IDENTIFICATION OF A PUTATIVE 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE FROM *PINUS TAEDA*: EVIDENCE FOR ROLES IN SIGNALING THE DEVELOPMENT OF COMPRESSION WOOD

by

JOHN ROBERT BARNES

(Under the Direction of Jeffrey F.D. Dean)

ABSTRACT

The gene encoding the protein 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) has been extensively studied in plant species because of its role in regulating the formation of the plant hormone, ethylene. Although the ACC synthase gene has been heavily studied in angiosperms, it has never been cloned from a gymnosperm species. This dissertation presents the cloning of a gene, PTACS-1, with high homology to ACC synthase, from *Pinus taeda* (loblolly pine), a commercially important tree in the southern U.S. Additionally, a shorter nearly identical transcript, PTACS-1s was identified. Expression of the PTACS-1 and PTACS-1s transcripts was measured using quantitative PCR in tissues throughout the tree, and in response to known elicitors of ACC synthase gene transcription. Further, the expression of PTACS-1 and PTACS-1s were monitored in response to known elicitors of compression wood, a reaction wood formed in

gymnosperms. Putative roles for PTACS-1 in compression wood development are discussed.

INDEX WORDS: ACC synthase, compression wood, ethylene biosynthesis, *Pinus taeda*, mechanical stress, gravisitmulation.

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JOHN ROBERT BARNES

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JOHN ROBERT BARNES

Major Professor: Jeffrey F.D. Dean

Committee: Bruce Bongarten
Claiborne Glover
Russell Malmberg
Scott Merkle

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

To my wife and family who always believed in me.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Loblolly pine

Loblolly pine (*Pinus taeda L.)* is one of the most important commercial tree species in the U.S. Its few branches, straight trunk and fast growth make it a nearly ideal tree for the forest products industry. Loblolly pine's native range stretches from southern New Jersey to Florida, and as far west as Texas (Fig. 1.1). It grows well in a variety of soils, and is known to regularly grow to heights of 100 ft. Loblolly pine is monoecious with red or yellow male strobili grouped as cylindrical shaped fingers at the base of the current year's branches. The female cones are purple-yellow and occur at the distal end of spring shoot flushes. Female cones are ovoid in shape with sharp spines. Loblolly pine needles, which typically occur in clusters of three, are approximately 5 inches long and dark green or yellow-green color.

As mentioned, loblolly pine is a prized tree for commercial uses as its straight trunk can be nearly devoid of knots for up to 30 ft, making it very useful for board lumber. Also, the tracheids and fibers in loblolly pine make it useful for pulpwood. Half of all loblolly pine harvested in Georgia in 2001 was for lumber, while just over a third was used for pulp (Johnson and Wells 2004). In 2001, approximately 500 million cubic feet of loblolly

pine and shortleaf pine were harvested for forest products in Georgia, which was well over half (55%) of all the softwoods harvested, and 42% of all trees harvested for forest products the forest products industry (Johnson and Wells 2004). As a result of loblolly pine's importance, it is one of the most heavily studied forest trees and the best studied of all conifers. Extensive genetic mapping and EST (expressed sequence tag) projects are helping to develop a basic understanding of the genes in this species and how they interact to affect tree growth and development (Devey et al. 1994, Allona et al. 1998, Lorenz and Dean 2002).

Wood production in gymnosperms

Wood, or secondary xylem, is produced through the division of meristematic cells in the vascular cambium in response to environmental cues perceived in the tree's crown. The vascular cambium, responsible for the new growth, is a lateral meristem present in the stem, or trunk, of a tree. The cambium is a single layer of cells, called initials, derived from the procambium, which develops from the apical meristem and promotes radial growth of the stem (reviewed in Plomion et al. 2001). However, because the initial cells are difficult to

identify directly, the cambium is usually referred to as the cambial zone, which can be from 6 to 40 cells thick (reviewed in Kozlowski and Pallardy, 1997b), and is composed of two types of highly vacuolated cells (Plomion et al. 2001). Fusiform initials give rise to xylem and phloem mother cells (reviewed in Plomion et al. 2001). Though periclinal divisions, these xylem and phloem mother cells divide in turn to give rise to functional xylem and phloem tissues. In addition, some cambial divisions generate ray initials, which later differentiate into vascular rays (reviewed in Kozlowski and Pallardy, 1997b).

Xylem tracheids and rays are the major component cells of conifer wood, accounting for 90-95% of wood cells. After the cambium divides to form xylem mother cells, the resulting cells undergo rapid cellular expansion. The degree and direction of expansion depend on the tree species in question. In angiosperms, vessel elements lengthen greatly, as well as expand both radially and tangentially (reviewed in Kozlowski and Pallardy, 1997b). Gymnosperm tracheids actually lengthen relatively little after division; however, due to their long initial length, they are typically longer than angiosperms vessel elements. Gymnosperm tracheids are six-sided, long and thin, with

thick cell walls. The cell walls of these tracheids begin to thicken soon after cell expansion. Their secondary cell walls are deposited in three layers, (S_1, S_2, and S_3, Fig. 1.2), and are composed of cellulose, pectin, hemicellulose, lignin, and small amounts of protein and lipids. Conifer phloem cells typically do not form secondary walls, which helps to distinguish phloem tissue (bark) from xylem tissue (wood). After deposition of the secondary cell wall, the xylem tracheids undergo apoptosis, leaving behind only the cell wall to serve for water transport and stem support.

The size, shape and composition of the xylem cell walls combine to impart the main properties of wood. The term "wood quality" has been used to describe the combination of these elements in regards to their utility as either board lumber or pulp (Karenlampi 1992). Wood quality has long been a focus of breeding strategies in the forest products industry as it directly correlates to the amount of usable wood that can be obtained from each tree.

Juvenile wood

Wood that is produced in the first 5 to 20 years of tree growth (depending on the species) is called juvenile wood. Juvenile wood is produced from immature cambium cells, and is characterized by fibers having shorter and

thinner cell walls and larger lumen diameters than those found in mature wood (Fig. 1.2). Chemically, juvenile wood tracheids have less cellulose and more lignin than do mature tracheids. Juvenile wood also has an increased microfibril angle, a greater occurrence of spiral grain and a larger percentage of compression wood (Bendtsen 1978) than does mature wood. All of these characteristics make juvenile wood undesirable for use in the forest products industry. Increased spiral grain and microfibril angle both lead to difficulties in pulping and warping of board lumber (Josa and Middleton 1994). The decreased size of juvenile secondary cell walls and their increased lignin content causes the tracheids to collapse into thinner ribbons during the pulping process yielding less pulp (Foelkel 1976). Specific gravity is also decreased in juvenile trees, leading to a 5 to 8 cubic foot disparity in the amount of woody material present per cord of cut wood (Thomas 1984). This disparity typically means higher prices are paid for less usable wood.

Compression Wood

Compression wood is a reaction wood formed in gymnosperms (reviewed in Timmell 1986). The tree utilizes compression wood to reorient the trunk from a leaning

position back to vertical, and to support horizontal branches. It is found at a higher percentage in juvenile wood than in mature wood. Compression wood is characterized by the eccentric pattern of growth rings, increased radial growth in the compressed area, and reddish wood color (Fig. 1.3). Compression wood is also formed in response to wind perturbation, snow, and other mechanical stresses, such as those occurring in leaning stems and on the underside of branches (Telewski and Jaffe 1986abc). The bulk characteristics of compression wood are based on both physical and chemical changes in the wood. Compression wood tracheids ultimately show many of the same characteristics of juvenile wood tracheids, only to a greater extent (Fig. 1.2). Compression wood tracheids are cylindrical instead of rectangular and have no S_3 layer in the secondary cell wall. The circular nature of compression wood tracheids leads to large intercellular spaces, which are usually not present in normal wood. Compression wood has dramatically shorter tracheids, which are believed to be the result of a higher rate of anticlinal divisions in developing compression wood (Timmell 1986). In addition, compression wood fibers contain 30-40% more lignin and 20-25% less cellulose than normal fibers. Compression wood also displays increased

phenolic content, characterized by an increase in the proportion of p-hydroxyphenylpropane units incorporated into the lignin (Timmell 1986). Increased microfibril angle is also a hallmark of compression wood fibers that make the wood very undesirable to the forest products industry due to the twisting and warping it causes in lumber (Josa and Middleton 1994).

Phytohormones and wood production

Although wood quality has a genetic component, it is also is influenced strongly by the environment (Lee 1999). Plant cell growth is affected by hormones, of plant growth regulators (PGRs) (reviewed in Kozlowski and Pallardy, 1997a), such as auxins, gibberellins, cytokinins, abscisic acid, and ethylene, all of which play roles in the regulation of cambial development and tracheid differentiation (reviewed in Little and Pharis 1995). Exogenous application of plant hormones has been observed to alter or affect all aspects of cambial growth and xylogenesis suggesting that variation in wood properties is at least in part controlled by hormonal changes (Mellerowicz et al. 2001). For example, Eriksson et al. (2000) demonstrated that increased levels of gibberellins result in larger and more abundant tracheids. Likewise,

Tuomainen et al. (1997) demonstrated that the auxin, indole acetic acid (IAA), functions as a "positional signal" affecting cambial growth rates and the duration of cell tracheid differentiation in hybrid poplar. Recently, microarray analyses demonstrated that most of the genes up-regulated during secondary xylem formation in *Arabidopsis* had cis-acting IAA responsive elements associated with their promoters (Ko et al. 2004). The authors also found that weight carried by the plant body was an important initiator of secondary xylem formation in the *Arabidopsis* model system (Ko et al. 2004).

Ethylene

Ethylene's effect on trees was noticed in the late 1800's when gas from street lamps was shown to cause defoliation of nearby trees. Ethylene, however, was not truly recognized as a hormone until 1901 when Dimitri Neljubov outlined the classic "triple response" in etiolated pea hypocotyls. The triple response is defined by increased radial growth, decreased lateral growth, and altered gravitropic response or epinasty. Since that time, ethylene has been shown to function in fruit ripening, abscission, senescence, wounding, and mechanical stress (Botella et al. 1995, Kato et al. 2000, Pandey et al. 2000,

Alexander and Grierson 2002, Patterson and Bleeker 2004). Ethylene is unique in that it is a gaseous hormone and, therefore, is thought to function in close proximity of its source. Although, much of the research on ethylene effects in plants has focused on fruit ripening (reviewed in Alexander and Grierson 2002), abscission (Patterson and Bleeker, 2004), and wounding (Kato et al. 2000), that research will not be the major focus of this review.

Ethylene biosynthesis

In plants, ethylene is produced via an offshoot of the methionine cycle (Fig. 1.4) where S-adenosyl methionine (SAM) is converted by 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) to 1-aminocyclopropane-1-carboxylate (ACC) and 5-methylthioadenine (MTA). ACC is then oxidized by ACC oxidase yielding ethylene, CO_2 and HCN. Synthesis of ACC is known to be the first committed step in ethylene production (reviewed in Wang et al. 2002).

SAM is the primary methyl donor in plants and 80% of methionine is converted into SAM by SAM synthetase (EC 2.5.1.6) (Ravanel et al. 1998). 5'-Methylthioadenosine, a by-product of the ACC synthase reaction, can be recycled by a methionine salvage pathway allowing ethylene to be produced without causing a large drain on methionine levels

within the cell (reviewed in Bleeker and Kende 2000). ACC, in addition to being oxidized to form ethylene, can be malonylated via a malonyl transferase to form an irreversible sink for ACC, which prevents conversion of ACC to ethylene under various conditions (reviewed in Fluhr and Matoo 1996).

Genetic screening and manipulation has made it possible to identify receptors and key members of the ethylene-signaling cascade. These studies have focused on mutants that lack an ethylene response (reviewed in Bleeker and Schaller 1996, Fluhr and Mattoo 1996, Kieber 1997, Wang et al 2002). Ethylene receptors will not be a major focus of this review. As a majority of the regulation of the ethylene pathway seems to occur through ethylene biosynthesis this will be the major focus of this review.

Ethylene and Xylem differentiation

Ethylene has been known to stimulate lateral growth in stems since its identification as a plant growth regulator in 1901. Neljubov showed that ethylene caused the so-called "triple response" (review Taiz and Zeiger 1998). However, the specific role of ethylene in cambial differentiation remains unclear. Ethylene levels are known to increase during active cambial growth and drop to minimal levels

during times of dormancy (Klintborg et al. 2002). Ethylene in loblolly pine is also known to stimulate the production of arabinogalactan-proteins, which are thought to be important in xylogenesis (No and Loopstra 2000). Although little is known about the contributions of endogenous ethylene to wood formation, experiments employing the ethylene releasing compound, ethrel (2-chloroethylphosphonic acid), have demonstrated an impact on xylogenesis. Eklund and Little (1995) studied the role of the auxin, IAA, and ethylene in controlling tracheid production by applying IAA and ethrel to stems. They found that cambial activation was associated with a rise in ethylene evolution. The authors concluded that, while ethylene did not directly stimulate cambial activity and tracheid production (as did IAA), it could stimulate production of tracheids when abnormally high levels caused accumulation of IAA.

As an expansion on the work by Eklund and Little (1995), Kalev and Aloni (1999) made longitudinal cuts in hypocotyls of *Pinus pinea*, and applied ethylene (via ethrel application) to the separated pieces of hypocotyl. They found that ethylene application caused the production of tracheids through site-specific accumulation of auxin, similar to the findings of Eklund and Little (1995). Kalev

and Aloni (1999), however, found that the newly produced tracheids were reoriented from axial to lateral positions within the hypocotyls. They also found that ethylene induced the conversion of parenchyma cells to tracheids on the cut hypocotyl surface in the absence of auxin. These results led to their postulation that ethylene is involved in formation of rays.

Ethylene was found to be produced not only in the cambial zone, but also in the transition zone between heartwood and sapwood (Nelson 1978). Heartwood, which is found in the core of the tree, has no living cells and is normally non-conductive. Heartwood develops in the oldest xylem, and is typically darker than the sapwood due to the incorporation of polyphenolic compounds into the tissue. Ethylene has been implicated as a major signal governing the transition between heartwood and sapwood (Shain and Hillis 1973). Solid evidence for ethylene serving as a causative agent for heartwood formation was obtained by Nilsson et al. (2002). In the experiment, holes bored into *Pinus sylvestris* trunks were treated with ethylene, carbon dioxide, and nitrogen (to simulate hypoxia). The gases were sealed in the trunk by fitting a steel tube into the hole and capping it with a stopper. Gas injections were carried out over 6 months during the active growing season.

Ethylene was found to cause the largest spread of "induced discolored wood" compared to the other treatments, while treatments with CO_2 and N_2 did not have significant differences in levels of induced discolored wood when compared with trees that were wounded by drilling (Nilsson et al. 2002). In addition, discolored wood stimulated by ethylene contained resin acids, pinosylvin, and pinosylvin monomethyl ether, which are all known indicators of heartwood formation (Nilsson et al. 2002). Therefore, it seems clear that ethylene promotes heartwood formation.

Ethylene and Compression wood

Kalev and Aloni's (1999) study with split hypocotyls also supports the idea that ethylene may be involved in compression wood formation. Compression wood is characterized in part by extensive radial growth and reduced longitudinal growth, conditions that mimic aspects of the ethylene triple response. Compression wood is also known to contain more and larger ray tracheids than normal wood (Timmell 1986), which is similar to the ethrel-stimulated production of rays seen by Kalev and Aloni (1999). Savidge et al. (1983) previously demonstrated elevated levels of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in compression

wood tissue. Additionally, recent two-dimensional electrophoresis studies has shown increased levels of ACC oxidase in compression wood compared to normal wood (Plomion et al. 2000). Telewski and Jaffe (1986abc) found that mechanical perturbation of tree stems led to increased ethylene emissions and the formation of wood with compression wood-like characteristics (increased spiral grain and increased lignin deposition in tracheids). Recently, Du et al. (2003) showed that ethylene evolution was significantly increased on the compression wood side of *Metasequoia glyptostroboides* after the trees were tilted at 45° for two weeks, and similar results were found when the trees were tilted in the opposite direction. In addition, they found that eccentric radial growth occurred in the same sections that emitted the highest levels of ethylene (Du et al. 2003). However, in many cases ethrel applications to conifers have not mimicked the wood formation or tracheid production typifying compression wood (Yamamoto and Kozlowski 1987, Eklund and Little 1996, and Little and Eklund 1999).

ACC synthase

ACC synthase (S-adenosyl-L-methioadenosine lyase, EC 4.4.1.14) is a low abundance, highly labile enzyme of the

ethylene biosynthetic pathway. It was first purified from tomato pericarp tissues, and from which it was determined to be a 50kD protein (Bleecker et al. 1986). The ACC synthase crystal structure was solved by Capitani et al. (1999). The enzyme was identified as being active as a homodimer (Alexander et al. 1994) in which each monomer displays two distinct domains, similar to the aspartate-aminotransferase (AATase). The enzyme has a αβα sandwich domain composed of a 7-strand β-sheet surrounded by 9 α-helices. The second, smaller αβ domain consists of 5 β strands with 5 α-helices coordinated around them (Capitani et al. 1999). The ACC synthase active site was shown to have a structure similar to that found in AATases (Huai et al. 2001). The enzymes were found to have 11 completely conserved residues in common, all of which are essential for enzyme activity. The active site was found to reside in a cleft between the two structural domains and along the monomer face involved in dimerization. The main differences in structure found between the ACC synthases and AATases is the presence of helix 4 in AATases and the presence of helix 2′ in ACC synthases.

ACC synthase catalyses the reaction converting SAM to ACC (Fig 1.5).

SAM + PLP → MTA + ACC

A putative catalytic mechanism for ACC synthase was proposed based on the tomato crystal structure solved by Huai et al. (2001) (Fig 1.5). The model suggests that Arg^{412} holds SAM next to the internal aldimine of the cofactor PLP, while Tyr^{240} removes the proton of Lys^{278} to form an external aldimine. In the next step, Tyr^{152} breaks the C-γ-S bond of SAM via nucleophilic attack and, while Arg^{157} and Arg^{412} hold SAM in the correct conformation, this releases MTA. A covalent intermediate is presumed to form between Tyr^{152} and the C-γ of SAM, which is then converted to ACC-aldimine. Evidence for the covalent intermediate has been reported (Ramalingham et al. 1985, Satoh and Yang 1989, Capitani et al. 1999). In the final step, Lys^{278} attacks the C4' of PLP to release ACC. This mechanism is very similar to that of other PLP-dependent enzymes, with the exception of the γ elimination (step 3), which appears to be unique to ACC synthase.

Regulation of ACC synthase at the protein level occurs through suicide inhibition in which its substrate (SAM) can become covalently linked to the active site, thereby disabling the enzyme (Yip et al. 1990) (Fig 1.5). This side reaction facilitated characterization of the ACC synthase active site by radiolabeling with [^{14}C]SAM followed by $NaB^{3}H_4$ reduction. After trypsin digestion, a 12 aa radio-

labeled peptide was isolated and found to have the sequence H_2N-S-L-S-K-D-L-G-L-P-G-F-R-COOH, where the lysine residue was labeled. Additional active site residues were proposed from comparison with the active site of AATases, both by identification of residues strictly conserved between the two classes of enzymes (White et al. 1994), as well as residues confirmed by crystallographic evidence to be positioned where they could take appropriate part in the catalysis reaction (Capitani et al. 1999).

Suicide inhibition of the ACC synthase enzyme occurs through a β,γ-elimination of MTA leading to the formation of L-vinylglycine (L-VG) (Satoh and Yang, 1989). L-VG is a known inhibitor of several PLP-dependent enzymes besides ACC synthase (Rando 1974, Soper et al. 1977). Based on the crystal structure of the L*ycospersicum esculentum* ACS co-crystallized with L-VG, L-VG is not covalently bound to the ACS monomer (Huai et al 2001). However, Capitani et al. (2002) contested evidence of non-covalently bound inhibition by L-VG based on the structure of the *Malus domestica* ACS co-crystallized with L-VG. Their evidence supported L-VG covalently linked to the enzyme at Lys^{273} via a ketamine intermediate.

In most plants ACC synthase comprises a small gene family that consists of four groups defined by differences

in their isoelectric points (pIs) as determined by isoelectric focusing (Fluhr and Mattoo, 1996). The diverse members of the ACC synthase gene family are thought to play differing physiological roles within the plant (e.g. Liang et al. 1992). Evidence for differing physiological roles was demonstrated by Tsuchisaka and Theologis (2004b) using promoters for the known *Arabidopsis* ACC synthases paired with reporter genes. The different ACC synthase gene promoters had differing responses to IAA, wounding and other treatments, suggesting that the different family members serve in different capacities. In addition, several of the ACC synthase promoters were shown to have overlapping expression patterns with other ACC synthase promoters. The overlapping expression of multiple ACC synthase homologs in the same tissue suggested that: 1) heterodimerization of the ACC synthase might occur in these tissues and serve as a way to regulate enzyme activity or 2) heterodimers previously identified in *E. coli* complementation studies (Tsuchisaka and Theologis 2004a) might actually exist and function *in planta.*

With respect to ACC synthase genes responding to physical stress, Botella et al. (1992) identified three different auxin-induced ACC synthase genes in etiolated mung bean hypocotyls, one of which (AIM-1) was rapidly

(within 10 min) induced by bending stress (Botella *et al.* 1995). Similarly, an ACC synthase homolog, Am-ACS3 was recently shown to be induced in gravistimulated snapdragon stems (Woltering et al. 2005). Am-ACS3 was also highly induced by IAA, but not ethylene (Woltering et al. 2005). The finding that AM-ACS3 is IAA-responsive fits the theory that asymmetric distribution of auxin is a major causative agent for the gravitropic response seen in stems. In addition this finding is in line with a previous finding of Woltering et al. (1991) that ethylene, and a steep gradient of ACC are found in gravistimulated stems. That earlier study suggested a 100-fold increase in the amount of ACC synthase activity in gravistimulated stems.

