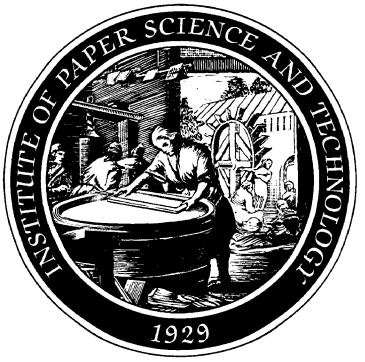

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Transcript Profiling: A Tool to Assess the Development of Conifer Embryos

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SUMMARY

Embryogenesis is a phase of development that has been understudied at the molecular level, especially in conifers. Somatic embryogenesis, the asexual propagation of embryos, provides insights into development and has major applications in the forestry industry. Loblolly pine is the most important commercial species in the United States; however, it is recalcitrant to somatic embryogenesis methods. An appreciation of gene expression and the development of 'expression markers' would allow us to follow conifer embryogenesis more closely and to gain some insight into the metabolic states of zygotic and somatic embryos. We have employed differential display to identify genes whose mRNA abundance changes over the course of development. We have isolated around 500 cDNAs and we propose to establish a database of transcript levels in somatic and zygotic pine embryos over the course of development using cDNA arrays. Approximately one-third of our cDNAs have similarity to sequences in the Genbank. Our intention is to gain insight into cell physiology and biochemistry by identifying inducible transcripts. This information will form the basis of testable hypotheses regarding manipulation of embryo development in tissue culture. Experiments deriving from transcript profiling will provide insight into development. The 'expression markers' will allow a classification system more closely tied to metabolic state and the integration of this data into ongoing physiological research will lead to improved protocols for somatic embryogenesis.

Key Words: Loblolly Pine, Embryogenesis, Gene Expression, Suspensor

INTRODUCTION

Somatic embryogenesis is an attractive experimental tool for studying plant development. It has great potential for the clonal propagation of superior trees and is integral to tree genetic engineering programs (Handley, 1995; McCullough and Dean, 1997; Tzfira et al., 1998). The recalcitrance of certain key tree species *in vitro* is thus particularly frustrating. Since the goal of somatic embryogenesis is to permit healthy development of embryos outside of the maternal tissue, it has been assumed that a medium whose composition resembles that of the seed has the greatest likelihood of supporting embryo development. Thus, tissue culture media have been created based on measurements of the composition of conifer seeds (reviewed by Tautorus et al., 1991; Attree and Fowke, 1993). The successes of these media, however, have been variable and results often have been very genotype-dependent.

Progress in somatic embryogenesis has been limited by the paucity of knowledge about molecular events in embryo growth and development. The measurements of trace elements, metabolite pools, hormone levels and osmolarity at different stages of development (Litvay et al., 1985; Teasdale et al., 1986; Feirer, 1995; Minocha et al., 1995; Minocha et al., 1999; Kapik et al., 1995; Pullman, 1997) supply useful data but provide only a broad overview of cell composition; details of molecular activities remain obscure. Molecular events within a developing embryo are the consequences of a program of gene expression. By identifying which genes are active during embryo development and charting their induction and repression, we can make inferences about the induction and repression of metabolic pathways. Further, as genes are transcribed the

appearance of particular mRNAs provides 'molecular markers' indicating the progress of an embryo through development. By comparing the transcript accumulation pattern of embryos growing *in vivo* and *in vitro*, differences and similarities in their metabolic states may be illuminated.

New molecular tools enable the cDNA cloning of mRNAs from different stages of development. The sequential induction of suites of genes leads to qualitative and quantitative differences in mRNA pools during different phases of growth, which has afforded researchers a strategy for isolating cDNA copies of these differentially accumulating transcripts. In these experiments, conducted in angiosperms, the mRNA population of the experimental tissue was copied by reverse-transcription into cDNA and cloned. This cDNA library was then screened against probes derived from mRNA isolated from experimental and control tissues. The differential hybridization to these probes led to identification of cDNAs of differentially expressed genes (Baker et al., 1988; Skriver and Mundy, 1990; Bray et al., 1990). Initially, work in conifers replicated that in angiosperms, and gene expression studies implied some conservation at the molecular level in both sequence and regulation (reviewed by Misra, 1995). More recently, novel sequences have been identified through the differential screening of cDNA libraries (Misra et al., 1996; Jarvis et al., 1996; Dong and Dunstan, 1996, 1997, 1999). A total of forty or fifty cDNAs for embryo-expressed cDNAs have now been isolated in various laboratories and are being characterized on a gene by gene basis. The screening methods employed, however, favor isolation of cDNA clones of genes whose mRNA level differ markedly between the phases of development considered; subtler modulations in mRNA levels are often overlooked in such studies. Many of

the cDNAs were isolated in late embryogenesis and represented storage-product genes whose transcripts were abundant at that time.

