cDNA CLONING OF WATER DEFICIT-INDUCIBLE GENES FROM LOBLOLLY PINE

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Abstract.--Pine trees produce new proteins when growing under water deficit and this is achieved principally by activating formerly quiescent genes. Determining the identity of these genes, how they sense changes in the environment and the role their proteins play in drought tolerance is important for an appreciation of tree growth under stress and for emerging biotechnologies. We have isolated cDNA clones (DNA copies of the mRNA) of drought-induced genes from Loblolly Pine (Pinus taeda). DNA sequencing and computer aided comparisons reveal strong similarities between these pine clones and stress-induced genes in other plants, particularly those involved in lignin synthesis.

Keywords: gene expression, drought, cDNA cloning, Pinus taeda L., stress proteins, O-methyl transferase, SAM synthetase, ABA.

INTRODUCTION

Drought is one of the principal environmental stresses affecting tree growth. Whether episodic or perennial, drought impairs development and productivity and renders trees susceptible to secondary, normally, sub-lethal insults. In extreme cases drought will destroy large areas of forest thus contributing to land erosion and to desertification. Drought stress is the most common cause of pine seedling mortality in both naturally regenerated and planted stands in the U.S. (Anonymous 1986). In one long term study drought accounted for 57% of first year seedling mortality (Williston 1972)

The physiological effects of water shortage upon trees have been investigated extensively (Pallardy 1981, Newton et al. 1985,1990, Valluri et al. 1988, Vance and Zaeer 1988) and the development of drought-tolerant, high-performance trees has been pursued through breeding programs (Wakeley 1954, van Buijtenen et al. 1974, Newton et al. 1986). Currently these approaches are being supplemented by the molecular techniques of genome mapping to assist in identifying traits with particular chromosomal fragments (Neale et al. 1989, Tauer et al. 1992). As classical and modern approaches converge there is a growing need for information on the molecular effects of environmental stress on trees but little information is available at present.

By contrast the molecular biology of drought in herbaceous plants is an active field. Alteration in gene expression in plants growing under water-deficit has been observed for many crop species (Heikkila et al. 1984, Ramagopal 1987, Guerrero and Mullet 1988) and the isolation of clones from

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cDNA libraries, using altered expression as a selection criterion, has permitted the identification of many stress-inducible proteins (Skriver and Mundy 1990, Bray 1991, Olsen et al. 1992, Bohnert et al. 1992). In some cases functions have been ascribed to the putative polypeptides bases on sequence similarities to previously characterized proteins (Singh et. al. 1989, Borkird et al. 1990, Downing et al. 1992). A number of these drought-inducible genes show homology to genes expressed in maturing embryos (Dure et al. 1981, Baker et al. 1988, Dure et al. 1989). Conversely, many of the Late Embryogenesis Abundant (Lea) proteins (Galau et al. 1986, Baker et al. 1988) are induced in the vegetative tissue of mature plants during periods of dessication or by exogenous application of ABA (Gomez et al. 1988, Mundy and Chua 1989, Close et al. 1989, Bray 1990). No function has been elucidated for the majority of the water deficit-inducible proteins, however, the strong sequence conservation among species has prompted speculation as to their role in dessication tolerance (Dure et al. 1989, Dure 1993).

Work from our laboratories has shown that dehydration does alter the pattern of protein synthesis in loblolly pine (Valluri et al. 1988, 1989; Vance et al. 1988; Funkhouser et al. 1993), an expected result and one which is consistent with other plants. We have sought to identify these proteins through cDNA cloning. Here we report the construction of a cDNA library from roots of a drought-stressed loblolly pine, selection and tentative identification of genes. A number of clones with striking sequence similarity to wound/pathogen-induced proteins have been recognized, however, most of the pine clones have structural features or patterns of expression which distinguish them from their homologs.

METHODS

Plant Growth and Water Status

Plants were acclimatized by irrigating daily with Reverse Osmosis (RO) water for seven days. Water was then withheld, in staggered fashion, so that different sets of plants were deprived for various periods. Control plants were watered continuously. All plants were harvested pre-dawn on the same day. Plant pre-dawn water potential was determined with a pressure bomb (Scholander et al. 1964) and thermocouple psychrometry with methods described previously (Emadian and Newton 1989; Castro-Jimenez et al. 1989; Newton et al. 1989). A branch from each plant was used for measurement, the rest of the plant was harvested, leaves, stem and roots were frozen separately.

