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Somatic Embryogenesis: A Model for Early Development in Higher Plants

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INTRODUCTION

The ability to produce morphologically and developmentally normal embryos and, indeed, whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis, resides uniquely within the plant kingdom. Since the initial description of somatic embryo production from carrot callus cells more than 35 years ago (Steward et al., 1958), this unique developmental potential has been recognized both as an important pathway for the regeneration of plants from cell culture systems and as a potential model for studying early regulatory and morphogenetic events in plant embryogenesis.

The last 5 to 10 years have witnessed an explosion in the number of species that can now be regenerated from cell culture into whole plants through somatic embryogenesis. The literature contains hundreds of references describing the specific manipulations required to effect somatic embryo development from a variety of agronomically and horticulturally important plants. Although this is obviously an extremely important application of the process of somatic embryogenesis, it is not the focus of this review. Rather, this review will focus on the use of somatic embryogenesis as a model system for understanding the regulation of gene expression required for the earliest developmental events in the life of a higher plant: the development of the fertilized zygote into a mature embryo.

The events of fertilization and subsequent embryo development normally occur deep within maternal tissues. The early embryo is minute and is surrounded by both endosperm and maternal cells. Although the morphological description of embryo development has been extensively recorded through microscopy, molecular and biochemical analyses of early embryogenesis have been hampered significantly by this physical inaccessibility. As a consequence, we know very little about the genes that are necessary for early embryogenesis in higher plants and even less about their regulation. This is beginning to be remedied by recent intensive efforts to genetically identify genes required for early embryogenesis in model systems such as Arabidopsis and maize (see West and Harada, 1993, this issue); many interesting mutants have been identified that may provide entry points into molecular analyses of major morphogenetic events in embryogenesis. These analyses would be greatly enhanced by the availability of cell, tissue, and developmental stage–specific markers of important events in the differentiation of cells and the establishment of the major tissue systems of the plant, which occur early in embryogenesis. In addition, once genes have been identified that are essential for embryogenesis, the subsequent analysis of their regulation would be greatly facilitated by the availability of an appropriate in vitro model system that is not limited in tissue quantity or accessibility. The somatic embryo system represents just such a model system. This review will summarize the process of somatic embryogenesis and will address the strengths and limitations of somatic embryos as potential models for studying early events in plant embryo development.

SOMATIC EMBRYO INDUCTION AND DEVELOPMENT

The original descriptions of somatic embryogenesis came from observations of carrot cells in culture, and carrot has remained the primary experimental system for studying somatic embryogenesis. Recent studies of Dudits et al. (1991) have highlighted the utility of alfalfa microcallus cells as an alternative system, particularly for studying the induction of embryo development from cultured cells. Somatic embryos are induced from cultured callus cells by a relatively simple manipulation of the culturing conditions, as summarized in Figure 1. In carrot, this generally involves (1) the establishment of a callus cell line from small hypocotyl pieces cut from sterilely germinated individual seeds, (2) the selection of an embryogenic subpopulation of the cultured cells through sieving or gradient fractionation, (3) the removal of auxin from the culture medium, and (4) the dilution of the cells to a relatively low density. The overall embryogenic potential of a culture is highest when the culture is relatively young (i.e., within the first year of its life) and resides primarily within a subpopulation of the culture that has been termed "proembryogenic masses" (PEMs; Halperin, 1966) or "State 1" cell clusters (Nomura and Komamine, 1985). These cell clusters can be selected out of the total population by sieving the culture and/or by density gradient fractionation so that the enriched population is greater than 90% embryogenic and relatively synchronous in development, at least through the early stages of morphogenesis. Although synchrony breaks down as the culture continues to develop, more advanced embryo stages can be isolated from less synchronous

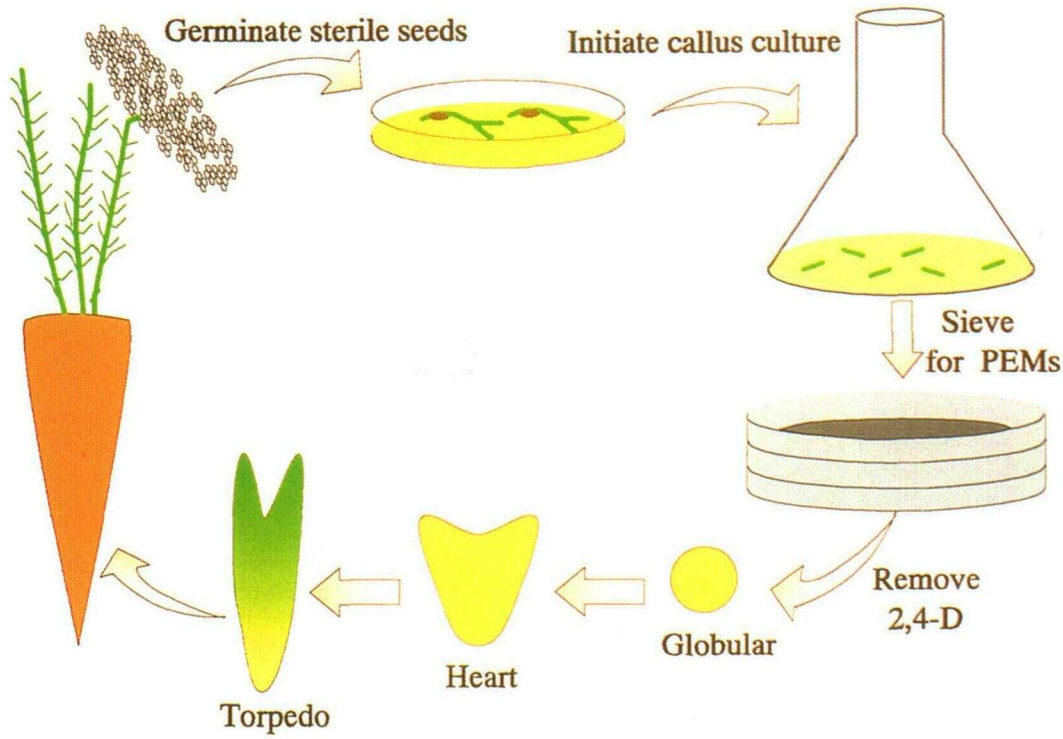

Figure 1. Summary of the Culturing of Carrot Somatic Embryos.

cultures by resieving the culture and selecting for larger embryos. Using this basic procedure, gram quantities of any given stage of embryogenesis can be easily isolated.

