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Cloning of genes developmentally regulated during plant embryogenesis

(somatic embryogenesis/differential immunoadsorption/expression vectors)

JUNG H. CHOI*, LIANG-SHI LIU†, CHUMPOL BORKIRD, AND Z. R. SUNG

Departments of Genetics and Plant Pathology, University of California, Berkeley, CA 94720

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ABSTRACT **Genes specifically induced during somatic embryogenesis may play key roles in plant embryo development. An antiserum against an extract of carrot somatic embryos revealed a few rare antigens induced at the onset of embryogenesis. Through differential immunoadsorption techniques, we purified antibodies against the embryo-specific antigens and probed a phage λ gt11 library of cDNA from carrot somatic embryos. This paper describes three distinct cDNA clones that hybridize to embryo-specific RNAs. Monospecific antibodies, purified by affinity to the recombinant phage fusion proteins, confirm that the cloned cDNAs encode unique embryo-specific peptide antigens. One 50-kDa protein correlates with embryogenic ability in cultures of other plant species, including cereals.**

Since Steward *et al.* first reported somatic embryogenesis in 1958 (1), it has emerged as an attractive system for investigating fundamental issues in plant development (2). Cultured carrot cells proliferate in a chemically defined medium in the presence of a synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D). Upon removal of 2,4-D, single cells or small clusters of cells initiate organized cell divisions to give rise to spherical, globular-stage embryos. These acquire polarity and differentiate tissue layers to become heart-stage embryos. They then elongate to become torpedo-stage embryos capable of "germinating" and eventually regenerating fertile plants. This process resembles the morphogenesis of zygotic embryos (3).

The ease of obtaining large quantities of somatic embryos in synchronous cultures (4) makes the critical events of early embryogenesis available for molecular analysis. But from a developmental point of view, an equally important advantage of somatic embryogenesis is that the unorganized cell clusters can provide controls for the housekeeping and cell growth that accompany somatic embryo development. Thus, a differential comparison of developing embryos against unorganized cell clusters should identify those gene products associated solely or preferentially with embryonic organization and morphogenesis.

Furthermore, in reverse genetic approaches, such as expression of antisense RNA (5), the disruption of embryogenesis without affecting unorganized growth can discriminate those genes with specific developmental functions from genes with nonspecific proliferative functions. Thus, in theory, carrot somatic embryogenesis should provide opportunities to analyze the molecular bases for organized versus unorganized growth, acquisition of polarity, differentiation of tissue layers, and segregation of meristems.

Nevertheless, the proteins and genes specifically associated with somatic embryogenesis have been difficult to isolate. Two-dimensional protein gel comparisons (6–8) have shown that the vast majority of the resolved proteins remain constant between somatic embryos and unorganized cell clusters. This apparent similarity of gene expression may reflect the embryogenic competence of the cultures in 2,4-D. Such cell clusters, described frequently as "proembryogenic masses" (9), may be committed to the embryogenic program but diverted by 2,4-D from achieving embryonic organization and morphogenesis (2).

Still, either only a few new proteins suffice to execute the embryogenic program, or most new proteins synthesized are too low in abundance to be detectable. In any case, isolation of molecular probes for these proteins and their genes seemed impracticable by traditional techniques (10). Instead, the recent development of phage expression vectors (11) offers an alternative for obtaining cDNA clones for rare mRNA species using antibody probes. Antibodies also hold potential in themselves as investigative tools for cytological localization, quantitation, and ultimately for disrupting the function of their specific antigens.

We report here the use of antibody adsorption and epitope selection techniques to isolate both cDNA clones and monospecific antibodies against rare developmentally regulated proteins, without extensive protein purification steps. We also present results from the initial characterization of the isolated clones, examining their expression at both the RNA and protein levels.

MATERIALS AND METHODS

Cell Cultures. HA, a haploid line of carrot cells (*Daucus carota* L., cv. Juwarot), was routinely propagated in B5 medium (GIBCO) supplemented with 1.0 mg of 2,4-D per liter (12). Unorganized cell clusters were subcultured at 14-day intervals into fresh medium at an initial density of Klett 40 (Klett 1 = 2×10^4 cells per ml) (13). Embryonic cultures were initiated at Klett 4 after filtration through a 170-μm nylon mesh. Staged embryos were prepared by the method of Giuliano *et al.* (4).

