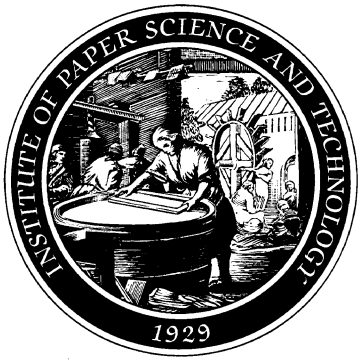

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Differential Display: A Tool to Follow Natural and Somatic Embryo Development in Loblolly Pine

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DIFFERENTIAL DISPLAY: A TOOL TO FOLLOW NATURAL AND SOMATIC EMBRYO DEVELOPMENT IN LOBLOLLY PINE

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ABSTRACT

Somatic Embryogenesis has great potential for clonal production of conifer embryos to meet the increased demands of the Pulp and Paper Industry. The quality of embryos, however needs improvement. The similarities and differences between somatic and zygotic embryos are unclear and, to date, gene expression studies have focused on a few proteins, expressed late in development. We have used the technique of Differential Display to examine gene expression throughout the development of somatic and zygotic embryos. Many similarities and differences in gene expression were uncovered. Seventy seven somatic and over one hundred zygotic cDNA 'bands' have been cloned and their sequences are being determined. This technique is rapid, simple and sensitive and can provide insights into the physiology of conifer embryogenesis.

INTRODUCTION

The US Pulp and Paper Industry has a need to ensure a continuing supply of high quality, low cost fiber. The rapidly growing, and quickly renewable forests and plantations in South America and Southeast Asia provide an economic challenge to the industry and a spur to scientists investigating factors affecting growth and production.

Somatic Embryogenesis, a technique for generating plant embryos from a piece of donor tissue, has great potential for meeting the fiber demands of the Pulp and Paper Industry (1,2,3). Somatic Embryogenesis may be used to produce millions of clonal copies of superior trees which have been selected in the field for qualities such as growth rate, hardiness, density or form. The technique may be applied to the propagation of rare, exotic or environmentally sensitive trees. The cryopreservation of somatic embryos and their subsequent revival permits flexibility in the timing of their deployment.

Somatic Embryogenesis in conifers, however, has its' limitations; embryogenesis is very dependent on genotype (3) and even with the best embryogenic material, the quality of embryos, their vigor and form, is poor compared to zygotic embryos (2, 4). Improvement in Somatic Embryo quality has been the goal of a research program at IPST for over a decade.

Somatic Embryogenesis can be divided into four stages;
1. *Initiation*, which occurs most efficiently where conifer seeds provide immature embryos as a starting tissue.
2. *Multiplication*, in liquid culture, where embryos may grow and multiply 2-6 times per week. 3. *Maturation*, on solid media in the presence of plant hormones and osmoticum.
4. *Germination* to produce plantlets which can be transferred to soil and ultimately can be planted in the field.

It has been estimated that 30 000 different genes are involved in plant embryo development (5,6). The process of Somatic Embryogenesis seeks to mimic the natural development of zygotic embryos, that is, to permit the natural program of gene expression to occur *in vitro*. The differences in the quality of somatic and zygotic embryos attests to shortcomings in laboratory culture methods, however the degree to which somatic and zygotic embryos are different, the points at which their development begins to differ and the nature of these critical developmental differences are largely unknown. Monitoring and comparing gene expression during the development of somatic and zygotic conifer embryos would provide invaluable insights into their differing physiologies. Identification of genes whose expression differed in somatic and zygotic embryos, would allow inferences to be drawn about their respective biochemistry and from this understanding, tissue culture conditions to be modified in an informed manner.

To date, the principal indices of embryo development have been morphology, size and weight. Monitoring biochemical changes in conifer embryos has been restricted by the limited quantity of embryo material available and has focused on the accumulation and deposition of lipids (7) and of a handful of seed storage proteins which are expressed late, during the maturation phase of embryogenesis (8,9).

