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Somatic Embryogenesis: Morphogenesis, Physiology, Biochemistry and Molecular Biology

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ABSTRACT Somatic embryogenesis has become a major tool in the study of plant embryology, as it is possible in culture to manipulate cells of many plant species to produce somatic embryos in a process that is remarkably similar to zygotic embryogenesis. Traditionally, the process has been studied by an examination of the ex vitro factors which influence embryo formation. Later structural, physiological and biochemical approaches have been applied. Most recently, molecular tools are being used. Together, these various approaches are giving valuable information on the process. This article gives an overview of somatic embryogenesis by reviewing information on the morphogenesis, physiology, biochemistry and molecular biology of the process. Topics covered include a brief description of the factors involved in the production of embryogenic cells. Carrot cell suspension is most commonly used, and the development of a high frequency and synchronous system is outlined. At the physiological and biochemical levels various topics, including the reactivation of the cell cycle, changes in endogenous growth regulators, amino acid, polyamine, DNA, RNA and protein metabolism, and embryogenic factors in conditioned medium are all discussed. Lastly, recent information on genes and molecular markers of the embryogenic process are outlined. Somatic embryogenesis, the best example of totipotency in plant cells, is not only an important tool in studies in basic biology, but is potentially of equal significance in the micropropagation of economically important plants.

Key words: Amino acid metabolism, carrot cell cultures, cell cycle reactivation, conditioned medium, DNA/RNA and protein metabolism, embryo development, embryogenic cells, embryogenic factors, molecular markers, polyamine metabolism

Introduction

Somatic embryogenesis in plants leads to the formation of embryos and ultimately plantlets from non-sexual cells and without sexual fusion. As such, it is the best example of totipotency in plants. This phenomenon, first proposed by Haberlandt (1902), states that all living cells possess the potential to regenerate entire organisms. Somatic embryogenesis, in the form of apomixis, is widely observed in nature (see Sharma and Thorpe 1995), and is presently reported to take place in numerous species of angiosperms, both monocots and dicots, and in gymnosperms in vitro (see Brown et al. 1995; Dunstan et al. 1995; KrishnaRaj and Vasil 1995; Thorpe and Stasolla 2000). The capacity to form somatic embryos in vitro in a large number of species has made it a useful and versatile tool in the study of basic aspects of embryogenesis, as well as in application in micropropagation, and genetic transformation of agronomic, horticultural and forestry species. This paper discusses basic aspects of somatic embryogenesis, namely morphogenic, physiological, biochemical and molecular studies on the process. Much of the information available on these basic aspects of somatic embryogenesis has come from studies with carrot cultures.

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Somatic embryogenesis: morphogenesis

Under in vitro culture conditions, somatic embryogenesis is achieved successfully through the judicious selection or manipulation of the inoculum, proper choice of the medium, including growth-active substances, and the control of the physical culture environment. Details on these morphogenic aspects of somatic embryogenesis can be found elsewhere, including Evans et al. (1981), Thorpe (1982, 1994), Ammirato (1983), Bhojwani and Razdan (1983), Merkle et al. (1995), and Thorpe and Stasolla (2000).

Various plant parts have been used to produce somatic embryos. For many species the explant of choice is the plant embryo, which consists of cells already possessing embryogenic competence (termed pre-embryogenic determined cells or PEDCs [Sharp et al. 1980; Evans et al. 1981]). Other plant parts more differentiated must be induced to become embryogenic, (termed induced embryogenic determined cells or IEDCs). These differences are a reflection of their current gene expression program. For achieving the IEDC state, cells which are quiescent or committed to some function in the plant must be re-programmed into the embryonic state. This often requires the formation of a callus through several rounds of cell division, and many factors both in planta and ex planta have been shown to influence the performance of the selected inoculum (see Thorpe and Stasolla 2000). Not unexpectedly genotype also plays an important role. Carrot tissues undergo somatic embryogenesis via the production of IEDCs.

The most commonly used medium for somatic embryogenesis is the Murashige and Skoog (MS 1962) salt formulation with additives including B-vitamins, a reduced form of nitrogen (usually amino acids and/or adenine), a carbon/energy source (usually sucrose at 2-4% w/v) and phytohormones/plant growth regulators. In manipulating somatic embryogenesis, early studies indicated that for many species exogenous auxin was an absolute requirement for the induction of embryogenic cells (ECs), also referred to as embryogenic masses or clumps, proembryos, proembryonic tissue, etc. These ECs will often develop into somatic embryos by transfer to a medium without or with a reduced level of auxin (see Nadar et al. 1978; Fujimura and Komamime 1979a; Vasil and Vasil 1986). The most commonly used auxin is 2,4-dichlorophenoxyacetic acid (2,4-D). Some species also require a cytokinin, and in a few cases, other growth active substances have been found to be necessary for or to enhance embryo development (see Thorpe and Stasolla 2000).

There are many aspects of the culture environment that can influence somatic embryogenesis in cultured tissues. These include (a) the physical form of the medium, i.e. liquid or semi-solid, (b) pH, (c) humidity, (d) light, (e) temperature, and (f) the gaseous atmosphere (Thorpe 1994). There is limited information on how these various factors and others, such as osmotic stress and culture density, influence somatic embryogenesis (see Thorpe and Stasolla 2000).

One of the most important attributes of somatic embryogenic tissue of many species is its ability to proliferate indefinitely (Merkle et al. 1995). The proliferative process has been termed secondary, recurrent or repetitive embryogenesis. The phenomenon is of potential importance for mass clonal propagation and in gene transfer technology. Proliferation of embryogenic cells takes a number of forms and is influenced by a variety of factors, including those involved in EC induction and embryo formation. Auxin is the best documented factor involved with continuous proliferation of ECs, but there is an apparent interaction of auxin with reduced nitrogen (Mohan Ram et al. 1982).