RNA Isolation

Conventional methods of RNA preparation are often inefficient when applied to pine due to the high levels of resins and phenolics in pine tissue, especially needles. We have developed a method for preparing RNA which is free from impurities and suitable for enzymatic manipulation (Chang et al. 1993). This method was used in this study.

cDNA Library Construction and Molecular Methods

The root tissue of an 8 month old seedling with a water potential of -1.1 MPa was chosen for cDNA library construction. Poly(A)$^+$ RNA was isolate

from total RNA using the PolvA Tract magnetic sphere system (Promega, WI). A cDNA library was prepared using the Stratagene 1-ZAP cDNA Synthesis kit according to manufacturer's recommendations (Stratagene, CA). Standard molecular methods were used in other cases (Sambrook et al. 1989)

DNA Sequencing and Data Analysis

DNA was sequenced by the Sanger method using Sequenase™ (USB, Cleveland, OH) in the DNA Technologies Laboratory, Texas A&M University. Computer searches of the NIH Genbank and Swiss Protein databank were performed. Data was analyzed using the DNA Star program (Madison, WI).

RESULTS AND DISCUSSION

Isolation and Expression of the Drought-Regulated Genes

A cDNA library of $7.2x10^6$ pfu was constructed from poly A(+) RNA isolated from the roots of a 5 month old, water-stressed pine seedling. Clones of drought stress-regulated genes were isolated by differential screening of about 15,000 independent plaques essentially as described by Adair et al. (1992). From 28 putative water stress-responsive clones identified in a primary screening, 15 were confirmed by Northern analysis. Six distinct clones which contained cDNA molecules whose size was similar to that of the mRNA to which they hybridized, were sequenced.

The gene corresponding to clone LP1 is induced by water deficit in both needles and roots. In stems, however, the expression appears to be constitutive (data not shown). In needles, the expression drops sharply as water stress increases; in roots, this diminution is gradual. The sequence and transcript size predict a 5' untranslated region of around 300 nucleotides for this mRNA (data not shown). LP1 has a long open reading frame beginning with an ATG which could encode a polypeptide of 224 amino acids. The predicted sequence of this protein bears strong resemblance (40% identity, 62% similarity) to a Caffeoyl-CoA-3-O-Methyltransferase from parsley (Fig. 1, Schmitt et al. 1991). In parsley, the mRNA for Caffeoyl-CoA-3-O-Methyltransferase has a 5' untranslated region of about 300 nucleotides (Schmitt et al. 1991). The parsley protein has been implicated in plant defense responses to pathogens and is part of the phenylpropanoid pathway (Pakusch et al. 1989, 1991, Hahlbrock and Scheel 1989). Caffeoyl-CoA-3-O-Methyltransferase converts Caffeoyl-CoA to Feruloyl-CoA, a precursor of lignin (Lewis and Yamamoto 1990, Sederoff and Chang 1991). In parsley, this enzyme is induced by fungal elicitors and is implicated in the synthesis of ferulic esters for cell wall reinforcement, a defense response to pathogen attack (Pakusch et al. 1989, 1991). Evidence for this pathway in pine has not yet been furnished. In parsley cell culture, induced expression is rapid and transient (Schmitt et al. 1991), in loblolly pine seedlings subjected to water stress a more sustained induction is apparent (data not shown).

