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Gene expression during somatic embryogenesis – recent advances

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Somatic embryogenesis is a remarkable illustration of the dictum of plant totipotency. During embryogenic induction of cells, there is differential gene expression resulting in synthesis of new mRNAs and proteins. This genetic information in turn elicits diverse cellular and physiological responses that are involved in ‘switching over’ of the developmental programme of the somatic cells. Various model systems have been widely investigated to understand the mechanisms of gene regulation during this developmental process, and an array of genes activated or differentially expressed during so-

matic embryogenesis have been isolated employing various molecular techniques. Nonetheless, the precise mechanisms controlling plant gene expression and the detailed steps by which these genes direct the plant-specific process of somatic embryogenesis remain far from being clearly understood. Thus, future trends involve characterization of development-specific genes during somatic embryogenesis to provide a deeper insight in understanding the mechanisms involved during differentiation of somatic cells and phenotypic expression of cellular totipotency in higher plants.

SOMATIC embryogenesis is the developmental restructuring of somatic cells towards the embryogenic pathway, and forms the basis of cellular totipotency in higher

plants. This developmental switching involves differential gene expression conferring on the somatic cells the ability to manifest the embryogenic potential^{1,2}. Somatic embryogenesis thus involves a plethora of molecular events encompassing not only differential gene expres-

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sion, but various signal transduction pathways for activating/repressing numerous gene sets, many of which are yet to be identified and characterized.

The advent of molecular techniques has been crucial in identification of genes that exhibit differential activity, e.g. construction of cDNA libraries, differential display analysis, subtracted probe analysis, PCR and its modifications, etc. Quite expectedly, various structural and functional genes like the hormone responsive genes, homeobox genes, *LEA* genes, genes coding for chitinases, kinases regulating somatic embryogenesis and many others have been identified and characterized. Although many of these have overlapping functions, an attempt has been made in this presentation to categorize them and provide a lucid understanding of this developmental process.

The present review is thus an effort to collate information on various somatic embryo-specific genes that have been identified during the last few years (Figure 1). It also focuses on the newer information and insights gained on the role of various molecules involved in regulation of the process of somatic embryogenesis. The reader is referred to other excellent reviews on the developmental and genetic aspects of somatic embryogenesis¹⁻⁶.

Housekeeping genes

A number of genes have been identified that play a significant role during cell division and cell wall formation at various stages of embryo differentiation⁷. Due to active

cell wall synthesis in embryogenic tissues along with an increased activity of the genes involved in cell cycle regulation⁸, the proteins encoded by these genes are usually characterized by proline repeat motifs. They are also rich in glycine content and show resemblance to either the cell wall proteins or those involved in auxin-influenced developmental processes.

Many genes involved in the gene expression machinery have also been identified. Although genes coding for actin and tubulin are also expressed in non-embryogenic cells, during embryogenesis enhanced cell wall and membrane formation results in an increase in the expression of these genes as well^{1,9}. Similarly, there is enhanced expression of two histone-coding genes, *H3-1* and *H3-11*, during alfalfa somatic embryogenesis in response to auxin treatment¹⁰. A globular embryo-specific cDNA encoding for elongation factor-1 α , *CEM1* is reported in the actively dividing cells. The encoded protein functions in the interaction of the aminoacyl tRNA with ribosomes during the synthesis of proteins for housekeeping chores in the cell¹¹. Another gene *CEM6*, isolated by subtractive hybridization, is specifically expressed at the pre-globular and globular stages of carrot somatic embryo formation. The amino acid sequence of the protein encoded by *CEM6* is glycine-rich and has a hydrophobic signal-sequence-like domain, attributing it a role in cell wall biogenesis during embryogenesis¹².

DNA topoisomerase I is a key enzyme involved in various processes of DNA metabolism. Balestrazzi and co-workers^{13,14} have isolated partially overlapping DNA topoisomerase I-encoding cDNAs in carrot. Southern

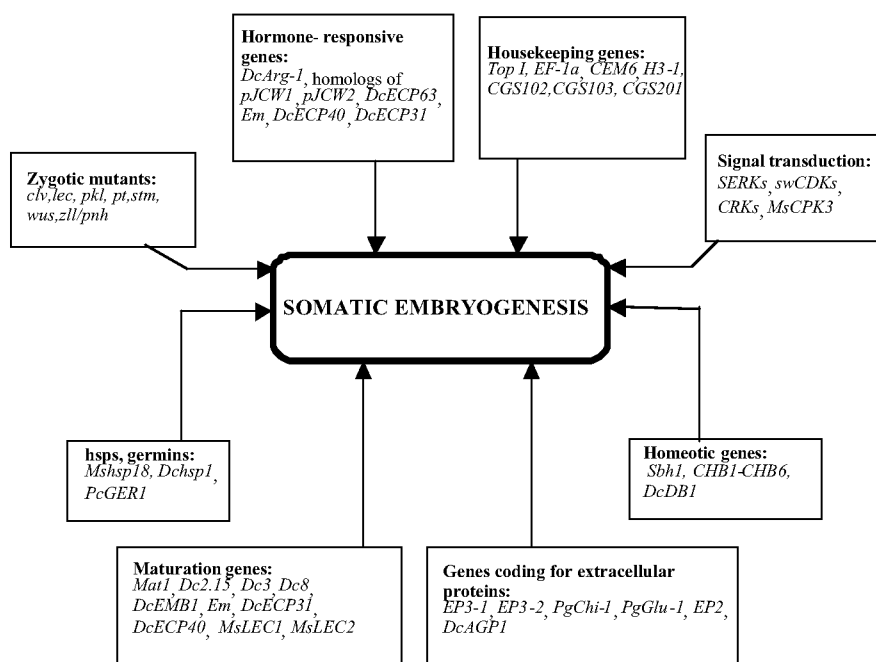


Figure 1. Schematic representation of various genes identified influencing somatic embryogenesis in higher plants.

analysis revealed the presence of single copy gene. The levels of *top1* transcript increased during cell proliferation in the 2,4-D-induced carrot hypocotyls. The transcript accumulation enhanced with proliferation of provascular cells and, histologically, *top1* transcripts were maximally expressed during torpedo stage of somatic embryogenesis, exemplifying the association of increased *top1* gene expression during cellular proliferative activities such as mitotic divisions.

The idea that cell cycle genes play a key role in somatic embryogenesis is substantiated with identification of plant cyclin cDNAs expressed during carrot somatic embryogenesis¹⁵. A *cdc2* protein kinase cDNA (*cdc2MS*) has been isolated from alfalfa. The encoded protein shares 64% identity with the yeast and mammalian kinases and shares the common features of the *cdc2* protein kinases as well. *cdc2MS* can complement a temperature-sensitive *cdc2* mutant of fission yeast. Also, the transcript levels of *cdc2MS* were found to be higher in alfalfa shoots and auxin-induced suspension cultures⁸. Recently, a cDNA of an early auxin-inducible gene, *OsIAA1*, was sequenced and characterized from the rice cDNA library using cyclin *cycAt* as probe, indicating that it may have a putative role in cell division¹⁶.

Expression pattern of three carrot cDNA clones coding for the three isoforms of the enzyme glutamine synthetase (*CGS102*, *CGS103* and *CGS201*) was investigated during somatic as well as zygotic embryogenesis. Transcript levels of *CGS102* and *CGS201* increased during the early stages of somatic embryogenesis and developing seeds, whereas *CGS103* was expressed only in the later stages of seed development and senescent leaves, and was absent in somatic embryos or young leaves. The expression of *CGS102* and *CGS201* declined in the presence of medium supplemented with glutamine as nitrogen source, indicating transcriptional regulation of GS activity. This also signifies the involvement of a common regulatory system for nitrogen metabolism in somatic and zygotic embryogenesis¹⁷. A glutathione S-transferase has also been isolated from chicory leaf tissues exhibiting early stage of somatic embryogenesis¹⁸.

