeptides, as well as the proper folding of newly synthesized proteins and protein complexes. This was demonstrated using two-dimensional protein electrophoresis, which showed that dedifferentiation and subsequent somatic embryogenesis are associated with complex changes in the protein pattern (for recent publications, e.g., Gianazza et al., 1992; Sallandrouze et al., 1999). Regulation of protein degradation is attracting increasing attention as an important regulatory mechanism in cell cycle progression and development in eukaryotic cells, including those of plants (Udvardy, 1996; Genschik et al., 1998; Criqui et al., 2000). Auxin signalling has been shown to be at least partially based on the degradation of specific proteins (Leyser, 2001; Rogg and Bartel, 2001). Many of the early plant responses to auxin are apparently mediated by members of a family of auxin responsive AUX/IAA proteins that dimerize with, and thus inhibit, members of the auxin response factor (ARF) family of transcription factors (as review see, e.g., Walker and Estelle, 1998). AUX/IAA proteins are unstable and their degradation is triggered by an ubiquitin–protein ligase complex (SCF type) that is regulated by modification with an ubiquitin-related protein (Gray et al., 1999; Gray and Estelle, 2000). Recent genetic and biochemical data indicate that auxin accelerates the degradation of the already short-lived AUX/IAA proteins to derepress transcription by ARF proteins (Gray et al., 2001).

The ubiquitin–proteasome pathway of protein degradation is responsible for the degradation of most cell proteins; not only regulators and short-lived proteins, but also those that serve structural roles and are long lived (Rock et al., 1994). Ubiquitin, a small peptide of 76 amino acids, can be covalently linked to other proteins and serves as a biochemical tag that marks proteins for degradation (Ingvardsen and Veierskov, 2001). Dedifferentiation of mature tobacco cells was shown to be accompanied by a sharp increase in ubiquitin gene expression (Jamet et al., 1990). This elevation in ubiquitin gene activity may be related to cellular reorganization during dedifferentiation and might be required for the selective destruction of proteins associated with the previous, differentiated cell state. In line with this observation, it has recently been reported that, in the case of reactivated tobacco protoplasts, entry into the DNA replication phase could be prevented by MG132, a specific inhibitor of the ubiquitin–proteasome pathway, but not by leupeptin, a protease inhibitor (Zhao et al., 2001).

The overall changes in cell metabolism and the initiation of a new developmental program generate the need for new proteins that must be folded and assembled properly to ensure their functions. Protein folding is controlled by special proteins exhibiting chaperone activities, including so-called heat shock proteins or HSPs (Morimoto, 1998). In addition to their expression during the heat shock response, which can also be considered as a transient state when cellular reprogramming is required, many HSP genes have been reported to be developmentally regulated (for reviews, Dudits et al., 1995; Schoffl et al., 1998). For example, the expression of heat-shock genes occurred during embryogenesis from somatic cells in alfalfa and carrot (Dudits et al., 1991; Kitamiya et al., 2000). Although the induction of somatic and mi-

crospore embryogenesis involves the application of some kind of stress, the developmental phase-dependent expression of HSPs, during zygotic embryogenesis and seed maturation, indicates that these proteins may have additional functions apart from those necessary to cope with environmental stresses (for review, Schoffl et al., 1998). For example, in sunflower, expression of class II small HSPs is roughly parallel with storage protein and lipid accumulation, whereas expression of class I small HSPs coincides with seed desiccation (Coca et al., 1994). In general, it can be hypothesized that the developmentally regulated appearance of HSPs is associated with their chaperone function, promoting the assembly of newly synthesized proteins during developmental transitions (for reviews, Dudits et al., 1995; Schoffl et al., 1998).

Cell division and differentiation: being under control

In attempts to understand the molecular levels of the transition from the somatic to embryogenic cell type, the regulators of the cell cycle can be considered as key determinants during both dedifferentiation and embryo formation. Recent developments in the field of plant cell cycle research provide some clues to their roles. The core of the cell cycle machinery is well conserved among eukaryotes, but obvious differences also exist (for review, John, 1996). For example, in plants, the synthesis of the new cell wall following cytokinesis has to be integrated into the events of the cell cycle, which increases the complexity of regulation (for review, Sylvester, 2000). The formation of special cytoskeletal structures, such as the preprophase band (PPB) and the phragmoplast, defines the plane of cell division. In the absence of cell migration, these cellular components strongly influence morphogenesis in plants (reviewed by Hemerly et al., 1999; Sylvester, 2000; Meijer and Murray, 2001). It is also well accepted that the regulation of plant cell division is highly dependent on external signals affecting plant development and morphogenesis (see as reviews, e.g., Dudits et al., 1998; den Boer and Murray, 2000).

In plants, both the G1-S and G2-M phase transitions can be controlled by changes in environmental factors. *Rhizobium*-induced formation of root nodule primordia from alfalfa cortical cells is initiated by the division of cells in G0/G1 phase (Yang et al., 1994), while in *Arabidopsis* lateral root development, G2-arrested pericycle cells respond to auxin treatment and form a lateral root primordium (Beeckman et al., 2001). Tobacco microspores can be accumulated in the G2 phase as a result of sucrose starvation stress that induces their embryogenic development (Touraev et al., 1996). Alfalfa leaf protoplast populations treated with high concentrations of 2,4-D are also enriched in cells with nuclear DNA content characteristic of G2 cells (T. Pasternak, P. Miskolczi and A. Fehér, unpublished results). These observations indicate that developmental changes in cells, induced by environmental or developmental signals, are dependent on the original DNA content and differentiation state of the cells.

The progression of cells through the different phases of the cell cycle is regulated primarily by the activity of different cyclin-dependent kinase (CDK) complexes in all eukaryotes, including plants (for reviews, Mironov et al., 1999; Mészáros et al., 2000). The expression of the gene coding for the kinase component of the complex (Cdc2-related kinase) is induced by auxin (Hirt et al., 1991) and present not only in dividing plant cells, but also in division-competent cells (Hemerly et al., 1993), thus representing a marker for the degree of differentiation. It is well known that *in vitro* grown plant cells, with few exceptions, require exogenous growth regulators, namely auxin and cytokinin, for sustained cell division. In embryogenic alfalfa leaf protoplasts, the Cdc2MsA protein re-appeared in response to auxin treatment; however, the histone H1 phosphorylating activity of this protein was dependent on post-translational modifications that required the presence of cytokinin (Pasternak et al., 2000). These modifications may include the de-phosphorylation of the CDK protein on Thre14/Tyr15 regulatory residues (this mainly operates at G2-M transition, but a similar role in G1-S transition cannot be excluded), or by the induction of the expression of the regulatory cyclin subunit cyclin D3 (at G1-S transition) (for review, John, 1996).

Although the activation of cell division is a prerequisite for embryogenic induction, the activities of the cell division-regulating cdc2-like kinase complexes are similar in alfalfa protoplasts grown under either embryogenic or non-embryogenic conditions, in spite of the fact that earlier activation of the division of embryogenic cells has been observed (Fehér et al., 2002; Pasternak et al., 2002). Bögre et al. (1990) also reported that leaf protoplast-derived cells of an embryogenic *versus* a non-embryogenic alfalfa line were activated earlier. Decreased cell doubling time has

been associated with the start of somatic embryogenesis in carrot (Warren, 1980). In the alfalfa leaf protoplast system, high 2,4-D concentration or stress-induced embryogenic competence were associated with small cell size and earlier cell cycle activation (Pasternak et al., 2002). In carrot cell cultures, a 2,4-D concentration-dependent switch between elongation and division has also been observed and it was demonstrated that 2,4-D inhibited the elongation of cells, not directly, but as a consequence of promoting their division (Lloyd et al., 1980). In the apical meristem, the files of smaller cells divided faster as compared to their larger neighbours, although cell size and cell cycle time are not always correlated in the same manner (reviewed by Dudits et al., 1998). Cell size, cell morphology and the timing of cell activation have been reported to be correlated with earlier endogenous auxin synthesis in alfalfa leaf protoplast-derived cells (Pasternak et al., 2002). It can be speculated that this increase in endogenous IAA level can be responsible for accelerating the dedifferentiation process of an earlier activation of the division cycle, preventing cell elongation.