Cassava leaf explants and somatic embryos were generously provided by Laszlo Szabados of the Centro Internacional de Agricultura Tropical (Cali, Colombia). Peach samples were furnished by P. Morgens, R. Scorza, and J. Cordts of the U.S. Department of Agriculture North Atlantic Area Appalachian Fruit Research Station (Kearneysville, WV). Protein samples from embryogenic and nonregenerating cultures of maize (derived from B73 zygotic embryos) were furnished by Keith Lowe of Stauffer Chemicals (Richmond, CA).

Preparation of Antiserum. Somatic embryos of HA, consisting of a mixture of stages from globular to advanced torpedo, were harvested after 17 days in culture by vacuum

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Abbreviation: 2,4-D, 2,4-dichlorophenoxyacetic acid.

*Present address: School of Applied Biology, Georgia Institute of Technology, Atlanta, GA 30332.
†Present address: Institute of Genetics, Academia Sinica, Beijing, People's Republic of China.

filtration through Miracloth. The embryos were ground in a mortar and pestle in phosphate-buffered saline containing 1.0 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride. The extract was centrifuged at 12,000 rpm for 15 min at 4°C in a Sorvall SS-34 rotor. The pellet was washed twice and resuspended in 2 vol of phosphate-buffered saline. New Zealand White rabbits were immunized subcutaneously and intramuscularly with 1.0 ml of a 1:1 emulsion of carrot extract with complete Freund's adjuvant. After four booster injections with incomplete Freund's adjuvant at biweekly intervals, the rabbit was sacrificed and exsanguinated by cardiac puncture.

Adsorption of the Antiserum. Nitrocellulose filters (Sartorius) were saturated with extracts from unorganized cell clusters, prepared by grinding in 50 mM Tris·HCl, pH 6.8/1 mM dithiothreitol/0.1% $NaDodSO_4$. A 0.5-ml aliquot of the antiserum diluted in 10 ml of blocking buffer (20 mM Tris·HCl, pH 7.3/0.15 M NaCl/0.1% Tween-20/3% bovine serum albumin) was incubated repeatedly with several such filters.

Polyacrylamide Gel Electrophoresis. Carrot somatic embryos or unorganized cell clusters were collected by vacuum filtration on Miracloth, weighed, and ground in an equal volume of 2× Laemmli sample buffer (14). The mortar was rinsed with another volume of distilled water, and the rinse was added to the sample. The samples were heated at 65°C for 5 min and then centrifuged for 5 min on a Microfuge. The supernatant was loaded on 7.5–15% acrylamide linear gradient gels unless otherwise indicated. Gels were stained with silver (15) or blotted onto nitrocellulose paper (16) in transfer buffer containing 0.1% $NaDodSO_4$ and 20% methanol. Sample loadings were adjusted to give equal silver staining intensities.

Protein Blot Analysis. When desired, sections of the protein blots were stained overnight with 0.1% Pelikan Fount India Ink in TBS-Tween (20 mM Tris·HCl, pH 7.3/0.15 M NaCl/0.1% Tween 20) (17). Otherwise, the blots were treated for 2 hr in blocking buffer at room temperature, incubated overnight with primary antibody diluted in blocking buffer, washed 4 or 5 times with TBS-Tween, then treated for 8–14 hr with horseradish peroxidase-conjugated goat anti-rabbit gamma globulin (affinity-purified from Bio-Rad). The color development used 4-chloro-1-naphthol (18).

Screening the Phage λ gt11 Library. The details of the carrot cDNA library construction have been published (19). The library was screened by following published protocols (20) except that peroxidase-conjugated secondary antibody was used as described for protein blots. The adsorbed antiserum gave clear signals over background without further adsorption against *Escherichia coli* proteins.

