GENE EXPRESSION IN ORGANOGENIC TISSUES OF LOBLOLLY PINE

(*Pinus taeda* L.)

By

YAN ZHANG

Bachelor of Agronomy

Beijing Agricultural College

Beijing, P. R. China

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Thesis Approved:

Thesis Advisor

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

1.1 Goals and Objectives

Loblolly pine (*Pinus taeda L.*) ranks high among the natural resources of the South. Traditionally, forest tree breeders used methods for crossbreeding or selection breeding to develop new and improved forest stocks. However, those methods are tedious, costly, and of limited success because of the long generation time and large size of forest trees (Karnosky 1981, Ledig *et al.* 1985).

Over the last several decades, vegetative propagation has become an important regeneration method for trees, as both a research tool and an operational management practice (Talbert *et al.* 1993, Huang, *et al.* 1993). Haissing (1989) found loblolly pine could be produced by tissue culture through one of the two different developmental pathways: organogenesis or somatic embryogenesis. Although somatic embryogenesis, especially for loblolly pine, still remains a major challenge, organogenesis has become a reliable technique for *in vitro* regeneration in many forest species (Haissig 1989). Most of the research on organogenesis has focused on manipulation, including the selection of medium and explant source and control of the culture environment. Few studies have

reported on the basic aspects of *de novo* organogenesis and regulation at the molecular level.

The overall goals of this research are to clarify the genetic and molecular mechanisms of the developmental process of shoot organogenesis and the regulation of the development pathway. The purpose of this study is to understand the genetic control of the developmental process of *de novo* organogenesis and provide information and tools for manipulation of shoot regeneration in loblolly pine. Key genes specifying organogenic transition will be identified and cloned, and the functions of the cloned genes will be determined. To accomplish the objectives of this project, the following research activities were planned:

1. Establish explants that do or do not consistently initiate shoot primordia *in vitro*.
2. Prepare poly (A^+) mRNA to clone genes differentially expressed at the shoot-forming stage.
3. Amplify major genes that are directly involved in shoot differentiation from different tissue sources using the PCR-based differential display technique.
4. Confirm expression of genes using Reverse Northern analysis.
5. Obtain full-length genes by screening a loblolly pine cDNA library constructed from shoot-forming tissue. Isolate and characterize the structures of those identified genes.

1.2 Significance

Loblolly pine ranks high among the natural resources of the South. Southern yellow pine has provided an average harvest of some 10 billion board feet a year and at least half of this has been loblolly pine (Zobel 1992). Today, the growing stock volume of this species stands at 90 billion board feet. Foresters, land managers, landowners, industrialists, and many others have seen the benefits that loblolly pine has already provided and they are becoming increasingly aware of the innumerable possibilities the future holds. This potential requires developing both strategies and technologies for increasing forest productivity and biomass production.

This research concentrates on molecular studies of the transition of cells from the quiescent state to organ differentiation, which leads to shoot formation and plant regeneration. Information obtained from this research will extend our knowledge of morphogenetic control and differentiation and explore molecular mechanisms for *de novo* organogenesis, which will provide a base for expansion of the application of biotechnology to woody plants for enhanced production of renewable resources. The practical significance of the research is to allow or accelerate the mass production of superior clones and selected genotypes of loblolly pine and to increase production of biomass for commercial forestry and fuel.

Organogenesis in plants is a complicated developmental process. There is at present not much information about it. However, recent studies of the molecular genetics of flowering have yielded knowledge about molecular mechanisms of flower development (Markley *et al.* 1993). This breakthrough suggests success in studies of plant organogenesis. Recently, in a related study with rice, a cDNA clone specific to

plant regeneration was isolated from embryogenic cultures through differential screening of an embryogenic cDNA library (Mizobuchifukuoka *et al.* 1996). Organogenesis, the other aspect of organized development, also forms a unipolar structure; thus both developmental pathways should at least share some common characteristics. All the research mentioned above provides fundamental support and suggests a high probability of success for this research.

Shoot apical meristem is believed to be generative tissue and the continual activity of the apical meristem results in shoot development (Aitken-Christie *et al.* 1988). Shoot apical meristem plays an important role in *de novo* organogenesis and other developmental processes. In loblolly pine, apical meristematic tissue of young seedlings has great potential to develop *de novo* shoots (Huang *et al.* 1995). Using apical meristems of loblolly pine, this work will focus on elucidating molecular mechanisms controlling *de novo* organogenesis. Loblolly pine has received a lot of research attention because of its value. All this work makes it the logical choice among conifer species as a model system for studies of plant development and regeneration. The information from this research should ultimately make significant contributions to both fundamental understanding and practical applications.

1.3 Literature Review

1.3.1 Regeneration of Trees by Vegetative Propagation

The world demand for wood products is expected to rise sharply over the next few decades. To meet this growing demand there will be an increasing need for large numbers of trees of improved quality and shortened rotation. Forest trees can be regenerated by either sexual or asexual propagation. Traditionally sexual reproduction of seedlings for reforestation has been the most important means of propagation in pine. Since the report of plantlet regeneration in *Pinus palustris* Mill via organogenesis from mature zygotic embryos (Sommer *et al.* 1975) was published, significant progress has been made in the vegetative propagation of conifers. Now vegetative propagation *in vitro* has become an important tool for regeneration of forests (Haissig 1989, Patel and Thorpe 1986).

In vitro propagation not only allows plant multiplication at a rapid rate but also production of desirable genotypes in large quantities for commercial markets. Such propagation has special value because it allows regeneration of large numbers of plants with genetic homogeneity. Also vegetative propagation is a critical component of many genetic improvement programs for higher plants, including traditional breeding and genetic engineering. Zobel (1992) concluded that the most promising use of tissue culture and biotechnology was to supply desired genotypes with large numbers developed by the tree breeder.

Von Arnold (1991) concluded that there were three different regenerative pathways used for the *in vitro* propagation of conifers. These are; (1) regeneration

through somatic embryogenesis, (2) regeneration from adventitious meristems (organogenesis); and (3) regeneration via the proliferation or enhancement of axillary bud development of existing meristems.

Somatic embryogenesis is the *de novo* production of structures resembling zygotic embryos, derived either from organized tissues or from callus. Somatic embryos must be bipolar and have no vascular connection to the source tissue. Somatic embryos may be derived either through direct or indirect embryogenesis. In direct embryogenesis, embryos are formed essentially by multiplication of a zygotic embryo explant. Indirect embryogenic systems involve dedifferentiation of nonembryonic tissue to form a callus from which somatic embryos arise (Sharp *et al.* 1980).

Organogenesis is the *de novo* production of plant organs from tissues or calli. In many cases, initial explants are induced to form callus and subsequently shoots. The shoots are then elongated, and rooted (Gladfelter and Phillips 1987). Production of plantlets from axiallary shoots is a method to excise shoots from source tissue and root them individually to multiply the original genotype.

Vegetative propagation through all three routes of regeneration has been widely reported for hardwood forest trees. During the last decade, more research priority was placed on commercially important coniferous species. Loblolly pine (*Pinus taeda* L.) is one of those species which has been studied world wide (Baker and Langdon 1990, Frampton 1992, Gupta and Durzan 1991, Huang and Tauer 1996). Producing over half of the total pine volume harvested in the Southern US, loblolly pine is the most important softwood species in the southern pine region. It occurs almost throughout the entire South and Southeast (Ellis *et al.* 1992). The tree makes rapid growth on a variety of

soils, and the wood is valued for pulp and structural materials. Therefore, many forest products companies are interested in developing commercial vegetative propagation systems for loblolly pine. Manipulation for *in vitro* regeneration of loblolly pine has been studied in many laboratories. Major activities involve searching for regenerable materials and developing regeneration methods. Various tissue culture procedures have been applied to regenerate loblolly pine. These include induction of adventitious shoots from cotyledons (Mehra-Palta *et al.* 1978), somatic embryogenesis via immature zygotic embryos (Becwar *et al.* 1990, Gupta and Durzan 1987), and organogenesis from shoot apical meristems (Bergmenn and Stomp 1992). Although some successes have been seen in these areas of tissue culture research, there are obvious limitations associated with each system because they all use seeds or seed-derived materials. Shoot primordium can only initiate from limited cells on cotyledons, and shoot multiplication frequency is low. Somatic embryogenesis is still in the research phase and is far from operational. Furthermore, success of these systems depends on the initial explant type. Some types of explants respond well to shoot induction, whereas others do not, indicating that these cultured materials must acquire competence to respond to organized development.

Although there are several known technical difficulties, resistance from forest nursery operations, and a lack of experience in the production of rooted cuttings (Boulay, 1987), the future of vegetative propagation technology appears to be bright because of its utility as an important tool in tree improvement.

1.3.2 Organogenesis of Woody Plants

Plant organogenesis refers to a developmental process where organ primordia, such as buds, are initiated on an explant in response to triggering by exogenously applied plant growth regulators (Thorpe 1990). The primary advantage of organogenic regeneration methods is that some of these methods may have the potential for a high frequency of plantlet production in a short period of time. Callus can be grown in large quantities with little demand for inputs of labor and space, and adventitious shoot production can be used to achieve high multiplication rates. The phenomenon of *de novo* organogenesis has been observed in a variety of tissue culture systems. Many plants can now be propagated by *in vitro* organogenesis (Ahuja 1993, Wagley *et al.* 1987).

The process of organogenesis can follow two major developmental sequences in conifers: direct or indirect organogenesis (Hicks 1980). Indirect organogenesis would be used when there is an initial production of callus tissue from the primary explant, followed by the appearance of meristemoids (Torrey 1966). It is believed that the fate of these meristemoids is not determined and they can produce either shoot or root primordia. This method is presently of limited use for plantlet regeneration in conifers because of low regeneration rate (Ahuja 1993). However, a low frequency of plantlet regeneration has been achieved in *Larix x eurolepis* hybrids (Laliberte and Lalonde 1988).

The process of direct organogenesis is defined as organ formation directly from a primary explant in the absence of an intervening callus stage (Hicks 1980). The predominant morphogenic route reported for adventitious shoot formation in conifers has been through direct organogenesis (Thorpe and Biondi 1984). The procedure typically followed to accomplish plantlet regeneration by direct organogenesis can be divided into

four steps: (1) initiation of shoot meristems, (2) development and elongation of shoot buds, (3) root meristem initiation and development, and (4) plantlet acclimation to the ambient environment (Thorpe and Biondi 1984).

Shoot apices, needles, hypocotyls, epicotyls, cotyledons, zygotic embryos, dormant buds, needle fascicles, and lateral buds have been used as explants for the induction of adventitious bud meristems (John 1983, Thorpe and Biondi 1984). Generally, shoot organogenesis is not as easy as root organogenesis. There are several limitations of vegetative regeneration via shoot organogenesis for many pine species. The inability to induce shoot differentiation from meristematic cells and adventitious shoot formation from other explants is often the limiting factor for regeneration of plants from tissue culture (Huang *et al.* 1991). For woody species, tissue maturation is often accompanied by a reduced capacity for regeneration. Thus it is difficult or even impossible to vegetatively propagate many woody plants by the time they are old enough to evaluate them for superior traits. Present regeneration success with conifers depends on the age of explant, tissue source, genotype and species chosen. Totipotency often appears to be limited in callus culture to a few cells identified as meristemoids.

Research interest and needs in clonal propagation have extended studies into the molecular level to correlate organogenesis with changes in biochemical metabolism and genetic events within the target tissue (Bertrand-Garcia *et al.* 1992, Mandaci *et al.* 1994, Stabel *et al.* 1990). Huang and Tauer (1996) showed correlation between histone variation and organogenesis. Changes in gene expression during shoot organogenesis in loblolly pine have been demonstrated (Huang *et al.* 1995).

Much physiological, biochemical and tissue culture research has been focused on understanding control of shoot organogenesis, which plays an important role in plant regeneration. Environmental influences on shoot organogenesis can be dramatic and are thought to be relatively well understood, at least in terms of manipulating culture conditions for shoot induction (Hicks 1994, Thorpe 1990). However, the fundamental developmental biology of the shoot organogenesis process is not well understood. Major work has been done with non-woody species, and it is believed the organogenesis process shares some common features between woody and non-woody species as well as with gymnosperms (Thorpe 1990). In addition, woody and non-woody species respond to similar culture conditions for induction of shoot organogenesis in culture. This suggests that the mechanisms controlling the developmental process of organogenesis is highly conserved among plants, raising questions about how this process is regulated and how the genes involved interact.

1.3.3 Genetic Studies on Loblolly Pine

Recently, tree improvement has extended studies to the molecular level to characterize the genetic basis of gene expression patterns. Sewell and Neale (1995) constructed a consensus map for loblolly pine using restriction fragment length polymorphisms (RFLPs) as genetic markers. This consensus map contains many known and characterized genes within the 12 chromosomes of loblolly pine, and could serve as the foundation for present and future genetic studies (Sewell *et al.* 1995). Also genomic and cDNA clones of two genes encoding arabinogalactan-proteins (AGPs) were isolated and characterized. AGPs are highly glycosylated proteins thought to play important roles

in plant development, and were found to be abundant in differentiating xylem of loblolly pine (Loopstra *et al.*1995). A water deficit stress inducible gene (1p5) of loblolly pine has been cloned (Dilip *et al.* 1995). The predicted translation product of the 1p5 gene is rich in glycine (40%) and serine (20%), and appears to be a cell wall targeted protein with a possible function in cell wall reinforcement (Dilip *et al.* 1995). To increase the rooting ability of *in vitro* propagation, molecular and cellular events during adventitious root initiation in loblolly pine cuttings has been studied by Goldfarb and his colleague (Goldfarb *et al.* 1995). They found the ability of a cutting to respond by forming roots is not just dependent on the availability of auxin, but by some other determinant of cellular competence. They cloned genes from loblolly pine with sequence similarities to the auxin-induced genes from pea, soybean, and *arabidopsis.* Their research indicates that at least one of the genes is induced by auxin in hypocotyl cuttings. In addition, a cDNA library with the mRNA differentially transcribed in the bud-forming apical meristems of loblolly pine has been constructed (Huang and Tauer 1996). A number of cDNA clones were identified when the shoot-forming cDNA library was screened with probes made from several known genes which are involved in cell division and proliferation, as well as in cellular metabolic events (Huang and Tauer 1996). DNA sequence analysis and predicated amino acid sequences revealed cDNA clones with homology to several genes of interest. One cDNA clone encoding a histone (H4) gene shows a relatively high homology with that of tomato, having 73.1% identity at the DNA level and 99% identity at the amino acid sequence level respectively. Increased abundance of the H4 transcripts in the proliferating meristems suggests that it plays an important role in the shoot initiation processes that preceeds rapid cell division. Other recognized cDNA clones

include several plant genes, such as genes coding for a chromosomal replication initiator protein and a cysteine protease involved in cellular metabolism (Huang and Tauer 1996).

Like most other conifers, loblolly pine is very difficult to regenerate *in vitro*, particularly for long-term cultured cells or suspension cultures. Compared with nontreated controls, biotechnological manipulations, which precede regeneration, decrease regeneration ability (Huang and Tauer 1996). In searching for regenerative tissues, much research effort has been directed toward apical meristems of plants.