Many treatments lead to the formation of somatic embryos that appear morphologically normal, however, for some species, these embryos may not germinate or subsequently convert into normal plantlets (somatic seedlings or emblings). For others, precocious germination may occur prior to the completion of embryo formation, leading to poorly developing plantlets. Thus manipulation of the culture medium and environment to prevent precocious germination and produce physiologically normal mature somatic embryos is a requirement. Different salt formulations, which differ in the type, concentration and balance of nitrogen, in particular, are used at different strengths. As well, specific amino acids; including glutamine, proline and serine, may be important. Carbohydrate supply during embryo maturation also appears to be important for embryo quality and number (Merkle et al. 1995). While sucrose (up to 6% w/v) is the most commonly used carbohydrate during embryo maturation, other carbohydrates, such as maltose, may be more effective in some species. The role of

carbohydrate is partly osmotic. A major medium component for many species, especially gymnosperm, is abscisic acid (ABA). Other factors including high antioxidant levels, low O_2 levels, ethylene biosynthetic inhibitors, light quality and quantity have all been shown in influence somatic embryo quality and quantity (see Thorpe and Stasolla 2000).

The accumulation of reserve substances is a normal part of zygotic embryo development (Bewley and Black 1985). These are laid down during seed maturation and are needed following germination for early seedling growth. Such reserves are used for a similar purpose in somatic embryos, which, in addition, lack an associated endosperm or megagametophyte. Most of the studies on reserve accumulation in somatic embryos have been concerned with storage protein and lipid accumulation. Very few studies have investigated starch accumulation (Thorpe and Stasolla 2000). The effects of the various morphogenic treatments used to produce somatic embryos can have a profound effect on the histology, particularly of the meristem, and their subsequent germination and somatic seedling development (see Yeung and Stasolla, this volume).

As an example of the in vitro approaches used for somatic embryogenesis, the method used with carrot will be briefly outlined. Carrot (*Daucus carota* L) callus can be initiated from any part of the carrot, although petiole and taproot segments are the most commonly used explants (Ammirato 1984). These are usually placed on MS basal salts and vitamins with 1- 4.5μM 2,4-D. To initiate embryogenic suspension cultures, callus is transferred to liquid medium of the same composition. Suspensions with cell clusters of various sizes are subcultured every 14-21 days. For somatic embryo development, such cell clusters are transferred to auxin free medium. To improve the efficiency of embryo formation and to enhance its usefulness as a tool in basic research, Komamine and his associates (Fujimura and Komamine 1979a; Nomura and Komamine 1985) developed a high frequency and synchronous embryogenic system with carrot. Heterogeneous cell populations from carrot suspensions were fractionated by sieving through nylon screens and subsequent density gradient centrifugation in Ficoll. Small, round and cytoplasm-rich com-

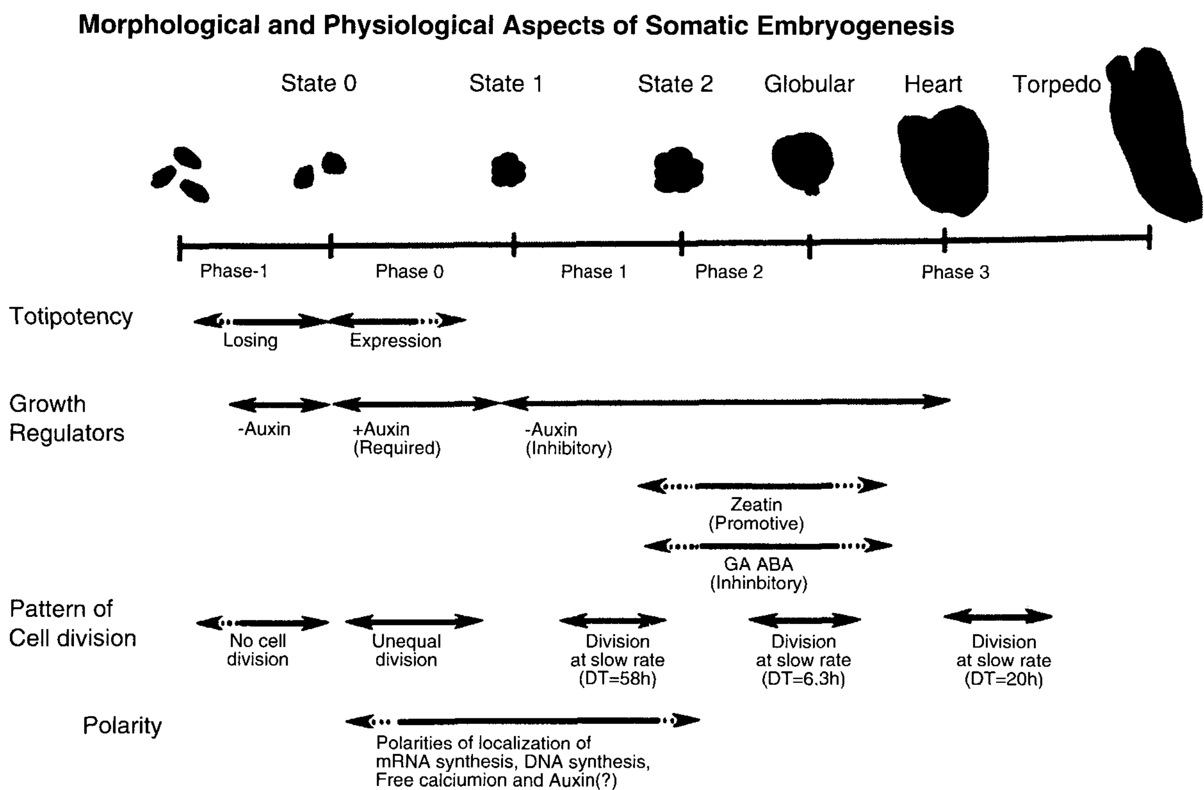

Figure 1. Summary of key morphological and physiological events occurring during the induction of embryogenic cells and their development into somatic embryos in the high frequency and synchronous carrot cell culture system. Figure kindly provided by Prof. A. Komamine, The Research Institute of Evolutionary Biology, Tokyo, Japan.