We wished to gain a larger overview of gene expression during development and to identify genes expressed at earlier times of development. To this end we have employed the techniques of differential display to identify genes expressed at different times of development. The identification of such genes will yield 'molecular markers' which can be used to chart progress through development. We have isolated, cloned, and sequenced over 500 of these. We have gridded these clones as DNA arrays and evaluated the expression of these selected genes over different phases of development. Further, we have employed these techniques in a study of suspensor function to monitor gene expression in different embryonic tissue.

MATERIAL AND 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 (Pullman and Webb, 1994). 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 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 and Pullman (1995) or with modifications in media mineral composition. Somatic embryos were grown in cell suspension culture medium 16 and maturation medium 240 (Pullman and Webb, 1994). Resulting somatic embryos were selected, staged (Pullman and Webb, 1994), and sorted into vials containing the same stage. Somatic embryos were stored at –70°C.

Differential Display and Cloning of cDNA Bands

Differential Display was performed and cDNA bands were isolated and cloned according to Xu et al. (1997).

DNA Arrays and Hybridization

Cloned cDNA was spotted onto membranes using a manual pin spotter (V&P Scientific, San Diego, CA). Probes were made and hybridization conducted as described in Cairney et al. (1999). For cDNA probe preparation, polyA RNA was isolated using polyT coated magnetic beads (Xu et al., 1997) and reverse transcribed followed by PCR amplification (SMART cDNA synthesis kit, Clontech, LaJolla, CA) as described in Cairney et al. (1999). Hybridization was performed in a 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 washed once at 42°C for 30 min.

RESULTS

Somatic Embryogenesis in Loblolly Pine

Somatic embryogenesis offers the advantage of rapid embryo multiplication in a small space. The disadvantage is that, while fairly efficient for many plants, the process is difficult in pine trees of commercial importance. Yields of pine embryos are low; their quality is poor; and there are problems in regenerating trees. Understanding and improving somatic embryogenesis is a major research effort at IPST. The tissue culture process used in our laboratories is reported more fully by Pullman et al. (1998) and is briefly described below.

Initiation. Conifer somatic embryogenesis involves the culture of zygotic seed embryos, usually from breeding programs, to start or initiate a culture. Many pine embryos undergo a natural multiplication process in the seed called cleavage polyembryony. Through the use of plant hormones, we can continue cleavage polyembryony to form masses of early-stage somatic embryos.

Multiplication. After initiation, somatic embryos are moved to another medium to increase their numbers (Pullman and Webb, 1994). This can be done on a gelled or liquid multiplication medium, which contains plant hormones to continue cleavage polyembryony and somatic embryo multiplication. Suspension cultures, in liquid media, have the advantages of increasing growth rates, decreasing variation, and being easy to automate for transfer from one medium to another. Cultures may replicate 2-6 times weekly, rapidly producing large numbers of somatic embryos.

Maturation. Once a sufficient population of embryos has been grown on the multiplication medium, embryos are placed on a development and maturation medium (Pullman and Webb, 1994). A different set of plant hormones and environmental cues are required to continue development of the early-stage somatic embryos.

Germination. The last step is embryo germination and acclimation to achieve growth out of the tissue culture vessel to produce somatic seedlings ready for planting in the field.

To enable the process to be described more exactly, a refined staging system was developed which classifies the embryos, based on morphology, into nine stages of development (Pullman and Webb, 1994). While a generation of loblolly pine plantlets has been achieved through somatic embryogenesis (Pullman et al., 1998), losses occur at every stage and the process is not sufficiently efficient for commercial purposes. To gain insight into the metabolic state of zygotic and somatic embryos over the course of development, we have applied a series of molecular tools to illuminate conifer embryogenesis.

Transcript Profiling by Differential Display

Differential display is a reverse transcription - PCR (RT-PCR) technique which permits the comparison of different mRNA populations without the need for cloning (Liang and Pardee, 1992; Liang et al., 1993). An estimated 30,000 genes are expressed over the course of embryogenesis (Okamuro and Goldberg, 1989; Thomas, 1993). In differential display, reverse transcription is conducted using an 'anchored' oligo dT primer, which included two additional nucleotides at the 3' end. This sequence modification ensures that the primer binds at the junction

of the polyA tail and the body of the mRNA. Further, the sequence at the 3′ end causes the primer to bind to a subset of the mRNA population, which is subsequently reverse-transcribed into 'first-strand' cDNA. A portion of this cDNA is then used for PCR where the second arbitrary primer (AP) is added. Since the AP binds to a subset of the cDNA, the combination of anchored primer and arbitrary primer will amplify a sub-population of the original mRNA population. Repetition of the reaction with different primer combinations will ultimately permit representation of the complete mRNA population of the tissue under study.

