Chapter 9
Somatic Embryogenesis

1. INTRODUCTION

During the course of evolution, many plant species have evolved different methods of asexual embryogenesis, including somatic embryogenesis, to overcome various environmental and genetic factors that prevent fertilization. Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos. Somatic embryos resemble zygotic embryos morphologically. They are bipolar and bear typical embryonic organs. However, they develop via a different pathway. Somatic embryogenesis occurs to a limited extent under natural conditions, within ovules (e.g., *Paeonia*) and more rarely on leaves (e.g. *Asplenium* and *Kalanchoe*). Since the first observation of somatic embryo formation in *Daucus carota* cell suspensions by Steward *et al.* (1958) and Reinert (1958) the potential for somatic embryogenesis has been shown to be characteristic of a wide range of tissue culture systems in plants. During the past 40 years, somatic embryogenesis has been described in a large number of plant species. New species and modified methods are continuously reported and described and their number continuously increases. Methods for bringing about this kind of morphogenesis are also steadily being modified and improved. Somatic embryogenesis can probably be achieved for all plant species provided that the appropriate explant, culture media and environmental conditions are employed. In this chapter we shall highlight important aspects of somatic embryogenesis. We shall not give details for different species since this field of research is developing very fast and it is important to search for the latest information; this can easily be done on the web. Somatic embryos are used as a model system in embryological studies. However, the greatest interest of somatic embryos is centred in its practical application for large-scale vegetative propagation, particularly because of the possibility to scale up the propagation by using bioreactors (see Chapter 1). In addition, in most cases, somatic embryos or embryogenic cultures can be cryopreserved, which makes it possible to establish gene banks. Embryogenic cultures are also an attractive target for gene transformation.

2. PLANT EMBRYOGENESIS

Before starting to work with somatic embryogenesis, it is crucial to have a basic knowledge about the embryology of the species of interest. Plant embryogenesis begins with the zygote and passes through a stereotyped sequence of characteristic stages. Although considerable morphogenesis occurs after seed germination, the embryonic phase is crucial as it is here that meristems and the shoot-root body pattern are specified. Angiosperms and gymnosperms became separated about 300 million years ago. Since their embryology differs in many aspects we shall briefly describe embryology in one angiosperm, *Arabidopsis*, and one gymnosperm, *Pinus* (Fig. 9.1).

2.1. FERTILIZATION

One of the characteristics of angiosperms is the process of double fertilization, where both male gametes participate in an act of fusion: one unites with the egg cell to form the diploid zygote from which the embryo develops, while the other gamete fuses with the central cell of the embryo-sac which then develops into the triploid endosperm. The egg cell is polarized, having a nucleus at the cytoplasm-rich chalazal pole while the micropylar pole is highly vacuolated (Russell, 1993). The microtubular cytoskeleton and the location of actin microfilaments within the cytoplasm are also polarised (Dodeman *et al.*, 1997). The cytoplasm is predominantly maternally inherited in angiosperms, although there are species with uniparental, paternal or biparental cytoplasmic inheritance (Dumas *et al.*, 1998). Typically, an angiosperm zygote is briefly quiescent after karyogamy. The endosperm is first syncytial and later becomes cellular in most taxa (Otegui and Staehelin, 2000). The endosperm plays a role in embryo nutrition as it accumulates reserves of starch, proteins and lipids. Genetic analysis suggests that maternal and endosperm tissues may regulate each others' development (Lopes and Larkins, 1993).

Gymnosperms display single fertilization since only the larger male gamete migrates through the egg cytoplasm and fuses with the egg nucleus in the centre of the egg cell while the smaller male gamete degenerates. The zygote is surrounded by neocytoplasm consisting of a large fraction of the egg and

E. F. George et al. (eds.), Plant Propagation by Tissue Culture 3rd Edition, 335–354.

male nucleoplasm, and some of the male cytoplasm (Singh, 1978). Typically, in gymnosperms, cytoplasmic inheritance has a biparental character with paternal inheritance of chloroplasts and maternal inheritance of mitochondria (Neale and Sederoff, 1989). The megagametophyte originates from the megaspore and undergoes development prior to fertilisation. The first phase of megagametophyte development is characterised by an extensive series of cell-free nuclear divisions. Then, wall formation begins at the periphery and proceeds towards the centre until the entire megagametophyte is cellular (Gifford and Foster, 1987). As the embryo begins to differentiate, the cells of the megagametophyte accumulate reserves of starch, proteins and lipids. In the early stages of development, the embryo is nourished by the egg cytoplasm through the suspensor and it is only later that it begins to draw upon the cells of the megagametophyte. However, the bulk of these reserves are held over by the megagametophyte for use by the germinating embryo (Raghavan and Sharma, 1995).

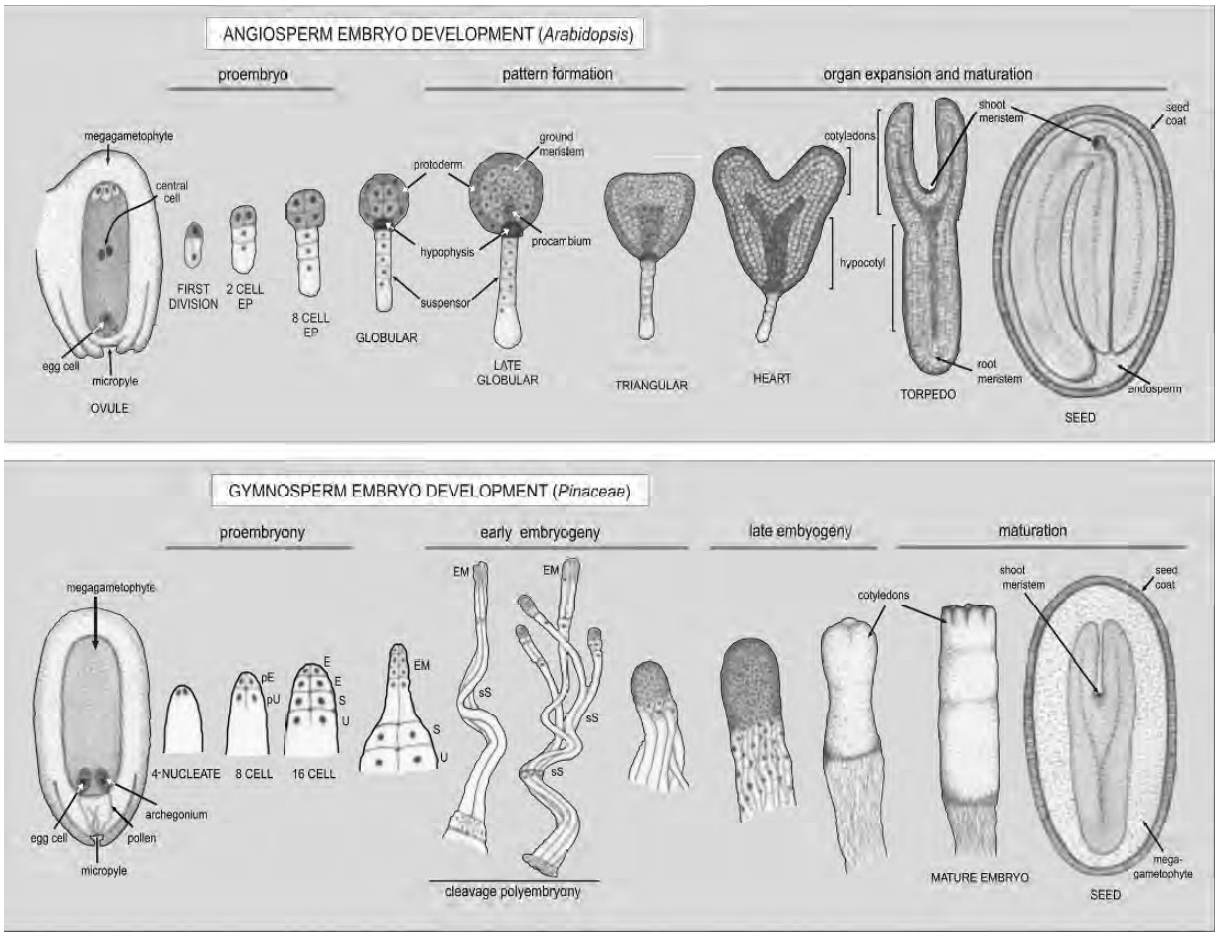

Fig. 9.1 Schematic overview of angiosperm *(Arabidopsis)* and gymnosperm *(Picea)* embryo development. The illustrations are not to scale. Drawings were prepared based on Goldberg *et al.* (1994) and Giffort and Foster (1987). Abbreviations: EP - embryo proper, pU - primary upper tier, pE – primary embryonal tier, E - embryonal tier, S - suspensor tier, U - upper tier, EM – embryonal mass, sS - secondary suspensor.