In this paper we report the use of Differential Display to monitor gene expression in somatic and zygotic embryos. We describe a technique which we have developed which allows us to follow the expression of hundreds of genes throughout the course of development using very small amounts of tissue, in some cases a single embryo. Clear differences in gene expression between somatic and zygotic embryos can be seen and over 100 cDNAs have been cloned for further analysis.

MATERIALS AND METHODS

Plant Materials

Zygotic Embryos: Loblolly pine (*Pinus taeda*) cones were collected weekly from Boise Cascade's breeding orchard near Lake Charles, Louisiana. Cones from tree BC-1 were shipped on ice to IPST and received within 24-48 hours of collection. Cones were opened and seeds collected for isolation of embryos. Seeds were cracked using a hemostat, pried open with the aid of a scalpel, and the integument and nucellus tissue removed from the ovule. The female gametophyte was slit, pried open and the dominant embryo or mass or embryos removed. Embryos were quickly observed through a dissecting microscope and evaluated for stage (4). Stage 9 embryos were also categorized by the week they were collected; 9.1 (Stage 9, week 1) 9.2 (Stage 9, week 2) etc. Staged zygotic embryos were then placed in a cryostorage vial partially emersed in liquid nitrogen. Twenty similar staged embryos were collected per vial. Frozen embryos were stored at -70 °C until analyses were performed.

Somatic Embryo Development: Cultures of somatic embryos for loblolly pine were initiated as described by Becwar et al. (10) or with modifications in media mineral composition. Somatic embryos were grown in cell suspension culture medium 16 and maturation medium 240 (4). Resulting somatic embryos were selected, staged (4), and sorted into vials containing the same stage. Somatic embryos were stored at -70 °C until analyses were performed.

Differential Display

Poly(A) RNA was isolated from embryos using mRNA Direct kit (Dynal) according to manufacturer's instructions with modifications. Frozen embryos, 20 µg in each tube, were ground in 200 µl of the lysis/binding buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM EDTA, 1% SDS, 5 mM DTT, pH 8.0) with a plastic pellet pestle (VWR) powered by a cordless drill (SKIL 2236). The lysate was centrifuged for 15 min. at 14000 rpm at 4°C to remove the debris. The oligo(dT) beads were fully suspended and 8 µl was taken for each embryo sample. The beads were washed twice with the lysis/binding buffer on a magnetic stand (Promega), suspended in 200 µl of the same buffer and mixed with the clear embryo lysate. The mixture was left on ice for 5 min. and then on a magnetic stand at room temperature for 3 min.. The buffer was removed by a pipettor. The beads were suspended in 100 µl washing buffer and transferred to a 200 µl PCR tube. The beads were then washed, using the magnetic stand, twice with washing buffer, once with washing buffer without LiSD and once with 1x RT buffer (25 mM Tris-Cl, 38 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, pH 8.3).

Nineteen µl RT reaction mixture (1x RT buffer, 20 mM dNTP) was added to the washed poly(A)-RNA-beads. The reaction was heated to 65°C for 10 min., cooled on ice, and 200 units of MMLV reverse transcriptase (Promega) was added. The RT was performed at 37°C for 1 h. and the reaction was heated to 95°C for 4 min. storage at -20°C.