SOMATIC VERSUS ZYGOTIC EMBRYOGENESIS

The development of somatic embryos closely resembles that of zygotic embryos both morphologically and temporally. As summarized in Figure 2, embryos of the first recognizable stage, the globular stage, generally grow out of the small cell clusters selected for somatic embryogenesis within 5 to 7 days after carrot cells are shifted to auxin-free medium. In some cases, a small suspensor-like region can be seen, although this is more easily visualized in embryos developing on solid media than in liquid cultures that are shaken (Halperin and Wetherell, 1964). After 2 to 3 more days of isodiametric growth, the globular stage is followed by an oblong stage (Schiavone and Cooke, 1985), which signals the shift from isodiametric to bilaterally symmetrical growth and the beginning of the heart stage. The globular-to-heart transition is clearly marked by the outgrowth of the two cotyledons, the elongation of the hypocotyl, and the beginning of the development of the radicle. These processes continue through the torpedo and plantlet stages, and by ~2.5 to 3 weeks after induction, plantlets can be identified that contain green cotyledons, elongated hypocotyls, and developed radicles with clearly differentiated root hairs. These plantlets will continue to grow in liquid culture, or they can be transplanted to solid medium for regeneration of whole plants.

The similarity between zygotic and somatic embryogenesis is both striking and remarkable, considering that somatic embryos develop completely outside both the physical constraints and the informational context of maternal tissue. The fact that structurally and developmentally normal embryos can develop from somatic cells demonstrates that the genetic program for embryogenesis and its elaboration are totally contained within the cell and can function completely in the absence of gene products from the maternal environment. Although the hormonal content of callus growth medium may somehow mimic some natural signal to initiate embryogenesis, it is clear that the morphology and size of the various embryo stages are completely intrinsic in the embryogenic program and are not controlled by any environmental information or spatial limitations.

EMBRYO DEVELOPMENT AND HORMONES

Although it might appear that the removal or decrease in auxin is the trigger of somatic embryo development, the situation

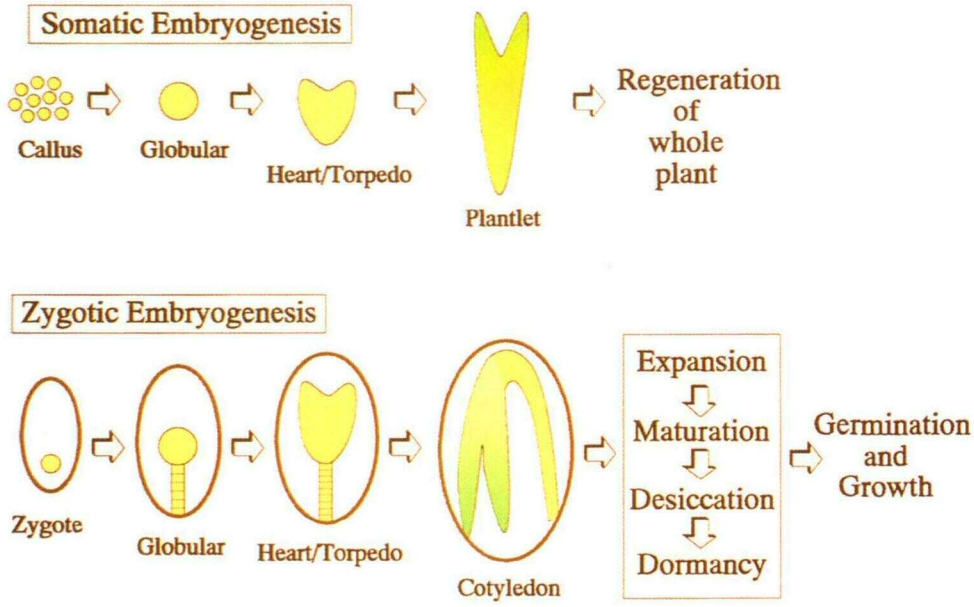

Figure 2. A Comparison of Somatic and Zygotic Embryogenesis.

Morphologically and developmentally, somatic embryos and zygotic embryos are most similar from the globular stage through the torpedo stage. Somatic embryos do not experience desiccation or dormancy, but rather continue to grow into fully differentiated plantlets.

is not quite so simple. A significant literature on auxin biosynthesis, metabolism, and transport in embryos that has grown out of extensive analysis of carrot somatic embryos (Schiavone and Cooke, 1987; Michalczuk et al., 1992a, 1992b) shows that auxin appears to play important roles both in the induction of embryo development in culture and in the subsequent elaboration of proper morphogenesis in embryo development.

The role of exogenous auxin in somatic embryo induction appears to depend on the nature of the explant used in the experiment. For example, petiole explants (Ammirato, 1985), hypocotyl explants (Kamada and Harada, 1979), and single cells isolated from established suspension cultures (Nomura and Komamine, 1985) require exposure to exogenous auxin for 1, 2, or 7 days, respectively, before they are competent to undergo embryogenesis upon auxin removal. Whether auxin treatment stimulates more than just cell proliferation as a prerequisite to embryogenesis is unclear. Microcallus cells of alfalfa require only a short (a few minutes to a few hours) pulse of auxin before they are competent to initiate embryogenesis in hormone-free medium (see Dudits et al., 1993, for review).

Although the process of embryo induction from cells in culture is not understood, it is now generally believed that in the continued presence of auxin, the PEMs within the culture synthesize all the gene products necessary to complete the globular stage of embryogenesis and that the PEMs also contain many other mRNAs and proteins whose continued presence generally inhibits the elaboration of the embryogenesis program. It follows that the removal of auxin results in the inactivation of a number of genes such that the embryogenesis program can now proceed. The observation that some carrot cell lines are able to develop to the globular stage but not beyond in the continued presence of auxin (Halperin and Wetherell, 1964; Borkird et al., 1986) suggests that new gene products are needed for the transition to the heart stage and that these new products are synthesized only when exogenous auxin is removed.