2.2. STAGES DURING EMBRYO DEVELOPMENT

Embryo development in angiosperms can be divided into two main steps (Dodeman *et al.,* 1997):

1. Embryogenesis *sensu stricto*, beginning with the zygote and finishing at the cotyledonary stage.

The development through the globular, heart, torpedo and cotyledonary stages can be divided into a sequence of different stages representing three major events (Goldberg *et al*., 1994):

i. Asymmetric division of the zygote, giving rise to a small apical cell and a large basal cell.
ii. Specific pattern formation, which takes place in the globular embryo.
iii. Transition to the cotyledonary stage which coincides with the initiation of the root primordium followed, in dicots, by the shoot primordium.

2. Maturation of the embryo followed by germination.

The sequence of embryo development in gymnosperms can be divided into three phases (Singh, 1978):

1. Proembryogeny - all stages before the elongation of the suspensor.
2. Early embryogeny - all stages after elongation of the suspensor and before establishment of the root meristem.
3. Late embryogeny - intensive histogenesis including establishment of the root and shoot meristems.

These three steps in gymnosperm embryo development correspond to the stages described above for embryogenesis *sensu stricto* in angiosperms and are followed by the period of embryo maturation.

2.3. ASYMMETRIC DIVISION OF THE ZYGOTE/PROEMBRYOGENY.

In angiosperms, the zygote divides a few hours after fertilisation at which time it already has a highly polarized appearance, displaying uneven distribution of organelles and metabolites (Goldberg *et al*., 1994). Such cytological and physiological polarity in the zygote affects the ultrastructure of the daughter cells. The first division is almost invariably asymmetric and transverse, cutting off a large vacuolated basal cell and a small, densely cytoplasmic terminal cell. The organogenic part of the embryo is derived from the terminal cell with little or no contribution from the basal cell. The asymmetric pattern of the first division is an important feature for the initiation of the embryogenic developmental pathway. It has been suggested that asymmetrical division is probably the consequence of nuclear migration from the central region of the cell to the periphery (Duditis *et al*., 1995).

There are four types of proembryogeny in gymnosperms, of which the conifer type is the most common and is interpreted as a basal plan for gymnosperm embryogeny (Wehmeyer *et al*., 1993). A common feature of gymnosperm embryo development is the free nuclear stage which, with the exception of the dicotyledonous genus *Paeonia* (Yakovlev and Yoffe, 1957), is not seen in any other plant group. The number of free nuclei differs among species. In *Picea* and *Pinus*, which represent the conifer type of embryogeny, four nuclei are present at the free nuclear stage. Proembryo development begins when the fertilised egg nucleus divides into two, then four, free nuclei contained within a dense region of cytoplasm (neocytoplasm). The four free nuclei are arranged in a tier at the chalazal end of the archegonial sac. After division, two tiers of four nuclei each are formed, the primary embryonal tier and the primary upper tier which soon become cellular. Internal divisions of these two tiers produce four tiers of four cells each. The lower two tiers constitute the embryonal tier, the tier above it is called the suspensor tier. Cells of this tier may elongate to form the primary suspensor; they are often dysfunctional but can show some meristematic activity. The uppermost tier degenerates. The upper four cells of the embryonal tier elongate to form a functional suspensor (called an embryonal suspensor) and the lower four cells of the embryonal tier form the embryonal mass. The proembryo stage lasts approximately one week.

2.4. PATTERN FORMATION IN THE GLOBULAR EMBRYO/EARLY EMBRYOGENY

In angiosperms the divisions of the zygote up to the globular stage embryo are in predictable planes (Raghavan and Sharma, 1995). When first formed, the embryo proper consists of a mass of relatively undifferentiated cells. Soon, however, changes in the internal structure of the embryo result in the initial development of the tissue system of the nascent plant. Two critical events take place after the embryo proper is formed (Goldberg *et al*., 1994):

1. the specification of the radial pattern with three primordial tissue layers, and
2. the differentiation of the regions along the apical-basal axis from which embryonic organ systems generate.

The first tissue to differentiate is the protoderm which is formed by periclinal divisions of cells of the early globular embryo. The formation of the protoderm, which restricts cell expansion, is essential for the remaining developmental phases (Dodeman *et al.,* 1997). Subsequent cell differentiation events within the embryo proper result in the production of an inner procambial layer and a middle layer of ground meristem cells. Differentiation of these three

tissue layers establishes a radial axis in the globular embryo (Goldberg *et al.,* 1994).

Three major regions can be distinguished along the axis of apical-basal polarity: an apical region giving rise to the shoot meristem and the cotyledons, a central region including hypocotyl, embryonic root (radicle) and the initials of the root cap and a basal region corresponding to the hypophysis, which gives rise to the quiescent centre of the root meristem and the central root cap (Jurgens *et al*., 1994).

During early embryogeny in gymnosperms, cells of the lower embryonal tier divide, creating an embryonal mass. The basal cells of the embryonal mass continue to divide predominantly in a transverse plane and elongate, contributing to the thick secondary suspensor. The lack of the restriction of cell divisions means that even the surface layers continue to divide periclinally as well as anticlinally, preventing differentiation of a distinct protoderm (Singh, 1978). However, this outer cell layer does function as a protoderm (Romberger *et al.,* 1993). The club-shape early embryo enlarges rapidly filling the corrosion cavity that forms in the female gametophyte (Owens and Molder, 1984).

2.5. ESTABLISHMENT OF ROOT AND SHOOT MERISTEMS/ATE EMBRYOGENY.

In dicotyledonous species, the root primordium emerges at the end of embryo pattern formation. Cotyledons are specified from two lateral domains at the apical end. The formation of cotyledons results in the characteristic heart stage. At the same time the hypocotyl region of the axis begins to elongate. Morphogenic changes during this period are mediated by differential cell division and expansion (Goldberg *et al*., 1994). The shoot meristem forms later in embryogenesis from cell layers localised in the upper axis between the two cotyledons. In monocotyl-edonous species both the root and the shoot meristems are laid down in a lateral fashion rather than distally. As a result, the axis of the mature embryo does not correspond to the axis of the proembryo (Mordhorst *et al*., 1997). The distal region above the shoot meristem greatly expands to form the scutellum. Late embryogeny in gymnosperms is a period of histogenesis and organo-genesis. Early during this period, the root and shoot apical meristems are delineated and the plant axis is established. The root apical meristem forms near the centre of the embryo first as a root-organisation centre. The shoot meristem originates at the distal part of the globular embryonal mass and it is relatively superficial compared to root organisation centre. The cotyledon primordia arise in a ring around the distal end of the embryo. At this stage provascular tissue and cortex also differentiate. In *Pinaceae*, the protoderm covers only the shoot/hypocotyl region but in other gymnosperms it covers the entire surface of the embryo (Romberger *et al*., 1993).

2.6. MATURATION.

A remarkable change occurring during this period is that the developmental programme switches from pattern formation to storage product accumulation in order to prepare the young sporophyte for dormancy and postembryonic development. Following the previous period of cell division and histodifferentiation, there is a period of embryo development in which the major cell expansion and storage product deposition occur called the maturation phase. The maturation program is responsible for:

1. synthesising large amounts of storage products,
2. inducing water loss,
3. preventing premature germination, and
4. establishing a state of dormancy (Goldberg *et al.,* 1994).

The rate of synthesis and deposition of storage proteins, lipids and starch increases and results in cell expansion in both cotyledons and axis. Cell vacuoles exhibit a specialised behaviour during maturation in that they split up and dehydrate to give rise to protein bodies and aleurone grains (Dodeman *et al*., 1997; Bethke *et al*., 1999). At the end of the maturation phase seeds enter dormancy (Goldberg *et al*., 1989). An essential regulator of the process is ABA. The concentration of ABA peaks in abundance during late embryogenesis (Bewley and Black, 1994), modulating gene expression, at least at the transcriptional level. The mature seeds are classed as orthodox or recalcitrant (Engelmann, 1991). The embryos of orthodox seeds undergo maturation drying while recalcitrant seeds do not and are generally dessication intolerant. The majority of angiosperm and conifer seeds are of the orthodox type. Orthodox seeds can be further divided into quiescent or dormant type (Bewley and Black, 1994). Quiescent seeds can germinate after addition of water while dormant seeds require additional factors to germinate. Orthodox seeds are more resistant to diverse conditions and can survive in more extreme environments.

