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REVIEWS AND PERSPECTIVES

Developmental Biology of Embryogenesis from Carrot Culture

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Since Steward and Reinert independently discovered somatic embryogenesis in carrot culture (Reinert, 1959; Steward et al., 1958), it has been a model system for understanding plant embryogenesis. The development of somatic embryos resembles zygotic embryogenesis and exhibits the characteristic morphogenetic stages: globular-, heart-, and torpedo- stages. Many established cell lines of carrot can produce millions of somatic embryos in liquid cultures that are essentially free of undifferentiated tissues. Embryogenesis can be controlled experimentally to achieve synchronized development; uniform embryonic stages can be isolated *en masse* (Giuliano et al., 1983). These unique features of carrot culture make possible the study of biochemistry and genetics of early embryogenesis. This paper does not provide a complete review; rather, it attempts to outline the questions addressed and the approaches taken in the study of carrot embryology.

1. The role of 2,4-D

Most early studies on the external factors and physiological conditions that influence embryogenesis are directed at the question of what external factors

control embryogenesis. Among the various factors, e.g., nitrogen sources (Wetherell et al., 1976), carbohydrates (Verma and Dougall, 1977), various growth regulators, ethanol (Tisserat and Murashige, 1977), etc., the synthetic auxin, 2,4-dichlorophenoxyacetic acid is the most important external factor which universally controls all carrot cultures. Two,4-D is believed to be necessary for the acquisition of embryogenic potential at the time when the explant proliferates into callus tissue. The callus tissue of carrot cultures already possesses embryogenic potential. The presence of 2,4-D prevents the development of embryos, and results in unorganized growth. Upon the removal of 2,4-D, embryogenesis can take place. However, in certain carrot cultures, e.g., cultures initiated from wild carrots, 2,4-D does not prevent the initiation of embryogenesis. Halperin and Wetherell (1964) reported that "in the cultures maintained in 1.0 mg/1 2,4-D, mature embryos never developed, although immature embryos could be found up to the globular stage. These globular embryos are merely large, rounded masses of meristematic cells." When embryogenesis is scored within one week of development, it is possible to obtain nearly 100% globular embryo formation in medium containing 2,4-D, provided the cells are cultured at very low density, 2×10^4 cells/ml (Sung and Okimoto, 1981). Thus, the natural tendency of carrot cultures is toward embryogeny and 2,4-D is merely an inhibitor. The control over embryogenesis is mostly endogenous, but initially medium change (see Section 4) and cell interaction (in simplistic terms, cell density) play important roles. Once embryogenesis is initiated, 2,4-D can prevent the transition from globular- to heart-stage.

2. Biochemical phenotypes that appear in temporal fashion

To search for the internal control of morphogenesis, carrot biologists have turned to the biochemical changes that occur during carrot embryogenesis. Enzymatic activities in the embryonic and callus cultures have been compared. Several pyrimidine biosynthetic enzymes (Ashihara et al., 1979), ornithine transcarbamylase (Baker et al., 1983), arginine decarboxylase, and S-adenosyl methionine decarboxylase activities (Montague et al., 1979; Feier et al., 1984; Feinberg et al., in press) all show increased activity during early embryogenesis.

While the enzymatic activities discussed above show quantitative changes, several qualitative properties were also found. The embryonic tissues express tyrosinase activity and are capable of inactivating alpha-amanitin (Pitto, unpublished results) and cyclohexmide (Sung et al., 1981) but not the callus tissues. The biochemical basis of the inactivation is being characterized. While it is difficult to conceive that these traits play a role in embryo development,

they are excellent candidates for studying the mechanism of developmental regulation (see Section 4).

The synthesis and accumulation of the relatively abundant proteins have been examined by two-dimensional gel electrophoresis (2D-PAGE) (see Choi and Sung, this issue). In general, about 2% of the 800 detectable proteins differ between the callus and embryonic tissues. To study proteins that are specific to embryonic stages, approximately 50 embryos of the three morphogenetic stages were hand-picked, labelled with ^{35}S-methionine, and the soluble proteins extracted and separated by native-SDS 2D PAGE. This method resolves about 200 individual radioactive spots; few unique proteins specific to the morphogenetic stages were detected (Okimoto and Sung, unpublished results). These studies show that no major pattern of protein modulation as seen in nematode and *Dictyostelium* (Johnson and Hirsch, 1979) occurs during plant embryo development. In view of the fact that both callus and embryonic tissues are proliferating tissues, and that the callus tissues are committed to an embryogenic fate, they are likely to be similar in structural and functional proteins. The few proteins that do differ between the two tissues are intriguing. However, they cannot be assumed to be the internal factors that control embryogenesis. They may play a role in embryogenesis if the biochemical change 1) occurs prior to morphogenesis, and 2) is shown to be required for development (see Section 3).