Affinity Purification of Monospecific Antibodies. Fusion proteins were partially purified from lysogenized phage (20) by precipitation in 40% saturated ammonium sulfate. After the precipitate was resuspended in 0.1 M 4-morpholinepropanesulfonic acid (Mops), pH 6.8/0.15 M NaCl/0.01 M $MgCl_2$/0.5% 2-mercaptoethanol and dialyzing in 0.1 M Mops (pH 6.8), the fusion proteins were coupled to Affi-Gel 10 (Bio-Rad) for affinity chromatography (21). Crude antiserum (0.1 ml) was diluted in blocking buffer and applied to the affinity column. The runoff was collected and reapplied to the column several times. After the bound antibody was washed with 20 column volumes of blocking buffer, it was eluted with 5 mM glycine, pH 2.3/3% bovine serum albumin/0.15 M NaCl/0.3% Tween-20 (22). The eluted fractions were immediately neutralized with 1/10th volume of 0.5 M Na_2HPO_4.

RNA Blots. Poly(A)$^+$ RNA was electrophoresed on 1% agarose gels containing formaldehyde and blotted onto nitrocellulose filters (23). Nick-translated, ^{32}P-labeled probes were prepared from recombinant pUC18 plasmids (24) containing carrot cDNA sequences subcloned from the purified recombinant phage. Filter hybridizations were performed (23) in 50% formamide, containing 5× NaCl Cit (1× = 0.15 M NaCl/0.015 M sodium citrate), 5× Denhardt's solution (0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and 0.1 mg of poly(A) per ml at 45°C for 36–48 hr. After hybridization, the filters were washed successively for 30 min each in 2× NaCl/Cit, 1× NaCl/Cit, 0.5× NaCl/Cit, and 0.1× NaCl/Cit, all containing 0.1% $NaDodSO_4$ at 65°C.

RESULTS

Antiserum Highlights Developmentally Regulated Proteins. Rabbits were immunized with a crude pellet fraction obtained at 15,000 × *g* from a homogenate of unsynchronized carrot somatic embryos. Immunochemical staining of protein blots revealed that this antiserum recognizes a large number of antigens in both unorganized cell clusters and somatic embryos (Fig. 1). However, several antigens showed differences in accumulation between the two cultures. The bands at 37, 44, and 185 kDa appeared only during unorganized growth in medium containing 2,4-D. Conversely, a major cluster of bands at 20 kDa accumulated only in embryo cultures, and bands at 66 and 68 kDa as well as a few minor bands were present at higher levels in embryos than in unorganized cells.

Very few antigens, and none of the differentially expressed antigens, persisted in differentiated seedling tissues. This result indicates that most embryonic antigens are not involved in the differentiated functions of leaves, petioles, and roots. Many embryonic antigens also disappeared when cultures with 2,4-D reached limiting density and cells entered stationary phase.

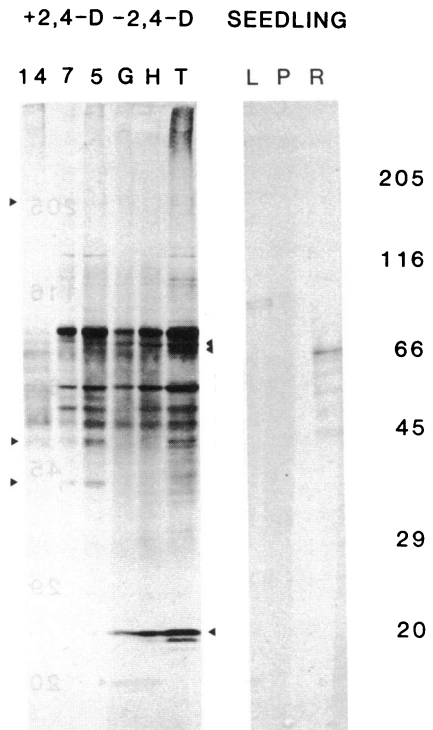

FIG. 1. Immunoblot of carrot extracts probed with antiserum against carrot somatic embryos. A culture of HA cells in 2,4-D was sampled at days 5, 7, and 14 after subculture. Somatic embryos were morphologically staged (lanes: G, globulars, 100–120 μm; H, hearts, 170–200 μm; T, torpedos, 250–300 μm) as described. Juwarot (parental cultivar for HA) seedlings were frozen in liquid nitrogen and then divided into leaves (lane L), petiole (lane P), and root (lane R) fractions. Each seedling had 2 or 3 leaves and hairy roots approximately 2 inches long. Sizes are shown in kDa.