2.7. SUSPENSOR SYSTEMS

The suspensor is a dynamic temporal structure with important functions during embryogenesis (Yeung and Meinke, 1993). The suspensor functions early in embryogenesis and it then undergoes programmed cell death (Nagl, 1976; Meinke, 1995). It appears from extensive structural, biochemical, and physiological studies with a variety of angiosperms that the suspensor plays an active role early in development by promoting continued growth of the embryo proper. Being active nutrient transporters and important sources of plant growth regulators such as auxins, gibberellins, cytokinins and ABA (Raghavan and Sharma, 1995). during early stages of embryogenesis, suspensor cells often display increased transcriptional activity (Marsden and Meinke, 1985; Forino *et al*., 1992; Yeung and Meinke, 1993). Compared to the cells of the embryo proper, suspensor cells contain more RNA and proteins and synthesise them more efficiently than embryo cells of the same age.

At the same time as the suspensor promotes growth of the embryo proper, suspensor growth is inhibited by the embryo proper (Mardsen and Meike, 1985; Yeung and Meinke, 1993). The developmental potential of the suspensor is often greater than its normal developmental fate. This potential is revealed only when the inhibitory effect of the embryo proper is disturbed, which leads to misregulation of programmed cell death in the suspensor, as evidenced from two *Arabidopsis* embryo specific mutants. One mutant (*twin*) displays abnormal proliferation of suspensors giving rise to multiple embryos (Vernon and Meinke, 1994; Zhang and Somerville, 1997), while another mutant *(raspberry*) fails to undergo the transition from globular to heart stage and continues to proliferate both in suspensor and embryo proper regions (Yadegari *et al*., 1994).

However, somatic embryogenesis can sometimes occur without simultaneous development of a normal suspensor suggesting that either the suspensor does not play a crucial role in embryo development or components in the culture system replace the need for a suspensor. The non-embryogenic single cells present in embryogenic cultures of *Daucus carota* have been shown to have a stimulating effect on embryogenesis. Thus the suspension-cultured cells that have retained certain aspects of suspensor cells may possibly take over the role of the suspensor cells *in vitro*.

3. REGULATION OF EMBRYO DEVELOPMENT

During embryogenesis, the zygote undergoes a complex series of morphological and cellular changes that establish the morphogenetic pattern of the plant and the meristematic tissues required for post-embryonic development. Large numbers of genes must be expressed in a highly coordinated manner to ensure that the single-celled zygote develops into an organised, multicellular structure.

More than 3 x 10^4 different genes are expressed in embryos and seedlings (Thomas,1993). It was estimated that about 3500 different genes are necessary to complete embryo development. Of these, ca. 40 genes may direct the formation of all body pattern elements in the *Arabidopsis thaliana* embryo (Mayer *et al*., 1991). However, progress in studying gene regulation during embryo development is limited by inaccessibility of the embryo, particularly at the early stages of embryogenesis. This has to some extent been overcome by the use of *in vitro* fertilisation, somatic embryogenesis and embryo defective mutants which have greatly increased our understanding of the regulation of embryo development.

3.1. ESTABLISHMENT OF CELL FATE IN THE EMBRYO

There are two mechanisms for generating cell fate diversity: unequal division of a polarized cell and position-dependent cell fate determination (Laux and Jurgens, 1997). Both these mechanisms operate during embryo development. The correct cell division planes play a crucial role during embryogenesis since division of a polarised cell partitions the cytoplasm and any regulatory molecules contained therein. As a result of asymmetrical divisions the daughter cells may inherit different cytoplasmic determinants and thereby acquire different fates. One transcription factor encoded by the *SHORT-ROOT* (*SHR*) gene, which is required for the asymmetric cell division responsible for formation of ground tissue (endodermis and cortex) as well as specification of endodermis in *Arabidopsis* roots, has been suggested to participate in a radial signalling pathway (Helariutta *et al*., 2000).

The final cell fate is determined by positional information and/or by events established earlier on and transmitted down cell lineage. However, except for the early epidermal cell fate, no evidence has been

found for the existence of a rigid cell lineage in plants. Irish and Sussex (1992) suggested the term "probability map" rather than "fate map" to emphasise the absence of a rigid cell lineage. Cell position rather than previous developmental history is considered to be essential for the formation of somatic tissues (Dawe and Freeling, 1991). Therefore, in general terms, the establishment of cell fate during embryo development involves localised activity of specific regulatory proteins, as a consequence of the localised expression of particular genes (Lindsey and Topping, 1993). Although cell-cell communication is thought to play an important role in patterning of the plant embryo, the underlying molecular mechanisms is not understood. Two mechanisms have been proposed: signalling across the cell surface via leucine-rich repeat receptor serine-threonine kinases, and exchange of molecules through plasmadesmata (see review by Mayer & Jurgens, 1998).

Clonal analysis has indicated that stem cell fate is specified by positional information and imposed on the cells that currently reside at the summit of the shoot meristem. Only the progeny of the stem cells that stay at this position remain pluripotent, whereas daughter cells that leave this position differentiate (Mayer *et al*., 1998; Bowman and Eshed, 2000). Molecular mechanisms co-ordinating these two ant-agonistic processes in the *Arabidopsis* shoot meristem are now known to be controlled by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes (Schoof *et al.,* 2000).

3.2. EMBRYO MUTANTS

Mutagenesis and the subsequent identification and analysis of mutants have yielded several classes of mutant forms arrested at different stages of embryo development (Lotan *et al*., 1998, with refs.). Most screens have been done in *Arabidopsis*. Genetic analysis of different mutants leads to the conclusion that three basic elements of embryonic development, viz. pattern formation, morphogenesis and cytodifferentiation, are regulated independently.

Many of the mutants arrested early in development are likely to be affected in basic housekeeping functions that first become essential during early stages of development. Those which have been characterised are involved in basic functions such as biotin biosynthesis (*bio 1,* Shellhammer and Meinke, 1990), cell division and cell expansion (*EMB30*, Shevell *et al*., 1994) and intron splicing (*sus2*, Meinke, 1995). Other mutants are likely to be defective in genes that play a more direct role in plant growth and development. However, in some cases it may not be possible to make a clear distinction between housekeeping and regulatory functions because many genes are likely to perform cellular functions which are directly related to both growth and morphogenesis.

One class of mutations includes the suspensor mutants (*twin*, *raspberry*), which are an indirect consequence of altered balance between suspensor and embryo proper development (Vernon & Meinke, 1994; Yadegari *et al*., 1994; Zhang & Somerville, 1997). Disruption in signals between suspensor and embryo proper causes the suspensor to take on an embryo-like fate. Analysis of *raspberry* mutants, which are blocked at the globular stage but still differentiate tissue layers in their correct spatial context, indicates that tissue differentiation can take place independently of both organ formation and morphogenesis (Yadegari *et al*., 1994).

During embryo development three spatial domains are formed along the longitudinal axis: the apical domain composed of the cotyledons, shoot apex, and upper hypocotyl, the central domain including the majority of the hypocotyl and the basal domain consisting primarily of the root (West and Harada, 1993). Evidence for these domains was obtained by analysis of several apical-basal mutants, such as *gurke*, *fackel*, *monopteros* or *gnom* lacking one of the embryonal domains. Moreover, these domains can develop independently of each other (Goldberg *et al*., 1994). Although detailed mechanisms of this apical-basal axis formation are not yet clear, it has been postulated that they may originate from an intrinsic polarisation of the zygote, with the surrounding tissue possibly influencing the orientation of the axis (Mayer & Jurgens, 1998).

Analysis of *gnom* and *monopteros* mutants sheds light on the importance of auxins in pattern- and organ formation. The *MONOPTEROS* gene encodes a transcription factor, possibly involved in auxin signalling. A model was proposed in which a gradient distribution of auxins in the globular embryo is essential in mediating the transition from radial symmetry to bilateral symmetry, finally leading to shoot meristem formation (Fisher and Neuhaus, 1996). The GNOM protein was proposed to regulate vesicle trafficking required for the coordinated polar localisation of auxin efflux carriers which in turn determines the direction of auxin flow (Steinmann *et al*., 1999).

Nine recessive mutations derived from four independent loci causing the deletion of the shoot apical meristem (SAM), have been characterised in rice (Satoh *et al*., 1999; Chapter 8). Analysis of these mutants showed that differentiation of radicle and scutellum is regulated independently of SAM but that of coleoptile and epiblast may depend on SAM.