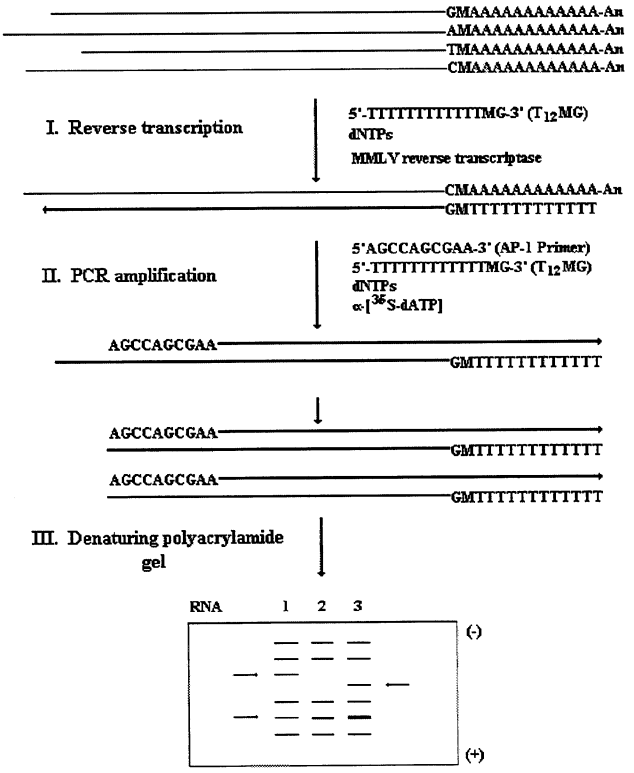

Figure 1. Principle of Differential Display. (Adapted from Genhunter Corporation, 1994). The upper four lines represent mRNA molecules with different ribonucleotides adjacent to the poly A tail. Reverse transcription of RNA is conducted using an anchored primer which hybridizes to a subset of the mRNA, selected on the basis of the terminal two ribonucleotides.

Differential display PCR was performed in a 20 µl reaction containing 1x PCR buffer (Perkin Elmer), 2 µM dNTP, 1 mM T_{12}MN anchored primer, 0.2 mM arbitrary primer (10-mer), 0.5 units of *Taq* DNA polymerase and 1 µl a^{35}S-dATP (Amersham). Cycling conditions were 94°C for 30 sec., 40°C for 1 min., 72°C for 2 min., 40 cycles. The PCR products were separated on pre-cast sequencing gel (Stratagene). The gels were rinsed with water and dried. Dilute ^{35}S-dATP with ink was spotted at the corners as alignment markers. The gels were then exposed to BioMax films overnight.

Cloning of DNA Fragments from Differential Display

The bands of interest were identified and marked on the X-ray film. The film was aligned with the markers on the gel. The gel underneath marked bands was cut with a scalpel blade and the cut region was picked up after applying 0.5 ml of water. The gel fragments were used directly in PCR to amplify the cDNA. The PCR condition was similar to that of RT-PCR. The only changes were that the α^{35}S-dATP was omitted and that the concentration of dNTP was 200 µM. The PCR products were purified with Chroma Spin-100 columns (CLONTECH) and ligated with pCR2.1 vector (Invitrogen). After transformation in DH5a *E. coli*, 5 white colonies for each construct were checked for the size of insert DNA by PCR using M13R and M13F primers. Three clones that contained plasmid DNA with a insert of the estimated size of the original differential display band were further examined by digesting the colony PCR products with restriction enzymes MseI and Nla3. The clones that yielded different digestion patterns were selected and their plasmid DNA extracted using Wizard plus SV Miniprep (Promega). The insert DNA was sequenced at the DNA Core Facility, University of Missouri-Columbia. Sequencing data was managed by a software named CSE (**C**loned **S**equence **E**ditor) developed in our lab.

Southern Blotting

PCR was performed using the Miniprep DNA to amplify the insert region. Five µg of PCR product in 15 µl were mixed with 3.3 µl 3.0 M NaOH and incubated at 65°C for 30'. After cooling to room temperature and mixing with 20.5 µl 20xSSPE, 1 µl was spotted on duplicate membranes (Hybond-N+, Amersham). The DNA was cross-linked to the membrane in a CL-1000 UV-linker (Upland, CA) for 2 min., pre-hybridized with hybridization buffer (0.5 M Na-phosphate, pH7.2, 5% SDS and 10 mM EDTA) at 65°C for 30 min. Probes were made by PCR using the RT as templates. The
50 µl PCR reaction mix contained 1x PCR buffer (Perkin-Elmer), 5 µl dATP+dGTP+dTTP (5 mM each), 5 µl T12MN (0.04 µM each), 5 µl AP primer (2 µM), 2 ml template (RT-beads), 2 unit *Taq*, and 5 µl; ^{32}P-dCTP (Amersham). The cycle conditions were the same as that for differential display. The PCR probes were purified using NICK column (Pharmacia,) according to manufactures instructions. Hybridization was performed in 3 ml hybridization buffer in a hybridization oven at 65°C overnight. The membrane was then washed three times with 0.1x hybridization buffer at room temperature for 10 min. and once at 42°C for 30 min.