Cell division and differentiation are developmentally related processes. The complex relation between differentiation and cell division, however, might differ in the various stages of plant development, both *in vivo* and *in vitro*. *In planta*, the fusion of differentiated gametes activates a series of cell divisions in the zygote, resulting in embryo formation through structural and functional differentiation into suspensor cells and embryo proper cells, followed by the establishment of tissue layers (protoderm, basal tissue and vascular primordium), as well as root and shoot meristems (Jurgens, 1992). The sustained cell division in the meristems provides a continuous supply of cells undergoing differentiation processes for organogenesis, and the establishment of the plant body during the whole ontogenic development of a plant.

In vivo, the reactivation of the cell cycle can be a starting point for new organ initiation caused by changes in hormonal homeostasis (e.g., lateral root formation) or by external signals (e.g., root nodule formation in response to Nod-factor). Asymmetric distribution of cell contents among daughter cells, as a result of asymmetric cell division, can be decisive for their further differentiated cell fate (for review, Scheres and Benfey, 1999). An essential switch in developmental pathways may be facilitated in dividing cells by a more open chromatin structure, altered metabolism and physiological homeostasis, compared to differentiated cells (see later). A correlation between cell division competence and developmental determination was indicated by studies on trichome initiation in *Arabidopsis* (Larkin et al., 1997). Trichomes are normally initiated in a field of dividing cells and the ectopic overexpression of the regulators of trichome initiation can also result in trichome formation, exclusively in proliferating tissues (Lloyd et al., 1994; Hulskamp and Schnittger, 1998).

In vitro cell cultures can be initiated from explants of either meristematic origin or from differentiated organs. In both cases, the artificial initiation and maintenance of cell division is a prerequisite for the generation and the establishment of the dedifferentiated, meristematic cell fate. In cell cultures, the dividing cells can follow alternative developmental pathways such as unorganized callus growth, root and shoot initiation or somatic embryo formation. In the last case, division of somatic or dedifferentiated cells generates a cellular state similar to that of the zygote, formed after the fertilization of the egg cell.

Therefore, although cell division and differentiation are frequently considered to be mutually exclusive processes (in differentiated somatic cells, division is arrested while the dedifferentiated cell fate is linked with the capacity for sustained division both *in vitro* and *in planta*), their relation is much more complex. Cell cycle progression and differentiation are related but divergent processes in animal cells as well, and their regulation is linked by components having important regulatory roles near the G1/S cell cycle transition point (Gao and Zelenka, 1997; Studzinski and Harrison, 1999). Some of these components, such as the retinoblastoma (Rb) repressor proteins, the E2F-type transcription factors, as well as D-type cyclins and CDK inhibitors (CKIs), exist in plants as well (for reviews, e.g., Gutierrez, 1998; Mironov et al., 1999; Mészáros et al., 2000; Stals and Inze, 2001), indicating the existence of similar mechanisms. This is further supported by some experimental observations. In *Arabidopsis*, leaf aging and differentiation are associated with increased expression of the ICK1 CDK inhibitor protein (Wang et al., 1998). In maize leaf, the regions of older differentiated cells contain more Rb protein than younger tissues (Huntley et al., 1998). Ectopic expression of the *cyclinD3* gene caused abnormal meristem and leaf development in transgenic plants (Riou-Khamlichi et al., 1999). Overexpression of the same protein in calli induced differentiation and greening and prevented shoot regeneration (Riou-Khamlichi et al., 1999).

Although additional evidence is required, it can be hypothesized that several key regulators of cell cycle/differentiation control play key roles in somatic embryogenesis through coordinated interactions with hormonal, environmental and developmental signalling pathways.

Chromatin remodelling: open for changes

Chromatin structure, the DNA organization around basic nuclear proteins (histones), is intrinsically involved in the regulation of nuclear processes, such as DNA repair, replication and especially transcription (for review, Varga-Weisz and Becker, 1998). Chromatin structure changes in a dynamic way and is continuously remodelled during development. Chromatin-dependent gene silencing is a common mechanism for maintaining the differentiated state of cells. Thus chromatin remodelling is necessarily linked with cellular dedifferentiation and the switching of cell fate.

It can be hypothesized that chromatin remodelling plays two major roles during the early stages of somatic embryogenesis. Dedifferentiation requires unfolding of the supercoiled chromatin structure, in order to allow the expression of genes inactivated by heterochromatinization during differentiation, and subsequent chromatin remodelling can result in the specific activation of a set of genes required for embryogenic development. Below we summarize some of the recent experimental observations supporting this hypothesis.

In tobacco protoplast-derived cells, probing of the chromatin structure by nuclease digestion, as well as the accessibility of DNA by the interchelating fluorescent dye, propidium iodide, revealed two separated periods of chromatin decondensation. The first took place during protoplast isolation and was linked to dedifferentiation, while the second one was induced by plant hormones, auxin and cytokinin, and linked to the reactivation of cell division (Zhao et al., 2001). In this model, dedifferentiation (first phase of chromatin decondensation) represents a transitory stage when cells become competent to switch cell fates. However, their further fates depend on hormonal signals: in the absence of auxin, cells went through apoptosis, auxin and cytokinin were both required for cell division, while auxin treatment alone resulted in differentiation (Zhao et al., 2001).

Similar observations were made with leaf protoplast-derived cells of alfalfa. The structure and size of the nucleus and especially the ratio of the volumes of the nucleus and nucleolus, as well as the stainability of the chromatin with fluorescent dyes, could be correlated with auxin/cytokinin-dependent phases of cell reactivation and division (Figure 1) (Pasternak et al., 2000). Both studies indicated that the first phase of chromosome decondensation was partial.

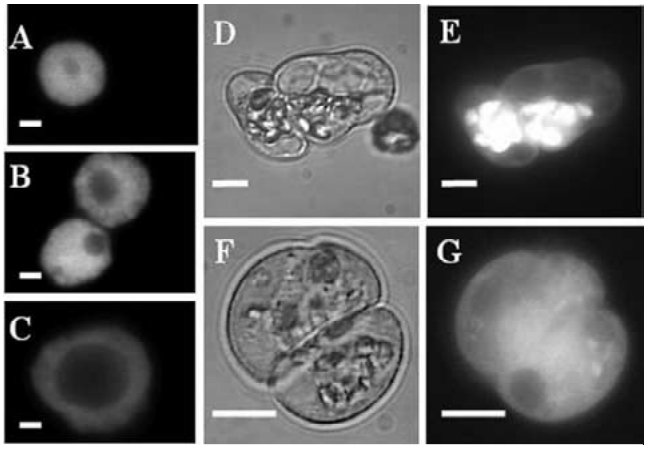

Figure 1. Cellular markers of dedifferentiation and totipotency in alfalfa leaf protoplast-derived cells. (A–C) Nuclear structure as a marker of activation of leaf protoplast-derived alfalfa cells. 4′,6-diamidino-2-phenylindole (DAPI)-stained isolated nuclei of: (A) Differentiated leaf cell; (B) 1-day-old protoplast-derived cell; (C) 3-day-old dividing protoplast-derived cell. Dedifferentiation is associated with an increase in the size of the nucleus and nucleolus that is the most pronounced in dividing cells (see Pasternak et al., 2000). Bar represents 1 μm. (D–G) Cellular distribution of the pH-dependent fluorescent dye fluorescein diacetate (FDA) is dependent on the cell type developed from alfalfa leaf protoplasts. In elongated, non-embryogenic cells (D) FDA accumulates in the functioning chloroplasts (E), while in the compact, cytoplasm rich embryogenic cells (F) it remains in the cytoplasm (G). Bar represents 10 μm.

In *Drosophila*, a large number of genes, known collectively as genes of the Polycomb (Pc) group, regulate the accessibility/inaccessibility of particular chromosomal regions. In plants, Pc-like genes play an important role in suppressing the development of unfertilized seeds (Chaudhury et al., 2001). In *medea* (*mea*) and *fertilization independent endosperm* (*fie*) mutants, endosperm, and sometimes embryo, development is initiated in the absence of fertilization (Ohad et al., 1996; Grossniklaus et al., 1998). The mutated proteins contain domains similar to *Drosophila* polycomb proteins: MEA has a domain similar to the SET domain of *enhancer of zeste* and FIE shares homology with the WD domain of *extra sex combs* (Ohad et al., 1999). On the basis of homology with their animal counterparts, plant Pc-like proteins may be involved in chromatin remodelling. This suggestion is supported by experiments based on the overexpression of the chromodomain of a *Drosophila* Pc-protein in tobacco. The fused GFP protein could target specific chromatin regions and caused a variety of morphological phenotypes in the transgenic plants, indicating interference with endogenous chromatin-regulating mechanisms (Ingram et al., 1999).