Thus, a number of essential genes associated with various important cellular activities such as cell cycle regulation and housekeeping of the cell have been studied during somatic embryogenesis, and many more genes may be expected to be isolated in the near future.

Hormone-responsive genes

Auxin-inducible genes

Auxins have emerged as one of the potent initiators of somatic embryogenesis^{1,19}. At the molecular level, it has become apparent that auxin-induced growth and development involves changes in gene expression²⁰.

Auxin application to excised organs, cell cultures and whole plants can lead to rapid accumulation of numerous mRNAs, thus leading to isolation of several of these corresponding cDNAs from a variety of plant systems²¹⁻²⁴.

Heat shock proteins (hsps) have also been found to be expressed during somatic embryo development in response to hormones such as 2,4-D (refs 25, 26). *Dchsp-1* is one of the auxin-induced genes that is homologous to low molecular weight hsps and exhibits constant expression throughout somatic embryo development. It is also responsive to other hormones as well as stress conditions known to induce somatic embryogenesis. Another gene, *Dcarg-1*, homologous to auxin-regulated genes, is auxin-specific and its transcript is detectable only during the early induction period and not during the subsequent developmental phases of embryo differentiation²⁶.

Isolation of two such variable auxin-responsive genes in carrot somatic embryos indicates that at high concentrations, the growth hormone may be perceived as a stress condition. Dudits and co-workers⁴ reported that treatment of alfalfa embryogenic cells with a high concentration, of 2,4-D for a short period induced somatic embryogenesis. With this treatment, alfalfa cells proceed from the G₁ phase to the S phase in the cell cycle, as judged from the expression of cell cycle-related *cdk* and cyclin genes resulting in the formation of somatic embryos. The exposure to auxin shock serves as a trigger, inducing cell division in the epidermal cells and promoting their further differentiation to somatic embryos. Induction of somatic embryogenesis as a short-term response to auxin has also been reported from wheat leaf bases²⁷. Thus even a small pulse of auxin is sufficient for induction of competent cells to trigger embryogenesis^{4,5,27,28}.

It has also been noted that transition of the globular embryo to the heart-stage embryo and its further development requires either a low level of auxins or their complete absence. Three partial cDNA clones (Nos 43, 87, 93) have been isolated from cell clusters of the earliest stage of carrot somatic embryogenesis using differential display analysis. Their transcripts preferentially accumulate in the embryogenic cell clusters formed after treatment with 2,4-D (ref. 29). The deduced amino acid sequence of the No. 43 and No. 93 cDNA clones showed homology with thaumatin-like protein and precursor of the proline-rich Dc 2.15 protein respectively.

A class of auxin-induced genes – *SAUR* (*small auxin up-regulated*) genes, *pJCW1* and *pJCW2*, were first identified in soybean³⁰. When used as probes, these clones indicate that auxin specifically induces accumulation of mRNAs hybridizing with these sequences²¹. Such auxin-responsive cDNAs can thus serve as an effective tool for screening the embryogenic potential of embryogenic and non-embryogenic lines. In case of two auxin-responsive cDNA probes, *pJCW1* and *pJCW2*, the transcript levels

declined in the older alfalfa somatic embryo cultures, indicating an age-related change in the morphogenic potential. In sweet potato also, the newly induced embryogenic callus lines produce competent embryos that convert readily into plantlets, while the older cultures fail to do so. This could be explained by desensitization of auxin responsiveness as a central feature of reduced embryogenic competence in callus lines following prolonged exposure to 2,4-D (ref. 31).

A change in the methylation status is also observed when carrot embryogenic cells are treated with exogenous auxin, and apparently, an optimal level of methylation is required for the normal development of somatic embryos as hypermethylation and hypomethylation both cause immediate and irreversible block of embryogenesis³².

ABA-inducible genes

ABA is a plant growth hormone that regulates several processes during embryogenesis and seed formation, and is known to accumulate in various other plant parts in response to abiotic stresses such as drought, freezing and salt stress³³. Endogenous ABA content has been shown to peak during embryo maturation and decrease to low levels in the dry desiccated seed. The mechanism by which ABA regulates gene expression involves transcriptional as well as post-transcriptional events, such as transcript processing, mRNA stability, translational control, protein activity and turnover. There are also indications that secretion of proteins can be regulated by specific signal molecules such as growth hormones, calcium and cAMP.

Late embryogenesis abundant (LEA) proteins are also ABA-inducible, and several cDNAs of embryo specific/embryogenic cell proteins have been isolated and characterized. *DcECP31* (ref. 34), *DcECP40* (ref. 35), *DcECP63* (ref. 36) from carrot and *Arabidopsis AtECP31* (ref. 37), *AtECP63* (ref. 38) encode for LEA proteins with increased expression during the torpedo stage of somatic embryos but not in seedlings, implying their regulation by ABA and other unknown embryo-specific factors.

Comparison and functional analysis of the various ABA responsive genes has led to the identification of ABA-responsive elements (ABREs) in plants³⁹. At present, more than twenty functional ABREs have been identified in the ABA-inducible promoters, with a 8–10 bp core sequence of ACGT⁴⁰. ABREs are related to many environmental signals, therefore combinatorial interaction between the ACGT core motifs and other regulatory sequences in the promoter may determine the signal specificity. The *ECP* (extracellular protein) genes expressed during somatic embryogenesis also have ABREs in their promoter regions, which contain a con-

served motif (ACGT core motif) and a *Sph* box (CATGCATG) that has been identified as a motif mediating gene activation of the maize anthocyanin regulatory gene *C1*. *C-ABI3* gene, homologous to the *ABI3* gene of *Arabidopsis*, has been isolated and characterized from carrot somatic embryo cDNA library⁴¹. The gene codes for a transcription factor that is detected in embryogenic cells, somatic embryos, and developing seeds. *C-ABI3* has a conserved B3 domain that is known to bind *Sph* box; however, it does not bind directly to *DcECP31* promoter. This suggests that VP1/*ABI3* factor, including *C-ABI3* may act as coactivator that interacts with the G-box via protein–protein interaction with G-box-specific DNA-binding proteins⁴². Promoter deletion analysis in *DcECP31* with a transient assay system using protoplasts from embryogenic cells of carrot has revealed a –250 bp upstream region for embryo-specific and ABA-inducible activity, while the distal (–670 to –390 bp) and proximal regions (–140 to –50 bp) are essential for the ABA-inducible expression⁴³. Using deletion analysis, base substitution mutagenesis and electrophoretic mobility shift assay (EMSA), two *cis*-elements (regulatory), motif X (CACACGTGGG) and motif Y (CACACGTATC) have been identified. Motif X is essential for ABA inducibility and works in co-operation with motif Y. Only half of the promoter activity was observed on mutation in motif Y. There could thus be some combinatorial interactions amongst the various motifs for achieving optimal promoter activity. The ACGT motif is an important sequence typical for binding of bZIP transcription factor⁴³.

Thus, the hormone responsive studies at the molecular level during somatic embryogenesis may play an important role in identifying various factors involved in the hormone-induced signal transduction pathway.

Embryo-associated signal transduction pathways

The perception of hormone stimuli and/or secondary messenger like calcium may trigger various signal transduction cascades in somatic embryos in a fashion similar to other developmental processes of higher plants. Various kinases have been identified and their significance unravelled in transducing the signal from the cell membrane to the action site. These protein kinases often undergo autophosphorylation for their activation and are involved in regulation of other successive transducer(s) in the signal transduction pathway. Three alfalfa somatic embryo genes (*ASET1*, *ASET2* and *ASET3*) have been found to be expressed at early embryonic stages in embryogenic lines and not in non-embryogenic lines and mature embryos. *ASET2* protein is predicted to code for several potential membrane-spanning domains and a potential phosphorylation site, attributing it a significant role in signalling pathway(s) during alfalfa somatic embryogenesis⁴⁴.