3. Biochemical phenotypes that may be important for embryogenesis

Two proteins detected on the 2D-PAGE have been characterized (Sung and Okimoto, 1981). In a wild carrot culture, W001C, these two proteins are not synthesized in the callus tissue, but are made in somatic and zygotic embryos (Sung, 1984). Thus, they have been called embryonic proteins. They are synthesized early in embryogenesis; in fact, they are induced as soon as the culture is transferred into the embryogenic medium. A mutant that cannot undergo embryogenesis does not synthesize these proteins in embryogenic medium. However, it is possible to find callus tissues that will synthesize these two proteins. Another mutant isolated from W001C, WCH105, is believed to be altered in the regulation of several functions (see next section); it expresses the embryonic proteins in the callus tissue (Sung and Okimoto, 1983). A cultivated carrot culture, HA, synthesizes the embryonic proteins in the callus, embryos, and roots, but not in the shoots and leaves (unpublished results).

The fact that these proteins are expressed in tissues other than the embryos does not exclude the possibility that they play a role in embryogenesis. The

developmental specificity of these proteins may be determined by the protein's spatial distribution in a particular tissue, or by its interaction with other proteins in the tissue. In order to demonstrate that they are not required for embryogenesis, it is necessary to find a mutant that does not synthesize these proteins in the embryo.

A biochemical phenotype that has been demonstrated to be necessary for embryogenesis is polyamine metabolism. Polyamines have been implicated in various growth and differentiation processes. In carrot culture, Montague et al. (1978) showed that polyamine concentrations change during embryogenesis. Feier et al. (1984) subsequently used difluoromethyl arginine an inhibitor of polyamine synthesis to inhibit embryogenesis. In order to demonstrate that polyamines have a role in embryogenesis distinct from the role in growth, we used a mutant cell line that does not form embryos but grows at the same rate as the wild type in embryogenic medium. The mutant does not show the characteristic increase in spermidine and spermine levels during the first three to six days of embryogenesis. The addition of these polyamines sometimes can improve and increase embryogenesis (Fienberg et al., in press, unpublished results). These results provide convincing evidence of a requirement for elevated spermidine and spermine concentrations for embryogenesis to occur. Based on these findings, it is now worthwhile to pursue the regulation of polyamine synthesis and the role of polyamines in embryogenesis.

Temporal phenotypes that do not play a role in embryogenesis nevertheless are useful for the following studies:

1. If biochemical changes occur at different times during development, they can be used as biochemical markers to mark the time of development.
2. For those markers that appear at the same time, they may be regulated by a common mechanism, thus providing a system to study coordinate regulation in development.
3. If the regulation of the biochemical phenotypes respond to the same signals that control embryogenesis, information gained from studying the biochemical phenotypes could potentially be extrapolated to understand morphogenesis.

4. The regulation of the temporal phenotypes

In studying the timecourse of the biochemical changes, it appears that many of the functions that show an increase during embryogenesis occur during early embryogeny, and some are induced as soon as cells are transferred into embryogenic medium. Subsequently, we found that as long as the medium is fresh, regardless of whether it contains 2,4-D, it can induce the appearance of the biochemical changes, i.e., the embryonic proteins, polyamine synthesis

(Montague et al., 1978), CH^i (unpublished results), and ornithine decarboxylase activity (Baker et al., 1983). If the cells are cultured under embryogenic conditions, the "expression" of these phenotypes continues. On the other hand, their expression is suppressed in callus culture. Sengupta and Raghavan reported an increase in the ratio of poly(A)$^+$RNA to RNA two hours following subculture. This phenomenon is termed "fresh medium effect."

