

ified fusion protein of the recombinant lysogens (Fig. 2). Precise temporal (1) and spatial expression of the gene products can be determined by the use of monospecific antibodies. In addition, using the antibody probes, it is possible to screen large numbers of plant samples and determine if there are homologous proteins expressed in other plant species. Based on tissue-specificity and temporal expression during embryogenesis, we identified a gene product characteristic of cells committed to, but not undergoing, embryogenesis, and several gene products that are associated with the transition from a radially symmetrical to a polarized, bilaterally symmetrical structure (1). One out of the three antibodies we studied detected three homologous proteins in somatic and zygotic embryos of other plant species (4).

A large scale fusion and screening project was conducted to isolate monoclonal antibodies detecting antigens associated with somatic embryos (Fig. 1). One of the monoclonals detected a nuclear antigen associated with cells capable of cell division. It is a protein of 45 kDa with a pI of 6.7, with a cellular concentration <0.01% of the total embryo proteins. Single cells that are undergoing terminal differentiation contain diminishing quantities of this antigen. In planta, the antigen is preferentially located in the nucleus and nucleolus of shoot meristems (9). Its presence in tissue culture cells, in meristems, and in embryos suggests that it is characteristic of cells in division, and not of embryogenesis per se.

In conclusion, we have identified molecular markers characteristic of the processes of cell division and embryogenesis. Gene products expressed preferentially in the embryogenic cell clusters before embryo morphogenesis might be markers for the induced and committed embryogenic state. Future and present studies aim to identify and characterize a larger set of molecular markers for all stages of embryo development. The role of these selected antigens will be studied by the interference of their function with antibody and of their expression with antisense RNA.

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Organogenesis in Vitro as a Developmental Process

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Plants have a remarkable propensity for asexual or vegetative propagation, and it is not surprising that this capacity extends to plant cells or tissues cultured in vitro (24). The "regenerative response" in vitro includes somatic embryogenesis as well as organogenesis (formation of shoots or roots from cultured tissue). It may be useful to make a further distinction, subdividing the organogenic response. In many instances, explants have the capacity to give rise to shoots, roots, or floral structures when cultured on a medium that supplies mineral salts, vitamins, and a carbon source, but is devoid of plant hormones. These processes may be termed "adventive organogenesis". Exogenously supplied plant hormones often can facilitate these processes, but are not absolutely required for organogenesis to occur. In these instances, the immediate precursors of the new organs are cells in the explant itself (5, 6). The other type of organogenesis is not adventive and involves a "dedifferentiation" of the explant, elaboration of callus tissue along the cut edges of the explant, and the induction of new organs from this newly formed callus tissue. Exogenously supplied phytohormone not only controls the process but is required for organogenesis to occur. This paper is concerned with the specifics of this last type of organogenesis; we suspect that the general principles derived from this work apply to all organogenesis in vitro. As much as organogenesis in vitro reflects the mechanisms of organogenesis in vivo, these general principles will also apply to the development in whole plants ex vitro.

Using pith explants from tobacco (*Nicotiana tabacum* L.), Skoog and Miller (15) first showed that organogenesis was governed by the balance of auxin and cytokinin in the tissue culture medium.

Media with a relatively large auxin : cytokinin ratio induce roots, those with a low auxin : cytokinin ratio induce shoots, and those with an intermediate auxin : cytokinin ratio induce unorganized growth as callus tissue. However this induction occurs, it is followed by morphological differentiation and development. Our interest focuses on the process of induction rather than the process of morphological differentiation and development. Specifically, we are interested in two questions. Does the phytohormone balance induce the formation of organs, in the sense that zoologists use the term "induce"? How much of the substantial amount of time between placing and explant into culture and seeing the newly formed organs is devoted to the induction process and how much is devoted to morphological differentiation and growth? In addition, we would like to know why some plants do not regenerate and whether molecular changes occur that could be diagnostic of partial progress through the entire process of organogenesis.

Organogenesis in *Convolvulus arvensis*

Shortly after Skoog and Miller's (15) demonstration of the controls on organogenesis from cultures of tobacco, Earle and Torrey (7) demonstrated the formation of shoots in vitro from plated suspensions of friable callus of the field bindweed (*Convolvulus arvensis* L.). Small pieces of the lamina of leaves from greenhouse-grown plants will form organs readily when cultured on quite simple media: shoots on shoot-inducing medium (SIM); roots on root-inducing medium (RIM); and callus on callus-inducing medium (CIM) (for details of the culture system and exact formulations of media see ref. 2).