Embryo studies are limited by small amounts of material and conventional RNA isolation methods involve unacceptable loss. We have developed a procedure for use with small amounts of material (Xu et al., 1997). Tissue is ground and mRNA is isolated in the presence of magnetic beads to which oligo-dT has been attached. These beads bind polyA mRNA from the cell homogenate. A magnet is used to draw the beads to the side of the tube, with the mRNA 'in tow'. Washing of the beads removes cell debris, and the beads, to which mRNA remains bound, are then used for RT-PCR directly.

Using a small amount of somatic embryo material, a clear, reproducible set of bands is produced. When differential display reactions, generated from embryos at different stages of development, are run side by side on a gel, we can observe the appearance and disappearance of mRNA species as bands on the gel. In Figure 1, differential display has been used to survey gene expression over 18 stages of zygotic embryo development. Several examples of bands, representing mRNAs, which are confined to early or late development, are shown. In addition, mRNAs that appear to be restricted to specific stages of development are noted. Comparison of

zygotic and somatic profiles shows similarities and differences between the somatic and zygotic patterns of gene expression (data not shown). Bands representing mRNAs, whose abundance changes over development, and bands that differ between somatic and zygotic embryos, have been cloned, analyzed, and sequenced. Approximately one third of these cDNAs show a strong similarity to sequences in the GenBank, thus permitting a tentative assignment of functions. Clones derived from differential display are often partial copies of the mRNA, consisting principally of the 3'end of the molecules, a region that is poorly conserved between species. The failure to note strong resemblance between two thirds of our clones and the sequences in the GenBank may, in part, reflect this fact. Our results, such as those shown in Figure 1, have for the first time permitted the identification of 'molecular makers' throughout the course of conifer embryogenesis.

Transcript Profiling by DNA Array Technology

Differential display is a useful technique for revealing differences in transcript abundance; however, problems of reliability with the technique, and the fact that, for our purposes, only a small subset of the mRNAs are of interest, led us to consider alternative assay methods. Stage-specific markers (dots in Figure 1) may yield information important for understanding embryo development and for modifying somatic embryogenesis protocols. It is important, therefore, to employ a robust assay technique which indicates mRNA abundance and is, as far as possible, insensitive to experimental variations such as sample preparation. DNA arrays permit the reliable examination of large numbers of cloned sequences. Gene arrays (micro-arrays) are being developed in many research programs as a key component of functional genomics (Lashkari et al.,

1997; Bouchez and Höfte, 1998). Large-scale sequencing of plant ESTs (expressed sequence tags, essentially cDNAs) has led to the discovery of many new gene sequences. The function of these new sequences may be inferred from gene expression studies together with the gain of function/loss of function studies in mutants or transgenics. High-throughput gene expression data are being obtained by transcript profiling using high-density gene micro-arrays. EST sequencing and transcript profiling methods can be applied to almost any biological system. In contrast, methods to test functions through gain/loss of function experiments on a large scale have been worked out only in a small number of model plant systems, including Arabidopsis and maize. Trees, particularly conifers, are not currently amenable to mutagenesis or gene tagging because of their long life cycle; this is generally due to the large genomes and the difficulty of transferring foreign DNA and regenerating transgenic plants. It is likely, however, that increasing information on new gene functions, that will be gained from model plant systems, will help assign function to many new genes isolated from trees.

An understanding of the timing and the 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. DNA arrays permit the evaluation of gene expression, potentially genome-wide, within an organism in response to altered growth conditions (Schena, 1996; Desprez et al., 1998; Marshall and Hodgson, 1998; Ramsey, 1998). Gene arrays may be comprehensive, consisting of a library of essentially unselected genes, cDNAs, primers (Marshall and Hodgson, 1998), or they may be more focused, consisting of selected clones (Cairney et al., 1997, 1999; Xu et al., 1997). The choice will depend on the nature of the experiment and the sensitivity of the detection system. Gene arrays are particularly useful for obtaining rapid and

quantitative information for transcript abundance of known and unknown genes in developmental pathways, metabolic responses, or entire organisms that have not been extensively characterized (Schena et al., 1994; Lashkari et al., 1997). A large amount of information can be gained that allows the effect of tissue culture process alterations on mRNA accumulation to be viewed. The metabolic progress of an embryo through development may then be followed (Cairney et al., 1997, 1999). Tissues can be subdivided and assays conducted to reveal expression in different locations.