The apical meristem is a specialized tissue composed of morphologically undifferentiated cells that serve as progenitors for all shoot -associated structures (Kelly and Meeks-wagner 1995). This type of tissue is competent for shoot induction. Shoot apical meristem is believed to be generative tissue and the continual activity of the apical meristem results in shoot development (Aitken-Christie *et al.* 1988). Meristemic tissue may play an important role in *de novo* organogenesis and other developmental processes. Shoot apical meristem also possesses the unique property of continuous development, and should therefore be expressing unique genetic functions (Fleming *et al.* 1993, Medford 1992). Some molecular genetics research has focused on characterization of the genetic basis and gene expression patterns in apical meristems, but most studies on the apical meristems relate to floral transition from vegetative meristems. Many genes associated with flower development have been cloned from apical meristems where they are preferentially expressed (Markley *et al.* 1993, Peng and Iwahori 1994). However, few studies on apical meristems have focused on shoot regeneration (Huang *et al.* 1995). In loblolly pine, apical meristematic tissue of young seedlings has great potential to develop *de novo* shoots (Huang *et al.* 1995). It is assumed that the apical meristem is not

only the source of signals modulating developmental processes but also the site of perception of signals for determining the developmental pathway of the apical meristem itself. Although the fate of cells produced within the apical meristem is relatively clear, much is unknown about both the genetic events that occur and the regulatory factors that are involved in cell differentiation.

Organogenesis in plants is a complicated developmental process and there is little information on the basic aspects of organ differentiation. However, this molecular study was designed to help us understand the series of cellular, biochemical, and genetic events involved in organ development. Study of the molecular genetics of flowering (Markley *et al.* 1993) has evolved at a rapid rate, and the breakthrough in understanding flower development suggests success in studies of plant organogenesis. Recently, in a related study with rice, a cDNA clone specific to plant regeneration was isolated from embryogenic cultures through differential screening of an embryogenic cDNA library (Mizobuchifukuoka *et al.* 1996). Furthermore, patterns of protein synthesis in organogenic and non-organogenic cultures were assessed. Differences in the protein profile were found using polyacrylamide gel electrophoresis, with a few new protein bands observed in the bud-initiating tissues. This is evidence that specific proteins were synthesized during shoot differentiation, and it suggests that the newly synthesized proteins are necessary for specific cellular metabolism leading to formation of adventitious shoots (Huang and Tauer 1994). Shoot apical meristems of loblolly pine proved to be competent to respond to induction for organogenesis (Huang and Tauer 1994). The study showed that a set of novel mRNAs was rapidly synthesized after the meristems were placed on shoot-induction medium (i.e., a modified LP containing 2.5 to

5 mg/l benzylaminopurine). The results also showed apparent differences in gene expression between treated and non-treated meristems. The newly synthesized mRNAs could serve as modulators to activate cells and as messages to signal the shift from simple cell proliferation to organ differentiation (Huang and Tauer 1996).

1.3.4 PCR Based Differential Display

The PCR method was devised and named by Mullis and colleagues at the Cetus Corporation (Mullis & Faloona 1987). It is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. Although the principle has been described for 20 years, the use of PCR was limited until a heat-stable DNA polymerase became widely available (Chien *et al.* 1976).

With the advent of heat stable DNA polymerase, the PCR has rapidly become one of the most widely used techniques of molecular biology. It is a rapid, inexpensive and simple means of producing microgram amounts of DNA from minute quantities of source material, and it is relatively tolerant of poor quality template (Taylor 1991). Many variations on the basic procedure have now been described and applied. For example, the PCR has had a major impact on the diagnosis and screening of genetic disease and cancer, and on the rapid detection of slow growing microorganisms and viruses, such as HIV. Use of PCR has established a central role in the human, animal, and plant genome projects. In most molecular biology laboratories, the PCR has been routinely used in processes such as probe preparation, clone screening, mapping and subcloning, preparation of sequencing templates, and also for more advanced applications such as cloning very low abundance transcripts and gene recombination (McPherson *et al.* 1991).

Starting materials for gene analysis and manipulation by the PCR may be genomic DNA, RNA, and nucleic acids from archival specimens, cloned DNA, or PCR products themselves. The requirements of the reaction are simple: deoxynucleotides to provide both the energy and nucleosides for the synthesis of DNA, primer, template, a buffer containing magnesium and heat stable DNA polymerase. The availability of heat stable DNA polymerase gives the PCR two major advantages. First, replenishment of polymerase after each heating step is not required. Second, annealing of the oligonucletide primers is more specific and DNA synthesis is more rapid at higher temperatures.

With each cycle of heating and cooling, the amount of DNA in the region flanked by the primer will double. The number of cycles required for optimum amplification varies depending on the amount of starting material and the efficiency of each amplification step. Generally, 25 to 35 cycles should be sufficient to produce 100ng to 1ug of DNA of a single copy human sequence from 50ng of genomic DNA (Taylor 1991). A final incubation step at the extension temperature (usually 72C) results in fully double-stranded molecules from all products (Taylor 1991). Because the PCR is a highly sensitive and rapid technique for detecting extremely small amounts of a specific DNA or RNA, many new methodologies have been developed based on it.

The mRNA differential display (DD-PCR) is a newly developed method, reported by Liang and Pardee (1992) to select for novel genes expressed in mammary tumor cells but not in normal cells (Liang *et al.* 1992). Since the development of the differential display process in 1992, the application of this process has expanded into almost all areas of science. Researchers have use differential display to successfully isolate differentially

expressed cDNAs from mammalian tissues, such as mammary tumor cells (Liang *et at.* 1992), from glucose induced bovine aortic smooth muscle cells (Nishio *et al.* 1994) and from a variety of other tissues.

The differential display method is based upon comparisons of most mRNAs expressed in two or more cell populations, by running their reverse transcribed PCR products on sequencing gels in adjacent lanes. The differential expression can be readily detected by eye, the band cut out of the gel, eluted, and after PCR amplification used directly as a probe in Northern blot analysis. The process includes the reverse transcription of the mRNAs with oligo-dT primers anchored to the beginning of the poly(A) tail, followed by the PCR reaction with a second primers arbitrary in sequence. The amplified cDNA sub-populations of 3' termini of mRNAs as defined by this pair of primers are size separated on a DNA sequencing gel. By changing primer combinations, 15,000 individual mRNA species from a mammalian cell may by visualized. Differential display provides the fingerprinting for mRNA analogous to 2-D protein gel electrophoresis to visualize proteins. The mRNA samples analyzed side by side would allow differentially expressed genes to be identified and probes to be recovered and used to clone their cDNAs or genomic DNA (GenHunter, Nashville, TN).

The mRNA differential display method has several technical advantages over existing methods. Differential display is based on PCR and DNA sequencing gel electrophoresis, which are simple technologies. Differential display is a sensitive method because 5 ug of total RNA is enough to cover all the anchored oligo-dT primers used in all combinations with 80 arbitrary primers. This would cover the majority of mRNAs in an eukaryotic cell. Researchers found 90-95% of the bands of mRNA differential display

are reproducible from run to run; in addition, more than two RNA samples can be compared at one time, revealing genes unique to a process instead of cell type specific genes, therefore identification of the gene is built in. Both dominant genes (oncogenes) and recessive genes (tumor suppressor genes) can be detected simultaneously. Another advantage of differential display is the speed of this technique. The pattern of a mRNA differential display can be obtained in two days and one can follow success at each step.

The PCR based differential display technology was used for mammalian tissues first (Liang *et al.* 1992). Presently, more and more researchers are successfully using this method (Goormachtig *et al.* 1995). Wilkinson et al (1995) identified genes involved in ripening strawberry fruit by differential display. The genes related to cotton fiber development were isolated by this method (Song-Ping 1995). Furthermore, this technique has been used to study pathogen stress in alfalfa (Truesdell & Dickmean 1997) and genes regulating sucrose in cultured rice cells (Tseng *et al.* 1995). Differential display has also been used to study plant developmental processes, cloning new potential markers of *in vitro* tomato morphogenesis (Torelli *et al.* 1996) and identifying developmentally regulated genes during somatic embryogenesis in eggplant (Momiyama *et al.* 1995).

1.3.5 Gene Cloning and a cDNA Library

Recombinant DNA technology has revolutionized molecular biology and genetics. Today any segment of DNA can be isolated and replicated to provide sufficient copies of a gene to study its structure and expression. In addition, cellular systems can be designed to produce large quantities of a particular biological substance. Recombinant

DNA techniques are being used in new industrial and medical technologies, and in agriculture for the development of new varieties of plants. The core process of DNA recombination technology is gene cloning. Once a gene and its regulators has been cloned there is almost no limit to the information that can be obtained about the structure and expression of that gene.

The basic steps in a gene cloning experiment are several: A fragment of DNA containing the gene to be cloned is inserted into a vector to produce a chimera or recombinant DNA molecule. The vector transports the gene into a host cell. Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place. After a large number of cell divisions, a colony, or clone of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule (Brown 1995).

There are very few areas of biological research that have not used gene cloning, with PCR, and the recombinant DNA techniques. Agricultural research in particular has benefited greatly from gene cloning, which provides a new dimension to crop improvement. For example, gene cloning enables directed changes to be made to the genotype of a plant, circumventing the tedious phenotypic selection processes inherent in conventional breeding (Yoder and Goldsbrough 1994). Cloning can lead to developing plants that resist insect attack (Fischhoff 1987) or plant pathogens by transformation with cloned genes (Truve 1993).

The first libraries of complementary DNA (cDNA) clones were constructed in the mid-to-late 1970s using RNA-dependent DNA polymerase (reverse transcriptase) to convert poly (A^+) mRNA into double-stranded cDNA suitable for insertion into prokaryotic vectors.

A cDNA library represent the information encoded in the messenger RNA (mRNA) of a particular tissue or organism. Each organism and tissue type has a unique population of mRNA. The information encoded by the RNA is converted into a stable DNA duplex (cDNA) and then inserted into a self-replicating vector. A representative cDNA library contains full-length copies of the original population of mRNA from each organism or tissue type.

The starting material for any representative plant cDNA library is a supply of mRNA from the plant tissue of choice. The quality and quantity of the mRNA used is of fundamental importance to the construction of a cDNA library. The cDNA synthesis may be achieved using one of two methods. Short oligonucleotides containing 12 to 20 deoxythymidines act as primers for reverse transcription of mRNA. The second method of cDNA synthesis is random priming. Studies have indicated that sequences close to the 5' end of very long mRNAs are more readily cloned using this method. (Brown 1995). The double-stranded cDNA molecules obtained from the reaction of DNA polymerase-I are then inserted into a plasmid or a phage vector by attaching artificial restriction enzyme sites onto the ends of the cDNA. After packaging into phage particles or plasmid vectors, the library is completed and ready for screening.

Once the library has been plated out, the phage plaques are transferred to duplicated nitrocellulose filters or nylon membranes. Incubating these nitrocellulose

replicas with a ^{32}P labeled nucleic acid probe constitutes the library screening. Generally, the probe could be a cloned DNA fragment of the target gene (e.g. A DD-PCR fragment), and this is used to search for clones that contain additional sequences flanking the clone fragment. In other cases, a closely related gene may have already been cloned and can be used as a probe for the gene of interest using conditions that allow partially matching sequences to hybridize.

After an individual plasmid containing a single cDNA fragment has been obtained, Southern blot analysis with the probe is required to confirm its identity. The cloned gene is then sequenced, and this sequence is compared with all sequences in the database in GenBank using a computer search. One can determine if this clone encodes the protein of interest or a previously unknown protein.

This technology has developed into a powerful and universal tool in the isolation, characterization, and analysis of both eukaryotic and prokaryotic genes.

CHAPTER II

MATERIALS AND METHODS

2.1 Establishment of Shoot Apical Meristemic Culture

In order to clone shoot organogenesis regulation genes by comparing their differential expressions within non-organogenic tissue, organogenic cultures of loblolly pine at different developmental stages were developed using plant tissue culture.

2.1.1 Preparation of Seedlings of Loblolly Pine

Seeds of loblolly pine family H-17 were used in our experiment. Seedlings were obtained from sterilized seeds which were germinated for five to seven days at room temperature on sterile 0.8% agar-solidified water in petri dishes.

Seed sterilization:

1. Pine seeds were soaked in 1% fungicide solution for 10 hours with agitation.
2. The seeds were rinsed with sterile distilled water to remove the fungicide.
3. Seeds were surface disinfected in 30% hydrogen peroxide (H_2O_2) for 10 minutes with agitation.
4. The H_2O_2-treated seeds were rinsed three times with sterile distilled water (1-5-1 minutes).

5. The wet seeds stood in flask for 6 hours or overnight.
6. After six hours, steps 3 and 4 were repeated.
7. The empty nonviable seeds that float were discarded. Surface sterilized seeds were placed into petri dishes for seed coat removal.
8. Seed coats were removed using sterile forceps and scalpel. Coat removal is necessary for germination of the seeds.
9. Seeds were further sterilized by soaking in 0.5% mercuric chloride ($HgCl_2$) in a 50 ml. flask for 10 minutes with agitation.
10. Seeds were rinsed three times with sterile distilled water (5 minute each time).
11. Seeds were transferred to a petri dish and sterilized by soaking in 15% chlorox (commercial bleach) for 5 minutes with agitation.
12. Seeds were rinsed three times with sterile distilled water (1-5-1 minutes).
13. Sterilized seeds were transferred onto 0.8% water agar medium in petri dishes (25 seed / dish) with sterilized forceps.
14. The dishes were sealed and incubated in continuous light at 20-25 °C for 5-7 days until germination.

2.1.2 Preparation of Shoot Apical Meristemic Tissue

When the hypocotyls had grown out of the megagametophyte approximately 5 mm., the seedlings were removed from the agar plates and prepared for shoot apical meristemic tissue induction.

Preparation of tissue for shoot apical meristem induction

1. The young seedlings were removed from agar plates and the megagametophytes were removed from the seedlings.
2. The seedlings were surface sterilized using 15% Clorox (commercial bleach) for 10 minutes with agitation.
3. The seedlings were rinsed three times with sterile distilled water (1-5-1 minutes).
4. The cotyledon, radical and most of the hypocotyl were excised.
5. The shoot apical meristem (1mm in length) was inserted up-side down into LP medium (Tables 2.1, 2.2) in a petri dish.
6. The dishes were sealed and grown in continuous light at 20-25 °C.
7. Samples of the shoot apical meristemic tissues at three days, one week, two weeks and three weeks were picked and stored in -80 °C for RNA isolation.

Table 2.1. Stock solutions for LP medium

Stock	Components	Amount added	Total volume (ml)
Nitrates	$Ca(NO_3)_2.4H_2O$	60.0 g	500
	NH_4NO_3	20.0 g	
KNO_3	KNO_3	90.0 g	500
Sulfates	$CuSO_4.5H_2O$	12.5 mg	500
	$MgSO_4.7H_2O$	18.0 g	
	$MnSO_4$	0.69 g	
	$ZnSO_4.7H_2O$	0.43 g	
PBMO	H_3BO_3	0.31 g	500
	KH_2PO_4	13.5 g	
	Na_2MoO_4	10.64 mg	
Halides	$CoCl_2.6H_2O$	1.25 mg	500
	KI	4.0 mg	
Inositol	Myo-Inositol	25.0 g	250
Thiamine HCl	Thiamine HCl	10.0 mg	
Fe-EDTA	$FeSO_4.7H_2O$	1.5 g	500
	Na_2EDTA	2.0 g	

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Table 2.2. Composition of LP medium (500 ml)

Nutrient Components	Amounts (from stocks)	Final Concentration (mg/l)		Treatment
H_2O	435 ml			
Nitrates	2.5 ml	$Ca(NO_3)_2.4H_2O$	600	
		NH_4NO_3	200	
KNO_3	2.5 ml	KNO_3	900	
Sulfates	2.5 ml	$CuSO_4.5H_2O$	0.125	Autoclave
		$MgSO_4.7H_2O$	180	
		$MnSO_4$	6.9	
		$ZnSO_4.7H_2O$	4.3	
PBMO	2.5 ml	H_3BO_3	3.1	Sterilized
		KH_2PO_4	135	
		Na_2MoO_4	0.1064	
Halides	2.5 ml	$CoCl_2.6H_2O$	0.0125	
		KI	0.04	
Fe-EDTA	2.5 ml	$FeSO_4.7H_2O$	15	
		Na_2EDTA	20	
Agar	4 g			
H_2O	30 ml			
Inositol Thiamine HCl	5 ml	Myo-Inositol	1000	Filter
		Thiamine HCl	0.4	
Sucrose	15 g			Sterilized
BAP 1000	1.25 ml			
NAA 1000	5 ml			
pH	5.5			

2.2 RNA Isolation

To compare the expression patterns of different development stage tissues, the RNA of shoot apical meristemic tissues and other control tissues was isolated.