The formation of a SAM is the outcome of a successive patterning process initiated very early in embryo development. Once the SAM is established, expression of the *WUSCHEL* gene defines a group of cells that function to specify overlaying neighbours as stem cells (Mayer *et al*., 1998). The *CLAVATA* (*CLV1* and *CVL3*) and the *SHOOT MERISTEMLESS* (*STM*) genes specifically regulate shoot meristem development in *Arabidopsis*. *CLV* and *STM* have opposite functions: *clv1* and *clv3* mutants accumulate excess undifferentiated cells in the shoot meristem, while *stm* mutants fail to form the undifferentiated cells of the shoot meristem during embryonic development (Clark *et al*., 1996, with ref.). *CLV1,* which encodes a receptor kinase (Clark *et al*., 1997) acts together with *CLV3* to control the balance between meristem cell proliferation and differentiation (Fletcher *et al.*, 1999).

Arabidopsis mutants with enlarged SAMs such as *clv* and *primordia timing* (*pt*) give rise to stable embryogenic cultures (Mordhorst *et al*., 1998). The authors suggest that an increased population of noncommitted SAM cells are responsible for facilitated establishment of somatic embryogenesis. Genetic studies have revealed that, in *Arabidopsis*, the *ABA-INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*) and *LEAFY COTYLEDON1* (*LEC1*) loci play major roles in regulating maturation (Kurup *et al*., 2000). All three promote embryo-specific processes and simultaneously repress germination. They interact to regulate several processes during seed maturation, including accumulation of chlorophyll, desiccation tolerance, sensitivity to ABA and expression of storage proteins (Parcy *et al.,* 1997; Wehmeyer & Vierling, 2000). *FUS3* and *LEC1* regulate abundance of the ABI1 protein. However, the *lec1* mutation is pleiotropic and by analysing the effects of the *lec1* mutation on embryo development it was shown that *LEC1* is an important regulator of embryo development that activates the transcription of genes required for both embryo morphogenesis and cellular differentiation (Lotan *et al*., 1998).

3.3. GENE EXPRESSION DURING EMBRYOGENESIS

Although the newly formed zygote contains both maternal and paternal genetic information, it seems that the activity of many genes acting during early embryo and endosperm formation may depend solely on transcription from the maternally inherited alleles since the paternal genome is initially silenced (Vielle-Calzada *et al*., 2000).

The general feature underlying the expression of embryo-specific mRNA sequences is that they appear and decay at various times during embryogenesis. This indicates that the expression of each set of genes is controlled by specific regulatory signals. What causes the activation of specific genes at specific stages of embryogenesis is not well understood.

The involvement of plant homeobox genes in embryogenesis was first demonstrated by the analysis of the *Arabidopsis stm* mutant. *STM* encodes a KNOTTED1 (KN1)- type homeodomain protein and is expressed in the region of SAM during embryogenesis (Smith *et al*., 1995). Localised expression of other *KN1*-type homeobox genes has also been observed around the region in which the SAM develops early in embryogenesis (Sentoku *et al*., 1999). Another example is the *ATML1* gene, belonging to the homeodomain-leucine zipper (*HD-Zip*) transcription factors, which is expressed specifically in the apical cell after the first division of the zygote. Later, at the globular stage, the expression of *ATML1* is restricted to the protoderm (Lu *et al*., 1996). Furthermore, a family of HD-Zip genes with cell-layer-specific expression patterns defining subdomains of the embryo and certain meristems have been isolated from maize (Ingram *et al*., 2000). An embryo-specific zinc finger protein gene, *PEI1*, plays an important role for the globular to heart-stage transition (Li and Thomas, 1998).

Characterisation of gene expression during embryo development, maturation and germination has led to the identification of distinct classes of developmentally regulated genes in angiosperms (Dure III 1985; Goldberg *et al*., 1989). These genes can be divided into five major classes as follows:

- *Class 1*. Constitutively expressed genes whose products are present at all stages and have functions required during normal plant growth. These genes have housekeeping functions essential for many plant cells, including those in the embryo.
- *Class 2*. Embryo-specific genes whose expression is restricted to the embryo proper, and ceases prior to or at germination.

- *Class 3*. Genes highly expressed during early embryogenesis until the cotyledonary stage.
- *Class 4*. Genes representing seed protein genes, expressed during the expansion of cotyledons and seed maturation.
- *Class 5*. Genes expressed abundantly in later stages of embryogenesis until seed maturation. The genes are activated by ABA.

Seed proteins, class 4, are encoded by several multigene families and are very abundant during the maturation stage of embryogenesis – representing up to 50% of the embryonal mRNAs at the midpoint of maturation. In the embryo the expression is localised within specific cells of the cotyledons and axis and is absent from the surrounding non-embryogenic seed tissues. Seed protein genes are regulated by both transcriptional and post-transcriptional processes (Goldberg *et al.*, 1989).

Among class 5 genes, late embryogenesis abundant (LEA) protein genes predominate (Dure III *et al*., 1989; Thomas, 1993). LEA proteins share several common characteristics. They are all hydrophilic and contain a large number of uncharged and hydroxylated amino acids. They are thought to protect cellular membranes and proteins during desiccation and the subsequent period of dormancy. Since these genes can be induced in other parts of the plant by ABA or application of various stresses, their expression is not embryo-specific.

4. GENERAL ASPECTS OF SOMATIC EMBRYOGENESIS

Somatic embryos can either differentiate directly from the explant without any intervening callus phase or indirectly after a callus phase (Williams and Maheswaran, 1986). Explants from which direct embryogenesis is most likely to occur include microspores (microsporogenesis), ovules, embryos and seedlings.

A special case of direct somatic embryogenesis, is the process which usually is classified as secondary embryogenesis. Secondary embryogenesis is termed continuous, recurrent or accessory, when the first formed somatic embryo fails to develop into a plant but instead gives rise to successive cycles of embryos, secondary, tertiary etc. Secondary embryos develop directly from epidermal and subepidermal cells of the cotyledons or hypocotyls (Thomas *et al*., 1976). In some cases the formation of secondary embryos is of significant importance for increasing the yield of plants regenerated. If directly formed somatic embryos are converted into plants, no further multiplication is possible. However, it is often difficult to stop the process and consequently no, or only a few, normal plants can be regenerated. The distinction between direct and indirect somatic embryogenesis is unclear except for secondary embryogenesis. The main problem is that according to older hypotheses direct embryogenesis should take place from embryogenic pre-determined cells. In contrast, indirect somatic embryogenesis should take place from undetermined cells and an undifferentiated callus should first be formed. However, in reality the callus formed is either embryogenic or not. It is usually easy to distinguish between embryogenic and non-embryogenic callus based on morphology and colour. Embryogenic callus is composed of proembryogenic masses (PEMs). At present it is not known if the first formed PEM actually is an embryo which deviates from the normal embryonic development in response to plant growth regulators (PGRs) and proliferates. Owing to the difficulty in strictly separating direct and indirect somatic embryogenesis, we will focus on what would be classified as indirect embryogenesis, where embryogenic callus is formed first (see Chapter 1). To execute this pathway efficiently, a number of critical physical and chemical treatments should be applied with proper timing. Although great progress has been made in the development of these treatments and understanding of their mechanism of action, it has also been revealed that some maturation treatments, coinciding with increased yield of somatic embryos, may cause adverse effects on embryo quality, thereby impairing germination and *ex vitro* growth of somatic embryo plants. Consequently, *ex vitro* growth of somatic embryo plants is under a cumulative influence of the treatments provided during the *in vitro* phase.

Plant regeneration via somatic embryogenesis includes five steps:

1. Intiation of embryogenic cultures by culturing the primary explant on medium supplemented with PGRs, mainly auxin but often also cytokinin.
2. Proliferation of embryogenic cultures on solidified or in liquid medium supplemented with PGRs, in a similar fashion to initiation.
3. Pre-maturation of somatic embryos in medium lacking PGRs; this inhibits proliferation and stimulates somatic embryo formation and early development.

4. Maturation of somatic embryos by culturing on medium supplemented with ABA and/or having reduced osmotic potential.
5. Regeneration of plants on medium lacking PGRs.