petent single cells (State 0) or embryogenic cell clusters (State 1), which consisted of less than 10 cytoplasm-rich cells, could be selected. State 0 single cells divided to give State 1 embryogenic clusters in the presence of auxin (2,4-D at 50 Mμwas optimal). State 1 embryogenic cell clusters differentiated into globular, heart and torpedo shaped embryos synchronously and at high frequency (between 80-90%) when they were transferred to auxin-free medium containing zeatin (0.1 μM), (see Figure 1).

Somatic embryogenesis: physiological and biochemical aspects

The appearance of a somatic embryo is the endpoint of a series of developmental steps involving cellular dedifferentiation and acquisition of competence in IEDCs, followed by cellular determination in all ECs and only finally by cellular differentiation (Christianson 1987). While most studies have been concerned with the morphogenesis of the process, fewer have dealt with the physiology and biochemistry of the process. A major problem with most systems is the lack of synchrony and the dilution effect during embryo formation (Thorpe 1982). As well, most of the studies have not been concerned with the whole process, as the usual approach has been to transfer embryogenically competent cells from a medium containing auxin to one without and following embryo development (Thorpe 1982; 1988). Thus, it is not always clear whether the parameters measured are related to the developmental process or merely a response to the effects of reduced auxin (Thorpe 1988; 1993). The carrot high frequency synchronous system of Komamine and his associates described earlier is proving extremely useful in gathering physiological, biochemical and molecular data on the somatic embryogenic process, but information obtained from other less efficient systems have, never-the-less, contributed to our understanding of the embryogenic process and also will be discussed.

Reactivation of the Cell Cycle

One of the basic features in initiation of somatic embryogenesis is the reactivation of the cell cycle in differentiated plant cells under the influence of external stimuli (Dudits et al. 1995). The artificially induced series of cell divisions open the way to a switch from somatic to embryogenic cell type that requires the coordinated expression of a set of genes and the post-translational modifications of the regulatory proteins also involved in the cell cycle control. Under defined circumstances, the reprogrammed transcription will generate a unique cellular state with specific functions that are needed to restart ontogenesis. Thus regulatory mechanisms in cell cycle control are of major importance, but only limited information is available about the cell cycle parameters in embryogenic cells.

A time course study of ^{3}H-thymidine incorporation into mesophyll protoplast-derived cells from leaves of embryogenic and non-embryogenic genotypes of alfalfa revealed that the maximum in the first incorporation peak occurred two days earlier in the embryogenic line (Bögre et al. 1990). This finding suggests that there might be a link between the accelerated activation of the cell cycle and embryogenic processes in somatic cells. Therefore, studies are needed on cell cycle control in relation to auxin-induced division and embryogenic response (Dudits et al. 1995).

Cell doubling time was shown to decrease at the start of somatic embryo development in carrot (Warren and Fowler 1978). Using their high efficiency and synchronous carrot system, Nomura and Komamine (1995) showed that in the early stage of the process, from State 1 cell clusters to embryos, three phases could be recognized. In Phase 1, during the first three days after transfer to auxin-free medium, undifferentiated cell division occurred slowly. In Phase 2, between 3 and 4 days, very rapid cell division occurred, leading to the formation of globular stage embryos, which subsequently developed into heart and torpedo shaped embryos. The cell doubling time in Phase 2 was only 6.3 hours (Figure 1). In addition, polarity and spatial distribution of cells at the S phase were characteristic of carrot cells (Komamine et al. 1990). In general, only a defined fraction of the cells divided in the presence of 2,4-D. On transfer to auxin-free medium, cells within the proembryogenic clusters continued to divide with a characteristic distribution of the daughter cells during embryo formation (Nishi et al. 1977). In contrast, non-embryogenic cells tended to be arrested at G1 due to auxin starvation.

Changes in Endogenous Growth Regulators

As indicated earlier, exogenous growth regulators play a prominent role in stimulating and inhibiting somatic embryogenesis, but their effects on endogenous levels of the plant hormones are physiologically important. Endogenous auxin, indole-3-acetic acid (IAA) was detected in embryos, but the level did not change significantly during embryo development (Fujimura and Komamine 1979b). A rapid decline in both free and conjugated 2,4-D metabolites was found within seven days after transfer from the auxin-containing medium (Michalezuk et al. 1992). In contrast, IAA levels remained relatively steady during that period, i.e. up to the globular stage, but it declined during further embryo development.

Exogenous GA_3 inhibited carrot somatic embryo development (Fujimura and Komamine 1975; Kamada and Harada 1979). Non-embryogenic cells and embryogenic cells in a medium with 2, 4-D showed high levels (2.9-4.4 [$\mu g.g^{-1}$ DW] of polar GA [GA_1 like] and low levels of less polar GA [GA_{4-7} like]). On transfer to auxin-free medium the developing somatic embryos contained low levels (0.2-0.3 $\mu g.g^{-1}$ DW) of polar GA, but 13-22 times higher levels of less polar GAs (Noma et al. 1982). In suspension cultures of a hybrid grape (*Vitis vinifera x V. rapestris*) free and highly water-soluble GA-like substances decreased on a dry weight basis during embryo development, but increased on an embryo basis (Takeno et al. 1983).