However, there is still controversy as to the role of ethylene and ACC synthase in the gravitropic response. Madlung et al. (1999) showed negligible differences in the bending response of an ethylene non-responsive mutant of tomato. The mutant showed similar responses to gravitropic stress as were seen in wild type tomato, even though the mutant could not perceive ethylene (Madlung et al. 1999).

That cyclohexamide often induces ACC synthase transcription (Zarembinski and Theologis 1993), fits with the observation that some Aux/IAA-inducible proteins appear to be regulated by a transcriptional inhibitor (Yamagami et

al 2003). All ACC synthases that demonstrated increased expression in response to IAA also responded to treatment with 50μM cyclohexamide. Some ACC synthase genes, however, display no increased expression after cyclohexamide treatment (Arteca and Arteca 1999) indicating that expression of some ACC synthase genes is more stable than others (Zarembinski and Theologis 1993).

ACC Oxidase

ACC oxidase, or ethylene-forming enzyme (EFE), long resisted cloning by traditional methods (reviewed in Kende 1993). Greierson et al (1985) translated different ripening transcripts and separated the products by SDS-PAGE. One of the translated products was a 35kDa peptide that was also identified in a cDNA screen of ripening versus green tomatoes as a clone designated pTOM13 (Slater et al. 1985). However, only after pTOM13 was cloned into yeast where it subsequently displayed EFE activity (Hamilton et al. 1991) was ACC oxidase truly identified. Almost simultaneously it was shown that *Xenipus lavis* oocytes injected with pTOM13 displayed EFE activity (Spanu et al 1991).

Using purification conditions similar to those used for flavanone-3-hydrolase (Hamilton et al 1991.), ACC oxidase was shown to be related to the 2-oxoglutarate-

dependent dioxygenase family (Ververdis and John 1991). Further purification of the ACC oxidase demonstrated that while the enzyme did not require 2-oxoglutarate for its reaction, it did require Fe^{2+} and ascorbate, as do other 2-oxoglutarate-dependent dioxygenases (Smith et al 1992). Dong et al. (1992) determined the correct stoichiometry of the reaction.

$$ACC + O_2 + \text{ascorbate} \xrightarrow{CO_2, Fe^{2+}} C_2H_4 + CO_2 + HCN + \text{dehydroascorbate} + H_20$$

Homology modeling of the apple ACC oxidase predicted the protein to have a compact jelly-roll motif, consisting of eight α-helices and 12 β-strands, similar in structure to isopenicillin-N synthase (Seo et al 2004). The model also displayed the presence of a highly conserved, facial triad motif (His-X-Asp-X-His) that forms a Fe^{2+} binding pocket in a number of enzymes. The predicted binding site lies within a group of antiparallel β-strands that forms a cleft in the heart of the enzyme (Seo et al. 2004). The ascorbate cofactor was modeled to hydrogen bond with Arg^{244} and Ser^{246} of the enzyme, as well as the Fe^{2+} atom (Seo et al 2004). The conserved Fe^{2+}-binding motif was located at positions His^{177}, His^{234}, and Asp^{179} (Seo et al 2004). These residues, along with Val^{159} and Thr^{157}, form the core of the active

residues within the proposed model of the ACC oxidase enzyme (Seo et al 2004).

Although ACC synthase is generally considered the primary regulator of the ethylene biosynthetic pathway, there is quite a bit of evidence for regulation of ACC oxidase as well. Differential expression of ACC oxidase has been shown in a number of plant tissues (Tang et al. 1994, Raz and Ecker 1999). For example, ACC oxidase has displayed increased transcript accumulation in senescing tissues (Yang et al. 2003), ripening fruits (Nakatsuka et al. 1997), wounded tissues (Liu et al. 1997) and IAA-treated plants (Yu et al. 1998), as well as tissues exposed to ethylene (Kim et al. 1998). On the other hand, some ACC oxidases are also expressed constitutively (Liu et al. 1997, Kim et al. 1998, Yu et al. 1998).

ACC oxidase has been linked to reaction wood production in woody species. Using two-dimensional electrophoresis, Plomion et al. (2000) found an ACC oxidase that was up-regulated in compression wood samples from *Pinus pinaster*. Likewise, ACC oxidase displayed increased expression on the tension wood side of *Populus tremula x P.tremuloides* (Andersson-Gunneras et al. 2003). While the latter study also found that ACC levels were higher in tension wood (upper side), ACC was significantly higher in

the opposite wood, leading to the postulation that ACC synthase expression was not specific to a particular side of the gravistimulated poplar stem. Thus, ACC oxidase expression was exposed as the primary determinant for asymmetric ethylene production in gravistimulated poplar stems.

Ethylene signal transduction

Transduction of the ethylene signal has been studied intensely in *Arabidopsis* using mutant screens to dissect the signaling pathway (reviewed in Kieber 1997; Wang et al. 2002). These studies have led to the discovery of a linear pathway from the ethylene receptor to the level of transcription factors. Although this review has concentrated on the biosynthetic side of the ethylene pathway and how those enzymes are regulated, it is important to note that this represents only half of the story.

The ethylene receptor was cloned from *Arabidopsis* by Chang et al. (1992), and was the first hormone receptor cloned from plants. Ethylene receptors (ETR1, ETR2, ERS1, ERS2 and EIN4 in *Arabidopsis*) all harbor domains similar to bacterial two-component receptors (reviewed in Lohrmann and Harter 2002) (Fig 1.6). These receptors are thought to

work as homodimers, although since ERS1 and ERS2 lack receiver domains, heterodimerization may occur in some cases (Hua et al. 1998). The ethylene receptors can be divided into two subfamilies. The ETR1-like subfamily has three hydrophobic stretches that are predicted to span the cell membrane (Wang et al. 2002). The carboxyl end of the ETR1 subfamily (ETR1 and ERS1) displays a region homologous to the histidine kinase domain of the bacterial two-component systems. ERS1, however, does not have a response regulator at the carboxyl-terminus. ETR2 subfamily members (EIN4, ETR2, and ERS2) have four predicted membrane-spanning regions at the amino terminus, as well as histidine kinase-homologous domains, but lack histidine kinase activity. Fankhauser et al. (1999) found that this receptor class exhibits Ser/Thr kinase activity.

Ethylene receptors function when ethylene binds to the N-terminal membrane-associated portion of the protein. Copper is a known cofactor for this binding, and Ran1, a copper transporter protein, supplies copper for the ethylene receptor. When ethylene is not bound to the receptor, an associated protein, CTR1, is active. CTR1 is a negative regulator of the ethylene response pathway, and has similarity to the Raf-family of mitogen-activated protein kinase kinase kinases (MAPKKK). Recent studies in

Medicago demonstrated homologues to the MAPK and MAPKK enzymes that complete the MAPK cascade, a signaling mechanism used in many eukaryotic systems (Ouaked et al. 2003) (Fig 1.6). EIN2, EIN3, EIN5 and EIN6 are positive regulators that act downstream of CTR1. The functions of EIN2, EIN5 and EIN6 are unknown, although it is known that EIN2 is a membrane-bound protein (Alonso et al. 1999). EIN3 is a transcription factor that regulates transcription of ETHYLENE RESPONSE FACTOR 1 (ERF1), another transcription factor that recognizes the GCC-box domains common in ethylene-inducible genes promoters (Fig 1.6).

Objectives and Summary of Research

The main goal of the work described in this dissertation was to extend our knowledge of the gene for the ethylene biosynthetic enzyme, ACC synthase, and to test its responsiveness to stresses implicated in the formation of compression wood in loblolly pine. Specific objectives were as follows:

- Clone the ACC synthase gene from loblolly pine
- Investigate the expression of the cloned ACC synthase gene in normally growing pine tissues
- Test ACC synthase expression in response to known elicitors of ACC synthase expression in other plants

- Investigate ACC synthase response to known elicitors of compression wood development
- Dissect whether ACC synthase is responsive to gravistimulation or mechanical stress

The resultant research is detailed in the following chapters:

Chapter 2 presents cloning of a full-length putative ACC synthase gene (PTACS-1) from loblolly pine, as well as a splice variant of that gene (PTACS-1s). Expression of these transcripts was measured in many tissues collected from loblolly pine trees.

Chapter 3 reports on PTACS-1 and PTACS-1s expression in response to known elicitors of ACC synthase and compression wood. The study also attempts to show whether PTACS-1 is primarily responsive to gravistimulation or mechanical stress.

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Figure 1.1. Map of Loblolly Pine (*Pinus taeda)* Native Range

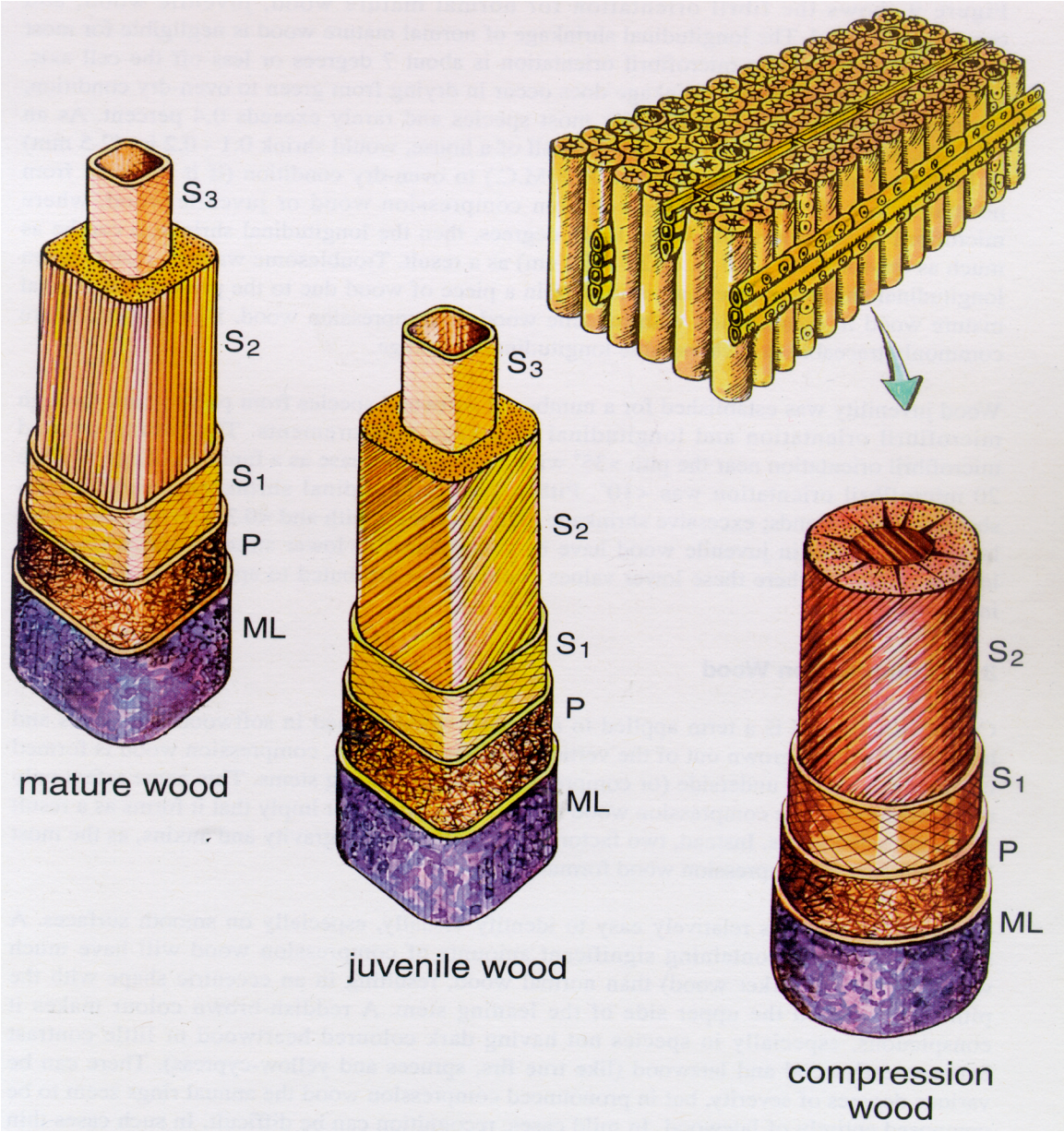

Figure 1.2. Diagram of tracheid cell wall makeup under high magnification. Shown is the Middle Lamella (ML), a lignin rich layer that holds fibers together, the primary cell wall (P) composed of lignin and cellulose, and the secondary cell walls (S_1, S_2, S_3) composed of tightly packed cellulose. (from Josza and Middleton 1994)

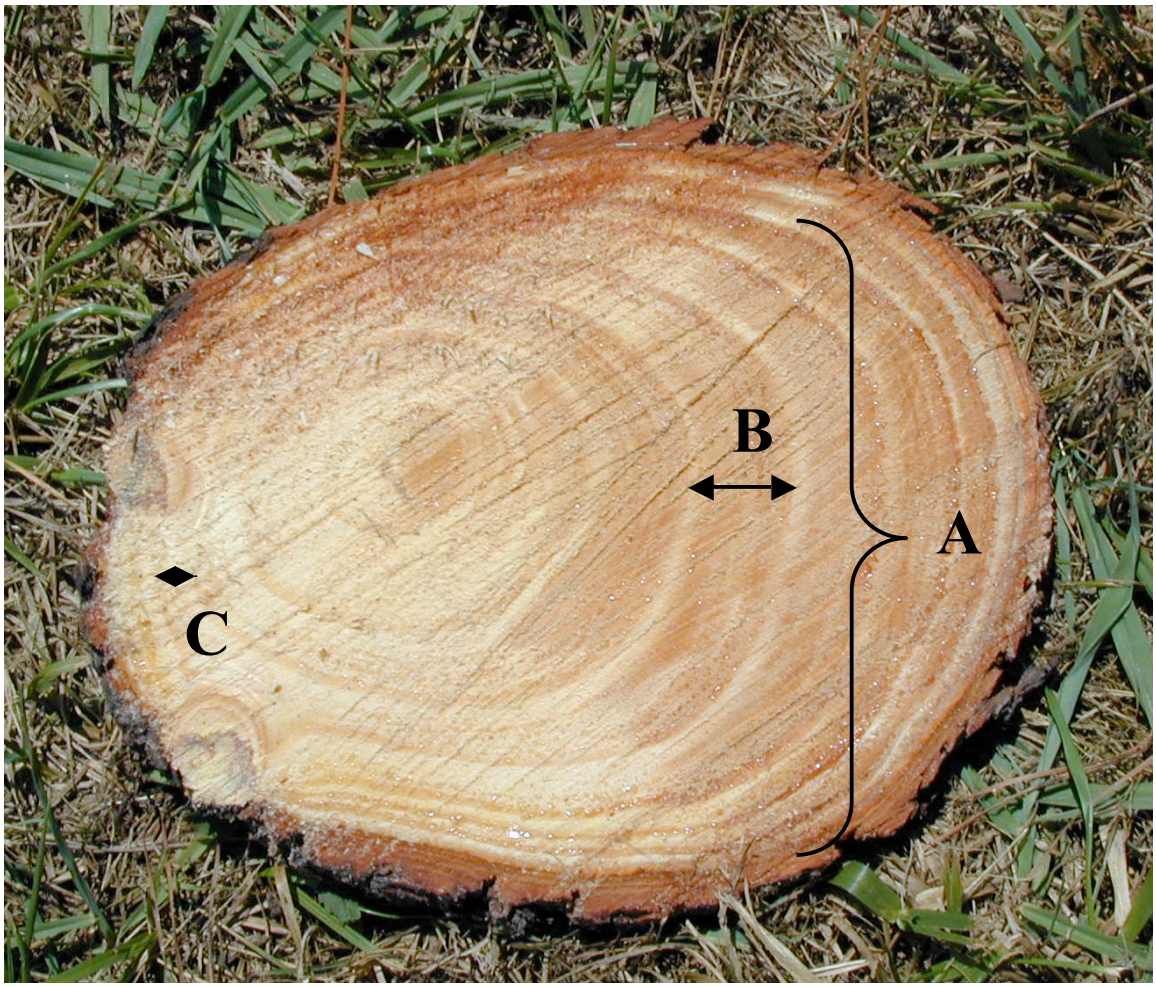

Figure 1.3. Cross section from a leaning loblolly pine stem (~10 years old). Compression wood is delineated in this cross section by the dark colored zone (A) and the increased ring width on the underside (B) versus the topside of the stem (C) seen in B and C.

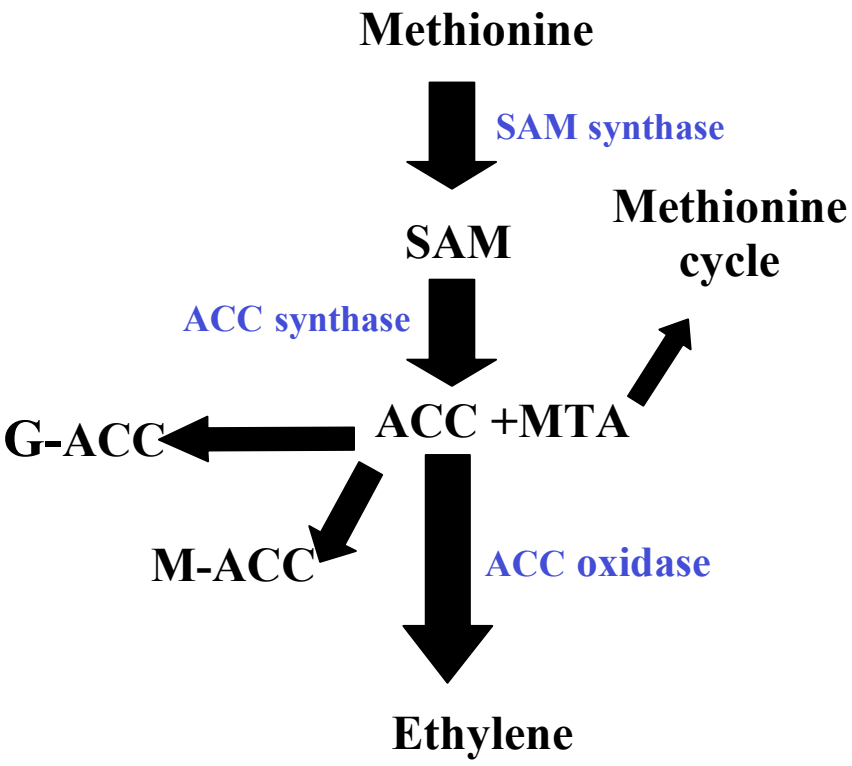

Figure 1.4 Pathway for ethylene biosynthesis. Enzymes involved in the pathway are colored Blue. Recreated from Fluhr and Matoo 1996

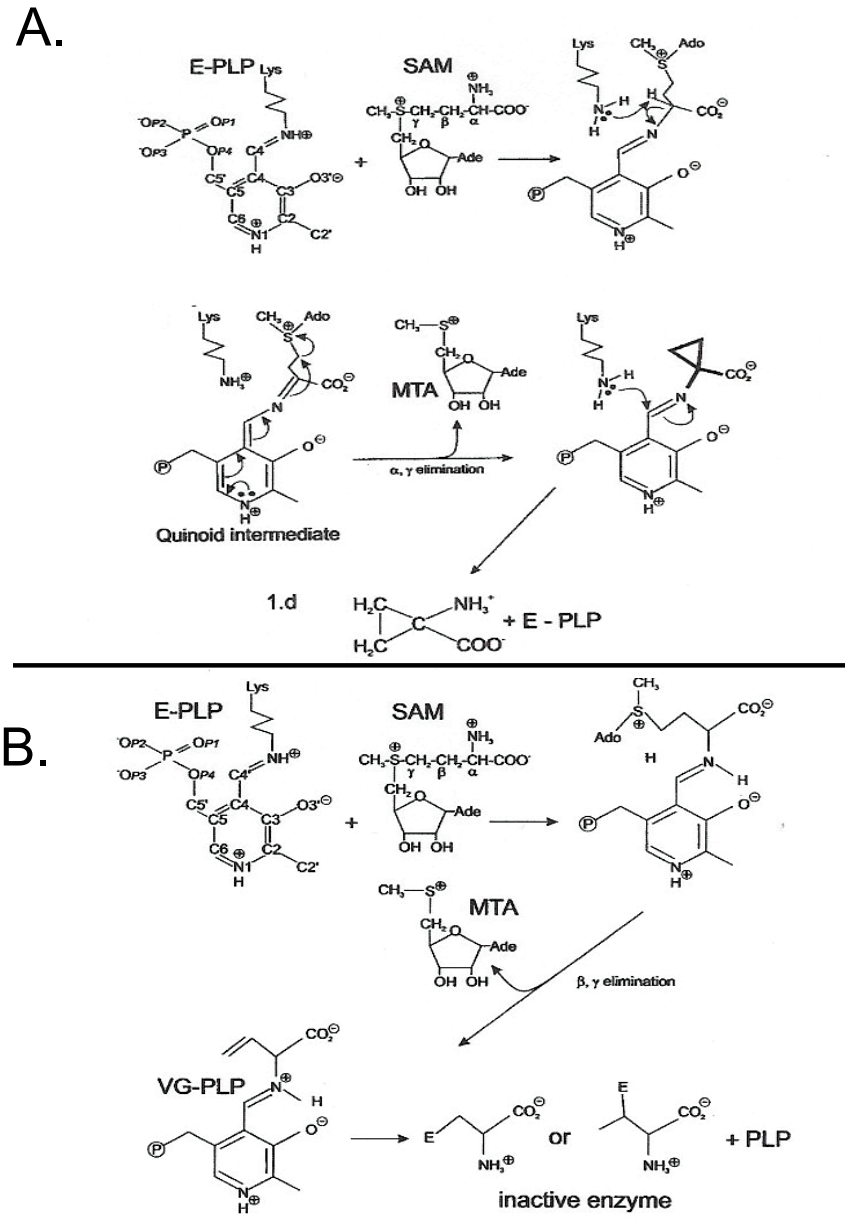

Figure 1.5 Mechanism of ACC synthase enzyme (A) and mechanism for suicide inhibition (B) of ACC synthase. Taken from Jakubowicz 2002.