For DNA arrays, deposition of DNA onto substrates is often conducted robotically, producing a grid of about 10,000 clones on a glass slide about 1.5 cm^2; however, the costs of such systems limit their availability. In our assays we have used a manual spotter (V&P Scientific Inc., San Diego, CA) which will deposit 384 clones on membranes of about 60 cm^2. Our clones are spotted in replicates of four, thus we have 1,536 spots per membrane serving as the substrate. Once spotted, the substrate is hybridized with a probe and reverse transcribed using RNA from the tissue of interest to produce a pool of labeled cDNAs thereby reflecting the mRNA population of that tissue (Fig. 2). Whichever deposition method is used, the cloned DNA on the substrate is present in excess; thus, the amount of probe hybridized reflects its abundance in the tissue being studied. Since the embryos we are studying are small and the isolation of sufficient RNA to conduct direct reverse transcription is difficult for all but the suspension culture and very late stages of development, we have isolated mRNA as described (Xu et al., 1997) and conducted RT-PCR to amplify the probe. Control experiments comparing direct reverse transcription to this RT-PCR approach have allowed us to develop protocols that reflect the mRNA profile in the tissue (Xu et al., in preparation).

Figure 3 shows a comparison of transcript abundance for stage 1 (suspension stage) and Stage 9 somatic embryos. The intensity of dots reflects the abundance of particular mRNAs. While many genes appear to be expressed at a similar level, pronounced differences in mRNA abundance are clear for several genes. These data give us qualitative information on the expression of different genes and this can be converted to quantitative information through the use of imaging systems. Such assays allow us to follow the changes in abundance of a particular mRNA at a particular time of development. Figure 4 shows three different transcript accumulation profiles for three embryo-expressed genes from loblolly pine. Signal intensity from DNA arrays were quantified using a Fuji BAS 1800 imaging system. The transcript level for gene LPS24 is fairly steady from stage 3 through stage 6 of somatic embryo development. The mRNA levels for gene LPS25 decline and mRNA for gene LPS26 becomes more abundant. By comparing zygotic and somatic embryos, a clearer observation of their differences can be made. Expression patterns have been confirmed for a number of these genes by Northern blotting and signal quantification (data not shown). Where gene identity is known, inferences can be made about the metabolic activity within the embryo, which may be confirmed experimentally. Such data can lead to tissue culture modifications for improved embryo development in vitro. The appearance and the disappearance of certain mRNAs can be used as molecular markers to monitor development either as a quality control tool, to ensure that an established protocol continues to be effective, or as an experimental tool, to reveal the effect of a new protocol on the pattern of gene expression. Hypotheses have been so advanced that favorable tissue culture conditions are those which produce somatic embryos which closely resemble zygotic embryos. DNA arrays provide a swift and sensitive assay to monitor the effect of such changes on transcript accumulation.

Transcript Profiling in the Suspensor

Gene expression studies using cDNA arrays are particularly useful to rapidly characterize the transcript profile of specific tissues or cell types that have not been well studied (Schena et al., 1996; Lashkari et al., 1997). We have begun using cDNA arrays to gain insight into the growth, development and function of the suspensor of pine embryos. In angiosperm plants, the suspensor is required for early embryo development, and three different roles have been attributed to the suspensor (Cionini, 1987; Yeung and Meinke, 1993): 1) The suspensor supports the embryo and positions it inside the seed. 2) It is a route for nutrient translocation, a conduit involved in the feeding of nutrients from the surrounding mother tissue to the embryo. 3) It synthesizes growth regulators and thus may control embryo growth and development. These roles highlight the importance of the suspensor in angiosperms but have only been partially verified in conifers. The suspensor usually arises from the first cell division of the zygote, which is typically an asymmetric one. The first cell suspensor is the larger basal cell; it continues to divide and forms a tissue that expands more rapidly than the embryo-proper. After this phase of rapid expansion, its cells differentiate, begin to produce gibberillins and other growth regulators (Cionini, 1987) and may accumulate storage products (Schwartz et al., 1997). Very little is known of the molecular processes of suspensor formation and function, and their regulation. There is growing evidence that the suspensor is central to embryo development and that overall its importance has been overlooked.

Recently, important findings have revealed that suspensor cells have the potential to generate an embryo. In *raspberry*, *twin* and *sus* mutants of *Arabidopsis*, the arrest of normal

development of the embryo-proper, early in development, is accompanied by transformation of suspensor cells which divide and differentiate to give rise to an embryo or an embryo-like structure (Yadegari et al., 1994; Zhang and Sommerville, 1997; Schwartz et al., 1994). The explanation proposed for these observations is that an inhibitory molecule is produced by the embryo in normal development, thus preventing further growth of suspensor cells. These and other studies of suspensor function and embryo development provide clear indications of a complex interaction between the suspensor and the embryo that evolves during development.

The suspensor of pines have unique and interesting features. Many conifers form relatively large suspensors that persist through most of embryo development although it remains a transient organ, as in all plants. In addition, the suspensor of certain conifers, including pines, is intimately involved in the process of cleavage polyembryony and the ensuing competition between the multiple resulting embryos (Spurr, 1949). Once the dominant embryo is established, the suspensor network may provide an effective channel through which it may actively suppress the subordinate embryos. The suspensor of the dominant embryo expands by secondary growth and becomes much larger than the dominant embryo. This secondary growth may be stimulated by the embryo-proper. Finally, pines and other conifers form well-developed suspensors in somatic embryogenic cultures (Jain et al., 1989; Becwar et al., 1991; Behrendt and Zoglauer, 1996) therefore it is straightforward to isolate suspensor tissue from somatic embryos. By contrast, the suspensor is often absent from somatic embryos of angiosperms, or when present, it typically lacks the features of the zygotic suspensor (Yeung and Meinke, 1993).