2.2.1 Decontamination of RNase

All items including glassware and plasticware to be used for experiments with RNA are treated to be essentially free of RNase and sterilized. Gloves were worn at all times during the preparation of materials and solutions for the isolation and analysis of

RNA as well as during all manipulations involving RNA. It is necessary to change gloves frequently.

Glassware treatment:

Glassware was treated by baking at 250 °C for 4 or more hours or at 180 °C for at least 8 hours.

Plasticware treatment:

1. Plasticware were soaked in a solution of 0.1% diethylpyrocarbonate (DEPC) for 2 hours at 37 °C.
2. Plasticware were then rinsed several times with sterile water and autoclaved for 15 minutes on liquid cycle to remove traces of DEPC.
3. Equipment that could not be sterilized by heating was soaked in 0.1% DEPC solution overnight and then rinsed thoroughly with sterile, DEPC-treated water.

Electrophoresis tank treatment:

1. The gel rig was cleaned with detergent solution, rinsed with water.
2. Dried with ethanol.
3. Treated with a solution of 3% H_2O_2 for 10 minutes, rinsed thoroughly with DEPC-treated water.

Water and solutions treatment:

1. All solutions were prepared using RNase-free glassware and chemicals reserved for RNA work.
2. The solutions were treated with 0.1% DEPC at 37 °C overnight with occasional vigorous shaking, then autoclaved.

3. After autoclaving, the hot solutions were swirled to remove residual CO_2 and DEP, DEPC breakdown products.

2.2.2 Simultaneous Isolation of RNA and DNA from Conifers

Isolation of good quality RNA from conifer tissues is often difficult because of the large amount of phenolic compounds, polysaccharides, and unknown secondary metabolites. A protocol for total RNA isolation from pine tissues was specially developed, so it could be used for combined RNA and DNA isolation.

Shoot apical meristemic tissues treated on organogenesis-induction medium for 3-days, 1-week, 2-weeks, 3-weeks and controls (hypocotyls, cotyledons, and radicals) were taken out off the -80 °C storage right before isolation.

<u>Isolation of total mRNA:</u>

1. 10-15 ml extraction buffer was warmed to 65 °C in a water bath. 2-mercaptoethanol was added into the buffer to a final concentration of 2%.
2. 1-2 g of tissue in liquid nitrogen was grinded to a fine powder with a mortar and pestle.
3. The frozen powder was mixed with 10-15 ml of extraction buffer and incubated for 15 minuets at 65 °C.
4. The incubant was extracted two times with an equal volume of $CHCl_3$-IAA, and phases were separated at 3.5K rpm at room temperature for 10 minutes in an SS34 rotor.
5. 1/4 volume 10 M LiCl was added to the supernatant, mixed gently, and precipitated 5 hours at -20 °C or overnight at 4 °C.

6. Centrifuged at 10 k rpm for 20 minutes and pellet was collected for isolating RNA. The resulting supernatant may be saved for DNA isolation.

7. The RNA pellet was dissolved into 500 μl of SSTE.

8. Extracted once with an equal volume of $CHCl_3$-IAA.

9. 2 volumes of ethanol to the aqueous phase were added and RNA was precipitated at -80 °C for at least 30 minutes or at -20 °C for 2 hours.

10. Spun 20 minutes in a microfuge at full speed to pellet RNA, and dried pellet was resuspended in the DEPC-treated sterile H_2O, the resultant RNA was ready to use.

Additional Steps For DNA Isolation:

1. DNA was precipitated from the supernatant from step 6 with one volume of isopropanol.

2. Spun at 10 k rpm for 20 minutes to collect DNA pellet.

3. The DNA pellet was re-suspended in 0.5 ml TE buffer.

4. The crude DNA sample was extracted with an equal volume of $CHCl_3$-IAA.

5. Aqueous phase was collected and precipitated with one volume of isopropanol.

6. DNA was pelleted by centrifugation and washed the DNA with 70% ethanol.

7. DNA was dissolved into an appropriate volume of TE buffer.

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Solutions and Buffers:

Extraction buffer:

		200 ml
2%	CTAB (hexadecyltrimethylammonium bromide)	4.0g
2%	PVP (polybinylpyrrolidinone K30)	4.0g
100mM	Tris-HCl, pH 8.0	2.42g
25mM	EDTA	1.86g
2.0M	NaCl	23.4g
0.5g/l	Spermidine	0.1g

(mixed, autoclaved, and stored at 4 °C , and 2% 2-mercaptoethanol was added just before use).

SSTE buffer:

		100 ml
0.6M	NaCl	3.5g
0.2%	SDS	0.2g
10mM	Tris-HCl, pH 8.0	0.12g
1mM	EDTA	37 mg

(mixed, autoclaved)

10M Lithium chloride (LiCl):

	100 ml
Lithium Chloride	42.39g
H_2O	80 ml

(autoclave)

Chloroform ($CHCl_3$): isoamyl alcohol (IAA) = 24:1

Isopropanol: commercial stock was used

2.2.3 RNA Formaldehyde Gel Electrophoresis

1. Formaldehyde gel (11x14 cm horizontal gel) was prepared

	100.0 ml	80.0 ml	30.0 ml
1 % agarose	1 g	0.8 g	0.3 g
DEPC-dH_2O	72 ml	57.6 ml	21.6 ml
10x gel-running buffer	10 ml	8.0 ml	3 ml
18 % formaldehyde (12.3 M)	18 ml	14.4 ml	5.4 ml

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Agarose was dissolved in DEPC-dH_2O in the microwave, DEPC-dH_2O, 10x gel-running buffer and formaldehyde were added (Sigma F1635), and swirled to mix. The gel was castled in the hood, and the gel was allowed to set at room temperature for at least 30 min.

2. RNA samples were prepared

	20 µl	50 µl
RNA (5-30 µg/lane)	x µl	x µl
10x gel-running buffer	2.0 µl	5.0 µl
1.7 % formaldehyde	3.5 µl	8.8 µl
50 % formamide (deionized)	10 µl	25 µl
DEPC-dH_2O	20-x-y	50-x-y

y = gel running buffer + formaldehyde + formamide

A "master mix" was prepared for multiple RNA samples by mixing all reagents except RNA's and then transferred an appropriate volume of mix into each RNA sample in a tube.

3. The samples were incubated at 65 °C for 15 minutes, and then quenched briefly on ice. All of the fluid in the bottom of the tube was deposited by spinning briefly (i.e., a few seconds).

4. The gel was pre-run at 80V for 5 minutes before the samples were loaded.

5. 2 µl of sterile, DEPC-treated gel-loading buffer was added to each sample. Additional 1 µl of EtBr solution (1 mg/ml in DEPC-d$H_2$0) was added to each RNA sample.

6. The samples were loaded into wells of the gel. A molecular marker (3-5 µg of BRL RNA ladder, 0.24-9.5 kb) was loaded into the outside lanes of the gel, and prepared as described for the RNA sample.

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7. The gel submerged in 1x formaldehyde gel-running buffer was run for a few hours at 80V or overnight at 20V. Although constant re-circulation of the buffer was not necessary for a short run, thė buffer should be re-circulated while run overnight.

8. Electrophoresis was stopped when the dye has migrated ½ the length of the gel.

Reagents and buffers;

10X formaldehyde-gel running buffer (1 liter)

Reagent	10X
MOPS (free acid, MW 209.3)	200 mM
NaOAC (anhydrous)	80 mM
EDTA (0.5 M, pH 8.0)	10 mM

41.86 g of MOPS (Sigma M8899) and 6.56 g of NaOAC were dissolved in 800 ml DEPC-treated water, pH to 7.0 with NaOH, and 20 ml of DEPC-treated EDTA was added. The volume was adjusted to 1 liter with DEPC-treated water and autoclaved.

Formaldehyde gel-loading buffer (sterile)

	10 ml
50 % glycerol	5 ml
1 mM EDTA (pH 8.0)	20 µl (0.5 M stock)
0.25 % bromophenol blue	25 mg
0.25 % xylene cyanol FF	25 mg
DEPC-dH_2O	5 ml

2.2.4 Removal of DNA Contamination from RNA

In order to use mRNA Differential Display technology to identify differentially expressed genes, it is crucial that the total RNA should be absolutely free of DNA

contamination. Dnase I from MessageClean™ Kit (GenHunter, Nashville, TN) was used to clean up the RNA samples.

Protocol:

1. Dnase I digestion

   Added in order

Total RNA in DEPC-H_2O	50.0 μl (10-50 μg)
10X Reaction buffer	5.7 μl
DNase I (10 unit/ul)	1.0 μl

   Mix well and incubate for 30 minutes at 37 °C.

2. 40 μl of Phenol/$ChCl_3$ (3:1) were added to remove proteins. Vortexed for 30 seconds and kept 10 minutes on ice.

3. Spun in an Eppendorf centrifuge at 4 °C at maximum speed for 5 minutes. The upper phase was collected.

4. 5 μl of 3 M NaOAC and 200 μl 100% EtOH were added and kept at -80 °C for more than 1 hour.

5. Spun for 10 minutes at 4 °C. The supernatant was carefully removed and RNA pellet was washed with 0.5 ml 70% EtOH (in DEPC water).

6. Spun for 5 minutes, and EtOH was removed. Spun briefly and the residual liquid was removed.

7. RNA was re-dissolved in 10 to 20 μl of DEPC-H_2O.

8. Quantitated by OD_{260} after 1:300 dilution of the RNA sample.

9. RNA was stored as 1 μg /μl aliquots at -80 °C, and diluted to 0.1 μg /μl with DEPC-H_2O right before used in differential display PCR reaction.

2.3 Differential Display Procedure

The mRNA Differential Display technology was used to identify genes which might be related to the shoot organogenesis. Shoot apical meristemic tissues (3-days, 1-week, 2-weeks, 3-weeks) were compared with non-organogenesis tissues (hypocotyl, cotyledon, radical) to identify tissue specific cDNA fragments by their differentially expressed patterns.

2.3.1 Reverse Transcription of mRNA

The seven different types of RNA, free of chromosomal DNA contamination, were freshly diluted. They were then reverse transcripted to cDNA with oligo-dT primers (Table 2.3) anchored to the beginning of the poly (A^+) tail. The 3' primer consists of 11 deoxythymidine residues plus one additional 3' base to provide specificity.

Protocol:

1. The all components were thawed, and set on ice.
2. Three reverse transcription reactions were set for each RNA sample for each of the three primers (M=A, G, or C)

Components (for 20 µl final volume)	
dH_2O	9.4 µl
5X RT buffer	4.0 µl
dNTP (250 uM)	1.6 µl
Total RNA (DNA-free)	2.0 µl (0.1 µg /µl)
$H\text{-}T_{11}M$ (2 uM)	2.0 µl

3. Thermocycler was programmed to 65 °C, 5 minutes-- 37 °C, 60 minutes-- 75 °C, 5 minutes --end.

4. The tubes were put into the thermocycler for 10 minutes at 37 °C. 1 μl MMLV reverse transcriptase was added to each tube, quickly mixed well by finger tipping and continued incubation.

5. At the end of reaction, the tube was briefly spun to collect condensation.

6. The tubes were stored at -20 °C for later use.

2.3.2 PCR to Amplify the cDNA and Label with ^{33}P

PCR reaction was use to amplify the amount of cDNA. The second 16 primers with arbitrary sequence (Table 2.3) anchored to the 5' end of the cDNA. Different combinations of 5' and 3' primers were used to generate a panel of PCR products which were labeled with ^{33}P for comparison of the seven RNA samples.

<u>Protocol:</u>

1. The components were thawed and set on ice.

   H-AP primer = primer #1 – primer #16. H-T_{11}M primer, M=A, G, C

   20 μl final volume for each primer set combination.

Components	Volume
dH_2O	9.2 μl
10X PCR buffer	2.0 μl
dNTP (25 uM)	1.6 μl
H-AP primer (2 uM)	2.0 μl
H-T_{11}M (2 uM)	2.0 μl
RT-mix from Reverse Transcription	2.0 μl
α-[^{33}P] dATP (1200 Ci/mmole)	1.0 μl
AmpliTaq (Perkin-Elmer)	0.2 μl
Total	20.0 μl

2. Mixed well by pipetting up and down.

3. 25 μl mineral oil was added to each tube.

4. PCR reaction was run as 94 °C, 30 second — 40 °C, 2 minutes — 72 °C, 30 seconds for 40 cycles — 72 °C, 5 minutes — end.

5. The tubes were stored at 4 °C for later use.

Table 2.3 Primers Used in mRNA Differential Display Reactions

H-T_{11}G	5'-AAGCTTTTTTTTTTTG-3'
H-T_{11}A	5'-AAGCTTTTTTTTTTTA-3'
H-T_{11}C	5'-AAGCTTTTTTTTTTTC-3'
H-AP1	5'-AAGCTTGATTGCC-3'
H-AP2	5'-AAGCTTCGACTGT-3'
H-AP3	5'-AAGCTTTGGTCAG-3'
H-AP4	5'-AAGCTTCTCAACG-3'
H-AP5	5'-AAGCTTAGTAGGC-3'
H-AP6	5'-AAGCTTGCACCAT-3'
H-AP7	5'-AAGCTTAACGAGG-3'
H-AP8	5'-AAGCTTTTACCGC-3'
H-AP9	5'-AAGCTTCATTCCG-3'
H-AP10	5'-AAGCTTCCACGTA-3'
H-AP11	5'-AAGCTTCGGGTAA-3'
H-AP12	5'-AAGCTTGAGTGCT-3'
H-AP13	5'-AAGCTTCGGCATA-3'
H-AP14	5'-AAGCTTGGAGCTT-3'
H-AP15	5'-AAGCTTACGCAAC-3'
H-AP16	5'-AAGCTTTAGAGCG-3'

2.3.3 Separation of Amplified cDNA Fragments by PAGE

The expression patterns of a group of cDNA from the same 5' and 3' end primers were compared by separating on a 6% denaturing polyacrylamide gel in TBE buffer. To insure making "bubble-free" gels, the glass plates must be thoroughly cleaned and the outer glass plate should be siliconized or coated before each use. Always wear gloves

while handling the glass plates during assembly to avoid fingerprints on the glass plates. Fingerprints will cause bubbles to form during gel casting. Polyacrylamide is a hazardous chemical and a neurotoxin. Always wear gloves, lab coat, and safety glasses while working with polyacrylamide.