Calcium-mediated signal transduction

Calcium, a second messenger in many hormone-regulated events, plays a key role in various cellular and physiological processes of higher plants^{45,46}. During any calcium-mediated signal transduction process, there is a general increase in the cytosolic calcium followed by perception of changes by calcium-binding proteins. These proteins upon binding to calcium, undergo conformational changes and get transformed into their activated form. This confers on them the ability to interact with a wide range of regulatory proteins^{47,48}. Calmodulin (CaM) has emerged as a prominent protein involved in mediation of calcium signalling in plants. The CaM proteins are encoded by a multigene family in carrot and other plant species⁴⁹⁻⁵¹. CaM is known to be regulated post-transcriptionally both by its ability to bind calcium and by specific methylation of Lys 115 (ref. 52).

The role of calcium has been well investigated in carrot somatic embryogenesis and calcium was found to be essential for morphogenesis of undifferentiated cells into somatic embryos at a threshold level of 200 μ M (refs 53 and 54). Calcium at a higher concentration had no effect on both viability as well as embryogenic potential of the culture. At lower concentrations or by chelation of residual calcium with EGTA, somatic embryogenesis was inhibited. Calcium channel blockers, verapamil and nifedipine, exerted an inhibitory influence on the embryogenic capacity. Surprisingly, the ionophore A23187, besides suppressing somatic embryo formation, also caused morphological deformities of the globular embryos. These observations together suggest the importance of exogenous calcium and maintenance of a calcium gradient for proper embryo development. Analysis of membrane-associated calcium and total calcium distribution using fluorescent dyes revealed changes in calcium distribution during embryogenesis, without alterations in the membrane-associated calcium concentration. Active calcium/calmodulin complexes have also been detected in the meristematic regions of heart- and torpedo-stage embryos, thus suggesting the regulatory role of activated calmodulin in embryonal regions showing rapid cell divisions^{54,55}. Expression of CaM mRNA has also been seen to increase upon induction of somatic embryos and remain constant thereafter. Genes coding for calcium-binding protein (*MsCa1*) also show an increase in the transcript levels after 2,4-D treatment and preferentially accumulate at early globular stages⁴.

A novel family of calcium dependent/calmodulin-independent protein kinases (CDPKs) was first characterized from soybean⁵⁶. Unlike calcium/calmodulin-dependent protein kinases, CDPKs are activated by direct binding of calcium. These kinases also have a C-terminal, CaM-like EF-hand motif that enables them to bind calcium, and causes conformational change and activation of the N-terminal kinase domain⁵⁶⁻⁵⁸. A cDNA encoding a typical

protein kinase homologous to other plant kinases has been screened from the carrot somatic embryo cDNA library⁵⁹. Unlike the previously known calcium-dependent related kinases (CRKs), this CRK exhibits divergence in amino acid sequence in the C-terminal domain, including non-conservative substitution in EF-hand motifs. Divergence is also observed at the junction domain existing between the catalytic domain and calmodulin-like domains. N-terminal possesses a myristoylation signal, signifying the involvement of protein targeting to membranes. Carrot somatic embryos express CRK mRNA and the protein at much higher level than the mature plant tissues. *CRK* gene sequences seem to be conserved among angiosperms, indicated by their presence in both monocot and dicot species, and is presumed to be phosphorylated by cyclin-dependent kinases, thus playing a pivotal role in cell-cycle regulation. Two CDPKs of 55 and 60 kDa have been identified in soluble protein extracts of embryogenic cultures of sandalwood⁶⁰. The proteins showed differential expression and were absent in plantlets regenerated from somatic embryos. The temporal expression of *swCDPKs* during globular stage of somatic embryos and zygotic embryos, seed maturation (endosperm development) and germination indicates their involvement in the process of differentiation and development. *SwCDPK* is post-translationally inactivated in zygotic embryos during seed dormancy and during precocious seed germination. In sandalwood embryogenesis, there is a four-fold increase in calcium levels during differentiation of pro-embryogenic masses into somatic embryos. Chelating agents arrest somatic embryo formation though the cells continue to proliferate, indicating the inhibition of calcium-mediated signalling pathways involving CDPKs and CRKs (ref. 61).

MsCPK3, another calmodulin-like protein kinase (CPK) has been isolated from cultured alfalfa cells⁶². The full-length cDNA encodes for 553 amino acid polypeptide of 60.2 kDa. *In vitro* phosphorylation assays revealed protein activation by calcium and inhibition by calmodulin antagonist (W-7). *MsCPK* gene expression increased during early phase of somatic embryogenesis. Though the gene activity could not be stimulated by kinetin, ABA or NaCl treatment, heat shock was able to induce its expression, suggesting its potential role in hormone and stress-activated reprogramming of developmental pathways during somatic embryogenesis⁶².

Somatic embryogenesis receptor kinases

Among the many cDNA clones isolated from carrot suspension cultures at various phases of growth (two months to four years) by cold plaque screening, differential display and RT-PCR, one of them (approximately 680 bp) exhibited homology with animal and plant receptor pro-

tein kinases. Since it is expressed in somatic embryo cultures, it came to be known as *somatic embryogenesis receptor kinase (SERK)* gene. The protein encoded by this cDNA contains an N-terminal domain with five leucine-rich repeats (LRRs) acting as a protein-binding region. One intracellular domain and LRR motif also contain potential N-glycosylation sites. A unique feature of SERK protein is the presence of proline-rich region between the extracellular LRR domain of SERK and the membrane-spanning region. This is a conserved feature of plant cell wall proteins known as extensins⁶³. Although a transmembrane domain is present in the amino terminal of the protein, it does not show any characteristic feature of signal peptides. The intracellular domain of SERK contains eleven sub-domains characteristic of the catalytic core of protein kinases, and the second half of the C-terminal motif may be mediating protein-protein interaction, a prerequisite for transmission of an intracellular phosphorylation cascade⁶³. SERK protein, like many other protein kinases, has autophosphorylation activity as demonstrated by bacterially expressed SERK fusion protein. LRR sequence of SERK shows homology with *Arabidopsis* RLK5 (ref. 64) and *Arabidopsis* *ERECTA* gene⁶⁵. The domains VI and VII appear as a serine/threonine kinase. Southern analysis reveals that there may be a single copy of *SERK* gene. Thus, *SERK* gene may serve as a characteristic molecular marker for differentiating between competent and noncompetent cells. The expression of *SERK* is observed from competent cell stage up to the globular stage of somatic embryos, but is not detectable in non-embryogenic stages of embryogenic cultures. In other plant parts, *SERK* mRNA was detected transiently in the zygotic embryo up to early globular stage and is absent in unpollinated flowers or other plant tissues. *SERK* promoter fused with the *LUC* reporter gene demonstrated that the elongating cells in carrot that express *SERKs* indeed have the ability to undergo somatic embryo formation.

Shah and co-workers⁶⁶ have reported the chemical characterization of a transmembrane receptor kinase from embryogenic carrot cell cultures. In order to study intrinsic biochemical properties of DcSERK protein, the kinase domain was expressed as a 40-kDa his-tag fusion protein in baculovirus insect cell system. The kinase domain fusion protein showed *in vitro* autophosphorylation at serine and threonine residues.

In *Arabidopsis*, five members of SERK family have been identified (*AtSERK1*, *AtSERK2*, *AtSERK3*, *AtSERK4* and *AtSERK5*). Expression studies of *AtSERK1* reveal specific expression in nucellus, megaspore and embryo sac. *AtSERK1* is also expressed during somatic embryogenesis. *AtSERK1* cDNA was fused to two different variants of green fluorescent protein (GFP), a yellow-emitting GFP (YFP) and a cyan-emitting GFP (CFP); both were transiently expressed in plant and insect cells. Confocal laser scanning microscopy revealed the pres-

ence of *AtSERK1*-YFP fusion protein at the plasma membranes of both plant and animal cells. It was thus suggested that LRRs and N-linked oligosaccharides may be required for the correct localization of *AtSERK1*-YFP protein. Homodimerization of *AtSERK1* was also carried out using yeast-protein interaction experiments and fluorescence spectral imaging microscopy. The YFP/CFP emission ratio revealed that without leucine zipper domain, *AtSERK1* is monomeric⁶⁷. Furthermore, the embryogenic competence of callus derived from the seedlings over expressing *AtSERK1* was three-four times higher when compared with the wild-type callus. This indicates that the protein encoded by the gene *AtSERK1* is sufficient to confer embryogenic competence in culture. Similarly, this gene mediates acquisition of embryogenic competence in the egg cell during zygotic embryogenesis⁶⁸.