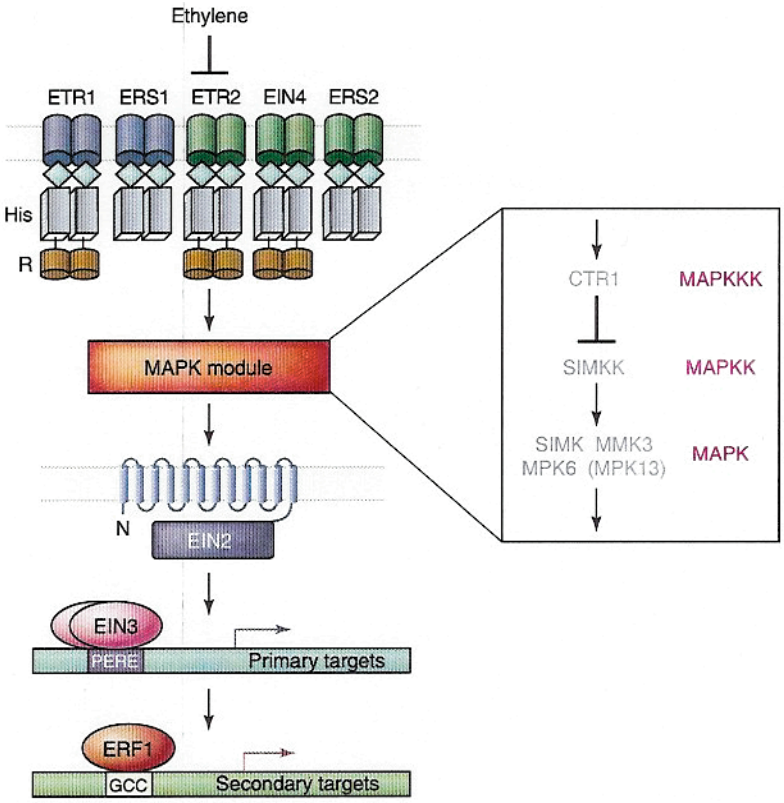

Figure 1.6. Ethylene signal transduction pathway. Taken from Chang 2003

CHAPTER 2

CLONING AND CHARACTERIZATION OF THE PUTATIVE ACC SYNTHASE GENE PTACS-1 FROM LOBLOLLY PINE[1]

[1] Barnes, J.R. and Dean J.F.D. to be submitted to *Canadian Journal of Forestry*

Abstract

Ethylene is a plant hormone known to play many roles in plant development and maturation. 1-Aminocyclopropane-1-carboxylate synthase is the rate-limiting enzyme in the biosynthesis of ethylene, and its transcription is tightly correlated with sites of ethylene production and action. To date no full-length ACC synthase genes have been isolated or studied in a gymnosperm species. This study describes isolation of the first putative ACC synthase from a gymnosperm, PTACS-1 from *Pinus taeda* (loblolly pine), an important commercial forest tree species. Also reported here is the isolation of a putative splice variant of PTACS-1 (PTACS-1s) that is missing 138 bp from the 5' end of the transcript. This study examines the differential expression of PTACS-1 and PTACS-1s in various tissues of loblolly pine.

Introduction

Ethylene is a phytohormone involved in many aspects of plant growth and development (Wang et al. 2002). Increases in ethylene production signal the onset of senescence, seed germination, and fruit ripening (Wang et al. 2002). Increased ethylene production has also been observed in response to wounding (Lincoln et al. 1993).

Ethylene biosynthesis is regulated mainly by the activity of 1-aminocyclopropane-1-carboxylate synthase (ACC synthase), the rate-limiting step in the ethylene biosynthetic pathway (Kende 1993). ACC synthase is an enzyme that converts S-adenosyl methionine (SAM) to ACC, the immediate precursor to ethylene (Wang et al. 2002). ACC synthase genes have been cloned from a number of plant species, and transcription is typically responsive to hormone treatment and wounding (Wang et al. 2002). ACC synthase is a short-lived enzyme regulated not only by rapid turnover of the mRNA, but also through suicide inhibition by a reaction by-product vinylglycine, that destroys enzyme activity (Feng and Kirsh 2000).

ACC synthase genes typically occur as small gene families; for example, a nine-gene family has been identified in the *Arabidopsis* genome (Liang et al. 1992). Families of similar size have been found in other plant species, such as mung bean and sunflower (Botella et al. 1992, Liu et al. 1998). Different, family members have been shown to respond specifically to different stimuli; for example Botella et al. (1995) showed that transcription of the mung bean (*Vigna radiata*) ACC synthase, AIM-1, was induced by bending stress.

No full-length ACC synthase genes have been cloned from any gymnosperm species. Although ACC synthase activity was measured in Scots pine (*Pinus sylvestis*) (Klintborg et al. 2002), the gene was not cloned. Herein we report the cloning of the first ACC synthase gene from a gymnosperm, specifically loblolly pine (*Pinus taeda L.*), and measurement of its expression in various pine tissues.

Materials and Methods

Plant Material and Nucleic Acid Extraction

Bare-root, open-pollinated seedlings of loblolly pine were obtained from the Georgia Forestry Commission. The trees, in one-gallon pots, were maintained in a greenhouse for two years before harvest with regular watering and fertilization. Roots, candles (new growth at the end of a branch), primary flush needles, secondary flush needles, photosynthetic bark, and xylem were collected and flash-frozen in liquid nitrogen. Tissues were ground by freezer mill to ensure complete breakdown of the tissue. RNA was extracted by the method of Chang et al. (1993). Genomic DNA was isolated from needles ground in liquid nitrogen and purified by centrifugation through a CsCl gradient (Sambrook et al. 1989).

Cloning of the Loblolly pine ACC synthase

RNA from loblolly pine new-growth candles treated with 1mM IAA for 1 hr at room temperature was used as a template for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) amplification using primers designed on the basis of an apparent loblolly pine ACC synthase transcript fragment found in the GenBank EST database (AWO64832). This EST was identified using a *Lycospersicon esculentum* (tomato) ACC synthase amino acid sequence to perform a sequence similarity search (Basic Local Alignment Search Tool – tBLASTx) of the loblolly pine database (http://pinetree.ccgb.umn.edu/).From this EST sequence, primers ACS1731s (5'GACTTGTGCCTTCCAGGTTT3') and ACS1967R (5'GACCCGCATTACTCTTCACA3') were designed using Oligo 4.0-s (Molecular Biology Insights Inc., Cascade, CO).
For RT-PCR, cDNA synthesis was performed according to the protocol (50 min at 50°C) provided with the superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) using an oligo-dT primer. The PCR reaction was done in a 20µl reaction volume with 1.5mM $MgCl_2$, 100nM primers, 2µM dNTPs, and 2 units of thermostable polymerase (Promega, Madison, WI). Thermocycling was done using the following cycle parameters: 95°C for 5min, then 35 cycles of 95°C for 30s, 51°C for 30s, and 72°C for 1min. Cycle sequencing reactions

were performed using Big Dye terminator chemistry (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol, and sequences were determined using an ABI 310 automated DNA sequencer. The 3' end of the cDNA was captured by 3' Rapid Amplification of cDNA Ends (3'-RACE) (Roche, Indianapolis IN) using primers ACS1967S (5'TGTGAAGAGTAATGCGGGTC3') and the AP primer (5'GGCCACGCGTCGACTAGTACTTTTTTTTTTTT3') (Frohmann et al 1988). PCR cycling conditions for 3'-RACE were the same as above, except the extension step was lengthened to 90s.

Obtaining a full-length clone of the cDNA proved difficult. Using an M13R primer specific for the pSL1180 vector used to construct an existing loblolly pine root cDNA library, most of the 5'end of the cDNA was captured by PCR using the gene-specific primer, ACS1797R (5'CTCAGCAAATGTCAAGATTC3'), under the following cycling parameters: 95°C for 5min, then 35 cycles 95°C for 30s, 55°C for 30s, and 72°C for 2min. A shorter transcript was also captured from this cDNA library using the same primers. Sequencing showed that it was identical to the putative ACC synthase, but with a 138 bp deletion. The full-length cDNA, including the 5' untranslated region (UTR), was finally captured using PCR walking of genomic DNA (Barnes 1994). Genomic PCR walking was accomplished by synthesizing

four ligated-anchor genomic DNA libraries as detailed in a protocol provided with the GENOMEWALKER kit (CLONTECH, Palo Alto, CA). Using the restriction enzymes DraI, EcoRV, PvuII, and StuI, 5μg of genomic DNA was cut to completion, and the restricted DNA was used as the basis for each of the four genomic libraries. An adaptor was ligated onto the ends of the restricted DNA. Primers, ACS621R (5'GGCACAGGGTATTACTGATCCCAA3') and ACS632R (5'AAGTAGAAGAGGGCACAGGGTATT3'), were synthesized and utilized in conjunction with the Adaptor primers from CLONTECH, AP1 (5'GTAATACGACTACTATAGGGC3') and AP2 (5'ACTATAGGGCACGCGTGGT3'), in a nested PCR reaction. This procedure amplified a fragment from the DraI-ligated anchor genomic library using the following thermocycling conditions for both rounds of amplification: 95°C for 5min, then 40 cycles of 95°C for 30 s, 60°C for 30s, and 72°C for 2min. All amplimers were cloned into the pCR II-TOPO vector following instructions in the Topo TA vector system kit (Invitrogen, Carlsbad, CA).

Sequence Alignment

Sequences were compiled, compared to exisiting sequences in target databases using BLAST, and aligned using the Wisconsin Package Software Ssuite, ver. 10.1

(Genetics Computer Group, Madison, WI). Similarity and identity shading was done using the multiple sequence alignment editor and shading utility, Genedoc, ver. 2.6.002 (Nicholas et al. 1997).

Quantitative PCR

Primers for quantitative PCR (QPCR) (Higuchi et al 1996) were designed using Beacon Designer, v 2.0 (PremierBiosoft, Palo Alto, CA). A partial sequence for a loblolly pine glyceraldehyde-3-phosphate dehydogenase (AN: BF777294) was used to design the primers for GapDH control reactions. This sequence was chosen because it appears to be from the 3′ end of the GapDH gene. The primer sets made to the PTACS-1 (ACS278F-1, 5′-TTGGCTCAAGTCTCATCC-3′and ACS390R-1, 5′-ATCTCAAGATTGGCATTCC-3′), PTACS-1s (1s gap shortF, 5′- GGCCGAAAATCAGGTAATGG -3′and 1s ACS408r, 5′-CTGGATAAGTAGGGTGATGG -3′), and GapDH (GapDH164R, 5′-CCGAATCCGTTGATAGCCACTT-3′and GapDH32F, 5′-GAAGGCATGTACGCGAGAACA -3′) were tested against both mixed cDNAs and plasmid clones. The products were then run on a 1% agarose gel in 1x Tris-acetate/EDTA buffer, pH 8, to make sure that no secondary PCR products or primer dimers were produced (data not shown).

DyNAmo SYBR Green qPCR Mastermix (MJ Research, Watertown, MA) was used for all QPCR experiments, following the manufacturer's protocol. All samples were replicated three times for the ACC synthase genes and twice for the GapDH controls. A standard curve based on known quantities of plasmid containing the loblolly pine ACC synthase cDNA was determined for each experiment. All experiments were run using a DNA Opticon thermocycler (MJ Research, Watertown MA) under the following cycling conditions (suggested by R.G. Rutledge, Canadian Forest Service, Laurentian Forestry Centre, Sainte Foy, Quebec): 95^{o}C for 10min, followed by 50 cycles of 95^{0}C for 1sec and 55^{o}C for 1min. The instrument was set to measure dye florescence at the end of each cycle. Initial primer concentration was 300nM for all reactions, and template cDNA for all samples was made using 300ng of Dnase-treated total RNA and Superscript III (Invitrogen) according to manufacturer's protocol. The first-strand RT reaction product was diluted 1.5 times, and 3uL was used for each QPCR reaction. The Cycle Threshold (cT) line was determined manually as the point where the R^2 value for the standard curve reached its highest value.

Modeling PTACS-1 and PTACS-1s

Models of the inferred PTACS-1 and PTACS-1s proteins, as well as the Arabidopsis aminotransferase (ATase) ACS12, were generated using the MODELLER program (Sali et al. 1995). The sequences were aligned with the available tomato ACC synthase for which a crystal structure was available. The available apple ACC synthase crystal structures Capitani et al. 1999, 2002, 2003) lacked information on the dimeriztion interfaces and were not used for model generation.

Results

Isolation of Putative ACC Synthase cDNA Clone PTACS-1 from Loblolly pine

Oligonucleotide primers based on a loblolly pine EST (AWO64832) encoding a putative ACC synthase were used to clone a putative ACC synthase gene by PCR. RNA isolated from loblolly pine candles treated with IAA was used as template in RT-PCR reactions, and a single band of the expected size was recovered, cloned into the PCRII-TOPO vector, and sequenced (data not shown). BLAST analysis indicated significant sequence homology to ACC synthase sequences in GenBank. Additional primers were based on sequence from the partial clone, and 3'RACE was used

capture downstream sequence through the 3′ untranslated region of the cDNA to the poly-A tail. However 5′RACE was unsuccessful in capturing the 5′ end of the gene.

Most of the upstream sequence was finally obtained using a loblolly pine root cDNA library provided by Dr. Walter Lorenz (University of Georgia) as template for additional PCR reactions using vector and gene-specific primers. Two amplimers differing in length by approximately 150 nucleotides were cloned and sequenced. Both products showed high homology to other ACC synthases and were identical, except for a 138 bp deletion in the shorter transcript (Fig. 2.1, boldface). However, neither fragment isolated from the library appeared to represent the complete 5′ end of the gene as neither encoded an obvious N- terminal methionine.

The final piece of the cDNA 5′ end sequence was obtained using genome-walking techniques (Barnes 1994) to amplify it from a genomic DNA template. With the complete sequence a translational start site (Fig. 2.1) was predicted as the first methionine in the longest open reading frame. A transcriptional start was predicted based on its proximity to an apparent TATA box (Fig. 2.1, underlined sites) predicted using Webgene Hctata (http://l25.itba.mi.cnr.it/~webgene/wwwHC_tata.html). As

additional evidence in support of the transcriptional start site, PCR reactions using primers based on genomic sequence upstream of the putative transcriptional start site failed to yield products when cDNA pools known to contain the gene of interest were used as template (data not shown).

Alignment of loblolly pine PTACS-1

DNA sequence alignments were generated using the putative loblolly pine ACC synthase DNA, PTACS-1, *Arabidopsis* ACC synthase homolog (ACS7 AF332390), two newly identified *Arabidopsis* ATases found to have sequence homology to ACC synthases (ACS 10, (AF348575, and ACS12, AF336920) (Yamagami et al. 2003), as well as ACC synthases from apple (*Malus domestica*, AY120897), mung bean AIM –1 (*Vigna radiata* Z11613), and snapdragon ACS-3 (*Antirrihum majus*, AF083816) (Fig. 2.2). Sequence similarities between PTACS-1 and the other ACC synthases are apparent across the length of the gene. In addition, PTACS-1 displayed complete conservation in the known active site residues for these enzymes (Yip et al 1990) (Fig. 2.2, 4^{th} underlined region). Unlike the *Arabidopsis* ATases, PTACS-1 contains residue Y^{192} (cognate to Y^{98} in apple), which is essential for ACC synthase activity (Tarun and Theologis 1998). Of interest is that PTACS-1s lacks this residue by virtue of

the 138 bp deletion. PTACS-1 also has a tyrosine residue, Y^{329} (cognate to Y^{233} in apple), a residue important for binding the ACC synthase cofactor, pyridoxyl-5-phosphate, but which is not conserved in the *Arabidopsis* ATases. PTACS-1 also maintains strong homology in region 6 of the conserved ACC synthase regions (Fig 2.2), where similarities with the *Arabidopsis* ATases break down.

Modeling of PTACS-1 and PTACS-1s

Molecular homology modeling was performed on the inferred PTACS-1 and PTACS-1s proteins. The structural models for these proteins were compared to the tomato ACC synthase crystal structure (1IAX dimer, 1IAY monomer) (Huai et al 2001), as well as a structural model developed for the *Arabidopsis* ACS12 ATase (Figs. 2.3, 2.4). The PTACS-1 structural model appears very similar to the tomato ACC synthase crystal structure with the PLP cofactor and the Y^{192} residues in very similar positions (Fig. 2.3). The Y^{192} residue resides on a surface loop that differs between the modeled structures for ACS12 and PTACS-1 (Fig. 2.4). This loop in the ACS12 model projects away from the monomer, and appears to impact the location of the strictly conserved position where Y^{152} resides in the ACC synthase dimeric structure. Tryosine^{329} in PTACS-1 also seems to be in the

same orientation as Y^{240} in the tomato protein. Although ACS12 has residue F^{290} in this position, a mutation of the corresponding residue in apple ACC synthase, Y^{233} to F leads, to a 24-fold reduction in ACC synthase activity (White et al 1994). ACS12 and ACS 10 do have a tyrosine (Y^{287}) in the conserved location for ATases (Fig. 2.4). The tyrosine position is conserved in ATases and establishes the mechanistic difference between ACC synthases and ATases by how the Schiff's base is positioned in the active site (White et al 1994).

Although PTACS-1s is missing the variable loop that contains the initial tyrosine residue conserved in ACC synthase, the rest of the model retains structural similarity to the ACC synthase structures. The N-terminal domains of PTACS-1, PTACS-1s and ACS12 could not be modeled successfully as they have insufficient structural homology to other proteins in the protein crystal structure database.

Expression of ACC synthase in various tissues

Quantitative PCR was used to determine tissue expression profiles of the putative ACC synthase, PTACS-1, and its putative splice variant, PTACS-1s. Because the sequence identity of PTACS-1 and PTACS-1s is so high, the

oligonucleotide primer, 37-gap-short-F, was designed so that it straddled the junction of the putative splice site. Primer sets for the two transcripts were absolutely specific for each gene under QPCR reaction conditions. PTACS-1 and PTACS-1s transcripts in RNA samples from roots, primary and secondary needles, candles, xylem, photosynthetic bark and fascicles were quantified (Figs. 2.5A, B), and GapDH was used as a housekeeping gene with which to normalize data between samples. Primary needle and the fascicle samples contained high levels of the PTACS-1 transcript. However, roots displayed the highest level of expression, which is consistent with data for ACC synthase genes from other plant species, as ethylene is known to be important for root elongation (Kende, 1993). An experimental repeat to verify the results for root expression showed a large variation in expression. The source of this variation is unclear, but it could be due to a variance in greenhouse watering or fertilizer application schedules.

Expression in PTACS1-s was also measured in all tissues sampled. Large variations in transcript levels were also seen in experiments to quantify the PTACS-1s expression. Differences in the culture of the first and second groups of trees that might have led to such large

variations in gene expression are not known, but could be due to variations in water or fertilizer application. Variation could also be single nucleotide polymorphism (SNPs) in the primer-binding site (R.G. Rutledge, personal communication), which could decrease binding of the PTACS-1s primers to template in mixed genotype samples, resulting in less product formation.

