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Natural and Somatic Embryo Development in Loblolly Pine

Gene Expression Studies Using Differential Display and DNA Arrays

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Abstract

In production biological systems, monitoring and controlling the growth environment is possible, but assessing the metabolic competency of the organism is more difficult. Somatic embryogenesis (SE), a tissue-culture method for multiplying embryos asexually, has great potential to capture at low cost the genetic gain from breeding and genetic-engineering programs. Loblolly pine, however, has proven recalcitrant in the production of somatic embryos suitable in quality for operational use. Many similarities and differences in gene expression were uncovered. We have modified a recent technique called differential display (DD) to examine gene expression, allowing the comparison of somatic and zygotic embryos. Over 400 cDNA "bands" have been cloned and their sequences determined. These bands can serve as "expression markers," providing rapid, simple, and sensitive assessment of embryo physiology and development. These techniques are applicable to many areas of research where monitoring specific levels of gene expression is important for evaluating the performance of a system.

Index Entries: Differential display; zygotic embryogenesis; somatic embryogenesis; loblolly pine.

Introduction

Determining conditions that promote growth of an organism and/or production of a desirable end-product is greatly facilitated by knowing the metabolic state of that organism. Online data permits flexibility and refinement of processes. With biological systems, controlling the environment with regard to pH, temperature, or substrate supply is possible, but the metabolic competency of the organism is more difficult to assess. Improvement protocols are lengthy and expensive. Here we describe a series of

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molecular techniques for monitoring gene expression during growth. While dealing with embryo development in loblolly pine *(Pinus taeda)*, these techniques are simple and swift and can be applied in many systems as a quality-control measure and can be extended to provide information for process improvement.

Embryogenesis is a complex developmental pathway that plays a pivotal role in the life cycle of higher plants. Development of embryos within seeds has been studied extensively in both angiosperms and gymnosperms, the value of this knowledge has direct impact on agriculture and forestry practices.

In contrast to zygotic embryogenesis (ZE), somatic embryogenesis (SE) is a technique for clonally producing embryos asexually in tissue culture. Scientifically, somatic embryogenesis can be a tool for the study of development *(1,2)*; practically, it can be a means of generating genetic diversity and propagating desirable genotypes *(3,4)*. This latter application of somatic embryogenesis is particularly important for the U.S. Forest Products Industry. Forest species have long generation times and propagation of high-value genotypes is difficult. Somatic embryogenesis has great potential for clonally multiplying valuable genotypes generated in tree-improvement programs or produced by genetic engineering *(4–6)*.

Large-scale production of embryos for operational use is occurring for some spruce and firs *(4)*; however, the process is very species- and genotype-dependent *(3,7)*. For loblolly pine, the most important commercial softwood in the Southern United States, the process is very inefficient and embryo quality is unsuitable for operational use. Nevertheless, many forest-products companies in the U.S. and the rest of the world, recognizing that SE will be a key feature of future tree-improvement programs, have intensive research programs in SE. Few laboratories have reported capabilities to generate loblolly pine plantlets through somatic embryogenesis *(8,9)*. A refined staging system that discriminates more clearly between the developmental stages of pine embryos has been developed *(8)*. This staging system allows improved accuracy in the evaluation of SE. Comparisons between loblolly pine zygotic and somatic embryos have indicated that somatic embryos did not develop beyond stage eight *(8)*. In order to speed improvement of somatic embryo quality, a new approach was needed.

In embryogenesis, the changes during development are subtle and difficult to observe visually. However, molecular events are dramatic and conspicuous. A fundamental understanding of the gene-expression patterns during zygotic-embryo development would allow improved comparison and monitoring of somatic and zygotic embryos. In turn, it was speculated that identification of normal and abnormal gene activity would provide data-based hypotheses for somatic embryo improvement. It has been estimated that 20,000 genes may be expressed during plant embryogenesis *(10)*. Many of these genes may be duplicated or nonessential. Jürgens et al. *(11)* estimated that 4000 genes may be essential for embryogenesis in *Arabidopsis*. Meinke *(12)* predicted only 500 genes that may be

identified in *Arabidopsis* through embryonic lethal and defective mutations. The genes that are active early in development are largely unknown, and to date sensitive methods for analysis have not been available.

