PROMOTERS AND REGULATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) OXIDASE GENES FROM LOBLOLLY PINE (*PINUS TAEDA* L.)

by

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(Under the Direction of JEFFREY F. D. DEAN)

ABSTRACT

This dissertation describes investigations into the gene structure and regulation of 1-aminocyclopropane-1-carboxylate (ACC) oxidase in loblolly pine (*Pinus taeda* L.). A cDNA clone, PtACO1, and its genomic sequence were isolated and characterized. The gene contained four exons separated by three introns. Quantitative PCR analyses showed that the PtACO1 transcript was expressed in a regulated fashion in various tissues of loblolly pine. Transcription of the gene responded to bending stress and IAA treatments. To study regulation of the ACC oxidase gene in more detail, the promoter region, upstream of the gene was isolated from loblolly pine genomic DNA. The promoter, designated pACO1, was recovered by PCR amplification along with a second promoter, designated pACO2, from another ACC oxidase gene. Using promoter-reporter gene constructs in transgenic *Arabidopsis* plants, these two promoters were shown to be differentially expressed in various *Arabidopsis* tissues. Both promoters were strongly expressed in rapidly dividing and expanding cells in several tissues, and particularly in roots. Subjecting the transgenic plants to a variety stresses and perturbations, it was found that the pACO1 promoter responded to fungal infection, acute bending stress and IAA treatment. The pACO2 promoter was upregulated by IAA treatment, and responded in an

asymmetric fashion during gravitropic response of stems. It was concluded that the ACC oxidases from loblolly pine from which these promoters were cloned were differentially expressed and could be up-regulated in response to bending stresses and hormone treatments. The potential relationships between bending stress, gravitropism, ethylene biosynthesis and auxin with respect to compression wood formation in conifers were discussed in light of these results. In addition to experimental work with the loblolly pine ACC oxidase, an extensive computational analysis was completed to place the pine gene within a detailed phylogenetic tree generated for the 2-oxoglutarate-dependent dioxygenase (2-ODD) genes identified in the completed *Arabidopsis*, rice and poplar genomes.

INDEX WORDS: Ethylene biosynthesis, 1-Aminocyclopropane-1-carboxylate (ACC) oxidase, gene promoter, bending stress, auxin, 2-oxoglutarate-depedent-dioxygenase.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Ethylene gas was first recognized as a plant growth regulator (PGR), or phytohormone, at the beginning of last century when Dimitri Neljubov outlined the classic "triple response" in etiolated pea (*Pisum sativum*) hypocotyls. The triple response is defined by increased radial growth, decreased hypocotyls elongation, and altered gravitropic response or epinasty in plants exposed to ethylene, and has been shown to be a reliable marker for ethylene sensitivity in plants (Guzmán and Ecker, 1990). The effect that ethylene has on plants has been extensively studied and many aspects of plant growth and development, as well as plant responses to environmental stress, have been related to this unusual gaseous hormone.

I. Ethylene and plant development

As a plant hormone, ethylene is involved in the many aspects of plant development, especially the initiation and termination of many physiological processes (Pratt and Goeschl, 1961). To explain the mechanisms by which ethylene regulates plant development, two systems have been proposed (for review, see Lelievre et al., 1997). System 1 is responsible for production of basal levels of ethylene present in vegetative tissues, as well as preclimateric and non-climateric fruits, while System 2 provides for the high levels of ethylene production associated with ripening of fruits and flowers (Oetiker and Yang, 1995). Brief outlines of the effects that ethylene has on the different plant tissues and developmental processes follow.

Seed germination

The process can be positively or negatively influenced by ethylene (reviewed in Esashi, 1988; Kepczynski and Kepczynska, 1997). As either an endogenous or exogenous agent, ethylene can stimulate seed germination in many plant species, including cocklebur (*Xanthium strumarium* L.) (Katoh and Esashi, 1975), lambsquarters (*Chenopodium album* L.) (Karssen, 1976), red root pigweed (*Amaranthus retroflexus* L.) (Schonbeck and Egley, 1980), cotton

(*Gossypium hirsutum* L.), maize (*Zea mays* L.), broad bean (*Vicia faba*) (Afifi et al., 1977), various broad-leaf weeds (Olatoye and Mall, 1973; Taylorson, 1979), and *Arabidopsis thaliana* (Kepczynski and Kepxzynska, 1997). The gas may act by stimulating germination of non-dormant seeds, for example in pea, or by breaking dormancy in seeds that exhibit embryo dormancy, for example in tobacco (*Nicotiana tabacum* L.) (Ketring and Morgan, 1969; Egley and Dale 1970; Whitehead and Nelson, 1992; Sutcliffe and Whitehead, 1995). It is thought that ethylene may cause these changes in seed dormancy by counteracting another phytohormone, abscisic acid (ABA) (Beaudoin et al., 2000).

