PLANT MOLECULAR BIOLOGY REPORTER
Volume 2, Number 3, Summer 1984 Pages 19-25

REVIEWS AND PERSPECTIVES

Two-Dimensional Gel Analysis of Carrot Somatic Embryonic Proteins

Jung H. Choi and Z.R. Sung

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

Somatic embryogenesis of carrot cells in culture offers a potentially elegant system for identifying and isolating those gene products with specific roles in development and differentiation. Under the proper conditions, the conversion of cells from non-embryonic to embryonic growth is quantitative, producing yields of early embryos sufficient for biochemical and molecular studies (Sung et al., 1979). The trigger for initiating the expression of embryonic functions is the transfer of cells into fresh medium lacking 2,4-D; the signal is therefore relatively simple and well-defined compared to most animal cell systems. Uniquely and most importantly, the non-embryogenic growth of these cells in suspension cultures supplemented with the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), provides a good control for cell growth and maintenance functions.

Proteins present in embryos but not in the non-embryonic cells are thus more likely to have "developmental" functions than "housekeeping" functions. Proteins with the opposite behavior, being absent in embryos but present in non-differentiating cells, may also play interesting roles as suppressors of development. Several such "embryonic" or "callus" proteins have already been identified on native/SDS two-dimensional gels (Sung and Okimoto, 1981

Offprint requests to: Jung H. Choi, Dept. of Plant Pathology and Genetics, University of California, Berkeley, CA 94720.

and 1983). However, these techniques resolved only about 200 proteins and were limited to the soluble, cytoplasmic fraction.

To extend the search for embryo-specific functions, particularly those that may be responsible for embryonic organization during growth, proteins were extracted from embryos and from non-embryonic suspension cultures and separated on urea-IEF/SDS two-dimensional gels (O'Farrell, 1975; Garrels, 1979). Such methods resolve approximately 1000 proteins, including the less abundant proteins which each constitute less than .01 percent of the total cellular protein content. The use of detergents during sample preparation and the isoelectric focusing dimension enables solubilization and resolution of most membrane proteins as well.

Methods

Parallel cultures of haploid (HA) cells (Smith et al., 1981) were initiated at low density (less than 4 Klett units by the turbidimetric method of Sung, 1976) into fresh B5 medium with and without 2,4-D. After 15 days of culture, cells were pipetted into sterile, disposable 15-ml graduated conical tubes (Corning) and allowed to sediment at unit gravity. The volumes were then adjusted to approximately 0.05 ml cells in 0.5 ml of the original medium. This procedure minimizes artifacts due to centrifugation and change to fresh medium (Sung and Okimoto, 1981). The cells were then incubated 4 h on a rotating agitator at 24°C in the presence of 0.125 mCi ^{35}S-methionine (Amersham SJ204).

Procedures for sample preparation and running the first dimension isoelectric focusing gels were adapted from O'Farrell (1975) and from Garrels (1979). After the labeling period cells were washed 3X in distilled water. The pelleted cells were resuspended in 0.1 ml extraction buffer (10 mM TrisHC1, pH 7.4, 30 mM KC1, 10 mM $MgC1_2$, 0.5% Triton X-100, 1.0 mM DTT) and transferred to a pre-chilled mortar. RNase (0.005 mg in 0.01 ml extraction buffer) and SDS (0.003 ml of a 10% solution) were added and the mixture ground for 30 seconds with a pre-chilled pestle. The homogenate was transferred to a 1.5 ml Eppendorf tube and immediately frozen on dry ice. Another 0.1 ml of extraction buffer was used to wash the mortar and added to the homogenate. The sample was lyophilized to dryness, reconstituted in 0.1 ml lysis buffer (O'Farrell, 1975), and stored at −80°C.

Isoelectric focusing gels were prepared and run according to O'Farrell, 1975, except that the acrylamide concentration was reduced to 3% and 1% Triton X-100 replaced the 2% NP-40. The second dimension was run essentially as described by O'Farrell (1975), using the Laemmli buffer system (Laemmli, 1970) and 9% acrylamide gels. The results were visualized by silver staining (Morrissey, 1981) and autoradiography.