Differential screening to isolate early embryo-specific genes would greatly facilitate the identification and study of genes important to early embryogenesis, but the inaccessibility and the small size of early-stage zygotic embryos so far have prevented such an endeavor. Studying the differences in somatic and zygotic embryos can illuminate developmental pathways. Although differences occur, somatic embryos generally parallel zygotic embryos in development. The manipulation of tissue-culture systems can provide insight into the regulation of development. For example, hormones may elicit gene expression and cause progress down a developmental path. In model angiosperm plants such as *Arabidopsis*, recent mutation studies have identified genes essential for embryogenesis, a number of which have been cloned and identified *(2,13)*. Gymnosperm embryogenesis, although resembling angiosperms in many ways, represents a distinct and important developmental scheme *(3,14)*. Work on gymnosperm embryogenesis has employed microscopy, biochemical, genetic, and tissue-culture tools to elucidate pathways. Although the genetic studies such as those conducted with *Arabidopsis* and maize are not possible in conifers owing to the long generation times, our recent advances are providing information on gene expression during embryogenesis of pines that will be applicable to gymnosperms and angiosperms.

Most gene-expression studies have been limited by the small quantities of material available and the insensitivity of techniques, and thus have focused on a few highly expressed genes, active late in development *(15,16)*. The earliest phases of development were inaccessible for this type of work; however, recent work using somatic embryos from carrot has provided a broader range of genes for analysis *(17)*. We have recently initiated a program to identify and clone genes expressed at every stage of embryo development in loblolly pine. By modifying differential display (DD) protocols, we have succeeded in cloning over 400 cDNAs for genes expressed at early, mid, or late stages of loblolly pine embryo development *(18,19)*. These protocols allow us to perform DD with a single pine embryo *(18)* and to examine the expression pattern of a broad spectrum of genes during zygotic or somatic embryo development. In addition, crucial genes active at specific points in embryo development can be identified, isolated, and studied.

Methods

Plant Materials

Zygotic Loblolly Pine Embryos

Loblolly pine cones were collected weekly from Boise Cascade's breeding orchard near Lake Charles, LA, and shipped on ice to IPST. 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 of embryos removed. Embryos were quickly observed through a dissecting microscope and evaluated for stage *(8)*. Stage 9 embryos were also categorized by the week they were collected: 9.1 (Stage 9, wk 1); 9.2 (Stage 9, wk 2); and so forth. Staged zygotic embryos were then placed in a cryostorage vial partially immersed in liquid nitrogen. Twenty similar-staged embryos were collected per vial. Frozen embryos were stored at –70°C until analyses were performed.

Somatic Loblolly Pine Embryos

Somatic embryos were collected at different stages of development. Cultures of somatic embryos for loblolly pine were initiated as described by Becwar et al. *(9)* or with modifications in media mineral composition. Somatic embryos were grown in cell-suspension culture medium 16 and maturation medium 240 *(8)*. Resulting somatic embryos were selected, staged *(8)*, and sorted into vials containing the same stage. Somatic embryos were stored at –70°C.