We have begun gene isolation and expression experiments in the suspensor of immature zygotic embryos. The embryo cDNAs arrays (described above, Cairney et al., 1999) were

screened to identify additional differentially expressed genes. The filters were hybridized with suspensor and embryo-proper cDNAs prepared from and using very small amounts of micro-dissected zygotic suspensor and embryo-proper tissue from different time points during development. The cDNAs were synthesized from poly A+ RNA isolated using magnetic polyT coated beads (Rosok et al., 1996) by using a PCR amplification method (SMART PCR cDNA synthesis, Clontech) and further labeled by random priming (READY-TO-GO DNA labeling beads, Pharmacia). Hybridization followed standard procedures, washing under high stringency and detection by X-ray autoradiography. The signal intensity on the X-ray film was used to determine the relative abundance of steady state RNA in the suspensor relative to the embryo head (Table 1). For the purpose of normalizing the signal intensity between hybridizations, we used three ribosomal proteins as internal standards. We compared the normalized signal intensity of suspensor and embryo-proper from embryos of developmental stage 3 (Pullman and Webb, 1994). Several genes appeared indeed differentially expressed between these two parts of the embryo. The result revealed that at this stage of development, steady state RNA of a storage protein gene (albumin) and other proteins normally associated with late embryo development, were abundant in the suspensor and yet only present at low levels in the embryo-proper (**Table 2).** These findings, although preliminary, point to a developmental pathway for the pine suspensor that is distinct from the embryo-proper with regard to timing of a suite of important genes. The early accumulation of albumin suggests a storage/feeding role for the pine suspensor that could involve production of storage proteins for future mobilization into the embryo-proper, consistent with recent angiosperm literature (Panitz et al., 1995). Hybridizations with cDNA probes prepared from the megagametophyte tissue further showed that patterns of gene expression of this reserve tissue show significant similarities with that of the suspensor.

CONCLUSIONS

The study of plant embryogenesis is fundamental to our appreciation of growth and development and to the production of our food and fiber. Somatic embryogenesis of conifers holds great promise for forest products industries as a method for multiplying trees with superior genotypes; however, many conifers are recalcitrant to this process compared to angiosperms. The ultimate understanding of embryogenesis depends on our knowledge of the genes that are expressed during embryo development. However, few embryo-expressed genes are known and the detection of gene expression in embryos is made difficult because the embryos are usually embedded deep in the plant tissues and are very small at early stages. To overcome this problem, we have developed an RNA differential display technique that uses minute amounts of plant tissue. These studies reveal numerous differences in mRNA profiles of somatic and zygotic embryos, as well as differences in the stage of development at which specific mRNAs appear. From differentially displayed bands, we have cloned over 500 cDNAs that are expressed at different times of embryo development. These cloned cDNAs have been placed in a high-density array and are used to detect the expression of corresponding genes in developing embryos. The detection method we have developed is highly sensitive and reproducible. Differences in the expression of these genes can be detected easily in a single embryo of loblolly pine. Somatic and zygotic embryo gene expression profiles have been compared, as have gene expression changes in somatic embryos in response to culture conditions.

Expression patterns from DNA arrays generally agree with those generated by differential display. Occasional differences, however, are observed, notably where apparent early- or late-specific genes appear to be constituitively expressed when assayed in arrays. These differences are most probably due to cross hybridization of related sequences, i.e., transcripts derived from different members of a multigene family. PCR-based techniques, such as differential display, select from among very similar sequences by virtue of specific pairing of primer and template sequence. Hybridization techniques, such as DNA arrays, which employ long probes and long targets, lack this fine discrimination. The ease of use and robust nature of DNA arrays recommend this technique for multigene studies, however a combination of assay methods, such as preselection of clones by differential display, or parallel monitoring of specific transcripts with specific primers may be important for gaining insight into gene regulation. Techniques which employ a panel of synthetic oligonucleotides, designed to different parts of an mRNA, offer increased discrimination (Lipshutz et al., 1998). The technical demands and expense of this approach however, have limited its adoption. Arrays of DNA clones, nonetheless, provide much information on mRNA abundance allowing subsequent investigations to be directed towards the understanding of particular genes or metabolic pathways.