Exogenous ethylene or ethrel and 2,4-D suppressed SE formation in carrot independently (Tisserat and Murashige 1977). Ethylene biosynthetic inhibitors, such as cobalt, nickel and salicyclic acid, or addition of silver nitrate (an inhibitor of C_2H_4 action) all increased carrot embryogenesis (Roustan et al. 1989; 1990a,b). Similar results were obtained with C_2H_4 biosynthetic or action inhibitors with rubber, but in addition, the use of culture closures that allowed free gaseous exchange was as effective (Auboiron et al. 1990). Somatic embryogenic potential in different carrot lines was inversely related to the C_2H_4 production capacity of the cultures (Feirer and Simon 1991). More recently, a stage-specific effect of C_2H_4 was observed (Roustan et al. 1995). Ethylene did not affect the inductive or late differentiation phases, but its application at the beginning of the embryo developmental phase was inhibitory for embryo formation. In contrast, C_2H_4 biosynthesis during the induction or developmental phases inhibited the thidiazuron-regulated SE formation in geranium (Hutchinson and Saxena 1996; Hutchinson et al. 1997). In monocots, such as corn, initiation of embryogenic callus from immature embryos was enhanced 3-to-10 fold when the C_2H_4 biosynthetic inhibitor, aminoethoxyvinylglycine (AVG) and action inhibitors, $AgNO_3$ and silver thiosulfate (STS), were incorporated into the medium (Vain et al. 1989). Regeneration ability was also increased three-fold by maintaining the EC on medium with AVG or $AgNO_3$.

Exogenous ABA was shown to improve embryo morphology in some species, e.g. caraway (Ammirato 1977), and reserve accumulation during somatic embryo formation in others, e.g. rapeseed (Taylor et al. 1990). However, it inhibited embryo development in carrot (Fujimura and Komamine 1975). The amount of endogenous ABA in cultured cells and globular stage embryos of carrot was low, but concentrations increased during further embryo development (Kamada and Harada 1981). ABA levels in developing somatic embryos of hybrid grape decreased from the globular stage to the mature stage (Rajasekaran et al. 1982). Chilling to 4C, which allowed normal germination of mature embryos, enhanced precocious germination in immature embryos of hybrid grape. Chilling led to a marked reduction of endogenous ABA. However, ABA has been shown to enhance conversion of carrot somatic embryos into plantlets (Nickle and Yeung 1994).

The above findings, outlining changes in the endogenous levels of the phytohormones and their correlative effects, clearly indicate a significant role for them in normal somatic embryo development.

Amino acid metabolism

The enhancement of carrot somatic embryo development by -alanine was shown to involve accelerated cell division during the earlier stages of the process (Kamada and Harada, 1979). As well, α-alanine was found to be quickly transformed to glutamic acid by alanine aminotransferase and utilized as a nitrogen source (Kamada and Harada 1984). Activity of the enzyme was observed in both cells and embryos, but it decreased with culture time. In alfalfa, the production of somatic embryos was also increased by the addition of alanine and proline (Skokut et al. 1985). However, synthesis of protein from

these and other amino acids was not quantitatively different. Similar metabolic activity was found in both regenerating and non-regenerating lines, but the levels of free amino acids were higher in the non-regenerating lines.

A ^{15}N-NMR spectroscopic study with carrot revealed that the proembryogenic masses (PEMs) contained resonances for histidine, amino sugars, glutamine, arginine, urea, alanine, α -amino nitrogen, serine, aliphatic amines and some unknowns (Joy et al. 1996). Similar resonances were found in various embryo developmental stages (Figure 2). However, resonances for arginine and aliphatic amines peaked during the globular and torpedo stages and were substantially reduced in germinating stage embryos. The dominant resonances found in non-embryogenic cells and germinating embryos were glutamine and γ-amino nitrogen. Amino acid analysis of the various embryo stages showed that glutamate, glutamine and arginine were the major contributors to the soluble amino acid profiles. During embryo development glutamate and glutamine continued to increase in concentration, whereas arginine and its related metabolites (ornithine and α-amino butyric acid [GABA]) were biphasic; increasing in globular and torpedo stage embryos and decreasing in germinating embryos. ^{14}C-labeled glutamine pools in non-embryogenic cells and germinating embryos were greatest compared to other embryo stages, whereas labeled GABA pools were greatest in globular and torpedo stage embryos. Taken together, these data indicate that the amino acid metabolism of each embryo developmental stage is distinct. These changes are shown diagramatically in figure 3. The findings also suggest that during carrot somatic embryo development, a switch takes place in metabolism, whereby the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway is predominant in non-embryogenic cells and germinating embryos. Furthermore, during early to mid-embryo development, metabolism utilizing the ornithine cycle is enhanced and predominant.