Differential Display

DD was performed according to Xu et al. *(18)*. Poly(A) RNA was isolated from embryos using mRNA Direct kit (Dynal) according to manufacturer's instructions with modifications. Frozen embryos, 5–100 mg in each tube, were ground in 200 µL of the lysis/binding buffer (100 m*M* Tris-HCl, 500 m*M* LiCl, 10 m*M* ethylenediamenetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), 5 m*M* dithiothreitol (DTT), pH 8.0) with a plastic pellet pestle (VWR, Bridgeport, NJ) powered by a cordless drill (SKIL 2236). The lysate was centrifuged for 15 min at 14,000 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, Madison, WI), 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. 100 µL of Washing Buffer containing LiDS (mRNA DIRECT kit, LiDS contains 100 m*M* Tris-HCl, pH 8.0, 0.15 m*M* LiCl, 1.0 m*M* EDTA, and 0.1% SDS) was added and the mix was transferred to a 200-µL polymerase chain reaction (PCR) tube. The beads were then washed once with 100 µL washing buffer with LiDS and once with 50 µL Washing Buffer (mRNA DIRECT kit, containing 100 m*M* Tris-HCl, pH 8.0, 0.15 m*M* LiCl, and 1.0 m*M* EDTA), then finally with 1X reverse transcription (RT) buffer (25 m*M* Tris-Cl, 38 m*M* KCl, 1.5 m*M* $MgCl_2$, 5 m*M* DTT, pH 8.3).

Nineteen microliters RT reaction mixture (1X RT buffer, 20 m*M* 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 U of MMLV reverse transcriptase

(Promega) were added. The RT was performed at 37°C for 1 h and the reaction was heated to 95°C for 4 min and stored at –20°C.

DD-PCR was performed in a 20 µL reaction containing 1X PCR buffer (Perkin Elmer, Norwalk, CT), 2 µ*M* dNTP, 1 m*M* T_{12}MN anchored primer, 0.2 m*M* arbitrary primer (10-mer), 0.5 U of *Taq* DNA polymerase and 1 µL a^{35}S-dATP (Amersham, Arlington Heights, IL). Cycling conditions were 94°C for 30 s, 40°C for 1 min, 72°C for 2 min, 40 cycles. The PCR products were separated on precast sequencing gel (Stratagene, La Jolla, CA). 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 DD

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, containing marked bands, was cut with a scalpel blade and the cut region was picked up after applying 0.5 µL 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 µ*M*. The PCR products were purified with Chroma Spin-100 columns (Clontech) and ligated with pCR2.1 vector (Invitrogen, Carlsbad, CA). 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 an insert of the estimated size of the original DD band were further examined by digesting the colony PCR products with restriction enzymes Mse1 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 (Cloned Sequence Editor) 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 min. After cooling to room temperature, 20.5 µL 20X SSPE (containing 5% gel-loading dye) the denatured DNAs were then blotted onto Hybond N+ membranes (Amersham) as arrays using a VP 386 pin blotter (V&P Scientific, Inc., San Diego, CA). Each DNA was dot-blotted four times as a quadrate on the membrane. The dots are ~1.2 mm in diameter and each of them contains about 30 ng DNA. The DNA was then cross-linked to the membrane at 120,000 mJ/cm2 in a CL-1000 UV-linker (Upland, CA) prehybridized with hybridization buffer (0.5 *M* Na-phosphate, pH 7.2, 5% SDS, and 10 m*M* EDTA) at 65°C for 30 min. Probes were made by Advantage PCR (Clontech, Palo Alto, CA) using the

first strand cDNAs generated by SMART cDNA synthesis kit (Clontech) as templates. The 50 µL PCR reaction mix contained 1X PCR buffer, 5 µL dATP+ dGTP+dTTP (5 m*M* each), 2-µL PCR primers (10 µ*M*), 5 µL template (first strand cDNA), 1 µL KlenTag enzyme mix and 5 µL ^{32}P-dCTP (Amersham). The cycle conditions were 94°C 2 min, 15 cycles of 95°C for 15 s, 52°C for 30 s, 68°C for 6 min. The PCR products were purified using NICK column (Pharmacia) according to the manufacturer's 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. The autoradiograph images were scanned and digitized using GelPro 3.0 (Media Cybernetics, Silver Springs, MD).