The molecular basis for ethylene-regulated gene induction during seed germination is still unclear, but among the genes demonstrated to be induced by ethylene during seed germination are a cysteine proteinase in chickpea (*Cicer arietinum* L.) (Cervantes et al., 1994), a class-I β-1, 3-glucanase in tobacco and pea (Leubner-Metzer et al., 1998; Petruzzelli et al., 1999), three members of the gene family encoding putative ethylene receptors (ETRs) in tomato (*Solanum lycopersicum*) (Lashbrook et al., 1998), the gene encoding the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in white lupin (*Lupinus albus*) (Bekman et al., 2000), and the gene encoding the last step in the ethylene biosynthetic pathway, ACC oxidase (Rodriguez-Gacio and Matilla, 2001; Petruzzelli et al., 2000). Other PGRs, such as gibberellin (GA) and jasmonic acid (JA) also take part in regulating gene expression during seed germination (Staswick et al., 1992; Ritchie and Gilroy, 1998), and differential gene expression in tissues such as seeds and leaves appears to be controlled by the complex interactions between regulatory networks of multiple PGRs (Wu and Bradford, 2003). Down-regulation of the ethylene-signaling pathway by glucose signaling through hexokinase (HXK) and the GIN1 (Glucose-Insensitive)

gene product has been shown to stimulate germination, as well as cotyledon and leaf development (Zhou et al., 1998).

Leaf morphology

Ethylene is an important regulator of leaf ontogeny in higher plants (Osborne, 1991). Ethylene evolution from the leaf apex has been reported in several plant species (for review, see Osborne, 1991) and its primary role in developing leaves of dicotyledonous plants appears to limit cell expansion in younger leaves (Osborne, 1991; Kieber et al., 1993; Lee and Reid, 1997). Ethylene similarly inhibits cell elongation, and affects cell size and shape in etiolated seedlings (Ortuño et al., 1991; Sánchez-Bravo et al., 1992). Ethylene has also shown to induce the differentiation of its own target cell class in bean (*Vigna radiata* L.) (McManus et al., 1998). Ethylene produced at distinct leaf developmental stages in white clover (*Trifolium repens*) induces separate responses, from modulation of leaf growth in the apex to controlling senescence in mature leaves (Hunter et al., 1999). Exogenously applied ethylene can stunt axial growth, and at the cellular level this is correlated with a switch in microtubule alignment with respect to the long axis of the cell, which allows lateral bulging rather than axial elongation (Lloyd, 1994). Ethylene is also involved in meristem identity and leads to a significant reduction in the number of cells in the shoot apical meristem (Hamant et al., 2002).

Stem elongation

Classically, ethylene is considered an inhibitor of growth because of its ability to reduce cell elongation (Abeles et al., 1992; Smalle and Van Der Straeten, 1997). However, under certain conditions, ethylene can stimulate growth in specific tissue types in a variety of different species. For example, in darkness, ethylene typically inhibits hypocotyl elongation, but upon exposure to light, ethylene appears to promote hypocotyl elongation in some cases (Smalle and Van Der

Straeten, 1997). Ethylene formation in submerged stems is a prerequisite for the growth response in deepwater rice (*Oryza sativa* L.) and *Rumex palustris* where stem and petiole elongation is stimulate by ethylene under flooded conditions (Kende et al., 1998; Voesenek and Blom, 1999). The following chain of events appears to lead from submergence to accelerated internodal growth: 1) the level of O_2 inside submerged stems decreases (Raskin and Kende, 1984); 2) submergence and reduced levels of O_2 induce ethylene synthesis (Métraux and Kende, 1983); and 3) ethylene enhances growth indirectly by increasing the activity of endogenous gibberellin (Raskin and Kende, 1984). Some terrestrial plants also show evidence of a positive growth response when exposed to ethylene, although it has been suggested that ethylene may induce these responses within minutes and that they are generally short-term in nature (Sisler, 1990).