RESULTS AND DISCUSION

Differential Display is a Reverse Transcription - PCR (RT-PCR) technique which permits the comparison of different mRNA populations without the need for cloning. An outline of the technique is given in Figure 1 and details may be found in the original papers (11,12). Due to its simplicity and sensitivity this technique has found application in many field where gene expression differences between developmental stages or growth conditions are being studied (13,14). We have modified this procedure for use with small amounts of material. Tissue is ground and mRNA is isolated in the presence of magnetic beads to which oligo-dT is attached. These beads abstract the polyA mRNA from the cell homogenate. Washing of the beads removes cell debris, the beads, to which mRNA remains bound, are then used for RT-PCR directly (see Material and Methods). This technique circumvents problems associated with loss of RNA using conventional extraction methods (15).

Using small amount of Somatic Embryo material, a clear, reproducible set of bands is produced. When Differential Display reactions generated from Somatic Embryos of different stages are run side by side on a gel, we can observe the appearance and disappearance of mRNA species over the course of development (Fig.2). The activity of Early Expressed, Late Expressed, Stage Specific and Consituitively Expressed genes are apparent. By aligning the X-ray film with the original gel, bands can be located and subsequently isolated and cloned (see Material and Methods). To date we have isolated 77 bands which show differential expression on gels. Since many bands isolated are heterogeneous we classify cloned bands by restriction digest (Material and Methods). A single isolated band may generate several classes of clone (See Fig.3), only one of which may be a copy of a differentially expressed mRNA. To confirm the differential expression of the cloned bands, we spot a small amount of these cDNAs on membranes and hybridize them with a probe generated by asymmetric PCR (Fig. 3 and Material and Methods). False positives (e.g. band 'A', Fig. 3) are identified and excluded from further analysis. Clones of differentially expressed mRNA are sequenced. To date we have sequenced over 80 cDNAs and this number is increasing. Many of these cDNAs do not correspond to a sequence in the GenBank, perhaps because they represent mainly 3'UTR sequence, which is poorly conserved between species or perhaps because they are newly identified genes. Some positive identifications have been made however. Clone lps038 has 75% amino acid identity to Elongation Factor 2 (Xu and Cairney unpublished).

Differential Display has been used to survey gene expression in Zygotic Embryos of equivalent stages. Figure 4 shows 'Early', 'Late', 'Constituitive' and Stage-specific gene expression. Comparison of Zygotic and Somatic profiles shows similarities and differences between the somatic and zygotic patterns of gene expression. Bands, representing mRNAs, which differ between somatic and zygotic embryos have been cloned, analyzed and sequenced. Once more, many bands have no matches in the GenBank however some strong similarities to kinases, GTP-binding proteins, ribosomal proteins and storage proteins have been found (Xu and Cairney unpublished).

Limitations of Differential Display and Solutions

Differential Display is a very promising technique to examine gene expression in conifer embryos, however there are limitations on the inferences which can be drawn from the results.

a) Differential display indicates the presence of a particular mRNA but it does not reveal whether or not this mRNA is being translated. Several labs have shown that certain mRNAs in plant embryos are under translational control; the mRNA for 2S, 7S and 11S storage proteins are present early in development although these proteins are not being synthesized at this time (16). Similarly, heat shock protein transcripts are not translated in carrot callus, although mRNA is abundant, but are translated in globular stage embryos, although heat shock mRNA is scarce (17). Recently, the developmentally regulated distribution of transcripts between ribonucleoprotein and polysomal fractions have been observed in carrot embryos for a number of other mRNAs (18). This notwithstanding, Differential Display allows identification of mRNA species whose regulation can be studied in greater detail with subsequent isolation of the cDNA clones. The production of protein varies, to a large degree, with the mRNA concentration, changes in mRNA synthesis and degradation determining protein synthesis. The profile of expression generated by Differential Display is a tool for study which has not be available to scientists studying conifer embryogenesis. Whichever mode of gene regulation is operating, the technique offers new possibilities for their study.