Results

Mass Isolation of Genes Differentially Expressed in Loblolly Pine Zygotic Embryos

We have developed an RNA DD method *(18,19)* that is sensitive enough to produce more than 30 different DD banding patterns using as little as one embryo at mid- to late-stages of development. Similar results can be obtained from just a few early-stage embryos. This technique, which extracts mRNA directly from tissue homogenate using oligo(dT) beads, avoiding losses inherent in conventional RNA extraction methods, is fast, reliable, and low-cost. In the experiment shown in Fig. 1, DD is performed using RNA from somatic embryos (Genotype 260) and for comparison with RNA from zygotic embryos from tree BC-1. Genotype 260 is an embryogenic culture from a single loblolly pine seed. Staged embryos from this culture are presumed to all be genetically identical. BC-1 is a wind-pollinated tree that produces cones containing seeds all from the same mother, but from different and unknown pollen parents. Thus, a vial of 20 somatic embryos would produce banding patterns representing one genotype, whereas a vial of 20 BC-1 embryos would produce banding patterns representing 20 highly similar genotypes. Many mRNAs (indicated by bands) are present in both somatic and zygotic embryos; however, differences in gene expression at different stages of embryo development, as well as between somatic and zygotic embryos, can be easily detected.

Powered with this new technique, we have used more than 20 sets of primer pairs to perform DD of loblolly pine zygotic embryos at all developmental stages. We have produced more than 600 DD patterns that showed a total of more than 60,000 bands. More than 600 bands were identified that showed a changing pattern at different stages of embryo development. We have attempted to clone cDNAs from all these bands, and have isolated 417 cDNA clones. Their sequences were determined. To our knowledge, this is the largest collection of cDNAs related to plant embryogenesis.