Determination for shoot production

Does exposure to the phytohormone balance in SIM actually induce the formation of shoots? Induction, in its widest developmental sense, is seen as a change in the fate or destiny of a cell or group of cells. In the case of in vitro-shoot organogenesis from leaf explants, induction results in cells of leaves giving rise to cells determined or fated for shoot organogenesis. Acquiring this specific developmental state, determination, has an experimentally recognizable endpoint: the explant will produce shoots even if the inductive medium is replaced by basal medium.

In the experimental protocol, leaf explants are placed on shoot-inducing medium for various lengths of time. When the explants are removed to basal medium, only the determined explants will go on to produce shoots. A set of representative data is shown in Table 1. After 14 days on inductive medium, explants of a particular genotype, designated genotype 19, are determined and produce shoots even if transferred to basal medium. Replicate experiments give the same or closely similar values for the time of determination. (Interested readers can find data for other genotypes in ref. 2)

Competence for induction of shoot production

On a gross scale, shoot organogenesis from leaf explants is preceded by the formation of small amounts of callus at the cut margins of the explant. Indeed, histological investigation shows this callus to be the tissue from which shoots arise (See Fig. 2c). This elaboration of callus tissue is commonly referred to as the "dedifferentiation of the explant". Capacity to respond to the inductive effects of a medium is called competence. (See ref. 14 for an excellent review of competence, induction, and determination.) Measurements of the time of determination with our adaptation of the protocol of Walker et al. (22) include both the time required for determination as well as the time required to produce tissue "competent" for induction. A modification of our protocol can distinguish these two events. Callus-inducing medium (CIM) does induce and support the growth of callus from a wide variety of *Convolvulus* genotypes. Preculture on CIM before transfer to shoot-inducing medium can shorten the time required in culture on SIM to make explants determined for shoot formation. Results of such an experiment performed with genotype 19 are shown in Table 2. Explants are precultured on CIM for various times, then cultured for various lengths of time on SIM, and finally transferred to basal medium. The first row in Table 2 is our protocol for ascertaining the time of determination (Table 1). Genotype 19 requires 14 days for explants to become determined for shoot formation. Preculture on CIM media for 3 days shortens the time required on SIM by 3 days; increasing amounts of time on CIM further shortens the required length of culture on SIM. Finally, however, longer precultures on CIM do not further shorten the required culture on SIM (data not shown). This minimum time requirement for SIM is the time ac-

tually required for the induction of roots. Subtraction of that time from the total length of culture necessary to result in explants determined for shoot formation (Table 1) estimates the time for the explant to become competent for induction. Genotype 19 takes at least 7 days on SIM to become competent ("dedifferentiated"); other genotypes acquire competence after as little as 3 or 4 days (e.g., genotype 30, ref. 2).

Surprisingly, competence for organogenesis is not directly related to the extent of callus proliferation (nor is determination). Elegant experiments by Walker et al. (22) have shown that competence for embryogenic induction in alfalfa (*Medicago sativa* L.) is a function of the size of the cellular aggregates. Documenting callus proliferation by measuring fresh weights of explants from two widely divergent *Convolvulus* genotypes shows that growth of each is very similar, is exponential over the first 14 days on SIM, and that competence is not a simple function of "explant mass" (Fig. 1).

Histological events associated with determined state

Leaf explants of genotypes 23 were placed on SIM and removed to either basal medium or prepared for histological examination. After 7 days on SIM, the leaf explants showed the beginnings of cellular proliferation at the cut margins (Fig. 2a), and a more extensive proliferation in the regions near small veins (Fig. 2b). The pronounced files of cells are remarkably similar to sections through

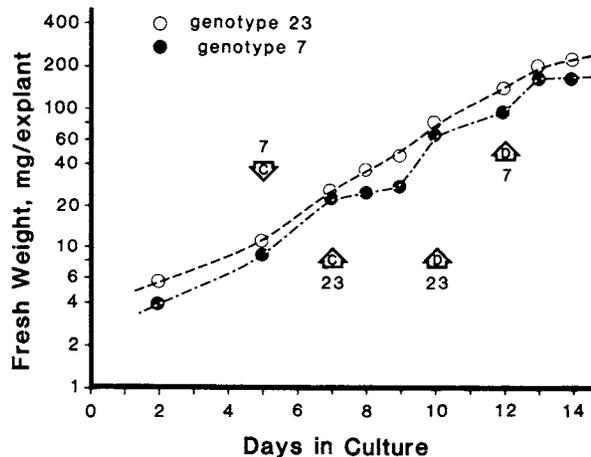


Fig. 1. Fresh weights of *Convolvulus* explants cultured on shoot-inducing medium (SIM). Two genotypes, 7 and 23, were chosen for their differences in both the time required to become competent and the time required to become determined for shoot formation. Neither competence nor determination is a simple function of cell mass (note logarithmic ordinate). Arrows containing the letters C and D mark the time each genotype becomes competent for induction and determined for shoot formation.