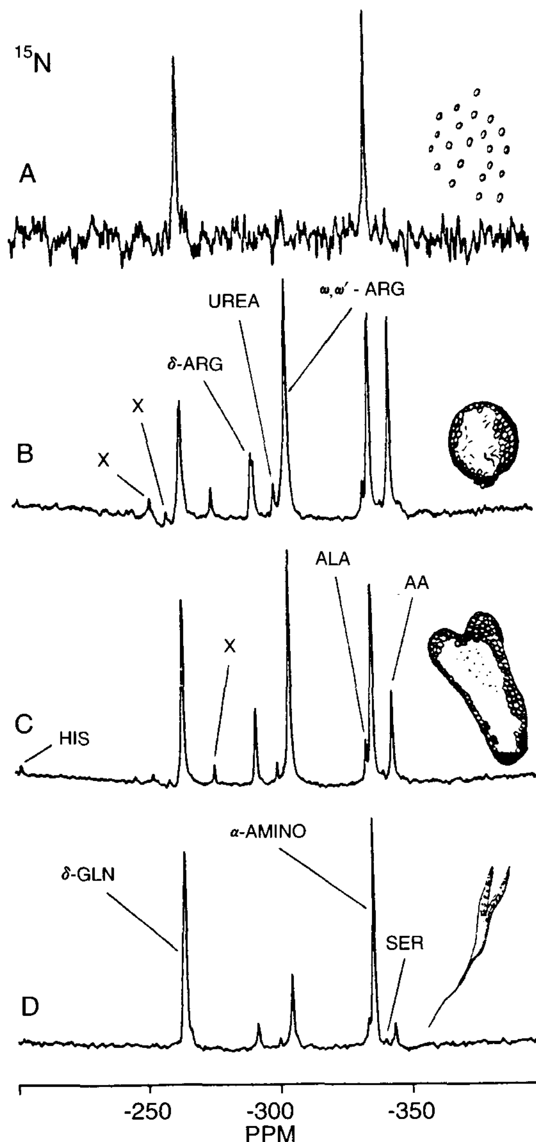

Figure 2. *Nitrogen NMR spectra from various somatic* embryo developmental stages of carrot cell suspensions. The cultures were grown in medium with ^{15}N-inorganic nitrogen for 10 days before sieving to obtain the different stages and the taking of spectra. ^{15}N-spectra from non-embryogenic cells (A), globular (B), torpedo (C) and germinating stage (D) embryos. AA, aliphatic amines; X, unknown. Reproduced with permission of Physiologia Plantarum from Joy et al. (1996).

Polyamine metabolism

Polyamines (PAs) are small aliphatic amines that have long been associated with somatic embryogenesis. Levels of PAs in developing carrot embryos were several times higher than in proliferating embryogenic cultures (Fieinberg et al. 1984). The activities of arginine decarboxylase and S-adenosylmethionine (SAM or AdoMet) decarboxylase (key enzymes in PA biosynthesis) were suppressed by auxin, but increased in cultures of developing embryos. In particular, putrescine and spermidine levels increased during carrot embryogenesis (Montague et al. 1978; Feirer et al. 1984; Mengoli et al. 1989). These findings indicate that increased PA levels are required for somatic embryogenesis in carrot.

Inhibitors of PA biosynthesis differed in their action during embryo formation. Partial inhibition of ornithine