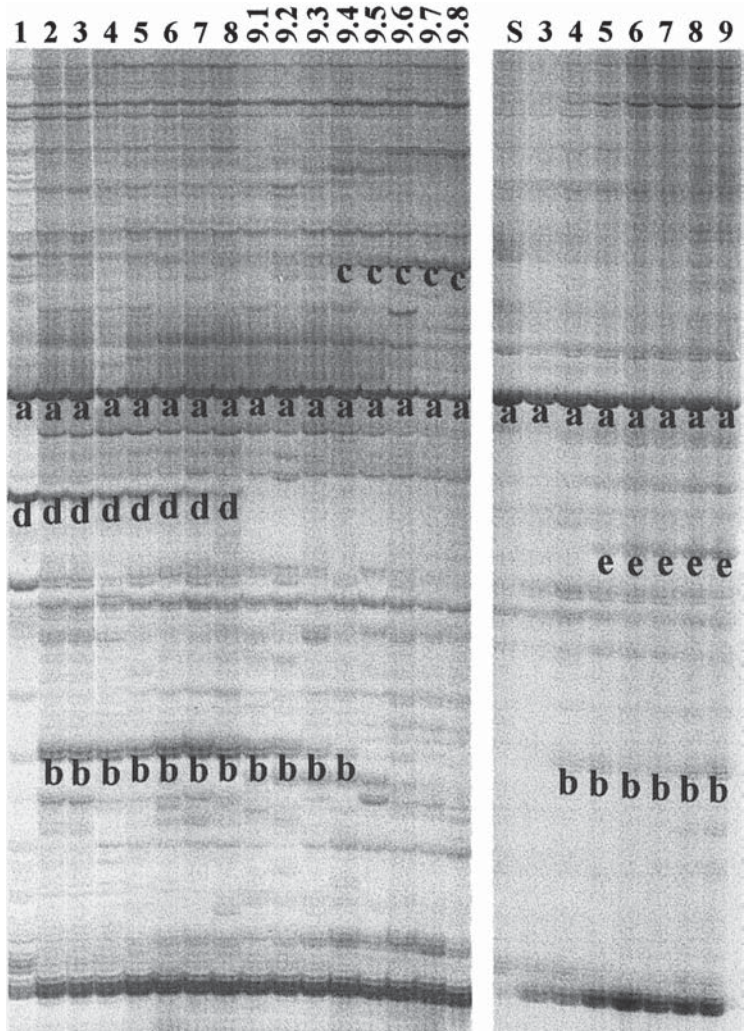


Fig. 1. Differential display of loblolly pine zygotic and somatic embryos at different stages of development. The zygotic embryos (left panel) used were from tree BC-1 and the somatic embryos (right panel) are of genotype 260. Primer pair T12VC-AP3 (GenHunter, Nashville, TN) were used in the PCR reactions. The numbers on the top of the lanes indicate the stages of the embryos used. The letters superimposed on the images mark different types of banding patterns; **(A)** the band appeared in both embryos at all the stages; **(B)** early to middle stages in ZE and middle to late stages in SE; **(C)** late stages in ZE and absent in SE; **(D)** early stages in ZE and absent in SE; **(E)** present in SE but not in ZE.

Detection of Gene Expression by Micro-Array Assay

The presence or absence of bands in somatic and zygotic embryos (Fig. 1) could be explained in terms of sequence difference between genotypes. The multiplicity of genotypes in the zygotic samples would suggest that differences in banding patterns do reflect qualitative differences in

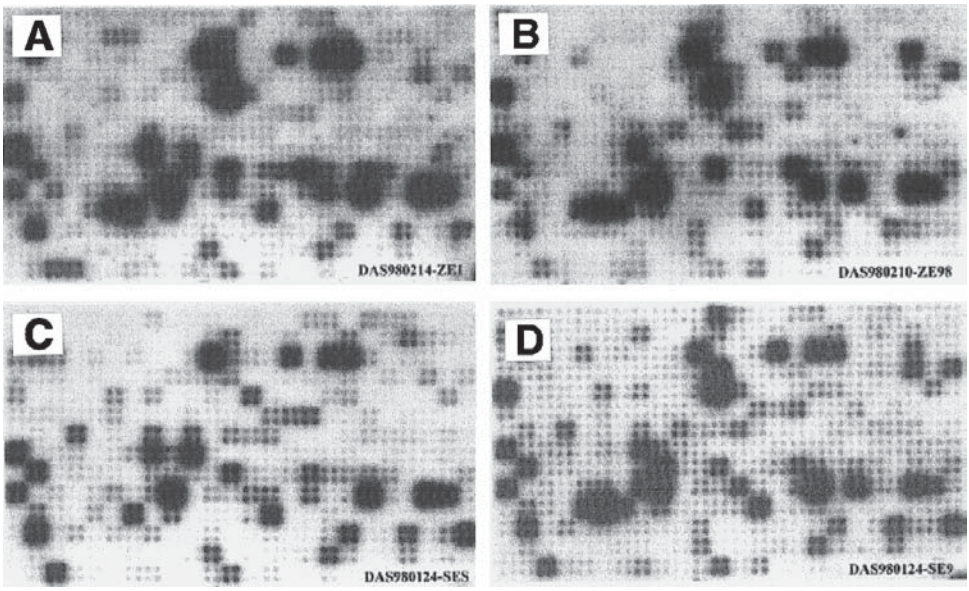
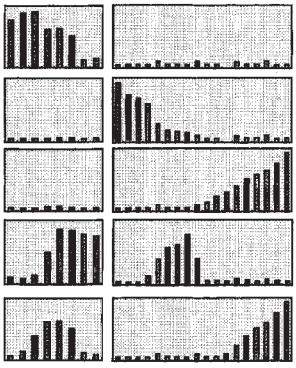


Fig. 2. Detection of gene expression by high-density array Southern hybridization. Cloned cDNAs (326) were blotted on a membrane as high-density arrays. Each cDNA was blotted four times as a quadrate. The membranes were hybridized to the total cDNAs derived from total mRNA isolated from zygotic embryos at stage 1 **(A)**, stage 9.8 **(B)**, somatic embryos at suspension stage **(C)**, and stage 9 **(D)**. Dark spots indicate high level of gene expression and light spots indicate low level of gene expression.