Table 1. Mean numbers of shoots produced from *C. arvensis* leaf explants exposed to shoot-inducing medium (SIM) for various lengths of time.^z

Mean no. of shoots										
Day of transfer from inductive medium ^y										
0	3	5	7	10	12	14	17	19	21	
0.00	0.00	0.00	0.00	0.00	0.00	0.44	1.56	3.56	1.33	

^z Genotype 19, shoots counted when explants had been in culture for 3 weeks, n = 15 explants for each mean.

^y Explants are transferred from SIM to basal medium.

Table 2. Mean numbers of shoots from *C. arvensis* explants exposed to callus-inducing medium (CIM), then shoot-inducing medium (SIM) before transfer to basal medium.^z

Mean no. of shoots											
Days on CIM	Days on SIM										
	0	3	5	7	10	12	14	17	19	21	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.44	1.56	3.56	1.33	
3	0.00	0.00	0.00	0.00	0.67	0.78	2.00	3.22	3.78	2.56	
5	0.00	0.00	0.00	0.00	0.11	1.00	1.56	2.89	2.78	4.11	
7	0.00	0.00	0.00	0.22	1.22	0.11	0.56	0.89	0.22	1.00	

^zGenotype 19, shoots counted when explants had been in culture for 3 weeks, n = 15 explants for each mean.