Once embryogenesis has been induced, the role of auxin changes in that the embryos begin to synthesize their own auxin, possibly via an alternative pathway (Michalczuk et al., 1992a, 1992b). Moreover, several studies have revealed that proper polar transport of auxin is a prerequisite for normal morphogenesis (Schiavone and Cooke, 1987; Liu et al., 1993) beyond the globular stage. Interestingly, however, the dependence of morphogenesis on polar auxin transport appears to be different in somatic and zygotic embryos. In somatic embryos, treatment of embryos with inhibitors of polar auxin transport results in blockage of morphogenesis to the next embryo stage (e.g., inhibitor-treated globular stage embryos continue spherical expansion but not axis elongation and inhibitor-treated oblong stage embryos continue axis growth but do not initiate cotyledons; Schiavone and Cooke, 1987). Zygotic embryos, however, show a less dramatic effect of treatment with the same inhibitors of polar auxin transport. In *Brassica juncea* (Indian mustard), inhibition of auxin transport at the globular stage results in the production of mature embryos with a fused, cylindrical cotyledon rather than two separate cotyledons (Liu et al., 1993). However, treatment of more mature embryos has no effect on subsequent morphogenesis.

What is the basis of this difference in response between somatic and zygotic embryos? One suggestion (T. Cooke, personal communication) is that in zygotic embryos, morphogenesis is actually regulated by two overlapping mechanisms, one of which arises as a maternal effect or as a consequence of the polarized position of the embryo in the embryo sac, whereas the other is intrinsic in the embryo itself. This latter, intrinsic mechanism, which would be the only active mechanism in somatic embryos, would be dependent on polar auxin transport, whereas the former mechanism, which would exist in zygotic embryos, would promote some aspects of morphogenesis even in the absence of polar auxin transport. Although the existence of a dual system for auxin regulation of embryogenesis remains speculative, these results illustrate the potential of using somatic embryos to distinguish between intrinsic and extrinsic regulation of the process of embryogenesis. This may also be useful in understanding the influence of another plant hormone, abscisic acid (ABA), on the process of embryogenesis, as discussed below.

Clearly, the involvement of auxin in the induction and development of zygotic and somatic plant embryos is complex, but what is obvious is that the products of both processes, a globular embryo with the full potential for correct organogenesis, appear quite equivalent. It follows that the molecular events that dictate the differentiation and propagation of all of the major tissue systems in the developing embryo are likely very comparable, if not equivalent, in somatic and zygotic embryos. Because these events occur during the globular to heart/torpedo stages, it is therefore at these stages that somatic embryos can most clearly serve as a good model for embryogenesis and as a source of materials for biochemical and molecular analysis.

Beyond the torpedo stage, the processes of zygotic and somatic embryogenesis again diverge. Zygotic embryos move into the cotyledon stage, followed by the maturation stage during which substantial storage protein synthesis occurs, followed by preparation for desiccation and dormancy (see Thomas, 1993, this issue). Mature zygotic embryos dehydrate, a period of quiescence ensues, and ultimately a new program of postgermination development begins. A significant proportion of this desiccation–dormancy program appears to be hormonally regulated, primarily by ABA. In contrast, somatic embryos grow and differentiate continuously, apparently activating the shoot and root apical meristems with no obvious quiescent state. Because of the divergence between somatic and zygotic embryos at these later stages of development, the term "plantlet" embryo (versus "cotyledon" embryo) seems more appropriate to describe the fully differentiated somatic embryo.

Although somatic embryos do not desiccate and become dormant, they do synthesize and accumulate ABA (Hatzopoulos et al., 1990a), and they also express a number of genes that have been shown to be ABA inducible and that are generally associated with desiccation tolerance (e.g., *LEA* genes, discussed below). If these genes do, in fact, play a role in desiccation tolerance, then it would logically follow that the signal to prepare for desiccation is an intrinsic and anticipatory one, in that these genes are expressed in somatic embryos that will never experience desiccation. In contrast, the dormancy program is apparently not activated in somatic embryos, but rather appears to be induced extrinsically, possibly through a maternal signal, which could be simply a higher ABA concentration. This would be consistent with the observation that treatment of plantlet stage somatic embryos with exogenous ABA can induce a quiescent state similar to the dormancy of zygotic embryos (Ammirato, 1987). Clearly, our understanding of the role of ABA in embryogenesis is incomplete, but further analysis of ABA metabolism and influence in somatic embryo development should help to clarify the contribution of internal and external sources of ABA in the proper development of zygotic embryos.

GENE EXPRESSION DURING SOMATIC EMBRYOGENESIS

Gene expression during somatic embryogenesis can be evaluated either by isolating genes expressed in somatic embryos and subsequently identifying the function of those genes or by studying the expression of a variety of other genes isolated from nonembryo tissues in the hope that they may also play some role in embryogenesis. A number of genes have been identified that are enhanced in expression in developing embryos, and several of these are being used to analyze mechanisms of gene regulation during embryogenesis.

Genes Isolated from Somatic Embryos

The dramatic transition from unorganized callus cell growth to somatic embryo development, coupled with measurements that indicate active RNA synthesis (Fujimura and Komamine, 1980) during this transition, suggested that a substantial reprogramming of gene expression, presumably occurring at the transcriptional level, dictates this developmental switch. Early studies of Sung and coworkers showed that few changes were apparent in the abundant proteins that were being synthesized in somatic embryos compared to callus cells (Sung and Okimoto, 1981). These results were optimistically interpreted as showing that at least a few changes in gene expression could be observed by protein analysis, and it was speculated that even more changes may have occurred in the production of less abundant proteins or mRNAs. Several groups took a similar approach to trying to identify "embryo enhanced genes" from carrot somatic embryos. The basic experimental strategy relied on a comparison between genes and proteins being expressed in somatic embryos versus callus cells. Several of the genes that have been isolated from somatic embryos are summarized in Table 1.