RNA profile; however, the expression of our cloned DNA in the loblolly pine embryos needed to be confirmed, and their expression during embryogenesis of other species needs to be examined. Northern blotting is usually used to estimate the level of the expression of a gene. This technique requires a few μg of RNA for each probing. Because we have several hundred probes to deal with, such an approach would be impractical given the amount of tissue available. We have taken a reverse approach, spotting cloned cDNAs on a piece of membrane as individual dots and using mRNA from the embryo as probe to hybridize to the cDNA on the membrane. The amount of mRNA needed can be as little as 10 ng, which can be obtained from a single late-stage or a few early-stage embryos. In our protocol, the mRNA is converted to cDNA and the cDNA is then amplified to full length using PCR. Figure 2 shows typical results of this method. The expression of all the cDNAs on the membrane is determined by one easy hybridization. To minimize differences that might arise through errors in imprinting the membrane, each cDNA was imprinted four times; thus, we see quartets of identical signals in Fig. 2. The similarities as well as differences in the expression of those genes are clearly visualized when different embryo materials are examined.

Currently, we have performed high-density array Southern blots for both somatic and zygotic embryos at all the developmental stages. The



Gene expression pattern	Number of arrayed genes displaying this pattern
Expressed only in SE or increased expression in SE	29
Expressed only in early stages ZE	0
Expressed only in late stages ZE	11
Middle to late stages in SE, early to late stages in ZE	8
Middle to late stages in ZE, early to late stages in SE	0

Fig. 3. Comparison of gene expression in somatic and zygotic embryos among genes represented in array. Eighteen high-density array Southerns (Fig. 2) were performed for zygotic embryos and 8 for somatic embryos. The images were digitized using GelPro 3.0. Additional patterns were observed showing more quantitative differences; the patterns above are examples of different expression profiles that can be observed using DNA array techniques.

information that can be extracted from these gene-expression detection experiments is overwhelming. To better understand and present the data, we have digitized the images. Figure 3 is an example of summaries we made from the digitized data. Many genes expressed in the late stages of ZEs are not expressed in late-stage SEs. Many genes expressed late in SE are expressed in early to middle stages of ZE development. The table also shows that many genes are expressed in somatic embryos, but not in zygotic embryos.

Discussion

How can the information arising from these studies be used to improve the process of somatic embryogenesis or indeed other protocols, such as defining the optimal conditions for enzyme induction in an industrial process?

An understanding of the timing and location of gene expression and the function of the proteins encoded by differentially expressed genes can provide many insights into the biochemistry and physiology of a system (Fig. 4).

DNA arrays (also known as gene arrays, DNA chips, and in our case, dot array Southerns) permit the evaluation of the expression of hundreds or thousands of genes within an organism in response to altered growth conditions *(20–22)*. Gene arrays may be comprehensive, consisting of a library of essentially unselected genes, cDNAs, primers *(21)*, or may be more focused, consisting of selected clones, such as described in this article *(18,19)*. The choice will depend on the nature of the experiment and