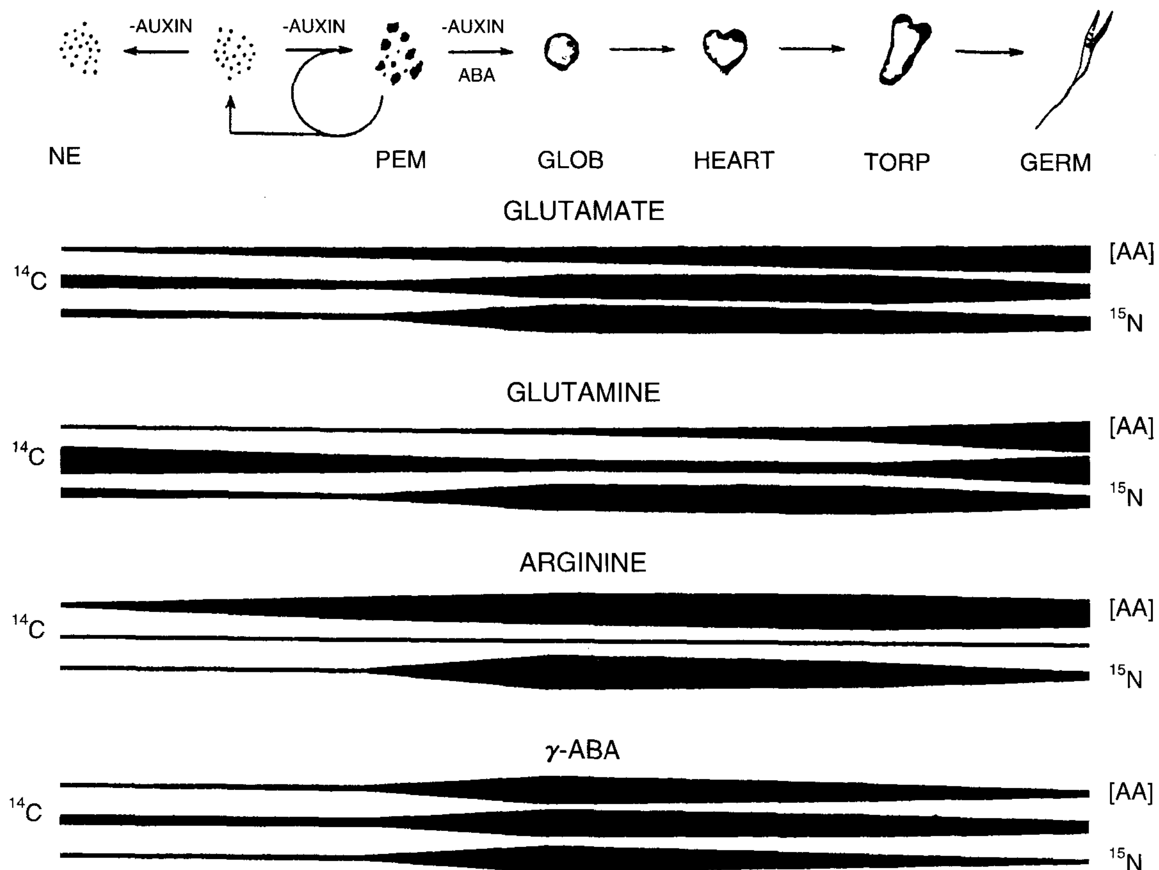

Figure 3. Diagramatic representation of relative changes in the major amino acid profiles in carrot cell suspensions during different stages of somatic embryogenesis. Cells were grown in culture for 10 days on ^{15}N-inorganic nitrogen before sieving to obtain the different stages. In contrast, ^{14}C-sucrose was fed to the isolated embryogenic stages for 3h before harvest. Metabolite levels are a comparison among the different embryogenic stages, and are relative to the greatest amount found (arbitrarily set at 100%). [AA], amino acid concentration, ^{14}C, carbon-14 content, ^{15}N, nitrogen-15 content, NE non-embryogenic cells. Reproduced with permission of Physiologia Plantarum from Joy et al. (1996).

decarboxylase only inhibited proliferative growth in embryogenic suspensions, but not embryo development (Robie and Minocha 1989; Minocha et al. 1991a); while complete inhibition diminished the inhibitory action of auxin on development. In contrast, inhibitors of arginine decarboxylase decreased the PA titer and SAM decarboxylase activity, thereby inhibiting embryo development (Feirer et al. 1984; Mengoli et al. 1989; Robie and Minocha 1989; Minocha et al. 1991a). Addition of polyamines in combination with their inhibitors allowed embryo development to proceed (Feirer et al. 1984). However, it should be noted that difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase, also counteracted the inhibitory effects of auxin transport inhibitors on embryo development (Nissen and Minocha 1993).

Methylglyoxal bis (guanylhydrazone) (MGBG), an inhibitor of SAM decarboxylase, completely inhibited somatic embryogenesis in carrot (Minocha et al. 1991b). MGBG caused a significant reduction in the levels of spermidine and spermine, while allowing increased accumulation of putrescine. Cellular levels of 1-aminocyclopropane-1-carboxlic acid (ACC), a precursor of ethylene, were considerably higher in the presence of MGBG. These results suggest that the interaction between ethylene and PA biosynthetic pathways through competition for the common precursor, SAM, plays an important role in the development of somatic embryos in carrot cell cultures (Nomura and Komamine 1995).

A further role for SAM in carrot somatic embryogenesis has been suggested (Munksgaard et al. 1995). S-adenosylhomocysteine (SAH) is the product of SAM when it donates its methyl group in reactions leading to DNA methylation. SAH, which is thought to inhibit DNA methylation, is generally low in mature plant cells due to SAH hydrolase. SAM increased 5 fold during somatic embryogenesis and a considerable increase in the

methylation index (SAM/SAH ratio) was also found. The authors proposed that the levels of SAM and SAH may be involved in the control of somatic embryogenesis by affecting the level of DNA methylation, which in turn might cause differential changes in gene activation.

More recent studies with eggplant further support the idea of a causative role for PAs in somatic embryogenesis (Yadav and Rajam 1998). Higher levels of PAs, particularly putrescine, due to higher arginine decarboxylase activity in leaf discs from the apical region (high embryogenic capacity) than from the basal region (with poor embryogenic capacity) were correlated with the differential embryogenic response. This finding was further supported by pre-treating the discs with putrescine and difluoromethylarginine to regulate the internal PA levels.

Conditioned medium and embryogenic factors

Although cell density has been shown to be important in somatic embryogenesis, as mentioned earlier, the actual factors promoting the morphogenic phenomenon have remained illusive. For example, conditioned medium could overcome the inhibitory effects of low cell density (Hari 1980; Smith and Sung 1985). Progress is being made in determining factors from conditioned media that might play a causative role in somatic embryogenesis. Much of this work has come from De Vries and his associates, who reported that a small number of proteins were secreted into the medium within two days of embryo initiation (De Vries et al. 1988). Some of these secreted proteins are thought to be related to the formation of embryogenic cells and somatic embryos. Analysis of these extracellular proteins showed that one of them EP1 was secreted only by non-embryogenic cells (van Engelen et al. 1991), whereas the extracellular protein EP2, identified as a lipid-transfer protein, was secreted only by embryogenic cells and somatic embryos (Sterk et al. 1991). Based on the extracellular location of EP2 and the expression pattern of the encoding gene, the authors proposed that EP2 might be involved in the transport of cutin monomers to the specific sites of cutin synthesis. A third protein (EP3) was shown to be capable of rescuing somatic embryos in a temperature sensitive mutant carrot cell line (ts11) at non-permissive temperature (De Jong et al. 1992). This ts11 mutant has a defect in the glycosylation of extracellular proteins (Lo-Schiavo et al. 1990). The rescue protein, consisting of at least three homologous isoenzymes, was determined to be an acidic endochitinase, resulting from a small family of class IV chitinase genes (Kragh et al. 1996). Of interest was the finding that the effect of the chitinase was mimicked by a *Rhizobium sp*-produced nodulation factor, a lipooligosaccharide (De Jong et al. 1992), which led to the suggestion that the chitinases were involved in the generation of signal molecules. Later studies (van Hengel et al. 1998) found that EP3 genes were expressed in embryogenic and non-embryogenic suspension cultures, including ts11, but not in somatic or zygotic embryos. EP3 genes are expressed in young fruit and in some endosperm cells of carrot seeds. They concluded that EP3 chitinases are involved in reinitiating cell division in embryogenic cells and embryos, as part of a nursing cell system that is required for but not restricted to embryogenesis.