In addition to these general differential screening approaches, other experimental strategies have allowed the isolation of a small number of additional genes that are up regulated in somatic embryos. For example, capitalizing on

Table 1. Characteristics of Genes Cloned from Carrot Somatic Embryos

Gene	Identity of Product	Expression Information: Callus[a]	Somatic Embryo[b]	Zygotic Embryo[c]	ABA[d] Increase	Library Construction	Reference
DC8	LEA (Grp3)	±[e]	+ + + (H)	+ + +	Yes, embryo	λgt11, embryo antisera to callus proteins	Borkird et al. (1986), Franz et al. (1989), Hatzopoulos et al. (1990a), Goupil et al. (1992)
DC49	Unknown	+	+ + +	ND[f]	ND		Choi et al. (1987)
DC59	Oleosin	±	+ + + (H)	+ + +	Yes, embryo		Hatzopoulos et al. (1990b)
DC3	LEA (Grp3)	±	+ + + (PEM,G,T)	ND	Yes, embryo and vegetative tissues	λgt10, cDNA from mixed embryo vs. callus cDNA	Wilde et al. (1988), Vivekananda et al. (1992)
DC5	Unknown	ND	+ + + (PEM,G,T)	ND	ND		
DC13	Unknown	ND	+ + + (PEM,G,T)	ND	ND		
EMB-1	LEA (Grp1)	±	+ + (G,H); + + + (T)	+ + + (seed)	ND	λgt10, cDNA from mixed embryo vs. callus cDNA	Ulrich et al. (1990), Wurtele et al. (1993)
ECP31	LEA (Grp4)	ND	+ + + (PEM); ± (G,H,T)	+ + + (H)	Yes, somatic embryos	λgt11, cDNA from PEMs, probed with PCR-amplified DNA for protein	Kiyosue et al. (1992, 1993)
ECP40	LEA (Grp2)	ND	+ + + (PEM); ± (G,H,T)	+ + + (H)	Yes, somatic embryos		
EP2	Lipid transfer	±	+ + +	+ + +	ND		De Vries et al. (1988), Sterk et al. (1991)
EP3	Chitinase	ND	+ + +	ND	ND		De Jong et al. (1992)
DC1.2, 2.26, 3.1	Unknown	ND	+	ND	ND	λgt10, cDNA from day 8 embryos vs. cDNA from callus plus day 20 embryos	Aleith and Richter (1990)
DC2.15	Proline rich	+	+ + + (G,H,T)	ND	ND		
DC4.2	Unknown	+	+ + + (H)	ND	ND		
DC7.1	Glycine rich	+	+ + (G,H)	ND	ND		
DC9.1	Glycine rich	+ + +	±	ND	ND		
DC10.1	Unknown	+	+ + + (G); + + (H,T)	ND	ND		
EF1-α	Elongation factor	+ +, Not translated	+ + + (G polysomes)	ND	ND	λZapII, cDNA to polysomal RNA from globular embryos vs. polysomal cDNA from callus	Apuya and Zimmerman (1992), N.R. Apuya (unpublished results)
ATP-2	ATPase	+ +, Not translated	+ + + (G polysomes)	ND	ND		
CEM1	EF1-α	ND	+ + (PEM); + + + (G,H,T)	ND	ND	λgt11, cDNA from day 5 embryos vs. callus	Kawahara et al. (1992)

[a] Callus generally refers to total callus cultures (i.e., embryogenic and nonembryogenic cells). If the culture was separated, the information for the PEMs is listed in the "Somatic Embryo" column.

[b] Somatic embryo stages include PEMs to plantlet stages. G, globular stage; H, heart stage; T, torpedo stage.

[c] Zygotic embryo analyses represent information from either in situ hybridization or RNA blot hybridization.

[d] ABA increase reflects an increase in mRNA abundance after treatment of somatic embryos (or other tissues as stated) with exogenous ABA.

[e] mRNA levels range from ± (detectable but not abundant) to + + + (much greater and maximal abundance); −, not detectable.

[f] ND, not determined.

the observation that embryogenic cultures secrete some unique proteins, Sterk et al. (1991) isolated genes encoding two extracellular proteins (EP), EP2 and EP3, that are enhanced during embryogenesis. Experiments designed to isolate post-transcriptionally controlled genes yielded two other genes, EF1-α and ATP2 (Apuya and Zimmerman, 1992). In total, 21 genes have been described, some of which have been characterized in greater detail than others. The identities or at least the properties of several of these genes have been described and are summarized in Table 1. Each of the classes of proteins that have been identified as being up regulated during embryogenesis has added to our understanding of both the process of somatic embryogenesis as well as the relationship between somatic and zygotic embryogenesis, as described below.

Late Embryogenesis Abundant (LEA) Gene Expression in Somatic Embryos

Several genes that are preferentially expressed in somatic embryos appear to have characteristics of a class of proteins called LEA proteins (Dure et al., 1981, 1989; Galau et al., 1986). LEA proteins are very hydrophilic proteins that are abundantly expressed late in zygotic embryogenesis in many plant species, including cotton (in which they were originally described), barley, rice, oilseed rape, and wheat (Dure et al., 1989). The timing of their expression in embryogenesis and their ABA inducibility have led to the suggestion that in zygotic embryos, they play a role in protecting the embryo during desiccation. The *LEA* genes isolated from carrot somatic embryos include representatives of all four described groups of LEA proteins, which are characterized by significant homology in either amino acid composition or general protein structure (Dure et al., 1989).

All of the carrot *LEA* genes that have been analyzed to date show detectable expression in callus cells (some, like ECP31 and ECP40, are highly expressed in the PEMs of callus cell cultures), and most of the *LEA* transcripts increase significantly in abundance in somatic embryos at the heart stage (Choi et al., 1987; Wilde et al., 1988; Franz et al., 1989; Kiyosue et al., 1992, 1993; Wurtele et al., 1993). Indeed, we have found the LEAs to be the most abundant and differentially expressed mRNAs in somatic embryos, as compared to callus cells (X. Lin, G-J. Hwang, and J.L. Zimmerman, unpublished observations), which probably explains why so many *LEA* genes have been isolated through differential screening approaches using carrot somatic embryos. All of the carrot *LEA* genes tested can be induced by ABA treatment of callus cells and somatic embryos (Hatzopoulos et al., 1990a; Goupil et al., 1992; Kiyosue et al., 1992, 1993; Vivekananda et al., 1992). However, it appears that only DC3, a group 3 *LEA*, can be induced in nonembryonic tissues by either ABA or desiccation stress, whereas the other LEAs that have been tested (DC8, DC59, ECP31, and ECP40) cannot be induced in nonembryonic cells. A further discussion of such genes and their regulation by ABA and other factors can be found in Thomas (1993, this issue).