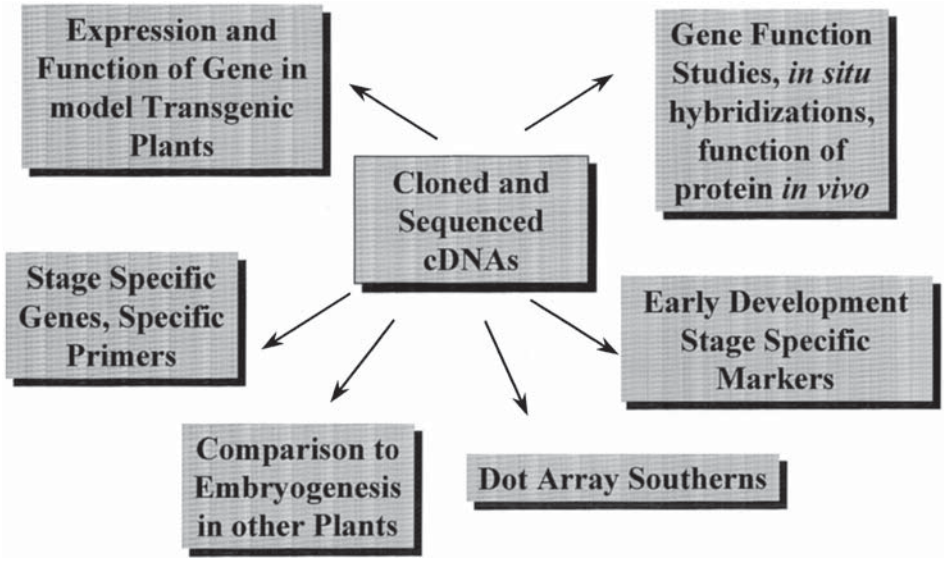


Fig. 4. Approaches with gene regulation studies arising from the cDNA cloning of genes expressed in embryos. See text for their applicability to process improvement.

the sensitivity of the detection system. A large amount of information can be gained that allows the effect of process alterations on mRNA accumulation to be viewed. The metabolic progress of an embryo through development may then be followed. Tissues can be subdivided and assays conducted to reveal expression in different locations. Such assays can be carried out without knowledge of the function or even the identity of the genes being assayed. Synthesis of protein from transcripts is not assayed in this system or in DD. Post-transcriptional regulation of expression must be determined by other experiments; however, assays that provide information on the differential accumulation of mRNA are instructive of themselves and provide materials for further study.

To demonstrate the ubiquity or uniqueness of a gene-expression profile in development, we can avail ourselves of the ability of related genes to cross-hybridize in dot array Southerns. If we use RNA from different genotypes or tree species, and hybridize at lower stringency, we can view cross-hybridizing mRNA and note their identity, time of appearance, and quantity compared to the loblolly pine controls. This technique might be applied to quite divergent species. Our sequence analysis shows very strong homologies between certain pine cDNAs and cDNAs from *Arabidopsis*, Tomato, Tobacco, and so forth. (Xu et al., unpublished). Such assays may forge a link between studies in tractable model plants, such as *Arabidopsis*, and commercially important plants such a pine or maize. In *Arabidopsis*, developmental mutants have been isolated but their overall effect is not known. By using our pine cDNA array systems we may be able to evaluate the effect of a mutation on embryo metabolism and physiology.

DD provides types of information similar to dot array Southerns. DD relies on sequence homology between arbitrary primers and target mRNA sequences *(23)*. Subsets of the expressed genes are viewed by using different sets of primer pairs and many repetitions are needed in order to evaluate the entire repertoire of mRNAs present in a given sample. The technique was formerly prone to problems of reproducibility; however, recent protocol changes have alleviated these difficulties *(24,25)*. One of the attractions of DD is the ability of the technique to discriminate between similar but nonidentical sequences. With dot array hybridization techniques, there is a possibility that mRNA derived from different gene family members may cross-hybridize. Changing the stringency of hybridization can diminish this possibility somewhat; however, a primer, specific for a sequence is a more precise tool. Where sequence information is known, specific primers can be designed and, using slightly modified protocols, the expression of a single mRNA or a small number of chosen mRNAs, can be followed. In this way, where a stage-specific mRNA is identified by differential display using embryos (or a process-specific mRNA identified in your assay), the band can be cut from the gel, cloned, sequenced, and primers can be designed *(18)*. If one is dealing with a family of genes, and sequence information is available, then primers specific for a family member may be designed. By designing several primers that do not cross-hybridize, spacing these primers such that in RT-PCR, each family member will generate a band of a recognizable size, the expression of different members of a gene family can be followed.

These gene-expression assays will allow us to develop a system for following embryo development that is more closely allied to metabolic competency than is the present system. Currently, the state of maturity of an embryo is determined based on a subjective evaluation of morphology. A system that could monitor the expression of key genes as stage-specific markers would allow protocol variations to be more quickly and confidently evaluated (Fig. 5).