Adsorption Enriches for Embryo-Specific Antibodies. To better resolve the embryo-specific antigens, we attempted to rid our antiserum of antibodies that reacted against antigens present in unorganized cell clusters. After three rounds of adsorption against extracts from unorganized cell clusters (see *Materials and Methods*), the antiserum gave the result shown in Fig. 2*C* when used to probe a protein blot.

The adsorption procedure removed almost all of the reactivity against the major antigens in unorganized cell clusters. The residual reactivity consists of minor antigens, whose low levels may be insufficient to remove their cognizant antibodies from the serum in a few rounds of adsorption.

In contrast, the adsorbed serum clearly highlighted the previously identified embryo-specific antigens at 68, 66, and 20 kDa. In addition, a prominent new embryo-specific antigen emerged at 57 kDa, along with numerous minor embryo-specific antigens. These latter were masked by the more abundant common antigens when the blot was probed with the original crude antiserum (Fig. 2*B*).

None of the embryo-specific antigens, except possibly the 20-kDa band, corresponds to a visible embryo-specific band in the silver-stained gel (Fig. 2*A*). Either the antigens themselves were too scarce to be perceived among other proteins in a crude extract or the embryo-specific epitopes were posttranslational modifications of proteins common to both types of cells.

Adsorbed Antiserum Identifies Putative Embryo-Specific cDNAs. The adsorbed antiserum was used to probe a phage λ gt11 library of cDNA sequences from carrot somatic embryos (19). In all, 22 recombinant phage producing antibody-reactive fusion proteins were isolated and purified from a total of 1.6 million phage screened. Since 12% of the phage in the library were recombinant, and of these 1 phage in 6 would have had the cDNA insert in the correct frame and orientation for proper expression, about 30,000 phage were expressing carrot cDNAs. Thus, the isolated sequences combined represent approximately 0.1% of the total cDNA population, and any single clone represents only on the order of 0.01%.

To examine the expression of these cDNA sequences at the mRNA level, the inserts were subcloned into pUC18 plasmids (24). Fig. 3 shows blot-hybridization experiments with poly(A)$^+$ RNA from carrot somatic embryos and unorganized cell clusters, probed with plasmids containing the indicated cDNA clones. The levels of RNAs homologous to three clones, clone 8, 49, and 59, were severalfold higher in the embryos than in the unorganized cell clusters by visual estimation. Hybridization to a carrot α-tubulin cDNA probe (25) was used as a control for a "constitutive" gene. The cellular content of the tubulin RNA was much higher than that of the three RNA species under study. Of the remainder of the original 22, clones 33 and 50 showed identical hybridization patterns and homology to clones 8 and 49, respectively (data not shown). Four other clones showed no clear differential expression (data not shown), and the rest are not yet characterized.

Developmental Expression of Individual Antigens. Reversing the approach taken in isolating the cDNA clones, we used the β-galactosidase fusion proteins synthesized by the recombinant phage to affinity-purify monospecific antibodies against each of the cloned antigens.

The monospecific antibodies were used to identify the cognate carrot proteins on protein blots and to examine their accumulation during development. Fig. 4 shows that the 66-kDa protein is immunologically related to the clone 8 antigen, is present in unorganized cell clusters at low levels but increases severalfold in globular-stage somatic embryos, and maintains elevated levels through the torpedo stage. Upon germination to form seedlings, the 66-kDa protein apparently disappears during the differentiation of leaves, petioles, and roots. Reproductive tissue from mature plants also lacks detectable amounts of this antigen, from flower buds to immature seeds. Since developing embryos comprise a nearly insignificant portion of the mass of the immature carrot seeds, this sample tests for the presence of the antigen in the endosperm and other nonembryonic tissues.