b) Primers used in Differential Display are short and specific for certain sequences, thus their use to compare gene expression in different genotypes may be limited. Certainly this is true and some of the bands present in one genotype and absent in another may be due, not to the absence of an mRNA, but to minor sequence differences which prevent short primers from annealing. This problem can be overcome by using cloned cDNAs, isolated through Differential Display, to probe gene expression by RNase protection assay and/or designing longer primers, based on the sequence of the cloned cDNAs which will amplify despite minor mismatches in the primer annealing sites (Johns and Cairney unpublished).

c) Susceptibility of PCR to artifact. This problem is addressed in our approach by confirming the identity of cloned bands by Southern (see Fig. 3) and Northern or Ribonuclease Protection Assay.

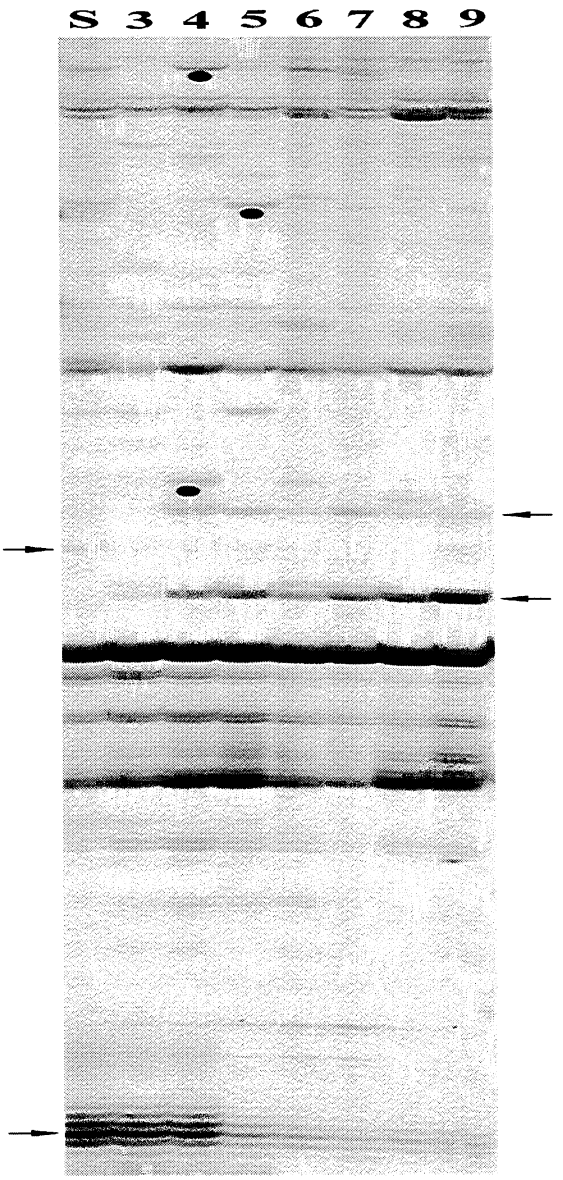

Figure 2. Autoradiograph (X-ray image) of typical RNA differential display of somatic embryos of loblolly pine. Poly(A) RNA was extracted from somatic embryos at suspension stage (S, including stage 1 and 2), stages 3 to 9 (numbers on top of the lanes). Differential display was

performed as described in Materials and Methods, using T12VG and 5'-GGTGCGATCC-3' primers. Each 'band' on the gel indicates the presence of a specific mRNA. Right arrows indicate mRNA species present at early stages; left arrows, late stages and black dots, one or two specific stages.