The ability to follow the expression of specific genes during the course of loblolly pine embryogenesis has many implications for research and process improvement. Genes expressed at specific phases of embryogenesis can now be identified and their temporal and spatial expression determined. Putative control regions may be investigated and their response to externally applied stimuli in tissue culture can be evaluated. Gene expression could be used as a tool to monitor somatic embryogenesis, that is, as a quality control tool to ensure successful protocols are being

reproduced. Inferences may be made from the induction of specific biochemical pathways during the course of development suggesting process improvements, which in turn could be monitored by gene expression assays. While conventional evaluation of new tissue culture protocols takes several months, gene expression changes can be viewed within a few days and may be used to predict potentially successful protocols, thus accelerating process improvement.

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TABLE 1. List of loblolly pine cDNA clones differentially expressed (relative to the embryo-proper) in the suspensor of zygotic embryos and in the megagametophyte based upon abundance of steady state RNAs of stage 3 embryos, determined in dot blot and cDNAs array hybridization experiments.

Gene[1]	Relative Transcript Abundance[1] Suspensor	Megagametophyte
Albumin 1	+	+
Elongation Factor-2 (Chlorella)		+
LEA protein (2 cDNAs)	++	+++
Oxygen evolving complex (2 cDNAs)	+++	
Plasma membrane ATPase		++
SnRNA associated protein	++	+
Transcription regulator IE63	++	+
Ubiquitin-like protein	++	
Yeast membrane protein	++	
9 cDNAs of unknown function	+	
8 cDNAs of unknown function	+	+
3 cDNAs of unknown function		+
Ecdysome-inducible protein E75	+	--
Actin		--
B2 protein from carrot	-	--
Dynamin-like GTP binding protein		-
EST similar to Insulin	-	
GA regulated protein GASA5	--	---
Histone 3	-	--
Malate oxidoreductase		--
Mouse embryonic ectoderm protein	--	
Protein kinase		---
Ribonucleoprotein Sm D3		-
Serine kinase (human)	-	
Translation elongation factor 1-alpha	-	-
Transmembrane WD 40 type 1 protein	--	
Voltage dependent anion channel		---
5 cDNAs with no database hits	-	
9 cDNAs with no database hits	-	-
12 cDNAs with no database hits		-

[1]Sequence probability scores range from 10^{-10} to 10^{-110}, most sequences have scores above 10^{-20}, with storage proteins having greatest similarity.

[2]Relative RNA abundance was determined by digitalization of X-Ray film and image analysis (Imagegauge, Fuji). To simplify the presentation, here the relative abundance is expressed as slightly more (+), more (++), much more abundant (+++) or slightly less (-), less (--), much less abundant (---), compared to the embryo-proper.

FIGURE LEGENDS

Figure 1. A differential display pattern of loblolly pine 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 late stages; left arrows, early stages and black dots, one or two specific stages. For purposes of clarity only a few differential display bands have been marked.

Figure 2. Schematic showing the principle of DNA arrays using a manual pin. The right panel shows results of two membranes containing an identical set of cDNA clones, hybridized with probes from suspension (stage 1) or stage 9 embryos.

Figure 3. Expression of the 326 cDNAs in loblolly pine somatic embryos at early and late stages. Messenger RNA was extracted from somatic embryos at suspension stage (Top panel) and stage 9 (Bottom panel), converted to cDNA. The first strand cDNAs were then amplified by PCR and hybridized to membranes containing 326 loblolly pine embryo cDNAs.

Figure 4. Graphical representation of transcript accumulation of three embryo-expressed genes from loblolly pine. DNA arrays of pine cDNAs were hybridized in separate experiments with probes derived from embryos at stage 3, 4, 5 and 6 of development, classified according to Pullman and Webb (1994). Relative signal intensity from DNA arrays was measured using a Fuji BAS 1800 imaging system.