Discussion

Ethylene biosynthesis has been extensively studied in angiosperms due to its diverse roles in fruit ripening, senescence, and abscission (Liang et al 1992, Park et al 1992, Clark et al 1997). A few studies have examined ethylene biosynthesis and its importance to gymnosperms (Klintborg et al. 2002); however, the genes for this pathway have, for the most part, not been studied. This is the first study to report cloning of a full-length ACC synthase gene from a gymnosperm. Using PCR, genomic and cDNA clones with homology to ACC synthase genes from other plants were recovered from loblolly pine. The predicted open reading frame was also similar to the sequences for two *Arabidopsis* ATases (Yamagami et al 2003). The predicted peptide for PTACS-1 is significantly longer than those for other reported ACC synthase sequences, having an N-terminal

extension similar to those predicted for *Arabidopsis* ATases, ACS12 (27aa shorter), and ACS10 (35aa longer). It seems apparent from the high homology shared by the ACS10 and ACS12 ATases and the ACC synthases in *Arabidopsis* that these enzymes must have shared a common ancestor. The loblolly pine PTACS-1 gene supports this notion because it by contains elements of both enzymes, e.g. the long N-terminus of the ATases and the conserved amino acid residues Y^{192} and Y^{329} that are critical for ACC synthase activity.

The transcriptional start site for PTACS-1 was predicted from genomic sequence. Although there is a methionine (M^{36}) encoded in the PTACS-1 that appears equivalent to the initiation codon in the *Arabidopsis* ACS12 gene, the longest open reading frame for PTACS-1 was considered to more likely encode the gene product. Web based prediction (webgene AUG_EVALUATOR, http://l25.itba.mi.cnr.it/~webgene/wwwaug.html) analysis of the gene sequence did not identify M^{36} as a preferred translational start site. Longer C-terminal tails in ACC synthases from other species compared to PTACS-1 means the overall difference in length between PTACS-1s and mung bean ACC synthase is only 43 amino acids.

The PTACS-1s transcript was found when a PCR reaction used to clone the PTACS-1 5′ end produced two bands from root cDNA templates. The PTACS-1s transcript displayed complete identity to the PTACS-1, except for a 138bp omission. This transcript is assumed not to be a cloning artifact due to the ability to amplify it from many different RNA sources. In addition, the PTACS-1s transcript has been identified in other expressed sequence tag libraries for loblolly pine (data not shown). The PTACS-1s transcript is assumed to be either a splice-variant or have arisen from a recent genetic duplication.

Evidence for PTACS-1s arising from a splice variant are the complete identity of PTACS-1 and PTACS-1s sequences outside the deleted sequence, and the putative residual intronic region remaining in PTACS-1 is AU rich (58%), which is a common feature of intron sequences (Lorkovic et al. 2000). However, the putative 3′ splice junction would require a non-canonical GAUCU/GU 3′ss sequence. It is not unknown whether such an acceptor sequence is common in gymnosperms. Likewise, the 5′ss (UU) sequence is unknown in gymnosperms with the exception of the $tRNA^{Tyr}$ for *Ginkgo biloba* (Akama et al. 1997); however, this $tRNA^{Tyr}$ would likely be spliced via different cellular machinery.

The other possibility, that PTACS-1s is a separate gene with high homology to PTACS-1, has not been verified due to the difficulties in genome walking into upstream surrounding sequence. Unfortunately, genome walking into the promoter region was unsuccessful, apparently due to the inability of PCR primers to distinguish between PTACS-1 and PTACS-1s, except over the splice junction.

Secondary transcripts from ACC synthase genes have been seen in other species (e.g. Olson et al. 1995, ten Have and Woltering 1997, Peck and Kende, 1998). One study showed that the protein translated *in vitro* from *Pisum satvium* ACS-1b (PS-ACS1b), the secondary transcript of the pea ACC synthase, PS-ACS1a, had no ACC synthase activity (Peck and Kende, 1998). The authors postulated that this protein could possibly form an inactive heterodimer that might add extra regulatory control to ACC synthase at the protein level.

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AACACTTTGCAACCTTGAGAATTCATAATTTGAAATCCGCTCATATCCTTCATAGCCTCAATCCTTTGAATGGTAGCTTC
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M~~T~~H~~D~~N~~S~~
ACCCATAGCCGCAGCGGCAGAAGCAGAATTGAAGAAGATAGACAGAGGGAGGAGGACAGAGCAAGAGGCCGAGGAACAGG
T~~H~~S~~R~~S~~G~~R~~S~~R~~I~~E~~E~~D~~R~~Q~~R~~E~~E~~D~~R~~A~~R~~G~~R~~G~~T~~G~
GGCAGTCATGAGGGTAATTGTCCCATTACAGGGCGTCGTGCAAGGGCGTGGAGGGGTCGTTTTGGGATCAGTAATACCCT
~A~~V~~M~~R~~V~~I~~V~~P~~L~~Q~~G~~V~~V~~Q~~G~~R~~G~~G~~V~~V~~L~~G~~S~~V~~I~~P~~C
GTGCCCTCTTCTACTTCCTTCAGCTCTACCTCAAGCGCCATCGCTCTGAGAGAGCCCCTCGTGAGGAGGTTCCTCCCCCG
~~A~~L~~F~~Y~~F~~L~~Q~~L~~Y~~L~~K~~R~~H~~R~~S~~E~~R~~A~~P~~R~~E~~E~~V~~P~~P~~P~~
CCTCGTAGGCCCTCCAGAAGTCCCCGGAAGCCTGCCTTGGTCTCTTCAAGGGCTCTCCATGTGATCGAGGACGAAAATTC
P~~R~~R~~P~~S~~R~~S~~P~~R~~K~~P~~A~~L~~V~~S~~S~~R~~A~~L~~H~~V~~I~~E~~D~~E~~N~~S~
CCCTTACTACAGGGGTTGGCAGGCATACAAGGAGAATCCCTATGATGCCGAACACAACCCGGATGGTATTATTCAAATGG
~P~~Y~~Y~~R~~G~~W~~Q~~A~~Y~~K~~E~~N~~P~~Y~~D~~A~~E~~H~~N~~P~~D~~G~~I~~I~~Q~~M~~G
GTTTGGCCGAAAATCAG**TTATCATTGGATCTAATTGAAGGTTGGCTCAAGTCTCATCCAGAGGTGTCATCATGGGAAGGG**
~~L~~A~~E~~N~~Q~~L~~S~~L~~D~~L~~I~~E~~G~~W~~L~~K~~S~~H~~P~~E~~V~~S~~S~~W~~E~~G~~
ATCAGTGATACGCCTTTGAGCTTAAGAGGAATTGCAAGCCATCAAGATTATTATGGAATGCCAATCTTGAGATCTGTAAT
I~~S~~D~~T~~P~~L~~S~~L~~R~~G~~I~~A~~S~~H~~Q~~D~~Y~~Y~~G~~M~~P~~I~~L~~R~~S~~V~~M~
GGCTGATTTTATGTGTGAAGTCATGAAGGCACCCTTCAATCCAGATCAAATAGTATTGACAGCAGGGGCTACTCCAGCAA
~A~~D~~F~~M~~C~~E~~V~~M~~K~~A~~P~~F~~N~~P~~D~~Q~~I~V~~L~~T~~A~~G~~A~~T~~P~~A~~I~
TAGAAATGCTCGGCTTTTGTTTGGCAGATCCAGGAAATGCATTTTTGGTTCCATCACCCTACTATCCAGCgtaaatattt
~E~~M~~L~~G~~F~~C~~L~~A~~D~~P~~G~~N~~A~~F~~L~~V~~P~~S~~P~~Y~~Y~~P~~A~~
Gaacctcttattttgcatgagtaggccattgataattaatgggaactatagttcataacttcatataaccgattataatg
Gttaatttgctcttttctttggatagctttctagttttgacatgctgagattctgttaaccggttatgttggatccatga
tgcgggtttttattcaactaaaacagagatctgtggcttttgcagTTTTTGCAGGGATCTTAAATGGCGTGCTGGTGTTG
~~F~~C~~R~~D~~L~~K~~W~~R~~A~~G~~V~~
AATTAGTACCTGTTCCTTGTCGCAGCAGTGACAACTTTAATGTGACAATAAATGCCCTAGACAGGGCTTTCAGTCAAACG
E~~L~~V~~P~~V~~P~~C~~R~~S~~S~~D~~N~~F~~N~~V~~T~~I~~N~~A~~L~~D~~R~~A~~F~~S~~Q~~T~
AAAAAGAGAAGGTTGACTGTTCGTGCTCTTCTCATATCAAATCCTTCCAACCCGGTCGGAAACATATTGGATTCAGCGAC
~K~~K~~R~~R~~L~~T~~V~~R~~A~~L~~L~~I~~S~~N~~P~~S~~N~~P~~V~~G~~N~~I~~L~~D~~S~~A~~**T**
ATTGACCATGCTCTTAGAGTTTGCAACAGAGAAGAACATTCATCTTGTATGCGATGAAATTTATGCAGGATCTGTGTACG
~~L~~T~~M~~L~~L~~E~~F~~A~~T~~E~~K~~N~~I~~H~~L~~V~~C~~D~~E~~I~~Y~~A~~G~~S~~V~~Y~~
ATAGCCCAAATTTCACTAGTATAGCAAAGGTTCTAGAGTCAGGTGAATTTGACAAGAGCAGAGTCCATATTATCTATGGC
D~~S~~P~~N~~F~~T~~S~~I~~A~~K~~V~~L~~E~~S~~G~~E~~F~~D~~K~~S~~R~~V~~H~~I~~I~~Y~~G~
ACATCTAAAGACTTGTGCCTTCCAGGTTTTAGAGTTGGAGTTATTTATTCTTACAATGATAAAGTCCTCAAAGCTGCTCA
~T~~S~~K~~D~~L~~C~~L~~P~~G~~F~~R~~V~~G~~V~~I~~Y~~S~~Y~~N~~D~~K~~V~~L~~K~~A~~A~~Q
GCAAATGTCAAGATTCTGCTCTGTTTCTTCTCAGACACAGCATTTGCTCATTAACATGCTTTCGGACGCCCAATTTATCC
~~Q~~M~~S~~R~~F~~C~~S~~V~~S~~S~~Q~~T~~Q~~H~~L~~L~~I~~N~~M~~L~~S~~D~~A~~Q~~F~~I~~
AGCAATATCTGAGTGAGAATCAGAGGAGGTTAAGGAAGGCATCTCTTGTACTCACTGAGGGATTGAAGGAAGCAGGCATT
Q~~Q~~Y~~L~~S~~E~~N~~Q~~R~~R~~L~~R~~K~~A~~S~~L~~V~~L~~T~~E~~G~~L~~K~~E~~A~~G~~I~
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~K~~Y~~V~~K~~S~~N~~A~~G~~L~~Y~~C~~W~~V~~E~~M~~G~~S~~L~~M~~P~~S~~Y~~S~~E~~K~~G~~E
GCTTAATCTTTGGGATAAATTATTGAATGAAGCAAAAATAAATGTCACTCCAGGATCTGCTTGTTATTGCATTGAACCTG
~~L~~N~~L~~W~~D~~K~~L~~L~~N~~E~~A~~K~~I~~N~~V~~T~~P~~G~~S~~A~~C~~Y~~C~~I~~E~~P~~
GATGGTTTCGCATCTGTTTTAGTACTCTGAGCTGCCAAGATATGACTGTGGCTTTACGGCGAATTCAAGAATTCTCCAAA
G~~W~~F~~R~~I~~C~~F~~S~~T~~L~~S~~C~~Q~~D~~M~~T~~V~~A~~L~~R~~R~~I~~Q~~E~~F~~S~~K~
CGTCGTAAACGTAGTTTGTAgggataattattcttgccaaaactttcaatcttgaaatcattctttttgcaaaaaaaaaa
~R~~R~~K~~R~~S~~L~~*
aaaaaa

Figure 2.1. Genomic DNA sequence of ACC synthase from *Pinus taeda*. The intron and untranslated regions are represented in lowercase letters. Putative TATA box sites are underlined, and the nucleotide sequence missing from the putative splice variant is shown in bold.

Figure 2.2. Alignment of deduced loblolly PTACS1 and PTACS1s polypeptides with ACC synthases and ATases. Sequences are as follows: *Arabidopsis* ACC synthase **at-acs7**(AF332390), *Arabidopsis* ATase **at-acs10** (AF348575), *Arabidopsis* ATase **at-acs12** (AF336920), mung bean ACC synthase **vr-aim1** (Z11613), snapdragon **am-acs3** (AF083816) and apple **md-acs3** (U73816). Identical residues are shaded black and conserved residues are shaded grey. The seven highly conserved regions of ACC synthase polypeptides are underlined with arrows (Yamagami et al 2003). The 11 residues strictly conserved between all ACC synthase enzymes are marked with circles. Black circles indicate differences between ACC synthase and both *Arabidopsis* AATase enzymes, gray circles indicate differences between ACC synthase and one *Arabidopsis* AATase enzyme, open circles are conserved in both.

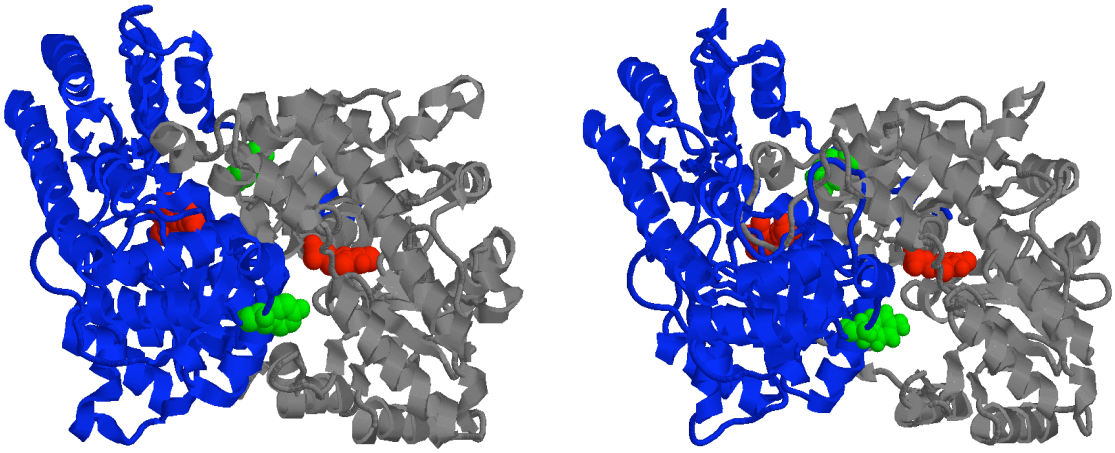


Figure 2.3. Homology model of the PTACS-1 transcript (B) and the Tomato 1IAX crystal structure (A) dimer. The individual monomers are colored Gray and Blue. The PLP cofactor is red, and Y^{192} (Y^{92} in tomato), which interacts with PLP, is shown in green.

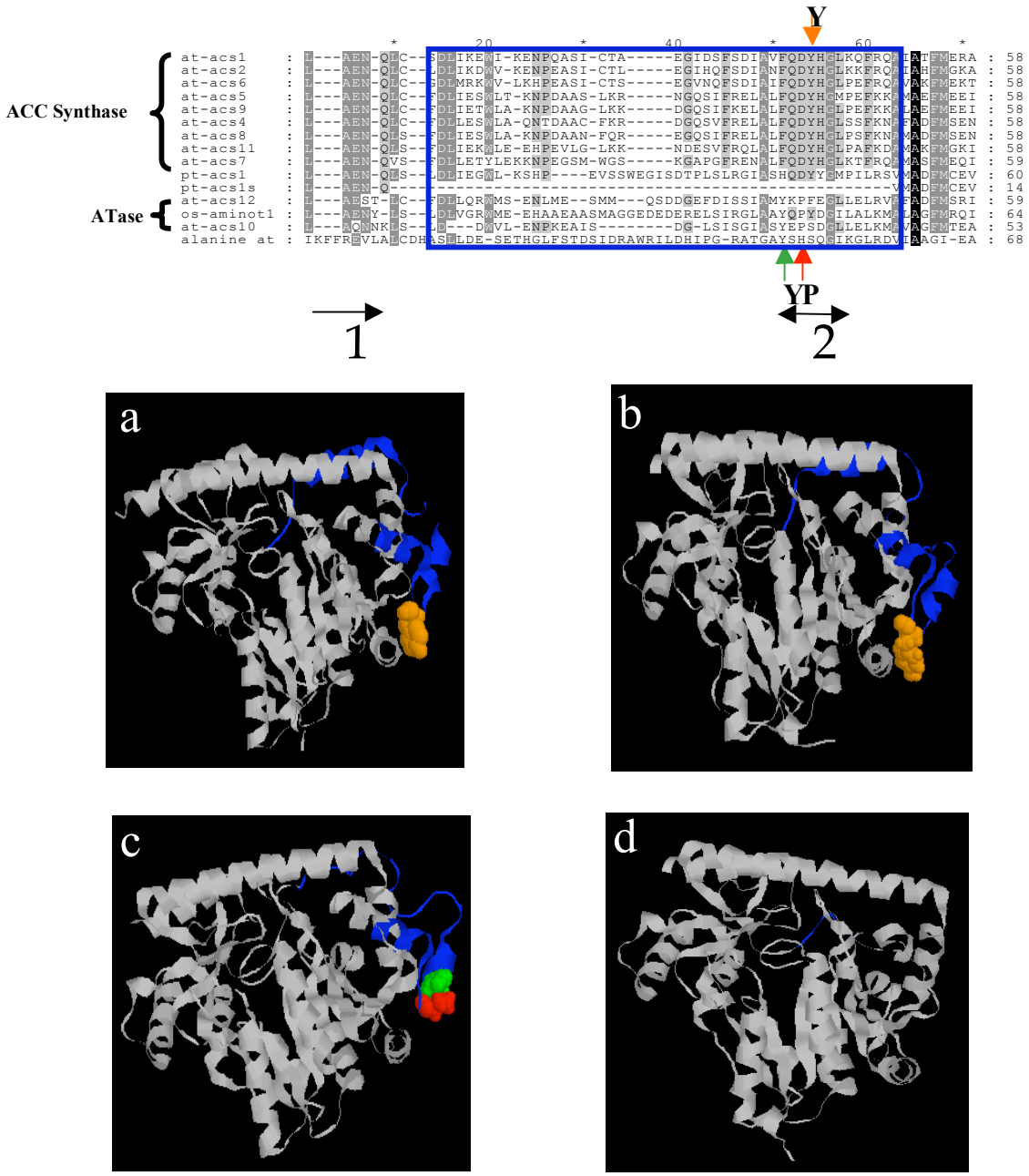
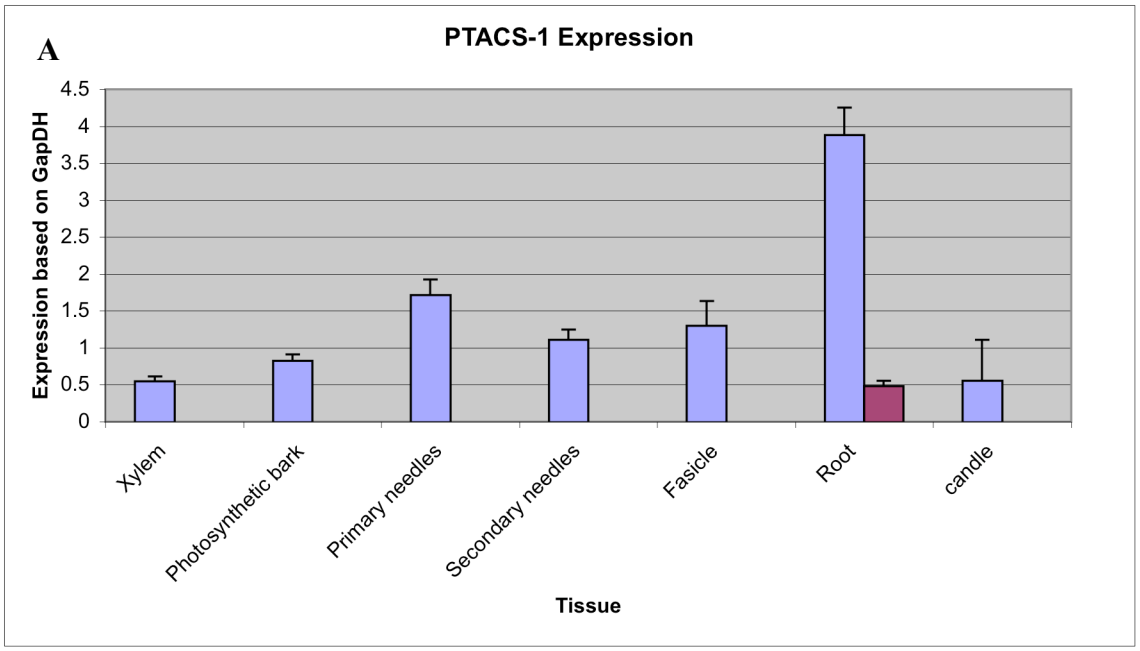