On a molecular basis, partial control of stem elongation plasticity in *Stellaria longipes* by ethylene (Emery et al., 1994) is attributed to the differential regulation of members of the ACC synthase gene family (Kathiresan et al., 1998). In etiolated hypocotyls of pea (*Pisum sativum*), where rapid lateral cell expansion and hypocotyl swelling are rapidly induced by ethylene, *Cel4* mRNA levels are doubled, suggesting that accumulation of these transcripts may be promoted directly or indirectly by ethylene (Brummell et al., 1997). Some studies also suggest that ethylene may regulate hypocotyl growth in *Arabidopsis* by interacting with responses to cytokinin, another PGR (Cary et al., 1995; Su and Howell, 1995).

Root architecture

Ethylene affects lateral root development, root hair initiation, and overall root elongation (Lynch and Brown, 1997; Dolan, 2001; Ma et al., 2001). Plants growing in sealed plates developed increased root hair densities, radial root expansion, decreased root elongation, reduced leaf expansion, and cotyledon epinasty, all phenomena associated with ethylene exposure

(Baskin and Williamson, 1992; Kieber et al., 1993; Masucci and Schiefelbein, 1994; Tanimoto et al., 1995; Smalle and van der Straeten, 1997; Pitts et al., 1998). After cell specification, ethylene and auxin intact to regulate root hair morphogenesis as supported by the following observations: 1) treatment of seedlings with ethylene or the ethylene precursor, ACC, triggers development of root hairs and blocking either ethylene biosynthesis or perception causes a reduction in the frequency of root hairs (Masucci and Schiefelbein, 1994; Tanimoto et al., 1995); 2) the root hair initiation mutants, *axr2* (Wilson et al., 1990), *axr3* (Leyser et al., 1996), and *ctr1* (Kieber et al., 1993), exhibit altered responses to auxin and ethylene; 3) the root hair initiation defective mutant, *rhd6*, can be suppressed by application of ACC or an auxin, indole-3-acetic acid (IAA) (Masucci and Schiefelbein, 1994); 4) mutants with altered responses to ethylene and auxin may also show defects in root hair length (Reed et al., 1993; Okada and Shimura, 1994; Pitts et al., 1998). Ethylene has different effects on the initiation and elongation of root hairs, leading to the proposition that the two responses have different sensitivities to ethylene. Thus, root hair initiation may require a higher level of ethylene than does the root hair elongation. Genetic studies indicate that different sets of gene products are critical for controlling root hair initiation and root hair elongation (Parker et al., 2000; Schiefelbein, 2000), which implies that the two ethylene-dependent responses in a single root hair cell result from the activation of different genetic pathways (Cho and Cosgrove, 2002).

Dormancy

Ethylene has been shown to release dormancy in various plant tissues (Esashi and Leopeld, 1969; Ketring and Morgan, 1969; Olatoye and Hall, 1973), and examples where ethylene has been observed to release of seed dormancy were mentioned above under “seed germination”. The involvement of ethylene in potato tuber dormancy regulation was first reported by Rosa

(1925). Depending on the concentration and duration of exposure, exogenous ethylene can either hasten or delay tuber sprouting (Rylski et al., 1974). Temporary treatment with exogenous ethylene was reported to stimulate the sprouting of partially dormant tubers (Alam et al., 1994), while endogenous ethylene has been shown to play an essential role in regulating potato microtuber dormancy (Suttle, 1998).

Abscission

The role of ethylene in leaf abscission has been well established since the early 1970s (Jackson and Osborne, 1970; Morgan and Durham, 1973; Poovaiah and Rasmussen, 1973; Poovaiah and Leopold, 1973), and ethylene is regarded as an endogenous regulator of abscission in most plants (Jackson and Osborne, 1970; Nooden and Leopold, 1978; Addicott, 1982; Morgan, 1984). The effect of ethylene on abscission has been extensively studied because the rate and extent of abscission has a major impact on crop productivity and value. The premature abscission of developing reproductive organs (flowers and/or fruits) can greatly reduce the overall yield of a crop. Conversely, abscission of mature weed seeds or fruits aids in both the dispersal of that weed and its ability to re-establish itself in subsequent seasons (Suttle, 1985).