In contrast to the carrot embryogenic cultures (see also Table 1), in *Dactylis glomerata*, video cell tracking has identified that in somatic tissues, competent cells are morphologically small, isodiametric, cytoplasmically-rich and arise from the provascular region. The developmental stages of both direct and indirect somatic embryo formation from the various segments of a leaf tissue have been tracked^{69,70}. The embryogenic cells of *D. glomerata* also express the *SERK* gene and whole mount *in situ*

Table 1. Some major genes expressed during carrot somatic embryogenesis*

Gene	Function	Reference
<i>CEM1, CEM6</i>	Housekeeping (elongation factor1- α)	12
<i>top1</i>	Housekeeping (topoisomerase I)	13, 14
<i>CGS102, CGS103</i> <i>CGS201</i>	Glutamine synthetase	17
<i>Dcarg-1, Dchsp-1</i>	Hormone-responsive (auxin-inducible)	27
<i>cDNA clones</i> <i>43, 87, 93</i>	Hormone-responsive (auxin-inducible)	29
<i>DcECP31, DcECP40,</i> <i>DcECP63, C-AB13</i>	Hormone-responsive (ABA-inducible)	34-36, 41
<i>DcSERK</i>	Somatic embryogenesis receptor kinases	66
<i>CHB1-CHB6</i>	Homoeobox containing genes	81-83
<i>EP3-1, EP3-2</i>	Extracellular proteins (chitinases)	92-94
<i>DcAGP1</i>	Extracellular proteins (arabinogalactans)	124
<i>EP2</i>	Lipid transfer protein	128
<i>Dc2.15</i>	Maturation gene	132
<i>Dc3, Dc8, DcECP31,</i> <i>DcECP40, DcEMB1</i>	LEA proteins	6, 139
<i>EMB-1</i>	Homology with <i>Em</i> gene	145

*The list is not exhaustive but enlists genes recently identified during carrot somatic embryogenesis. The reader is also referred to other reviews in this context by Zimmerman⁶ and Rao¹⁹³.

hybridization reveals that *SERK* is expressed differentially. *SERK* gene was expressed in competent cells to globular stage, but was not present in the clubbed-stage somatic embryos. In contrast to *Daucus carota*, the gene is also expressed in the shoot apical meristem (SAM) region of the protoderm, coleoptile and coleorrhiza. The probe used for *in situ* hybridization was an EST cDNA clone R2976 from *Oryza sativa*. Interestingly, this partial cDNA clone is 70% identical to the *D. carota* and *SERK* cDNA sequence. At the amino acid level, they share 82% identity. *Oryza* probe gave stronger signals than the *Daucus* probe and both exhibit similar spatial expression pattern, thus indicating that *SERK*-mediated embryo-specific path is operational in grasses as well.

Recently, two novel genes, *ZmSERK1* and *ZmSERK2* from maize (*Zea mays* L.) have been isolated using degenerate primers and PCR analysis⁷¹. These genes share all the unique features of the *SERK* family. Both the genes are present as single copy in maize genome, and exhibit 70% identity among each other at nucleotide level with similar intron/exon structure as that of the other *SERK*s identified. The tissue-specific expression studies of these two genes have determined preferential expression of *ZmSERK1* in male and female reproductive tissues with strongest expression in microspores, whereas *ZmSERK2* is uniformly expressed in all the tissues investigated. Both the genes are expressed in embryogenic as well as non-embryogenic cells. Thus, although all the members of the *SERK* family identified in dicot and monocot share similar exon-intron structure along with various similarities at protein level, mutant analysis studies would be required to determine a functional relationship amongst the various subgroups of *SERK* genes.

Homeobox genes

Homeobox genes were first identified in *Drosophila*^{72,73} and are the key regulatory genes controlling pattern formation and morphological differentiation in multicellular organisms. These genes by now have been well-characterized in plants such as maize, *Arabidopsis* and rice⁷⁴⁻⁷⁹. Homeotic genes contain a characteristic conserved nucleotide sequence called the homeobox. The encoded homeodomain codes a transcription factor with DNA-binding activities and is also associated with pattern formation in plants⁸⁰. The functional significance of homeotic genes in embryo development in insects, amphibians and mammals is extended to plants, substantiating further the mechanism responsible for the genetic control of development as a much more universal phenomenon than anticipated before⁷⁷.

A homeotic gene, *Sbh1*, has been identified and characterized by screening soybean cDNA somatic embryo library using maize *KNOTTED 1* (*KN-1*) as a heterologous probe. The 1.5 kb gene belongs to a small family of

homeotic genes. The amino acid sequence of the homeodomain has the characteristic helix-turn-helix structure and has similar invariant and conserved residues. The *Sbh1* protein bears overall 47% homology with the *KN-1* protein and 87.5% with the homeodomain. The putative leucine zipper motif is, however, absent in both *Sbh1* as well as maize *KN-1* that is otherwise a common feature of homeotic gene products. Expression of *Sbh1* mRNA is development as well as tissue-specific. There is increase in the levels of the transcript during transition from the heart-stage to torpedo-stage, when cotyledon differentiation and prevascular tissue are determined. mRNA is not detectable in nonembryogenic tissues, and other tissues though stem and hypocotyl exhibited weak expression. The expression pattern during somatic embryogenesis, in addition to its homology with *KN-1* gene, indicates its regulatory nature during vasculature biogenesis and as a significant player in the formation of somatic embryos⁵⁹.

Six homeobox-containing genes (*CHB1-CHB6*) from carrot somatic embryo and hypocotyl cDNA library have been identified using degenerate oligonucleotide probe of twenty-three bases belonging to HD-Zip family⁸¹⁻⁸³. Although the HD-Zip sequences encoded by these clones are similar to each other, the sequences beyond are extremely variable, preventing their cross-hybridization. Expression of the transcripts (1.3 kb to 2.3 kb) varies greatly from each other, both temporally and spatially during somatic embryogenesis as well as in mature seedlings. *CHB1* is expressed constantly in undifferentiated cell clusters, but notable is the enhanced expression of *CHB2* after globular stage with a maximum at heart-shape and early torpedo-stage of somatic embryo differentiation. Expression of the same decreases drastically in undifferentiated cultures. Whole mount, *in situ* hybridization has demonstrated the preferential accumulation of *CHB3*, *CHB4* and *CHB5* in the innermost cortical cell layers of the embryonal axis in torpedo-shaped embryos. The expression of *CHB6* is restricted to the procambial cells of heart- and torpedo-stage embryos. In the embryonic cotyledons and hypocotyl of the seedlings, mRNA of these later four genes was closely associated with the vascular tissues⁸⁴. This is a clear indication of their regulatory involvement in the differentiation of vascular tissue during embryonal development, as homeotic genes of *Arabidopsis Athb-8* (HD-Zip III family) and tomato *VAHOX1* (member of HD-Zip-I family) are also expressed during vasculature development^{85,86}. It is possible that these proteins may interact with each other and form heterodimers.

DcB1, a chromobox gene was isolated and characterized from cDNA library of embryogenic cell clusters of carrot. The cDNA (1.4 kb) encodes for conserved chromo domain containing protein of 392 amino acid residues⁸⁷. The chromo domain (37 amino acid in length) is present as a polycomb gene product, which is a repressor of homeotic genes and a heterochromatin protein I of *Droso-*

phila. This domain is postulated to function in the binding of proteins to chromatin. The differential expression of *DcCBI* mRNA revealed its increased expression during early stages (globular- and heart-stages) of somatic embryos, whereas low levels of transcript were detected in torpedo-shaped somatic embryo and during seed formation⁸⁷.