Figure 2.4. Tomato 1IAY crystal structure (a) monomer and homology models of PTACS-1 (b), *Arabidopsis* ACS12 (c), and PTACS-1s (d). The individual monomers have residues highlighted in the same color configuration as in the pileup above. The important tyrosines Y^{192} (Y^{92} in tomato) and Y^{149} for ACS12 are displayed as spacefill. The variable loop region is shown in blue. The conserved Proline residue in the new class of ATases is colored green. The pileup consists of all of the Arabidopsis ACC synthases a putative ATase from rice(BAA84790) and a known alanine ATase from *Arabidopsis*



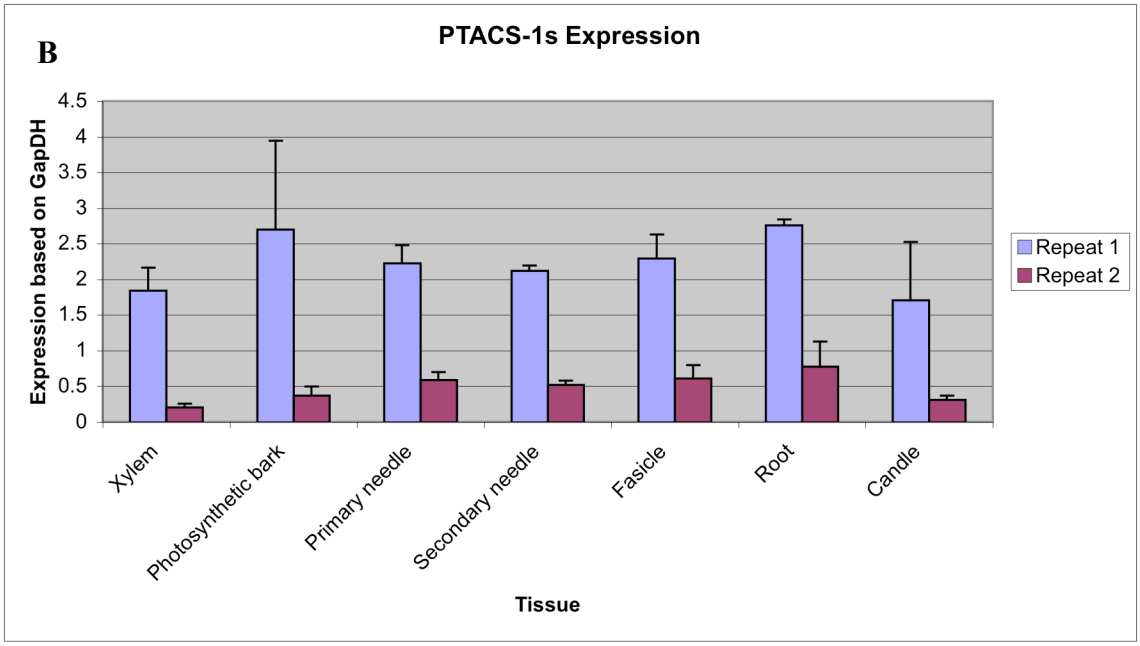


Figure 2.5. Expression of PTACS-1 (A) and PTACS-1s (B) relative to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase. Error bars shown are two times standard deviation

CHAPTER 3

ANALYSIS OF PTACS-1 AND PTACS-1S TRANSCRIPT RESPONSE TO KNOWN ACC SYNTHASE AND COMPRESSION WOOD ELICITORS[2]

[2]Barnes, J.R. and Dean, J.F.D. to be submitted to *Tree Physiology*

Abstract

The putative loblolly pine homolog of the ACC synthase gene (PTACS-1) is expressed in many tissues throughout the tree. However, nothing is known of its response to common ACC synthase gene expression elicitors, such as wounding, submergence, gravistimulation, bending, and auxin treatment. Using quantitative PCR (QPCR) the effect of these stresses on PTACS-1, and the related transcript, PTACS-1s, expression was measured. The expression patterns displayed by PTACS-1 suggest that it may have roles in signaling the production of compression wood.

Introduction

The phytohormone, ethylene, is known to play roles in fruit ripening, senescence, wounding, germination and root nodulation (reviewed in Johnson and Ecker 1998). In plants, ethylene is produced from 1-aminocyclopropane-1-carboxcylic acid (ACC), which is produced from *S*-adenosyl-L-methionine (SAM), through the activity of the enzyme, ACC synthase. ACC is subsequently converted to ethylene via the action of ACC oxidase. Production of ethylene is sustained by regeneration of SAM from 5-methylthioadenine (MTA), a byproduct of the ACC synthase reaction, and ATP. Primary regulation of the ethylene biosynthetic pathway typically

occurs at ACC synthase. ACC synthase controls ethylene production in response to stress (Morgan and Drew 1997), and ACC synthase expression is induced by such treatments as wounding (e.g. Kato et al. 2000), gravistimulation (Woltering et al. 2005), flooding (eg Shiu et al. 1998), chilling (Wong et al. 1999), and various plant growth regulator treatments (Tsuchisaka and Theologis 2004). ACC synthases usually occur as gene families whose members display differential expression in response to different treatments (Tsuchisaka and Theologis 2004).

There is good evidence that ethylene plays a role in the formation of reaction woods in trees. Compression wood, a type of reaction wood in gymnosperms, is formed in response to bending stress or when a tree must reorient itself from a non-vertical position. Compression wood, which is always found on the underside of leaning stems or branches, is characterized by eccentric growth rings, increased radial growth in the compressed area, higher phenolic content of the wood, increased numbers of vascular rays, and short, round tracheids that do not contain an S_3 cellulose layer (reviewed in Timmell 1986). Evidence for ethylene involvement in this altered developmental sequence includes an increased level of the ethylene precursor ACC in the compressed area (Savidge et al. 1983), increased

ethylene and IAA levels in the compressed area of *Metasequoia glyptostroboides* stems (Du et al. 2004), and elevated ACC oxidase in compression wood of maritime pine (Plomion et al. 2000).

Botella et al. (1995) found that mechanical strain induced an ACC synthase in mung bean. This ACC synthase, AIM-1, could be induced by IAA or bending of mung bean hypocotyls at a frequency of 1 Hz. In addition, it was found that expression of the AIM-1 transcript was a calcium-independent phenomenon. This type of response would be consistent with a response that might be expected if ethylene signaling related to reaction wood formation.

Expression of PTACS-1, a putative ACC synthase gene isolated from loblolly pine, was assayed using quantitative PCR (QPCR) to determine whether transcript levels respond to bending stress, similar to those seen for the AIM-1 transcript and consistent with possible involvement in compression wood formation. In addition, QPCR was utilized to determine whether PTACS-1 gene expression would respond to wounding, IAA, submergence, or gravity. A putative splice variant or recent duplication of PTACS-1, PTACS-1s, was assayed in parallel using QPCR to determine how its transcript reacted to bending, IAA treatment, and wounding.

Materials and methods

QPCR

Using the specific QPCR primers for PTACS-1 and PTACS1-s described in chapter 1, QPCR (Higuchi et al., 1996) was performed as previously described.

Plant material and RNA extraction

New growth candles were taken from *Pinus taeda* trees grown in 1-gallon pots from bare root stock obtained from the Georgia Forestry Commission. These plants were maintained in the greenhouse for over two years under ambient conditions with regular fertilization and water. Candles were used because endogenous expression of PTACS-1 was minimal in these tissues (see Chap. 1). In addition, candle production was prolific, allowing multiple time points to be taken from the same genetic background. Candles were vertically oriented on the tree, so compression effects should not have been present, and they were metabolically active, and thus contained abundant RNA.

For some experiments, seedlings were grown from "improved seed" provided by International Paper Company (Tuxedo Park, NY). Seedlings were maintained in tube containers or flats for 9 months in the greenhouse under a regular fertilization and watering regime.

RNA was extracted using the method of Chang et al. (1993), with the exception that isoamyl alcohol was omitted to improve yields (Lori Baker, personal communication).

Bending treatment

Candles on five trees (different genotypes) were bent to approximately 90^{O} from vertical using twine. Candles from each tree were collected for each time point. At harvest, candles were cut from the tree, stripped of any emerging needles, and flash-frozen in liquid nitrogen. Only areas of the candle involved in the bend were collected. Single candle experiments were used to assess genotypic influences, and were performed in the manner described above using two individual trees and only 1 candle per bending treatment.

IAA Treatment

The auxin, indole-3-acetic acid (IAA) (100 mM stock solution in ethanol), was used to treat seedlings at a final concentration of 1mM in water. Stems excised at the root:shoot junction were placed upright in a graduated cylinder and immersed in the IAA solution. For controls, cut stems were similarly immersed in water containing 1% ethanol, which is roughly equal to the amount of alcohol

coming from the IAA stock solution. Five stems were used for each time point. Needles were stripped from stems prior to flash-freezing in liquid nitrogen.

Wounding treatment

Candles that were still attached to loblolly pine trees were wounded by carefully crushing the tissue multiple times with pliers so that the tissue was evenly compressed, but without reorienting the candle from a vertical position. Five candles from five trees (different genotypes) were used for each time point. Needles were stripped before flash-freezing in liquid nitrogen.

Gravitropic experiment

Seedlings grown in tube containers were placed in a rack attached to a clinostat, and rotated at 2 rpm. These plants were arranged equidistant from the center of the clinostat, and were rotated along the axis of the stem. Stems were either bent 90^{o} or left unbent during the rotation (Fig 3.5a). Only stem tissue bewtween the root collar and the base of the crown was used for this study, and only tissue in the bent region of the stem was used for the bent time points (Fig 3.5a brackets). Five plantlets (different genotypes) were used for each time point.

Needles were stripped before flash-freezing in liquid nitrogen.

Results

PTACS-1 transcript levels showed a quick response to bending, more than doubling (2.3-fold) in expression within 5-10 min, and returning to normal levels within 3hrs (Fig. 3.1a). PTACS-1s, however, showed a much different pattern of expression in response to bending. This transcript was consistently repressed across the entire time course (Fig. 1b).

Experiments using single candles supported the observation of increased expression in response to bending stress, although the response was highly variable between genotypes (Fig. 3.2). Since the two trees used in this study were unrelated genotypes, differences in the expression response levels could have been due to genotypic differences. This response of PTACS-1 to bending appears similar to that reported by Botella et al. (1995) for AIM-1 gene expression in mung bean hypocotyls.

To investigate whether the increased expression of PTACS-1 was due to shearing and/or wounding of cells within the bent area, or represented a gravitropic response, two experiments were performed. To address the wounding

response, candles were wounded mechanically and transcript levels were evaluated (Fig. 3.3a). Wounding the candle in this manner did not produce significant increases in PTACS-1 expression. The expression of PTACS-1s, however, was greatly affected by wounding, showing a 32-fold increase in message levels after 10 min, before returning to basal levels at 1hr. The expression again jumped back up to 17-fold elevated levels at the three-hour time point (Fig. 3.3b).

Indole-3-acetic acid is usually a potent inducer of ACC synthase expression (e.g. Botella 1992). IAA also plays a role in wood development, acting as a positional signal affecting cambial growth rates (Tuomainen et al. 1997). This has led to the hypothesis that a change due to bending in the basipetal transport of IAA could lead to localized ethylene production and compression wood formation (Little and Savidge 1987).

To assess the effect of IAA on pine ACC synthase transcription, seedling stems were completely immersed in 1mM IAA in an upright position so as not to change the orientation of the cuttings. Gravistimulation is known to affect the production of some ACC synthases, such as AM-ACS3 from *Antirrhinum majus* (Woltering et al, 2005). The PTACS-1s transcript was monitored in parallel.

IAA increased the level of PTACS-1 transcript expression 2-fold (Fig. 3.4a). However the effect was short-lived, as expression dropped below basal levels after two hours. Submersion in water has been shown to affect expression of some ACC synthases (Rieu et al. 2005), but appeared to have little impact on PTACS-1 expression (Fig. 3.4a). IAA also affected the expression of PTACS-1s, but within a different time frame. PTACS-1s expression more than doubled at 3hrs, but returned to normal levels after 6hrs. However, this change in expression appears to be due to submersion as differences between the 3hr IAA treatment and 3hr submergence control samples were not significantly different. PTACS-1s also showed 5.5-fold increased expression at 8hrs of submersion (Fig 3.4b). This was unexpected, as the 8hr time point for IAA treatment did not show a similarly elevated response.

To investigate whether the increase in PTACS-1 expression in bending experiments was due to gravistimulation versus shearing stress, the following experiment was performed. Seedlings in tube containers were mounted on a clinostat and rotated around the stem axis. Trees were placed equidistant from the center of rotation and rotated at 2 rpm (Fig 3.5a). Because compression wood

can be induced by centrifugal force (Timmell 1986) the seedlings were spun slowly so as to minimize this potential problem. Figure 3.5b shows that the same increased expression in the ACC synthase was seen at 5 min (4-fold increase) after the seedlings were bent as was seen in normal bent plants kept upright (Fig. 3.5b). This suggests that ACC synthase expression was induced primarily by mechanical strain and not gravistimulation.

Discussion

PTACS-1, the putative ACC synthase gene from loblolly pine, was shown to have much the same response to bending and mechanical strain as did the AIM-1 transcript (Botella et al. 1995). The PTACS-1 gene showed increased expression under bending stress (>2 fold; Fig. 3.1a), and IAA treatment (2-fold Fig. 3.4a), but was not affected by wounding (Fig. 3.3a) or submersion (Fig. 3.4a). The response of PTACS-1 to bending appeared to be primarily a response to mechanical strain and not gravistimulation (Fig. 3.5b).

Compression wood is known to be caused by mechanical perturbations and bending (Telewski and Jaffe 1986abc), as

well as application of ethylene-releasing compounds, such as ethrel (for example, Du et al. 2003). The data presented here does not contradict the hypothesis that ethylene plays a part in compression wood formation. The putative ACC synthase gene shows a quick response to bending stress; however, the equally quick decay of this response suggests that other factors must be involved in the long-term processes leading to the formation of compression wood. Although this could be accomplished by auto-stimulation of another loblolly pine ACC synthase gene, as seen in *Arabidopsis* by Wang et al. (2005), we were unable to identify any other ACC synthase homologs in loblolly pine. It is important to note that due to the frequency of ACC synthase gene families in other species; thus, we feel is likely that additional ACC synthase genes remain to be found in pine.

The differential expression of PTACS-1s in response to wounding (Fig. 3.3b) and IAA treatment (Fig. 3.4b) was interesting. The strong induction by wounding (32-fold) suggests an important role in response to this stress. Interestingly, short, alternatively spliced transcripts have been seen for a number of ACC synthase genes in other plant species and treatments e.g. flooded tomato roots

(Olson et al. 1995) and developing carnation ovaries and styles (ten Have and Woltering 1997). The phenomenon was investigated for the ACC synthase gene transcripts, PS-ACS1a and PS-ACS1b, from pea (Peck and Kende 1998). The authors found that their ACC synthase probe hybridized to multiple transcripts in IAA-stimulated apical hooks of etiolated pea seedlings. The shorter transcript, PS-ACS1b, was found to arise from an alternative-splicing event of a transcript expressed from an internal promoter (Peck and Kende 1998). The PTACS-1b transcript protein product was shown to have no ACC synthase activity (Peck and Kende 1998) which was not surprising due to the absence the entire first exon, including a strictly conserved tyrosine residue (Y^{85}) integral to ACC synthase function. The fact that a short transcript occurs and is highly expressed in a wide range of plants, tissues, and treatments suggests that the transcript product is important and should be investigated further.

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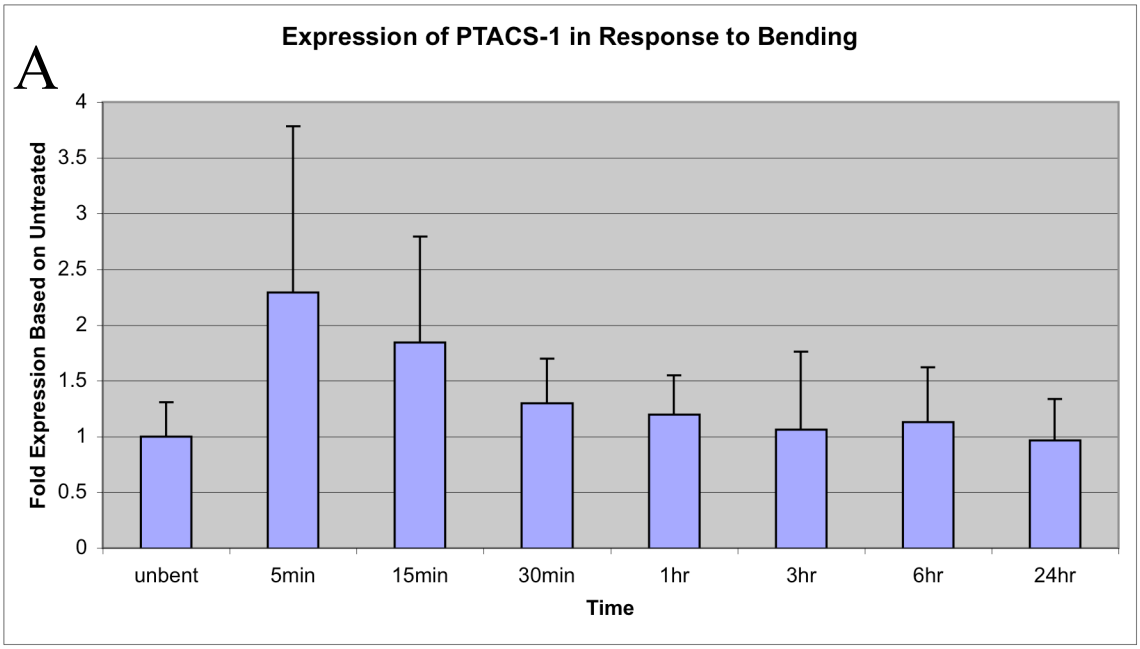


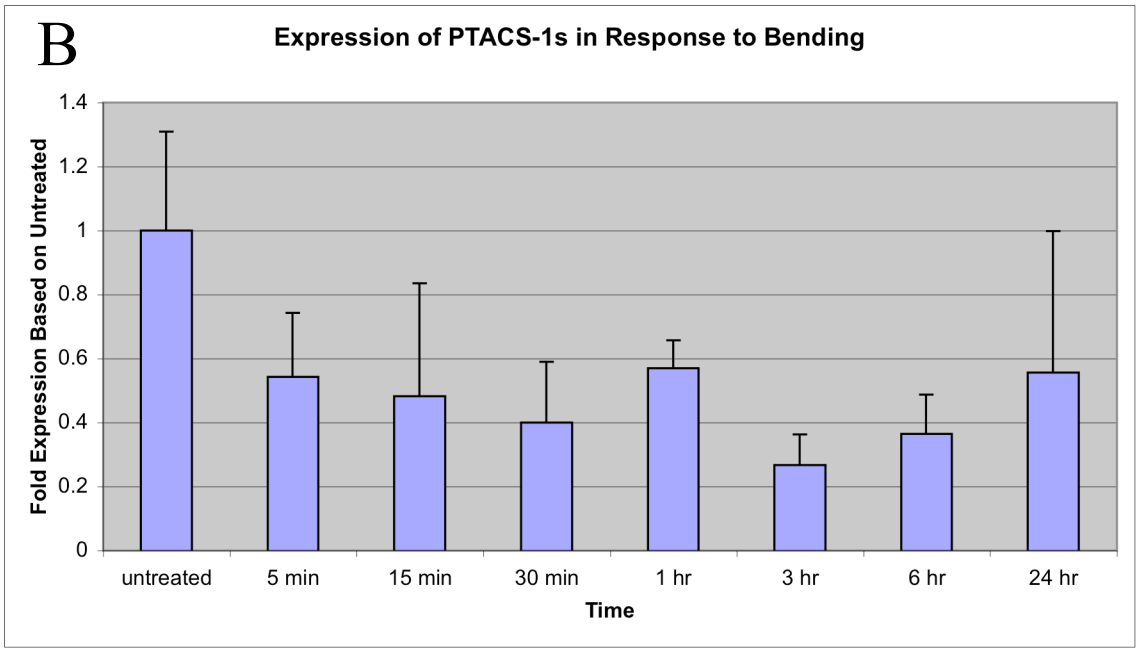


Figure 3.1. Expression of PTACS-1 (**A**) and PTACS-1s (**B**) in response to bending in candles. QPCR data is shown as fold change in expression compared to the untreated candles. Five candles from five trees were used for each time point and the experiment was run twice.