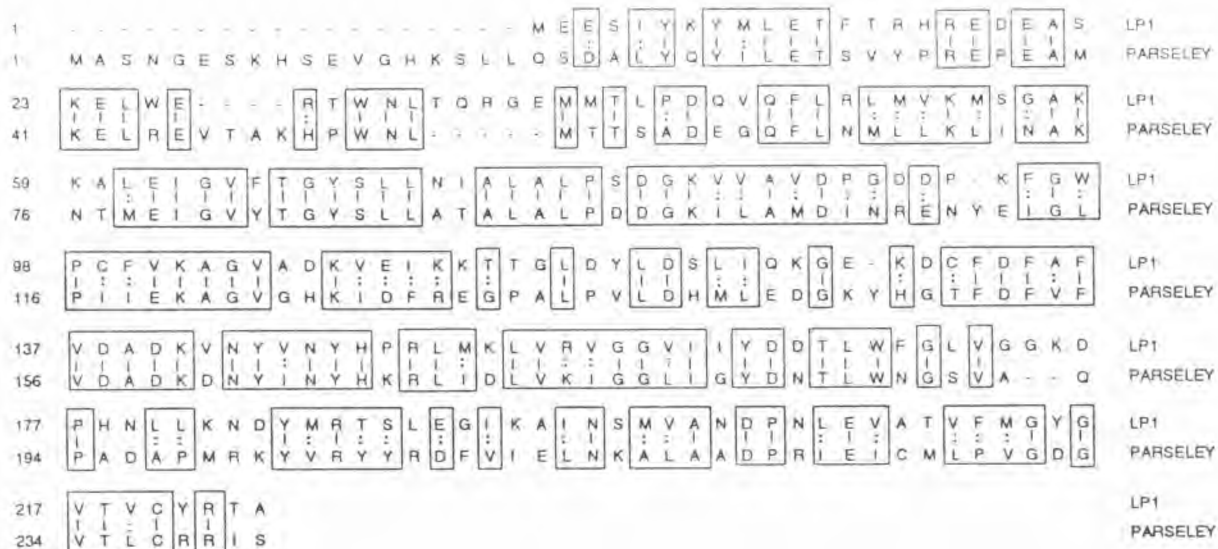

Figure 1. Amino acid homology between predicted polypeptides from loblolly pine clone LP1 and parsley Caffeoyl-CoA-3-O-Methyltransferase (Schmitt et al. 1991). Solid lines indicated identical residues, dotted lines, similar residues.

A number of cDNA clones of putative O-Methyltransferases have been isolated recently. Those from parsley, alfalfa and aspen were described as biphasic Caffeic Acid O-Methyltransferases capable of converting both caffeic acid to ferulic acid and subsequently of methylating 5-hydroxy ferulic acid to form sinapic acid (Bugos et al. 1991, Gowri et al. 1991). An O-Methyltransferase from Ice plant has been shown to convert myo-inositol to ononitol, a precursor of pinitol, a cyclic sugar with osmoremedial properties (Vernon and Bohnert 1992). When all five predicted polypeptides are aligned (Fig. 2) we see a striking dichotomy; parsley and pine forming one class of sequence while alfalfa, aspen and ice plant form the second class, with little homology between the groups. Within the second group we have enzymes which have quite different substrate specificities thus the sequence similarity of the alfalfa and aspen enzymes to the OMT from ice plant is most notable. This feature may reflect the broader substrate specificity of the "biphasic" enzymes. It would be of great interest to determine the ability of the alfalfa and aspen enzymes to methylate myoinositol.

LP2 is water deficit-inducible in all tissues with maximal expression occurring in the stems. Expression remains high in all tissues even under severe stress (approximately 30 days without water). The putative protein encoded by this clone is virtually identical to S-adenosylmethionine synthetase from a number of plant species (Fig. 3, Peleman et al. 1989, Larsen and Woodson 1991, Kawalleck et al. 1992).

S-adenosylmethionine synthetase (SAM synthetase) catalyses the biosynthesis of S-adenosylmethionine from methionine and ATP (Tabor and Tabor 1984). S-adenosylmethionine is a co-factor in numerous biochemical reactions acting

