ISOLATION AND CHARACTERIZATION OF WATER DEFICIT STRESS INDUCIBLE cDNAS AND THEIR GENOMIC COUNTERPARTS FROM *Pinus taeda* (LOBLOLLY PINE)

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Abstract. Water deficit stress (WDS) is one of the most important factors affecting trees in forest stands. To survive the stress, plants activate certain genes of which the translated products are assumed to play a major role in tolerance. Previously we have cloned and characterized 3 genes. Here we describe another WDS induced gene.

The predicted translation product of 1p5 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. In order to understand the function *in vivo*, *1p5* gene has been cloned behind a CaMV 35S promoter and used in transformation studies.

Also the genomic clones of *1p3* and *1p5* genes were isolated from a lambda library and sequence analysis was done. A 1.1 kb fragment of *1p3* and 2.3 kb piece of lp5 upstream of the transcription start site have been cloned in front of *gus* genes and these constructs have been used in the transformation of tobacco. Preliminary data indicate that the lp5 promoter is functional in cell suspension tissue. Analyses of sequence data and results of transformation studies are presented.

This study will enable us to understand the molecular mechanism involved in stress tolerance and gene regulation in forest trees.

Keywords: *Pinus taeda*, tater deficit stress, cDNA, promoter analyses, transformation.

INTRODUCTION

Pinus taeda is an important conifer in the reforestation program in the semi-arid regions of U. S. and especially in Texas. These trees are well known for their rapid growth, high biomass production and drought tolerance among conifers. Still many seedlings are lost in the field due to lack of water (Williston, 1972), and the losses can amount to several million

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dollars annually. Further studies have revealed WDS as the cause for up to 90% of the variation in annual ring-width of conifers in the humid temperate and arid climates (Zahner, 1968). Perry et al., (1994) reported that water deficit stress was responsible for more than half of the variation in stem volume index at the end of the first growing season in pine seedlings.

Plants cope with WDS by avoidance, postponement and/or tolerance (Kramer, 1983). Because of their immobility, plants that cope WDS by postponement or tolerance have to make some metabolic and/or structural adjustments to survive the stress (Ho and Sachs, 1989). Techniques of molecular biology now have opened new avenues to study these adjustments in detail at the gene level. An understanding of these alterations and their regulation facilitate breeding of new and improved plants through genetic engineering and formulation of better management strategies for drought tolerance to increase productivity of plants.

Although many advances have been made in the study of stress induced responses of plants, namely gene expression, little is known about these mechanisms in *P. taeda.* Thus, this study was undertaken to gain an insight into genes induced by water deficit stress in loblolly pine. We have cloned several WDS induced cDNAs isolated through differential screening of a WDS root cDNA library. These clones isolated from loblolly pine - named 1p were sequenced and their expression patterns have been analyzed by Northern screening. Three of them were described previously. We report here two other clones not described before and the isolation and characterization of genomic clones of two of the above described WDS cDNA clones.

METHODS

Plant Growth and Water Status

Full-sib *P. taeda* L. seedlings grown to heights of 30 to 45 cm with an age of 8 to 13 months were used in the experiments. One week before WDS treatments began, all seedlings were acclimatized by daily irrigation with reverse-osmosis water. The seedlings were then separated into 5 different groups and water was withheld, in staggered fashion so that different groups were deprived for various periods. All plants were harvested predawn on the same day. Immediately before harvest, the water potential of a medium-aged needle fascicle from each seedling was measured using a Scholander pressure chamber. Then each seedling was separated into root, stem and needle portions and immersed in liquid nitrogen. These tissues were stored at -80^{0} C for subsequent nucleic acid isolation from individual seedlings that had attained targeted water potentials.

Nucleic Acid Isolation

The RNA extraction was done according to protocol of Chang et al., (1993). The DNA was isolated using the procedure developed by Doyle and Doyle (1990).

Northern and Southern Analysis

The Northern and Southern analysis were performed according to Maniatis et al., (1982). Each sample in Northern analysis is represented by 10 lig of total RNA, and in Southern blots 15 lig of total DNA, digested with the given enzyme were run/lane in gel electrophoresis. All gels were photographed after ethidium bromide staining, for mobility measurements, and future references. The probes were made using a random primed labeling kit (USB).

Genomic Library Construction and Molecular Methods

A genomic library of loblolly pine was created in bacteriophage Lambda GEM 11 obtained from Promega (Madison, Wisconsin), with a titer of 8 X 10^5. The library was screened with the 1p cDNA clones to isolate the genomic counterparts after plating at a density of 2000 pfu/plate on a *E. coli* KW251 lawn.

The positive plaques were isolated separately and were further screened by secondary and tertiary plaque lifts.