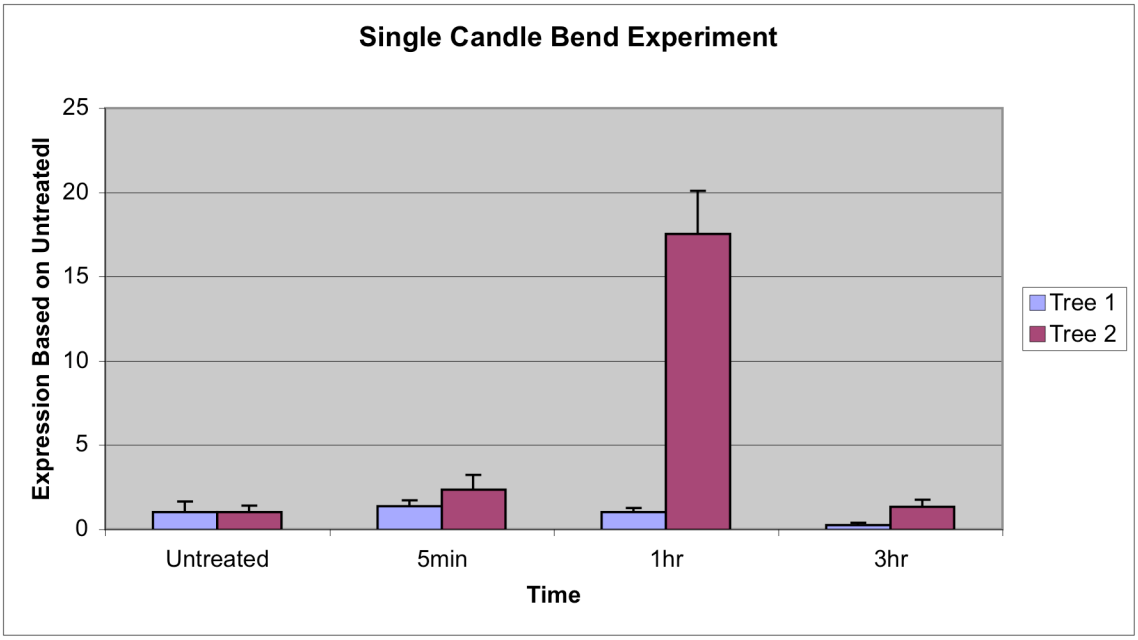
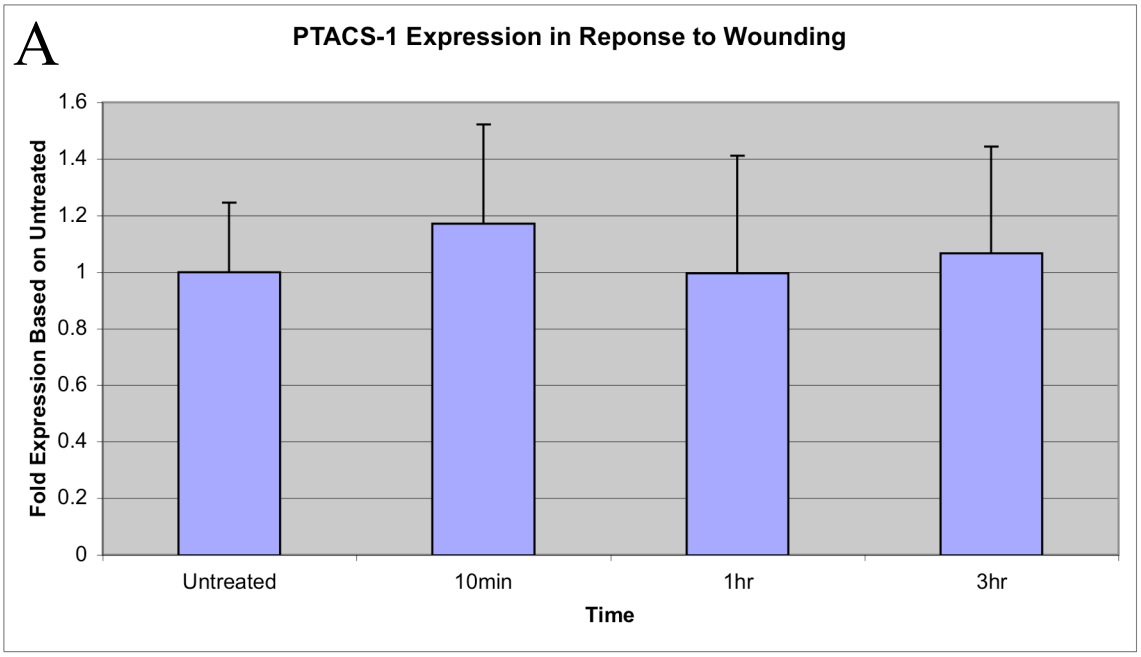


Figure 3.2. Expression of PTACS-1 in response to bending in a single candle. QPCR data is shown as fold change in expression compared to the untreated candles.



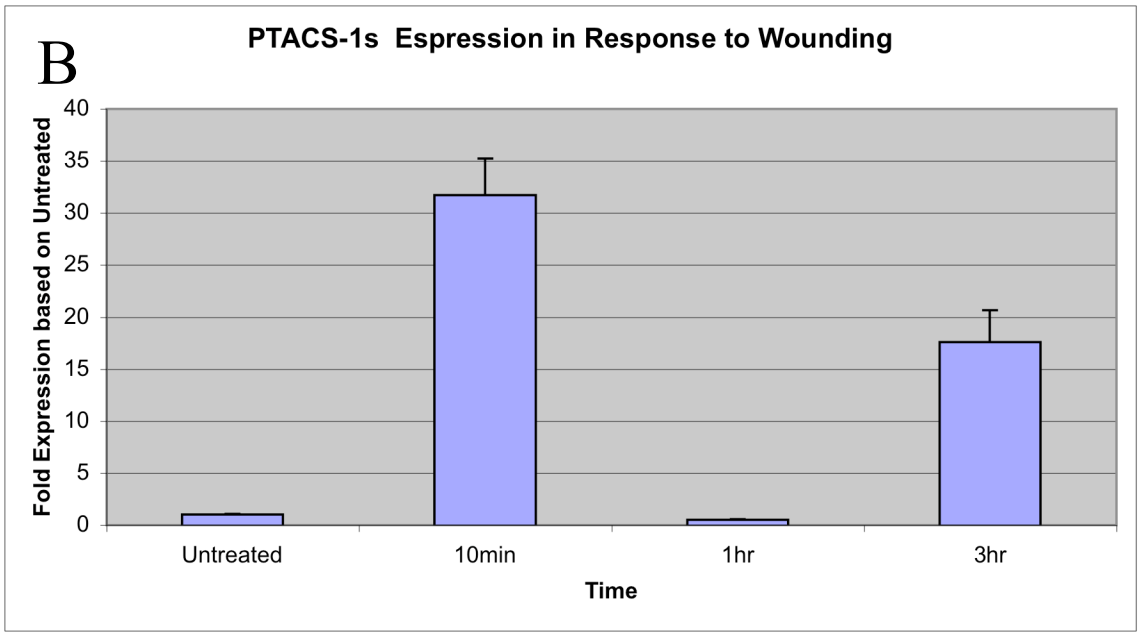
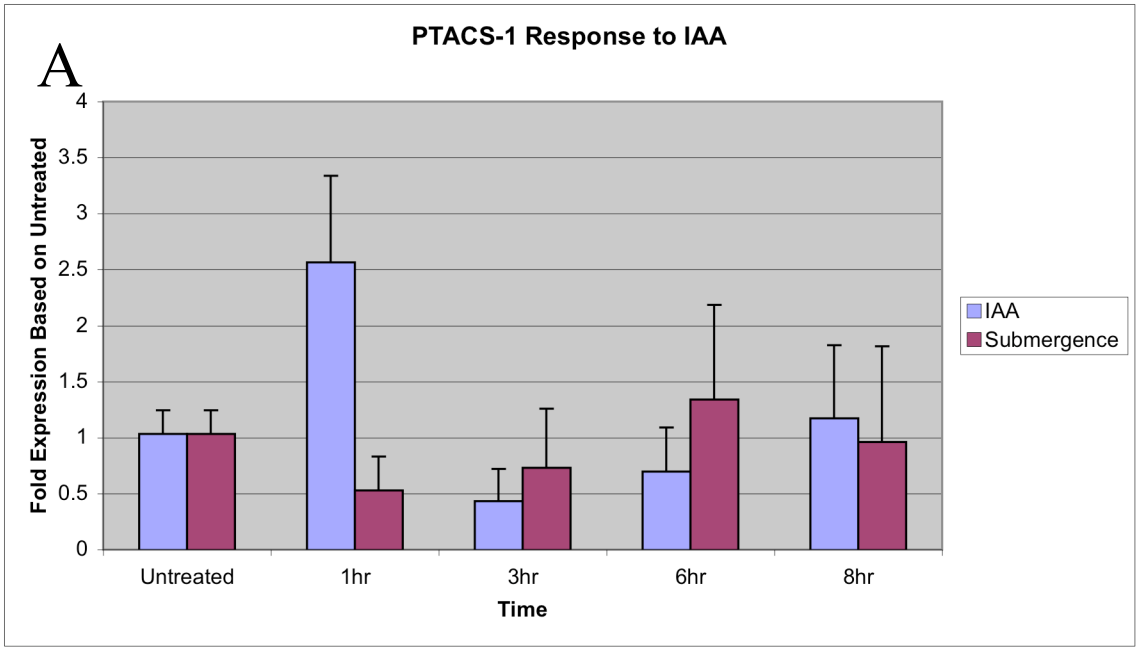


Figure 3.3. Expression of PTACS-1 (**A**) and PTACS-1s (**B**) in response to wounding in candles. Wounding was done mechanically and candles were compressed by squeezing plies down their length. QPCR data shown as fold expression based on the untreated candles. Five candles from five trees were used for each time point and repeated.



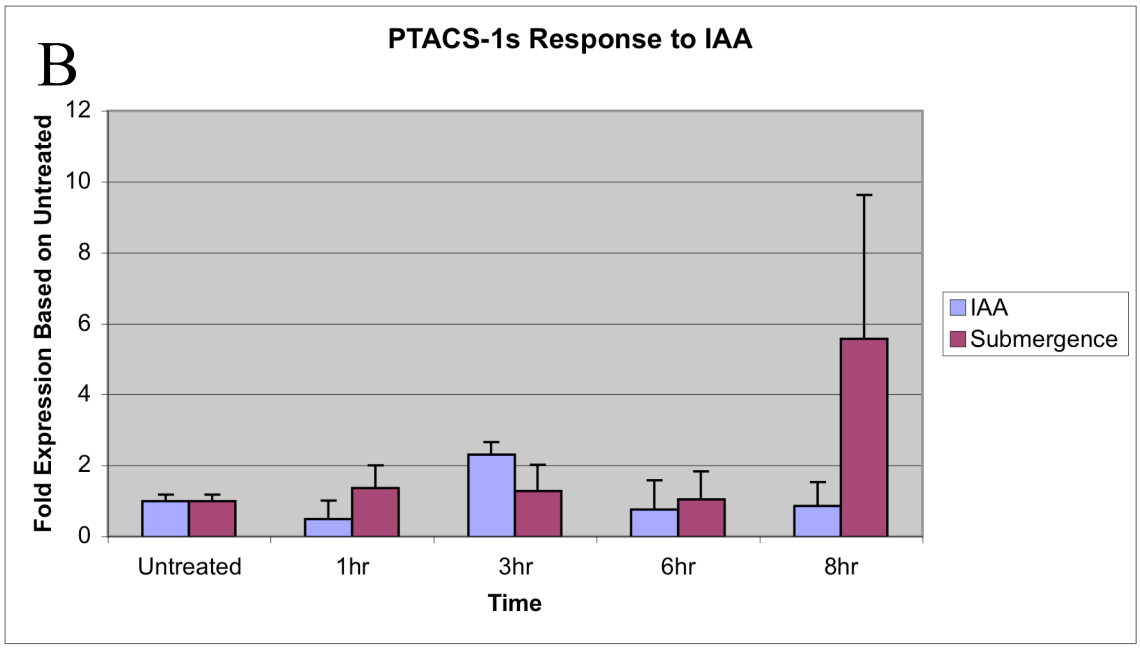


Figure 3.4. Effect of 1mM indole-3-acetic acid and submergence on PTACS-1 (**A**) and PTACS-1s (**B**) expression. Plantlets were submerged in IAA solution or 1% ethanol. Data is shown as fold change in expression based on the untreated seedlings. Five seedlings were used for each time point, and the experiment was repeated once.

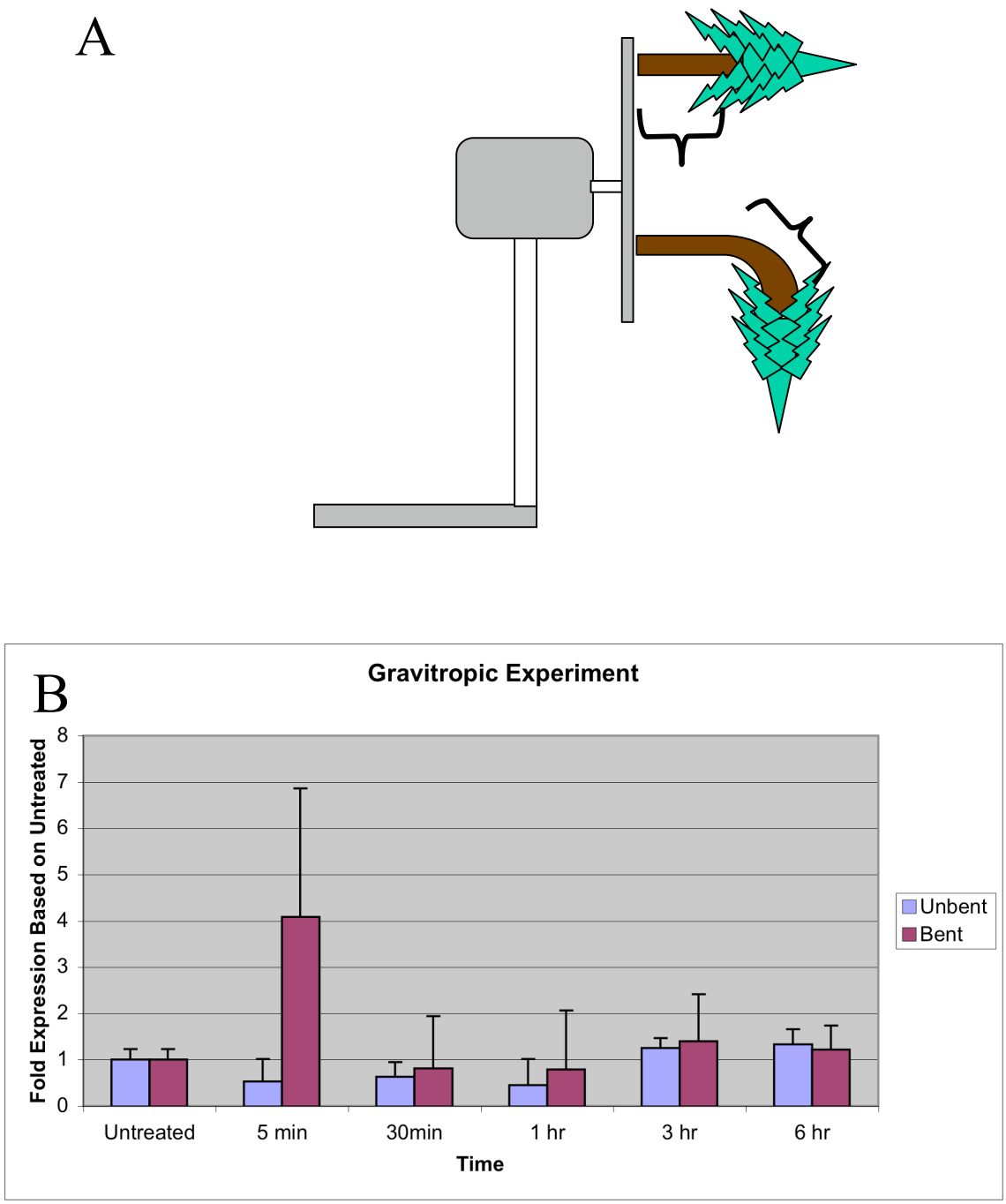


Figure 3.5. A. Cartoon of loblolly seedlings mounted on clinostat. Trees were spun at a rate of 1 revolution every 30sec. Seedling were bent or not and spun for the appropriate time. Brackets designate harvested portion. **B.** QPCR data from time course with the loblolly seedlings mounted on the clinostat. Data shown as fold expression based on the untreated seedlings. Five seedlings were used for each time point and repeated.

CHAPTER 4

CONCLUSIONS AND DISCUSSION

This dissertation presents the cloning of a putative ACC synthase, PTACS-1, from loblolly pine, as well as a putative splice variant of that transcript, PTACS-1s. The PTACS-1 coding sequence is 1798 bp long and displays high homology to ACC synthases, as well as a class of aminotransferases (ATases) recently discovered by Yamagami et al. (2003). The PTACS-1 genomic sequence contains one intron that is 215 bp in length. The PTACS-1 gene was shown to have low to moderate expression (roughly equivalent to that of loblolly pine GapDH) in a sampling of pine tissues. Roots were the only tissue in which high levels of PTACS-1 ACC synthase expression were seen (~4x greater than GapDH).

The shorter, but highly homologous transcript, PTACS-1s, was discovered during cloning of the PTACS-1 gene, when PCR amplification of the 5' end of the gene from a cDNA library generated two distinct bands. DNA sequencing demonstrated that the sequences were identical, except for a 138 bp deletion in the first exon. Thus, PTACS-1s is 1660 bp in length and is thought to be the product of either alternative splicing or an extremely recent gene duplication event. The 138 bp sequence that is omitted from PTACS-1s is A-U rich (58%), and while it does display a typical splicing branch site, the sequence is located 11 nt

away from the 3′ splice site instead of the 5′ end. In addition, the putative "intron" in PTACS-1s does not display typical intron splice junction conservation as the ends of the putative spliced region have the sequence AG/TTATC 5′ss and GATCT/GT 3′ss. Thus, while the putative exonic ends are typical of angiosperm plants, the intronic ends are not (Lorkovic et al. 2000). That said, there is little or no information available on intronic sequence conservation in gymnosperms, especially as it relates to alternative splicing. A UU nucleotide pair has been described for the 5′ss of a *Ginkgo biloba* $tRNA^{Tyr}$ splice junction (Akama et al. 1997), but there is no mention of an intronic sequence in gymnosperms ending in the sequence CT3′ss. In *Euglena gracilis,* however, intervening sequences of the ribulose-1,5-bisphosphate carboxylase/oxidase (rbcS) genes were found to have non-conserved 5′and 3′ splice sites, suggesting the presence of novel recognition components in the classical spliceosome (Tessier et al 1995). Further investigation will be necessary to determine whether or not these non-classical splice junctions are common in gymnosperms.

It remains possible that PTACS-1s is actually a separate gene, but this seems unlikely because the coding sequence and 3′UTR of PTACS-1s are identical to the coding

sequence and 3'UTR of PTACS-1. Unfortunately, the promoter and 5' UTR region of the PTACS-1s gene could not be isolated from genomic DNA using several different PCR techniques, likely due to the high identity of the two transcripts, which left only the putative splice junction as a primer site for genome walking experiments. In pea, the transcript PS-ACS1b, an alternative transcript of PS-ACS1a, is expressed via an internal promoter (Peck and Kende, 1998). This would be different mechanism from the PTACS-1s transcript as PTACS-1 and PTACS-1s share the same putative translational start.

PTACS-1s was expressed in all tissues sampled. Differences in expression seen in the two experimental sets (Fig. 2.5b) could be due to the presence of a single nucleotide polymorphism (SNP) in one of the primers used to amplify PTACS-1 for QPCR quantitation. A SNP in one of the primers can affect primer efficiency, causing mispairing of the primer and affecting the amount of transcript quantified. This possibility would explain how the trend in both data sets was the same, but the relative expression levels changed dramatically. Additionally, the trend of the PTACS-1 gene to be stimulated to a large extent and fall drastically in subsequent time points (eg. Fig 3.3b.) could

be explained by the presence of a SNP in a primer used to monitor the expression of PTACS-1s.

PTACS-1 appears to be an ACC synthase, and not one of the new class of ATases discovered by Yamagami et al. (2003), due to conservation of known active site residues, Y^{192} and Y^{329} (which correspond to Y^{92} and Y^{240} in tomato). These residues are known to be important for ACC synthase function, with mutagenesis of Y^{92} leading to loss of enzyme activity, and loss of Y^{240} leading to a 24-fold reduction in activity (White et al. 1994). Modeling of the *Arabidopsis* aminotransferase ACS 12 and PTACS-1 proteins shows that Y^{192} resides on a flexible loop that varies greatly between the PTACS-1 and ACS 12 models. The ACS 12 loop projects out from the dimerization face of ACC synthases, and would be likely to affect contact in a dimeric ACC synthase structure (Fig. 2.4). However, due to the limited study performed as yet on these ATases, it is unclear whether they would be expected to dimerize *in vivo*.