A novel carbohydrate epitope in the cell walls of certain single cells in embryogenic, but not in non-embryogenic, suspension cultures of carrot has been located using a monoclonal antibody (Pennell et al. 1992). Expression of this epitope is regulated during initiation, proliferation, and prolonged growth of the suspension cultures, such that changes in its abundance always preceded equivalent changes in embryogenic potential. The epitope is expressed in the cell walls of three types of single cells and one type of cell cluster and are developmentally regulated. The switch of one of the single cell types to somatic embryogenesis was accompanied by the immediate dissipation of the cell wall epitope. This epitope is therefore a cell wall marker for a very early transitional state in the developmental pathway of carrot somatic embryogenesis. Further studies showed that some cells arising from epitope-containing cells by asymmetric division, are without the epitope, but can be made to form somatic embryos if cultured in conditioned medium from the epitope-containing cells (McCabe et al. 1997). This suggests that soluble signal molecules are involved in somatic embryogenesis and that cell-cell interaction, e.g., within embryogenic cell clusters, is important for embryo development.

Changes in DNA, RNA and protein metabolism

The induction of embryogenic tissue and the subsequent development of somatic embryos requires a repro-

gramming of the cultured material. Thus DNA and RNA metabolism and protein synthesis have been investigated during these processes and in particular during development of somatic embryos (Nomura and Komamine 1995). Polarity of DNA and RNA synthesis, mRNA, free Ca^{2+} ion and probably auxin appeared in the late phase of State 0 cells to State 1 cell clusters; thus making the acquisition of polarity a very early event in the embryogenic process (Figure 1). Active DNA synthesis was observed during the formation of globular embryos (Fujimura et al. 1980). Prior to active DNA synthesis, the turnover rate of RNA and protein increased substantially. As confirmation, it was observed that the activities of both the de novo and salvage pathways of pyrimidine nucleotide biosynthesis increased during embryogenesis accompanying active RNA synthesis (Ashihara et al. 1981). RNA synthesis increased initially and then decreased. The rate of rRNA synthesis was lower during embryo development than during EC proliferation in the presence of auxin, while the reverse was observed for the rate of Poly $(A)^+$ RNA synthesis (Sengupta and Raghawan 1980). Rates of protein synthesis also increased after transfer to auxin-free medium. SDS-PAGE and immunoblot analysis of total protein extracts indicated the presence of low amounts of globulins (storage proteins) in globular and mature somatic embryos of maize, but these increased following ABA maturation treatment (Thijssen et al. 1996). As well, although accumulation of soluble proteins, and 7s globulins, the predominant storage proteins in oil palm, increased rapidly during the early stage of somatic embryo development, they declined later, indicating lack of proper maturation of the oil palm embryos (Morcillo et al. 1998). Using their high frequency and synchronous carrot embryogenic system, Normura and Komamine (1995) showed that there was active turnover of RNA and protein during Phase 1 (1st 3 days in auxin-free medium) and synthesis of new species of mRNA and protein in Phases 1-2 (up to globular stage). In Phase 2, active DNA synthesis occurred, in part due to a reduction in replicon size. As well, high template activity and relative changes in components of histone were also observed in Phases 1-2.

Stress is also thought to play a role in the induction of embryogenic tissue (Dudits et al. 1995). As possible evidence, Pitto et al. (1988) observed characteristic differences in the pattern of heat shock proteins (HSPs) at different developmental stages of carrot embryogenesis. Also in carrot cultures, the globular stage somatic embryos accumulated considerably less heat shock RNA in comparison to the embryo at later stages or to cultured cells after heat shock (Zimmerman et al. 1989). Analysis of the level of HS 17.5 mRNA indicated the lack of transcriptional induction of this heat shock gene in globular carrot embryos. A possible link between the embryogenic response and HSPs is also supported by the finding that the temperature sensitive non-embryogenic carrot mutant (Ts59) is defective in the phosphorylation of a heat shock protein (Terzi and Lo Schiavo 1990). In alfalfa, a small heat shock gene (Mshsp18) was found to be expressed in early globular and heart stage embryos (Györgyey et al. 1991). The authors proposed that this HSP may serve as a molecular chaperone with an assembly function during the developmental switch for the initiation of embryogenic cells.

Somatic embryogenesis: molecular markers and genes

During embryogenic cell formation and subsequent embryogenesis, there is a shift in metabolism, as indicated earlier. These shifts precede and are coincident with the developmental process, and are therefore indicative but not proof that differentiation is occurring at the molecular level (Thompson and Thorpe 1990). There have been many attempts to analyze gene expression during the embryogenic process, and with improvements in the methods of molecular analysis, slow but steady progress is being made in increasing our understanding of the process (Thorpe and Stasolla 2000).

Two dimensional gel electrophoresis has revealed that specific proteins are detectable at different stages of the embryogenic process. For example, two "embryogenic" proteins that were coordinately regulated were observed during carrot embryo development (Sung and Okimoto 1981, 1983; Choi and Sung 1984). Other studies showed changes in specific polypeptides during embryo development (Hatzopoulos et al. 1990). A set of cDNA recombinants that represent genes with differential expression in PEMs or during somatic embryogenesis was detected in carrot (Wilde et al. 1988; De Vries et al. 1988). Another carrot cDNA detected both somatic and zygotic-specific mRNA, with increased transcripts in

heart-stage somatic embryos (Borkird et al. 1988; Franz et al. 1989). Differential screening of a carrot cDNA library identified clones representing genes that encode for glycine-rich proteins that are induced during the embryogenic stages (Aleith and Richter 1990), and a cell protein which can serve as an early marker for somatic embryogenesis (Kiyosue et al. 1992). As well, a variety of auxin-responsive genes have been identified in alfalfa cell suspension during the transition to embryo differentiation and different stages of embryo development (Dudits et al. 1995).