Seauencina of DNA and Data Analysis

The sequencing of cDNA and genomic clones were done using a combination of a manual and Dye-Deoxy automated sequencing method. Sequencing reactions were performed using a Sequitherm kit (Epicenter Technologies) or a Dye-Deoxy sequencing kit (Applied Biotechnologies Inc) in a theimocycler.

The sequences were analyzed by the MacVector program (Kodak). Computer searches of the NIH Genbank and Swiss Protein Databank were also performed.

RESULTS AND DISCUSSION

Isolation and Characterization of WDS Induced Gene *1p5,* and Genomic counterparts of *1p3* and *1p5*

Previously, we have isolated 28 putative WDS induced clones by differential screening of a WDS root cDNA library. Out of these gene ip5 was sequenced and characterized.

The cDNA clone of *1p5* consists of 981 bp (data not shown) and the Northern analysis (Fig 1) indicate the message to be about 1 kb. This gene is mainly expressed in roots and to a lesser extent in stems, with very little or no expression in needles. There is an open reading frame that corresponds to a translation product of 205 amino acids (Fig. 2) with a calculated molecular weight of 17,264, and a pI of 6.39. It is very rich in glycine (41%) and serine (20%) and has no proline, tryptophan and histidine residues. When aligned to sequences at the GenBank, the putative LP5 protein showed the greatest similarity to silk fibroin heavy chain protein of *Bombyx mori,* and glycine rich cell wall structural proteins from *Arabidopsis,* petunia and *Phaseolus.* Thus, LP5 appears to be a cell wall related structural protein.

There is evidence for this assumption to be correct in the predicted amino acid sequence. First, it has a very hydrophobic amino terminal that is 17 (5th - 21st) amino acids long which is characteristic of a signal peptide. The peptides with such a signal peptide are translocated to endomembranes during translation and are secreted out of the cell unless an endoplasmic retention signal is found. The second feature that indicates that LP5 is a cell wall protein is the 9 direct repeats of a GXGXGY sequence in the carboxy terminal. All of the above mentioned proteins to which LP5 is similar have been predicted to have an anti parallel, ß pleated sheet structure. According to this model, the residues alternating with glycine have all their bulky side chains projecting on the same side of the ß pleated sheet. The primary sequence of LP5 allows the formation of a structure similar to the above with all the tyrosine residues having their side chains on one side of the ^{9} pleated structure (Fig. 3). Third, Lei and Wu (1991) who also isolated a cell wall targeted GRP, proposed the reinforcement of the cell wall as the function of their protein based on the exposed tyrosine residues in the direct repeats. It has been shown that the peroxidases can form isodityrosine bonds between two tyrosines in the same protein or in two different proteins (Liyama et al., 1994) forming a network that serves as a matrix for linkage of proteins with polysaccharides or polyphenols, thus reinforcing the cell wall (Epstein and Lamport, 1984). The possibility of crosslinking between LP5 and other cell wall proteins and/or lignin via tyrosine residues in the 13 pleated sheet structure exists as tyrosine residues are placed in alignment to each other as described by de Oliveira et al.(1990).

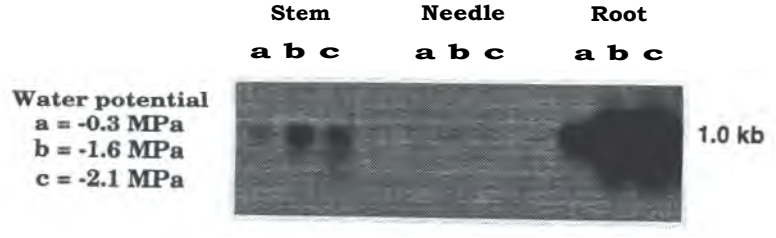

Figure 1. Northern Analysis of *1p5*.

MSKQKLLIFAAMAGLLFACAAVESRIARSDLGLDLGGGLGLGVGVGAGLGLGGGSASGSGSGSGS
GSGSGSGAGSAAGSGSGSGAGSGAGSYAGSGAGNGGGQGRGSGSGYGSGSGYGAGNGNGNGYGAG
SGYGAGNGNGNGYGAGSGSGSGSGSGSGRGYGSGSGTGSGYGSGSGSGYGNGSGSGSGYGAGDDG
SNEGASGGGY

Fig. 2. The predicted translation product of 1p5. The putative signal peptide is given in bold letters. The tyrosine residues with potential to be involved in crosslinking are underlined.