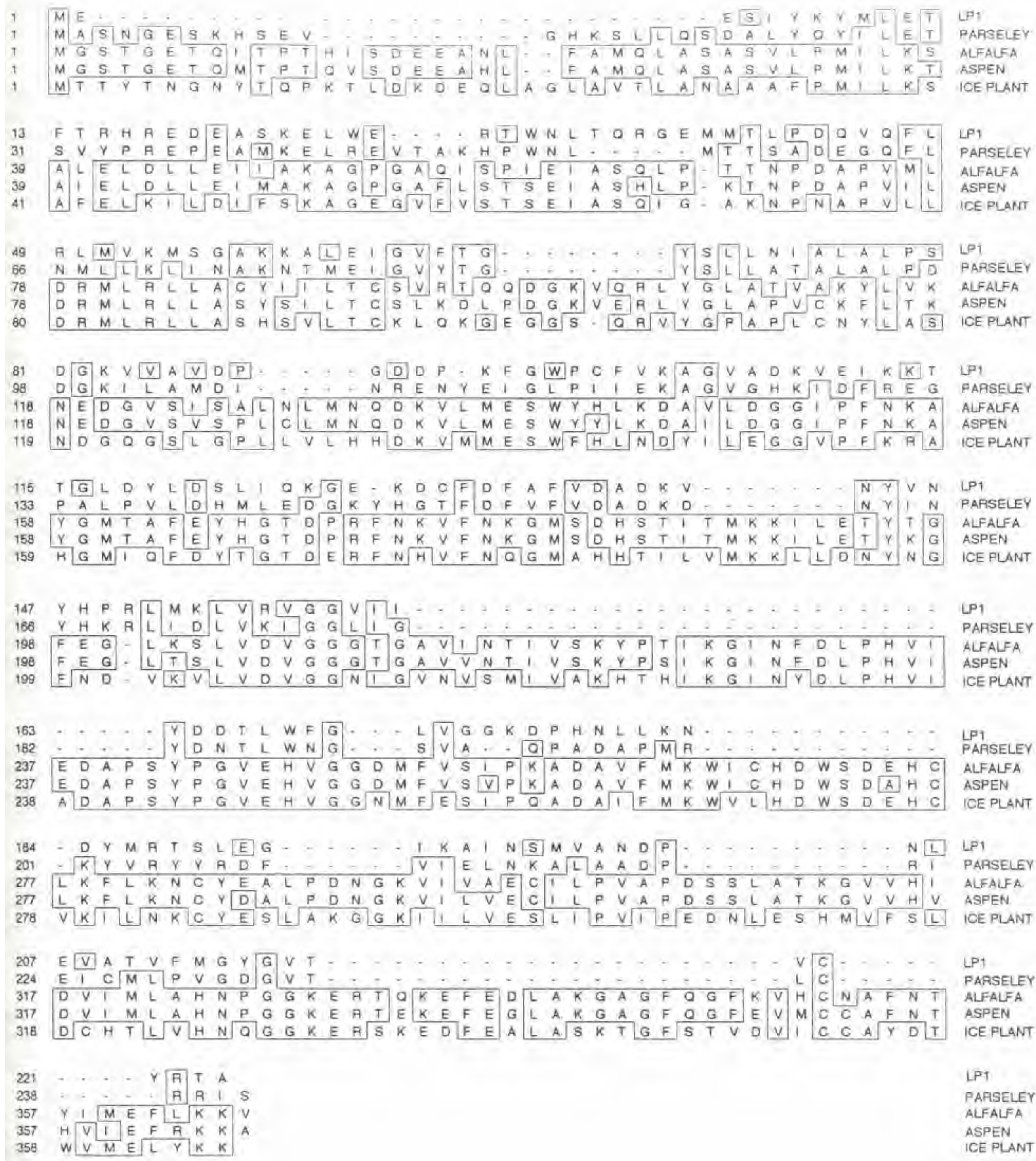

Figure 2. Sequence comparison of 0-methyltransferase proteins from loblolly pine, LP1, parsley (Schmitt et al. 1991), alfalfa (Bugos et al. 1991), aspen (Gowri et al. 1991) and ice plant (Vernon and Bohnert 1992). Identical residues of one group are boxed.