The mutation named *fertilization independent seed* (*fis2*) caused the same phenotype as *mea* or *fie*, but *fis2* encodes for a zinc-finger transcription factor (Chaudhury et al., 1997; Luo et al., 1999). It is hypothesized that FIS2 forms a complex with MEA and FIE and recruits them to particular promoter regions which regulate the transcriptional activation of downstream genes (Schnittger et al., 1999; Grossniklaus et al., 2001).

Additional experimental evidence highlights the importance of regulating chromatin structure in developmental transitions. The *Arabidopsis* mutant, *pickle* (*pkl*), has a phenotype characterized by the postembryonic expression of embryo specific markers and the spontaneous regeneration of somatic embryos in its roots (Ogas et al., 1997). The product of the *pkl* gene was characterized as a chromatin-remodelling factor that represses gene expression and regulates the developmental transition from embryogenic to vegetative state (Ogas et al., 1999).

The above experimental findings strongly support the view that the initiation of embryogenic development is based on a release from transcriptional repression by the signal of fertilization. Whether MEA- and FIE- or PKL-like proteins and other regulators of chromatin remodelling play a similar role during somatic embryo development as they do during early seed development, and whether the down-regulation of their function is sufficient or only a necessary condition in the induction of somatic embryogenesis, remain interesting questions to be answered.

Embryogenic cells: what do they look like?

In order to be able to study the earliest phases of somatic-to- embryogenesis, good markers are required that indicate the acquisition of embryogenic competence in the induced cells. These markers must be universal to allow the comparison of different embryogenic systems. In addition to serving as markers, they may also be useful in revealing specific cellular processes related to the somatic-to-embryogenic transition and the determination/description of cellular states.

Cell morphology: an early marker of embryogenic competence

Formation of embryogenic cells can be correlated with characteristic morphological changes. The fate of embryogenic carrot cells was followed by video cell tracking by Toonen et al. (1994). The single cell fraction (<22 μm) of the established embryogenic cell culture contained cells that could be classified into five morphological groups. Although all cell types were capable of developing into somatic embryos with varying efficiency, the highest frequency was observed in the case of small, spherical, cytoplasm-rich cells. In another approach, Nomura and Komamine (1985) fractionated single carrot cells by density gradient centrifugation. They obtained a fraction of small, isodiametric, cytoplasm-rich cells that could initiate embryo formation with 90% frequency. This cell type was designated as State 0, or embryogenic competent cells which, in the presence of auxin (2,4-D), formed the State 1 embryogenic cell clusters, consisting of less than 10 cytoplasm-rich cells (Nomura and Komamine, 1995). These clusters differentiated into globular embryos on auxin-free medium. In contrast, in cell cultures initiated from petioles of *Medicago sativa*, single cells were unable to develop into somatic embryos and the fraction trapped between 224- and 500-μm meshes represented embryogenic cell clusters consisting of small, rapidly dividing cells (Xu and Bewley, 1992). Similar observations have been made in the case of *Picea abies* (Filonova et al., 2000).

In another approach, the expression of a gene coding for the 'somatic embryogenesis receptor kinase' (SERK) was used as a marker to define embryogenic cells in the activated hypocotyl explants of carrot (Schmidt et al., 1997). In this case, a class of elongated cells on the explant surface was reported to be competent for embryogenesis. In *Dactylis glomerata* leaf explants, using the expression of the *Dactylis* SERK-homologue gene as a marker of competence, exclusively small, isodiametric cells with rich cytoplasm proved to be competent and could develop into somatic embryos. In established embryogenic cell cultures, however, a few percent of elongated, vacuolated cells also expressed the marker (Somleva et al., 2000).

Pennell et al. (1992) using the JIM8 cell wall epitope as another molecular marker for embryogenic competence of carrot cells, hypothesized that small, cytoplasm-rich cells expressing this epitope represent a transitional cell state: they are formed from non-embryogenic cells and they either elongate and die, or divide and form the initial cell of the somatic embryo.

In alfalfa, leaf protoplast-derived cells cultured at different 2,4-D concentrations can develop into either embryogenic or non-embryogenic cell types. Similar morphological traits could be recognized when protoplasts of embryogenic and non-embryogenic genotypes were cultured at the same 2,4-D concentration. In both cases, embryogenic cells were small, spherical and densely cytoplasmic, while non-embryogenic ones were elongated and highly vacuolated (Bögre et al., 1990; Dudits et al., 1991; Fehér et al., 2002; Pasternak et al., 2002) (Figure 1D,F). Embryogenesis competent cells formed in chicory leaf explants were also characterized by dense cytoplasms (Blervacq et al., 1995).

It can be suggested therefore that in most embryogenic systems, including gymnosperms (Egertsdotter and von Arnold, 1998), this morphology is associated with embryogenic competence (for review, Yeung, 1995). The observed differences in the correlation of cell morphology and embryogenic competence in certain experimental systems can be ascribed to the different explants and experimental approaches used.

Physiological competence: pH and Ca^{2+}

All living cells use a network of signal transduction pathways to harmonize environmental stimuli with the completion of developmental programs. The 'cross talk' between different signalling cascades related to development, metabolism, environmental sensing, etc., results in very complex responses to diverse and multiple stimuli, even if a relatively small number of messengers are involved. In plant cells, the list of messengers used by signalling pathways includes, among others, pH and Ca^{2+}. Here we discuss their significance during the induction of somatic embryogenesis.

pH

Numerous studies have demonstrated that changes in cytoplasmic pH (pHc) occurred during metabolic and developmental transitions in a large variety of cells. In *Dictiostelium discoideum*, cytoplasmic pH determined different cell differentiation pathways (Gross et al., 1983). pHc increase is necessary for transition of cells from G0 to G1 and into S-phases in yeasts, echinoderm eggs, protozoa, slime moulds and mammalian cells (for review, Frelin et al., 1988). Tumorigenic Chinese hamster cells had a pHc of 0.2 pH units higher than that of normal cells (Ober and Pardee, 1987). Preventing the increase in cellular pH in sea-urchin eggs, by different methods, blocked pronuclear movements, diminished protein synthesis and prevented cleavage (Swann and Whitaker, 1990). In contrast, alkalinization of the cytoplasm with ammonia could reactivate the egg, and induced cyclin synthesis, $p34^{cdc2}$ phosphorylation and finally DNA replication (Whitaker, 1990; Schomer and Epel, 1999).

In plants, there is only a limited number of examples of the physiological role of long-term changes in cellular pH (as a review, Kurkdjian and Guern, 1989; Pichon and Desbiez, 1994; Bibikova et al., 1998). In *Bidens pilosa*, Pichon and Desbiez (1994) found that cytoplasmic pH was correlated with cell division. Alkalinization promoted the cell cycle in the meristematic region of the hypocotyl, while acidification inhibited it. Initiation of root hair cells in *Arabidopsis* could also be characterized by the acidification of the apoplast and the alkalinization of the cytoplasm (Bibikova et al., 1998).

The transition from the somatic to the embryogenic cell state is a complex process that includes dedifferentiation, cellular reactivation, division and metabolic, as well as developmental, reprogramming. Characteristic changes in intracellular pH are hypothesized to be associated with this transition. A relationship between medium (and cellular) pH and developmental state has been suggested by experiments with

wounded carrot zygotic embryos cultured in the presence of 1 μM NH_4Cl (Smith and Krikorian, 1990a, b). NH_4Cl-induced cellular alkalinization allowed the establishment of a continuous culture of preglobular stage proembryos. Medium pH was decreased in these cultures (down to pH 4), which was likely correlated with increased pHc. The development of embryos could only be advanced if the medium pH was raised to approximately pH 5.7. It had previously been shown that cells of an alfalfa embryogenic-type callus had higher average pHc values compared to cells from a non-embryogenic type (Schaefer, 1985).

In alfalfa leaf protoplast-derived cells, cytoplasmic and vacuolar alkalinization and medium acidification were shown to be correlated with the activation of cell division (Pasternak et al., 2002). Although a clear relationship between embryogenic competence and the degree of cytoplasmic alkalinization could not be established in this direct somatic embryogenesis system, small, cytoplasm-rich embryogenic cells had a tendency to exhibit higher vacuolar pH values in comparison to the non-embryogenic vacuolated cells (Pasternak et al., 2002). It is supposed that the large difference in the vacuolar pH of the embryogenic and non-embryogenic cell types is related to the difference in the vacuolar functions (for reviews, Wink, 1993; Marty, 1999; Ratajczak, 2000), linked to the fate of these cells: elongated, differentiated cells have large, central, lytic vacuoles with more acidic pH, while the small, dedifferentiated cells have several small storage-type vacuoles.