The high frequency carrot somatic embryogenesis system has allowed Komamine and his associates to investigate the process in detail. Their early studies detected two proteins that appeared and two that disappeared in developing embryos prior to the globular stage (Fujimura and Komamine 1982). Further studies led to the detection of four polypeptides, a protein (21D7) and 2 mRNAs during induction of embryogenic cells and cell cluster formation (Nomura and Komamine 1995). The protein, 21D7, has been determined to a component of the 26S proteasome, which is associated with cell division (Smith et al. 1997). Two other proteins (CEM1 and 6) have also been characterized. CEM1 has high homology with Elongation Factor 1α, which is essential for elongation of polypeptides during protein synthesis; and its transcripts are mainly localized in actively dividing areas of the developing embryo (Kawahara et al. 1994). CEM6, which encodes a glycine-rich protein, is especially expressed in the peripheral cells of globular and torpedo-staged embryos (Sato et al. 1995). Three additional genes (CHB2, 4 & 5) are homeobox-containing genes (Kawahara et al. 1995), which play a key role as switches in the control of developmental processes in various organisms. Another set of genes (CAR3, 4, 5 & 6) are expressed during carrot somatic embryogenesis. Their expression, together with that of other molecular factors during the different stages of somatic embryo development is indicated in figure 4.

Concluding thoughts

Somatic embryogenesis is the clearest example of

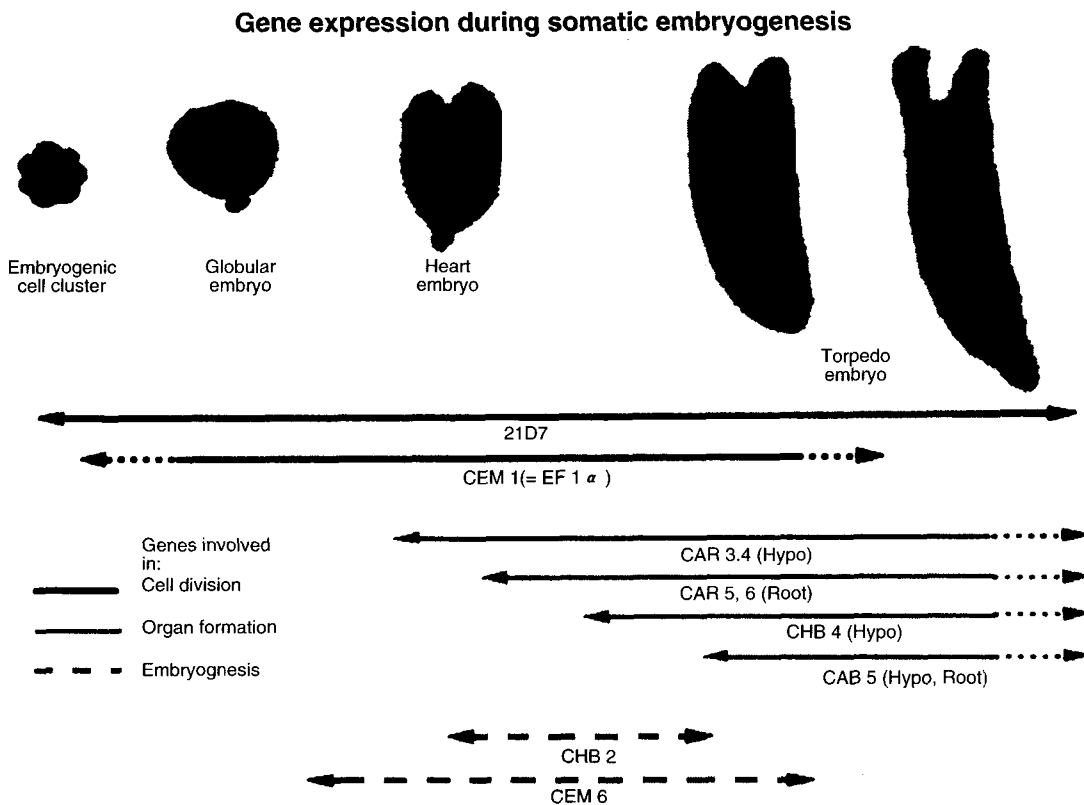

Figure 4. Gene expression during the different stages of somatic embryogenesis in the high frequency and synchronous carrot cell culture system. Figure kindly provided by Prof. A. Komamine, The Research Institute of Evolutionary Biology, Tokyo, Japan.

totipotency in plants. It has become a very important tool in examining the early events in the process, as cells in culture can be much more easily manipulated than the zygote within an ovule. The process has been studied at different levels of complexity from the morphogenic to the molecular. Although much of the information available deals with embryo formation from embryogenic cells, increasing information on the initial stages of EC formation has begun to emerge at these various levels. In particular, during the last decade, much information emphasising the biochemical and molecular biological aspects of the process has begun to emerge. The high frequency and synchronous carrot system (Figure. 1 & 4) has played a prominent role. While at present, much of the molecular data can at best indicate markers for the process, the functionality of these genes are slowly being elucidated. With the rapid development and improvement of molecular techniques, dramatic increases in information is about to occur. However, traditional biochemical approaches in describing pathway involvement in the different stages of the process is providing the background into which gene function through its production of "embryogenic" proteins will be increasing placed. This increase in information will allow for greater understanding of this morphogenic process and is therefore of great value per se. Nevertheless, the importance of this information also lies in its potential application to recalcitrant species, and with fused protoplasts and DNA-transformed cells of economically important plants, which cannot, at present, be regenerated into plantlets. Thus, studies on somatic embryogenesis, from both the basic and applied perspectives, will continue to make significant contributions to plant biology in this new millennium.

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