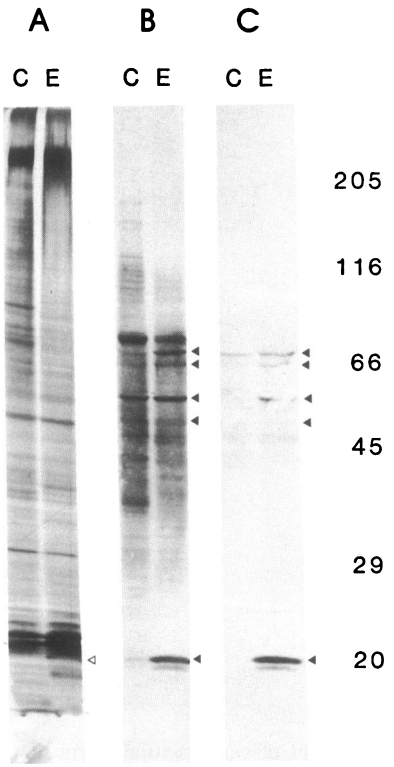

FIG. 2. Comparison of original antiserum versus antiserum adsorbed against immobilized extracts of unorganized cell clusters. Proteins from unorganized cell clusters (lanes C) and from somatic embryos (lanes E) were separated by $NaDodSO_4$/PAGE and stained with silver (*A*) or were blotted onto nitrocellulose and probed with the original antiserum against somatic embryos (*B*) or with adsorbed antiserum (*C*).

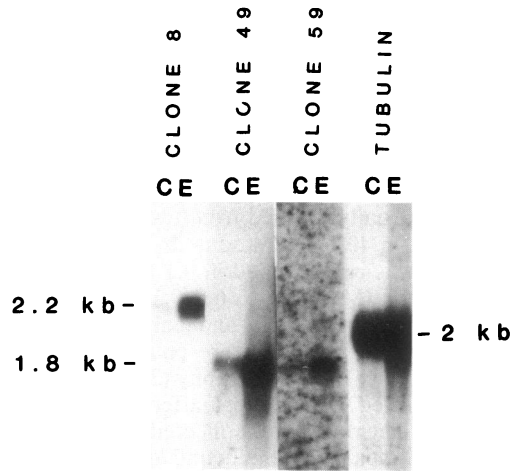

FIG. 3. Blot hybridization of cloned cDNA probes with poly(A)$^+$ RNA from somatic embryos and unorganized cell clusters of carrot. Each lane contains 5 μg of polyadenylylated RNA, processed and probed with nick-translated plasmids as described. Filters were hybridized in 50% formamide containing 5× NaCl/Cit, 5× Denhardt's solution, and 0.1 mg of poly(A) per ml at 45°C for 36–48 hr and then washed for 2 hr at 65°C with decreasing salts, ending with 0.1× NaCl/Cit. Lanes: C, unorganized cell clusters; E, somatic embryos.

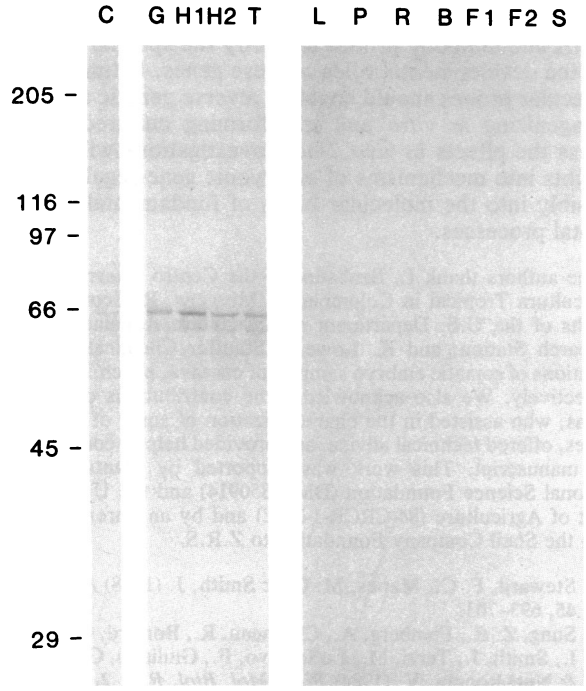

FIG. 4. Developmental expression of the 66-kDa embryonic antigen. Protein blots of the indicated samples were probed with antibodies affinity-purified against the clone 8 fusion protein, as described. A 9% acrylamide gel was used to separate the proteins in each sample. Lanes: G, carrot globular somatic embryos (<120-μm diameter); H1, heart-stage embryos (120- to 170-μm diameter); H2, late heart-stage embryos (170- to 200-μm diameter); T, torpedo-stage embryos; L, seedling leaf; P, seedling petiole; R, seedling root; B, flower buds; F1, early flowers; F2, late flowers; S, immature (green) seed pods. Sizes are shown in kDa.