The existence of both homeo- and chromo-box genes in plants thus suggests that regulatory mechanisms of developmental genes in plants may resemble those in *Drosophila*. Moreover, identification of these groups of genes paves the way for the isolation and identification of many more homeotic genes, and further study in this area can edify some of the important molecular events that take place during plant embryo pattern formation.

Expression of extracellular proteins

Extracellular proteins play a significant role in the development of angiosperm embryogeny^{88,89}. These proteins and changes in their expression pattern have been associated with induction as well as initiation of somatic embryogenesis^{90,91}. Carrot cell cultures secrete a wide spectrum of proteins in the medium, a process that contributes to the conditioning of the medium. The conditioned medium in turn is reported to have promotory effect on the initiation of somatic embryogenesis. In fact, these proteins are not necessarily secreted by the embryogenic cells, but can also be released by the nonembryogenic cells; for instance, extracellular protein EP1 is secreted only by the nonembryogenic lines^{92,93}. Another extracellular secreted protein, EP3 is a chitinase by function⁹⁴ and is known to lift the arrest of somatic embryo growth in the temperature-sensitive carrot mutant cell line *tsII*, at the non-permissive temperature conditions. This acidic chitinase belongs to a small family of class IV chitinase genes⁹⁵. The homologous isoenzymes of EP3 are encoded by at least four *EP3* genes. Two of the isoenzymes encoded by these genes *EP3-1*, *EP3-2*, have been purified and are shown to have subtle differences during embryo formation in the newly initiated mutant *tsII* culture. Class II endochitinase and a heterologous class IV chitinase from sugar beet fail to rescue the *tsII* mutants, thus implying the existence of differences in the specificity of chitinases in terms of their effect on somatic embryogenesis. EP3 protein is, however, neither secreted by somatic nor zygotic embryos, but is rather expressed in the integumentary cells of fruits and endospermal cells. Thus it may have a 'nursing role' during zygotic embryogenesis, which is imitated by suspension cells from which somatic embryos are formed⁹⁶.

Roots of leguminous plants are known to produce chitinases during their interaction with *Rhizobium* sp. These pathogen-related proteins control the biological activity of the 'Nod' factors by cleaving and inactivating

them. They are associated with the regulation of plant morphogenesis and cell division. Chitinases are also involved in the generation of signal molecules essential for embryogenesis in *tsII*. Sensitivity of *tsII* to chitinases coincides with a transient decrease in the amount present in the otherwise functional set of proteins⁹⁷. A 38-kDa extracellular SER (somatic embryogenesis-related) protein has been identified and characterized during chicory somatic embryogenesis and, interestingly, the polypeptide coding for β -1,3-glucanase is expressed at higher levels in the medium of embryogenic lines than in the nonembryogenic lines.

In *Cichorium*, among the three cDNAs, *CG1*, *CG2* and *CG3*, isolated by RT-PCR from leaf fragments cultured under embryogenic conditions⁹⁸, *CG1* codes for a 38 kDa β -1,3-glucanase and its expression is found to be high in embryogenic cells. However, *CG1* expression decreased with the decrease in callose deposition in the cell walls of the surrounding embryogenic cells as well as in the young embryos, signifying the association of *CG1* gene with the process of somatic embryogenesis. This also indicates that the proteins encoded by such embryo-specific genes may have a potential role in callose degradation during somatic embryogenesis⁹⁸⁻¹⁰⁰. Thus most of the extracellular proteins studied during somatic embryogenesis code for a class of chitinases or glucanases which are not only associated with early stages of embryo development but may also play a significant role in providing the nursing conditions for somatic embryo induction and cell wall degradation.

Arabinogalactans

Arabinogalactan proteins (AGPs) are proteoglycans (10% protein and more than 90% carbohydrate) found in higher plants and liverworts. They are commonly found in the cell membrane, cell matrix and cell walls and are rich in hydroxyproline, alanine, glycine and serine. The carbohydrate chains are mainly attached to the serine and hydroxyproline residues¹⁰¹. AGPs are unique to different plant organs and are development/stage-specific. Although the biological function(s) of AGPs remain uncertain, different hypotheses have been proposed; for example, AGPs may be involved in cell proliferation^{102,103}, cell expansion^{104,105} and regulation of somatic embryo development^{89,106-109}.

The first cDNA *AGP Pcl1*, encoding an AGP core protein, was reported from pear suspension cultures¹¹⁰ and lately, at least six more AGP-coding cDNAs have been identified and characterized in various systems. It has been shown that the presence of specific proteins can be correlated with the morphology of somatic embryos and proteinaceous compounds in conditioned cultures can influence the cellular differentiation process¹¹¹. Knox and coworkers¹¹² demonstrated polymorphism of AGPs dur-

ing the development of carrot roots. With the help of immunological techniques, it has been possible to characterize AGPs associated with somatic embryo development. JIM4 monoclonal antibodies against AGPs were localized in the protoderm of early somatic embryos, the provascular tissue of the root apex and the cotyledons¹¹³. Therefore, AGPs may be involved in establishment of pattern formation during embryogenesis. The embryogenic potential of old carrot cultures that had gradually lost the embryogenic capacity can be restored by carrot seed extracts containing AGPs, thus indicating the role of specific AGPs for induction of embryogenesis. In Norway spruce, the morphology of less-developed somatic embryos (group B) was influenced by concentrates of extracellular proteins of more developed (group A) embryogenic cell lines¹¹¹. Seed extracts could, however, stably convert B cells into A-type embryogenic cells and influence their morphology, whereas concentrated extracellular protein enhanced only aggregation of B-type cells. The total amount of AGPs as well as the composition of AGPs vary among different cell lines, as demonstrated by crossed electrophoresis and various anti-AGP monoclonal antibodies (JIM4, JIM8, JIM13 and Mac 207). It is thus possible that AGPs are involved in cell-to-cell contact since their structural and physical properties render the cells more adhesive, making them capable of associating with other macromolecules.

Purified AGPs from carrot embryogenic suspension cultures, even at nanomolar concentrations, can influence to reinduce nonembryogenic cells to embryo-forming cells^{89,106}. Pennell and co-workers¹¹⁴ suggested that there may be some correlation between the level of JIM8 reactive cells and the number of somatic embryos formed, but later revealed that most somatic embryos develop from JIM8 negative cells, reflecting the embryogenic competence of the entire culture rather than marking single individual competent cells¹¹⁵. This further shows that acquisition of embryogenic competence involves cells or product of cells that are themselves not able to develop into somatic embryo, but have the nursing function for the development of carrot somatic embryos.

Immunocytochemistry has established correlation between the presence of JIM4/JIM8 AGP epitopes and various stages of somatic embryos¹¹³. Even though no relation between the expression of the JIM8 cell wall epitope and the ability to develop somatic embryos has recently¹¹⁵ been observed, other investigators proved that it was possible to manipulate somatic embryogenesis by addition of exogenous AGPs. In chicory embryogenic cultures induced from roots, addition of β -D-glucosyl Yariv's reagent in the medium inhibits somatic embryogenesis in a concentration-dependent manner by binding to the extracellular AGPs; however, the effect was reversible. Immunofluorescence and immunogold labelling with MAbs revealed that AGPs are localized to the outer cell walls of the peripheral cells of the globular embryos.

Binding of β -D-glucosyl Yariv's reagent to cell-wall AGPs of rose suspension cells also inhibited the growth in a reversible fashion, probably due to suppression of the cell cycle in combination with prevention of cell expansion. These observations support the proposed role of AGPs in cell growth and division¹⁰².