The abscission process initiated by ethylene involves cell separation and cell wall breakdown in the abscission zone tissue. To accomplish this, ethylene induces the production of hydrolytic enzymes required for cell separation by regulating RNA and protein synthesis (Horton and Osborne, 1967; Morrė, 1968; Abeles, 1968; Abeles, 1969). Ethylene also appears to regulate the secretion of enzymes, such as pectin methylesterase, into the cell wall (Abeles et al., 1971). Horton and Osborne (1967) reported that cellulase activity was localized in the separation layer of bean explants abscission zones and that ethylene could increase levels of cellulase activity. Ethylene appears to lower the impact of auxin in the abscission zone by changing its synthesis,

transport, destruction, and/or conjugation, and this appears to make the abscission zone cells more susceptible to separation events (Beyer and Morgan, 1971; Morgan, 1976). The idea that ethylene regulates abscission in part through its ability to inhibit auxin transport was strengthened by the following observations: 1) ethylene-mediated auxin transport inhibition and abscission are both reversible processes; 2) ethylene-mediated auxin transport inhibition and abscission exhibit a similar sensitivity to temperature; and 3) the effectiveness of ethylene in both processes is significantly reduced by auxin pretreatment (Beyer, 1973). The physiological balance between auxins and ethylene has been shown to regulate the timing and the extent of leaf abscission (Jackson and Osborne, 1970; Morgan, 1984).

Although most of these studies have focused on ethylene, considering the gaseous nature of ethylene, it has also been suggested that the primary abscission-inducing factor may, in fact, be ACC, the immediate precursor of ethylene. ACC transport from roots to shoots was shown to play an important role in controlling water stress-induced leaf abscission in Cleopatra mandarin (*Citrus reshni*) seedlings (Tudela and Primo-Millo, 1992). The mechanism implies that roots have an important role in regulating the water relations of plants because abscission of leaves reduces the transpiring area (Kozlowsky, 1973; Addicott, 1982).

Floral development and pollination

Although some studies have suggested that ethylene can play a role in the transition of vegetative meristem identity to floral meristem identity (Galston and Sawhney, 1990), the best studied impacts of ethylene on floral processes occur after flowering. Pollination initiates fruit development, as well as the senescence of floral organs that become obsolete after pollination has occurred. In many flowers, the initial response to pollination is an early increase in ethylene production by the stigma, and this is often followed by increased ethylene production from

ovaries and petals. Pollination-induced ethylene produced by different floral organs is responsible for coordinating a variety of pollination-associated events, such as floral color changes, ovary growth, senescence of the perianth, and ovule development (Stead, 1992; for review, see Larson et al., 1993; Woltering et al., 1994). Ethylene has also been shown to play a regulatory role in post-pollination developmental events (Larson et al., 1993; O'Neill et al., 1993; Zhang and O'Neill, 1993; O'Neill, 1997; O'Neill and Nadeau, 1997). A small amount of ethylene, together with auxin, regulates ovary growth and ovule differentiation, but high levels of ethylene result in cell death in the ovary (Zhang and O'Neill, 1993). Ethylene appears to play an important role in regulating the yellowing of sepals after harvest, since chlorophyll loss is associated with an increase in floret ethylene synthesis (Tian et al., 1994). Ethylene also plays a role in the wilting of petunia petals (Whitehead et al., 1984; Woltering et al., 1997) and ovule development in tobacco (De Martinis and Mariani, 1999). Ethylene is also an obvious candidate as controller of senescence timing in most flowers (Leshem et al., 1986; Borochov and Woodson, 1989).

Fruit ripening

The impact of ethylene on fruit ripening is perhaps the most studied of all the effects this PGR has on plants (Hansen, 1943; Kidd and West, 1945;Olson et al., 1991; Yip et al., 1992; Lincoln et al., 1993; Klee, 2004; and as reviewed in Alexander and Grierson 2002). Ethylene accelerates the ripening of many fruits, including tomatoes, and is a normal metabolite in ripening of most fruits (Hartman, 1959). Denny (1924) was perhaps the first to demonstrate this, showing that ethylene increased the respiratory activity, as well as hastened the decomposition of chlorophyll, in the rind of mature green lemons, and this was coupled with accelerated onset of decay.

Besides the processes mentioned above, there is evidence for ethylene involvement in many other aspects of plant growth and development. For example, ethylene can induce swellings of mesocotyl in some seedlings (Camp and Wickliff, 1981), and it has a regulatory role in some types of programmed cell death (Young and Gallie, 2000). Ethylene has a negative effect on nodule formation in leguminous species (e.g., Grobbelaar et al., 1971; Goodlass and Smith, 1979; Lee and LaRue, 1992). Ethylene is not only a negative regulator of Nod factor signaling in root epidermis, but local production of ethylene controls where in the cortex nodule primordia are formed (Geurts and Bisseling, 2002). Hook formation and epinasty are two examples of differential growth where ethylene is considered to have major role in regulation (Abeles et al., 1992; Peck et al., 1998). Ethylene also appears to regulate cucumber (*Cucumis sativus* L.) floral sex determination by influencing two different ethylene receptors independently (Yin and Quinn, 1995).

