Chapter 7

Embryogenic Plant Cells in Microgravity

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ABSTRACT

Plant development entails an orderly progression of cellular events both in terms of time and geometry. There is circumstantial evidence that in the controlled environment of the higher plant embryo sac gravity (g) may play a role in shaping embryo development. It follows that normal embryo development may not occur reliably and efficiently under the weak microgravity (μg) environment of space. More attention must be given to studying the many aspects of reproductive biology in the space environment in order to be confident that higher plants will not only survive, but produce large numbers of viable embryos in a “seed to seed to seed” type experiment. Until the time arrives when successive generations of flowering plants can routinely be grown and intensively studied, the best we can do is to utilize acceptable surrogate systems and begin, piece-meal, to accumulate information on important aspects of plant reproduction in microgravity. Cultured cells can play an important role in these activities since they can be grown to be morphogenetically competent and can be evaluated as to their ability to simulate embryogenic events usually identified with fertilized eggs in the embryo sac of the ovule in the ovary. Cultured cells can also be manipulated with relative ease. The extreme plasticity of such demonstrably totipotent cell systems provides a means to test environmental effects such as μg on a potentially “free-running” entity without the constraints or confines of an embryo sac. The successful manipulation and management of plant cells and propagules in space also has significance for exploitation of biotechnologies in μg since embryogenic systems, perforce, are an important component of plant genetic engineering manipulations.

INTRODUCTION

Since all biological development has evolved in the presence of Earth’s 1g vector, it has been argued that gravity must play a role in plant development. As far back as 1960 in his book Plant Morphogenesis Edmund Sinnott queried whether the plant body as we know it could develop in the absence of specific gravitational stimuli or cues (Sinnott, 1960), but knowledge in this area is still very rudimentary. Even so, the term gravimorphogenesis is increasingly being used to describe the phenomenon studied in the slowly emerging discipline of the relationship of gravity to development. Key questions as they apply to plants and the μg environment that need to be addressed include — Do cells require gravity and/or other orienting forces at any stage for normal development to occur? What constitutes the or a minimal gravimorphogenetically sensitive or responsive unit? Can an embryogenic or multicellular totipotent unit function as a gravireceptor? Can pulses at small g levels be enough to compromise or ruin a gravimorphogenesis-type experiment in space (cf. Krikorian and Levine, 1991 and references there cited)?

By using in vitro test systems at different levels of initial organization which employ cells that can yield somatic embryos and from these grow to fully formed plants, one should be able to determine the required developmental complexity and pinpoint the threshold levels where the first detectable gravisensitive responses relative to embryogenesis and development emerge. The μg environment of space offers unique opportunities to try to erase “accumulated” or evolution-derived gravitational sensitivities or mechanisms in multicellular systems and to reapply g signals to developmentally more simple and/or naive systems, even single cells, that have been multiplied through successive generations in a μg environment. Such investigations could illuminate fully the relationship of gravity to both genetics and development. As opportunities for flight experimentation increase, and especially as Space Station Freedom and other long-duration near-0 g environments become available for comprehensive gravimorphogenetic testing, it should be possible to validate the prediction that gravity has a significant effect on early morphogenesis. Whether gravity is a morphogenetic determinant and has a direct effect on embryogenic development is another matter and can only be speculated upon at this time.

POLARITY, GRAVITY, AND EMBRYO DEVELOPMENT IN PLANTS

The early cell divisions that derive from the zygote and generate the multicellular mass, which ultimately leads to orderly differentiation into organs, are extremely important to organized development. Anatomical and morphological studies of embryogenesis in a variety of lower and higher plants have demonstrated that the earliest division planes establish directionality for axis growth (Hardham, 1982).
The initial divisions seem to be especially significant since their appearance often provides the first external sign that polarity has been achieved (Brawley and Robinson, 1985). In certain plant embryos polarity may be evident in the zygotic cytoplasm prior to the initial division, but for most the axis of growth is fixed when the zygote is partitioned (cf. Wardlaw, 1955, 1965a and b; Raghavan, 1986). Even so, it is suggestive that axial elongation of initially non-polarized protoplasts can be oriented by electric fields (White and Overall, 1989) and the polarization of microtubules is a noteworthy feature of that elongation (cf. Moroz, 1984; Robinson, 1985; Todd, 1989).