Protocols:

1. The plates were cleaned thoroughly and siliconed by spreading 5 ml silanizing reagent (5% γ-coate + ice cold 100% EtOH) .
2. The glass plate sandwich was assembled.
3. The gel was castled without air bubbles. The gel was polymerized for at least two hours or overnight.
4. The buffer chamber was filled with 1 x TBE. The gel was pre-run for 1 hour till the temperature of the buffer reached 55 °C.
5. 5 μl of each sample were mixed with 2 μl of loading dye and incubated at 80 °C. The gel was loaded and run.
6. The gel set was disassembled and dried at 70 °C for 2 hours. The gel was exposed to x-ray film at -80 °C for 2 days.

2.3.4 Re-amplification of cDNA Fragment

After developing the film, auto-radiographs were oriented with the gel. Bands with differential expression patterns were located by punching through the film with a needle at the four corners of each band. Bands were cut out with a clean razor blade for re-amplification.

Protocol:

1. The gel slice was soaked along with the 3M paper in 100 μl dH_2O overnight.

2. The samples were boiled with cap of the tube tightly closed for 15 minutes.

3. Spun for two minutes to collect condensation, and the gel and paper debris were pelleted.

4. The supernatant was transferred to a new microfuge tube.

5. 10 μl of 3 M sodium acetate, 5 μl of glycogen (10 mg/ml) and 450 μl of 100% EtOH were added. Kept on dry ice or in a – 80 °C freezer for 30 minutes.

6. Spun for 10 minutes at 4 °C to pellet the DNA.

7. The supernatant was removed. The pellet was rinsed with 200 μl ice-cold 85% EtOH. Spun briefly and the residual ethanol was removed .

8. The pellet was dissolved in 10 μl of dH_2O and re-amplificated in 2 μl. The rest was saved at -20 °C.

9. The same primer set was used for re-amplification.

10. The components as following were added for 20 μl reaction:

H-AP primer = primer #1 – primer #16.
H-T_{11}M primer, M= A, G, or C

dH_2O	10.2 μl
10 x PCR buffer	2.0 μl
dNTP (250 uM)	1.6 μl
H-AP primer (2 uM)	2.0 μl
H- T_{11}M primer (2 uM)	2.0 μl
cDNA template	2.0 μl
AmpliTaq (Perkin-Elmer)	0.2 μl
Total	20.0 μl

11. 15 μl of the PCR samples were run on a 1.5% agarose gel and stained with Ethidium bromide.

12. The cDNA size was checked under UV light.

13. If the cDNA probe failed to be re-amplified in the first round of PCR, it could be amplified by another 40- cycle PCR reaction with 2 μl of the first - round PCR sample.

2.4 cDNA Cloning

In order to manipulate the cDNA fragments identified from Differential Display, we cloned them using the TA Cloning System (Invitrogen, Carlsbad, CA) and PCR-TRAP Cloning System (GenHunter, Nashville, TN).

2.4.1 TA Cloning Kit (PCRII)

The TA Cloning Kit with PCR 2.1 provides a quick, one-step cloning strategy for the direct insertion of a PCR product into a plasmid vector. The selection of the insert is base on the blue-white colony color identity caused by the insert mutageniesis of an marker gene.

Ligation Reaction:

1. Fresh PCR products were used for cloning.

2. The cDNA fragments were ligated into the PCRII vector by adding the following components (10 μl reaction):

dH_2O	5 µl
10 X ligation buffer	1 µl
PCRII vector (25 ng/ µl)	2 µl
Fresh PCR product (~10 ng)	1 µl
T4 DNA ligase	1 µl

3. Ligation reactions mix was incubated at 14 °C for at least 4 hours or overnight.

4. The ligation reactions were centrifuged briefly and placed on ice.

Transformation:

1. Appropriate number of vials of One Shot™ supplier cells were thawed on ice.

2. 2 µl of 0.5 M 2-mercaptoethanol was pipetted into each vial of the One Shot™ cells and mixed by stirring gently with pipette.

3. 1-2 µl of each ligation reaction was pipetted into cells and stirred gently with pipette to mix.

4. The vials were incubated on ice for 30 minutes.

5. The vials were heat shocked for exactly 30 seconds in a 42 °C water bath without disturbance.

6. The vials were placed on ice for 2 minutes.

7. 250 µl of SOC medium was added to each vial.

8. The vials were shaken at 37 °C in a shaker at 225 rpm for exactly 1 hour. The vials containing the transformed cells were placed on ice.

Analysis:

1. 50 µl and 200 µl from each transformation vial were placed on a LB plate containing 50 µg/ml ampicillin, X-Gal, and IPTG.

2. Incubated at 37 °C for at least 18 hours. Plate was shifted to 4 °C for 2-3 hours for color development.

3. 5 white colonies were analyzed for the presence and orientation of insert by restriction mapping.

2.4.2 PCR-TRAP Cloning System

This system utilizes a third generation cloning vector that features positive-selection for DNA inserts. Only vectors with a PCR product incorporated through blunt end ligation will have ampicillin resistance. Anything that grows on a tetracycline plate most likely will have the plasmid vector containing a DNA insert.

Ligation:

1. The reamplified PCR products from differential display were used directly for cloning without any purification or dilution.

2. For a 20 μl ligation reaction, components were added in the following order:

dH_2O	10 μl
Insert-ready PCR-TRAP vector	2 μl
10 X ligation buffer	2 μl
PCR product	5 μl
T4 DNA ligase (200 unit/ μ l)	1 μl

3. Mixed well by pipetting up and down. Allowed ligating at 16 °C overnight.

Transformation:

1. GH competent cells were thawed on ice for 10 to 15 minutes. 100 μl of GH-competent cells was aliquoted to 1.5 ml microfuge tubes and set on ice.
2. 10 μl of ligation mixture was added to the tube containing the competent cells and incubated on ice for 45 minutes.
3. The cells were heat shocked for 2 minutes at 42 °C and the tube was set back on ice.
4. 0.4 ml of LB medium was added and the cells were incubated at 37 °C for one hour.
5. Cells were mixed well by vortexing briefly.
6. 200 μl of cells was plated on each LB plate containing 20 μg /ml tetracycline.
7. The plate was incubated upside down overnight at 37 °C.
8. The tetracycline resistant colonies were scored and the plate was saved at 4 °C for further analysis.

Checking for Insert:

Checking for plasmid with DNA insert was done using PCR with a primer set that flanks the cloning site of the PCR-TRAP vector.

A. Colony Lysis

1. Using a marker pen, each Tet^R colony to be analyzed was numbered on the back of the plate.
2. 50 μl of colony lysis buffer was aliquoted into each numbered microfuge tube.

3. Each colony was picked with a clean pipette tip, and the cells were transferred into the tube with colony lysis buffer.

4. The tube was incubated at 95 °C for 10 minutes.

5. The tube was spun for 2 minutes to pellet the cell debris.

6. The supernatant was transferred into a clean tube.

7. It was used directly for PCR analysis or stored at - 20 °C for future amplification.

B. PCR Reaction

1. For each colony lysate the following components were added:

dH_2O	10.2 µl
10 X PCR buffer	2.0 µl
dNTPs (250 uM)	1.6 µl
Lgh primer	2.0 µl
Rgh primer	2.0 µl
colony lysate	2.0 µl
AmpliTaq (Perkin- Elmer)	0.2 µl

2. Mixed well and 30 µl mineral oil was added to each tube.

3. The PCR reaction was run as follows: 94 °C for 30 seconds -- 52 °C for 40 seconds -- 72 °C for 1 minutes for 30 cycles -- 72 °C for 5 minutes -- end.

4. All of the 20 µl PCR products were analyzed on a 1.5% agarose gel. The PCR product after colony- PCR was 120 bp larger than the original PCR insert due to the flanking vector sequence being amplified.

2.4.3 Small-Scale Plasmid DNA Isolation

Protocol:

1. A single bacterial colony was inoculated into 2.0 ml LB broth with antibiotic.

2. Grown at 37 °C with vigorous shaking (200 rpm) overnight.

3. About 1.4 ml of bacterial suspension was poured into a 1.5 ml Eppendorf tube.

4. Bacterial cells were pelleted in a microfuge at a full speed for 15- 30 seconds.

5. Supernatant was discarded and cell pellet was resuspended in 100 μl of GTE buffer.

6. Boiled RNase A was added to a final concentration of 20 μg / ml.

7. 200 μl SDS- NaOH solution was added.

8. Mixed gently by inverting the tube several times, and incubated on ice for 10 minutes.

9. 150 μl of ice-cold 3M NaOAC (pH 4.8) was added, mixed by gentle inversion, and incubated on ice for 5- 10 minutes.

10. The lysate was spun in a microfuge tube for 5 minutes.

11. Supernatant was carefully transferred into a clean tube.

12. The RNase and other proteins were removed by equal volume phenol extraction.

13. The upper portion was transferred into a clean tube without disturbing the inter-phase.

14. 450 μl of ice-cold isopropanol was added and mixed by inversion.

15. Plasmid DNA was pelleted and dried, and re-suspended in 10-30 μl dH_2O.

16. The DNA concentration was checked by OD_{260} with 1:150 dilution.

Solutions and Buffers:

GTE buffer:

50 mM glucose
20 mM Tris (pH 8.0)
10 mM EDTA

SDS-NaOH solution:

	2% SDS	
	0.4 N NaOH	

3M NaOAC (pH 4.8):

		100 ml
	5 M KoAC	60.0 ml
	Glacial ascetic acid	11.5 ml
	dH_2O	28.5 ml

Phenol

	Phenol and $CHCl_3$ (1:1)

2.5 Confirmation of Differentially Expressed cDNAs

To insure that the cDNAs were truly tissue-specific, the fragments cloned from Differential Display were confirmed by "Reverse Northern" and "Quick Blot Southern Analysis" before using them as probes to screen the cDNA library.

2.5.1 Reverse Prime cDNA Labeling

The radioactive labeling was performed for the first strand cDNA from mRNA by reverse transcription. The labeled cDNAs were used as probes to screen for the putative differentially expressed cDNA fragments isolated by Differential Display technology.

A pair of RNA samples was labeled by reverse transcription. The positive one was from the organogenic tissue (3 - weeks shoot apical meristem), and the cotyledon tissue was used as a negative check.

2.5.1.1 Reverse Transcription

1. The components were thawed and set on ice.
2. Two reverse transcription reactions were set in PCR tubes corresponding to each of the RNAs being compared.

3. The following components were added in order: (For 40 μl final volume)

dH_2O	X μl
5 X RT buffer	8 μl
dNTP (-C)	6 μl
T_{20} primer	4 μl
Total RNA (20 μg)	Y μl
α-[^{32}P] dCTP	5 μl

4. Mixed well by pipetting up and down.

5. The thermocycler was programmed to --65 °C, 5 minutes -- 37 °C, 60 minutes -- 75 °C, 5 minutes.

6. The tubes were put into the thermocycler and the incubation was started.

7. After the temperature had been at 37 °C for 10 minutes, the thermocycler was paused and 4 μl of reverse transcriptase was quickly add to each tube.

8. Mixed well by pipetting up and down and continued the incubation.

2.5.1.2 Removal of Un-incorporated α-[^{32}P]

To reduce the background on the blot, the un-incorporated α-[^{32}P] dCTP was removed from the labeled cDNA by gel filtration using a Sephadex G-50 spin column.

Protocol:

1. The bottom of a 1- ml disposable syringe was plugged with a small amount of sterile glass wool.

2. Sephadex G- 50 equilibrated in TE (pH 8.0) was added into the syringe by a glass pipette.

3. The column was spun down in a rotor for 3 minutes at 3,000 rpm.

4. Addition of Sephadex was continued until the packed column volume was 0.9 ml.

5. 0.1 ml of STE buffer was added and re-centrifuged at exactly the same speed and for exactly the same time as before.

6. All the waste liquid was disposed, and a decapped 0.6 ml tube was put inside the centrifuge tube.

7. STE buffer was used to take the DNA sample to a total volume of 0.1 ml, and applied to the column.

8. Centrifuged at exactly the same speed and for exactly the same time as before.

9. 100 μl of effluent was collected from the syringe in the decapped Eppendorf tube.

10. For short length DNA samples, the column was washed once with 100 μl STE buffer and two effluents were mixed together.

11. 2 μl of each labeled cDNA in a scintillation counter was counted to determine the efficiency of cDNA labeling.

2.5.2 Reverse Northern by Colony Hybridization

The positive and negative probes are hybridized with the cDNA colonies on replica membrane.

Protocol:

1. The tetracycline-resistant colonies were transferred from each plate to a nitrocellulose membrane.

2. Duplicated by pressing a second membrane on the first membrane with colonies side up.

3. The membrane with fewer cells was laid down on the top of LB plates containing tetracycline.

4. After an overnight incubation, the filters with Tet^R colonies were carefully removed.

5. The plasmids were denatured by floating the filters on 0.5 N NaOH/ 1.5 M NaCl for 5 minutes.

6. The DNA was denatured for 3 minutes by transferring the filters and floating them on 0.5 M Tris-HCl buffer (pH 7.0)/ 1.5M NaCl.

7. The filters were blotted dry on a piece of 3M paper.

8. The DNA was UV cross-linked onto the filter.

9. The filters were washed in 6 X SSC plus 0.5% SDS at room temperature for 15 minutes with vigorous shaking to remove the cell debris from the filters.

10. The duplicate filters were separated into "positive" and "negative" groups in separate containers and pre-hybridized overnight at 42 °C.

11. Two radioactively labeled cDNA probes were boiled for 10 minutes, and quenched on ice.

12. The hybridization solution was chanced and boiled single strand salmon sperm DNA was added.

13. Each probe was added to its corresponding container representing "positive" and "negative" groups, respectively.

14. Hybridized overnight at 42 °C.

15. The membranes were washed 15 minutes in 1 X SSC with 0.1% SDS at room temperature followed by washing at 60 °C for 5 minutes in 0.25 X SSC with 0.1% SDS. The membranes were checked frequently to avoid over-washing.

16. The membranes were blotted dry on a piece of 3M paper.

17. The duplicate membranes were placed on X-ray film at - 80 °C.

18. The films were developed and the result was evaluated by comparing the hybridization signals of the corresponding duplicate films.

2.5.3 Bi-directional Blotting Southern Analysis

The cDNA fragments from DD-PCR were digested from the vectors by restriction enzymes (EcoR I and Hind III) and separated in an agarose gel. Duplicate membranes were obtained by gel blotting. The organogenic tissue specific cDNA should hybridize with the positive probe.

Protocol:

1. 20 μg of plasmid DNA was digested with a restriction enzyme at 37 °C for 2-3 hours.

2. 2 μl of loading buffer was added to each sample.

3. 0.8 % agarose gel was added into 0.5 X TBE buffer.