As mentioned earlier, the assays previously described may be conducted in the absence of any knowledge of the function of the genes whose expression is being monitored. However, where we can assign identities and do have clues as to what the proteins encoded by these genes are doing, our appreciation of the embryos' metabolism is greatly enhanced; we can begin a process of constructing testable hypotheses. Assigning an identity to a cDNA isolated by DD and assigning a function to its protein, proceeds from an initial homology search conducted by computer. As with all such searches, identification must be tentative, but the results provide the basis for sets of experiments that will shed light on a particular protein. In a simple example, if an mRNA (cDNA band on a gel) appears in stage 6 zygotic embryos but not in stage 6 somatic embryos, and if that cDNA has strong similarity to the sequence of a particular sugar-hydrolyzing enzyme, we might infer the induction of that enzyme at stage 6. Although that is not certain, the inference can be verified; antibodies to that enzyme may be

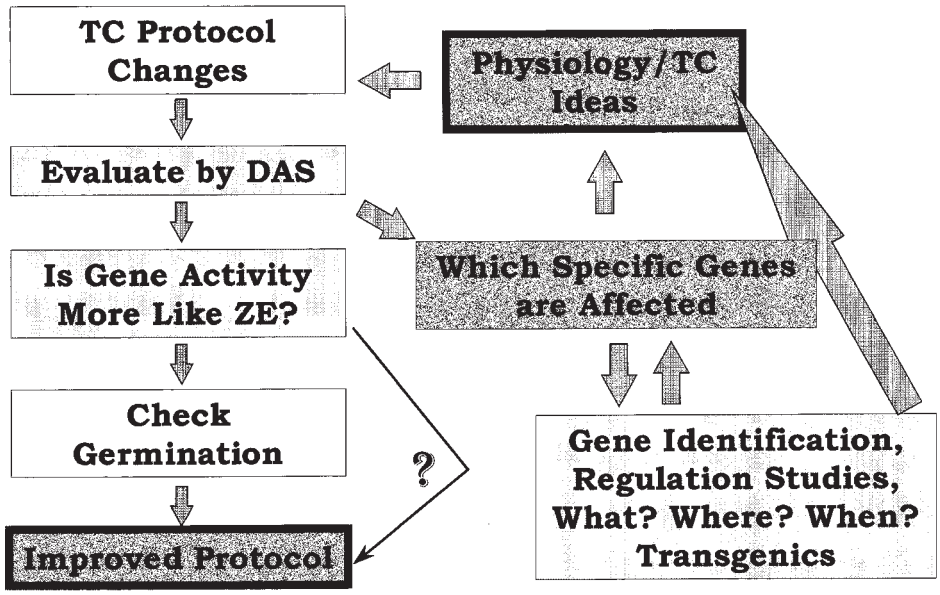


Fig. 5. Strategies for incorporating gene-expression studies into protocol improvement for somatic embryogenesis. DAS, dot array Southerns; TC, tissue culture; ZE, zygotic embryo.

available and if antibodies are not available, starting with the cDNA we have isolated, a protein could be produced and antibodies generated. Alternatively we may have a simple assay for the sugar. If these ideas prove correct, and that sugar is being metabolized in stage 6 embryos, we might then supplement our media with that sugar or its breakdown products at an appropriate stage of development and determine whether this step improves the quantity or quality of somatic embryos.

Further light on gene function will be shed by studies to localize expression to a particular tissue (e.g., *in situ* hybridization). The function of proteins whose sequence suggests a capability can be verified by expressing the protein from the cloned cDNA and assaying the purified product. The similarity of gene regulatory elements in different plants permits gene transfer and assay in heterologous systems *(26)*. Tree genes have been assayed in different systems *(27,28)* and the results have been useful in understanding biochemical pathways.

Process improvement for in vitro biological systems is often the result of empirical trial and error or mimicry of natural systems. An alternative is to assess the metabolic competency of the organism in order to promote growth or production of a desirable end product. The techniques presented here enable the researcher or production biologist to rapidly monitor specific levels of gene expression, which in turn can be used as a quality-control measure or to provide information for process improvement.

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