Immuno-magnetic sorting of single cells in a carrot embryogenic suspension culture using JIM8 and secondary antibody coupled to paramagnetic beads has been used to obtain pure populations, by McCabe and co-workers¹¹⁶. The cells in JIM8 (+) population are capable of forming somatic embryos, whereas cells of JIM8 (-) population fail to exhibit somatic embryo formation. In JIM8 (+) cell populations, certain cells (state-B cells) undergo asymmetric division resulting in JIM8 (-), i.e. state-C cells, and JIM8 (+), i.e. state-F cells. The state-C cells are competent to form somatic embryos in the presence of JIM8 (+) cells or their conditioned medium. This shows that there is some fragment or a part of AGP that is released extracellularly and is essential for the transition of the state-C cells into somatic embryos, and is involved in the signalling pathway for development of somatic embryos. Thus, a cell with a role in cell-cell communication and early cell fate selection can be marked by an epitope (presented by an AGP) in its cell wall.

Since AGPs are known to display developmentally regulated patterns of expression, they may be used for predicting emerging tissue patterns or developmental fate of cells¹¹⁷. Monoclonal antibodies (Mab) directed against carrot-seed AGPs yielded different AGP fractions by column affinity chromatography. There was two-fold increase in AGP fraction (ZUM18 AGPs) isolated with ZUM18 Mab in carrot embryogenic suspension culture, and an equivalent decrease in percentage of embryogenic cells with ZUM15 (Mab). The influence of ZUM15 AGPs was similar to the AGPs isolated from nonembryogenic suspension cultures. It is possible that the ratio of different AGPs in suspension culture determines the embryogenic potential, as is indicated from the studies in *D. carota* and *Cyclamen persicum*¹¹⁸. Diverse AGPs have promotory or inhibitory effects on carrot somatic embryogenesis. With the characterization of individual AGPs, they can be further manipulated in order to control more precisely the process of somatic embryogenesis.

Spatio-temporal expression of AGPs increases the possibility of their use as protein marker for detection of early stages of somatic embryos¹¹⁹. Protoplasts of carrot cells with reduced capacity for somatic embryogenesis could be retrieved partially with endochitinases and completely with AGPs pretreated with chitinases. This indicates the importance of *N*-acetylglucosamine and glucosamine association for the increased activity of AGPs. Also, the role of AGPs in cell division was reconfirmed, as they could re-initiate cell division of non-dividing protoplast subpopulation¹²⁰. Isolation and

sequencing of *AGP Pcl* and *AGP Nal* from pear cell suspension cultures and tobacco styles have been reported by Chen *et al.*¹¹⁰ and Du *et al.*¹²¹ respectively. Later, two more cDNAs coding for AGP have been identified from pear and tobacco cell suspension cultures^{122,123}. A carrot cDNA, *DcAGP1*, coding for the arabinogalactan protein has been recently isolated and characterized in carrot suspension cultures¹²⁴. *DcAGP1* codes for a novel and a non-classical AGP with similarity to basic proline-rich proteins and a PAC domain at the C-terminal. It has a histidine-rich basic domain and a cysteine-containing PAC domain found in a wide range of cell wall proteins, and is also characteristic of PRPs (pathogenesis-related proteins).

Lipid transfer proteins

Lipid transfer proteins (LTPs) are secreted extracellularly and may have a function in transporting phospholipids (cutin monomers) from their place of synthesis in ER to various cellular locations¹²⁵. Thus, they are indirectly involved in plant defence and prevention of water loss against environmental stresses, and some LTPs are also known to be ABA-inducible. The products of *LTP* genes typically lack tryptophan and are of small size (7–13 kDa), with 30–70% homology. These extracellular matrix inhabitants show controlled temporal and spatial expression in plant tissues. Expression of *LTP* genes is tightly associated with the first differentiated tissue of somatic embryos, i.e. protoderm differentiation. This outer layer exerts a regulatory role in controlling cell expansion during the embryo development programme¹²⁶. Expression of the *LTP* gene products is restricted to the peripheral layers of young tissues and developing embryos^{127–129}.

EP2 cDNA was the first to be isolated and characterized from carrot embryogenic cultures. It was later revealed that the gene encodes for a lipid transfer protein and is secreted extracellularly¹²⁸. The protein is not only expressed in embryogenic cell cultures but also in the shoot apex of seedlings, developing flowers and maturing seeds. The gene is uniformly expressed in the pro-embryogenic masses, whereas the expression diminishes in the non-embryogenic cell lines. The arrest of somatic embryos of the temperature-sensitive mutant, *tsII*, can be rescued by the extracellular addition of EP2 in the nutrient medium. This indicates that these mutants are defective in epidermis formation. EP2 may be expressed in the subepidermal layers for protective functions. *In situ* hybridization also demonstrates the localization of EP2 expression to the protodermal layer of somatic and zygotic embryos; thus EP2 may serve as an early marker for the detection of embryogenesis¹²⁸. In *Camellia* leaf cultures, during induction of direct somatic embryogenesis, deposition of cutin was found to be necessary for

normal somatic embryogenesis to occur, suggesting the involvement of *LTP* genes¹³⁰.

A reporter system has been developed in carrot to study the expression pattern of the *LTP* genes during somatic embryogenesis. The *Arabidopsis*, *LTP1* promoter is fused with the firefly luciferase coding sequence¹³¹, and the expression pattern of the reporter gene was identical to *EP2* gene from carrot. The use of semi-automated video cell tracking established that all the somatic embryos were derived from *AtLTP1* luciferase-expressing cell clusters, confirming further the role of *LTP* genes in the progression of somatic embryogenesis.

Maturation genes

During the maturation stages of somatic embryo differentiation, there is a rapid alteration in the gene expression programme; a variety of genes are 'turned on' or proteins synthesized *de novo*. The genes expressed during this period are maturation stage-specific and bear similarity with the zygotic embryo maturation genes. *Dc2.15* gene in carrot somatic embryos is maximally expressed at the heart stage and torpedo stage. Utilizing various PCR-based techniques, five distinct promoter sequences have been identified. Interestingly, they all contain a GATA binding site, which was originally found in human *HOX* gene involved in blood cell maturation. Expression studies with these promoters and *GUS* reporter gene revealed that only two of the promoter structures showed drastic increase in the *GUS* activity during the torpedo stage, while others remain silent/inactive throughout embryogenesis¹³².

Liu and co-workers¹³³ studied the expression of maturation gene *Mat1*, whose transcript level increased with desiccation and disappeared upon rehydration. However, lipoxygenase gene expression was also turned on during maturation of soybean somatic embryos. The lipoxygenase transcripts were detectable in germinating embryos, but were absent in non-germinating somatic embryos¹³⁴.

Late embryogenesis abundant proteins

During the terminal stages of zygotic embryogenesis as well as maturation, there is dramatic dehydration of the embryos and seeds, which is characterized by increased accumulation of ABA. At the molecular level, there is expression of specific genes whose products are in abundance and are capable of surviving the period of desiccation. Since these genes are expressed in the later stages of embryo maturation, they came to be known as late embryogenesis abundant (LEA) protein genes. Early-methionine labelled protein (Em), an abundant cytosolic protein in late, maturing wheat zygotic embryos, was the first LEA protein to be identified¹³⁵. Later, the *Em* gene

was cloned and characterized^{136,137} and its homologues have been identified in plants, including carrot and mungbean¹³⁸. All *LEA* genes share high sequence homology and are regulated by ABA. *LEA* genes play a similar key role in desiccation tolerance in different species. The characteristic features of the *LEA* genes are described by their premature induction and expression by exogenous ABA treatment. During somatic embryogenesis, *LEA* members were first identified in carrot somatic embryos – *Dc3*, *Dc8*, *DcECP31*, *DcECP40*, *DcEMBI* (refs 6 and 139). These genes were assigned as either early or late molecular markers of somatic embryogenesis depending upon their temporal and spatial expression pattern during various stages of somatic embryo differentiation. *Dc8* gene is expressed in the embryo and endosperm of zygotic embryos, and the mRNA expression pattern is similar between somatic and zygotic embryos^{140,141}. However, it was later found that the expression of *Dc8* is associated with but not dependent on somatic embryogenesis¹⁴². Using *Dc8* as a heterologous probe, *BP8*, an embryo-specific *LEA* gene has been identified in birch tree¹⁴³.