4. The samples were loaded into the gel and a DNA size marker was included.

5. The DNA was run towards the anode at 50-80 V until the bromophenol blue dye reaches 2/3 of the length of the gel.

6. The gel was stained in 1 μg/ml ethidium bromide solution for 10 minutes.

7. The DNA was visualized on the UV transilluminator, and the gel was photographed alongside a ruler.

8. The gel was incubated in 500 ml of 0.25 M HCl for 15 minutes until the blue dye turns yellow.

9. The gel was rinsed twice in dH_2O.

10. The DNA was denatured by placing the gel in 0.5 M NaOH for 30 minutes.

11. The gel was neutralized in 1M NH_4OAC for 20 minutes twice.

12. The gel was rinsed in 6 X SSC briefly.

13. A bi-directional blot (Figure 2.1) was set.

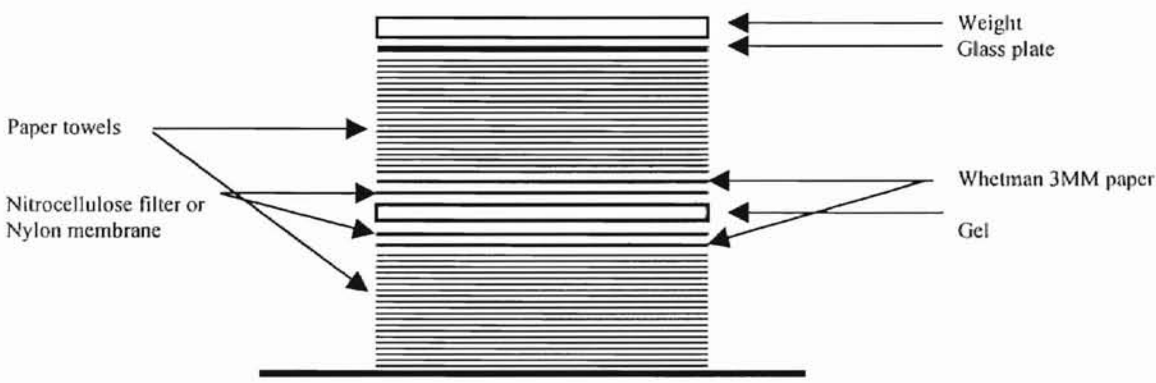

Figure 2.1 Bi-Directional Bloting Diagram

14. The cDNA was transferred to the membranes for 2 hours.

15. The membranes were briefly rinsed with 6 X SSC.

16. The DNA was UV cross-linked onto the filter.

17. The duplicate filters were separated into "positive" and "negative" groups in separate containers and prehybridized overnight at 42 °C.

18. Two labeled cDNA probes (+ and -) were boiled for 10 minutes and quenched in ice.

19. The hybridization solution was changed and boiled single strand salmon sperm DNA was added.

20. Each probe was added into its corresponding container representing "positive" and "negative" groups, respectively.

21. Hybridized overnight at 42 °C.

22. The membranes were washed 15 minutes in 1 X SSC with 0.1% SDS at room temperature followed by washing at 60 °C for 5 minutes in 0.25 X SSC with 0.1% SDS.

23. The membranes were blotted dry on a piece of 3M paper.

24. The duplicate membranes were exposed to a X-ray film at -80 °C.

25. The films were developed and the result was evaluated by comparing the hybridization signals of the corresponding cDNAs.

2.6 cDNA Library Screening

The confirmed cDNA fragments, which showed organogenic-specific, were used as probes to screen the organogenic cDNA library to obtain the full-length organogenesis genes.

2.6.1 Random Prime DNA Labeling

Protocols:

1. The cDNA (50-100 ng in a total volume of 6.96 μl H_2O) was denatured in a

microfuge tube at 95 °C for 5 minutes.

2. Quenched on ice immediately after boiling.

3. The following was added to a microfuge tube on ice (for 25 μl):

Denatured DNA	6.96 μl
10 mg/ml BSA	1.0 μl
RPL buffer	11.4 μl
50 μCi ^{32}P-dCTP	5.0 μl
Klenow (2.5 unit)	0.5 μl

4. The reaction was allowed at room temperature for at least three hours to overnight.

5. The reaction was stopped by adding 2 μl of 0.2 M EDTA (pH 8.0).

6. Incorporated and unincorporated nucleotides were seperated by spin-column procedure (See 2.5.1.2).

7. Samples were measured in scintillation counter.

8. The radio-labeled probe was stored at -20 °C until it was needed.

Solutions and Buffers:

OL Buffer:
1 mM Tris, pH 7.5
1 mM EDTA, pH 8.0
90 OD units /ml hexamers (Pharmacia, pd $(N)_6$, 27-2166-01)

HEPES: 1 M, pH 6.6 with NaOH, filter- sterilized and stored at 4 °C.

dNTP solution:		0.5 ml	store at -20 °C
250 mM	Tris, pH 8.0	125 μl 1M Tris, pH 8.0	
25 mM	$MgCl_2$	12.5 μl 1 M $MgCl_2$	
50mM	BME	1.75 μl β-mercaptoethanol	
0.1 mM	dATP	5.0 μl 10 mM dATP stock	
0.1 mM	dGTP	5.0 μl 10 mM dGTP stock	
0.1 mM	dTTP	5.0 μl 10 mM dTTP stock	
	H_2O	346 μl	

RPL Buffer:

Solutions HEPES/OL/dNTP were mixed in a ratio of 100 : 28 : 100.

2.6.2 Screen the cDNA Library

Dr. Y. Huang has established the cDNA library from shoot organogenic tissues in our laboratory.

2.6.2.1 cDNA Library Tittering

Protocols:

1. 50 ml culture of XL1-blue MRF cells were grown on LB medium containing 0.2 % maltose + 10 mM $MgSO_4$, grow overnight at 30 °C shaking at 200 rpm.
2. The cells were re-suspended in 0.5 original volume with sterile 10 mM $MgSO_4$.
3. 50 ml bacteria XL1- blue MRF' cells were spun down at 6,000 g for 20 minutes.
4. The bacteria cells were re-suspended in 5 ml 10 mM $MgSO_4$, and it was set on ice.
5. The library stock was diluted by 1X SM to three concentrations (10^{-2},10^{-4}, and 10^{-6}).
6. Mixed with 200 μl bacteria host cell (XL1- blue MRF').
7. Incubated in 37 °C water bath for 17 minutes.
8. The melted top agarose was kept in 65 °C water bath, and warm up the NZY plate (100 x 15 mm) at 37 °C.
9. 3 ml top agarose was quickly mixed with bacteria and phage at 45-50 °C, and

mixed well by inverting the tubes.

10. The mixture was poured on the NZY small plates, make smooth surface and dry the plates.
11. The plates were incubated at 37 °C for 4 - 5 hours.
12. The number of phage plaques were counted.

2.6.2.2 First Screening

Protocol:

1. 250 μl of host cells and 8 ml top agarose were plated on large 150 mm NZY plates (> 2 day-old) to 50,000 pfu/plate (30 μl library phage at 10^{-4} dilution).
2. The plates were incubated at 37 °C. Grow for 4-5 hours.
3. The plates were chilled for 1 hour at 4 °C to prevent the NZY top agarose from sticking to the nitrocellulose membrane.
4. Transferred onto a nitrocellulose membrane for 2 minutes.
5. A needle with waterproof ink was used to prick through the membrane and agar for orientation.
6. A second duplicate nitrocellulose membrane was made by transferring as before, but for 4 minutes.
7. The membranes were treated as follows (process on 3 mm papers)
   - A. Denatured for 5 minutes in 1.5 M NaCl/0.5 M NaOH.
   - B. Neutralized for 7 minutes in 1.5 M NaCl/0.5 M Tris (pH 8.0)
   - C. Rinsed 2 minutes in 2xSSC buffer solution.

8. Blot-dried briefly on an absorbency paper

9. The DNA was UV cross-linked to the membranes twice using the auto-crosslink setting (120,000 μJ of UV energy).

10. The stock agar plates of the transfers were stored at 4 °C to use after screening.

11. The pre-hybridization solution was pre-heated to 50 °C without the salmon sperm DNA.

12. The salmon sperm DNA was boiled for 10 minutes, quenched on ice and then added to the warm pre-hybridization solution.

13. The membranes (4 ml/ membranes) were prehybridized at 42 °C for 4 hours to overnight.

14. The hybridization solution was changed and new denatured salmon sperm DNA was added.

15. The labeled probes #10 and #11(1 X 10^6 – 5 X 10^6 counts / ml of hybridization solution) were boiled for 10 minutes, and quenched on ice.

16. Hybridized with probes #10 and #11 at 42 °C for 20 hours.

17. The membranes were washed at 52 °C in 1000 ml washing solution for 20 minutes, the washing solution was changed and the membranes were washed for another 10 minutes.

18. Membranes were exposed to film for 50 hours at -70 °C.

2.6.2.3 Secondary Screening

Protocols:

1. The membranes were oriented to the film and using the marked "dots" where the needle poked through the strongest "putative" clones were determined.
2. A Pasteur pipet and an inverted yellow pipet tip were used to transfer the clone into 1 ml of SM buffer and 20 μl of chloroform.
3. Vortexed and briefly spun. The phage stocks were stored at 4 °C.
4. The membrane was stripped at 65 °C for 2 hours for probe #14 and #17 hybridization.
5. Diluted and titered with host cells as before (see section 2.6.2.1) to determine the concentrations.
6. Plated on small 100 mm NZY plates (> 2 day-old) to 200 -500 pfu/plate with phage stock (at 10^{-4} dilution) plus 200 μl of host cells and 3 ml top agarose.
7. The plates were incubated at 37 °C, and grown for 4-5 hours.
8. The plates were chilled for 1 hour at 4 °C.
9. Made lifts from plates as before (2.6.2.2).
10. Pre-hybridization and hybridization were performed as before (2.6.2.2).

2.6.2.4 *In Vivo* Excision

Protocols:

1. The plaque of interest was cored from the plate and transferred to the tubes containing 500 μl of SM buffer and 20 μl of chloroform.

2. The tubes were shaken to release the phage particles into the SM buffer.

3. The tubes were incubated overnight at 4 °C.

4. An overnight culture of XL1-Blue MRF' and SOLR cells were grown in LB broth at 30 °C.

5. The XL1-Blue MRF' cells were spun down at 1,500x g. The cells were re-suspended by 10 mM $MgSO_4$ to an $OD_{600} \geq 1.0$.

6. The following components were combined in a 50-ml conical tube:

   200 µl XL1-Blue MRF' cells
   250 µl of phage stock
   1 µl of the ExAssist helper phage (> 1 X 10^6 pfu/µl)

7. The tube was incubated at 37 °C for 15 minutes.

8. 3 ml of LB broth was added then shaken at 37 °C and 100 rpm for 2.5 hours.

9. The conical tube was heated at 70 °C for 15 minutes and then spun at 4,000 x g for 15 minutes.

10. The supernatant was decanted into a sterile conical tube. This stock contained the excised pBluescript phagemid packaged as filamentous phage particles. Stored at 4 °C.

11. The SOLR cells were spun down at 1,500x g. and re-suspended by 10 mM $MgSO_4$ to an $OD_{600} = 1.0$.

12. 50 µl phage supernatant stock and 200 µl SOLR cells were combined and incubated in a tube at 37 °C for 15 minutes.

13. 25 µl of the combination solution was plated on LB plate with 50 µg /ml ampicillin, the plates were incubated overnight at 37 °C.

14. Ampicillin resistant single colonies were selected and 4 ml culture was made for DNA isolation.

Media and Reagents:

NZY Agar (1L)
1L NZY broth, adjust the pH to 7.5 with NaOH
15 g of agar
Autoclave, pour into petri dishes avoid bubbles.

NZY top agarose (1 L)
1L of NZY broth
Add 0.7% (W/V) agarose.

Hybridization and Prehybridization Solution (350 ml)
17.5 ml 100x Denhardts
87.5 ml 20x SSPE
3.5 ml 10% SDS
4.35 ml 10 mg/ml ss DNA (denatured, sonicated salmon sperm DNA)
237.15 ml dH_2O

Washing Solution (1L)
740 ml dH_2O
250 ml 20 X SSPE (5 X SSPE)
10 ml 10% SDS (0.1% SDS)

Stripping Solution (1L)
5 ml1 M Tris, pH 8.0 (5 mM)
0.4 ml 0.5 M EDTA, pH 8.0 (0.2 mM)
0.5 g Na-Pyrophosphate (0.05%)
1.0 ml of 100 X Denhardts (0.1 X)
Add dH_2O to 1 L

2.7 Molecular Characterization of the Identified cDNA Clones

The isolated full-length genes cloned from cDNA library were next sequenced. After sequencing, we compared the identities of these genes with the database in GenBank to determine the function of the genes.

2.7.1 Southern Blot

The organogenesis related genes were isolated using the small- scale plasmid DNA isolation method (see section 2.4.3). Southern blot analysis was used to confirm the genes picked from the cDNA library had homology with the probes.

Protocols:

1. 20 µg of plasmid DNA was digested with restriction enzymes (*EcoR I* and *XhoI*) at 37 °C for 2-3 hours.
2. 2 µl of loading buffer was added to each sample.
3. 0.8 % agarose gel was prepared in 0.5 X TBE buffer.
4. The samples were loaded onto the gel and a DNA maker was included.
5. The DNA was run towards the anode at 50-80 V until the bromophenol blue dye reached 2/3 of the length of the gel.
6. The gel was stained in 1 µg/ml ethidium bromide solution for 10 minutes.
7. The DNA was visualized on a UV transilluminator, and the gel was photographed alongside a ruler.
8. The gel was incubated in 500 ml of 0.25 M HCl for 15 minutes until the blue dye turns yellow.
9. The gel was rinsed twice in dH_2O.
10. The DNA was denatured by placing the gel in 0.5 M NaOH for 30 minutes.
11. A DNA transfer assembly was set as shown below (Figure 2.2):
12. The buffer tank was filled with transfer buffer (0.5 M NaOH) and allowed the transfer to proceed overnight.

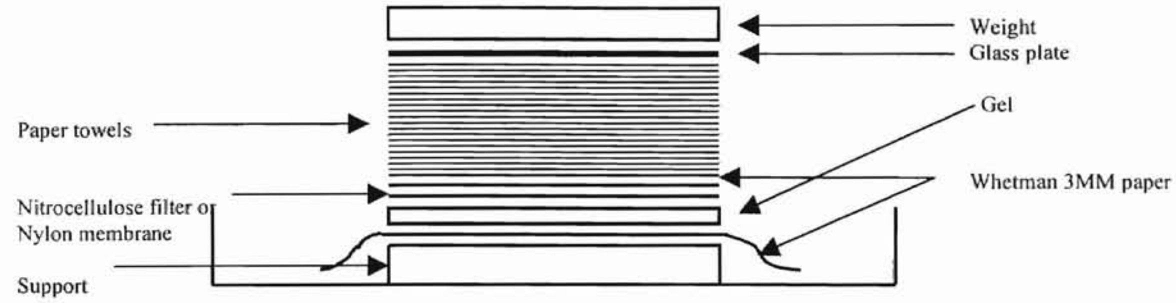

Figure.2.2 Southern Blot Analysis Diagram

13. The wet paper towels were replaced, and more transfer buffer was added if necessary.
14. The transfer set was carefully disassembled.
15. The filter was neutralized in 500 ml of 1.0 M NaCl/ 0.5 M Tris (pH 7.2) at room temperature for 15 minutes.
16. The DNA was fixed to the filter by UV cross-linking.
17. Pre-hybridized in 50 μl /cm^2 of hybridization solution at 42 °C for 4 hours.
18. The hybridization solution was changed and boiled salmon sperm DNA was added.
19. The ^{32}p labeled cDNA probes were boiled for 10 minutes, quench them on ice.
20. The probe was added and hybridized overnight at 42 °C.
21. The membranes were washed for 20 minutes at 42 °C followed by washing 10 minutes at 52 °C and 5 minutes at 62 °C, and the radioactivity counting was checked frequently.