In the same leaf protoplast system, buffering of the medium pH with 10 mM MES prevented embryogenic cell formation under inductive conditions (Pasternak et al., 2002). Increased transport across cell membranes, inhibited by extracellular MES, might be an important process in the metabolic reprogramming of embryogenic cells. Embryogenic cells accumulate starch (Yeung, 1995) and have vacuoles characterized by low transparency and strong staining by toulidene blue, indicating high protein content, likely to be storage proteins (Pasternak et al., 2002).

Another marker of dedifferentiated, embryogenic alfalfa cells is the intracellular distribution of fluorescein diacetate (FDA). This pH-indicator fluorescent dye is detectable exclusively in the cytoplasm of embryogenic competent cells, in contrast to its accumulation in the chloroplasts of the highly vacuolated, elongated cells (Figure 1) (Pasternak et al., 2002). We can assume that FDA accumulation in the chloroplasts is related to functional electron transport (photosynthesis). The lack of establishment of this transthylakoid pH gradient in the embryogenic competent cells may indicate the rapid dedifferentiation of chloroplasts and the loss of photosynthetic functions in these cells. Metabolic properties of plastids have been shown to differ among leaf and embryo cells of *Brassica* (Eastmond and Rawsthorne, 1998).

Ca^{2+}

Ca^{2+} functions as a key regulator of many cellular and physiological events in plants (for review, Sanders et al., 1999). Current models of Ca^{2+}-mediated signalling emphasize the significance of a transient change, usually an increase, in cytoplasmic calcium concentration, followed by the perception of such changes by calcium-binding proteins. The involvement of Ca^{2+} in a wide variety of stimulus-response pathways in plant cells raises several questions concerning how the same messenger can regulate the different responses. The amplitude, duration, frequency and location of the Ca^{2+} signal can be considered as key features in the determination of different messages.

The increase in intracellular Ca^{2+} concentration after fertilization of egg cells has been demonstrated in both animal and plant cells (Stricker, 1999; Antoine et al., 2000). In brown algae (Robinson et al., 1999) as well as in flowering plants (Antoine et al., 2000), it was hypothesized that gamete fusion-induced calcium influx plays a direct role in egg cell activation.

Although similar studies at the level of individual cells have not been performed during the induction of somatic embryo development, the dependence of somatic embryogenesis on external and internal calcium concentrations has been demonstrated in different systems. In an embryogenic carrot cell suspension, it was shown that an upward shift in the exogenous calcium concentration (e.g., from 10^{-3} to 10^{-2} M) at the time of transfer to auxin-free embryo differentiation medium increased the number of somatic embryos approximately 2-fold (Jansen et al., 1990). It was also observed that the elevated calcium concentration counteracted the inhibitory effect of 2,4-D on embryo development (Jansen et al., 1990). Overvoorde and Grimes (1994) reported that exogenous Ca^{2+} concentration higher than 200 μM is required for optimal embryo formation. The application of either Ca^{2+}-channel blockers or the Ca^{2+} ionophore

A23187 inhibited embryo initiation. These data indicate that exogenous Ca^{2+} and the maintenance of cellular Ca^{2+} gradients are required for proper embryo development *in vitro*.

Endogenous Ca^{2+} levels were determined during sandalwood somatic embryogenesis by Anil and Rao (2000). Transfer of cells of the proembryogenic cell mass (PEM) onto growth regulator-free medium to promote embryo development resulted in a several-fold increase in the uptake of exogenous Ca^{2+}, as well as the Ca^{2+} level in the symplast. This could be blocked by chelating exogenous calcium ions, which arrested embryo development, but still allowed cell proliferation to continue. Blocking calcium-mediated signalling by W7 resulted in an 85% decrease in embryo formation frequency. This response indicated that calmodulin or Ca^{2+}-dependent protein kinase could be involved in this process (Anil and Rao, 2000). Although there are data showing that calmodulin levels are increased in dividing cells (e.g., Perera and Zielinski, 1992), there were no significant changes detected in calmodulin levels (Overvoorde and Grimes, 1994; Dudits et al., 1995) or calmodulin methylation and calmodulin-binding protein levels (Oh et al., 1992) during carrot, sandalwood and alfalfa somatic embryo induction. However, an unknown Ca^{2+}-binding protein was reported to be transiently induced in the early phase of somatic embryo induction in an alfalfa cell culture by Dudits et al. (1995).

Additional data support that Ca^{2+}-dependent protein kinases (CDPKs) are involved in the signalling pathways during the formation of somatic embryos. In sandalwood, two CDPKs could be detected in protein extracts of embryogenic cell cultures (Steenhoudt and Vanderleyden, 2000). Their strong Ca^{2+}-dependent activities were detected in PEMs as well as in somatic embryos of different stages, but not in regenerated plantlets (Jackson and Casanova, 2000). The expression of the MsCDPK3-encoding gene has been shown to increase during the early phase of 2,4-D-induced embryogenesis from cultured alfalfa cells (Davletova et al., 2001). The role of Ca^{2+} in the establishment of cellular polarity during embryogenesis in plants is discussed below (Pattern formation: the establishment of polarity section).

Pattern formation: the establishment of polarity

In seed plants, the egg cell and zygote exhibit apical–basal polarity (Russell, 1993; Vroemen et al., 1996, 1999; Scheres and Benfey, 1999). Polarized development of the zygote and the embryo includes the formation of shoot and root meristems, which maintain the apical–basal polarity throughout plant development (Jurgens, 2001). These meristems are groups of undifferentiated cells and serve as cell sources for tissue and organ differentiation. In the absence of cell migration, the morphogenesis of plants is based on co-ordinated cell division and elongation followed by differentiation. Studies on root development, with the help of laser ablation of individual cells, have revealed that position, rather than lineage, determined patterning and cell fate (van den Berg et al., 1997, 1998). Position of a plant cell is largely determined by its contacts with neighbouring cells as a result of the orientation (plane) of cell division and cell elongation during tissue formation (van den Berg et al., 1997, 1998).

The initial zygotic division in higher plants is asymmetric (Scheres and Benfey, 1999). This event establishes the basic polarity of the plant which probably determines subsequent pattern formation (Jurgens et al., 1997). In *Arabidopsis*, where embryonic cell divisions are very regular, the *gnom* mutation, which affects the first asymmetric division, causing abnormal pattern formation (Busch et al., 1996). However, in the *fass* mutant, in spite of disorganized cell divisions, all pattern elements were formed in the embryo. Nonetheless subsequent development was distorted and resulted in dwarves and malformed plants (Torres-Ruiz and Jurgens, 1994). Thus, although embryogenic patterning may not involve specific cell divisions except the initial asymmetric division, final form does. Not only the pattern, but also the rate of cell division can be an important factor in developmental pattern formation. Expression of the dominant negative form of the cell cycle regulatory CDK (cdc2aDN) of *Arabidopsis* in embryos resulted in a range of altered phenotypes (Hemerly et al., 2000). The most severe phenotype was characterized by unrecognizable tissue organization, while in other cases some tissues were missing and the apical/basal (but not the radial) symmetry was distorted.

Much less is known about the establishment of polarity during somatic embryogenesis and there is controversy surrounding the role of the initial asymmetric cell division. In alfalfa, where leaf protoplasts can be used to induce direct development of embryogenic cells (Dijak and Simmonds, 1988; Song et al., 1990; Dudits et al., 1991), 2,4-D stimulates the

formation of asymmetrically dividing cells in a concentration- (Pasternak et al., 2002) and genotype- (Bögre et al., 1990) dependent manner, and the type of division determines further development. In the *Cichorium* hybrid 474, leaf cells can be induced to form somatic embryos directly by stress treatments (Blervacq et al., 1995). In this system, morphological asymmetry of the dividing embryogenic cells was not observed (Blervacq et al., 1995). In carrot, somatic embryos were induced in a heterogeneous cell population and proembryogenic cell mass (PEM) was formed from various initial cell types, as observed by the use of video cell tracking (Toonen et al., 1994). However, Nomura and Komamine (1985) showed that isolated small, cytoplasm-rich carrot cells, after an unequal first division and polarized synthesis of macromolecules, have the ability to develop into somatic embryos. In certain *Arabidopsis* ecotypes, leaf protoplast-derived embryogenic cells may exhibit a cell division pattern reminiscent of the first division of the zygote (Luo and Koop, 1997). Further evidence has been provided in experiments where subpopulations of carrot cells were labelled by the monoclonal JIM8 antibody, which recognizes a cell surface epitope (McCabe et al., 1997). The JIM8-positive cells went through an asymmetric cell division that resulted in daughter cells with different antibody staining characteristics dependent on their further fate. The JIM8+ daughter cell died while the cell devoid of the surface epitope was embryogenic and formed a somatic embryo.