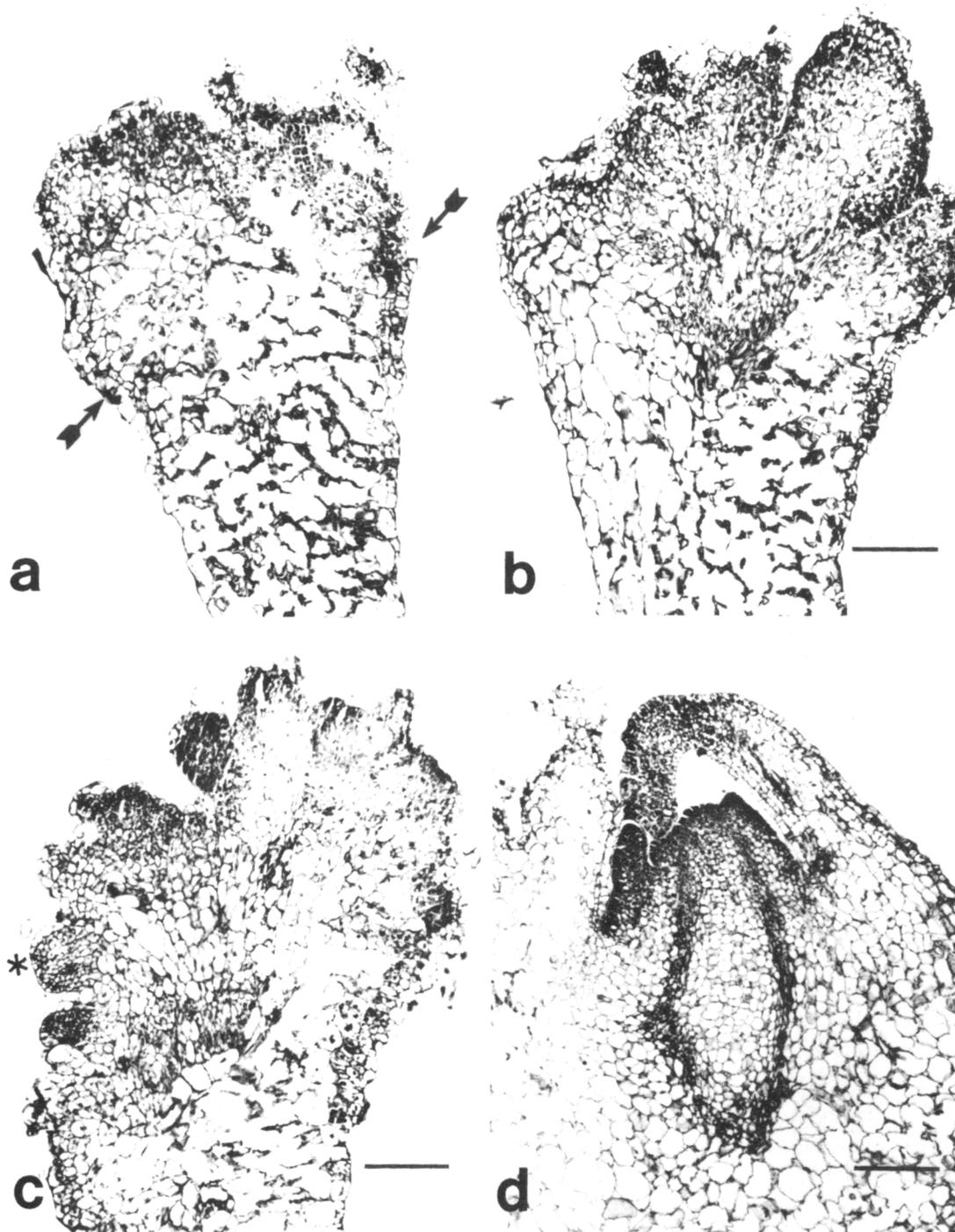


Fig. 2. Callus growth and shoot formation in *Convolvulus* revealed by paraffin-embedded material of known developmental age, sectioned at 10 μm and stained with safranin-fast green. (a) Explant after 7 days on SIM. Arrows mark the edge of the leaf explant; left-facing side was in contact with the medium. (b) Another section from an explant after 7 days on SIM. Such explants contain cells or groups of cells fated or determined for shoot formation; left-facing side was in contact with the medium. Asterisk marks an area with a well-defined outermost layer of box-like cells; this region may be the beginnings of the single-layered tunica plus corpus organization typical of *Convolvulus* shoot apices. (d) Newly formed shoot apex from explant cultured on SIM for 12 days; uppermost surface was in contact with the medium. Scale bars = 100 μm .

Table 3. Mean numbers of shoots produced from *C. arvensis* leaf explants exposed to acetylsalicylic acid (ASA) at different times².

Media sequence	Mean no. of shoots									
	Day of transfer									
	0	2	3	6	7	8	9	10	13	14
SIM to SIM + ASA	0.20	0.00	0.00	0.00	0.00	0.20	0.80	0.13	1.13	1.07
SIM + ASA to SIM	2.90	1.87	4.53	1.00	0.00	0.07	0.00	0.00	0.00	0.00

²Genotype T10, ASA at 2×10^{-5} M, shoots counted when explants had been in culture for 3 weeks, n = 15 explants for each mean.

undifferentiated callus seen by us and by others (13, 16). Equivalent explants moved from SIM to basal medium at day 7 proved not to be determined; i.e., did not go on to make shoots.

After 10 days on SIM, the callus proliferated at the leaf explant margins shows the presence of meristemoids (19), similar to those depicted by Maeda and Thorpe (13). The asterisk marks a structure that might be the beginning of a shoot apex, a tunica-carpus arrangement of cells without any associated leaf primordia (Fig. 2c) (*Convolvulus* has a single-layered tunica). Explants moved from SIM to basal medium at day 10 proved to be determined for shoot production. After an additional 2 days on SIM, well-formed shoot axes could be seen (Fig. 2d), complete with several leaf primordia and young leaves or leaf-like structures. Shoots from *Convolvulus* explants arise from the part of the callus in contact with the medium just as they do in tobacco (13). Careful observation reveals that the epidermis of the new shoot apices is contiguous with the outermost cell layer of the callus mass; indeed, preliminary observations reveal the presence of cutin not only along the new epidermis, but also along the outermost surface of the callus. Green and Brooks (8) argue for a primary organizing role for the epidermis in the formation of new apices, indirectly suggesting that apex formation is a superficial phenomenon. Our data would support both contentions.

Subdividing the induction process

Exogenous phytohormones induce, in the classical sense, explants to give rise to shoots or roots. Control over the type of organ is governed by the balance of the phytohormones and the control is exerted from the time the explant becomes competent for induction until the time when induction is completed and the explant contains cells or groups of cells determined for shoot or root development. That period is a substantial stretch of time, some 7 to 10 days, in most of the genotypes we have examined (3).