EMBI cDNA from carrot is expressed only in embryogenic tissues during the transition of globular- and torpedo-stage embryos and accumulates specifically in the meristematic regions¹⁴⁴. *EMBI* shows strong homology with *Em* genes isolated from wheat embryos, and may be involved in providing protection against dehydration during embryo maturation. Using RT-PCR, a homologue of *Em* gene has been identified in wheat somatic embryos¹⁴⁵. Twenty-fold high transcripts were observed in mature somatic embryos than the non-embryogenic cultures, and the gene is also expressed in mature zygotic embryos. Both Northern as well as RT-PCR revealed temporal regulation of the gene during somatic embryogenesis, thus indicating the use of *Em* genes as a marker to discriminate between direct and indirect embryogenesis during the initial period of culture¹⁴⁵.

Genes coding for lectins and storage proteins

Lectins are carbohydrate-binding proteins that are ubiquitous in microbes, animals and plants¹⁴⁶. The functions of plant lectins have, however, remained enigmatic although they have been assigned various roles during symbiotic recognition, seed storage, defence against predators and pathogens, growth regulation¹⁴⁷ and mediation of recognition between pistil and pollen¹⁴⁸, and various other plant recognition phenomena. Citrus seed storage protein, citrin, shows differential expression during embryogenesis. The cDNA clone coding for citrin is expressed at the early globular stage in the zygotic embryos whereas the somatic embryos expressed the transcript in the later stages, and only 10–20% level of the encoded peptides were observed in the polyembryonic seeds¹⁴⁹. Differen-

tial expression of lectins during various stages of somatic and zygotic embryo development has highlighted their importance in embryogeny of alfalfa. Later stages of embryos show increased accumulation of *MsLEC1* and *MsLEC2* mRNAs. Severe abnormalities were observed during somatic embryogenesis of *LEC1* and *LEC2* antisense plants. There is a strong possibility that mRNAs of *MsLEC1* and *MsLEC2* are critical for alfalfa embryo development, and their function may also involve growth regulation during embryogenic pattern formation¹⁵⁰.

Miscellaneous

Since carrot is the model system for investigation on somatic embryogenesis, it is interesting to note that most of the genes from different categories have been isolated from this system (see Table 1). However, some additional genes with accessory/incidental role in somatic embryogenesis have also been characterized (also see Figure 1).

Heat shock proteins

A range of heat shock proteins (hsps) have been isolated and studied for their expression pattern in zygotic embryos. In somatic embryos also, many of the genes coding for hsps are expressed differentially. It has been observed that heat shock treatment can arrest growth of globular embryos, whereas when other somatic embryo developmental stages are encountered with the same stress, they recover completely and the embryo continues to grow. The globular embryos exhibit lesser synthesis and accumulation of low molecular weight *hsp* mRNAs than other developmental stages or undifferentiated callus cells¹⁵¹. This can be attributed to failure of *hsp* gene transcription induction, while retaining the capability of regulating mRNA sequestration¹⁵². The stage-specific synthesis of hsps in carrot embryogenic cultures has been reported previously by Pitto *et al.*¹⁵³ and in tobacco cell suspension cultures by Kanabus and co-workers¹⁵⁴.

Mshsp18-1 and *Mshsp18-2* are the two cDNAs isolated from alfalfa suspension cultures, and both encode for small hsps belonging to *hsp17* family¹⁵⁵. The two cDNAs show 92% identity at the protein level and also share a homologous stretch of amino acids at the C-terminal region with *hsp22*, *hsp23* and *hsp26* from *Drosophila*. This region contains GVLTV motif characteristic of members of small hsps. *Mshsp18* mRNAs are not detectable in roots and leaf tissues, but low levels could be detected in microcallus suspension (MCS). The transcript level of *Mshsp18* could be enhanced by elevated temperature, CdCl₂ treatment and osmotic shock in cultured cells. Also *hsp18* mRNA level was detected to be high in somatic embryos derived from MCS. These studies together indicate that hsps may have a specific role during developmental switching in plant cells¹⁵⁵.

Another cDNA, *Dchsp-1*, coding for low molecular weight hsp has been isolated and characterized from the auxin-induced carrot hypocotyls. The transcript levels of *Dchsp-1* were abundant in carrot hypocotyl explants after auxin treatment, and show response to stresses such as heavy metals and high concentrations of cytokinins²⁶.

Germins

Germins are developmentally regulated proteins that were first discovered in wheat during germination. They are resistant to denaturation and proteases, and bind ionically to the cell wall. Their oxalate oxidase activity confers them a role in plant development and defence responses. Germin and germin-like proteins (GLPs) have been classified into a large gene family named cupins, by the presence of characteristic β -barrel core structure. Germins and GLPs have been proposed to play a significant role during somatic and zygotic embryogenesis^{156–158}. Interestingly, sequencing of wheat germin genome fragment *gf 2.8* and functional analysis of its promoter sequence, has revealed presence of numerous putative auxin-responsive elements (AuxREs). Also, the level of germin coding genes is enhanced transcriptionally upon auxin (such as 2,4-D) stimulation¹⁵⁹. Investigations of genes encoding auxin-binding proteins (ABP) in peach show significant similarity with GLPs¹⁶⁰. Genes encoding germins and GLPs have been isolated from various gymnosperms, monocots and dicots. However, *PcGER1* is the first sequenced and characterized cDNA isolated from somatic embryos from caribbean pine. *PcGER1* has an open reading frame of a 220-amino acid polypeptide sequence with a putative N-glycosylation site on Asn-69. The signal peptide of 24 amino acids supports the hypothesis of its apoplastic location. The N-terminal 20-amino acid sequence of the predicted mature protein is identical to one of extracellular pine GLPs, GP111. The genomic fragment *gf 0.8*, is identical to cDNA sequence and the coding region is uninterrupted by introns as in wheat *gf 2.8* and *gf 3.8* genomic clones¹⁵⁷. *PcGER1* is expressed in somatically quiescent and active zygotic embryos of caribbean pine and is absent in all the non-embryogenic lines as well as the female gametophyte. Detection of ionically bound cell-wall GLPs in the preglobular somatic embryos in *Pinus caribea* and not in non-embryogenic callus suggests their utility as molecular marker of somatic embryogenesis. It is also proposed that GLPs may be involved in initiation and termination of cell wall expansion during somatic embryogenesis.

Zygotic mutants and their significance in elucidation of mechanisms of embryogenesis

Mutational approach has greatly augmented our understanding of the functional activity of numerous genes

which could not be investigated by other techniques. Mutations in genes regulating flower development or embryo development and pattern formation have been studied and have significantly aided in identification of many novel and specific classes of genes.