CONCLUSION AND PROSPECTS

The cloning of genes expressed during embryo development can shed great light on the process. Most molecular biology research has been conducted with angiosperms.
The inaccessibility of early stage embryos has led to the adoption of somatic embryos as a model system for studying zygotic embryo development (18). In carrots, 'conditioned media' from somatic embryo cultures can promote somatic embryogenesis (20,21). This observation has led to the identification and cloning of genes for extracellular proteins responsible for some of this activity. The extracellular protein EP3 can rescue the temperature-sensitive carrot mutant cell line, allowing it to proceed through development at a normally, non-permissive temperature. EP3 has since been shown to be a glycosylated acidic endochitinase (22). Protein EP2 is a Lipid Transfer Protein. Cloning and expression analysis has shown that EP2 is restricted to the protoderm of both somatic and zygotic embryos. Thus, although the expression of EP2 is not specific to embryos, it is a very useful marker for the establishment of the epidermal layer (23). Although Somatic Embryos differ from Zygotic Embryos in the Carrot system, the analysis of gene expression and cloning of genes associated with particular genes expressed at different times of development has produced useful markers for following development in both somatic and zygotic embryos (18,19).

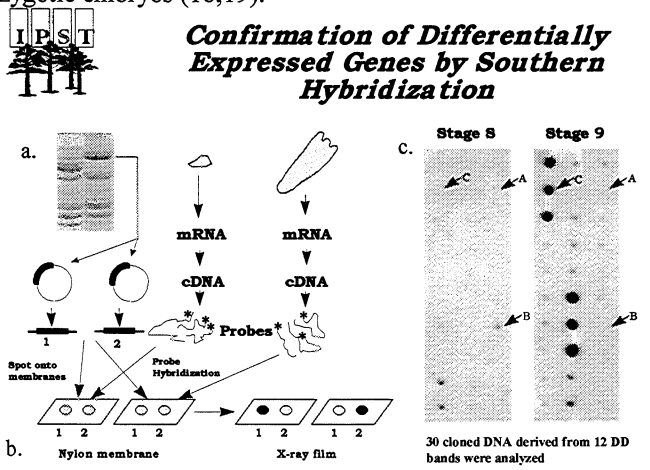

Figure 3. "Southern Blotting" of cDNA clones using probes derived from Early and Late Stage Embryos. a) A band of interest is cloned. b) Clones are spotted onto membranes and probes prepared by Asymmetric PCR as described in Materials and Methods. c) X-ray film of the membranes. Arrow 'A'; false positive, Arrow 'B', cDNA clone of mRNA present Early in Development; Arrow 'C', cDNA clone of mRNA present Late in Development.

Genes expressed late in development, particularly Late Embryogenesis Abundant (LEA) proteins have received much attention, both in terms of the structure of the protein (24) and the regulation of gene expression (6). The expression of Storage Proteins has been followed in maturing embryos (25). In both cases the proteins are fairly abundant and their expression is late in development. While studies with these proteins has been illuminating, the early events in development remain shrouded. In the work reported here, we have shown that the activity of developmentally regulated genes can be revealed by Differential Display. This technique can provide an expression profile for comparison of somatic and zygotic embryos, or somatic and zygotic embryos of different genotypes. The reproducibility of healthy development in different preparations culture media can be assessed, thus Differential Display could be employed as a 'Quality Control' technique in Tissue Culture Laboratories. The cloning and sequencing of cDNAs generated in Differential Display will allow physiological insights as the appearance and disappearance of identifiable mRNA (and in further analysis protein) can be followed. *In situ* hybridization to mRNA in embryo sections, using cDNA probes generated by Differential Display, will refine our understanding of developmentally regulated gene expression in conifers. One further, related application is the generation of protein markers to follow development, through *in vitro* translation of mRNA from cloned cDNAs and the subsequent generation of antibodies which may be used to follow the appearance of surface markers by in situ Western analysis.

In summary the technique of Differential Display offers unique possibilities for understanding and improving the process of Somatic Embryogenesis in conifers.