Most likely, the developmental process leading to the determined state involves some kind of gene action, and, as such, should be amenable to further analysis through the induction, recovery, and characterization of mutants. Enrichment selection techniques allowed the application of such an approach to the dissection of the process of somatic embryogenesis in carrot (1). We have been unable to recover similar temperature-sensitive (*ts*) mutants of shoot organogenesis in *Convolvulus*. There are many reports of substances that inhibit organogenesis in vitro, however. Some of these substances are general toxicants, but some should represent specific interferences with the process of organogenesis. With *ts* mutants, shifts from permissive to nonpermissive temperatures can identify the time in development when the mutant gene acts (or fails to act) (17); similarly, shifts to and from permissive and nonpermissive media can identify stage-specific inhibitors of the process of shoot organogenesis (3). These transient sensitivities to certain inhibitors identify the phenocritical times in development, the epigenetic crises sensu Waddington (20). They allow us to demonstrate that, although a single phytohormone balance controls shoot induction, the induction process is composed of discrete steps.

The experimental protocol is quite simple. A preliminary experiment reveals the concentration of a compound that just gives complete inhibition of shoot regeneration, but allows the explant to stay green, produce callus, and appear "healthy". Explants are placed on SIM with or without the inhibitor, and shifted to the other medium after various lengths of time in culture. After an appropriate amount of time (3 weeks in our system), explants are observed and the number of shoots counted and recorded. When the test compound is a general toxicant, only the "no-treatment control" explants make shoots. When the test compound is a stage-specific inhibitor, a reciprocal pattern of shoots and no shoots results from the two series of treatments. Acetylsalicylic acid (ASA) lowers fever in human beings and is known to cause a number of effects in plants (12); it is also a stage-specific inhibitor of shoot regeneration from *Convolvulus* explants. Explants of genotype T10 moved to SIM + ASA after the seventh day in culture will form shoots (Table 3). Explants moved from SIM + ASA by day 7 will also form shoots; but any transfer sequence that has the explant exposed

to SIM + ASA on day 7 results in no shoots. This response is evidence for an event in the regeneration process, sensitive to inhibition by ASA, which occurs on the 7th day of culture. Independent estimates of the time explants of genotype T10 become competent for induction and become determined for shoot formation reveal that this transient sensitivity to ASA occurs during the induction process. Examination of a number of compounds in this way allowed the discovery of stage-specific sensitivities to triiodobenzoic acid (TIBA), sorbitol, ribose, ammonium ion, and ASA in the process of the vitro shoot organogenesis from leaf explants of *C. arvensis* (3). All these sensitivities occur between the time the tissue becomes competent for induction and the time the tissue becomes determined for shoot production (2). As such, they identify steps in the process of organogenic induction, rather than in the processes of morphological differentiation and development.

These sensitivities as well as the events competence and determination occur at characteristic times in each genotype. Although we have not located each of the five sensitivities in every genotype, all evidence to date suggests that shoot organogenesis in any genotype includes all five phenocritical times (3).

Resume

The process of in vitro shoot organogenesis from leaf explants of *Convolvulus* is now seen to include a series of discrete steps. Leaf explants are placed on SIM. An initial dedifferentiation process results in the formation of competent callus tissue along the cut edges of explant (2). Shoot organogenic induction proceeds under the influence of the phytohormone balance in SIM.

This specific induction process is itself a multistep process, as evidenced by discrete, transient sensitivities to TIBA, sorbitol, ribose, ASA, and ammonium citrate (3). The end result of the induction process is cells or groups of cells fated or determined for shoot formation; these cells then undergo morphological differentiation and growth to result in shoots growing from the callus (Fig. 3).

The question of nonregeneration

The regeneration of shoots in vitro is a developmental process comprised of distinct phases, at least some of which are multistep (Fig. 3). A single culture medium needs to satisfy the requirements of all three phases if it is to result in organogenesis from the explant. Such "consensus" media do exist for a large number of species and cultivars. However, some cell cultures, cultivars, or entire plant species will not produce shoots or roots in vitro in response to any of an extensive list of auxin/cytokinin combinations. Our view of organogenesis suggests the possibility that at least some cases of nonresponsiveness to culture in vitro are simply due to the failure of one "happy medium" to elicit competence for induction and to induce organogenesis. This suggestion has proven true. Leaf explants from a number of seed-derived individuals of *Convolvulus* do not make shoots when cultured on SIM. Short precultures on RIM, a medium with the wrong phytohormone balance for shoot formation, followed by culture on SIM results in explants from those nonregenerating genotypes that give rise to large numbers of shoots (4). The same kind of approach, short precultures on SIM, followed by culture on RIM, can result in root formation from explants of genotypes that do not make roots when cultured on RIM alone (4).