Much attention has recently been directed toward analyzing the phenomenon of somatic embryo polarity (Brawley et al., 1984) but there is still inadequate understanding of the factors that direct the planes of early cell divisions. Nothing is yet known about the genetic component of polarity expression in embryos. In general, the relationship between molecular, cellular, and environmental mechanisms in establishing or modifying polarity is obscure. However, such data as are available support the view that initiation of polarity and determination of the plant axis is one of the earliest events in embryogenesis (Figure 1). The data further support the concept that factors influencing polarity can alter the embryo axis and subsequently affect the specific pattern of plant development (cf. Wardlaw, 1955; Barlow and Carr, 1984; White and Overall, 1989; Osborne, 1989).

Examples may be found where either internal and external factors, or both, play a role in determining polarity. In free floating plant zygotes such as those of the brown alga Fucus, polarity can be influenced by a variety of environmental factors including light, temperature, nutrients, pH, and mineral gradients (cf. Brownlee and Wood, 1986 and references there cited). Whether the microgravity space environment would alter any determinative influence of gravity on embryo polarity in a single cell system like the Fucus zygote would be of great interest (cf. Quatrano, 1990). It is interesting to note that wall-less cells (protoplasts) of Fucus can establish an axis but the orientation is labile. Indeed, cell wall is required for the fixation of the embryonic axis and only when new wall is produced is the axis irreversibly determined (Kropf et al., 1988).

In evolutionarily higher systems, there also is evidence that externally imposed gradients can determine polarity (Rathore et al., 1988). The development of zygotes in archegonia or embryo sacs is potentially complicated by the presence of surrounding maternal tissues (see Willemse, 1981 for a discussion of polarity, megasporogenesis, and megagametogenesis). In those cases where the surrounding tissue has an effect, whether or not the influence is physical or physiological or both is not known. Indeed, the evidence suggesting that treatments which affect the relationship between enclosed zygotes and surrounding tissue can alter polarity and subsequent development of the embryo is not extensive (Wardlaw, 1965a and b; Osborne, 1989).

Although some attempts have been made to assess more directly the possible role of gravity in the induction of embryo polarity and axis determination by means of devices such as clinostats that neutralize g, the studies have been by no means definitive. In work where centrifugation was used, stratification of the cytoplasm was sometimes seen. In some cases the initial partitioning of the embryo and its later organization was altered, while in other cases there were no changes. Satisfactory control experiments were not always conducted, and the significance of much of the published observations is not clear (Beams and King, 1939; Ootakai, 1963). In studies with the lower vascular plant Isoetes, zygotes were grown in situ in archegonia fixed in an agar substrate in various positions with respect to gravity. Others were grown on horizontal clinostats to determine if embryo orientation (development) was influenced (La Motte, 1937). These studies are not particularly sophisticated, but results show that embryo polarity and the orderly segmentation pattern leading to normal development of the plant axis was altered. There are no other examples known to this author where embryogenesis is altered by changing the g vector, and clearly there is insufficient evidence to permit any firm conclusions to be drawn on how widespread gravity effects on plant embryogenesis might be.

While not directly concerned with gravimorphogenetic effects of g on plant embryogenesis, one can call attention here to two of a number of simple systems where morphoge-
PLANT EMBRYOGENESIS

Netic determinants (including gravity) seem to be able to operate at the level of a "single" (albeit coenocytic) cell. The freshwater green algae Chara and Nitella have relatively large intermodal cells that are multinucleate and show strong polarity as to the way in which they regenerate "leaves," "stems," and "roots." The intermodal cells have long been recognized as being able to regenerate whole organisms when they are isolated from the parent plant (and apparently only when they are separated) and were thought initially to have a fixed or unalterable specific polarity (cf. Osterhout, 1952) in terms of where these organs could emerge from an explanted node. A switch apparently exists in the stem line cells at the node, however, which permits normally rhizoid-generating cells to develop into "shoot"-generating cells when they are inverted, i.e., when their g vector is reversed. Sandan (1955) had shown years ago that light (more accurately darkness) could, in fact, reverse morphogenetic polarity of intermodal (for all intents and purposes "single") cells. Apparently these highly plastic systems are able to respond to a number of morphogenetic signals, including gravity.

As to single cell systems from higher plants that can generate embryos, some data from work in our laboratory suggest that gravity might be important in somatic embryogenesis. It follows that this might be extrapolated from somatic embryogenesis to zygotic embryogenesis. From this one could hypothesize that plant embryo polarity, axis determination, and pattern development could be adversely affected in the μg environment of space (Krikorian, 1989).