22. The membranes were blotted dry on a piece of 3M paper.

23. The membranes were placed on x-ray film at -80 °C.

24. The films were developed and the result were evaluated by comparing the hybridization signals to the gel picture.

Solutions and buffers:

Denaturation buffer:

0.5 M NaOH

Neutralization buffer:

1.0 M NaCl
0.5 M Tris- HCl, pH 7.2

20 X SSPE: (1L)

175.3 g NaCl
27.6 g NaH_2PO_4-H_2O
7.4 g EDTA, pH 7.4

100 X Denhardt's:

2% Ficoll (400 K)
2% PVP (360 K)
2% Bovine serum album, BSA (fraction V)
Filter and store aliquots at -20 °C.

Prehybridization-mix:

5 X SSPE
5 X Denhardts
100 μg/ml salmon sperm DNA
0.1% SDS

Hybridization- mix:

Prehybridization - mix containing 10^6- 10^7 cpm/ml denatured probe

Washing Solution:

5 X SSPE
0.1 % SDS

2.7.2 Isolation of the Plasmid DNA Using Wizard System

High quality plasmid DNA were isolated using Wizard™ Plus Minipreps DNA Purification Systems.

Protocols:

1. 4 ml of cells was pelleted by centrifugation at 10,000xg for 10 minutes.
2. The cell pellet was completely re-suspended in 300 μl of cell resuspension solution.
3. 300 μl of cell lysis solution was added and mixed by inverting the tube 4 times.
4. 300 μl of neutralization solution was added and mixed by inverting the tube several times.
5. The lysate was centrifuged at 10,000xg in a microcentrifuge for 5 minutes.
6. 1 ml of re-suspended resin was pipetted into each barrel of the inicolumn/syringe assembly.
7. All of the cleared lysate was carefully removed from each miniprep and transferred to the barrel of the minicolumn/syringe assembly containing the resin.
8. The stopcocks were opened and a vacuum was applied to pull the resin/lysate mix into the minicolumn.
9. When the entire sample had completely passed through the column, the vacuum was broken at the source.
10. 2 ml of the column wash solution was added to the syringe barrel and the vacuum was re-applied to draw the solution through the minicolumn.

11. The resin was dried by continuing to draw a vacuum for 30 seconds after the solution had been pulled through the column.

12. The minicolumn was centrifuged at 10,000 x g for 2 minutes to remove residual column wash solution.

13. The minicolumn was transferred to a new microcentrifuge tube.

14. 30 μl of water was applied to the minicolumn and wait 1 minute.

15. The tube was centrifuged at 10,000 x g in a microcentrifuge for 20 seconds to elute the DNA.

2.8 Gene Expression Analyses

To demonstrate the expression of the cloned genes, the Northern Blot analysis was performed.

2.8.1 Synthesis of Probes

Two probes were synthesized. The first one was obtained by *EcoR I* and *Xho I* restriction digestion of plasmid DNA. The second probe was synthesized by PCR reaction with a pair of primers selected from a EMB11-similar gene. Both of them were then purified by glass wool column. And then they were labeled with ^{32}P by the Random Primer DNA Labeling method (Feinberg and Vogelstein 1982). The following protocols shows the glass wool column purification.

<u>Protocols:</u>

1. A small hole was made on the 0.5 ml tube and a stab of glass wool was put at the

bottom. This tube was put into a 1.5 ml tube.

2. The bands were cut from agarose gel and put into the 0.5 ml tube.
3. The DNA fragment was spun down at 12,000 rpm for 5 minutes.
4. 20 µl TE buffer or sterilized water were added and spun again at 12,000 rpm for 5 minutes.
5. All the solution was collected.
6. Added to the DNA solution volume 2.5 times 100% ethanol and 0.1 times of total volume 3M NaOAc to precipitate the DNA.
7. The tube was put into -80 °C for at least 10 minutes.
8. The DNA was pelleted at maximum speed for 5 minutes.
9. The liquid was poured out and the pellet was washed out with 75% ethanol once.
10. The tube was spun at maximum speed for 3 minutes.
11. The DNA pellet was air-dried for 10 minutes.
12. The DNA pellet was dissolved into 10 µl dH_2O.
13. A mini gel was run to check the concentration of the purified DNA.

2.8.2 RNA Isolation and Formaldehyde Gel Electrophoresis

Seven type of RNA samples were isolated from shoot apical meristemic tissues (3-day, 1-week, 2-week, 3-week) and non-meristemic tissues (cotyledon, hypocotyl, radical). The isolation protocol is same as 2.2.2. The RNAs were separated by formaldehyde gel electrophoresis by the same protocol in 2.2.3.

2.8.3 Northern Blot Analysis

Protocols:

1. The RNA gel was destained in 200 mM NaOAc (pH=4.0) for 20 minutes.
2. The membrane was pre-wet in H_2O and 2xSSC.
3. The transfer was set-up as Southern blot (Figure 2.2).
4. The buffer tank was filled with transfer buffer (20xSSC) and allowed the transfer to proceed overnight.
5. The membrane was washed in 5xSSPE briefly after blotting.
6. The DNA was fixed to the membrane by UV cross-link.
7. Pre-hybridized in 50 ul/cm^2 of prehybridization solution at 65 °C overnight.
8. The ^{32}P labeled cDNA probes were boiled for 10 minutes, and quench on ice.
9. The probes were added into the hybridization solution, and hybridized at 42 °C overnight.
10. The membranes were washed at 65 °C for 10 minutes. The radioactivity counting was checked frequently. The membrane was rinsed in 2x SSC.
11. The membranes were blotted dry on a piece of 3M paper.
12. The membranes were placed on x-ray film at -80 °C for 2 days.
13. The films were developed and the result was evaluated.

Solutions and Buffers:

Prehybridization Solution:

	400 ml
5x SSPE	100 ml 20x SSPE
5x Denhardt's	20 ml 100x Denhardt
0.2 % SDS	8 ml 10% SDS
100 ug/ml denatured ssDNA	4 ml 10 mg/ml ssDNA

Hybridization Solution:

	200 ml
50 % formamide	100 ml formamide
5x Denhardt's	10 ml 100x Denhardt
5x SSPE	50 ml 20x SSPE
0.2 % SDS	4 ml 10 % SDS
100 ug/ ml denatured ssDNA	2 ml 10 mg/ml ssDNA

Washing Solution:

2x SSC
0.5 % SDS

CHAPTER III

RESULTS AND DISCUSSION

3.1 Establishing Shoot Organogenic Culture

Organogenesis in plants is a complicated developmental process. Even though organogenesis has been a reliable technique for *in vitro* regeneration in many forest species, there is limited practical methodology available for loblolly pine organogenesis.

Organogenic cultures of loblolly pine family H-17 (Supplied by Western Gulf Forest Tree Improvement Cooperative) have been established from shoot apical meristems in our laboratory, allowing study of shoot organogenic culture in the laboratory condition. The germination rates of loblolly pine family H-17 seeds are shown in Table 3.1.

Table 3.1 Germination Rate of Loblolly Pine Family H-17 Seeds

Time (days)	6	7	8	9	10	11
Germination rate (%)	14	27	38	50	56	60

Generally, the seedlings were excised to develop organogenic cultures after germinating 5-7 days. We found that surface-sterilized seeds did not germinate uniformly if they had been stored at 4 °C. There was about a 3 week difference between the first seed germination and the last one. This phenomenon did not occur with the

surface-sterilized seeds stored at room temperature. We proposed that the non-uniform germination was caused by low temperature, which breaking dormancy in the seed. Pine seeds display highly variable germination behavior when sown following extraction or storage. The type and degree of dormancy vary among species, geographic sources of the same species, and lots within the same source. Germination is greatly improved and hastened by first subjecting the seeds to cold stratification, especially if the seeds have been stored (Krugman and Jenkinson 1974).

In loblolly pine, apical meristematic tissue of young seedlings has great potential to develop *de novo* shoots (Huang *et al.* 1995). The maturation of the tissues often reduces the capacity for vegetative regeneration and many plants are impossible to propagate vegetatively after the developmental state of the explants is determined (Torrey 1966). Shoot apical meristem plays an important role in *de novo* organogenesis and other developmental processes. It is believed that shoot apical meristem is a generative tissue and continuous activity of the apical meristem results in shoot development (Aithen *et al.* 1988).

Shoot apical meristem development was monitored to determine the optimum stage for initiation of organogenic culture. When the hypocotyls had grown out of the megagametophyte 5 mm, the seedlings were removed from the agar plates to induce shoot organogenic tissues. Plant growth regulators played an essential role in the organogenesis process. LP medium containing 2.5 mg/l BAP and 0.01 mg/l NAA gave relatively high initiation frequency (2%) of shoot organogenic tissue.

Auxins and cytokinins are known to affect gene expression in relation to a variety of phenomena (Hathway 1990), but their action in organogenesis is unknown (Hicks 1993).

3.2 Identification of Shoot Organogenesis-regulated mRNAs

To investigate shoot organogenesis at the molecular level, the total RNAs from different tissues and tissues at different stages of development were isolated (Fig. 3.1). We utilized the PCR differential display technique on the seven developmentally different RNA samples to identify putative shoot organogenesis-regulated genes. The cDNAs were made from 3-day, 1-week, 2-week, and 3-week organogenic tissues. Non-organogenic tissues hypoctyl, cotyledon and radical tissues were used as control.

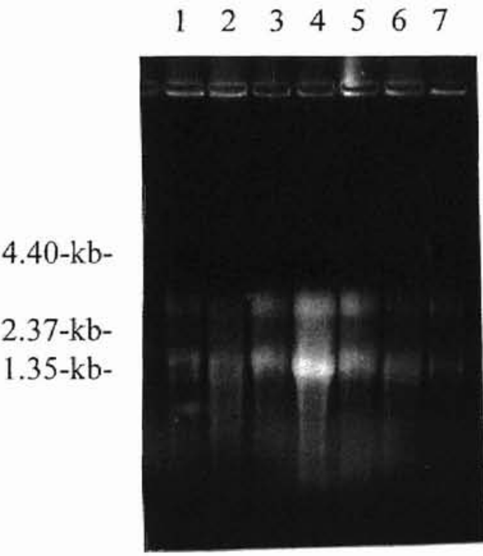

Figure 3.1 RNA pattern of loblolly pine seedling tissues. 1). 3-day shoot apical meristemic tissue, 2). 1-week shoot apical meristemic tissue, 3). 2-week shoot apical meristemic tissue, 4). 3-week shoot apical meristemic tissue, 5). Cotyledon, 6). Hypocotyl, 7). Radical.

PCR-based differential display is a method originally developed to identify differentially expressed genes in paired mammalian cell populations. The procedure involves PCR amplification of sub-populations of first-strand cDNAs using a given set of short oligonucleotide primers. The 3' primer, which consists of 11 deoxythymidine residues plus one additional 3' base to provide specificity, is used for reverse transcription of mRNA subsets. The 5' primer is a decamer of arbitrarily-defined sequence. Different combinations of 5' and 3' primers are used to generate a panel of PCR products for two or more DNA samples that can be compared on a polyacrylamide gel for distinct differences.

In our experiment, first strand cDNAs were synthesized from the total RNA by reverse transcription of the mRNAs with poly A^+ oligo-dT primers (A, G, and C). The cDNA was amplified by the PCR reaction with 16 pairs of primers arbitrary in sequence. Table 2.3 shows the sequences of all primers used. A total of 48 reactions were run for each RNA sample. The PCR products were labeled with ^{33}P-dATP and resolved on a denaturing polyacrylamide gel and the pattern of bands for the seven samples compared for each primer combination. Primer composition and the combination of primers used had a significant impact on the banding patterns that were observed. Fifty-one cDNA fragments that showed differential expression patterns crossing tissue types were identified. Table 3.2 shows the primer combinations and expression patterns of the cDNA fragments that were read from the differential display gel. In this table 3d, 1w, 2w, 3w represents 3-day, 1-week, 2-week, 3-week shoot apical meristemic tissues respectively. R, H, and C refers to the radical, hypocotyl and cotyledon. By comparing the density of the bands, the differentially expressed genes could be identified easily.

Figure 3.2 shows an area of a polyacrylamide gel where a putative organogenesis-regulated cDNA fragment was detected. The first strand cDNAs were synthesized from 3-week shoot apical meristemic tissue RNA using 5'-AAGCTTTTTTTTTTTA-3' as the primary oligonucleotide. The resultant cDNA product was PCR-amplified in the presence of ^{33}p-dATP using "12" as the 5' primer. The arrow points to a band where there was distinctly more product amplification with RNA isolated from shoot apical meristemic tissue than from non-organogenic tissue. In addition, the amount of cDNA product from the later developmental stage (3-week) shoot apical meristemic tissue was greater than that from the tissues of earlier developmental stages. This result suggests that this RNA may control not only the initiation of organogenesis but the developmental process as well. The 51 differentially expressed cDNA fragments identified were excised from the dried gels, eluted into buffer, and re-amplified for cloning. We also sequenced some of the cDNA fragments with significant differential expression patterns to provide information about gene function.

Table 3.2 DD-PCR cDNA Expression Patterns (primer sequences showed in Table 2.3)

Band No.	Primer combination	Organogenic tissue				Non-organogenic tissue		
		3d	1W	2W	3W	R	H	C
1	H-AP1/H-T_{11}A	+	++	+++	++++	+	/	/
2	H-AP1/H-T_{11}A	++	+++	+++	++++	+	--	/
3	H-AP1/H-T_{11}A	--	+	++	+++	--	--	/
4	H-AP1/H-T_{11}A	--	+	+	+++	+	--	/
5	H-AP1/H-T_{11}A	--	+	++	+++	--	/	/
6	H-AP1/H-T_{11}A	+	++	++	+++	/	/	/
7	H-AP1/H-T_{11}A	+	++	+++	+++	--	--	/
8	H-AP4/H-T_{11}A	--	++	++	+++	/	--	/
9	H-AP4/H-T_{11}A	+	++	+	+++	--	--	/
10	H-AP11/H-T_{11}A	+	++	++	++++	+	--	--
11	H-AP12/H-T_{11}A	++	+++	+++	++++	--	--	+
12	H-AP12/H-T_{11}A	+	++	++	+++	--	--	--
13	H-AP5/H-T_{11}A	/	+	++	++++	--	/	--
14	H-AP7/H-T_{11}C	--	+	--	++	/	/	/
15	H-AP5/H-T_{11}C	/	/	/	++	/	/	/
16	H-AP5/H-T_{11}C	/	/	/	++++	/	/	/
17	H-AP5/H-T_{11}C	--	/	+++	++++	/	/	/
18	H-AP5/H-T_{11}C	/	--	--	++	/	/	/
19	H-AP5/H-T_{11}C	/	/	+++	++++	--	/	--
20	H-AP5/H-T_{11}C	+++	+++	++++	++++	/	--	/
21	H-AP4/H-T_{11}C	--	++	+++	++++	--	/	/
22	H-AP6/H-T_{11}C	+	+	/	+++	/	--	--
23	H-AP6/H-T_{11}C	--	--	/	+++	/	/	--
24	H-AP8/H-T_{11}C	+++	+++	+++	++++	/	/	--
25	H-AP8/H-T_{11}C	++	+++	+++	+++	/	--	/
26	H-AP8/H-T_{11}C	+	++	++	++	--	/	/
27	H-AP8/H-T_{11}C	++	++	+++	+++	/	--	--
28	H-AP11/H-T_{11}C	++	+	++++	++++	--	--	--
29	H-AP11/H-T_{11}C	+	++	+	+++	+	--	--