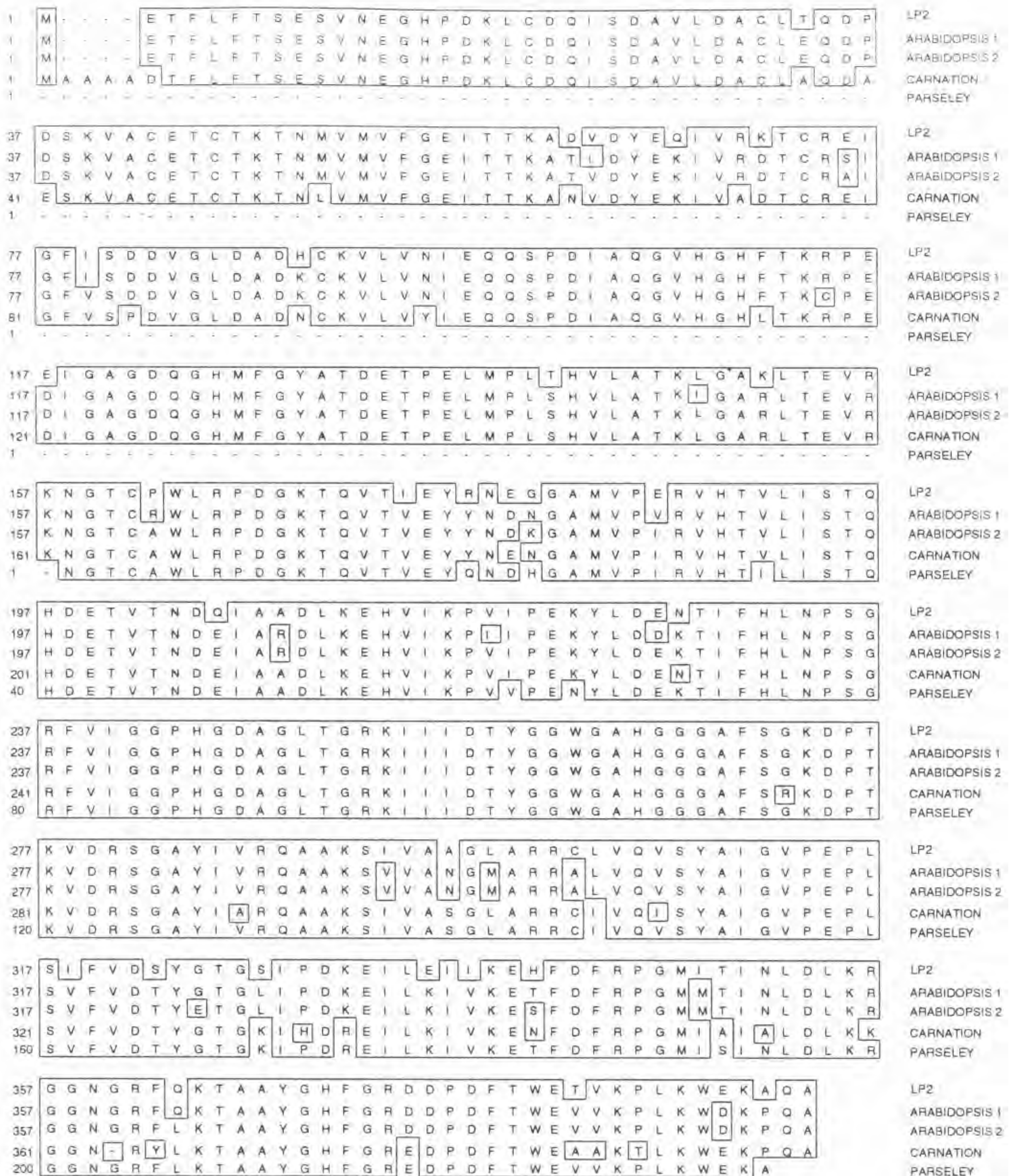

Figure 3. Protein sequence comparison of LP2 to SAM synthetase proteins from <u>Arabidopsis</u> (Peleman et al. 1989a,b), Carnation (Larsen and Woodson 1991), and parsley (Kawalleck et al. 1992). Identical residues are boxed.