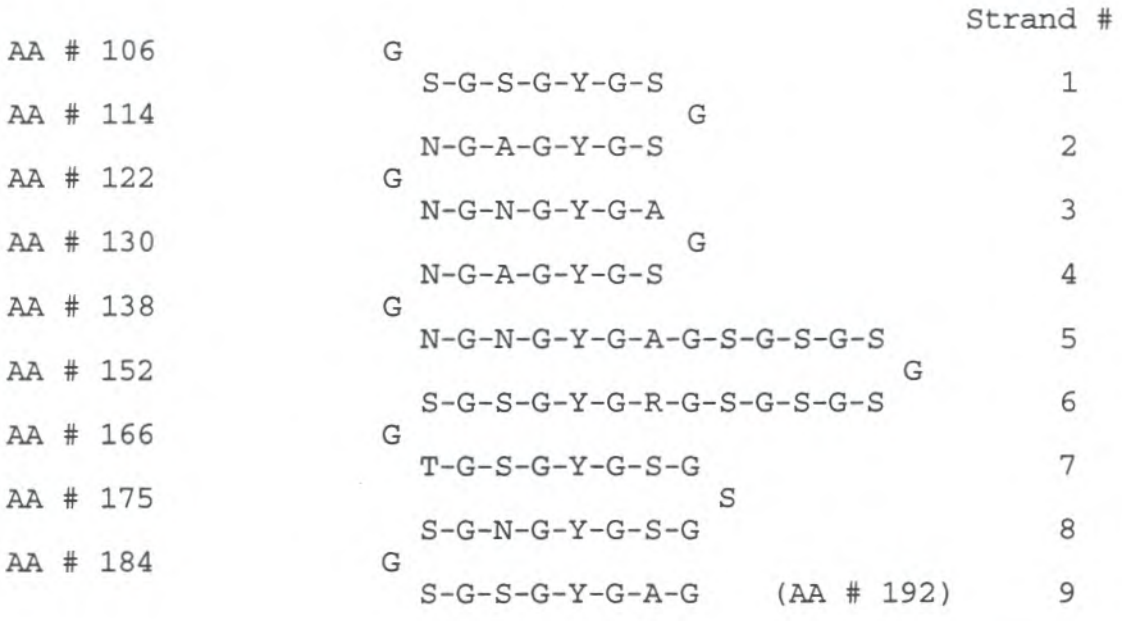

Figure 3. The proposed anti parallel ß pleated sheet structure of the LP5 at the direct repeats.

In order to confirm the function of LP5 in-vivo, the *1p5* cDNA clone was cloned behind a CaMV 35S promoter, and was inserted into pBI121 in between ***nptII*** and *gus* genes. This vector was then inserted into ***Agrobacterium tumefaciens*** LBA4404, and was used in transformation of tobacco leaf discs. More than twenty different, stable transformed, GUS positive tobacco plants were obtained after selection on kanamycin media. These were grown to maturity, and were allowed to seed. The seeds were collected and analyzed for both GUS activity and kanamycin resistance. Almost all GUS activity was limited to the phloem tissue in all transformed plants. This may be due to the 35S promoter which has the origin in a pathogenic virus which are adapted to live in the vascular system. Northern analysis indicated the presence of transcripts for both ***gus*** and 1p5 in the transformed leaves (Fig. 4). No differences were observed in the phenotype of the transformed plants compared to nontransformed. Southern analysis indicated the possible incorporation of two copies of T-DNA in one of the plants and the presence of /p5 homologs in tobacco. Chi Square analysis of the number of seedlings resistant to kanamycin compared to kanamycin sensitive seedlings in the Fl generation of transformed plants confirm this observation. Furthermore, GUS activity was observed in root hairs of the seedlings in the Fl generation. These studies are continuing to determine the function of LP5 in pines.

In our quest to characterize the regulatory elements in the 5' upstream sequences of *1p3* and *1p5*, Southern analysis indicated that the *1p3* gene belongs to a multi-gene family and that *1p5* has

only two members in it's family in loblolly pine. During the screening for genomic clones, 2 clones representing *1p3* and one representing 1p5 were isolated.

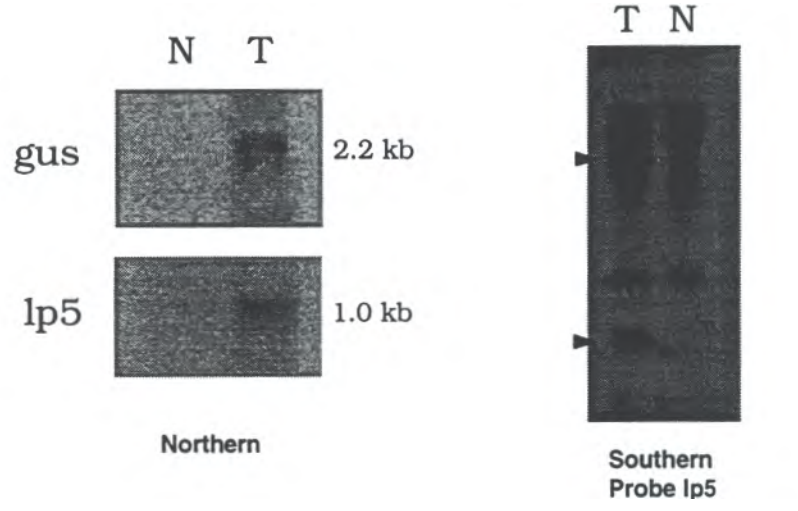

Figure 4. Northern and Southern analysis of transformed tobacco. Northern was done with leaf RNA and probed with *gus* and *1p5.* Southern was done on BamHI digested total DNA. Arrows indicate possible *1p5* inserts in the transformed genome. N = Nontransformed, T = Transformed.