It is very likely that the establishment of polarity is an important event, not only for zygotic, but for somatic embryogenesis as well. Even if morphological asymmetry is not obvious, the unequal distribution of cellular determinants can be decisive in the determination of cell developmental pathways following division. The polarity of the egg cell is evident from the position of the nucleus at the cytoplasm-rich chalazal pole, while the micropylar pole is highly vacuolated (Russell, 1993). The microtubular cytoskeleton is particularly dense near the nucleus and has no specific orientation. The actin microfilaments have a similar conformation. In alfalfa, protoplast-derived embryogenic cells were also characterized by disordered microtubules when compared to non-embryogenic control cells (Dijak and Simmonds, 1988). The positioning of the nucleus was also peripheral in these cells, indicating the early establishment of cellular polarity in response to the electric stimulation used to induce embryogenic development. The observation that the application of an electric field to alfalfa protoplasts enhanced direct embryogenesis (Dijak and Simmonds, 1988), raised the question as to whether there is need for a directional signal to establish polarity in somatic cells that will become embryogenic. It is interesting to note here that 2,4-D, one of the most efficient inducers of somatic embryogenesis in many plant cultures, has been shown to prevent electrical polarity in cultured tobacco cells (Goldsworthy and Mina, 1991). However, when 2,4-D was removed and replaced by indoleacetic acid (IAA), the percentage of electrically polar cells significantly increased. This may explain why 2,4-D blocks the development of somatic embryos at the preglobular stage in carrot cultures, and why its removal or decreased concentration is required for the development of embryogenic polarity.

Although there are some systems where a polar input (directional signal) is not needed to achieve cell polarity, external signals are usually required to establish the polar axis, not only in plant cells, but in other eukaryotes as well (Cove, 2000). The best-studied plant systems, in this respect, are the brown algae, *Pelvetia* and *Fucus* (Cove, 2000; Souter and Lindsey, 2000). The egg cells of these species are spherical and fertilization induces the polar outgrowth of a rhizoid. The first cell division generates a cell plate that is perpendicular to the long axis of the polar zygote and separates the rhizoid and thallus cells. The sperm entry generates a signal which determines the polar axis, however the alignment of this axis can be changed by directional light until the polar axis is fixed (Kropf et al., 1999). In *Pelvetia*, it is suggested that a rhodopsin-like protein is the photoreceptor sensing the light gradient and controlling cellular cGMP levels (Robinson and Miller, 1997; Robinson et al., 1998). Unequal distribution of Ca^{2+} channels and the established Ca^{2+} gradient are also important in early determination of the axis (Pu and Robinson, 1998). A calcium influx was triggered in the vicinity of the sperm entry site and subsequently spread to the whole egg cell during the *in vitro* fertilization of maize (Antoine et al., 2000).

In sunflower protoplast-derived cells, as indicated by the fluorescent probe DMBodipy-PAA, there was a translocation of Ca^{2+} channels, which depended on the division type (Vallee, 1997; Xu et al., 1999). In freshly isolated protoplasts, the probe was strictly localized to distinct points on the plasma membrane. This compound also labelled cytoplasmic strands and a region around the nucleus in cultured protoplast-

derived cells. When cells divided, the staining was localized homogenously in the plane of cell division in symmetrically dividing cells, but marked only a peripherical ring around the site of cell division during asymmetric division (Vallee, 1997; Xu et al., 1999). Later, when embryoids developed from the asymmetrically divided cells, the Ca^{2+} channel probe was clearly localized on basal part of the embryoid, as was the case with zygotic embryos (Vallee, 1997; Xu et al., 1999).

There is evidence that the polar localization of F-actin filaments and the polarized secretion of cell wall material play roles in axis fixation (Fowler and Quatrano, 1997; Belanger and Quatrano, 2000; Vroemen et al., 1999). The role of polarized secretion in the establishment of cellular polarity was verified by the disruption of this process by Brefeldin A, an inhibitor of Golgi function (Capitanio et al., 1997). The *gnom* mutant of *Arabidopsis* provided some experimental evidence for the existence of similar mechanisms of axis fixation in higher plants. As was already mentioned, this mutant does not establish the proper plane of cell division required for the oblique asymmetric division of the zygote. The gene affected by the *gnom* mutation was cloned and characterized as coding for a guanidine nucleotide exchange factor of a small GTP-ase belonging to the ARF family (Busch et al., 1996; Steinmann et al., 1999). These proteins are known to be involved in vesicle movement and secretion (Jackson and Casanova, 2000).

Auxin is transported in a polar manner along the shoot–root axis, which requires efflux carriers such as PIN1 (for review, Palme and Gälweiler, 1999). Asymmetric localization of PIN1 develops from a random distribution in Arabidopsis during early embryogenesis, as a result of actin-dependent cycling between the plasma membrane and endosomal compartments (Geldner et al., 2001). In *gnom* mutant *Arabidopsis* embryos, the localization of the putative auxin efflux carrier protein, PIN1, is disturbed (Steinmann et al., 1999). BrefeldinA, a vesicle trafficking inhibitor, was also shown to abolish the polar localization of PIN1 and, consequently, the polar transport of auxin (Geldner et al., 2001). The role of polarized auxin transport was implicated in the establishment of apical–basal patterning in *Fucus* embryos, as well (Basu et al., 2002).

The importance of polarized secretion during somatic embryogenesis was indicated by the asymmetric distribution of the JIM8 cell wall epitope in carrot cell cultures (see above). In addition, in experiments where BrefeldinA was used to treat embryogenic carrot cells, the polar development of somatic embryos was blocked, but could be restored by the application of conditioned medium containing secreted proteins (Capitanio et al., 1997). During the last few years, a large body of experimental data has accumulated on the role of extracellular proteins on somatic embryo induction and development.

Top secret(ed): secreted proteins and the embryogenic cell

Callose deposition in walls of embryogenic cells and the formation of a glycoproteic extracellular matrix around superficial cells of globular embryos can serve as cytological markers of cell reactivation and embryogenic differentiation (Dubois et al., 1990, 1991; Pedroso and Pais, 1995). In chicory, embryogenic competent leaf cells had thicker walls, exhibiting a brighter autofluorescence of cellulose under UV light, compared to non-reactivated mesophyll cells (Blervacq et al., 1995). In the case of direct embryo formation from leaf explants of *Camellia japonica*, as an early marker of the morphogenetic response, cell walls underwent characteristic changes, including the deposition of callose on the surfaces of induced cells (Pedroso and Pais, 1995). This was followed by the deposition of other materials (cutin) characteristic for somatic embryogenesis, where the involvement of lipid transfer proteins is presumed. Expression of lipid transfer proteins is a well-known early marker of somatic embryo induction in different systems (Sterk et al., 1991; Schmidt et al., 1997; Sabala et al., 2000). It is also a broadly used marker of embryo differentiation, as it is also linked to the formation of the protoderm layer in developing somatic and zygotic embryos (Thoma et al., 1994; Vroemen et al., 1996; Toonen et al., 1997a).

Arabinogalactan proteins (AGPs) are a class of proteoglycans, widely distributed throughout the plant kingdom, that have been implicated in diverse processes of plant growth and development (for review, Showalter, 2001), including somatic embryogenesis (for review, Vroemen et al., 1999). Although their exact functions are not clear, AGPs are presumably involved in molecular interactions and cellular signalling at the cell surface. Their role during somatic embryo formation was demonstrated by the addition of the β-D-glucosyl Yariv reagent (βGlcY), which interacts with AGPs, to the culture medium. This compound altered the morphogenetic response (root

formation instead of embryogenesis) in the case of carrot (Thompson and Knox, 1998) and blocked somatic embryo formation in the case of *Cichorium* (Chapman et al., 2000). Promotory and inhibitory effects of certain exogenous arabinogalactan protein fractions in carrot cultures were reported by Toonen et al. (1997b). The addition of isolated carrot seed AGPs to old, non-embryogenic cell lines re-induced their embryogenic potential (Kreuger and van Holst, 1993). Seed AGPs promoted somatic embryo maturation in Norway spruce (Egertsdotter and von Arnold, 1998). Several antibodies have been prepared against diverse plant AGPs and used to mark specific cell types (for reviews, Knox, 1997; Showalter, 2001). In *Cichorium*, immunofluorescence and immunogold labelling studies localized AGPs to the outer cell walls of globular somatic embryos, but they appeared internally in later developmental stages. AGPs were also abundantly present in the culture medium (Chapman et al., 2000). In embryogenic maize cells, the extracellular matrix surface network has also been shown to contain AGPs, as well as the JIM4 arabinogalactan protein epitope, that were not present on the surface of non-embryogenic cells (Samaj et al., 1999).