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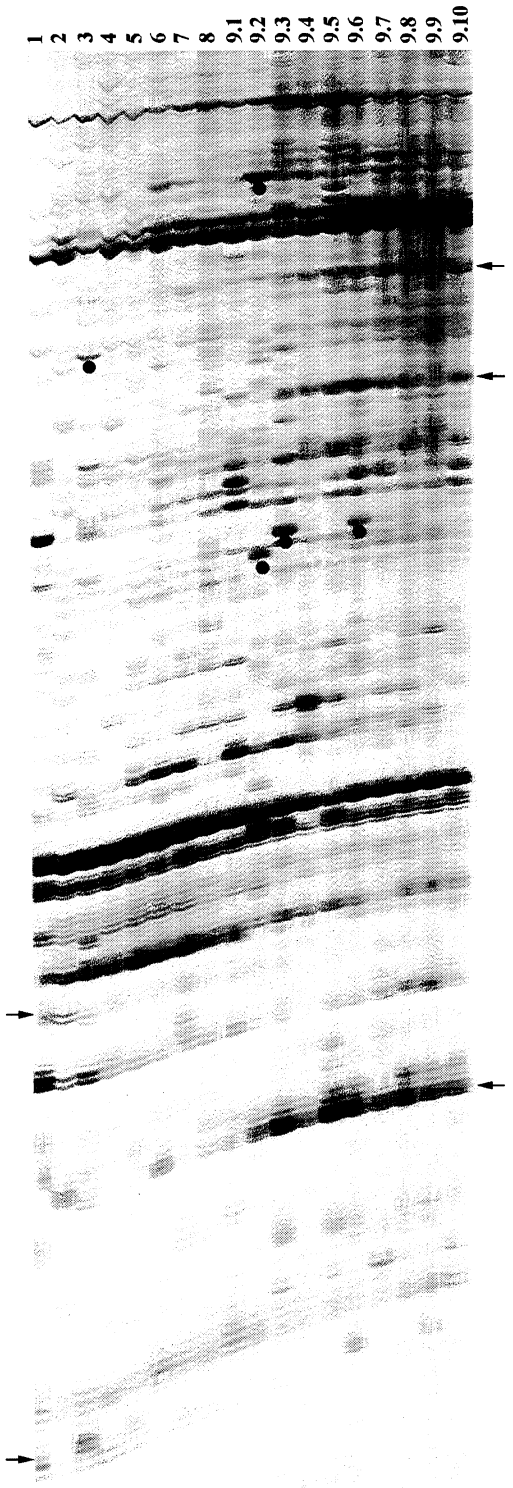

Figure 4. A differential display pattern of zygotic embryos at different developmental stages. Poly(A) RNA was extracted from zygotic embryos from stage 1 to stage 9.10 (numbers on top of the lanes). Differential display was performed as described in Materials and Methods, using T12VG and 5'-GGTGCGATCC-3' primers. Right arrows indicate mRNA species present at early stages; left arrows, late stages and black dots, one or two specific stages.

REFERENCES

1. Tautorus, T.E., Fowke, L.C., and Dustan, D.I., *Can. J. Bot.* "Somatic Embryogenesis in Conifers" 69: 1873-1899 (1991)

2. Attree, S.M. and Fowke, L.C., *Plant Cell Tiss. Org. Cult.* "Embryogeny of gymnosperms: advances in synthetic seed technology of conifers" 35: 1-35 (1993)

3. Handley, L.W., Becwar, M.R., Chesick, E.E., Coke, J.E., Godbey, A.P., and Rutter, M.R., *TAPPI R&D Division Biological Sciences Symposium, October 3-6, 1994, Minneapolis, Minnesota.* "Research and Development of Commercial Tissue Culture Systems in Loblolly Pine, *Pinus taeda* L." Pages 47-55 (1994)

4. Pullman, G. S. and D. T. Webb. *TAPPI R&D Division Biological Sciences Symposium, October 3-6, 1994, Minneapolis, Minnesota.* An embryo staging system for comparison of zygotic and somatic embryo development. Pages 31-34. (1994).