These responses of otherwise nonresponsive cultivars are evidence that the induction of organogenesis is controlled as Skoog and Miller (15) have described, and that the lack of organogenesis under standard conditions results from the failure of cultures to acquire competence for induction.

Changes in mRNA populations

Recent advances in the techniques of molecular biology have resulted in a detailed picture of gene regulation during the growth and development of plants. Not too surprisingly, perhaps, the structural differences that allow morphologists to distinguish the various parts of plants are reflected in differences in the proteins or mRNA

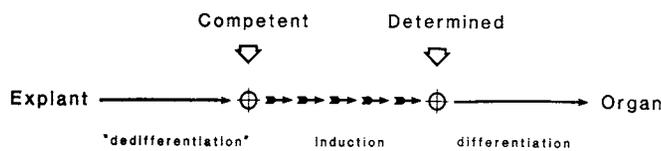


Fig. 3. Phases in the organogenic process.

species in those parts (10, 11). The induction phase in organogenesis from tissues cultured *in vitro* is a developmental process that necessarily precedes morphological differentiation (see ref. 21 for discussion of this point). Nevertheless, induction occurs under the influence of exogenous phytohormone (15); applied phytohormones have been shown to elicit specific mRNAs in a number of cases (see e.g., refs. 9 and 18), and such molecular species could prove to be exceptionally useful in both marking the process of organogenesis and in identifying those points in the process at which nonregenerating genotypes are blocked or diverted.

The process of root formation from leaf explants cultured *in vitro* lends itself to examination at the molecular level. Root caps of mature roots secrete "slime", a mucopolysaccharide; the process of root formation *in vitro* must involve the appearance of an mRNA for this protein (23).

Like shoot formation *in vitro*, root formation from cultured leaf explants is controlled by exogenous phytohormone, occurs in the callus tissue produced along the cut edges of the explant, and, as can be demonstrated, includes the acquisition of competence for induction, followed by induction, resulting in cells or groups of cells fated or determined for root formation. These events all precede the appearance of root primordia in the tissue (ref. 23; Christianson and Warnick, unpublished data). A series of explants of known developmental stage can be extracted for mRNA, that RNA used as a template *in vitro* for the synthesis of radioactively-tagged proteins, and those proteins separated by gel electrophoresis and visualized by autoradiography. These patterns of bands can be referred to as "translation profiles" and reflect the mRNA populations in the original explants (23). The leaf explants have a characteristic translation profile; a new, distinctly different translation profile is present within 2 days of culture on RIM. This profile persists unchanged through the acquisition of competence and through the induction process. On the day the explants first contain cells determined for root formation, a rapid change in translation profiles occurs to establish a profile closely similar to one from seedling roots. This change in profile occurs even though no roots or root primordia can be seen in the tissue for several more days and even though the explants contain only a few clusters of determined cells buried in a large mass of callus tissue (23).

Explants of genotypes that do not produce roots when cultured on RIM exhibit a leaf translation profile and then a profile "closely similar" to the profile that other genotypes exhibit during induction. This second profile then persists (23). The differences between regenerating and nonregenerating genotypes are not reflected as differences in certain abundant mRNAs; these experiments do not lead to "diagnosing" nonregeneration. These experiments do demonstrate the extreme rapidity of changes in cultured tissue. At a molecular level, the "dedifferentiation" of the explant is complete by the second day in culture (23).

Conclusion

Viewing organogenesis *in vitro* as a developmental process is the complement to more usual approaches treating it as a physiological response to the right combinations of plant hormones and nutrients. It has provided insights that allow otherwise "impossible" cultivars to regenerate. Not only is the composition of the medium important, but also the amount of time the explants see the medium. The observation that "dedifferentiation" of the explant, measured at the

molecular level or as the acquisition of competence, is quite rapid and is not synonymous with substantial amounts of callus proliferation may prove to be important for fresh approaches to old problems. If brief precultures on SIM, a medium rich in cytokinin, develops competence to respond to RIM and to make roots from nonresponsive leaf explants (4), perhaps hard-to-root rootstocks might be potentiated, not with auxin synergists, but by brief precultures with seemingly inappropriate media rich in cytokinins.

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