SYSTEMS FOR STUDYING EMBRYOGENESIS IN SPACE

A study of the influence of a μg environment on the early events of reproductive cell and zygote development could contribute substantially to a general understanding of regulatory factors in early plant morphogenesis. Equally important, results from such a study could provide a beginning for a clearer understanding of the behavior of plants grown in the environment of space (cf. Keefe and Krikorian, 1983; Krikorian et al., 1984; Halstead and Dutcher, 1984, 1987). For this type of developmental analysis, intact flowering plants are not, in my view, the preferred material to study. Intact whole plant systems are exceedingly complicated to work with, especially from the developmental perspective. Even if studies must eventually be carried out with whole plants, especially if they are to be grown for applied purposes, there are a number of questions that should be answered before reliable plant growth can be achieved in space. There is a lack of reliable information concerning most aspects of reproductive biology in the space environment. For most flowering plants nothing is known about pollen tube growth, sperm cell migration, and the fertilization mechanism as they occur in a μg environment (cf. Halstead and Dutcher, 1984, 1987 and references there cited). Regrettably, for the foreseeable near-term, the duration of most spaceflights will be relatively short and thus the possibility of carrying out "seed to seed to seed" types of experiments (cf. Keefe and Krikorian, 1983; Krikorian, et al., 1984) will be limited even using so-called tachyplants or fast-cycling plants such as the crucifer Arabidopsis (cf. Ivanov, 1974; Meyerowitz, 1987).

The reality of being able to reliably generate certain types of embryo-lethal mutants of Arabidopsis (Meinke, 1986) leads one to speculate that mutants with stages of embryogenesis sensitive to gravitational influences or polarity perturbations or changes might well be identifiable. Figure 2 shows the progressive development of an Arabidopsis embryo in the ovule (Müller, 1963). The implications of gravitational sensing and/or transduction blocks at different points for continued or normal development will be apparent. The value of such experimental systems for studying whole plant development in space and on Earth, if they were available, cannot be overstated. For the time being, and until such mutants become available, efforts are justifiably placed on the study of partial and surrogate systems.

The approach in our laboratory has been to concentrate on the development of cultured plant cell systems which are capable of undergoing organized development (i.e., somatic embryogenesis) in vitro. Such systems offer several advantages. For instance, relatively large numbers of cells and organizing units can be manipulated in small volumes. Excision of developing embryos from seeds in equivalent numbers would be very difficult, if not impossible. Certainly, removal of fertilized eggs or zygotes from the embryo sac in the ovule is out of the question. Indeed, it will be a landmark achievement when a zygote so removed can be nurtured to full maturity. In addition to such practical considerations, we have adopted the view that in vitro systems involving totipotent or morphogenetically competent cells present other advantages for providing answers to questions involving higher plant development — especially in space. Free cells in vitro, unlike zygotes in the environment of the embryo sac in ovules, should be more responsive to potential perturbations such as those that might exist in μg. We hypothesize that in such cells there should be no pre-
set or "learned" sensitivity to factors or conditions such as those encountered in the highly controlled environment of an embryonic sac other than that extant in the "genetic program" of the test system. In short, the exaggerated potential for eliciting a μg-associated expression of the plasticity of development and growth in an in vitro system involving embryogenic cells should provide a valuable means of probing environmental and nutritional impacts caused by complex interactions which may be encountered in space. Certainly one would anticipate that the perception and developmental expression of precise signals would be altered in μg (cf. Jennings and Trewavas, 1986; Schlichting, 1986; Osborne, 1989).

As cells differentiate in a multicellular embryo, they become part of organs that are morphologically and functionally different from one another even though the cells are presumed to contain identical genetic information. Questions that one can pose include — Does g exert a definitive influence on somatic embryogenesis? Do plant embryos require gravity or other orienting forces at any stage of their normal development? To what extent and in what ways does gravity influence embryo form? Are there specific blocks in embryogenesis in a low g environment? What constitutes the receptor(s) in g-sensitive developing embryogenic systems? What are the thresholds for gravity sensing in embryogenic systems? Are there effects of the space environment and μg on mitosis and chromosome behavior that adversely affect embryo development? If so, what are the developmental consequences? Does g play a determinative role in differentiation by affecting the relative composition of the cells to one another or by affecting the constituents within cells? In short, one is asking from several perspectives the broad question — To what extent and in what ways does gravity influence plant form?