Another AGP epitope recognized by the JIM8 antibody was originally described as a marker of the very early transitional stage of cultured carrot cells after embryogenic induction (Pennell et al., 1992). Subsequently, it was shown that most embryos develop from cells lacking the JIM8 epitope (Toonen et al., 1996), and that the AGP fraction containing the JIM8 epitope actually has an inhibitory effect on somatic embryogenesis in carrot (Toonen et al., 1997b). Finally it was found that the JIM8 epitope marks a specific small, spherical cell type which asymmetrically transferred the JIM8 epitope, following cell division, to a $JIM8^-$ embryogenic and a $JIM8^+$ apoptotic cell type (McCabe et al., 1997). Cultured cells, sorted on the basis of JIM8 labelling, were attached to secondary antibody-linked paramagnetic beads and were used to demonstrate that the JIM8 epitope represents a soluble signal produced by $JIM8^+$ cells to stimulate embryo development from $JIM8^-$ cells (Pennell et al., 1995; McCabe et al., 1997).

The first observations, indicating that extracellular, secreted proteins may have important roles during somatic embryo development, were derived from studies on the *ts11* temperature sensitive carrot mutant. *ts11* somatic embryos were arrested at the globular stage with aberrant protoderm formation at the restrictive temperature (32 °C) (de Jong et al., 1992). Conditioned medium from cultures of wild type cells (Lo Schiavo et al., 1990) and a 32-kDa acidic endochitinase, purified from the same medium (de Jong et al., 1992), complemented the mutation and allowed normal protoderm formation and development at 32 °C. Cytological investigations highlighted that the *ts11* mutation was associated with defects in the secretory characteristics of the cells, and not exclusively with the reduced level of the endochitinase (Baldan et al., 1997). In embryogenic cultures of *Cichorium*, the major secreted proteins were also identified as chitinases, glucanases and an osmotin-like protein, all of which accumulated at a significantly higher level in embryogenic compared to non-embryogenic cultures (Helleboid et al., 2000b). Similarly, in embryogenic alfalfa cultures, alterations in the levels of extracellular proteins homologous to the carrot endochitinases have been reported following the removal of 2,4-D (Poulsen et al., 1996). Recently, van Hengel et al. (2001) reported that carrot AGPs contained glucosamin and *N*-acetylglucosamine that were sensitive to endochitinase cleavage. It was also demonstrated that the embryogenic competence of protoplasts could be restored and enhanced by the application of AGPs, endochitinase-cleaved forms of which were more efficient (van Hengel et al., 2001). It can be hypothesized that chitinase-modified AGPs are extracellular molecules capable of controlling or maintaining the embryogenic competent cell state (van Hengel et al., 2001).

Genes/proteins involved in the formation of embryogenic cells: who plays the game?

The processes during which somatic cells acquire embryogenic competence obviously involve the reprogramming of gene expression patterns. As was discussed above, the morphology, physiology and metabolism of the cells are significantly altered due to dedifferentiation, activation of cell division and a change in cell fate. All of these changes are dependent on the inactivation of genes functioning in differentiated cells and the activation of those required for the above processes. Obviously, the overall reprogramming of gene expression has to be governed by regulator genes, including those encoding transcription factors. Genes regulating changes in the developmental fate of plant cells have attracted the interest of

plant biologists. It is not surprising, therefore, that since the beginning of plant molecular biological research, there have been attempts in many laboratories to identify genes which may render somatic cells embryogenic. Interestingly, although it was generally believed that somatic embryogenesis would serve as a model to identify key regulators in zygotic embryo development, recent studies of seed and embryo development mutants seem to provide the clues to an improved understanding of the basic regulation of somatic embryogenesis.

Approaches to identify genes activated during the induction phase of somatic embryogenesis

The most common approach to identify somatic embryogenesis-related genes is to compare gene expression in somatic embryos to that of callus cells. This approach resulted in the identification of a few abundant transcripts linked to specific stages of embryogenesis, rather than to the induction period of embryogenic development (for review, Zimmerman, 1993). More recently, similar experiments have resulted in the identification of a set of genes showing differential transcript accumulation during somatic embryo development in conifers (Dong and Dunstan, 1996, 1999).

As this review focuses on the very early stage of somatic embryogenesis, when the developmental transition actually takes place, only those experiments that aimed to isolate genes activated during the induction period will be described. During the last years, more progress has been achieved in this field due to improvements in experimental techniques, including tissue culture systems, as well as mRNA isolation and cDNA synthesis.

Most of the results are derived from the three best-studied systems: carrot, alfalfa and chicory. In carrot, the phase of embryogenic induction can not be clearly defined. Lin et al. (1996) used an approach whereby the mRNA population of globular embryos was compared to that of seedlings. The sensitivity of the cDNA library screening was improved by using a subtracted probe enriched for globular embryo enhanced transcripts. Thirty-eight cDNA clones, representing genes with altered expression during somatic embryogenesis, were identified. The majority of proteins encoded by these genes could be classified into the categories of cell wall proteins, enzymes, pathogenesis-related (PR) proteins, heat-shock proteins, late-embryogenic abundant (Lea) proteins, oleosins, a globulin-like protein, histones, ribosomal proteins, elongation factor 1α and ubiquitin fusion protein. The expression patterns of these clones were highly variable and could serve as markers of the developmental stages of the embryos. However, most could also be detected in undifferentiated callus cells. This limitation showed that the strategy used for the screening was not appropriate to identify genes characteristic for the very early stages. This was considered by Sato et al. (1995), who used cDNA prepared from undifferentiated callus cells to subtract cDNA from proglobular cell clusters developed 3 days after the transfer to hormone-free medium. However, only one of the nine identified clones proved to be specifically characteristic for somatic embryo formation. This clone, named as CEM6, was identified as a glycine-rich protein with a possible function in cell wall synthesis.

The determination of the transcripts of the earliest-activated genes during carrot somatic embryo induction was the goal of the experiments carried out by Yasuda et al. (2001). They used the differential display reverse transcription polimerase chain reaction (DDRT PCR) technique to compare mRNA populations in embryogenic and non-embryogenic cultures. Three cDNA clones were isolated. Clone #43 was preferentially expressed very early in the embryogenic cell clusters and its expression declined in globular embryos. It was also expressed in hypocotyls, the only tissue from which somatic embryos could be initiated, and not in other organs or tissues. This cDNA coded for a thaumatin-like protein and it was suggested that its expression was related to the stress response of the plant cells. Clone #93 represented a cDNA coding for a homologue of the DC 2.15 proline-rich protein (Holk et al., 1996), but without the characteristic Pro-X motifs. The expression of this clone, unlike the heart-shaped embryo-enhanced Dc 2.15, was characteristic of early embryogenic cell clusters, but could also be detected at later stages and in suspension cultured cells. It was suggested that clone #93 represents a transduction factor during somatic embryogenesis. A similar expression pattern could be ascribed to clone #87, which had no homologous sequence in the databases.

Schmidt et al. (1997) followed a more extensive approach. Three different screening techniques were used to search for genes specifically active in embryogenic *versus* non-embryogenic carrot cultures: differential cDNA library screening, cold plaque screening and differential display. All putative, dif-

ferentially expressed clones obtained from the three types of experiments were subjected to a second screening based on a dot-blot reverse Northern hybridization. Differential screening resulted in 30 putative clones, but only one of them could be verified as a gene exclusively expressed in the embryogenic cultures, after the second screening. Six clones derived from differential display RT PCR were sequenced and two of the fragments represented the lipid transfer protein (LTP) gene previously identified as a marker of early embryogenesis and protoderm formation (Sterk et al., 1991; Toonen et al., 1997a). More interestingly, one of the 26 clones obtained after cold plaque screening proved to be the cDNA of a gene expressed at a very low level, but in a very specific way, in embryogenic cultures. The sequence of this cDNA clone showed homology to plant and animal receptor kinases and was named as somatic embryogenesis receptor kinase (SERK). Characteristics of this putative regulator of the somatic-to-embryogenic transition will be discussed later (Genes in transition. Genes/proteins involved in embryogenic cell formation section).