The *1p3* genomic clones were members of that gene family and did not represent the exact cDNA clone. But the predicted translational products were very similar to each other. There is an intron in one clone and the other is being characterized. The *1p5* gene did not have any introns and matched base to base to the cDNA clone, indicating that it is the exact genomic counterpart of the *1p5* message.

The 5' transcription start sites of *1p3* and *1p5* genes were located by primer extension. It appears that the start site of the *1p5* transcript is flexible within 3 adjoining bases about 25 bases downstream from a putative TATAA box. A convenient EcoRI site is located just a base pair upstream of the predicted transcription start site. A 2.3 kb HindIII:EcoRI fragment containing the whole 5' upstream sequence was subcloned in front of the *gus* gene. Likewise, a 1.2 kb 5' upstream sequence of the *1p3* genomic clone was also cloned in front of *gus* in pB1221. Both these vectors were used in biolistic transfoLmation of slash pine suspension cultures using DNA coated gold particles. Both *1p5* and *1p3* promoters were functional when tested, and gave similar number of blue spots (positive for GUS with X-Gluc) against a CaMV 35S promoter (Data not shown). When the bombarded cells were placed on media with mannitol concentration adjusted to have an osmotic potential of 1.1 MPa, there was a marked increase in the number of blue spots observed (Fig. 5). This increase was dramatic with the *1p3* promoter compared to the *1p5* 5' upstream sequence (Data not shown).

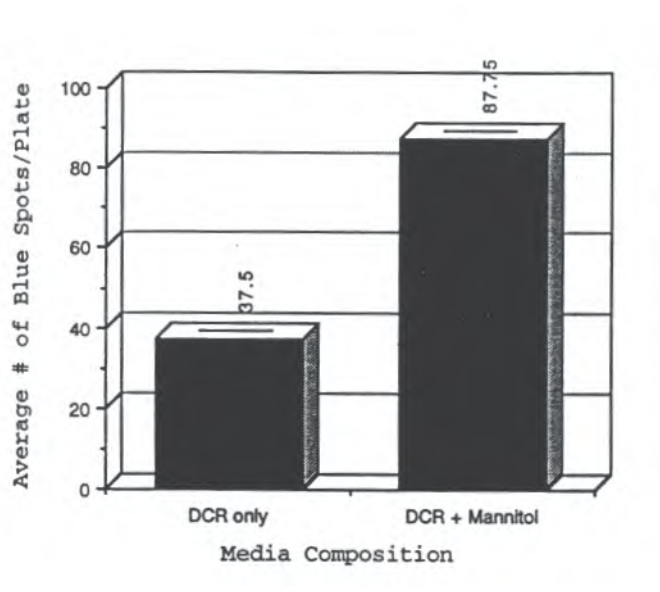

Figure 5. GUS activity of cells bombarded with *1135* promoter:gus construct under water deficit stress.

Modern molecular techniques have proven successful in manipulating genomes to produce transgenic plants of a desired character. These approaches are now being applied to forest species. Plantlets of loblolly pine have been regenerated from tissue culture (Gupta and Durzan 1986), although the efficiency of plantlet regeneration is often low.

However, the ability to propagate plant cells and produce fertile plantlets in vitro has perhaps its greatest potential when applied to the techniques of gene cloning and transfer. Towards this end a number of important conifer genes have been cloned. These findings raise the possibility of performing transgenic experiments with pine genes in 'model' plants, thus gaining some understanding of the function of a sequence before choosing to transfer it into pine tissue.

Transient expression of foreign genes in pine tissue has been achieved in a number of laboratories (Newton et al., 1992) and in some cases stable transformation has been achieved (Sederoff et al., 1986). The regeneration of a fertile transgenic plantlet, however, has proven more difficult but recent reports of success using gene gun technology (Newton et al., 1992) suggest that this goal is attainable, and ultimately should become routine.

The data presented here, together with that cited above, affirm the usefulness of molecular biological methods as tools to study the physiology of these formerly recalcitrant species. These data also indicate the potential for genetic modification of forest species to produce enhanced water deficit stress resistance.

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