Results and Discussion

Figures 1A and 1B show photographs, respectively, of parallel, 15-day old cultures of non-embryonic, undifferentiated cells in medium containing 1.0 mg/l 2,4-D, and of embryos developing in medium without 2,4-D. The embryos consist of a mixture of globular, heart and early torpedo stages. The culture containing 2,4-D consists of relatively smaller, irregular clumps showing no discernible organization.

In comparing the gel patterns of proteins extracted from the two cultures, it is important to keep in mind that the only starting variable is the presence or absence of 2,4-D. Though by 15 days the two cultures have quite different morphologies, the growth rates have been similar, yielding similar fresh weights. Differential depletion of the medium or cell densities should not be important factors in this analysis. The differences in their two-dimensional gel patterns should then be attributable to the presence or absence of the auxin, 2,4-D, and to the embryonic organization and development in one culture versus the lack of organization and development in the other.

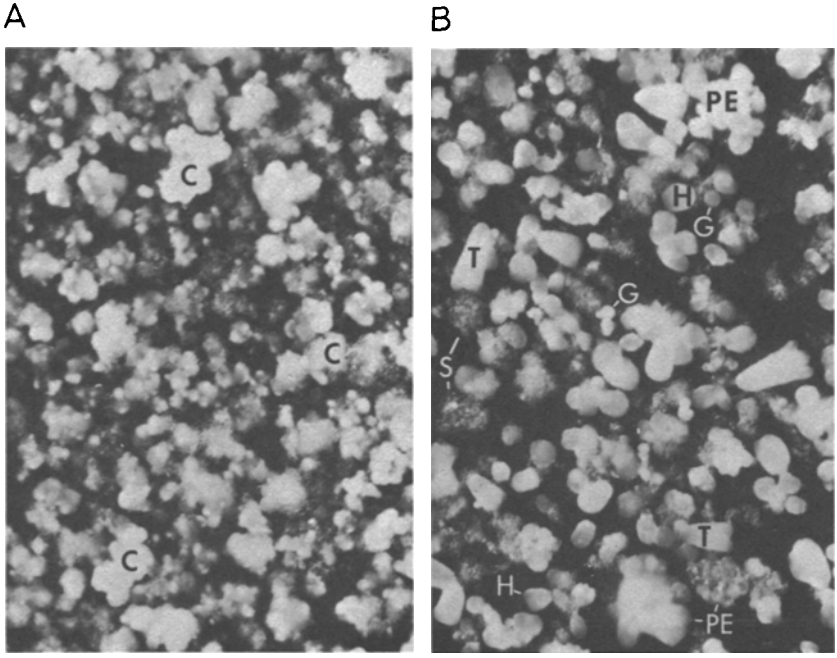

Figure 1. Morphology of haploid carrot cells 15 days after subculture at low density in medium (A) with 1.0 mg/l 2,4-D and (B) without 2,4-D. C: undifferentiated callus-like clumps; G: globular embryos; H: heart-stage embryos; T: torpedo-stage embryos; PE: polyembryos; S: elongated, nondividing and undifferentiated cells.

Figure 2 shows typical two-dimensional gel patterns obtained from these cultures. Silver-stained gels (2A and 2B) show the accumulated proteins present in the cells at this time, whereas the autoradiographs (2C and 2D) show the rates of synthesis. Arrows point to putative embryo-specific proteins, while open symbols enclose putative callus-specific proteins. Arrows and symbols of the same shape identify the same region in each gel or autoradiograph.

In general, relative intensities of proteins in the silver-stained gels correspond well with their relative intensities in the autoradiographs. However,