In addition to the analysis of regulatory mechanisms governing the expression of *LEA* genes in embryogenesis, an investigation of the distribution and timing of EMB-1 *LEA* gene expression in somatic and zygotic embryos of carrot has been performed (Wurtele et al., 1993). In situ hybridization analysis showed that EMB-1 mRNA begins to accumulate uniformly in both somatic and zygotic globular embryos, which is significantly earlier in development than the time of maximum production of LEA proteins. As development proceeds to the heart stage in both zygotic and somatic embryos, EMB-1 mRNA accumulates to higher levels and begins to show a polarity of distribution, with hybridization predominantly over the peripheral regions of the embryo. In mature zygotic embryos, very high levels of EMB-1 mRNA are seen, primarily associated with the procambium and shoot and root apical meristems. In plantlet stage somatic embryos, EMB-1 also accumulates predominantly in the meristematic cells, but at lower levels. There is no detectable EMB-1 mRNA in the endosperm, developing seed coat, or developing carpels or fruit of zygotically produced seeds, and EMB-1 mRNA does not accumulate in any cell type of young plants. The apparent difference in levels of accumulation of this *LEA* mRNA in somatic and zygotic embryos could support the idea that zygotic embryos experience a secondary signal (possibly a pulse of ABA) from the maternal environment that could not only enhance *LEA* gene expression, but could also signal the beginning of the dormancy program.

In situ localization experiments have thus shown that the expression pattern of at least the EMB-1 gene in carrot somatic embryos is analogous to its expression pattern in zygotic embryos of carrot (Wurtele et al., 1993). This result provides important validation of the use of somatic embryos as a model for studying embryogenesis. The fact that *LEA* mRNAs can be detected at much earlier stages in carrot somatic embryos than in cotton zygotic embryos may be a reflection of the fact that so much more young embryo tissue can be sampled in somatic embryo systems in which laborious dissection is not necessary, or, possibly, that in situ hybridization allows a more direct comparison between somatic and zygotic embryos. It would be instructive to perform in situ hybridization analyses of *LEA* gene expression in other species, such as cotton, for which RNA blot analysis suggests expression later in embryo development.

Somatic embryos provide a useful backdrop for analysis of the intrinsic hormonal influences on embryo induction, morphogenesis, and maturation preceding desiccation and dormancy. If the entire process is hormonally regulated, the activation of the entire signal transduction pathway must be triggered by the embryo itself rather than from any maternal interaction, because this is totally lacking in developing somatic embryos.

Secreted Proteins Produced by Somatic Embryos

Several years ago, it was observed that conditioned medium from somatic embryo cultures could promote somatic

embryogenesis (Hari, 1980; Smith and Sung, 1985). In addition, it was shown that secreted EPs could rescue embryogenesis in a temperature-sensitive (*ts*) variant carrot cell line (Lo Schiavo et al., 1988; De Jong et al., 1992). Exploiting the observation that important proteins appear to be secreted from carrot somatic embryo cultures, clones encoding some of these EPs were isolated (De Vries et al., 1988; Sterk et al., 1991). One of these clones, EP2, was identified as encoding a lipid transfer protein whose function was suggested to involve the transport of lipids or other apolar molecules out of cells. The expression of carrot EP2 is spatially regulated, showing restriction to cells of the protoderm of somatic embryos and epidermal cells of leaf primordia and floral organs (Sterk et al., 1991). Thus, although the expression of EP2 is not embryo specific, this gene can serve as a useful marker for the establishment of the epidermal cell layer. Other EPs, such as EP1 and EP3, have been similarly isolated from media of somatic embryo cultures (Van Engelen et al., 1991; De Jong et al., 1992). EP3 encodes a glycosylated acidic endochitinase (De Jong et al., 1992) that is able to restore embryo development to a *ts* somatic embryo defective cell line, *ts11* (see below). EP1, which has a region of homology with *Brassica S* locus glycoprotein genes (Van Engelen et al., 1991; Van Engelen and De Vries, 1992), is not expressed in embryogenic cell clusters or somatic embryos but rather is produced by the nonembryogenic cells of a callus culture. It has been suggested that these secreted proteins likely play a role in the regulation of cell expansion, which is critical to the maintenance of the integrity of the epidermal layer in embryos and other tissues, and to the proper establishment of shape and form, which is largely controlled by cell expansion (Van Engelen and De Vries, 1992; Sterk and De Vries, 1993).

Other Genes Isolated from Somatic Embryos

Several other genes have been isolated from cDNA libraries constructed from mRNA isolated from somatic embryos, as summarized in Table 1. Although none of these has been extensively characterized, the properties of a few of them are noteworthy. The DC59 clone, originally described by Choi et al. (1987), has been shown to encode a lipid body membrane protein (also called oleosin) that, although distinct from the LEA proteins, is also ABA inducible (Hatzopoulos et al., 1990a). Molecular analysis of this gene has identified regions within its 5′ end that interact with nuclear factors present only in embryo extracts; these regions show sequence similarity to regions 5′ to the DC8 LEA gene (most likely the ABA-responsive regions; Hatzopolous et al., 1990b). In addition, several of the clones isolated by Aleith and Richter (1990) have unusual amino acid sequences. DC2.15 has a core of repeating Pro-X motifs, and DC7.1 and DC9.1 are glycine rich. The two glycine-rich proteins also appear to have the potential for interesting secondary structures, such as membrane-spanning α-helical domains at both the amino and carboxyl termini and extensive β-pleated sheet structures in their cores. Although these proline- and glycine-rich proteins bear resemblance to certain cell wall proteins (Chen and Varner, 1985; Condit and Meagher, 1986; Keller et al., 1988), nothing is known about the specific functions of these proteins in embryogenesis or at other times in the development of plants. It would not be surprising if these proteins are cell wall components, because cell division and concomitant wall synthesis are very active during embryogenesis. Each of these genes, and the others described by Aleith and Richter (1990), is regulated in abundance during somatic embryogenesis. Their patterns of expression, where known, are summarized in Table 1.