Fresh medium not only controls the biochemical phenotypes, but also promotes embryo development in the wild carrot culture, W001C. By changing the medium every day, cells cultured at high density in 2,4-D grow as tightly packed tissue rich in cytoplasm. This morphology represents the embryonic stage prior to the formation of globular-stage embryos. It exhibits cellular organization, but there is no formation of an epidermis. This phenomenon suggests that fresh medium triggers the initiation of embryogenesis to the stage of organized growth; subsequent development is prevented by the presence of 2,4-D and the high density culture (Borkird and Chorneau, unpublished results).

The detection of these phenomena requires the use of cultures whose callus tissues are friable and contain few "embryogenic clumps." Some carrot cultures that are more heterogenous in cell population and contain large embryogenic clumps do not respond to fresh medium as described. For example, the haploid culture, HA, synthesizes the embryonic proteins, expresses tyrosinase activity, ama^i and CH^i in the callus tissue. One explanation is that the callus tissue of this culture is arrested at the organized growth stage; thus, it no longer exhibits the switch in gene expression typical of the transition from unorganized to organized growth.

The study of mutants altered in the expression of these functions has contributed to our understanding of the regulation of these early embryonic functions. A mutant selected to express ama^i in both callus and embryos was found to express CH^i also in a constitutive manner (Vergara et al., 1982). Similarly, WCH105 that was selected to inactivate CH in callus tissue has simultaneously expressed ama^i and embryonic proteins, but lost the ability to synthesize some callus-specific proteins (Sung and Okimoto, 1983). The close association of these embryonic properties as demonstrated by the pattern of their expression in mutants and various carrot cultivars indicates that they are regulated by a common mechanism. But the callus and embryonic functions are regulated in opposite manner, i.e., the synthesis of the callus proteins ceases as the embryonic functions are expressed and vice versa. Since they respond to fresh medium and to 2,4-D in the same manner that embryogenesis does, they provide a model system for understanding the regulation of embryo development.

The abilities to inactivate CH and ama, however, are different properties

because ama^i is a dominant trait, whereas CH^i is a recessive trait as analyzed in the somatic hybrids. The coordinate expression of these functions point to a general switch mechanism that simultaneously "turns on" a large number of embryonic functions. If these functions are expressed in zygotic embryos as the embryonic proteins, they could either appear simultaneously following fertilization or at later but separate times during embryogeny. In this light, fresh medium either mimics the event of fertilization or acts as an artificial signal that precociously initiates the expression of those functions which are committed to express later in development.

5. Developmental mutants blocked in embryogenesis

Since the ultimate information of nearly all biological processes lies at the gene level, another approach to the search for morphological control is to isolate mutants defective in development. The phenotypes of developmental mutants could be 1) arrest at one morphogenetic stage, unable to advance to subsequent stages, 2) complete disruption of organizational capability resulting in unorganized callus tissues, or 3) switch in developmental potential resulting in alternative morphology. The last phenotype is difficult to select and probably to analyze if abnormal morphology is the consequence. A filtration-enrichment and screening procedure has been used to select for mutants impaired in embryogenesis (Breton and Sung, 1982; Giuliano et al., 1983). Mutants exhibiting the first and second phenotypes were isolated. To facilitate the characterization of the mutations and identification of the gene products involved, conditional mutants were sought. Normal embryogeny takes place between the temperature regimes of 18 C and 32 C. It is possible to isolate mutants that form embryos at low temperatures, but not at 32 C (temperature-sensitive mutants). Thus, embryogenic cultures grown at 32 C were filtered through 100 micron meshes to remove cells that had developed into embryos. The filtrate consisted of cells incapable of growth in embryogenic medium, small embryos blocked at early developmental stages, and callus tissues. Upon further screening, the temperature-sensitive mutants impaired in development (*ts-emb*$^-$) can be distinguished from those that are not temperature-sensitive, those that are impaired in growth, and the habituated tissues.

Because selection is aimed at the isolation of mutants blocked at early embryogeny, *ts-emb*$^-$ lines blocked at the transition from unorganized to organized growth, from organized growth to globular-stage, and from globular- to heart-stage embryos have been found. The stability of these phenotypes can be demonstrated by testing the *ts-emb*$^-$ of the callus tissue initiated from the plantlets regenerated at 24 C. However, most of these cell lines including the parental strain cannot develop normal flowers; thus the trans-

mission of these traits through the progeny cannot be tested. Genetic characterization of the *ts-emb*$^-$ lines will have to rely on somatic hybridization.