Figure 1

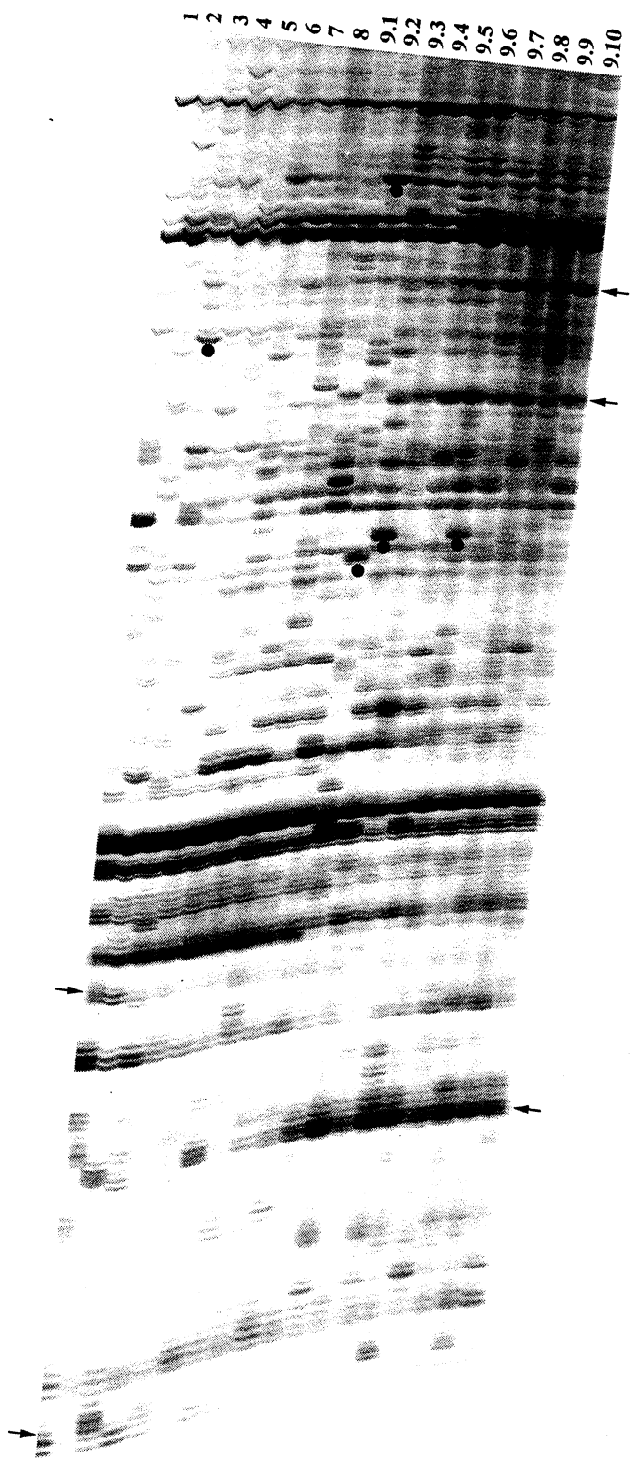


Figure 2

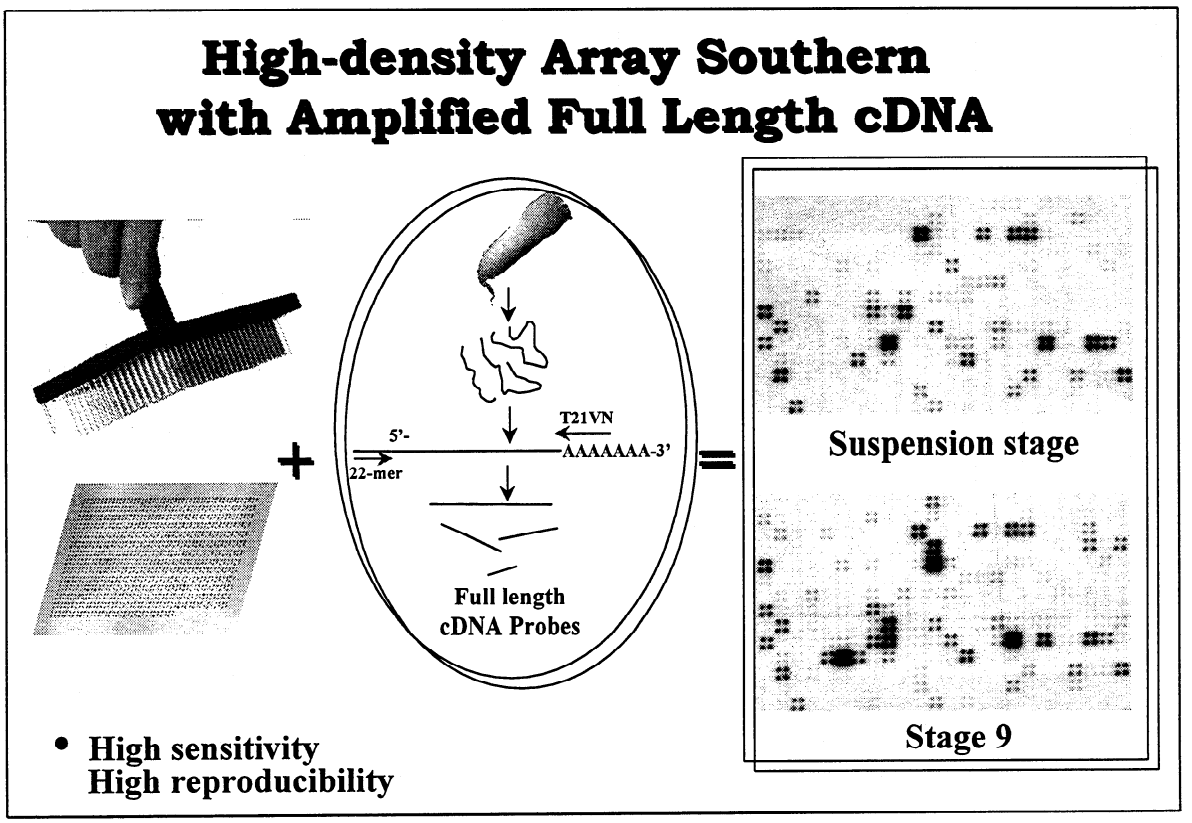

High-density Array Southern
with Amplified Full Length cDNA
T21VN
5'-
AAAAAAA-3'
22-mer
Full length
cDNA Probes
Suspension stage
Stage 9
• High sensitivity
High reproducibility