In addition to the isolation of several genes by virtue of their enhanced transcription and abundance in total RNA of somatic embryos, a few genes have been identified that are translationally enhanced in somatic embryogenesis. These include the translation elongation factor EF1-α and the β subunit of ATP synthase, ATP-2 (N.R. Apuya and J.L. Zimmerman, unpublished data). A clone encoding EF1-α was also isolated by more standard differential hybridization by Kawahara et al. (1992). Recent efforts in my laboratory have resulted in the isolation of five more genes that appear to be translationally controlled in somatic embryos of carrot (X. Lin, G.-J. Hwang, and J.L. Zimmerman, unpublished data).

Although the extent, significance, and mechanism(s) of post-transcriptional regulation of gene expression in plant embryo development are only beginning to be addressed, several studies suggest that there may be some similarities to animal embryogenesis, in which translational activation of stored or "maternal" mRNAs is the primary level of gene regulation in early development of nonmammalian embryos. Stored mRNAs appear to support embryo development until the eight-cell stage in the fern *Marsilea vestita* (Kuligowski et al., 1991), and maternal mRNAs are activated in early zygote development in *Fucus* (Masters et al., 1992). In addition, Pramanik et al. (1992) demonstrated that storage protein synthesis in alfalfa somatic embryos is translationally regulated; the mRNA is present but restricted to the nontranslated mRNP pool early in embryogenesis (globular-to-torpedo stages) and is then shifted to the polysome pool at the cotyledon stage. Moreover, post-transcriptional regulation, including differential transcript stability, has been suggested to be an important component of embryo mRNA accumulation in zygotic embryos (Walling et al., 1986). It is likely that further study will reveal that each aspect of post-transcriptional regulation (transcript processing, stability, translatability, etc.) contributes significantly to the process of early embryo development in plants.

Expression of "Nonembryonic" Genes during Somatic Embryogenesis

In addition to using somatic embryos as a means of isolating genes that are regulated during embryogenesis (and that may therefore be important in that process), somatic embryos have also been used to assess the expression of genes isolated from nonembryonic tissues. Particular attention has focused

on both cell cycle genes and histone genes. Callus suspension cultures of alfalfa and the somatic embryos derived from them are proving useful in studying the relationship among auxin, the reactivation of rapid cell cycling, and the induction of genes associated with the cell cycle (Hirt et al., 1991). In alfalfa, the induction of embryogenesis is quite different from that in carrot, in that alfalfa cultures are subjected to a brief pulse of relatively high concentrations (100 μM) of the synthetic auxin 2,4-D prior to being transferred to hormone-free medium for the development of embryos. In alfalfa cultures, this auxin pulse induces both active cell division, and, for the first few days of embryo development, the accumulation of mRNAs (two to three different size transcripts) for the *cdc2* protein kinase (Hirt et al., 1991). These mRNAs then decrease in abundance. Much of this work has recently been reviewed by Dudits et al. (1991, 1993). No analogous studies have been conducted in carrot in which induction of somatic embryogenesis does not require this high auxin pulse.

GENERATING MOLECULAR MARKERS FOR CELL DIFFERENTIATION IN EMBRYOGENESIS

One extremely important use of the somatic embryo system in studying development is in the generation of molecular markers for events in cell and tissue differentiation during the formation and development of the embryo. Such markers are essential for an understanding of the developmental consequences of embryo mutations that are being identified and characterized in other systems such as Arabidopsis. Some of the genes that have been cloned from carrot somatic embryos, such as EP2, have turned out to be useful markers of differentiation (of the dermal tissue system, in the case of EP2). Indeed, the EP2 gene serves to illustrate an important point: this gene is not embryo specific, but it is enhanced in expression in embryogenesis and, due to its specific localization, it can still represent a very useful molecular marker.

Early attempts at cloning genes expressed in somatic embryos were designed with the bias that genes that are important for embryo development should either not be expressed at all in callus cells or should be greatly reduced in mRNA abundance in callus. It now appears, however, that callus cells, and particularly the PEMs contained in the callus, are already expressing many of the genes that will be expressed through at least the globular stage of embryogenesis. Therefore, any gene cloning scheme that involves a differential or subtractive hybridization step comparing embryo cDNA with callus cDNA will likely eliminate many gene sequences that could represent potentially useful molecular markers, particularly for early events in embryogenesis.

One alternative approach to the isolation of useful markers in embryo development is simply to use genes that have been cloned from other time points in development during which expression of the gene appears to be tissue or cell-type specific. For example, genes whose expression is localized to the vascular system in leaf tissue might be useful in identifying provascular cells in early embryos. The characterization of the expression patterns of such genes by in situ hybridization to embryo sections could be informative.

Another alternative approach to gene isolation that my laboratory has taken recently is to identify clones that are induced in globular embryos as compared to 7-day-old seedlings. Superimposed upon this comparison was the use of only polysome-associated mRNAs rather than total RNA. A "subtracted probe" was made using seedling polysomal cDNA hybridized to globular polysomal cDNA. This probe allowed the isolation of 50 different clones (X. Lin, G.-J. Hwang, and J.L. Zimmerman, unpublished data) that included many genes that had been isolated previously and that are included in Table 1, among them DC8, DC59, DC3, EMB-1, EP2, and EF-1α. In addition, the screen identified several genes that had been previously isolated and characterized in nonembryonic tissues, such as histone genes and other genes of metabolism. Moreover, we were able to identify several undescribed or novel sequences that are enhanced in embryos compared to seedlings. The location and timing of expression of each of these genes during somatic embryogenesis are currently being evaluated through in situ hybridization. It is our hope that some of them may prove to be useful markers for specific cell types within the developing embryo.