4.1. INITIATION OF EMBRYOGENIC CULTURES

Somatic cells within the plant contain all the genetic information necessary to create a complete and functional plant. The induction of somatic embryogenesis must then consist of the termination of a current gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression programme. One possible mechanism for downregulation of current gene expression is DNA methylation, which is influenced by auxins (Lo Schiavo *et al*., 1989). It has also been proposed that PGRs and stress play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression. This results in a series of cell divisions that induce either unorganized callus growth or polarized growth leading to somatic embryogenesis (Dudits *et al*.,1995). The initiation of the embryogenic pathway is restricted only to certain responsive cells which have the potential to activate those genes involved in the generation of embryogenic cells. Only a few cells in the primary explant are competent for embryogenic induction, which according to Dudits *et al.* (1995) may be the result of varying auxin sensitivity of these cells. Alternatively it may be due to the presence of different auxin receptors, one responsible for cell division as such, and another responsible for the asymmetric division that generates an embryogenic cell (Lo Schiavo, 1994). Two mechanisms appear to be important for *in vitro* formation of embryogenic cells: asymmetric cell division and control of cell elongation (De Jong *et al.,* 1993; Emons, 1994). Asymmetric cell division is promoted by PGRs that alter cell polarity by interfering with the pH gradient or the electrical field around cells (Smith and Krikorian, 1990). The ability to control cell expansion is associated with polysaccharides of the cell wall and corresponding hydrolytic enzymes (De Jong *et al*., 1993; Emons, 1994; Fry, 1995).

Somatic embryogenesis has been described as being genetically determined. There are major genotype or cultivar differences for this trait. The choice of explant can in some species be of paramount importance for obtaining embryogenic cell-lines (Lo Schiavo, 1995, with references). Plant species which are capable of expressing their embryogenic potential regardless of the explant include *Daucus carota* and *Medicago sativa*, while for many other species embryonic or highly juvenile tissue has to be used as the explant. The pattern of the developmental response of cultured tissue is epigenetically determined and is influenced by the stage of development of the plant, the nature of the explant etc. (Litz and Gray, 1995).

Requirement for auxin or other PGRs for the initiation of somatic embryogenesis is largely determined by the developmental stage of the explant tissue. Usually an embryogenic callus is formed in the medium containing auxin. A nodular tissue is produced by the proliferation of PEMs, the process resembling a callus-like growth. Synthetic auxins are usually used for initiating embryogenic cultures. One mechanism whereby auxin may regulate embryogenesis, is through acidification of the cytoplasm and cell-wall (Kutschera, 1994). Most commonly used is 2,4-D (2,4-dichlorophenoxyacetic acid) at a concentration of one to ten micromolar. However, in some cases it is necessary to combine different auxins (see Chapter 5). Initiation of embryogenic cultures by cytokinin alone is relatively rare, but for many species it is important to combine auxin and cytokinin. In most species a low concentration of cytokinin (e.g. 0.1 to 1.0 μM) in the culture medium tends to stimulate the initiation of embryogenic cultures. At higher concentrations cytokinins may have a negative effect. Initiation can often be stimulated by either altering the concentration of exogenous applied auxins or the molar ratio of auxins to cytokinins. However, thidiazuron (TDZ) alone has been found to substitute for both auxin and cytokinin in many species (Murthy *et al*.,1998, with refs.). It was suggested that TDZ induces somatic embryogenesis by modulating the endogenous level of both auxin and cytokinin. In some cases a higher rate of somatic embryogenesis is obtained with TDZ than with other PGRs. Only in a few instances has ethylene been reported to stimulate somatic embryogenesis. For example, ethylene promoted somatic embryogenesis from leaf disks of *Coffea* cultured in the presence of isopentenyladenine as the sole exogenous PGR (Hatanaka *et al*., 1995).

More recently the application of the new generation of growth regulators such as oligosaccharides, jasmonate, polyamines and brassinosteroids (see Chapter 7) has proved to be useful for initiating somatic embryogenesis in many plant species.

In some species a remarkable stimulation of initiation of somatic embryogenesis has been

achieved with compounds other than PGRs. Reduced forms of nitrogen supplied to the medium are important; ammonium or amide nitrogen often stimulate somatic embryogenesis (see Chapter 4). The type of carbon source has also been reported to affect initiation in many species (see Chapter 4).

Wounded zygotic embryos of *Daucus carota* can initiate embryogenic cultures on hormone-free medium. The cultures can be maintained as unorganized embryogenic cell masses on hormone-free medium at pH 4.0, containing NH_4^+ as the sole nitrogen source (Smith and Krikorian, 1990).

4.2. PROLIFERATION OF EMBRYOGENIC CULTURES

Once embryogenic cells are formed, they continue to proliferate forming PEMs. Auxin is required for proliferation of PEMs but is inhibitory for the development of PEMs into somatic embryos (De Vries *et al*., 1988; Nomura and Komamine 1985; Filonova *et al*., 2000a). The degree of embryo differentiation which takes place in the presence of auxin varies in different species. It should be noted that depletion of auxin in the culture medium starts after only a few days. Consequently, if the cultures are not transferred to fresh medium each week, somatic embryos will start to develop.

Embryogenic callus is maintained in a medium similar to that used for initiation. The cultures can be maintained and increased on semi-solid medium. However, for large-scale propagation it is usually better to establish suspension cultures. In addition to the fact that proliferation rate is higher in suspension cultures, another advantage is that the cultures become more synchronized. In suspension cultures single cells and cell aggregates develop as separate structures. Thus, the cells can easily be separated by sieving or centrifugation and thereafter subcultured and manipulated as required. Low pH is essential for maintaining cultures in the proliferation phase. During each subculture period the pH of the medium drops, frequently from 5.8 to around 4. When preglobular stage PEMs of *Daucus carota* are subcultured on hormone-free medium buffered at pH 5.8 they promptly continue their development to globular, heart, torpedo and cotyledonary stage embryos (Smith and Krikorian, 1990). Embryogenic cultures of some species and some genotypes can be subcultured for a prolonged period on medium containing PGRs, and still retain their embryogenic potential i.e. the capacity to produce mature somatic embryos which can develop into plants. However, the risk of increasing the rate of somaclonal variation and diminishing the embryogenic potential accumulates with prolonged culture. In our laboratory we do not use embryogenic cultures which have been proliferating for more than six months. As soon as the embryogenic cell-lines have been established they are cryopreserved and successively thawed for use in different studies.

4.3. PRE-MATURATION OF SOMATIC EMBRYOS

Comprising a link between proliferation of PEMs and organized embryonic development, and at the same time separating these stages, PEM-to-embryo transition plays an important role. It seems likely that the inability of many embryogenic cell lines to form well-developed somatic embryos is to a great extent associated with disturbed or arrested PEM-to-embryo transition. Until the embryos have reached the appropriate developmental stage they should not be exposed to maturation treatments. Synthetic auxins, such as 2,4-D, which are particularly effective for promoting establishment and proliferation of embryogenic cultures, are usually not metabolized by the cells to the same extent as natural auxins. Hence, for stimulating further growth of the somatic embryos it is necessary to transfer the embryogenic cultures to medium lacking auxin. With the depletion of auxin the block on the expression of those genes required for the transition to the heart stage is removed (Zimmerman, 1993).

When cultures are transferred from proliferation medium to medium stimulating embryo development they consist of a mixture of single cells and cell aggregates. To synchronise the development, the cells can be washed and/or sieved.

4.4. MATURATION OF SOMATIC EMBRYOS

During the maturation stage somatic embryos undergo morphological and biochemical changes. The storage organs, the cotyledons, expand concomitant with the deposition of storage materials, the repression of germination and the acquisition of desiccation tolerance (Thomas, 1993). Somatic embryos accumulate storage products that exhibit the same characteristics as those of the zygotic embryos. The storage products are also targeted to the correct subcellular compartments (Merkele *et al*., 1995). However, the amount of a particular storage product, as well as the timing of its accumulation can differ between somatic and zygotic embryos (Merkele *et al*., 1995; Yeung, 1995). The synthesis and deposition of storage and late embryogenesis abundant (LEA) proteins during somatic and zygotic embryogenesis

are usually regulated through ABA- and water-stress-induced gene expression (Dodeman *et al.,* 1997).

In some species it is necessary to treat the embryogenic cultures with ABA, usually at concentrations of 10 to 50 μM, to stimulate maturation; for reasons unknown, this is especially the case for conifers. In other cases ABA is used for reducing the process of secondary embryogenesis or for inhibiting precocious germination. In general, treatment for one month is optimal. Prolonged treatment usually increases the number of mature embryos formed. However, long exposure to ABA can have a negative after-effect on plant growth (Bozhkov and von Arnold , 1998). Many other factors such as ethylene, osmotic stress, pH and photoperiod have been reported to influence somatic embryo maturation in different species.

Maturation of seed embryos is generally terminated by some degree of drying, which results in a gradual reduction of metabolism as water is lost from the seed tissue and the embryo passes into a metabolically inactive, or quiescent, state. The lapse into metabolic quiescence resulting from desiccation is important; in addition to conferring tolerance to extreme environmental stresses, it permits broad dispersal of seeds through time and space (Kermode, 1990). Hydration of the orthodox seed leads to its germination, which is an immediate response to a switch from the maturation programme to the germination programme. In contrast, recalcitrant embryos are unable to survive desiccation and do not cease development during maturation.