Unlike the clone 8 antigen, the 50-kDa protein identified by the clone 49 antibody showed faint levels of expression in seedling roots, which contain a higher proportion of rapidly dividing meristematic cells than do the leaves and petioles (Fig. 5*A*). However, the accumulation of this protein was much greater in cultured cells and higher in embryos than in unorganized cell clusters.

Clone 59 apparently encoded the protein for the major 20-kDa embryo-specific antigen seen in Figs. 1 and 2. The monospecific antibody demonstrates that this antigen cluster is immunologically distinct from all other proteins in carrot somatic embryos, unorganized cell clusters, or seedling tissues (Fig. 5*B*).

Expression in Other Plant Species. Somatic embryos of cassava, peach, and maize were tested along with their nonembryogenic callus cultures and a differentiated tissue as controls for developmental regulation. Zygotic embryos and leaf of Johnson grass also were examined.

The monospecific antibodies purified against the clone 59 and clone 8 fusion proteins failed to cross-react with any species besides carrot (data not shown). However, as Fig. 6 shows, all of the plants tested, including maize and Johnson grass, accumulated a 50-kDa protein in their embryos that cross-reacted to the antibody against the clone 49 carrot antigen. Moreover, the expression of this antigen was significantly induced in embryos over callus cells in all cases. The difference between the nonregenerable and embryonic maize cultures was particularly striking. Surprisingly, the immature leaves of cassava, sprouted from somatic embryos, also contained significant levels of this antigen, although the mature leaves from Johnson grass did not.

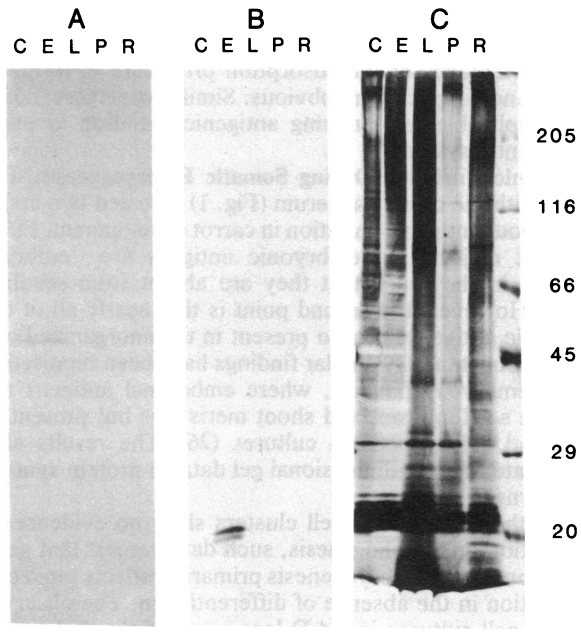

FIG. 5. Expression of the 50-kDa and 20-kDa embryonic antigens. (*A* and *B*) Protein blots were probed with antibodies affinity-purified against the clone 49 fusion protein (*A*) or the clone 59 fusion protein (*B*). (*C*) Silver-stained gel of the same samples. Lanes: C, unorganized cell clusters; E, somatic embryos; L, P, and R, seedling leaves, petioles, and roots.