Other tissue culture systems, where embryos can develop directly from somatic cells, were considered to be more appropriate for the isolation of genes regulating early events of somatic embryogenesis. One of these systems is based on a chicory genotype (*Cichorium* hybrid '474'), where somatic embryos develop directly and synchronously on leaf explants under specific conditions (Blervacq et al., 1995). The approaches to identify genes activated during the early phases of chicory embryogenesis resulted in the identification of cDNAs of a β-1,3-glucanase (Helleboid et al., 2000a) and other pathogenesis-related proteins and a non-symbiotic haemoglobin (Hendriks et al., 1998). Although the activities of these genes were characteristic for the transition of somatic cells to embryogenic cells as being related to stress, metabolism and cytoplasmic reorganization (membrane trafficking), it is very likely that none of them is a key determinant of embryogenic cell fate.

Direct somatic embryogenesis is well established for various alfalfa species and genotypes. More advantageously, there are also many closely related genotypes that are non-embryogenic under the same conditions. It is not surprising, therefore, that many attempts have been made to identify early embryogenesis-related genes in these species. Giroux and Pauls (1997) compared mRNA populations of established cultures of embryogenic and non-embryogenic *Medicago sativa* L. genotypes using a conventional differential cDNA library screening approach. Prior to mRNA isolation, cells were fractionated and the 230-μm fraction enriched in embryogenic cells was used to prepare the cDNA library and the probes. The screening resulted in three cDNA clones (*ASET1–3*) specifically present in cells of the embryogenic genotype. Two of the cDNA clones could be sequenced, but clear functions could not be ascribed to either of them based on the sequence information. The clone ASET1 was partial and coded for a hydrophobic protein. The ASET2 cDNA was full length and coded for a protein with structural similarities to integral membrane proteins. It was suggested that these proteins could be important in membrane related signalling events.

In *Medicago falcata*, somatic embryo development can be induced in leaf explants by parallel wounding and 2,4-D application (Denchev et al., 1991). mRNA samples were isolated at different time points, following the induction of direct somatic embryogenesis, and converted to cDNA prior to RNA arbitrarily primed PCR (RAP-PCR) by Fowler et al. (1998). Two different primer combinations were used and the cDNA fragments with differential accumulation during embryo induction were identified and sequenced. Most of the clones had no significant homology to database sequences and the expression patterns of only two of them have been verified. The clone A1.4 was characterized as a calnexin homologue with a potential chaperone function (Huang et al., 1993). Its expression might be regulated in a manner similar to that of heat shock protein genes, also characteristic of somatic embryogenesis (see before). Chaperone activities might be important due to the intensive synthesis of many new proteins during the reactivation and developmental transition of somatic cells. The clone A2.5 exhibited homology with a family of multidrug resistance genes, like the yeast SNQ2 gene (Servos et al., 1993). It was expressed only at the time of globular embryo formation. The role of these proteins during somatic embryogenesis needs to be clarified.

Differential screening of a cDNA library made from embryogenic *Medicago sativa* cell cultures, treated with a high 2,4-D concentration to induce somatic embryogenesis, and screened with cDNA from un-induced cells maintained in the presence of NAA, resulted in the isolation of cDNAs coding for small heat shock proteins (Dudits et al., 1991) a proline-rich protein (Györgyey et al., 1997) and a

calcium-binding protein with unknown function (Dudits et al., 1991).

In another approach, PCR-based cDNA subtraction was used to identify differentially expressed genes in induced and non-induced *Medicago falcata* leaves (Russinova et al., 1998). Due to the sensitivity of the approach, a very high number of clones were identified. They were classified into 'early' (from the induction onward), 'medium' (from 3 days after the induction) and 'late' (expression after 5–10 days) expression categories. The sequences obtained revealed the presence of many regulatory genes, such as genes of transcription factors, kinases, the phosphatase PP2C, and auxin-induced genes. Several ribosomal proteins and others related to translation and post-translational protein processing were also represented, as well as proteins linked to stress response (pathogenesis related and ABA-responsive proteins, etc.). In this system, somatic embryos were formed on leaf pieces as a result of the combined action of wounding and 2,4-D. However, many of the identified genes could be induced by wounding or 2,4-D alone. That is why further analysis is required to elucidate the potential significance of the protein products of these genes during somatic embryogenesis.

In *Lycium barbarum* (Kairong et al., 1999) three transcripts were identified by differential display that were expressed in embryogenic calli and in early somatic embryos, but not in non-embryogenic callus culture. The partial sequences could not verify any significant homology to known sequences in the databases.

Genes in transition: genes/proteins involved in embryogenic cell formation

Of the above-mentioned cDNA sequences, there is only one gene known to play a role in the acquisition of embryogenic competence in plant cells. This is the somatic embryogenesis receptor kinase (SERK) gene isolated by Schmidt et al. (1997). In carrot, SERK expression was shown to be characteristic of embryogenic cell cultures and somatic embryos, but its expression ceased after the globular stage (Schmidt et al., 1997). It could also be detected in zygotic embryos up to the early globular stage, but not in unpollinated flowers or in any other tissue. Using the SERK promoter fused to the luciferase gene and video cell tracking, it was also shown that SERK-expressing single cells could develop into somatic embryos (Schmidt et al., 1997). The *Arabidosis* homologue of the carrot SERK cDNA has also been cloned as one of five members of a small gene family (Hecht et al., 2001). It was shown that *in planta*, AtSERK1 expression was first expressed during megasporogenesis and then in the functional megaspore, in all cells of the embryo sac until fertilization and in the embryo up to the heart stage. After this stage, expression was undetectable in any part of the developing seed. Low expression was, however, detected in adult vascular tissues. AtSERK1 gene expression was also observed in the shoot apical meristem and cotyledon of auxin-grown *Arabidopsis* seedlings used to initiate embryogenic callus cultures. These observations indicated that AtSERK1 expression was not restricted to embryogenic cells, but characteristic of those cells capable of rapid response to hormonal signals and competent to form somatic embryos or embryogenic cell cultures (Hecht et al., 2001).

Ectopic expression of the AtSERK1 cDNA under the control of 35S promoter did not cause any specific phenotype, however, the efficiency of the initiation of somatic embryos was increased by approximately 4-fold in the transgenic seedlings (Hecht et al., 2001). AtSERK1 gene expression was higher in cell cultures derived from the *altered meristem program 1* (*amp1*) mutant of *Arabidopsis*, which has enhanced embryogenic capabilities (Mordhorst et al., 1998; Hecht et al., 2001). These mutants also had an increased number of undifferentiated cells in their shoot meristem, and it was hypothesized that AMP1 activity is required to suppress embryogenic development (e.g., by suppressing SERK activity) after germination (Hecht et al., 2001).

The expression of a SERK homologue gene was also tested in *Dactylis glomerata*, where somatic embryogenesis could be initiated directly from leaf explants, as well as indirectly from leaf-derived callus tissues (Somleva et al., 2000). In this system, SERK expression was present in small, cytoplasm-rich isodiametric cells that were shown to form somatic embryos by cell tracking. In contrast to carrot and *Arabidopsis*, SERK expression was maintained beyond the globular stages of embryogenesis in meristematic regions (Somleva et al., 2000). Based on all of the above experiments, the expression of the SERK gene may be used as a marker of embryogenic competence. And the SERK may represent a possible candidate for mediating signals which are required to initiate the embryogenic development. Other leucine-rich repeat-containing receptor-like kinases are

known to be involved in different developmental processes in plants, such as the ARABIDOPSIS CLAVATA and ERECTA proteins (for review, Fletcher and Meyerowitz, 2000) and the *Petunia* PRK1 protein, which is involved in signalling during pollen development and pollination (Mu et al., 1994). For SERK, the possible ligands and the downstream targets have to be elucidated in order to define its exact role in embryogenesis.