Somatic embryos of the recalcitrant-type seed do not naturally enter a period of developmental arrest. They germinate precociously but the resulting plants have a low survival rate. It is possible to induce quiescence in somatic embryos of orthodox seed type species by dehydration treatment. Numerous attempts to improve the quality of somatic embryos have shown the stimulatory role of low osmotic potential in the maturation medium in embryo development, both in angiosperms (McKersie and Brown, 1996) and gymnosperms (Attree and Fowke, 1993). The effect mimics water stress naturally imposed on orthodox seeds during later stages of maturation. This allows embryos to survive severe dehydration when moisture content can diminish to as low as 5% (Bewley and Black, 1994). Different osmotic agents, including low (e.g., inorganic salts, amino acids and sugars) and high molecular mass compounds [e.g., polyethylene glycols (PEG) and dextrans] can provide low osmotic potential medium. However, *in vitro* osmotic stress induced by PEG with a M_r >4000 most closely approaches water stress observed in cells of embryos and plants subjected to dehydration conditions (Rains, 1989). The reason is that larger molecules of PEG are not able to pass through the cell wall while water is withdrawn, leading to a reduced turgor pressure and more negative intracellular water potential. Although the addition of PEG to the maturation medium in many cases has been shown to stimulate maturation, there are also reports showing adverse effects of PEG on embryo germination and early post-germinative root growth (Bozhkov and von Arnold, 1998). In *Picea abies* we found that PEG-treatment exerted a deleterious effect on embryo morphology and root meristem development.

In some species, the maturation treatment is followed by partial desiccation which substantially enhances somatic embryo germination frequency. It has been suggested that the desiccation reduces endogenous ABA content (Kermode *et al.,* 1989) or changes the sensitivity to ABA (Finkelstein *et al.,* 1985). It is well known that the sensitivity of seeds to imbibitional stress is controlled by the initial moisture content of the seed and the rate at which water is taken up (e.g. Pollock, 1969). The interaction of these factors has a dramatic effect on germination and subsequent seedling vigour. Usually, a high rate of water uptake i.e. quick imbibition has a deleterious effect on germination (Vertucci, 1989). Therefore, it is important to keep the initial rate of water uptake at a low level. Different methods are used for desiccation (see for example Roberts *et al*., 1990). The easiest way is to place the mature embryos individually in empty petri dishes and keep the humidity of the surrounding air at such a level that the embryos are first dehydrated and then hydrated at an appropriate rate.

4.5. REGENERATION OF PLANTS

Somatic embryogenesis is a complex process where the quality of the final product, i.e. survival and growth of regenerated plants, depends on the conditions provided at earlier stages, when mature somatic embryos are formed and germinate. Therefore, in order to develop mass propagation of somatic embryo plants, a dissection of critical factors that might contribute to *ex vitro* performance of plants is required. Only those mature embryos which have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation develop into normal plants. Somatic

embryos usually develop into small plants, comparable to seedlings, on culture medium lacking PGRs. However, there are cases when auxin and cytokinin stimulate germination. Furthermore, a marked alteration in basal medium is often required. For some species, inclusion of extra compounds, like glutamine and casein hydrolysate, is also required. When the plants have reached a suitable size they can be transferred *ex vitro*. In many reports it has been shown that somatic embryo plants grow in a similar way to plants derived from true seeds. However, for some species somaclonal variation is a problem. In general, the use of 2,4-D and/or a prolonged callus phase are responsible for inducing genetic as well as epigenetic variation.

5. CONDITIONING FACTORS REGULATING SOMATIC EMBRYOGENESIS

The mechanism that controls cell differentiation in plant somatic embryos is far from clear, although there is evidence that soluble signal molecules are involved. It has long been observed that conditioned medium from embryogenic cultures could promote embryogenesis. For example, conditioned growth medium from highly embryogenic cultures can induce embryogenesis in non-embryogenic cultures (Hari, 1980). Growth medium preconditioned by a high-density suspension culture can also induce embryogenesis in cells cultured at a low cell density (Smith and Sung, 1985; de Vries *et al.*, 1988). This ability of conditioned medium to sustain or stimulate somatic embryogenesis implies that secreted soluble signal molecules are important.

5.1. EXTRACELLULAR PROTEINS

Secretion of proteins into the growth medium of suspension cultures has been reported in several species. However, only a few reports show that some specific secreted proteins can influence the development of somatic embryos. One of the proteins that promoted somatic embryo development in embryogenic cultures of *Daucus carota* was identified as a glycosylated acidic endochitinase (de Jong *et al.*, 1992). The authors studied the temperature sensitive variant *ts11* where somatic embryogenesis at the non-permissive temperature is blocked at the globular stage. Addition of the endochitinase to *ts11* embryogenic cultures rescued embryogenesis and promoted embryo formation. In similar experiments, an endochitinase from sugar beet stimulated early development of somatic embryos in *Picea abies* (Egertsdotter and von Arnold, 1998). In embryogenic cultures of *Daucus carota*, expression of an endochitinase gene was related to a certain population of cells that might have a nursing role in embryogenesis (van Hengel *et al.*, 1998). The exact role of the chitinases in somatic embryogenesis is not clear, but it has been suggested that chitinases are involved in the cleaving off of signalling molecule(s) from an as yet unknown substrate.

5.2. ARABINOGALACTAN PROTEINS

Arabinogalactan proteins (AGPs) have also been found to be important for the development of somatic embryos. AGPs are a heterogenous group of structurally complex macromolecules composed of a polypeptide, a large branched glycan chain and a lipid (Majewska-Sawka and Nothnagel, 2000). They are distinguished by their high carbohydrate-to-protein ratio, often more then 90% carbohydrate. AGPs are present in cell walls and plasma membranes, and commonly found in culture media. Perturbation of AGPs was found to alter somatic embryogenesis, indicating the importance of AGPs for embryo development. For example, addition of Yariv reagent, a synthetic phenyl-glycoside that specifically binds AGPs, to the culture medium blocked somatic embryogenesis in *Daucus* and *Cichorium* (Thompson and Knox, 1998; Chapman *et al.*, 2000). Precipitating AGP molecules with an anti-AGP antibody had a similar inhibitory effect on somatic embryo formation (Butowt *et al.*, 1999). Furthermore, addition of AGPs to the cultures was found to promote somatic embryogenesis. AGPs isolated from *Daucus* seeds restored the embryogenic potential in old cell lines of *Daucus* that had lost the ability to develop somatic embryos (Kreuger and van Holst 1993). Similarly, AGPs isolated from seeds of *Picea* promoted formation of more developed somatic embryos in low embryogenic cell lines of *Picea* (Egertsdotter and von Arnold, 1995). AGPs isolated from seeds of *Daucus* were fractionated using their binding to the antibodies ZUM15 and 18 (Kreuger and van Holst 1995). The ZUM15-reactive AGPs were inhibitory for somatic embryogenesis, while the ZUM18 reactive AGPs drastically increased the percentage of embryogenic cells and subsequently the number of embryos. Furthermore, ZUM18-reactive AGPs from *Daucus* enhanced the frequency of somatic embryos in *Cyclamen* (Kreuger *et al.*, 1995), and AGPs from *Lycopersicum* promoted somatic embryogenesis in *Daucus* (Kreuger and van Holst 1996). JIM4-reactive epitopes of AGPs were

found in embryogenic cells of *Daucus carota* and *Zea mays* (Kreuger and van Holst, 1996; Samaj *et al.*, 1999). On the other hand, JIM8-reactive AGP-epitopes were found in a subpopulation of *Daucus carota* cells with specific "nursing" function during somatic embryogenesis (McCabe *et al.*, 1997). Using JIM8 antibodies, McCabe *et al.* (1997) found two types of cells in embryogenic cultures of *Daucus carota*, - those that have JIM8-reactive AGP-epitopes (JIM8-positive cells) and cells devoid of JIM8-reactive epitopes (JIM8-negative cells). Further studies revealed that JIM8-negative cells in an unfractionated suspension develop into somatic embryos. At the same time, an isolated subpopulation of JIM8-negative cells could not form embryos. However, isolated JIM8-negative cells developed into embryos when supplemented with media conditioned by JIM8-positive cells. The data indicate that an active compound is released from JIM8-positive cells into the medium. Analysis of the chemical structure of this compound revealed the presence of both carbohydrate and lipid parts (McCabe *et al.*, 1997). It has been suggested that in embryogenic cultures of *Daucus carota* AGPs give rise to an oligosaccharin(s) with signalling functions (Darvill *et al.*, 1992). The presence of a glycosyl-phosphatidylinositol lipid anchor on some AGPs supports the possibility that AGPs are putative precursors of signalling molecule(s) (Svetek *et al.*, 1999). Although the chemical structure of the oligosaccharin(s) remains obscure, an increasing amount of data implicates lipochitooligosaccharides (LCOs) as signalling molecules in somatic embryogenesis.