COSMOS CARROT CELL CULTURE RESULTS

Work done some time ago in connection with the Cosmos biosatellites 782 and 1129 using embryogenic carrot cells showed that while the broad events of somatic embryogenesis did occur, questions remained. In the first instance, the carrot cell system used for the Cosmos experiments involved the generation of so-called competent units, inducing them on Earth to produce "proembryos," slowing down their progressive development by use of low temperature, and finally, subsequently exposing them to space conditions at a permissive temperature in order to evaluate their capacity to express further embryogenic capacity. The fine point of detail to be appreciated is that the experimental carrot cells used were already developmentally determined. Indeed, they were proven by prior experiment to be capable of undergoing somatic embryogenesis under a wide range of test conditions. They could not be manipula-

lated to induce their morphogenetic capability de novo in space. Since pre-programmed cells were generated on Earth, and chilled to preclude further development into later stage embryos on Earth, we have argued that the cells could well have retained a "memory" of the Earth's gravitational environment. Exactly what is meant by a "memory" is obviously not clear and the presence of multicellular sensors are currently too complicated to evaluate experimentally with confidence except at very gross levels (cf. Björkman, 1988). How one might successfully "erase" such a "memory" is obviously an equally moot point, but it can be proposed that successive generations of morphogenetically determined plant cells should be grown and attempts should be made to induce them de novo in space in a μg environment.

Other criticisms could be raised. The Cosmos 782 experiment could not be repeated exactly on the Cosmos 1129 flight. A centrifuge was not available for use in flight. None of the materials was chemically fixed in flight. Only after satellite recovery and transport of samples to Moscow was fixation performed. Even now, only preliminary results have been reported because of reluctance to publish in detail inadequately repeated experiments (cf. Krikorian and Stewart, 1978, 1979; Krikorian et al., 1981).

However, calculations carried out on data derived from the 1 g centrifuge control in space and Cosmos somatic embryos under microgravity can provide some clues to what might be expected from further experiments (cf. Tables I and II). Here, the results of the scoring of the normalcy of the developmental pathway of embryogenically competent carrot cells and proembryogenic units to later stages of embryogeny are presented. Table I presents a chi-squared analysis of the total number of somatic embryos per stage as a function of stage. Table II presents a pair-wise comparison of the proportion of somatic embryos at each stage. The chi-squared analysis compares the two distributions of somatic embryos among the four stages and indicates that these

Figure 3. Stages in the somatic embryogenesis of carrot as used in the scoring of embryos obtained in Cosmos flights. a, b = stage 1; c, d = 2; e = 3; f = 4.
Table I. Contingency Chi-square Method of Analysis of Somatic Embryogenesis in Microgravity and on a 1 g Centrifuge in Space. Stages of embryo development were subjectively categorized as Stages 1 to 4. Analysis from data of Krikorian and Steward (1978).

<table>
<thead>
<tr>
<th>Stage</th>
<th>0 g</th>
<th>1 g</th>
<th>Σ</th>
<th>Percent of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obs.</td>
<td>Exp.</td>
<td>Dev.</td>
<td>X²</td>
</tr>
<tr>
<td>Stage 1</td>
<td>6105</td>
<td>6103.16</td>
<td>+ 1.84</td>
<td>.0006</td>
</tr>
<tr>
<td>(Heart shaped)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td>1680</td>
<td>1570.41</td>
<td>+ 109.59</td>
<td>7.65</td>
</tr>
<tr>
<td>(Torpedo shaped, &lt; .75 and 1.5 mm long)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dev.</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>X²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 3</td>
<td>760</td>
<td>843.80</td>
<td>- 83.80</td>
<td>8.32</td>
</tr>
<tr>
<td>(Advanced embryonic forms with distinct root between .75 and 1.5 mm long)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dev.</td>
<td></td>
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</tr>
<tr>
<td>X²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 4</td>
<td>470</td>
<td>498.53</td>
<td>- 28.53</td>
<td>1.63</td>
</tr>
<tr>
<td>(Small plantlets with well developed root, &gt; 1.5 mm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dev.</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>X²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ</td>
<td>9015</td>
<td>9015.90</td>
<td>- 0.90</td>
<td>17.60</td>
</tr>
<tr>
<td>% of total</td>
<td>51.90</td>
<td>48.10</td>
<td></td>
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</tr>
</tbody>
</table>