Temperature-shift experiments have been performed to determine the temperature-critical period of the alteration in these lines. In general, it is found that products required for a specific morphogenetic stage are functioning several days prior to morphogenesis (Giuliano, unpublished results). One of the problems associated with the study of developmental mutants is the difficulty in determining the biochemical basis of the defect. In this respect, the biochemical phenotypes associated with embryogeny have been useful in their characterization. Conversely, the *ts-emb*$^-$ lines can be used to analyze the role of these biochemical phenotypes in embryogenesis. A simultaneous alteration of development and the expression of the temporal phenotype confirms the close association of the two processes and helps in the study of the involvement of biochemical traits during embryogenesis.

6. Developmental inhibitors

Phenocopies of mutants blocked at embryo development can be obtained by using inhibitors (Table I). Some inhibitors are not only specific to embryogenesis—at the same concentration, they do not affect the growth of callus tissue—but are also specific to a particular developmental stage. For example, inhibitors of polyamine synthesis such as 200 μM dicyclohexylammonium sulfate (DCHAS) inhibit the formation of globular-stage but not callus growth. When added after the globular-stage is reached, DCHAS no longer affects embryo development (Fienberg, unpublished results). Such inhibitors apparently interfere with functions effective during specific developmental stages. There are other inhibitors that are not stage-specific, but are nevertheless more toxic to embryogenesis; presumably they act on functions effective throughout embryogeny. It is difficult to distinguish this type of inhibitor from general metabolic poisons that preferentially block embryogenesis because development requires a more stringent balance of cellular metabolism than unorganized growth, except in cases where the target of the inhibitor is known. For example, alpha-amanitin inhibits RNA polymerase II; recent evidence suggests the existence of isozyme forms of the enzymes. One is expressed in the embryos, the other in the callus and mature plants (Pitto et al., 1983b). When the callus form is mutated to become resistant to alpha-amanitin inhibition, the enzymes extracted from the embryos are still sensitive to alpha-amanitin.

Dudits et al. (1979) showed that bromodeoxyuridine (BUdR) blocked embryo development at different stages depending on the time of treatment. If BUdR is added at the onset of embryogenesis, cells grow as compact tissues; when added two days after embryogenesis, embryos can develop up to the

TABLE I. *Developmental specificity of inhibitors during somatic embryogenesis*

Inhibitor	*Developmental stage*						*Reference*
	callus→	*organized growth→*	*globular→*	*heart→*	*torpedo→*	*plantlet*	
2,4-D + high density (0.1 mg/l)	+	−	−	−	−	−	Sung and Okimoto, 1981
2,4-D + low density (0.1 mg/l)	−	+	+	−	−	−	Sung and Okimoto, 1981
Ethanol + 2,4-D (10 mM)	+	−	−	−	−	−	Chorneau, unpublished
Ethanol − 2,4-D (10 mM)	+	+	−	−	−	−	Chorneau, unpublished
Colchicine (1×10^{-6}M)	+	−	−	+	+	+	Borkird, unpublished
Abscisic Acid (0.1 mg/l)	+	−	−	+ +	+ +	−	Borkird, unpublished
Tunicamycin (5×10^{-6}M)	+	−	+	+	+	+	LoSchiavo, unpublished
DFMA (1 mM)	+	−	+	+	+	+	Fienberg, unpublished
DCHAS (1 mM)	+	−	+	+	+	+	Fienberg, unpublished
Cycloheximide (10 μg/ml)	−	+	+	+	+	+	Sung et al., 1981
Alpha-amanitin (50μg/ml)	−	+	+	+	+	+	Pitto et al., 1983
BUdR (6×10^{-6}M)	+	−	−	−	−	?	Dudits et al., 1979

"+" Callus growth or embryo development of the specific stage is not affected.
"−" Callus growth is inhibited or embryo development is prevented. The latter usually grow as callus.
"+ +" Stimulation of callus growth or embryo development.

globular-stage, etc. Since BUdR is incorporated into the DNA, it may operate at the genome level, e.g., chromatin organization, transcription, etc. This result suggests that the changes involving the genomic level occur very early, within two days for the globular-stage, within four days for the heart- and torpedo-stages. For *D. carota* L. var. Duwicki, the transition from callus tissue to the organized state of growth does not involve changes at the genomic level. The temperature-shift experiments, however, suggest that the involvement of proteins can occur at a later time at the functional level. For mutants arrested at the late-heart stage, the temperature-critical period occurs after the globular-stage.