SOMATIC EMBRYOS AS A GENETIC SYSTEM

The power of genetic approaches to understanding developmental systems has been elegantly demonstrated in several animal systems. The generation of single gene mutations that block the expression of essential developmentally regulated genes has revealed many of the basic principles of cell differentiation and pattern formation in eukaryotes. Typical genetic approaches involving mutagenesis and the identification of defective phenotypes cannot be easily applied to the somatic embryo system for several reasons: (1) the proportion of cells that actually enter and complete embryogenesis is naturally somewhat variable and decreases with increasing time in culture; (2) prolonged time in culture can lead to the accumulation of mutations (somaclonal variation; Widholm, 1984) that may at the least confound analysis of embryo mutations, if not obscure them completely; (3) unusual plantlet phenotypes, such as multiple cotyledons, are not unusual in any culture; and (4) most embryogenic cell lines have a limited embryogenic lifespan (usually in the range of 1 to 2 years), after which it may become impossible to generate any embryos. Thus, although large numbers of individuals can be analyzed using the cell culture/somatic embryo system, the background of genetic and epigenetic variation in somatic embryogenesis often obscures interesting mutant phenotypes.

One genetic approach has, however, been shown to be potentially useful in dissecting events in somatic embryogenesis: the identification and characterization of conditional

mutants. These are mutant cell lines that are capable of undergoing normal somatic embryogenesis at a permissive condition but are blocked in development when exposed to a restrictive condition (usually increased temperature). *ts* variants of carrot somatic embryogenesis have been used in two different experimental approaches.

ts Mutants Allow Gene Isolation

The *ts* cell line *ts11* facilitated the isolation of one embryo-enhanced gene. This mutant cell line will grow but not develop normally through somatic embryogenesis at 32°C (Giuliano et al., 1984); at the restrictive temperature, this line produces abnormal misshapen heartlike embryos that lack normal polarity. However, normal development can be restored by inducing embryogenesis in "conditioned medium" (i.e., medium that has previously supported a normal embryogenic line's development). Fractionation and characterization revealed that the component of conditioned media that restored normal embryogenesis is a secreted protein, EP3, that is a glycosylated acidic endochitinase (De Jong et al., 1992). Although this is the only reported example of the use of *ts* mutants to assist in gene isolation, the potential utility of this technique is high, so long as the mutation is stable and the line remains embryogenic at the permissive temperature. As described below, this is not always the case.

ts Mutants Can Define Interesting Developmental Transitions

The possibility of isolating *ts* variants in carrot somatic embryogenesis is highlighted by the identification of *ts* variants that are defective or arrested at particular time points in embryogenesis. Breton and Sung (1982) described three primary classes of developmental variants that become apparent when cells are induced to undergo somatic embryogenesis at the restrictive temperature: those blocked for growth, those that cannot initiate embryogenesis, and those that are blocked at the globular stage. Furthermore, Breton and Sung (1982) reported that the *ts* phenotypes were generally stable in callus culture for at least 12 months and that the phenotypes were maintained after plantlet regeneration and subsequent reculturing. One of these variants, *ts59*, is blocked at the globular stage of development when cultured at the restrictive temperature of 32°C but develops normally at 24°C. The developmental block can be imposed by exposure to 32°C at two separate time periods of development, the first during the early globular stage and the second during the globular-to-heart transition. The only observable difference at the protein level between the wild type and *ts59* mutant was a variation in the heat shock protein (hsp) profile (Lo Schiavo et al., 1988). The *ts59* mutant failed to phosphorylate some hsps in the 36 to 45 kD range. However, it has not been determined whether this difference in hsp profile has any direct relationship to the *ts* embryo-arrested phenotype of this line.

Other experiments, including those from my own laboratory, have also shown a number of potentially significant relationships among hsp gene regulation, somatic embryogenesis, and *ts* mutations. For example, two-dimensional protein analysis of other *ts* variants of carrot somatic embryo development (see below) revealed a number of reproducible deficiencies in the constellation of low molecular weight hsps that are produced by these embryo-arrested cell lines (Hwang and Zimmerman, 1989). In the most extreme case, only six small hsps remained in a *ts* variant, compared to the 20 different low molecular weight hsps typically synthesized by normal carrot cell lines. However, it is not known whether the embryo-defective phenotypes result from the absence of the hsps and consequent decreased thermotolerance (thus rendering the cell lines *ts* and embryo defective), or whether the embryo-defective phenotypes result from an independent mutation in an essential embryo gene, with the alteration in hsp production being coincidental and unrelated to the phenotypes.

The relationship between hsps and embryo development (in both plants and animals) is complex. Carrot somatic embryos exhibit an extreme sensitivity to heat shock during a specific portion of the globular stage of carrot somatic embryogenesis (Zimmerman et al., 1989). Indeed, further development is arrested by a relatively brief heat shock during the globular stage, and heat shock at other time points in embryo development induces a higher proportion of abnormal morphology at subsequent embryo stages. There are many analogous examples of heat shock sensitivity in early animal embryogenesis (see Zimmerman and Cohill, 1991, for a comparative review).

One of the conclusions that emerges from studies of both plant and animal embryogenesis is that for an embryo to be thermotolerant, most, if not all, heat shock genes must be transcribed, if not translated, and generally this is not accomplished until the zygotic genome is transcriptionally active. A second general observation is that heat shock exposure at certain specific periods of development can result in predictable, nonheritable developmental defects; in Drosophila these defects, termed "phenocopies" (see Peterson, 1990, for review), mimic many developmental mutations, such as *bithorax* (Gloor, 1947) and *forked* (Mitchell and Peterson, 1982). In mammals, heat shock of embryos has been linked with several neural and craniofacial defects (Walsh et al., 1987, 1989). Although the precise molecular basis for all these developmental defects remains to be determined, it is clear that heat shock can have a profound effect on the process of embryo development, and at least some of these effects may relate to heat shock gene regulation.

A related and more extensive description of *ts* variants in somatic embryogenesis was reported by Schnall et al. (1988). In addition to characterizing a larger number of variants, this study defined the temperature-sensitive period (TSP) for each of the variants to establish the timing of gene action during embryogenesis. Although many of the variants exhibited extended TSPs, some showed TSPs that were restricted to

one or two embryonic stages. In some cases, the TSP temporally preceded the appearance of the variant phenotype, indicating gene activity in advance of any obvious morphological change; in others, the TSP was essentially coincident with the visualization of aberrant development. Although this study identified variants for every developmental stage in embryogenesis, approximately half of the variant embryos were blocked prior to the globular-to-heart transition. This is similar to the preponderance of globular embryo defects observed in the analysis of embryo defective mutants of Arabidopsis (see West and Harada, 1993, this issue) and may imply that a number of new gene products are necessary for this developmental transition to occur.