Chi-square $X^2 = \Sigma (\text{Obs.} - \text{Exp.})^2 = 36.47$

Degrees of freedom = (2 - 1) / Exp (4 - 1) = 3

$p < 0.001$

Table $X^2$ (df3, p .001) = 16.27

Method of calculating expected values

\[
\text{Exp} (\text{Stage } 1, 0g) = (\% \text{ of total for Stage } 1) \times (\text{Total in 0 } g) = (0.6770) (9015) = 6103.16, \text{ etc.}
\]

Distributions are significantly different from one another with $p < 0.001$. Specifically, the data clearly show that the transition from one embryonic stage to another was slowed down. In µg, a greater proportion of embryos was at "stage 2" and fewer embryos had progressed to "stages 3" or "4." (See Figure 3 for the scoring stages used.)

Using an embryogenic system similar in principle to that found in carrot, Theimer et al. (1986) have reported increased biomass of embryonic structures generated in space in liquid cultures of anise, Pimpinella anisum. Most of the criticisms of experimental protocol raised in earlier paragraphs referring to the carrot experiments apply to their work with anise, however, and for me, their results also remain equivocal. Surely much more work in space will be needed to reinforce the preliminary studies and to resolve the unanswered questions.

A much improved in vitro system for carrot somatic embryogenesis is in the process of being developed at Stony Brook. It will provide a better opportunity to get definitive answers to questions as to whether development of cultured plant cells in space can occur with acceptable fidelity from a morphological, cytogenetic, and temporal perspective (cf. Smith and Krikorian, 1988, 1989, 1990a and b). The advantages of the new system include: (1) the simulation of zygotic embryogenesis with high fidelity; we now have the ability to expose cells that show no obvious polarity to the
space environment and to “turn on” the “embryogenic switch” in space by means of the very simple procedure of a change in medium; (2) the elimination of the need for external growth regulators to be manipulated at any stage of the process of obtaining or modulating embryogenic cells; (3) the achievement of 100% response, i.e., all preglobular stage somatic embryos yield proembryos and later stage embryos; (4) the elimination of mechanical cell sorting required to prepare test specimens, i.e., they are selected very early in the culture process; (5) the suitability for automation; (6) the potential for selection of adaptive cells or mutants; (7) control to provide an open-ended system so that new test specimens do not have to be prepared de novo for successive experiments. Not only will answers obtained from such a system be of interest to developmental plant biologists, but they will have significance for those seeking to use biotechnological procedures and manipulations in space for a variety of reasons (cf. Keeffe and Krikorian, 1983). Indeed, the ability to use and manipulate plant cells and other kinds of propagules in vitro reliably in space will be a necessary prerequisite to many projected or hypothesized commercialization schemes (cf. Krikorian, 1985).

COMMENTS

The foregoing seeks to emphasize the considerable opportunity to learn about embryogenically competent plant cells and their behavior in space. There is obvious importance to obtaining answers to such questions as — To what extent does the gravitational environment influence polarity, axis determination, and embryogenesis in vascular plants? Are the haphazard positions of the embryos and the abnormalities noted in megaspores grown on clinostats actually due to the effect(s) of g neutralization? Is there an influence of µg on the biochemical relationships between the embryo and nutrient supply, whether in situ in maternal tissue or in vitro in appropriately designed culture vessels and apparatuses designed to accommodate perception of “all” the “right” signals? These kinds of questions should suggest that the µg of space can provide a unique research environment in which to study development. The less sophisticated but perhaps more utilitarian question also may be raised — Do we have the required knowledge to grow generations of plants over protracted periods in space?

Interesting observations on decreased levels of cell division in roots after growth in space for a week have been made (Krikorian and O’Connor, 1984; Halstead and Dutcher, 1987). Chromosome aberrations such as fractures and breaks in cells of roots grown in space for relatively short periods are apparently not rare. The range of chromosomal disturbances detected suggest the mitotic apparatus may behave like a g sensor. It follows that we have a long way to go before we can be confident of being able to grow plants through successive generations (cf. Halstead and Dutcher, 1987) without adequate countermeasures being in place. There is no reason to suppose that during extended duration experimentation, protracted exposure to µg could not modify...
responses such as those involving embryogenesis in ways we have discussed. Precisely what countermeasures might be needed to achieve satisfactory embryo formation and growth could be determined once the abnormalities were adequately understood.

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REFERENCES