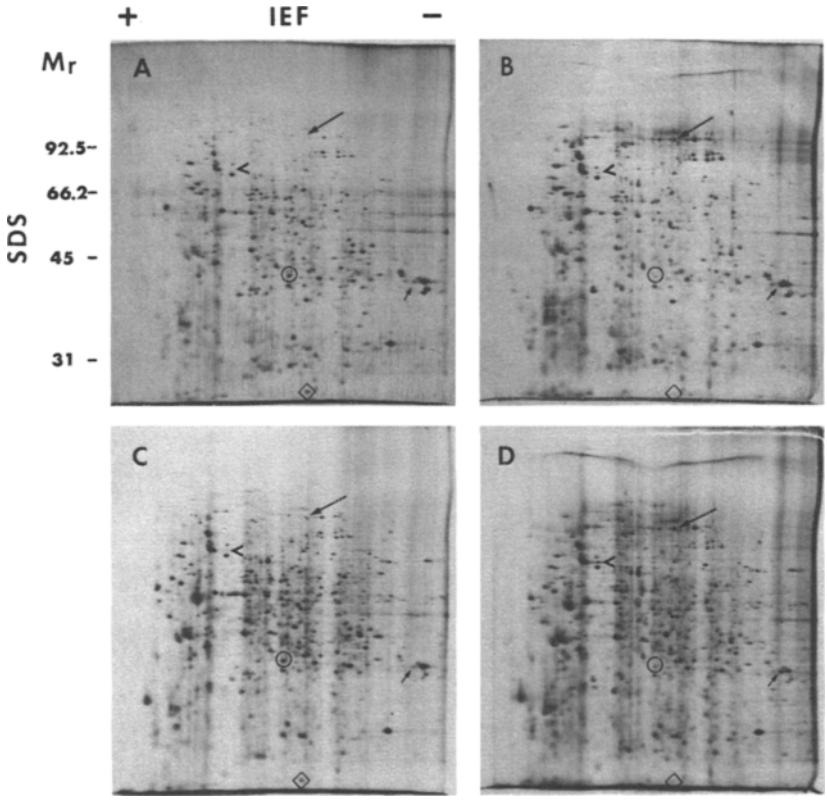

Figure 2. Two-dimensional gels of proteins extracted from 15-day-old cultures of haploid carrot cells. A: Cells grown with 1.0 mg/l 2,4-D; silver-stained gel. B: Embryos grown in the absence of 2,4-D; silver-stained gel. C: Autoradiograph of gel in (A). D: Autoradiograph of gel in (B). The locations of proteins specific to non-embryonic cultures are enclosed by open geometric symbols; whereas the locations of proteins specific to embryonic cells are indicated by arrows.

proteins with exceptionally rapid turnover should have higher relative intensities in the autoradiograph than in the silver stain, and proteins with exceptionally slow turnover should show the opposite. By comparing the two, one can obtain qualitative information on the relative half-lives of some proteins. The autoradiographs, resolving over 800 proteins compared to approximately 400-500 proteins in the silver stain, appear more sensitive. The autoradiographs also provide current information on which proteins are being translated and thus display changes in protein synthesis instantaneously. However, the silver stain should more accurately reflect the current protein composition of the cells and hence their developmental or functional state.

But the most striking conclusion after even a brief examination of these gels and autoradiographs is that, at this early stage, the embryonic protein profile is still extremely similar to the profile of proteins in undifferentiated and non-embryonic cells. Developmentally regulated and auxin regulated proteins together account for no more than 1-2% of the proteins resolved. A simple extrapolation of this figure, based on an estimate of approximately 10,000 polypeptides encoded by the typical eucaryotic genome, would indicate that only 100-200 proteins are responsible for the morphological organization and development seen in the early stages of somatic embryogenesis. If so, the vast majority of proteins, present in both the embryonic and non-embryonic cultures, must have common "housekeeping" functions not specifically related to either promoting or suppressing embryo development.

Such conclusions, of course, remain highly conjectural so long as they are based on a sample that includes only 10% of the total suspected number of proteins. Developmental proteins, playing subtle regulatory roles, may almost all belong to the category of extremely rare proteins which cannot be visualized with the present gel technology. Moreover, many proteins may have developmental functions but still be present in both embryonic and nonembryonic tissues, in different allosteric conformations, in association with different cofactors, or in different cellular compartments. Indeed, many possible flaws could invalidate such an extrapolation. Nevertheless, in *Dictyostelium*, estimates derived independently through genetic experiments have yielded similar numbers of no more than a few hundred developmental genes (Loomis, 1978).