Based on the close homology of ACC synthases to the aminotransferases found by Yamagami et al. (2003), it is reasonable to assume they share a common ancestor. A compilation of all available ACC synthase gene sequences from rice, tomato, pear, and *Arabidopsis*, along with the two pine transcripts PTACS-1 and PTACS-1s, the gravity-

stimulated ACC synthase gene, ACS3, from snapdragon (Woltering et al. 2005), the bending-stimulated ACC synthase gene, AIM-1, from mung bean (Botella et al. 1995), and the ACC synthase gene, MD-ACS3, from apple were analyzed using the phylogenetic program, PROMLK, which is part of the PHYLIP computer program for phylogenetic analyses (copyright 2000-2004, University of Washington, Seattle, WA). In addition, an alanine ATase, an aspartate ATase, the two ATases ACS 10 and ACS 12, from *Arabidopsis*, and a putative ATase from rice, were included in these analyses. The PROMLK program uses the maximum likelihood method for comparing amino acid sequence divergence to produce phylogenetic trees that presume a constant molecular clock. In this comparison, PTACS-1s and PTACS1 were divergent from the other ACC synthases and clustered with the ATases recently described by Yamagami et al. (2003) (Fig. 5.1). However, PTACS-1 is thought to function as an ACC synthase because, unlike the ACS 10 and ACS12 ATases, it displays complete homology to all eleven invariant residues in ACC synthases (Fig. 2.2). It also displays strong homology in all regions of known conservation in ACC synthases (Fig. 2.2), and models of PTACS-1 show that the loop region forming part of the dimer face is predicted to have the same orientation as a

comparable loop found in as the tomato ACC synthase crystal structure (Fig. 2.4). Most importantly, PTACS-1 shows conservation at Y^{192}, which corresponds to the conserved position found in ACC synthases and not the position conserved in the ATase class of enzymes. The positioning of these particular tyrosine residues lead to the major differences in the active site mechanics of the two classes of enzymes.

Rice also appears to harbor a gene with homology to the new class of ATases. This gene (AN: BAA84790) has an extended N-terminal sequence, similar to those seen in *Arabidopsis* ACS10 and ACS12, but unlike ACS10 and ACS12, the rice gene retains Y^{190}, which corresponds to the PLP-binding site (Y^{92} in tomato) that is also seen in PTACS-1. However, BAA84790 has a prolyl residue at position 189. This position is strictly conserved as an aspartic acid residue in known ACC synthases, while a prolyl residue at this position seems to be conserved in all members of the new class of ATases (Fig. 2.4) The prolyl residue would probably affect positioning of the Y^{190} significantly by producing a turn in the protein structure. Additionally, BAA84790 has a phenylalanyl residue at position 340, similar to what is seen in the *Arabidopsis* ACS12 ATase.

These changes suggest that BAA84790 may encode be an ATase, rather than an ACC synthase.

The primary differences between the ATases, ACS10 and ACS12, and the known ACC synthase genes center on residues known to function in coordinating the PLP cofactor (White et al. 1994, Huai et al. 2001, Capitani et al. 2003). The mechanistic difference between these two enzymes, in all likelihood, is due to the positioning of the PLP residue within the enzyme. Placement of the Y^{192} and Y^{340} residues is the only difference between the active site of aspartate aminotransferase and ACC synthase, affecting the relative orientation of the PLP in each enzyme (Eliot and Kirsh 2000). However, heterologous expression and enzyme activity assays will be required to study the differences between PTACS-1 and the ACS10 and ACS12 ATases more fully.

Modeling of the PTACS-1s transcript showed that it is missing the variable loop region mentioned previously. Other parts of the model are not affected by the 138 bp deletion that produces this shorter transcript. The N-terminal regions of PTACS-1, PTACS-1s and ACS12 could not be modeled because they did not show homology to known protein structures.

Quantitative PCR analysis of PTACS-1 transcript expression showed the putative ACC synthase to be induced

by IAA, which is a feature common to many ACC synthases. In addition, bending induced PTACS-1 gene expression, similar to the expression pattern for the AIM–1 gene in mung bean (Botella et al. 1995). The transient expression of PTACS-1 in response to bending suggests that this gene may not be the only ACC synthase involved in signaling for compression wood development. Although the PTACS-1 gene displayed rapid induction in response to mechanical stress, which is known to promote compression wood development, transcription of PTACS-1 returns to normal levels quickly (within 3 hrs). Compression wood formation likely requires a longer-lived, possibly continuous (in some circumstances), signal. However, ethylene can, in some plants, stimulate ACC synthase expression in an autocatalytic cascade. Thus, PTACS-1 induction could possibly start a prolonged response by inducing transcription of another ACC synthase family member. PCR amplification was attempted using genomic DNA as a template for degenerate primers designed to various conserved regions of ACC synthase genes, but these primers did not yield any additional products having homology to ACC synthases.

The gravistimulation experiment described in this study strongly suggested the PTACS-1 transcript to be

responsive to mechanical strain and not gravity. However, discussion of how gravity affects compression wood development has been argued through two theories. The Cholodny-Went theory maintains that IAA moves laterally from the upper part of the tree to the lower parts (Cholondy 1926, Went 1926). A bend in the stem leads to an asymmetric distribution of auxin and, therefore, leads to enhanced growth on the lower side, causing reorientation of the stem. More recently, the Cholondy-Went theory has been undercut as a possible explanation for reaction wood formation because of studies showing that IAA tends to remain constant on both upper and lower sides of some bent stems (ex. Hellegren 2003). The theory also has limitations in explaining how tension wood, the reaction wood in angiosperms, is formed on the opposite (upper) side of bent stems. However, the Cholondy-Went theory cannot be disputed by our data, as IAA treatment was shown to increase transcription of PTACS-1.

The second principal theory to explain reaction wood formation is that gravity is sensed at the cellular level by starch granules in amyloplasts (Leach and Wareing 1967). These starch granules are presumed to act as gravity "sensors" (statoliths) by settling to the lower side of the cell under the influence of gravity. However, starch

grains have never been shown definitely to act as statoliths, and statoliths have never been demonstrated in mature conifer stems (Timmell 1986). This theory does not seem to hold with the data from the gravitropic experiment for PTACS-1. Dedolph and Dippert (1971) demonstrated that they could make statoliths travel in a circular path by spinning plants on a clinostat, thereby making the statoliths essentially stationary within the rotating cell. PTACS-1 showed a rapid increase of transcription in "bent" stems undergoing slow rotation, suggesting that gravity was not the cause of the increase in PTACS-1 transcription.

A more satisfying explanation for the increase in PTACS-1 transcription in response to mechanical stimulation may be provided by the tensegrity model for cellular response to physical stress (Ingber 1993a,b). Tensegrity was first described by Buckminster Fuller in the architectural sense as a structure that when stressed mechanically would maintain its shape by transferring the stress from individual structural units to the coupled network (Fuller 1961). Fuller identified two classes of tensegrity structures, prestressed and geodesic (Fuller 1961, 1979). A prestressed model, depicted in Figure 5.2 as a piece by artist Kenneth Snelson, is characterized by holding its joints in a state of preexisting tensile stress

(reviewed in Ingber 2003ab). Inber (1993) has used this model to propose that intact cells can be considered prestressed tensegrity structures. In the cellular tensegrity model, cytoskeletal elements, microfilaments and intermediate filaments, provide tensional forces against which microtubules and extracellular matrix adhesions serve as forces that resist compression (Ingber 1993). The tensegrity theory was expanded to explain how mechanical stresses can lead to chemical signals within the cell. Wang et al. (1993) used magnetic microbeads attached to ExtraCellular Matrix receptors (ECM), such as integrins, to provide torsion on isolated cells with a magnetic field. In cells placed under torsion, these receptors displayed the ability to transfer mechanical signals across the cell membrane (Wang et al. 1993). Chen and Grimmel (1997) found that integrins mediated stretch-dependent activation of calcium signaling. Signaling via this mechanism is very rapid, on the order of 10 milliseconds (Chen and Grimmel, 1997). The tensegrity model would explain the quick induction of PTACS-1 transcription and the observation that mechanical stimulation induced transcription whereas wounding did not. It may be worth noting that transcription of the mung bean ACC synthase gene, AIM-1, while stimulated by mechanical pertubations (Botella et al. 1995), was not

stimulated by calcium influx, such as might have been expected from one common type of stretch receptor.

The increased transcription seen for the PTACS-1s gene in response to wounding suggests that this transcript may code for a functional protein. Shortened ACC synthase transcripts have been seen in other plant species and are differentially expressed in response to different treatments (Liu et al. 1992, Olson et al. 1995,ten have and Woltering 1997, Peck and Kende 1998). These gene products could be functioning to regulate ACC synthase activity by producing non-functional heterodimers of the enzyme, as suggested by Peck and Kende (1998). Recent findings by Tsuchisaka and Theologis (2004) support this possibility; however, the work of Yamagami et al. (2003) leaves the possibility that PTACS-1s could encode an active aminotransferase protein. Heterologous expression of the cDNA and activity assays of the expressed enzyme will be required to study this possibility more fully.

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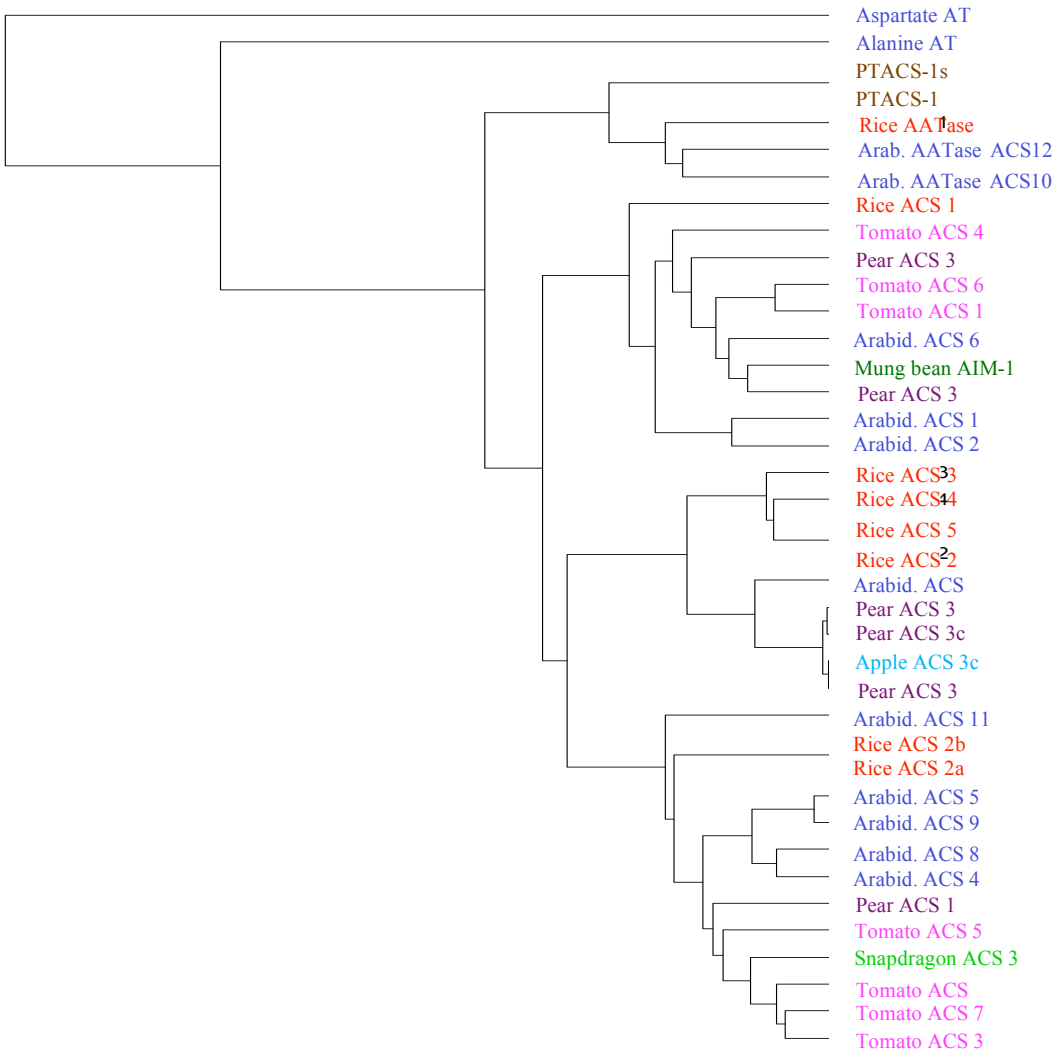

Figure 5.1. Phylogeny of ACC synthase and aminotransferase genes. The phylogenetic tree was generated using the ProMLK program.

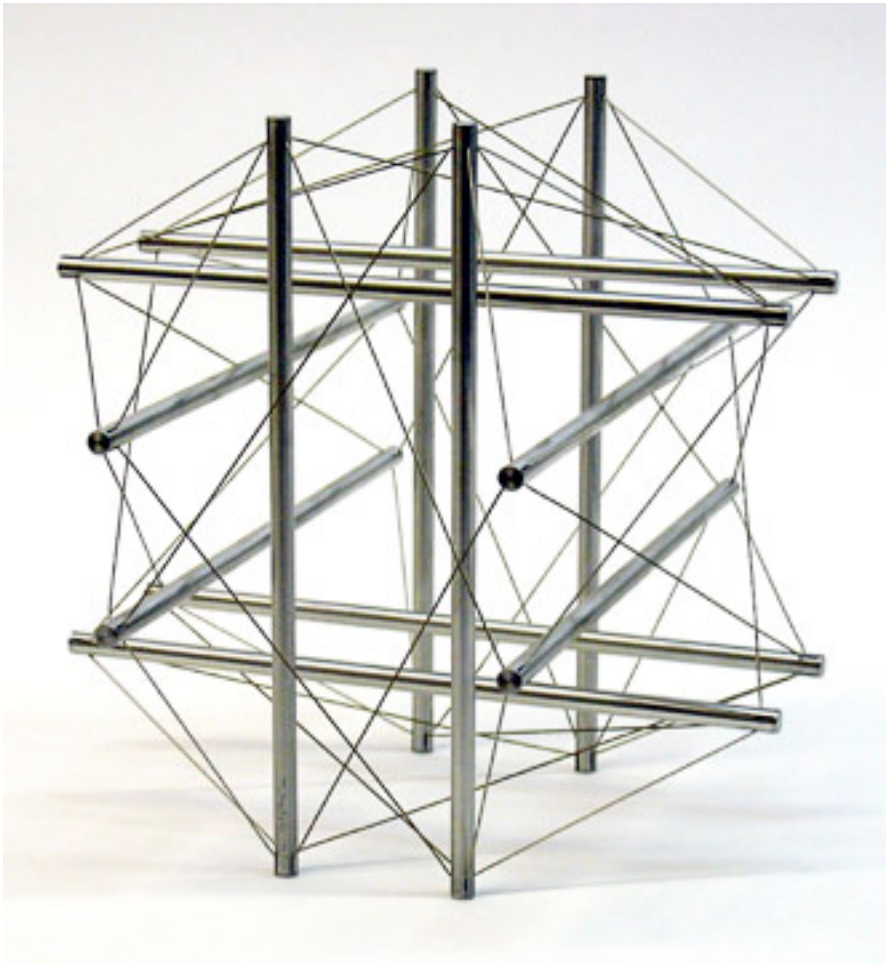

Figure 5.2. Prestressed tensegrity sculpture by Kenneth Snelson "Double Six" 1967

APPENDIX

ANALYSIS OF THE ROLE OF *LEAFY* AND *APETALA-1* GENES IN SOUTHERN HARDWOODS[3]

[3] Barnes, J.R., Wang, Y., Lorenz, W.W. Merkle, S.A., Covert, S.A. and Dean, J.F.D Published in *Proceedings of the 25th Biennial Southern Forest Tree Improvement Conference* July 11-14, 1999 New Orleans, LA

Abstract

In order to determine whether floral production could be manipulated in forest trees, two floral meristem identity genes from *Arabidopsis thaliana* (*Leafy* [LFY] from D. Weigel and *Apetala-1* [AP1] from M. Yanofsky) were placed under control of either a constitutive promoter or a copper-inducible promoter element and used to transform yellow-poplar (*Liriodendron tulipifera*). Cell lines were transformed by microprojectile bombardment and selected on 100mg/ ml kanamycin plates. Resistant cells were assayed for transgene integration by genomic PCR and for transgene expression by RT- PCR. In order to determine copy number, Southern blots were done on cell lines that were positive according to genomic PCR and RT-PCR tests. *In vitro* plantlets were regenerated from transformed cell lines showing integration, expression, and low copy number. Plants were then potted and placed in a greenhouse for a period of approximately two years. RT-PCR tests revealed that some constitutive AP1 lines lost expression during regeneration: *in vitro* plantlets (100% LFY, 80% AP1), and plants (100% LFY, 50% AP1). Phenotypic variations of the transgenic plants from wild type were seen in reduced size as well as a reduction in apical dominance. Thus far, none

of the plants have shown an ability to flower precociously. RT- PCR tests showed that the copper-inducible promoter element was constitutively expressed under tissue culture conditions even with no exogenous copper added to the medium. In addition, a partial cDNA representing a putative homolog of the LFY gene was recovered from in immature flower buds of yellow-poplar.

Introduction

Increased understanding of the molecular genetics underlying flower development in the model species, *Arabidopsis thaliana* has led to successes in the control of flowering through gene manipulation (Ma, 1994). This has raised the possibility that the same genes might be used to modulate flowering in forest trees. Tree breeding has always been a slow process due to the long lag to sexual maturity in most tree species. Chemical applications, as well as girdling and other mechanical stresses have been used to induce some species of trees to flower precociously. However, these methods often induce trees to make a phase change to a mature phenotype characterized by slow growth (Meilan 1997). In addition, not all juvenile trees flower in response to these treatments. If trees could be manipulated to flower precociously in a

reproducible manner, breeding cycles and generation time could reach levels comparable to that of agricultural crops.

Genes that control flower development also have potential uses in creating sterile trees. Many genes that have the potential to be very useful in forestry, such as insect resistance genes, carry with them the potential to be harmful to the ecosystem if planted stands of transgenic trees were allowed to interbreed with wild populations. These concerns have been heightened by recent observations that pollen from transgenic plants expressing the Bt toxin gene can be lethal to non-target species feeding on native plants proximal to transgenic stands (Losey et al. 1999). It will therefore be critical to develop techniques for creating trees that are sterile if we are to deploy genetically engineered trees. Note that testing sterility in such trees would be significantly less time consuming if flowering could reproducibly controlled.

Two genes in *Arabidopsis thaliana* that have been shown to reduce the lag time to flowering when over-expressed are LEAFY (LFY) (Weigel et al., 1992) and APETELA1 (AP1)(Mandel et al. 1992). The Arabidopsis LFY gene under control of the CaMV 35S promoter has been shown capable of inducing

precocious flowering in transgenic hybrid aspen trees (Weigel and Nilsson, 1995). The flowering time in the trees was shortened to only five months in the greenhouse from a period of 8-20 years in nature. Single flowers arose on the transgenic aspens at the axis where lateral shoots would normally occur in a wild-type seedling. In Arabadopsis, constitutive expression of AP1 resulted in a similar pattern of flowering with the lateral, as well as apical meristems forming single flowers.

We tested the LFY and AP1 genes from *Arabidopsis* to determine their appropriateness for engineering two southern hardwoods yellow-poplar (*Liriodendron tulipifera*) and sweetgum (*Liquidambar styraciflua*).

Materials and Methods

Tissue Culture and Transformation and Selection of Yellow-poplar and Sweetgum Cells

Protocols for tissue culture, transformation, selection, and regeneration have previously been described for yellow-poplar (Merkle and Sommer, 1986; Wilde et al., 1992) and sweetgum (Kim et al., 1997; Merkle et al., 1997).

Subcloning and Plasmid Manipulation

LFY and AP1 promoters and genes were graciously shared with us by Drs. D. Weigel (Scripps) and M. Yanofsky (U.C. San Diego), respectively. Both genes were sent to us in the *Agrobacterium*-based transformation plasmids pDW-139/LFY (unpublished) and pAM563/AP1 (unpublished). These plasmids both have the same general components such as NPT-II gene for kanamycin selection and the 35S CAMV promoter. Using microprojectile bombardment, these constructs (pDW-139/LFY and pAM563/AP1) were used to transform embryogenic yellow-poplar cells for flower induction studies.

Functions of the LFY and AP1 promoters with the ß-glucuronidase GUS reporter gene were prepared using appropriate restriction digests were ligated into pWLGUS. The pWLGUS plasmid was derived from the plasmid. pSL1180 (Pharmacia, Piscataway N.J.) by adding the GUS gene, 35S promoter, and the hygromycin resistance gene, hph, from PTRA-140 (unpublished). The constructs were used to transform sweetgum, as well as yellow-poplar.

In an effort to introduce a level of variable control over the flowering process, a copper-inducible promoter was also fused to the LFY and AP1 genes in a second set of vectors, pPMB7066 that has been previously in tobacco (Mett

et al. 1993). The copper-inducible LFY and AP1 constructs were only tested in yellow-poplar.