Table 3.2 DD-PCR cDNA Expression Patterns (continued)

Band No.	Primer combination	Organogenic tissue				Non-organogenic tissue		
		3d	**1W**	**2W**	**3W**	**R**	**H**	**C**
30	H-AP11/H-T_{11}C	+	+	+	+++	--	--	/
31	H-AP9/H-T_{11}G	--	--	--	++	--	/	--
32	H-AP9/H-T_{11}G	++	++	+++	+++	/	--	/
33	H-AP11/H-T_{11}G	--	--	+	++	/	/	/
34	H-AP11/H-T_{11}G	+	++	+	+++	/	/	/
35	H-AP11/H-T_{11}G	/	--	++	++++	/	/	/
36	H-AP12/H-T_{11}G	+++	+++	+++	++++	--	/	+
37	H-AP12/H-T_{11}G	+++	+++	+++	+++	/	/	--
38	H-AP12/H-T_{11}G	++++	++++	++++	++++	--	/	+
39	H-AP13/H-T_{11}G	++	--	++	+++	--	+	/
40	H-AP13/H-T_{11}G	+++	+++	+++	++++	/	--	--
41	H-AP14/H-T_{11}G	+	/	/	++	/	/	--
42	H-AP14/H-T_{11}G	--	--	+	+++	/	/	/
43	H-AP6/H-T_{11}G	+++	+++	+++	+++	/	--	+
44	H-AP7/H-T_{11}G	++	/	+	+++	--	/	/
45	H-AP7/H-T_{11}G	+++	++	+++	+++	/	/	/
46	H-AP7/H-T_{11}G	++	++	+++	+++	--	--	/
47	H-AP7/H-T_{11}G	+++	++++	++++	++++	--	--	/
48	H-AP8/H-T_{11}G	++	--	/	+++	--	/	/
49	H-AP8/H-T_{11}G	++	+	/	+++	--	--	--
50	H-AP8/H-T_{11}G	++	++	/	+++	--	/	--
51	H-AP8/H-T_{11}G	++	+	/	+++	/	--	/

++++: Very strong band
+++: Strong band
++: Medium band
+: Weak band
--: Very weak band
/: No band

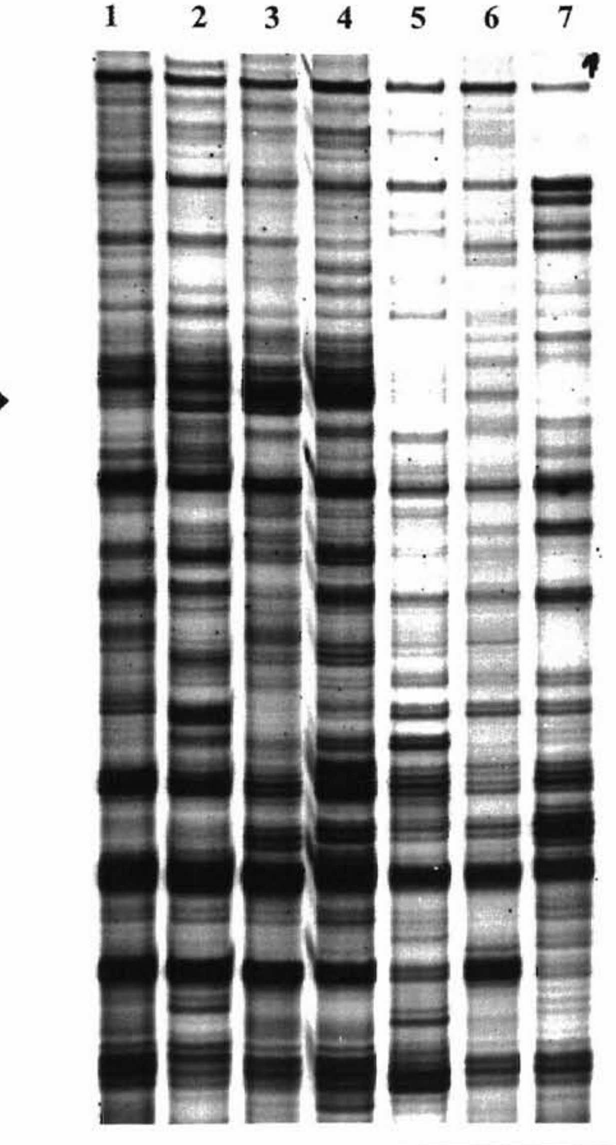

Figure 3.2 Comparison of the expression patterns between the samples from different tissue types (shoot apical meristems, cotyledon, hypoctyl and radical) suggests that the gene is specific for apical meristems. The expression patterns of different stages of apical meristem tissue show that a cDNA fragment (arrow) is associated with the organogenic development process. 1). 3-day shoot apical meristemic tissue, 2). 1-week shoot apical meristemic tissue, 3). 2-week shoot apical meristemic tissue, 4). 3-week shoot apical meristemic tissue, 5). Hypocotyl, 6). Radical, 7). Cotyledon.

3.3 Tissue-specific Expression

Reverse Northern was performed to verify the authenticity of the differentially displayed bands. The cDNA fragments corresponding to each of the selected bands were ligated into plasmid vector and transferred into *E. coli* cells. Antibiotic resistant colonies were transferred onto duplicate filters, and were then probed with ^{32}P labeled bulked cDNA, which had been reverse transcribed from RNA of the 3-week shoot apical meristemic tissue and cotyledon tissue. After washing and autoradiographing, the 3-week shoot apical meristemic tissue showed the same patterns with the original antibiotic plates. The plasmids with the cDNA insert did not hybridize with probes from cotyledon tissue (Fig 3.3). This result confirmed that the cDNA fragment cloned from the differential display gel was truly tissue-specific.

Tissue specificity of all the cDNA fragments was examined by the hybridization intensity of ^{32}P- labeled cDNA probes (3-week shoot apical meristemic tissue and cotyledon tissue) with cDNA fragments digested from the plasmid vectors. The results indicate that some of the cDNAs were shoot organogenic tissue specific.

Seven cDNA fragments (10, 11,14,17,24,39,45) selected from DD-PCR were confirmed as tissue-specific cDNA fragments. Thus these fragments were utilized as probes to screen the cDNA library to isolate the full-length organogenesis-regulated genes.

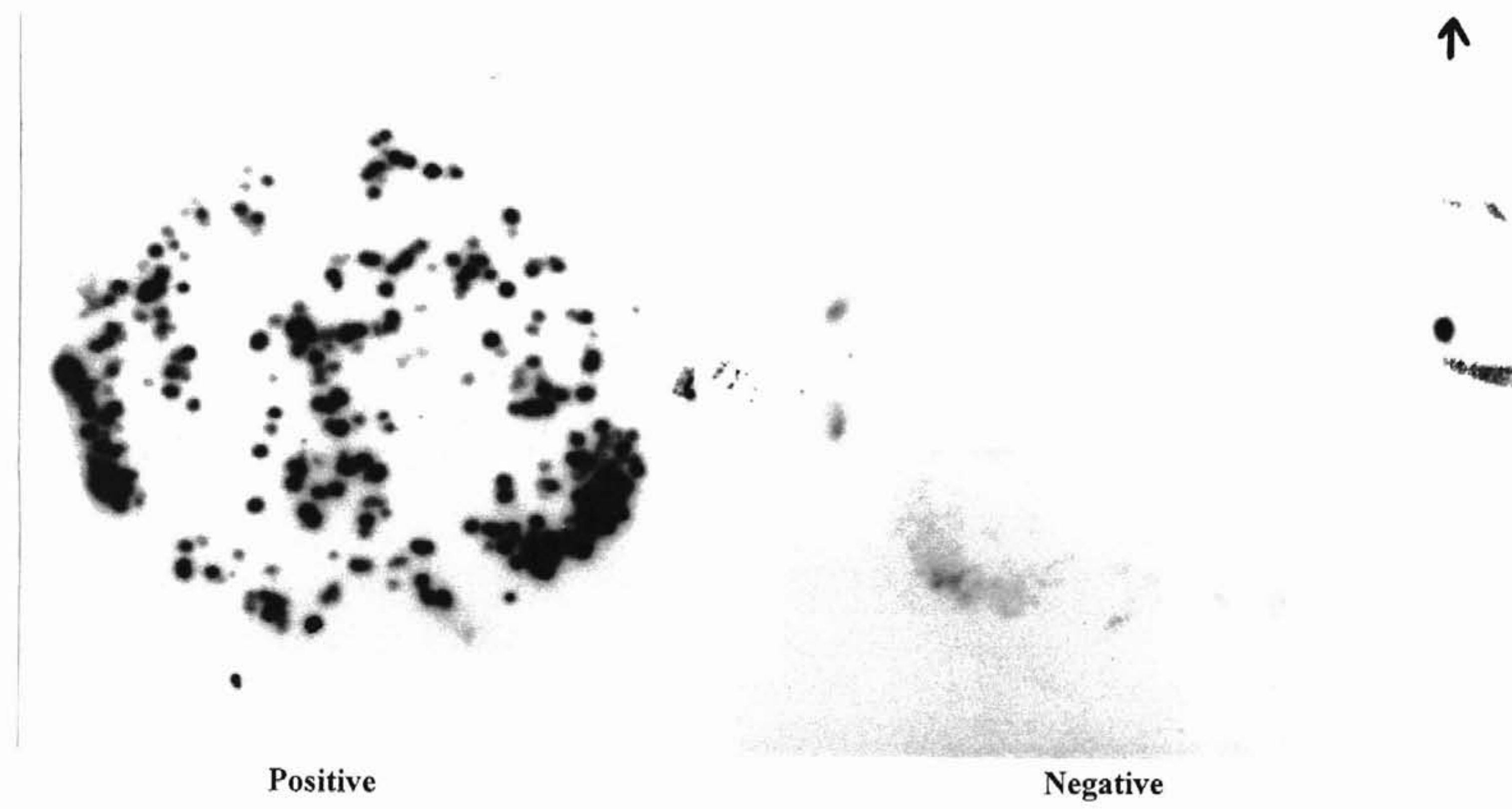

Figure 3.3 Reverse Northern was conducted using the labeled cDNA transcribed from total RNA as probes to hybridize with selected cDNA fragments from the differential display. Following hybridization positive results were found with 3-week shoot apical meristemic tissue, negative results were obtained when probing with cDNA from cotyledon tissue.

3.4 Sequence Analysis of cDNA Clones

By comparing the expressed patterns of these seven cDNA fragments cloned from DD-PCR, we chose four of them (10, 11, 14, 17) as probes to screen a cDNA library in order to obtain the full length shoot organogenesis related genes. This cDNA library contained shoot apical meristemic genes and was constructed by Dr. Y. Huang in the Forest Genetic laboratory, Oklahoma State University.

Eighty phage plaques homologous with probe #11 were collected from the secondary screening plates. Twenty-four of those plaques with strong signals were chosen for *in vivo* excision to get the genes. Southern blot analysis result suggests that twenty-one of those genes have homology with cDNA probe #11 cloned from the differential display gel.

After the cDNA clones were sequenced, we compared the sequence with the GenBank database. No genes related to organogenesis were obtained by probe #10, #14, and #17. However, we found one, which might have a function in the regulation of organogenesis using probe #11. This clone (11-4) was 1250 bp in length. Figure 3.4 shows the nucleotide sequence of this clone. It is uncommon that a poly A sequence is present in the middle of gene. We propose that this clone contain two genes. Comparison of the sequence of the first gene (11-4-A, which is from 1 to 655 bp) with the GenBank databases showed this region had 43 % homology with an antimicrobial gene (AMP1). Purified MiAMP1 inhibited the growth of a variety of fungal, oomycete and gram-positive bacterial phytopathogens *in vitro* (Marcus *et al.* 1997). The MiAMP1 gene may prove useful in improving disease resistance in transgenic plants. The second part of clone 11-4 (11-4-B, which is from 656 to 1250 bp) has 90% identity to the *Picea glauca*

late embryo abundant protein (EMB 11) mRNA (Figure 3.5).

There are at least three explanations for the structure of clone 11-4. First, this clone is a full-length gene containing two independent open reading frames and the expression of each is regulated by the other. Second, only the first part (11-4-A) expresses during the organogenesis development process and the second part (11-4-B) was ligated with it by chance during the cDNA library construction. Third, controversely, 11-4-A is not related by organogenesis and was ligated with 11-4-B, which has a function in organogenesis regulation.

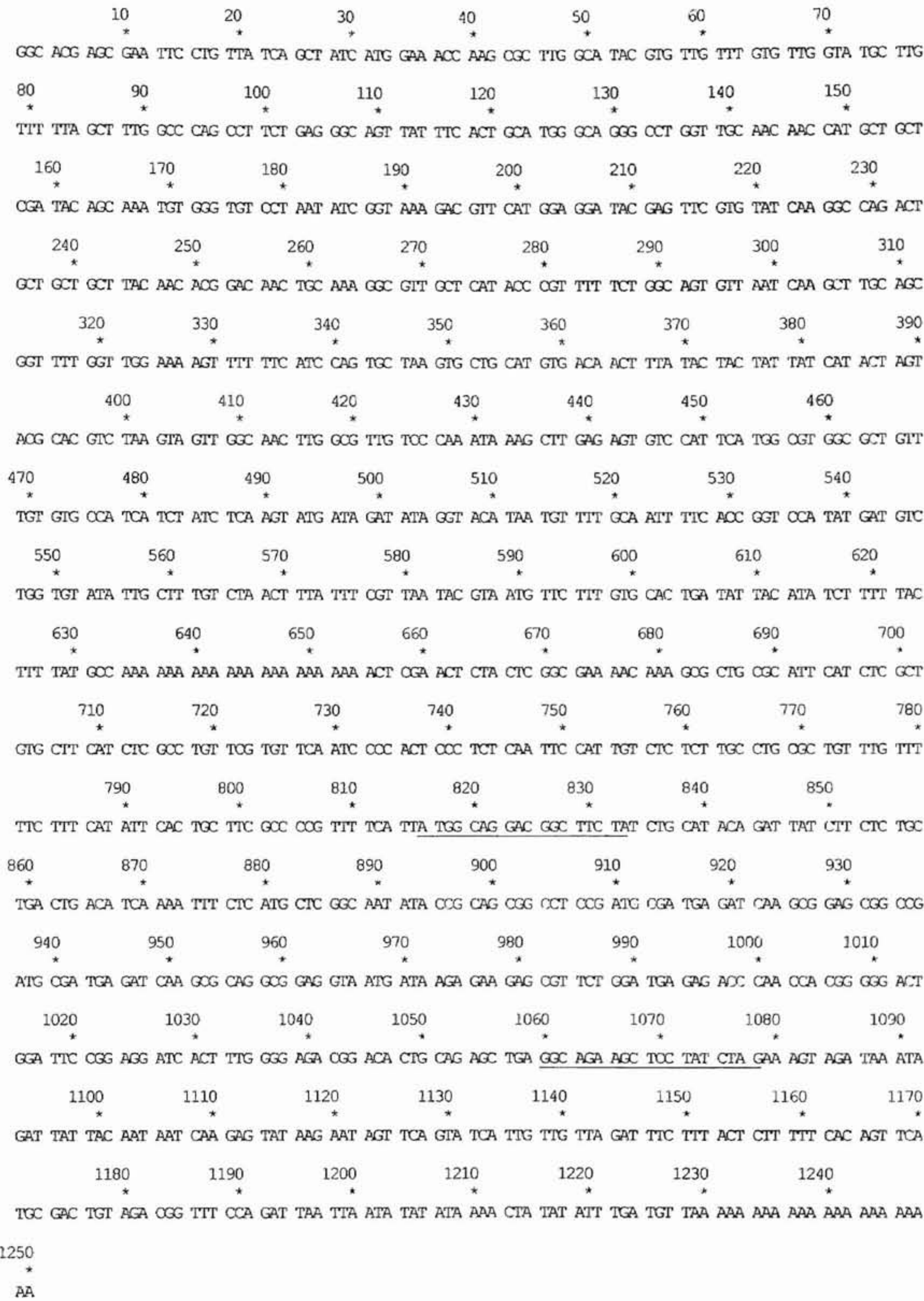

Figure 3.4 The DNA sequence of 11-4 obtained from the cDNA library. The full length of this clone is 1250 bp. The sequences of the primers are underlined.