While ectopic expression of the SERK gene was shown to enhance the embryogenic response of cultured cells induced to form somatic embryos, the ectopic overexpression of the *lec1* gene of *Arabidopsis* was shown to induce embryo development directly on vegetative tissues in the absence of any exogenous treatments (Lotan et al., 1998). The *lec1* gene was identified by studies of a mutation causing defects in embryo maturation and desiccation (Meinke, 1992; Meinke et al., 1994). Cloning and sequencing of this mutated gene revealed that it coded for a protein homologous to a subunit of a CCAAT box-binding transcription factor (Lotan et al., 1998). Further studies also revealed that the protein might function during early stages of embryogenesis in the suppression of suspensor proliferation, among other roles (Lotan et al., 1998). The *lec1* gene is expressed from the octan stage up to the late torpedo stage of embryogenesis. When the *lec1* cDNA was expressed in transgenic plants under the control of the 35S promoter, several abnormalities were observed, which indicated that embryo-specific programs were not completely shut off or had been reinitiated in the seedlings. This was confirmed by the ectopic expression of embryo-specific markers in these plants. More interestingly, two plants among the T2 progenies of the few fertile transgenic lines exhibited a phenotype in which embryo-like structures developed on their leaves. These structures expressed embryogenic markers, thus confirming the activation or maintenance of a gene expression program characteristic of embryogenesis (Lotan et al., 1998).

More recently, another gene, *lec2*, has been identified in *Arabidopsis*, having similar characteristics (Stone et al., 2001). The function of *lec2* was also shown to be required during both early and late zygotic embryogenesis. It also codes for a transcription factor, but has a plant specific B3 protein domain and as such, is similar to the transcription factors viviparous1/ABA insensitive3 and fusca3, acting primarily in developing seeds. Ectopic, postembryogenic expression of *lec2* in transgenic plants also resulted in the formation of somatic embryos and other organ-like structures and conferred embryonic characters to vegetative tissues (Stone et al., 2001). Thus, both *lec1* and *lec2* can be considered to be transcriptional regulators that can establish a cellular environment sufficient to initiate embryo development. Whether similar genes play key roles in the initiation of embryo formation during zygotic and *in vitro* induced somatic embryogenesis remains to be determined.

Another family of transcription factors, those containing the so-called MADS-box domain, are also important regulators of many plant developmental processes (Jack, 2001a). One of the best-described examples of their importance in plant development is the regulation of flower development and floral organ identity (Jack, 2001b). It was shown by Perry et al. (1999) that the MADS-box containing AGAMOUS-like 15 (AGL-15) transcription factor accumulated in embryos of diverse origin, including zygotic, apomictic, microspore-derived and somatic embryos. In addition to its high level in embryonic tissues, the subcellular localization of the protein was also changed when embryonic development began: its originally cytoplasmic localization became nuclear. Using promoter-GUS gene fusions, it was shown that its expression is not restricted to embryogenesis and it is more likely linked to a juvenile tissue state. This was confirmed by the ectopic expression of the AGL-15 cDNA, which caused a delay in many aging-related processes (Fernandez et al., 2000). These results indicated that although this protein is associated with embryogenesis, it has a more general function in plant development. Recently, a cDNA of another MADS-box containing transcription factor was identified by investigating differentially expressed genes during *Brassica* microspore embryogenesis (Boutilier et al., 2000). When this cDNA was overexpressed under the control of the 35S promoter in transgenic plants, ectopic formation of embryos and cotyledons on leaves was observed, due to which the gene was named *babyboom* (*bbm*). There are no data about its role during zygotic or somatic embryogenesis, but it can be supposed to have a general role during the different forms of plant embryogenesis.

Gene activation tagging was used by Zuo et al. (2002) to identify genes whose over-expression induces the formation of somatic embryos on *Arabidopsis* tissues without the need for external hormonal treatments. The identified allele, PGA6, was found to be identical to WUSCHEL (WUS), a homeodomain protein previously shown to be involved in specifying

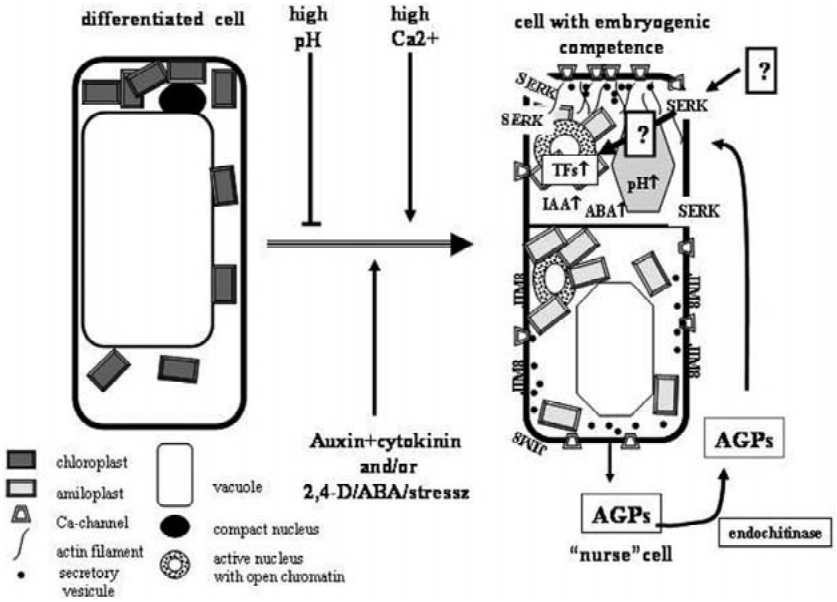

Figure 2. A schematic and generalized model of somatic embryogenic cell formation. The data are from different experimental systems and have not been experimentally proved to act together on the hypothetical way shown in the figure. The proper order and timing of the different events is not indicated to maintain simplicity. Differentiated cells, if subjected to appropriate signals and hormones (wounding, stress, auxin, cytokinin, ABA) dedifferentiate and divide in parallel with chromatin remodelling. General chromatin decondensation characterizes dedifferentiation while remodelling of chromatin structure might also be required for the initiation of the embryogenic path of development. Dedifferentiation and cell division are delayed by high medium pH and embryogenic cell formation is also dependent on cellular pH changes. Embryogenic competent cell is formed if the cell division-promoting auxin signal coincides with a stress signal. Effectiveness of inducing signals is dependent on the endogenous hormone levels. Formation of the embryogenic cell type is promoted by external Ca^{2+}. In embryogenic competent and embryogenic cells, polarity is established as indicated by the asymmetric distribution of the JIM8 arabinogalactan protein (AGP) epitope, the Ca^{2+}-channels, actin filaments and by polarized secretion of cell wall material. Division of embryogenic competent cells results in $JIM8^{+}$ nurse cells providing soluble signals (AGPs?) to promote development of embryogenic cells. The AGP-signal is generated by the contribution of endochitinases. Embryogenic competent cells are marked by the expression of the somatic embryogenesis receptor kinase (SERK). Neither the ligand(s) nor the targets of this putative receptor are known at present. At the end of the signalling cascade transcription factors like *bbm*, *lec1* or *lec2* are activated and they switch on the embryogenic pathway of development.

stem cell fate in shoot and floral meristems. WUS/PGA6 presumably promotes a vegetative-to-embryogenic transition and/or maintains embryonic stem cell identity.

Finally, as we have discussed above (Chromatin remodelling: open for changes section), regulators of chromatin remodelling likely play a key role in suppressing embryogenic developmental pathways, and thus can be considered as main regulators of the developmental switch during somatic embryo induction (for review, e.g., Grossniklaus et al., 2001).

Concluding remarks

Somatic embryogenesis is a unique experimental model to study developmental flexibility in higher plants. During the last few years there has been a considerable increase in the amount of information related to somatic embryogenesis gained in different culture systems, especially carrot, chicory, alfalfa and conifer tissue cultures. In spite of this accumulation of experimental data, we are still far from understanding the key events underlying the transition of differentiated somatic cells to the totipotent and embryogenic cell state. The recent identification of genes that are markers of switch in cell fate (like SERK) or are themselves capable of inducing embryogenic development in somatic cells (like *bbm*, *lec1* and *lec2*) has opened up new approaches to the question of developmental flexibility and determination in plants.

Interestingly, although somatic embryogenesis is considered to be a model proposed to understand early events of zygote development, investigations on

Arabidospsis embryogenesis mutants (*gnom*, *lec1* and *lec2*, *pkl*, etc.) have provided, and may still provide, important clues to somatic embryogenesis.

In addition to regulation at the level of gene expression, the importance of the physiological state of the cells, the presence of cell wall-derived or secreted signals, endogenous hormones and the interaction of different signalling cascades is evident from many studies. Based on these data, derived from different experimental systems, including those where somatic embryo development is directly induced on the surface of explants and those where the establishment of embryogenic cell cultures is necessary, we propose a model describing the present knowledge of the characteristics of an embryogenic cell. This schematic and generalized model of a 'virtual' embryogenic cell is shown in Figure 2.

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