Nucleic Acid Extraction

RNA extraction was carried out via one of three methods: 1) Grinding in Trizol reagent (Life Technologies Rockville, MD), 2) a modified Trizol protocol; or 3) a RNA isolation procedure developed for pine tissues. For cultured yellow-poplar cells, the Trizol protocol was carried out according to the manufacturers' instructions was sufficient to yield good quality RNA. However, genomic DNA contamination required that a DNase treatment be added after the extraction procedure. Yellow-poplar bud tissue required a modification to the Trizol protocol in order to recover clean RNA. In the modified protocol a blocking agent containing 4.0 M guanidine thiocyanate, 0.1M Tris-HCL (pH 7.5), and 1% ß-mercaptoethanol was ground along with the tissue in a liquid nitrogen-cooled mortar. The blocking reagent was added in a ratio of 1ml of reagent to every 2 grams of ground tissue to aid in the removal of phenolic materials. Otherwise, the procedure was performed according to the original protocol. For isolation of RNA from yellow-poplar leaves the method of Hughes and Galau (1998) was utilized. Integration of the transgene in the

transformed cell lines was verified by PCR using the DNA preparation protocol described by Klimyuk et al. (1993). DNA for southern analysis was prepared from tissue-cultured cells using a modified CTAB protocol found by Lasser et al. (1989).

Polymerase Chain Reaction

Reverse transcription-PCR was used to monitored expression of LFY and AP1 cell lines that displayed transgene integration. Two-step RT-PCR used Superscript (Life Technologies) reverse transcriptase and the appropriate gene specific primer for LFY (5337n) or AP1 (829n) was utilized in a separate step to prepare the template. This allowed for independent assessment of the degree of contaminating genomic DNA present in the samples. Two-step RT-PCR using and oligo-dT primer was used in homolog cloning experiments. Reverse transcriptase reactions utilizing Superscript enzyme were carried out according to the supplier's protocol with the reaction proceeding for 1hr at 48^{O}C in a thermocycler. One-step RT-PCR was used to screen AP1 and LFY expression in some cases, because it was faster than the two-step method. One-step RT-PCR was preformed using the Access RT-PCR system (Promega Madison, WI) according to the protocol provided

and reactions were primed with the oligonucleotides mentioned above.

Primers for screening transformed lines with the LFY and AP1 transgenes were developed from sequences deposited in gene bank. The screening primers for the LFY gene transformants were: (5004s) 5′-GGTACGCGAAGAAATCAGGAG-3′ and (5337n) 5′-CAGCTAATACCGCCAACTAAAG-3′. The primers for AP1 screening were:(349s) 5′-GCCGAAAGACAGCTTATTGCAC-3′ and (829n) 5′-TCCTCATTGCCATTGGATCATC-3′.

In experiments to amplify a LFY homolog from yellow-poplar using two-step RT-PCR the cDNA was primed with oligo-dT and subsequently amplified with the degenerate primers (LFY 490n) 5′-GTIGGTIACRTACCADAT-3′ and (LFY 250s) 5′-GARMGICARMGIGARCAYCCITTYAY-3′. The product was then reamplified with two nested primers (LFY 275s) 5′-GAYTAYYTITTYCAYYTITAYGA-3′ and (LFY 360n) 5′-ARIGGYTTRTARCAIGCYTGICKCCAY-3′. The product was cloned directly into the PCR 2.1 vector using the TA -Cloning Kit (Invitrogen), and colonies were screened by PCR using universal primers for the M13 reverse and T7 sites. The clone was subsequently sequenced in both directions and homology analyses were done using BLAST program available through the NCBI web site (http://www.ncbi.nlm.nih.gov/).

All PCR experiments included plasmid controls and a no-template control to control for contamination.

Southern Analysis

Yellow-poplar lines transformed with the LFY and AP1 constructs that showed transgene integration and expression by genomic PCR and RT-PCR screens were subsequently analyzed by Southern Blotting (Southern, 1975). Southern blots were probed using the AlkPhos Kit (Amersham) to label full-length versions of either the LFY or AP1 genes. Gels were run overnight and blotted to charged nylon membranes. All wash and probe steps were performed according to the supplied protocol from Amersham. The Southern blots were developed using CDPstar (Amersham) as described in supplier's protocol with exposure time of 2.5 hours.

GUS Staining

The GUS reporter gene was utilized to determine whether the LFY and AP1 promoters retained floral-specific expression patterns in yellow-poplar and sweetgum. Gus staining was carried out using the protocol described by Jefferson et al. (1992).

Greenhouse Care

Plants regenerated from transformed and control cell lines were maintained in a greenhouse under natural light for a period of two years. The plants were watered and fertilized regularly.

Results

Two different genetic lines of embryogenic yellow-poplar cells were transformed with the over-expression constructs (35S:LFY and 35S:AP1). The kanamycin-resistant lines were tested first for gene integration using genomic PCR. Lines that showed stable integration were retained for further study, all other lines were discarded. Relatively few kanamycin-resistant were discarded, suggesting that rearrangement events leading to loss of the Arabidopsis genes during integration were infrequent. In fact, only five resistant lines, all having received the AP1 construct, were shown not to contain the transgene and had to be discarded.

RT-PCR was used to test whether the integrated transgene was expressed properly in the transformed cell lines and the results of RT-PCR tests are summarized in Table 6.1. All of the cell lines harboring the 35S:LFY construct expressed the transgene. Sixteen of the 35S:AP1 lines lost transgene expression at the cell culture stage,

and fifteen of those sixteen lines had the same genetic background. Instability of the AP1 transgene expression was further demonstrated by loss of expression when plants were regenerated from two lines of the second genetic background.

In addition to loss of expression, lines harboring the AP1 construct also demonstrated what appeared to be reduced expression compared to lines transformed with the LFY transgene. They did not yield a strong signal in RT-PCR (faint bands Fig. 6.1) used to test for transgene expression performed under identical conditions for both LFY and AP1, even though the AP1 primers worked very well on the plasmid construct. Although suspect as a quantitative method, bands of similar intensity should have been apparent since both transgenes were driven by the same promoter. The observed difference in band intensity might have resulted from mispriming of the AP1 primers within the genome, or from the AP1 transgene being silenced in some way by the host cells. AP1 is a member of the MADS-box family of genes and mispriming could have occurred because of the strong sequence homology that the genes in this family display. However, mispriming does not satisfy our finding that expression from AP1 transcends in some cell lines was in several cases turned off by an unknown

mechanism (Table 6.1) We suspect that, in addition to mispriming, constitutive expression of the AP1 transgene product is deleterious to yellow-poplar cells, and as a consequence, the gene tends to be silenced over time in transformed cells.

Southern analyses were used to determine whether cell lines contained multiple copies of the LFY or AP1 transgene and lines having more than three repeating copies of the genes were not regenerated into plants (Fig 2). In general about 20% of the kanamycin-resistant cell lines contained only 1 or 2 copies of the repetitive transgene.

Yellow-poplar plants regenerated from cell lines harboring the 35S:GUS reporter gene fusion are indistinguishable from wild-type plants. Although such plants might have arguably served as the best control for this study, we opted to use untransformed, regenerated plants for comparison with regenerated transgenic plants.

Plants harboring the 35S:LFY and 35S:AP1 transgenes were compared to plants regenerated from untransformed lines of the appropriate host genotype. The plants have been maintained in the greenhouse for a period of over two years, and thus far, no flowers have been seen. However, the plants do have a distinctive phenotype (Fig 3). The 35S:LFY and 35S:AP1 transformants exhibit a reduced growth

rate and reduced apical dominance compared to wild-type plants. Also, the transformants appeared "bushy" due to shortened internode lengths. The "bushy" phenotype found in these transformed yellow-poplar trees is similar to what is seen in the *Arabidopsis* LEAFY mutant (Weigel et al., 1992). Therefore, it is apparent from the phenotype that the transgenes are playing a role in yellow-poplar meristem identity, as expected, and that the pathway affected is similar to the one in *Arabidopsis*. Phenotypic differences were not apparent in comparisons between plants harboring the two different transgenes, suggesting that both transgenes had similar effects. This finding supports the assumption that the overlapping roles played by the LFY and AP1 genes in *Arabidopsis* were reflected in yellow-poplar. Unfortunately, the transformed plants did not display any phenotypic characteristics desired by the forest products industry.

Because of the difficulties we had in maintaining constitutive expression of the AP1 gene in transformed yellow poplar, we attempted to use and inducible-expression vector based on elements of the copper-inducible response system from yeast (Mett et al. 1993). Kanamycin-resistant cell lines containing copper-inducible versions of the transgenes were tested using RT-PCR to determine under what

conditions the transgene would be expressed. LFY and AP1 transcripts were both expressed constitutively under normal tissue culture conditions (data not shown). In addition, plants regenerated from all lines harboring the copper-inducible AP1 transgene also demonstrated constitutive expression in soil under normal growing conditions (data not shown). Thus, the copper-inducible expression system appears to be too leaky for our desired uses in tissue culture or whole tree situations.

To identify elements that might be useful for the development of sterile trees, we tested tissue-specific actin promoters, as well as the LFY and AP1 promoters. The ACT2 promoter had been shown to express constitutively in *Arabidopsis* (An et al., 1996), while expression of the ACT11 promoter was limited to reproductive tissues (Huang et al., 1997). ACT2:GUS and ACT11:GUS fusions were used to transform yellow-poplar cells, and kanamycin-resistant colonies assayed for GUS expression staining showed that the ACT11 promoter was very active in embryogenic yellow-poplar cells. This highlights the difficulty in finding promoters active in reproductive tissues, but not in embryogenic cultures. Since most forest tree transformation systems utilize embryogenic cultures, such

promoters will be critical to the development of sterile trees.

LFY and AP1 promoter/GUS fusions were also examined for floral-specific expression in yellow-poplar and sweetgum. Both the LFY and AP1 promoters were active in embryogenic cells of yellow-poplar and sweetgum cells; thus, neither promoter would be useful for cell ablation strategies for sterility in forest trees.

Part of a putative LEAFY homolog was amplified from yellow-poplar using PCR. The clone was 288 base pairs in length and high homology to the 3' end of the Arabidopsis LFY gene (Fig 4). BLAST analysis showed that the isolated fragment was also very similar to a LFY homolog from *Populus balsamifera*, although results appeared somewhat skewed because a full-length gene from yellow-poplar was unavailable for analysis. Efforts to extend the clone to full length are continuing.

Conclusions

This study showed that the Arabidopsis LFY and AP1 promoters are expressed under normal tissue-culture conditions in embryonic cells of yellow-poplar and sweetgum. Therefore, neither of these is of much use for cellular ablation strategies in engineering sterility in

forest trees. Similarly, embryonic yellow-poplar cells might be useful in situations where a gene needs to be expressed in tissue cultured cells but not in regenerated plants. However, transgenic plants need to be analyzed in greater detail to determine whether the promoter is active in any other tissues. Studies of a copper-inducible promoter system found that the system was leaky throughout yellow-poplar tissue culture and regeneration. Thus, there is still a need to identify an inducible-promoter system that can routinely be used under the conditions required for genetic engineering of forest trees.

This study also showed that over-expression of the floral meristem identity genes, LFY and AP1, from Arabidopsis was not sufficient to cause precocious flowering in yellow-poplar within a reasonable time frame. Yellow-poplar transformed with 35S:LFY and 35S:AP1 plants have yet to produce floral organs of any kind after more than two years of growth in a greenhouse. This is in contrast to work done in hybrid aspen, which showed that expression of the LFY transgene could lead to flowering in a few months. The transformed yellow-poplar did exhibit a phenotype of reduced apical dominance, slow growth and shortened internodes. The phenotype seems consistent with the action of genes responsible for controlling meristem

identity. Thus far, hybrid aspen transformed with 35:S LFY has been the only tree to exhibit precocious flowering in response to a floral development transgene (Weigel and Nilsson, 1995). Precocious flowering is a must for researchers to properly test sterility in a reasonable amount of time. The finding that the meristem identity genes were insufficient to promote precocious flowering demonstrates the lack of knowledge in this area and that more research is needed to determine whether other transgenes, chemical applications, or mechanical stresses are required to determine whether precocious flowering can be achieved in forest trees.

Population increases will demand higher outputs from the forest products industry from reduced planted acreage and biotechnology is one of the most promising avenues for meeting the demand predicted in the future. Increased research into sterility and other aspects of flowering will be imperative if we are to deploy the genetically engineered trees necessary to meet the demand of future forestry.

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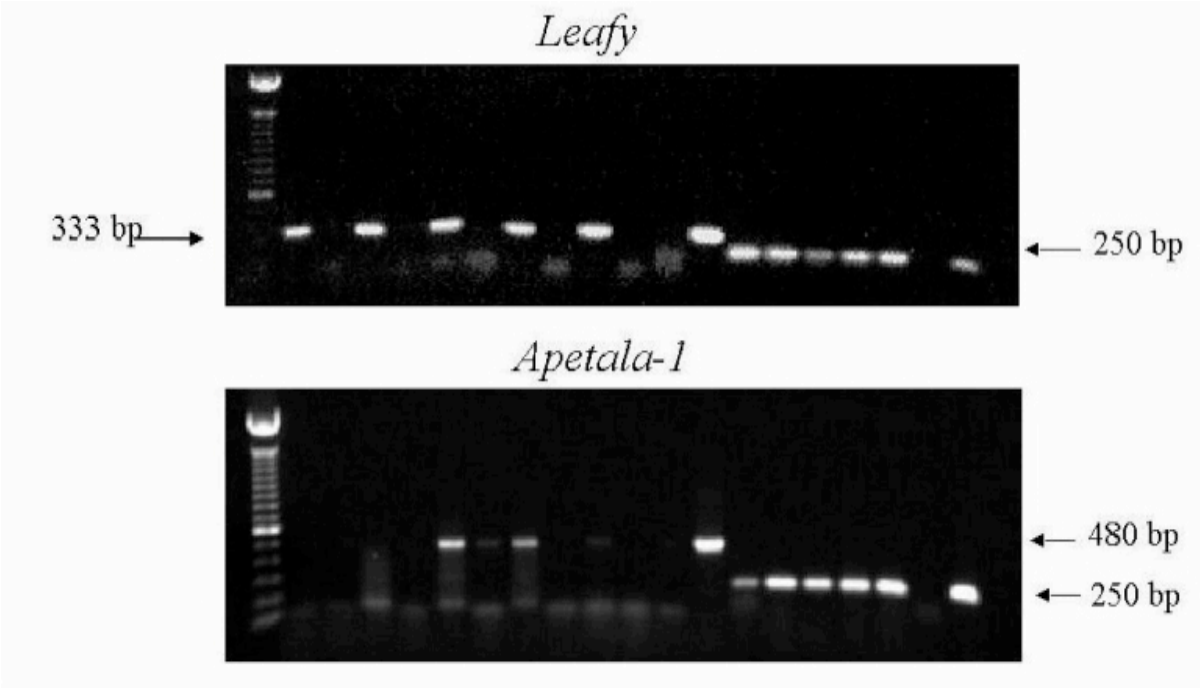

Figure 1: RT-PCR of LFY and AP1 genes in transgenic yellow-poplar. Both gels were loaded with alternating +RT and -RT. The 250bp band corresponds to the GAP-DH control used for each cDNA tested

* * *

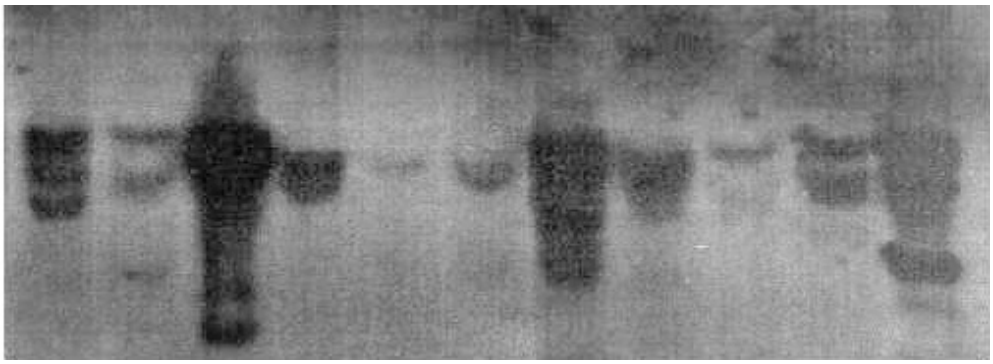

Figure 2: Southern analysis of the AP1 gene in 35S:AP1 yellow-poplar. To determine copy number, Southern blots were probed with the full length Arabadopsis LFY or AP1 gene. Asterisks denote transformants harboring only one or two copies of the transgene.

Figure 3: Comparison of 35S:AP1 and 35s:LFY yellow-poplar to a wild-type regnerat. Plants are all of the same age and have been grown in a greenhouse for a period of one and a half years.

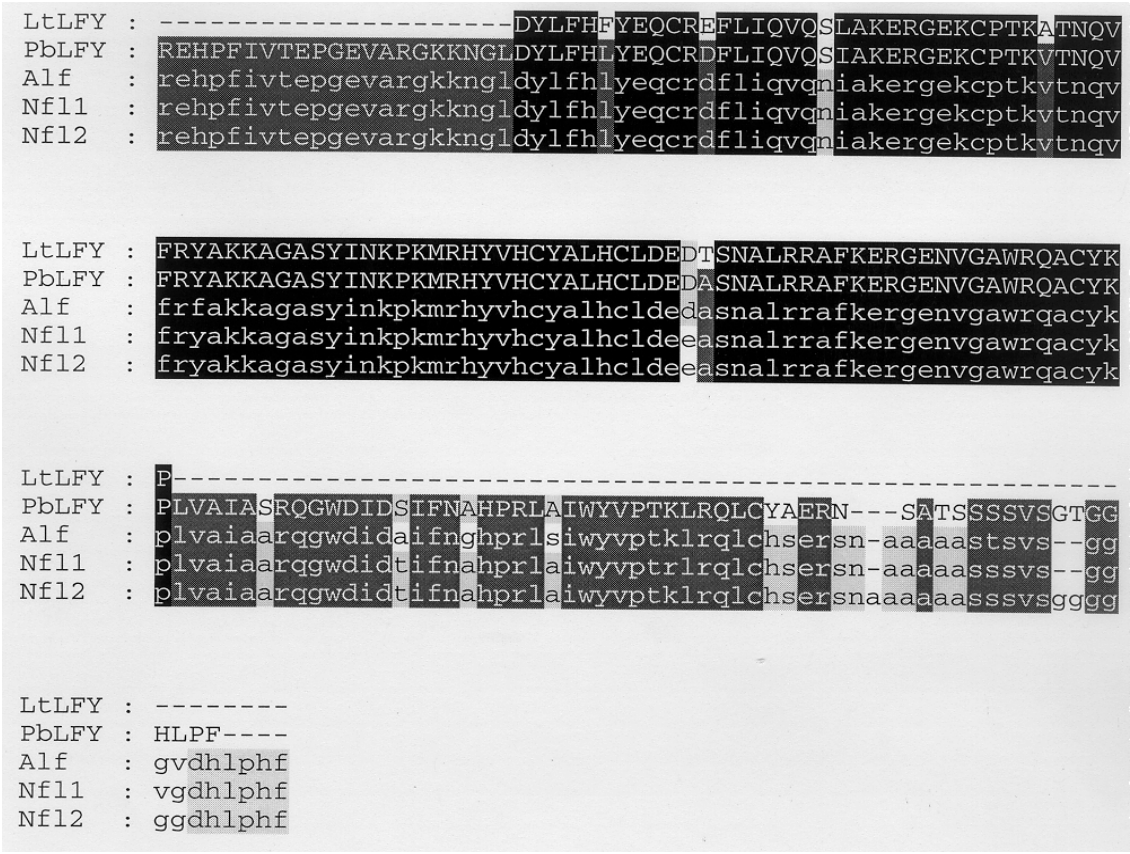

Figure 4: Sequence allignment of LFY homologs from yellow-poplar, P. balsamifera, Antirrhinum, and Tobacco.

Construct	Kanamycin-R	In vitro Expression	No Expression in Vitro	Lines and # of Regenerated Plants	Expression in Plants
35S:AP1; 91	11	A,C,E,F,G, J,H,K	B	A(4),B(10),E (15), F(15),C(6)	None in A
35S:AP1; 92 (1)	20	B,D,R	C,H,K,P	C(15),R(16)	None in C 80% in R
35S:AP1; 92 (2)	15	D,K,O,S	A,B,C,E,G, H,I, L,M,N,Q	NONE	NONE
35S:LFY; 91	13	A,B,C,E,F, I,J,L,M,N, O	NONE	C(16),E(4),A (13), J(15),F(15)	100% Expression in A,J,F,C

Table 1: Summary of RT-PCR experiments on35S:LFY and 35S:AP1 yellow poplar. LFY expression was detected in all of the 35S:LFY lines tested in cell culture and regenerated plants. AP1 expression was detected in 80% of cell cultures and 50% of regenerated plants. Letters designate uniquely transformed cell lines.