5. Okamuro, J.K. and Goldberg, R.B., "Regulation of Plant Gene Expression: General Principles" in *The Biochemistry of Plants*, Academic Press, Inc., Vol 15, p2- 82 (1989)

6. Thomas, T.L., *Plant Cell*, "Gene Expression during Plant Embryogenesis and Germination: An Overview" 5:1401-1410 (1993)

7. Flinn, B.S., Roberts, D.R., and Taylor, I.E.P. *Physiol. Plant.* "Evaluation of somatic embryos for interior spruce. Characterization and developmental regulation of storage proteins" 82:624-632 (1991)

8. Attree, S.M., Pomeroy, M.K. and Fowke, L.C., *Planta*, "Manipulation of conditions for the culture of somatic embryos of white spruce for improved triacylglycerol biosynthesis and desiccation tolerance. 187:395-404 (1992)

9. Hakman, I. Physiol. Plant. "Embryology in Norway Spruce (*Picea abies*). An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. 87:148-159 (1993).

10. Becwar, M. R., Nagmani, R., and Wann, S. R. *Can J For Res.* "Initiation of embryogenic cultures and somatic embryo development in loblolly pine (Pinus taeda)," 20: 810 (1990).

11. Liang, P. and Pardee, A.B. *Science*, "Differential display of eukaryotic messenger RNA by means of the Polymerase Chain Reaction", 257:967-971

12. Corton, J.C., and Gustafsson, J.A., *Biotechniques*, "Increased efficiency in screening large numbers of cDNA fragments generated by differential display" (1997)

13. Adati, N., Takashi, I., Koga, C., Kito, K., Sakaki, Y., and Shiokawa, K., *Biochim. Biophys. Acta,* "Differential display analysis of gene expression in developing embryos of *Xenopus laevis*", 1262:43-51 (1995).

14. Benito, E .P., Prins, T., and van Kan, J.A.L. *Plant Mol. Biol.*, "Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction" (1996)

15. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloninng: A laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

16. Pramanik, S.K., Krochko, J.E., and Bewley, J.D., *Plant Physiol.*, "Distribution of Cytosolic mRNAs Between Polysomal and Ribonucleoprotein Complex Fractions in Alfalfa Embryos: Stage-specific Translational Repression of Storage Protein Synthesis during Early Somatic Embryos Development" 99:1590-1596 (1992)

17. Apuya, N.R., and Zimmerman, J.L., Plant Cell, "Heat Shock Gene Expression Is Controlled Primarily at the Translational Level in Carrot Cells and Somatic Embryos" 4: 657-665 (1992).

18. Lin, X. Hwang, G-J. H., Zimmerman, J.L., *Plant Physiol.*, "Isolation and Characterization of a Diverse Set of Genes from Carrot Somatic Embryos", 112: 1365-1374 (1996).

19. Zimmerman, J.L. *Plant Cell*, "Somatic Embryogenesis: A model for Early Development in Higher Plants", 5:1411-1423 (1993).

20. Hari, V., *Z. Pflanzenphysiol.* "Effect of cell density changes and conditioned media of carrot cell embryogenesis" 96:227-231 (1980)

21. Smith, J.A., and Sung, Z.R. "Increase in regeneration of plant cells by cross feeding with regeneration *Daucus carota* cells" in Somatic Embryogenesis, M. Terze, L. Pitto and S.R. Sung, eds (Rome:IPRA) pp133-136 (1985).

22. De Jong, A.K., Cordewener, J., Lo Schiavo, F. Terzi, M., Vandederckhove, J., Van Dammen, A., and De Vries, S.C. Plant Cell, "A carrot somatic embryo mutant is rescued by chitinase" 4:425-433 (1992).

23. Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C., *Plant Cell* "Cell-specific expression of the carrot EP2 lipid transfer protein gene", 3:907-921 (1991).

24. Close, T. J. *Physiol. Plant.*, Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins.. 97:745-803.(1996).

25. Bewley, J.D., *TAPPI R&D Division Biological Sciences Symposium, October 3-6, 1994, Minneapolis, Minnesota.*, "Regultory events in Somati Embryogenesis - Transcription and Translation" Pages 132-137 (1994).