5.3. LIPOCHITOOLIGOSACCHARIDES

Lipochitooligosaccharides (LCOs) are a class of signalling molecules that promote division of plant cells. It has long been known that LCO signals, Nod factors, secreted by *Rhizobium*, induce cell divisions in the root cortex, leading to the formation of nodules that can then be colonized (Spaink *et al.,* 1991, Truchet *et al.,* 1991). Nod factors produced by different *Rhizobium* species uniformly consist of an oligosaccharide backbone of 1,4-linked N-acetyl-D-glucosamine (GlcNAc) residues varying in length between 3 and 5 sugar units, and always carry an N-acyl chain at the non-reducing terminus. This basic structure is essential for the infection leading to nitrogen-fixing nodules. At the same time, several lines of evidence suggest the involvement of LCOs in regulating somatic embryo development. Rhizobial Nod factors were found to stimulate somatic embryos of *Daucus carota* to proceed to the late globular stage (De Jong *et al.*, 1993) and promoted the development of larger PEMs from small cell aggregates and further somatic embryo development in *Picea abies* (Egertsdotter and von Arnold, 1998; Dyachok *et al.*, 2000). More detailed studies showed that Nod factors could substitute for auxin and cytokinin to promote cell division. Furthermore, the Nod factor-like endogenous LCO compound(s) have been found in the conditioned medium from embryogenic cultures of *Picea abies*. A partially purified fraction of LCOs stimulated formation of PEMs and somatic embryos in *Picea abies* (Dyachok *et al.,* 2002). Remarkably, both in *Daucus carota* and *Picea abies* embryogenic systems, rhizobial Nod factors could substitute for chitinases in their effect on early somatic embryo development (De Jong *et al.*, 1993; Egertsdotter and von Arnold, 1998), indicating convergence of the activated signalling pathways. Evidence for concerted action in somatic embryogenesis of AGPs, chitinases and GlcNAc-containing oligosaccharides has been obtained. GlcNAc-containing AGPs were isolated from immature seeds of *Daucus carota*. The addition of AGPs increased the number of somatic embryos in embryogenic cultures of *Daucus carota*, and preincubation of the seed AGPs with endochitinases further increased the frequency of embryo formation (van Hengel, 1998). Taken together, the data support a hypothesis that endogenous LCOs analogous in structure to rhizobial Nod factors are released from AGPs by the action of endochitinases and act as signal molecules stimulating the development of somatic embryos.

6. TRACKING OF SOMATIC EMBRYOGENESIS

In order to efficiently regulate the formation of plants via somatic embryogenesis it is important to understand how the somatic embryo develops. Ideally, such knowledge should be gained through the construction of a fate map representing an adequate number of morphological and molecular markers specifying distinct developmental stages within the whole process (Irish and Sussex, 1992, Strehlow and Gilbert, 1993). When constructed, the fate map showing the correct progression of somatic

embryogenesis would provide the basis for further analyses of specification, induction and patterning of the embryonic tissues and organs.

6.1. CONSTRUCTION OF FATE MAPS

Construction of a fate map in somatic embryogenesis can be based on two alternative approaches either using synchronous cell-division systems (Nomura and Komamine, 1985; Tsukahara and Komamine, 1997; Osuga *et al*., 1999), or time-lapse tracking of the development of individual protoplasts, cells and multicellular structures (Golds *et al.,* 1992; Toonen *et al.,* 1994; Filonova *et al*., 2000a; Somleva *et al*., 2000). The second approach usually yields more consistent data, because there is no need to use cell-cycle-affecting drugs and centrifugation treatments which otherwise might interfere with embryonic development. Another potential advantage of the time-lapse tracking technique is that the starting individual cell, or cell aggregate, can be pre-selected based on certain criteria, and that it is possible to perform simultaneous analysis of dynamics and distribution of injected molecular probes conjugated with low molecular weight fluorochromes or fused to green fluorescent protein.

6.2. ANGIOSPERMS

In angiosperms, *Daucus carota* is the best understood species in terms of developmental pathways of somatic embryogenesis and their molecular mechanisms. This fact is not altogether surprising since *Daucus carota* embryogenic cell suspensions are highly amenable to both synchronization treatments and time-lapse tracking analysis (Zimmerman, 1993; Toonen and de Vries, 1997). By using the latter approach, Toonen *et al*. (1994) have studied three important aspects of somatic embryogenesis:

1. cell types which possess embryogenic potential, (i.e. able to develop into somatic embryos),
2. major stages of somatic embryogenesis in common for different initial cell types, and
3. variations and similarities between the pathways of somatic embryogenesis initiated from different cell types.

Based on shape (spherical, oval or elongated) and the density of cytoplasm (i.e. distinction between cytoplasm-rich and vacuolated cells), the authors have classified single cells isolated from embryogenic *Daucus carota* suspensions into five types, and found that cells of all types can develop into somatic embryos, albeit with a different frequency (varying between 19 and 100 somatic embryos per 10,000 cells). Embryo formation, in all cases, proceeded through the same stereotyped sequence of stages, i.e., state-0, and 2 cell clusters, according to Komamine *et al*. (1990). Auxin is required for the formation of a state-0 cell cluster, while subsequent stages, state-1 and 2 cell clusters, occur following withdrawal of auxin. Interestingly, depending on initial cell type, embryogenesis could occur via three different pathways distinguished by a lack or presence of geometrical symmetry of cell clusters at all three above-mentioned developmental stages. Oval and elongated cells, which were always vacuolated, developed into somatic embryos via asymmetrical cell clusters. Spherical cells which were either vacuolated or cytoplasm-rich, developed via symmetrical cell clusters into somatic embryos. Cells of a more variable initial morphology first developed aberrantly shaped cell clusters which then transformed into somatic embryos (Toonen *et al*., 1994). These observations imply that organised growth and polarity may not always occur during *in vitro* somatic embryogenesis, suggesting that in somatic embryogenesis, auxin-induced PEMs may be intermediates between unorganised growth and conservative embryonic pattern formation.

6.3. GYMNOSPERMS

A time-lapse tracking technique has been used to analyse developmental pathway of somatic embryogenesis in *Picea abies* (Filonova *et al*., 2000). A representative example of how *Picea abies* somatic embryos form and develop is shown in Fig. 9.2. It is noteworthy that, in contrast to *Daucus carota*, when a single cell fraction was obtained through fractionation of crude suspensions of *Picea abies*, neither densely cytoplasmic nor vacuolated cells on their own could develop into somatic embryos. Therefore the tracking began with a few-celled aggregates, called PEM I-cell aggregates. They are composed of a small compact clump of densely cytoplasmic cells adjacent to a single vacuolated cell. When immobilised in a thin layer of agarose, under appropriate trophic conditions and an initial supply of auxin and cytokinin, a PEM I-cell aggregate formed an additional vacuolated cell, thereby progressing to the next stage, PEM II (day 3; Fig. 9.2). Evans blue staining intensity of the primary vacuolated elongated cell increased by day 3 and was higher compared to newly formed additional vacuolated cells. In one of the vacuolated cells within PEM II it was possible to detect, by using the terminal deoxynucleotidyl

transferase-mediated dUTP nick end labelling (TUNEL) assay, the first signs of nuclear DNA degradation during the somatic embryogenesis pathway (Filonova *et al*., 2000b). Successively, PEM II enlarged in size by producing more cells of both types, while maintaining a bipolar pattern (days 5-10; Fig. 9.2.). The polarity and the distinct centre of densely cytoplasmic cells were lost after day 10 owing to the increased proliferative activity of densely cytoplasmic cells. This process continued from day 15 onward leading to formation of PEM III (days 15, 20; Fig. 9.2), followed by transdifferentiation of somatic embryos from PEM III (day 25; Fig. 9.2). Coincidentally, with somatic embryo formation, the primary structure, viz. PEM III, degenerates through massive PCD. Withdrawal of plant growth regulators (PGRs) dramatically activates both embryo formation and PCD in PEMs (Filonova *et al*., 2000b). In cell tracking analysis, embryos started to develop in PGR-free medium, which supported embryonic development through almost the entire period of early embryogeny (day 25-45, Fig. 9.2). For further embryo development and maturation, agarose layers with early somatic embryos must be replenished with ABA-containing medium. The first visible response of early somatic embryos to ABA is that the embryonal mass became opaque (day 4 ABA; Fig. 9.2). This response was accompanied by the elimination of the suspensor through PCD (Filonova *et al*., 2000b). Thereafter embryos started to elongate (days 7-14 ABA; Fig. 9.2) and differentiate cotyledons (days 20-35 ABA; Fig. 9.2). The tracking was terminated when fully matured somatic embryos resembling their zygotic counterparts had formed (day 45; Fig. 9.2).