In contrast, other two-dimensional gel studies of protein synthesis in developing embryos, chiefly in animal systems, have shown much more extensive changes through early development (for example, see Johnson and Hirsch, 1979 on *C. elegans*; Brock and Reeves, 1978 and Ballantine, et al., 1979 on *Xenopus laevis*). Such significant discrepancies between animal zygotic embryo development and carrot somatic embryogenesis do not, however, present insurmountable problems in explanation. The former differs from the latter in

some important aspects. The early cleavage stage embryo subsists chiefly on its large stores of maternal proteins and mRNAs. Transcriptional activity is low, and translational activity is confined to maternal messages. Many housekeeping functions may be suppressed, or performed by stored, maternally inherited proteins without *de novo* synthesis. Major changes in the protein synthesis pattern occur during the blastula stage, when embryonic transcription begins, and again during gastrulation, which triggers the onset of biochemical differentiation of different tissues. In comparing the protein synthesis patterns among the various stages, investigators may be seeing the assumption of a greater number of housekeeping functions as well as the process of morphological development. Indeed, synthesis of the structural protein, actin, shows developmental induction at the gastrula stage in amphibians (Brock and Reeves, 1978).

In the experiments with carrot cultures described here, we compare two truly parallel cultures, starting from the same initial population and proceeding through similar rates of growth under chemically similar conditions. The housekeeping requirements of the two cultures are nearly identical; the major difference is the presence of auxin in one culture versus morphogenic development in the other. We therefore feel that the few changes in protein synthesis and accumulation seen here have a high likelihood of having an important association with morphogenesis.

In addition, that so few differences are seen by this analysis is more encouraging than disappointing. Such results focus interest and further research effort on a relatively few proteins of higher potential significance rather than presenting a bewildering array of proteins of questionable significance. Further investigations are under way, scrutinizing the behavior of these proteins in other carrot cultivars and cell lines, including mutant lines defective in embryogenesis (Breton and Sung, 1982; Sung, 1983). These studies are expected to confirm the utility of these embryo-specific proteins as markers of embryogenesis. Still other research efforts will focus on isolating in reasonably pure form sufficient quantities of these proteins for immunological and protein chemistry analyses. We hope, ultimately, to develop molecular and biochemical tools to probe the fundamental questions of morphogenesis and embryonic organization.

References

Ballantine, J.E.M., H.R. Woodland, and E.A. Sturgess (1979). Changes in protein synthesis during the development of *Xenopus laevis*. *J. Embryol. Exp. Morphol. 51:* 137-153.

Breton, A.M. and Z.R. Sung (1982). Temperature-sensitive carrot variants impaired in somatic embryogenesis. *Develop. Biol. 90:* 58-66.

Brock, H.W. and R. Reeves (1978). An investigation of *De Novo* protein synthesis in the South African Clawed Frog, *Xenopus laevis*. *Develop. Biol. 66:* 128-141.

Garrels, J.I. (1979). Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. *J. Biol. Chem. 254:* 7961-7977.

Johnson, K. and D. Hirsch (1979). Patterns of proteins synthesized during development of *Caenorhabditis elegans*. *Develop. Biol. 70:* 241-248.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London) 227:* 680-685.

Loomis, W.F. (1978). The number of developmental genes in *Dictyostelium*. In: *Molecular Basis of Cell-Cell Interaction*, R. Lerner (Ed.), New York, NY: Academic Press, pp. 497-505.

Morrissey, J.H. (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem. 117:* 307-310.

O'Farrell, P.H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem. 250:* 4007-4021.

Smith, J., I. Furner, and Z.R. Sung (1981). Nutritional and karyotypic characterization of a haploid cell culture of *Daucus carota*. *In Vitro 17:* 315-321.

Sung, Z.R. (1976). Turbidimetric determination of plant cell culture growth. *Plant Physiol. 57:* 460-462.

Sung, Z.R. (1983). Applications of two-dimensional gel electrophoresis in studies of gene expression during early plant development. In: *Two-Dimensional Gel Electrophoresis of Proteins*. Celis and Bravo (Eds.), Orlando, FL: Academic Press, pp. 397-413.

Sung, Z.R., J. Horowitz, and R. Smith (1979). Quantitative studies in normal and 5-methyltryptophan resistant cell lines of wild carrots. *Planta 145:* 339-345.

Sung, Z.R. and R. Okimoto (1981). Embryonic proteins in somatic embryos of carrot. *Proc. Natl. Acad. Sci. USA 78:* 3683-3687.

Sung, Z.R. and R. Okimoto (1983). Coordinate gene expression during somatic embryogenesis in carrots. *Proc. Natl. Acad. Sci. USA 80:* 2661-2665.