3.5 Organogenic Gene Expression

To confirm the hypothesis about the function of the cloned gene, a Northern blot analysis was performed. Seven types of RNA samples were isolated and transferred onto membrane. They were then probed by 11-4-A or 11-4-B separately. The 11-4-A gene was obtained by restriction enzyme digestion of the plasmid DNA and DNA electrophoresis. However, there was no useful restriction enzyme cutting site in 11-4-B. We synthesized a pair of primers which flank the open reading frame of 11-4-B (Figure 3.4 shows the sequence of the primers) and amplified this region to be used as a probe for Northern blot analysis.

Figure 3.7b is the result of Northern blot probed by 11-4-B. Compared with the RNA maker, the size of the resultant bands are 594 bp and it is almost the half length of the whole clone 11-4. Thus, this gene is most probably an independent gene and was linked with the first part during the cDNA library construction. Figure 3.6 shows the gene sequence of 11-4-B and the corresponding amino acid sequence.

Transcription study (figure 3.7) with RNAs synthesized in apical meristemic tissues (3-day, 1-week, 2-week, and 3-week) and non-meristematic tissues (cotyledon, hypocotyl, and radical) demonstrate that 11-4-B regulates the organogenesis associated activity at the transcriptional level and is expressed only in the apical meristemic tissues. The expressed amount of this gene increased significantly in 3-week apical meristematic tissue. It suggests that this gene may also control the organogenesis development processes.

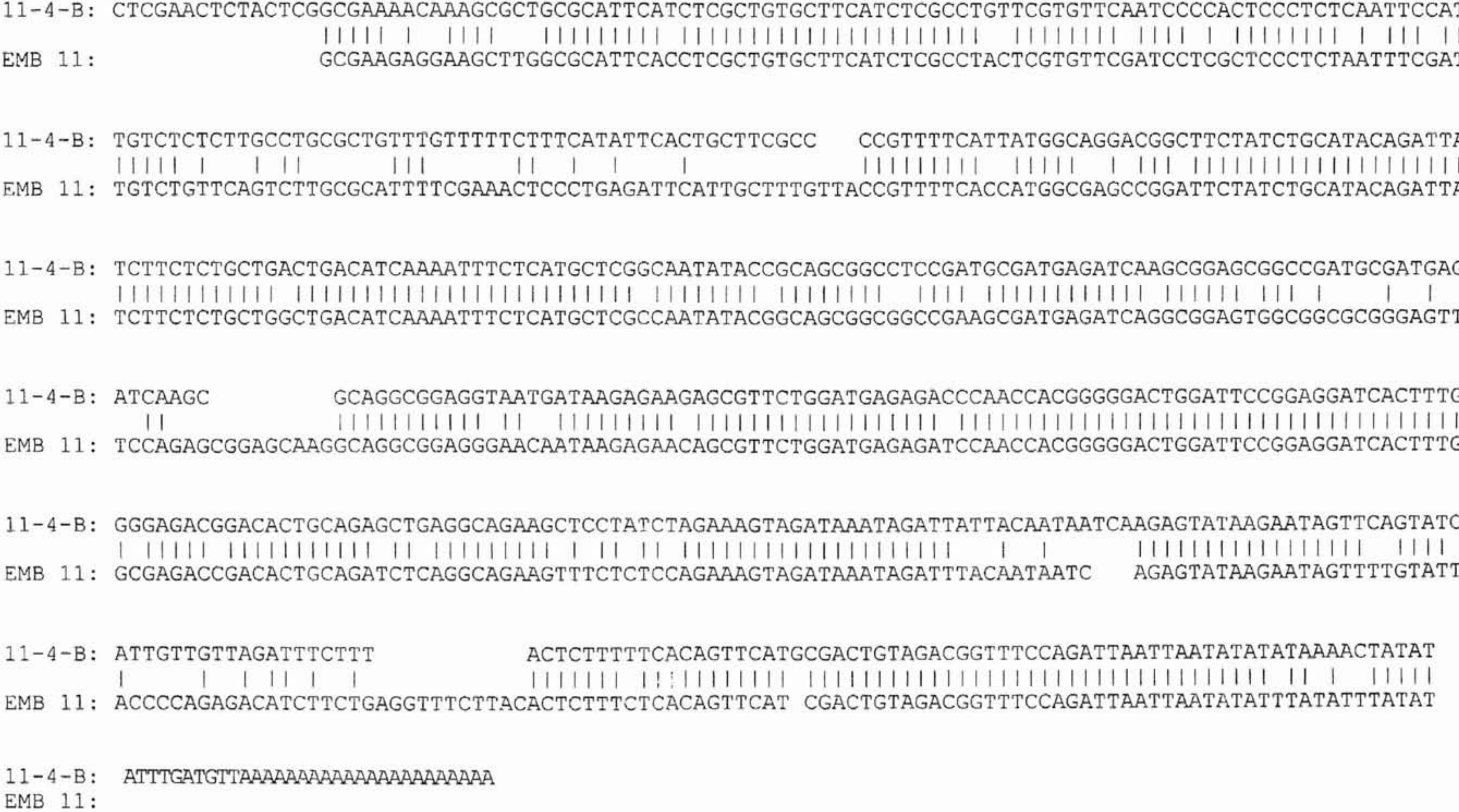

Figure 3.5 The DNA sequence comparison between 11-4-B and the *Picea glauca* late embryo abundant protein (EMB11) mRNA from the GenBank database. The homology between these two genes is 90%.

```
         10         20         30         40         50         60
          *          *          *          *          *          *
CTC GAA CTC TAC TCG GCG AAA ACA AAG CGC TGC GCA TTC ATC TCG CTG TGC TTC ATC TCG

         70         80         90        100        110        120
          *          *          *          *          *          *
CCT GTT CGT GTT CAA TCC CCA CTC CCT CTC AAT TCC ATT GTC TCT CTT GCC TGC GCT GTT

        130        140        150        160        170
          *          *          *          *          *
TGT TTT TCT TTC ATA TTC ACT GCT TCG CCC CGT TTT CAT T ATG GCA GGA CGG CTT CTA
                                                       M   A   G   R   L   L>

180        190        200        210        220        230
 *          *          *          *          *          *
TCT GCA TAC AGA TTA TCT TCT CTG CTG ACT GAC ATC AAA ATT TCT CAT GCT CGG CAA TAT
 S   A   Y   R   L   S   S   L   L   T   D   I   K   I   S   H   A   R   Q   Y>

240        250        260        270        280        290
 *          *          *          *          *          *
ACC GCA GCG GCC TCC GAT GCG ATG AGA TCA AGC GGA GCG GCC GAT GCG ATG AGA TCA AGC
 T   A   A   A   S   D   A   M   R   S   S   G   A   A   D   A   M   R   S   S>

300        310        320        330        340        350
 *          *          *          *          *          *
GCA GGC GGA GGT AAT GAT AAG AGA AGA GCG TTC TGG ATG AGA GAC CCA ACC ACG GGG GAC
 A   G   G   G   N   D   K   R   R   A   F   W   M   R   D   P   T   T   G   D>

360        370        380        390        400        410
 *          *          *          *          *          *
TGG ATT CCG GAG GAT CAC TTT GGG GAG ACG GAC ACT GCA GAG CTG AGG CAG AAG CTC CTA
 W   I   P   E   D   H   F   G   E   T   D   T   A   E   L   R   Q   K   L   L>

420        430        440        450        460        470
 *          *          *          *          *          *
TCT AGA AAG TA GAT AAA TAG ATT ATT ACA ATA ATC AAG AGT ATA AGA ATA GTT CAG TAT
 S   R   K>

480        490        500        510        520        530
 *          *          *          *          *          *
CAT TGT TGT TAG ATT TCT TTA CTC TTT TTC ACA GTT CAT GCG ACT GTA GAC GGT TTC CAG

540        550        560        570        580        590
 *          *          *          *          *          *
ATT AAT TAA TAT ATA TAA AAC TAT ATA TTT GAT GTT AAA AAA AAA AAA AAA AAA AAA A
```

Figure 3.6 Nucleotide and predicted amino acid sequences of 11-4-B (From 656 to1250 bp of 11-4). The start and stop codons of Open Reading Frame are underlined.

Although 11-4-B was not cloned directly by library screening, it might be included in the resultant of the differential display, colonies from the clone or even some plaques from the cDNA library and they were missed to be picked up by chance.

Transcriptional study of 11-4-A (Figure 3.7a) also demonstrated that 11-4-A and 11-4B are independent. The result shows that 11-4-A is expressed strongly in all shoot apical meristemic tissue, but very weak in the cotyledon, hypocotyl, and radical. There is no significant difference in the expression abundance between the different stages of the shoot apical meristematic tissues. Because the non-meristematic tissues were not cultured on LP medium, and plant response to the injury caused by excision may be affecting the gene expression. In order to demonstrate that this gene has a function in organogenesis regulation, except for the antimicrobial effect, further study is required to eliminate the effects from the medium.

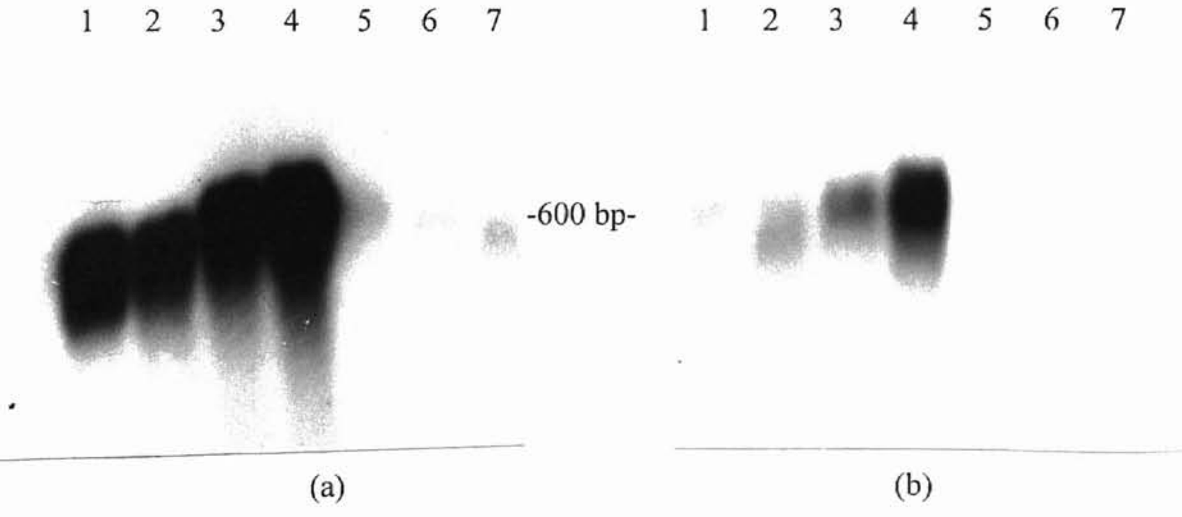

Figure 3.7 Gene expression patterns of 11-4-A (a) and 11-4-B (b). 1). 3-day shoot apical meristemic tissue, 2). 1-week shoot apical meristemic tissue, 3). 2-week shoot apical meristemic tissue, 4). 3-week shoot apical meristemic tissue, 5). Hypocotyl, 6). Cotyledon, 7). Radical. Total RNAs (40 ug) from all type of tissues were used for each line. Autoradiograms were exposed for ~ 40 hours.

CHAPTER IV

CONCLUSIONS

Loblolly pine (*Pinus teada* L.) is an economically important conifer species. We were trying to improve the *in vitro* propagation of loblolly pine by exploring the molecular mechanisms of *de novo* organogenesis. Because all the development processes are under genetic control, the purpose of this research was to clone organogenesis regulated genes from shoot apical meristemic tissue of loblolly pine.

Shoot organogenesis cultures of loblolly pine were established using shoot apical meristems obtained from young seedlings. By using this culture system (modified LP medium and long light), the induction rate of organogenic cultures was high. Organogenic cultures collected from various development stages were used for differential gene expression studies. Gene expression products (mRNA) associated with the organogenesis process was identified using PCR-based differential display technology. Fifty-one cDNA fragments with differential expression patterns and tissue specificity were cloned. Comparison of the expression patterns between the cDNA samples from different types of tissues (shoot apical meristems, cotyledon, hypoctyl and radical) suggests that the genes are tissue-specific. Furthermore, expression patterns of the selected cDNA appears to be associated with the shoot organogenesis. This result

suggests that mRNA differential display technology is a useful tool to clone differentially expressed genes involved in the development process of organogenesis in loblolly pine. Seven shoot-organogenesis specific cDNA fragments (10, 11, 14, 17, 24, 39, and 45) were confirmed using both Reverse Northern and Southern blot analyses, which were used as probes for screening of the organogenic cDNA library of loblolly pine. A number of cDNA clones have been obtained after we screened the organogenic cDNA library. One of these clones encodes a protein homologous with *Picea glauca* late embryo abundant protein (EMB11) mRNA. Its function may be involved in regulation of organ differentiation and meristematic cell activation. Another cDNA clone shows a homology (43%) with an antimicrobial gene (AMP1) in their amino acid sequences, but is highly expressed in shoot apical meristemic tissues of loblolly pine.

Histological studies of shoot formation in conifers showed that activities of meristematic centers or meristemoids developed from a few layers of epidermal and sub-epidermal cells of embryogenic tissue, produced bud primordia and finally adventitious shoots (Thorpe and Patel 1986). Finding out whether and when the regulatory genes are expressed in such meristematic centers or elsewhere is critical in understanding the functions of the cloned genes in such developmental processes. Therefore, one of the further works should be *in situ* hybridization or *in situ* PCR to determine specific tissue and cell types in which these genes are expressed.

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VITA

Yan Zhang

Candidate for the Degree of

Master of Science

Thesis: GENE EXPRESSION IN ORGANOGENIC TISSUE OF LOBLOLLY PINE (*Pinus taeda L*)

Major Field: Forest Resources

Biographical:

Personal Data: Born in Beijing, P. R. China, On August 31, 1972, the daughter of Mengwei Zhang and Demeng Kang.

Education: Graduated from Beijing Gangyuan High School, Beijing, P. R. China in July, 1990, received Bachelor of Science degree in Agronomy from Beijing Agricultural College in May 1994. Completed the requirements for the Master of Science degree with a major in Forestry at Oklahoma State University in May, 1998.

Experience: Employed as a laboratory technician, Biology Center, Beijing Forest University, 1994 to 1995; employed by Institute of Genetics, Chinese Academy of Science as a research assistant, 1995 to 1996; employed as a graduate research assistant, Department of Forestry, Oklahoma State University, 1996 to 1998.