Two of these variants were further characterized by protein analysis in an attempt to define some gene products that might be stage specific (Schnall et al., 1991). Although many differences in protein profiles were apparent in each of the variants, subsequent analysis revealed almost all of them reflected either cell line differences, age differences, or random variations, rather than differences due to developmental changes in protein synthesis (Schnall et al., 1991). It was therefore concluded that the changes that could be observed by protein analysis were not reflective of the genetic alterations in any of these lines. Unfortunately, all of the *ts* lines originally described by Schnall et al. (1988) were subsequently lost, either because they ultimately lost their embryogenicity at the permissive condition or because they lost the specificity of the variant phenotype at the restrictive temperature (T. Cooke, personal communication).

Limitations of Somatic Embryos in Genetic Analyses

The results of the protein analysis, coupled with the ultimate loss of the *ts* lines (or their embryogenicity), serve to highlight several properties of somatic embryos that must be recognized in considerations of somatic embryos as a genetic system. First, by virtue of their prolonged propagation in cell culture, the lines that produce somatic embryos likely contain and accumulate mutations that may ultimately confound any other genetic manipulations. Second, in most cases, the ephemeral nature of the embryogenic potential of any given cell line will severely restrict the time frame over which experiments can be conducted. This can be partially overcome by regular cycling of the cell line through embryogenesis and selection of normal plantlets for the reinitiation of the line as well as by whole plant regeneration and maintenance through vegetative growth and/or zygotic embryogenesis. Although the problems are not insurmountable, the propagation and maintenance of highly embryogenic cell lines is very labor intensive, as is the regeneration of plants from cell culture, and requires both finesse and vigilance. It is clear that the somatic embryo system of carrot can be manipulated genetically, but the questions one attempts to answer must be well considered and designed with an awareness of the vagaries and potential limitations of the somatic embryo system for this analysis.

MICROSURGICAL ANALYSIS OF SOMATIC EMBRYOGENESIS

Microsurgical manipulations have been used to characterize a number of aspects of patterning processes in both plant and animal systems (see Slack, 1983; Steeves and Sussex, 1989). These techniques have the potential to reveal such basic information as the source of pattern information, the degree of commitment or determination of particular cell or tissue types, and the contribution of context or environment to the developmental fate of a cell or tissue. Microsurgical manipulation has been applied to the carrot somatic embryo system with some interesting results. Studies by Schiavone (1988) and Schiavone and Racusen (1990, 1991) have revealed that when carrot somatic embryos are manipulated microsurgically, by either removing or grafting regions, the resulting embryo pieces or grafts retain developmental competence. For example, sections of the shoot pole comprising 25 to 90% of the original length of the torpedo embryo regenerated the root pole in approximately half of the cases; the regenerating root could be visualized within a few days of the surgery (Schiavone and Racusen, 1990). The regeneration of the shoot pole was more difficult to accomplish and was induced efficiently only when at least 90% of the axial length was removed; sections that contained a greater portion of the embryo length simply continued to grow as roots. Removal of one cotyledon resulted in the regeneration of one, or usually multiple, cotyledons. No growth of the removed cotyledon was observed. If both cotyledons were removed from the same embryo, either the cotyledons regenerated at the cut sites or the amputated region greened while a new shoot emerged along the surface of the midhypocotyl.

These experiments highlight the potential for developmental perturbation and recovery that exists in the somatic embryo system. However, the small size of even torpedo-stage embryos limits the utility and reproducibility of microsurgical manipulation. The types of manipulation that have been performed are relatively crude (although still remarkable), in that they have been limited to the deletion of relatively large portions of the embryos. Nonetheless, these experiments may lead the way for other types of manipulations, such as laser surgery or cell ablation experiments.

PROSPECTS FOR FUTURE STUDIES IN EMBRYO DEVELOPMENT USING SOMATIC EMBRYOS

The ability to generate essentially unlimited quantities of staged embryos through relatively simple manipulations in cell culture holds great promise for unravelling the complex process of plant embryo development. Perhaps the greatest potential for advancing our understanding of embryogenesis lies at the interface between somatic and zygotic embryogenesis systems. For example, although Arabidopsis remains an ideal

model system for the identification of developmental mutants in embryogenesis, subsequent molecular analysis of those mutants will always be complicated by the very small size of the embryo. Somatic embryos, by contrast, can supply milligram quantities of mRNA for developmental RNA blot analyses. Moreover, somatic embryos can allow analyses of transcriptional versus post-transcriptional regulation through quantitative nuclear run-off measurements and analysis of polysomal versus total RNAs (Zimmerman et al., 1989; Apuya and Zimmerman, 1992). Although these experiments are possible in zygotic embryos, through polymerase chain reaction amplification and other microtechniques, they are technically complicated enough that they will likely be avoided; indeed, the history of molecular analysis of embryogenesis suggests that this will be the case. In addition to facilitating a biochemical and molecular approach to embryogenesis, an understanding of the developmental consequences of any mutation in embryogenesis, such as disruption of epidermal development, defective placement or timing of provascular differentiation, or failure to establish apical meristems, would be greatly expedited by the availability of molecular markers for these differentiation events. The somatic embryo system has only begun to reveal its potential for generating such markers.

The availability of cell-specific markers could also allow the application of cell ablation techniques, such as those elegantly applied to impose male sterility (Mariani et al., 1990), resulting in the precise elimination of specific cells. These altered embryos could then be studied as they progress through development to determine whether other cells can take over for the ablated ones or the elimination of specific cells results in embryo lethality. Such experiments could initially be performed in somatic embryos and subsequently confirmed in zygotic embryos.

In summary, the molecular and genetic analysis of plant embryogenesis will reveal the mechanisms at work in the establishment of the polarity, the differentiation of the tissue systems, and the elaboration of the pattern that ultimately carries each species into the next generation. The analysis of somatic embryos can contribute significantly to this analysis. The system is not without its limitations, but, well used, holds the potential for significantly expediting our understanding of plant embryogenesis.

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