DISCUSSION

Cloning cDNAs for Developmentally Regulated Antigens. Our strategy for isolating cDNA clones for rare developmentally regulated antigens required specific antisera to probe a phage expression library. Rather than attempting to purify each of the embryo-specific antigens, we used a differential adsorption technique with the original crude antiserum. The resulting adsorbed serum reacted with only 0.1% of the carrot epitopes in the phage λ gt11 library. And of these, at least 5 of 22, or 20%, hybridize to embryo-specific RNAs. Thus the process, from initial characterization of the complex antigen population to probing an expression library with differentially

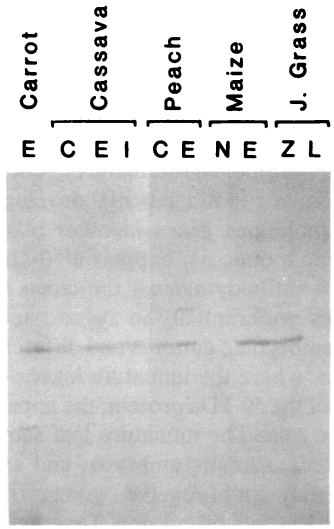

FIG. 6. Expression of the clone 49 antigen in cell cultures of other plant species. Protein blots were probed with antibodies affinity-purified against the clone 49 fusion protein. Lanes: E, somatic embryos; C, callus; I, immature leaf; N, nonregenerating cell culture; Z, zygotic embryos; L, mature leaf.

adsorbed serum, yields cDNA clones for even rare (0.01% of poly(A)$^+$ RNA) differentially accumulated messages.

The advantages of the adsorption procedure in terms of rapidity and simplicity are obvious. Similar strategies should prove applicable for studying antigenic variation in other experimental systems.

Antigenic Variation During Somatic Embryogenesis. The results with the crude antiserum (Fig. 1) revealed two major points about antigenic variation in carrot development. First, the great majority of embryonic antigens are "embryo-specific" in the sense that they are absent from seedling tissues. However, the second point is that nearly all of the embryonic antigens are also present in the unorganized cell clusters. Substantially similar findings have been reported in zygotic embryos of maize, where embryonal antigens are absent in seedling root and shoot meristems but present in callus and cell-suspension cultures (26). The results also corroborate the two-dimensional gel data on protein synthesis patterns (6).

Since the unorganized cell clusters show no evidence of organization or morphogenesis, such data suggest that gene expression during embryogenesis primarily reflects rapid cell proliferation in the absence of differentiation. The observation that cell cultures in 2,4-D lose many of the embryonic antigens when they reach high density and stop dividing reinforces this point.

Alternatively, the embryonic antigens may reflect the commitment or determination of cells to an embryonic program. Then the expression of these antigens in cultured cells would demonstrate that they have a similar state of commitment or determination. Examination of antigen expression in meristems, which also have a population of rapidly dividing, undifferentiated cells but are committed to different developmental programs, may help to clarify this issue.

Developmental Expression of the Cloned Antigens. The clones isolated in this study represent three distinct and unrelated proteins whose unusual patterns of expression correlate with embryonic organization and morphogenesis. Their lack of expression in proliferating but nonmorphogenic tissue makes it unlikely that these genes have a role in cell proliferation. Their lack of expression in seedling tissues suggests further that their roles are specific to the period of embryo development. Finally, the early induction and low abundance distinguish these proteins from seed storage proteins, which accumulate in massive quantities in the middle and late stages of embryogenesis (27, 28). Therefore, while the data presented here do not prove that these clones represent genes with significant roles in plant embryo development, they do argue against the most likely alternatives.

Because embryogenesis throughout the higher plants shares common morphogenetic features, one would expect that any gene involved in fundamental developmental events would have homologous genes in other plants. All tested species, including monocots, express a 50-kDa protein that crossreacts with antibody against the clone 49 antigen and that accumulates preferentially in zygotic and somatic embryos over nonembryonic cultures and differentiated organs. Even in cassava, where the immature leaves contain appreciable amounts of the 50-kDa protein, the apparent exception may support the rule. The immature leaf sample is derived from "germinated" somatic embryos, and explants of this tissue prove highly embryogenic, giving rise to somatic embryos within 10 days of culture. Together, these results imply that expression of the 50-kDa protein may correlate with embryogenic ability in both dicotyledonous and monocotyledonous species.

Further studies in progress will take advantage of the cDNA and antibody probes to clarify the specific functions and the developmental roles of these genes. Ultimately, the molecular probes should enable a reverse genetic approach, mutagenizing *in vitro* and transforming cultured cells to assess the effects *in vivo*. Such investigations will provide insights into mechanisms of embryonic gene regulation and possibly into the molecular bases of fundamental developmental processes.

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