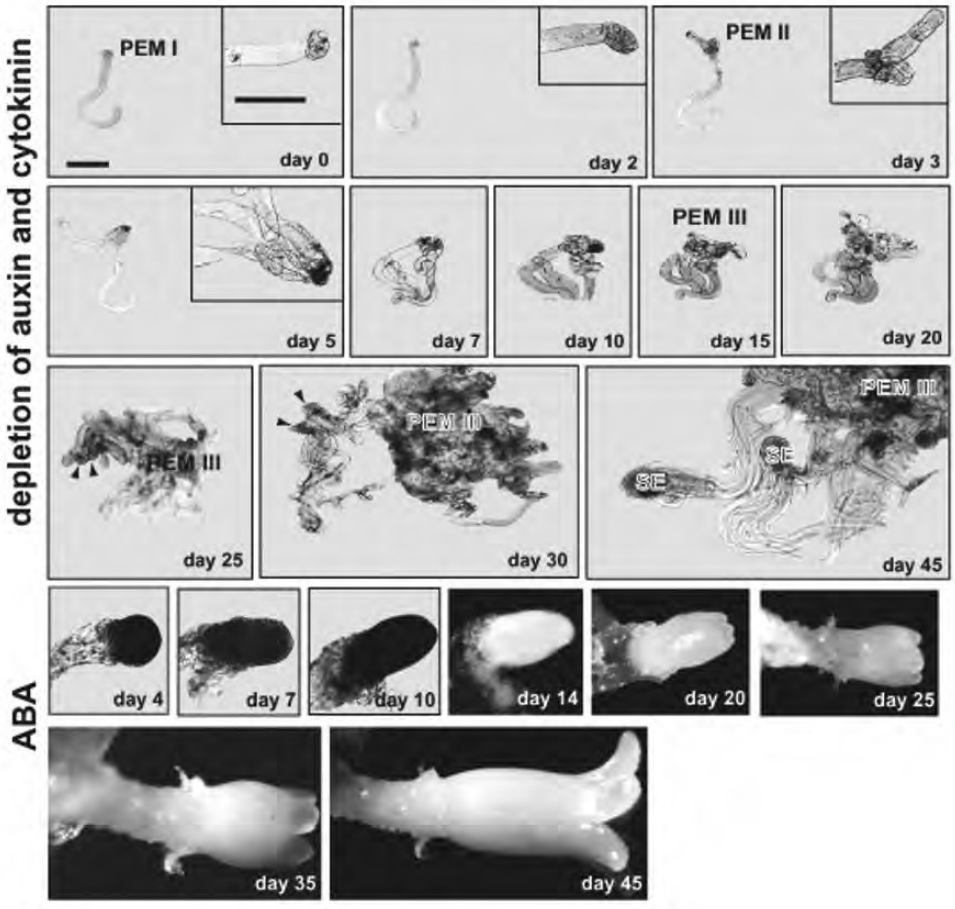

Fig. 9.2 Time-lapse tracking of somatic embryo formation and development in Norway spruce. The tracking was performed in a thin layer of agarose, first under gradual depletion of auxin and cytokinin and then following addition of ABA (for details see Filonova *et al.,* 2000a). Arrowheads denote somatic embryos (SE) transdifferentiating from PEM III. Bars = 250 µm.

6.4. MODEL FOR SOMATIC EMBRYOGENESIS

Based on the knowledge gained from studying the developmental pathway of somatic embryogenesis it is possible to construct a model of the process. The model has to be specific for each system. Below we give an example of a model for somatic embryogenesis in Norway spruce. Schematically shown in Fig. 9.3, somatic embryogenesis in *Picea abies* involves two broad phases, which in turn are divided into more specific developmental stages. The first phase is represented by proliferating PEMs,– cell aggregates which can pass through a series of three characteristic stages distinguished by cellular organization and cell number (stages PEM I, II and III), but can never develop directly into a real embryo. The second phase encompasses development of somatic embryos. The latter arise *de novo* from PEM III, and then proceed through the same, stereotyped sequence of stages as described for zygotic embryogeny of Pinaceae (Singh, 1978). Auxins and cytokinins are necessary during the first phase to maintain PEM proliferation, whereas embryo formation from PEM III is triggered by the withdrawal of PGRs. Once early somatic embryos have formed, their further development to mature forms requires ABA (Fig. 9.3). Each developmental stage within this fate map should be further characterised by a set of adequate molecular markers. What is emerging from our recent studies is that certain epitopes of arabinogalactan proteins and a lipid transfer protein are candidate markers (Dyachok *et al*., 2000; Filonova *et al*., 2000a, Sabala *et al*., 2000). Furthermore, a number of recently-described cytological and biochemical hallmarks of cell dismantling recruited during two waves of developmental PCD, viz. responsible for degradation of PEMs and elimination of suspensor (Filonova *et al*., 2000), will be an important adjunct to the fate map of *Picea abies* somatic embryogenesis.

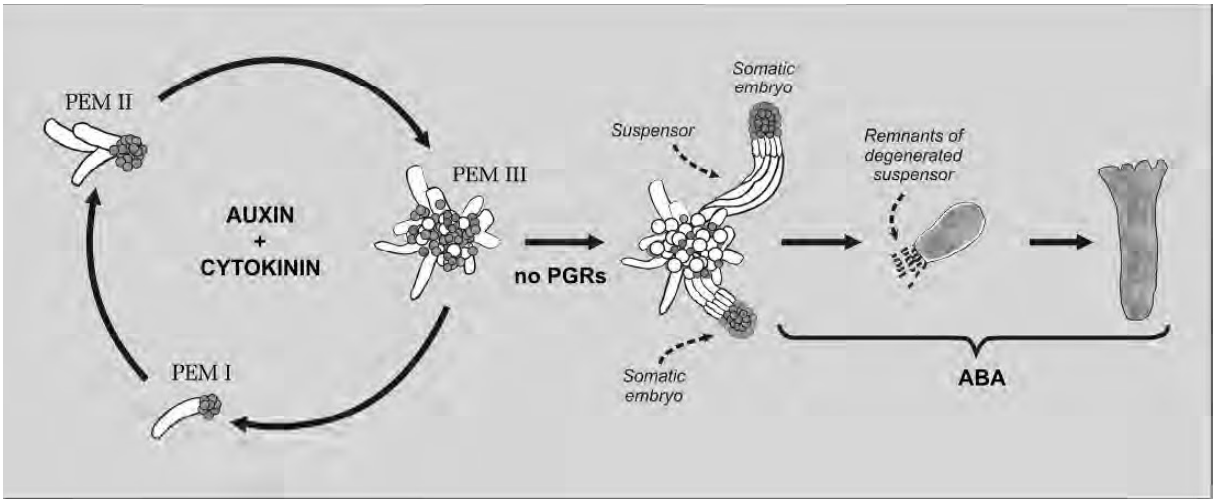

Fig. 9.3 A schematic representation of the developmental pathway of somatic embryogenesis in Norway spruce. Shown as dashed lines in the last but one stage of the pathway are the remnants of the degenerated suspensor in the beginning of late embryogeny.

REFERENCES

ATTREE S.M. & FOWKE L.C. 1993 Embryogeny of gymnosperms: Advances in synthetic seed technology of conifers. Plant Cell Tissue Organ Cult. 35, 1-35

BETHKE P.C., LONSDALE J.E., FATH A. & JONES R.L. 1999 Hormonally regulated programmed cell death in barley aleurone cells. Plant Cell 11, 1033-1045.

BEWLEY J.D. & BLACK M. 1994 *Seeds: Physiology of development and germination*. Plenum Press, New York.

BOWMAN J.L. & ESHED Y. 2000 Formation and maintenance of the shoot apical meristem. Trends Plant Sci. 3, 110-115.

BOZHKOV P. & VON ARNOLD S. 1998 Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. Physiol. Plant. 104, 211-224.

BUTOWT R., NIKLAS A., RODRIGUES-GARCIA M.I. & MAJEWSKASAWKA A. 1999 Involvement of JIM13 and JIM8-responsive carbohydrate epitopes in early stages of cell wall formation. J. Plant Res. 112, 107-116