In *Arabidopsis*, among the putative 500–1000 essential genes required for survival and successful reproduction, 40 appear to control the formation of embryo axis pattern elements^{126,161–163}. Also, the study of various embryo pattern-influencing zygotic mutants in *Arabidopsis* has enlightened the role of a variety of genes essential for normal embryo development. The analysis of mutants has shown that both apical–basal and radial patterns are independently established¹⁶⁴. Thus, radially arranged vascular, ground and epidermal tissues are present in mutants with abnormal apical–basal pattern, such as *gurke* and *fackel*¹⁶³. Studies have also been carried out regarding embryo lethal mutants. The *biol* and *raspberry* mutants are arrested in the zygotic embryo development during the transition of globular to heart stage^{165,166}, whereas *emb101*, *emb102* mutants show alterations in cytokinesis and cell wall formation¹⁶⁷. Radial axis mutants such as *knolle* (*kn*) and *keule* (*keu*) show abnormal radial patterning and homozygous mutant seedlings lack epidermis^{161,163}. The *gnom/emb30*, *short integument mutant* (*sin1*) of *Arabidopsis* show defects in the normal apical–basal axis or polarity determination, consequently leading to abnormal embryo formation. They also show abnormal first division and have either reduced or completely absent cotyledons^{163,168}. The *sin1* mutants, besides exhibiting funnel-shaped cotyledons or unorganized masses, have altered ovule formation and flowering times¹⁶⁸. Mutations in *monopteros* (*mp*), *gurke* and *pepino* influence early stages of embryo development (as early as octant stage); *gurke* and *pepino* influence the entire apical region resulting in elimination of one or both cotyledons and shoot apical meristem¹⁶⁹. Furthermore, the suspensor of *monopteros* mutant shows an aberrant organization, suggesting that the formation of root primordium requires participation of hypophyseal cells¹⁷⁰. Thus, root development depends upon both organized segmentation and on cell–cell interaction. This point is of major importance since primary root formation is often aberrant in somatic embryogenesis, where the two steps described are not so strictly defined¹²⁶. *Shoot meristem less* (*stm*), *mickey*, *fackel* (*fk*), *fass*, *zwillle* (*zel*), *hobbit* (*hbt*) are the mutants that show defective functioning or absence of shoot meristem in the maturing embryo^{163,167,170–172}. Thus, zygotic embryo developmental mutants are an important genetic tool facilitating understanding of mechanisms regulating morphogenetic and embryo differentiation events.

Somatic embryogenesis has become an important tool for the analysis of embryos developing outside the maternal environment. When combined with the availability of zygotic embryo mutants in *Arabidopsis*, this *in vitro*

system offers the possibility of manipulating the environment of the developing embryos and analysing effects in both the wild type and mutant embryos. The development of the zygote transits from the morphogenetic phase to the maturation phase, resulting in seed formation. Various cellular processes such as rapid synthesis and accumulation of storage proteins and lipids, also occur concomitantly. The embryo also acquires the ability to tolerate desiccation and establishes dormancy^{173,174}.

In *Arabidopsis*, *LEAFY COTYLEDON (LEC)* genes, *LEC1*, *LEC2* and *FUSCA3 (FUS3)* genes are seen to be involved in embryonic regulation during early and late phases of zygotic embryo development^{175,176}. These genes have pleiotropic effects¹⁷⁷⁻¹⁷⁹ and are required for normal development during morphogenesis as well as maturation phases. During early embryogenesis, *LEC* genes are required to specify suspensor cell fate and cotyledon identity^{180,181}, and are needed during maturation phase for the acquisition of desiccation tolerance and the expression of many maturation-specific genes. Consistent with the fact that conditions that promote maturation suppress germination, mutant embryos prematurely activate the post-germination programme. Thus *LEC* genes play a central role in controlling many aspects of zygotic embryogenesis and may function as regulators of morphogenesis. At the molecular level, *LEC1* shares extensive sequence similarity with the HAP3 subunit of CCAAT-binding transcription factor, implicating *LEC1* as a transcriptional regulator¹⁸¹. Ectopic expression of *LEC1* confers embryonic characteristics to seedlings and results in the formation of embryo-like structures on leaf surface thus indicating that the gene plays a role in conferring embryogenic competence to cells¹⁸¹. *LEC1* is proposed to be involved in establishing a cellular environment, promoting embryo development co-ordinating the morphogenesis and maturation phases¹⁸². Cloning of *LEC2* gene has shown¹⁸³ that it is expressed preferentially during embryogenesis and encodes a protein with similarity to other seed-specific transcription factors. Significant insight into the role of the gene was obtained by transgenic plants expressing *LEC2* ectopically, which results in the formation of somatic embryos. Besides somatic embryos, other organ-like structures and often embryonic characteristics to seedlings could also be conferred. These results suggest that *LEC2* is also a transcriptional regulator that establishes a cellular environment sufficient to initiate embryo development. Similarly, *FUS3* is also a regulatory protein: a B3 domain transcription factor that accumulates primarily during seed development. It is known to interact with RY promoter elements of seed gene promoters, and forms an essential component of the regulatory pathway controlling seed development^{182,184}.

Mordhorst *et al.*^{185,186} have also studied somatic embryogenesis in various zygotic mutants of *Arabidopsis*. When seeds of the *primordia timing* mutant (allelic to *hpt*, *cop2* and *amp1*) are germinated on 2,4-D medium,

stable embryonic cell cultures were observed. These somatic embryos arise from the abnormally enlarged shoot apical meristem (SAM) and exhibit typical patterning characteristic of zygotic embryos, though they have a higher and more variable number of cells. Embryonic cell clusters were also obtained from another mutant, *clavata (clv)*, with enlarged SAMs. A double mutant, *ptclv* with additive effects on SAM size shows even higher frequency of seedlings producing embryogenic cell lines. It was thus speculated that a larger-than-normal number of dividing and non-committed meristematic cells in the SAM facilitates the establishment of somatic embryogenesis in *Arabidopsis*. In contrast, Mordhorst *et al.*¹⁸⁷ also found that embryogenic cell cultures could be obtained from the mutants lacking an embryogenic SAM, *shoot meristemless (stm)*, *wuschel (wus)* and *zwill/pinhead (zll/pinh)*. The phenotype of the somatic embryos was similar to their zygotic counterparts, suggesting that the two might have an indistinguishable developmental programme and also that a functional SAM may not necessarily be required for somatic embryogenesis in *Arabidopsis*.

Another interesting mutant is the *pickle (pkl)* mutant of *Arabidopsis* which displays embryogenic traits after germination, as this mutant fails to repress embryonic features. Primary roots of *pkl* sometimes fail to develop normally after germination and instead exhibit embryonic differentiation characterized by gene expression of seed storage proteins and accumulation of large amounts of neutral lipids¹⁸⁸. Cloning and characterization of *PKL* locus reveals that it encodes a putative CHD3 protein, a chromatin-remodelling factor that is conserved in eukaryotes and is involved in repression of transcription. It is possible that *PKL* is a significant component of gibberellic acid-modulated development pathway that represses embryonic identity during germination and thus prevents re-expression of the embryonic development state, as otherwise, *LEC1*, the seed-specific transcription factor promoting embryonic identity is derepressed in *pickle* roots¹⁸⁹.

All these reports are suggestive of the fact that mutations in genes influencing SAM or *pkl* embryonic cells persist and probably through a default mechanism are capable of forming somatic embryos resembling zygotic embryos under favourable conditions. *clv1* being a putative member of a signalling pathway, indicates signal transduction pathways also playing an important role¹⁹⁰. If the default mechanism hypothesis holds true, then mutations in many genes can lead to an enhanced somatic embryo phenotype, which will be an attractive proposition for understanding the various pathways regulating embryogenic expression.

Concluding remarks

Studies in the field of plant somatic embryogenesis have progressed much farther than the prediction of plant cell

totipotency by the German botanist, Haberlandt in the early 1900s. The journey towards unearthing and analysing the underlying events of plant development actually initiated with the discoveries by Steward *et al.*¹⁹¹ and Reinert¹⁹² in carrot. Till date, carrot remains the workhorse for molecular characterization of cellular totipotency in plants^{1,2}.

These studies have clearly shown that somatic embryogenesis follows a unique developmental pathway regulated by temporal and spatial patterns of gene expression. Since the recognition of various regulatory mechanisms responsible for orchestrating this reprogramming of gene expression relies primarily on extensive molecular cloning complemented by functional analysis employing transgene technology, somatic embryogenesis serves as an excellent *in vitro* model system for such investigations. Comparison of the sexual and asexual embryo development pathways can also be supplemented by the information gained from the vast repertoire of zygotic developmental mutants¹⁶⁷. Plant embryogenesis has thus graduated from an era of descriptive and experimental embryology to that of molecular embryology. Meeting the challenge ahead would, however, entail multi-disciplinary approaches like laser and cell ablation of specific cell types, subcellular localization of gene products and manipulation of immature embryos in culture. Combined with the information generated through various genome projects, the elucidation of the mysteries of plant development would undoubtedly be in sight and provide an analytical understanding of the enigmatic totipotency in higher plants.

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