Figure 3

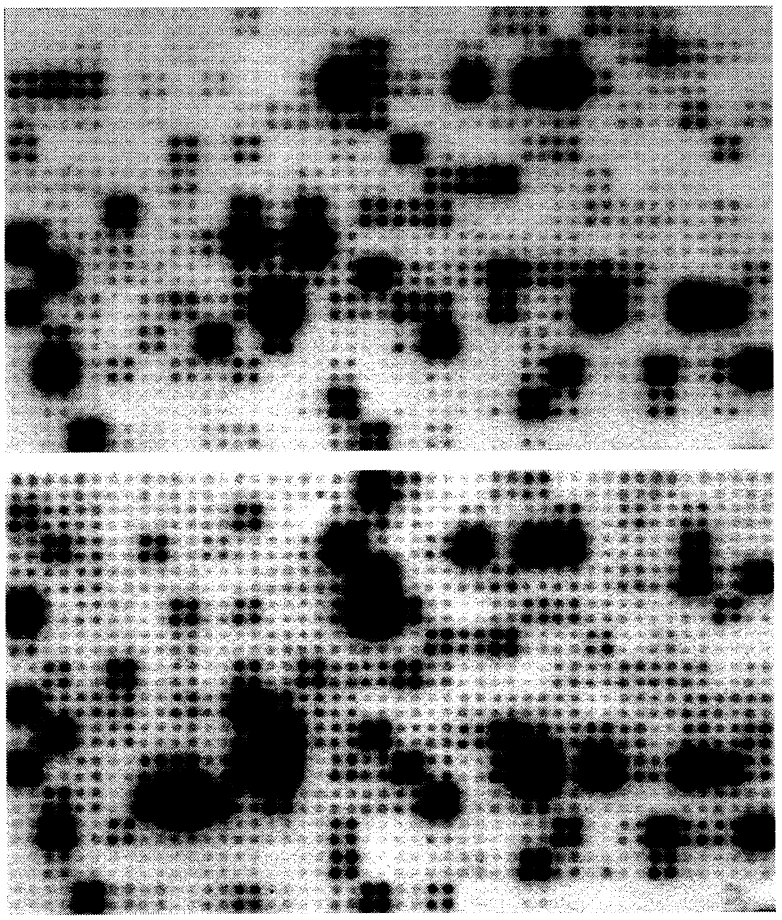
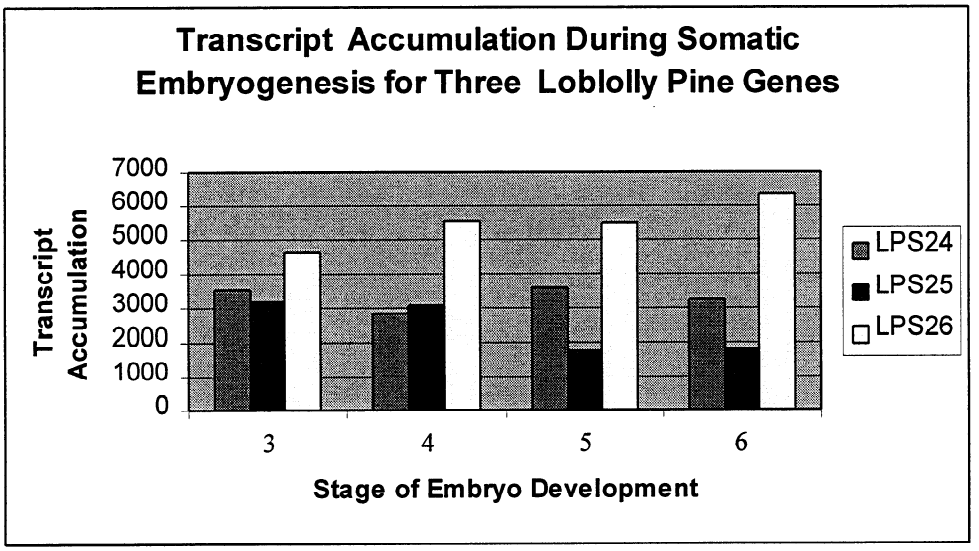


Figure 4


Transcript Accumulation During Somatic Embryogenesis for Three Loblolly Pine Genes
Transcript Accumulation
7000
6000
5000
4000
3000
2000
1000
0
3
4
5
6
Stage of Embryo Development
LPS24
LPS25
LPS26