7. Mechanism of morphogenetic transition (see diagram)

Because of the faithfulness of the morphogenetic sequence, it is commonly assumed that a "genetic program" like that of a biochemical pathway underlies developmental changes and that products of the first step are responsible for the subsequent steps (Model A). This hypothesis is supported by the fact that it is possible to block development at specific morphogenetic stages and that the removal of restrictive conditions such as 32 C or stage-specific inhibitors, within a reasonable period of time, permits subsequent development.

While the appearance of morphogenetic stages implies that embryogeny is comprised of discrete steps, development is actually a continuous process. It has been repeatedly observed that no definite sequences of cell lineage are associated with proembryo growth (Halperin, 1966). It is thus logical to propose an alternative hypothesis for the mechanism involving quantitative accumulation of information. Based on the simultaneous expression of a number of functions following the onset of embryogenesis, one might imagine that development could involve a single shift of gene expression at the beginning. The "developmental momentum" is built up by measuring the quantity of gene products, or the number of cells within the cell cluster. When a certain quantity is reached, communication within the tissue is established. As a result of this cellular interaction, the tissue acquires the ability to switch to the next stage (Model B). For example, it is generally assumed that the acquisition of polarity marks the transition from globular- to heart-stage. Nuti-Ronchi et al. (unpublished results) used the distribution of esterase activity, a marker for the commitment of stelar differentiation, to assay for the developmental stages. Callus tissues showed little detectable esterase activity; at the young globular-stage, the enzyme activities were localized in one area of the periphery, which extended into the cotyledons as the embryos developed. If development was arrested, the esterase activity became centrally located in the enlarged globular embryos. Eventually vascular tissues were found in the middle of the tissue and no normal embryos could be produced. The

DIAGRAM: *Possible Principles of Developmental Mechanism*

Model A	*ModelB*
1	1
↓	↓
a	a b c d
↓	↓
b	aabbccdd
↓	↓
c	aaaabbbbccccdddd
↓	↓
2	2
↓	↓
d	a b c d e f
↓	↓
e	aabbccddeeff
↓	↓
f	aaaabbbbccccddddeeeeffff
↓	↓
3	3
↓	↓
g	b c d e f g
↓	↓
h	bbccddeeffgg
↓	↓
i	bbbbccccddddeeeeffffgggg
↓	↓
4	4

Model A represents a step-wise mechanism of gene expression. Gene products are depicted as "a," "b," "c," etc. which are responsible for morphogenetic stages, "1," "2," "3," "4." Model B shows a burst of gene expression at the onset of embryogenesis followed by gradual increase in the amount of the same products. When the products reach a critical level, communication within the tissue is established, thus resulting in differentiation which may or may not involve new gene expression. Possible mechanisms for the forces causing differentiation are hormone gradient, electric polarity.

cytochemical marker provided a useful tool to study the spatial changes that correlate with normal embryogeny. It seemed that not only organized growth but also a critical size is required for proper cellular communication to establish polarized localization of esterase activity. Further, although the interference of the polarity blocks morphogenetic transition, it does not prevent growth. The enlarged globular embryos can no longer develop normally, however, because of the abnormal site of the vascular tissue formation. There is also a maximal size for cellular communication to maintain organization. The enlarging globular embryos eventually disintegrate into callus tissues.

Conclusion

The emphasis on the biology and biochemistry of carrot culture has established a sound background for studying developmentally regulated gene expression in early embryogenesis. There are functions which can be simultaneously induced and suppressed by simple experimental conditions. We have mutants altered in the regulation of these functions. Some of these functions are shown to be indispensable for embryogenesis. Furthermore, they respond to the same external factors that control embryogenesis. It will be intriguing to find out if similar mechanisms are involved in regulating biochemical functions and morphological differentiation.

The biology of carrot culture has advanced from phenomenology to experimental embryology. The capability to block embryogenesis by inhibitors, by mutations, or by epigenetic variations, means that we can ask questions not just about correlative, but also causal relationships between functions and morphology. Some experimental evidence is presented to explain the mechanisms of development, but no one hypothesis prevails. In the immediate future, we can search for molecules which show temporal and spatial changes. We hope further studies of the relationship between gene expression and development will contribute to understanding "the grammar that lies between the genetic space and the organismic space" (Lewin, 1984).

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