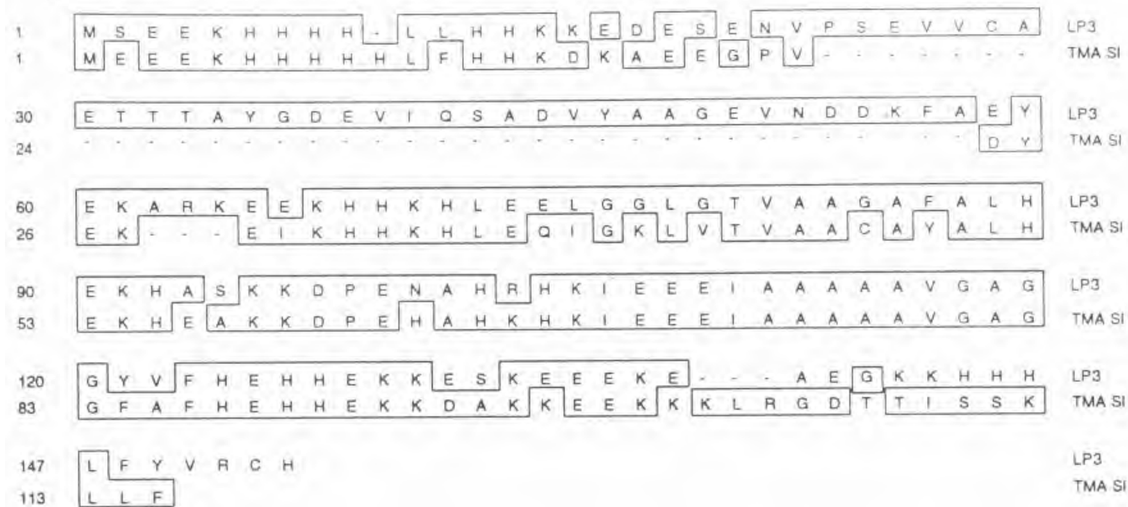

Figure 4. Protein sequence comparison between loblolly pine clone, LP3, and ABA-inducible tomato clone, TMA S1.

as a methyl donor to proteins, lipids, polysaccharides and nucleic acids (Tabor and Tabor 1984), it is also an intermediate in the synthesis of ethylene (Yang and Hoffman 1984). The ubiquity of the enzyme has led to a designation of the SAM synthetase gene as a 'housekeeping gene' but surpris ingly the gene shows strong cell specificity being highly expressed in stem, root and callus of *Arabidopsis,* with little expression in leaf (Peleman et al 1989). Peleman et al. (1989) suggested that SAM synthetase expression was highest in lignifying tissue. Recently, in parsley, induction of SAM synthetase gene expression by fungal elicitors has been demonstrated (Kawalleck et al. 1992). Since lignification is a plant defense response these two observe tions seem consistent and compatible with our own demonstration of SAM synthetase induction in response to environmental stress. By contrast the requirement for S-adenosylmethionine in ethylene synthesis appears small; SA, synthetase mRNA levels decline markedly in climateric carnation petals (Woodson et al. 1992).

Clone LP3 is expressed predominantly in roots. Levels of mRNA are detected in needles and stems but at much lower levels though the pattern of induction appears similar in all tissues with different water potentials for maximal expression. A polypeptide of 153 amino acids is encoded by LP3 which has 49% identity, 56% similarity to a tomato protein TMA SN1 (Genbank Acc # L08255). This tomato protein is reported to be ABA-inducible and expressed during fruit ripening. Interestingly, the putative loblolly pine protein possesses a region of 34 amino acids (Pro 24 to Ala 58) which is absent from the tomato homolog. If we exclude this region and re-consider only the matc the identity rises to 62%.

Levels of the phytohormone ABA rise in plants subjected to water stres (Hartung and Davies 1991) and the effects of water stress on gene expression often can be mimicked by the exogenous application of ABA (Skriver and Mundy 1990). Further, the induction of drought-responsive genes is depressed in mutants deficient in ABA synthesis (Cohen and Bray 1990, Bray 1991). These

observations have led to proposals that ABA acts as the cellular mediator of stress (Zevaart and Creelman 1988) though the observation that some drought-responsive genes are insensitive to ABA suggests the existence of multiple transduction paths and/or additional induction factors (Xu et al. 1990, Bostock and Quatrano 1992).

Concluding Remarks

The cDNA clones described in this paper identify genes induced by water stress in loblolly pine which show strong sequence similarity to genes induced by wounding and pathogen attack in other plants. The putative polypeptides synthesized by loblolly pine have distinctive features. The sequence differences between LP1 and 0-methyltransferases from other sources are intriguing (Fig. 2). The substrate specificity of the LP1 protein is currently being determined and this should allow a more accurate definition of the role these proteins play in stress physiology. The induction and sequence of LP2 (SAM synthetase) is consistent with recent findings. The LP3 protein has yet to be defined. These results, combined with our earlier findings on promoter structure and function (Newton et al. 1992, Funkhouser et al. 1993) reveal great similarities at the molecular level between pine and herbaceous plants. These demonstrations indicate a likely success in the expression of pine genes in rapidly regenerating transgenic model plants. Such gene transfer experiments are being conducted in our laboratories and should permit the evaluation of pine gene function for both research and applied purposes.

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