II. Ethylene and plant stress responses

Ethylene biosynthesis is increased in response to stimuli such as wounding, pathogen attack, hypoxia, and water deficit (for review, see Abeles et al., 1992; Morgan and Drew, 1997). Thus, ethylene is regarded as an important stress hormone. In general, increased ethylene biosynthesis slows down plant growth until the stress is removed. At the level of gene expression, ethylene induces transcription of many genes in response to a multitude of environmental and developmental stimuli (Klee, 2004). Responses to three such stimuli are briefly recounted here.

Hypoxia

Endogenous ethylene levels rise greatly in plants experiencing hypoxia (Atwell et al., 1988; He et al., 1996; Drew, 1997). In rice, ethylene production in response to hypoxia is increased through the induction of ACC synthase (S-adenosyl-L-methionine methythioadenosine-lyase),

the enzyme usually considered to catalyze the key regulatory step in the ethylene biosynthetic pathway (Yang and Hoffman, 1984; Cohen and Kende, 1987; Kende, 1987). The increased ethylene production is thought to cause the rapid internodal elongation that raises rice stems above flood waters (Kende, 1987). Transcript levels of *Arabidopsis* genes involved in ethylene biosynthesis and perception, as well as target genes, significantly increase in response to hypoxia (Liu et al., 2005). Through the induction of alcohol dehydrogenase (ADH) during later stages of hypoxia, ethylene can promote long-term structural adaptation, such as formation of aerenchyma and adventitious roots, in certain species (He et al., 1996; Lorbiecke and Sauter, 1999; Drew et al., 2000; Peng et al., 2001). Drew et al. (2000) proposed a signal transduction pathway for hypoxia responses in which hypoxia stimulates ethylene production, which in turn activates a signal transduction cascade involving phosphoinositides and Ca^{2+}. More recently, increases in ethylene formation during hypoxia was connected to the haemoglobin/nitric oxide cycle (Manac'h-Little et al., 2005) where NO seems to have a stimulating effect on ethylene biosynthesis (Dordas et al., 2003a, 2004; Igamberdiev et al., 2004). This could indicate participation of haemoglobin in an acclimation response to low oxygen levels where it regulates levels of NO and ethylene, delaying apoptosis and aerenchyma formation (Igamberdiev et al., 2005). From studies of the differential expression of ACC synthases (ACSs) in Arabidopsis, Peng et al. (2005) proposed that at least two signaling pathways were triggered during hypoxia in this species. One pathway acts through AAR1 and AAR2 genes and leads to the activation of ADH and ACS9. The other pathway leads to the activation of the ACS2, ACS6, and ACS7 genes. Activation of these genes leads to increased ethylene productions in both roots and shoots. This increased ethylene contributes to further activation of both ADH and ACS9, which results in the inhibition of ACS2 gene expression in roots.

Pathogen attack

Ethylene is produced during most if not all host-pathogen interactions in plants (Paradies et al., 1980; Boller, 1991; Avni et al., 1994), yet the role of ethylene in pathogen defense is not completely clear. Depending on the type of pathogen and host plant species, ethylene can have opposing effects on disease progresssion. Experiments utilizing exogenous application of ethylene and genetic analyses using various ethylene-insensitive mutants found ethylene to enhance pathogen resistance in some case, while decreasing it in others (El Kazzaz et al., 1983a; El-Kazzaz et al., 1983b). The ethylene-insensitive Arabidopsis mutant *ein2-1* showed increased susceptibility to the necrotrophic fungal pathogen *Botrytis cinerea* (Thomma et al., 1999), as well as the bacterial leaf pathogen *Erwinia carotovora* pv. Carotovora (Norman-Setterblad et al., 2000). Thus, while ethylene produced after pathogen infection may provide an important defensive signal in certain plant-pathogen interactions, and it may be detrimental in others.

Genes activated by ethylene in response to pathogen attack are possibly the most extensively studied among the different classes of ethylene-responsive genes. This class includes basic chitinase, β-1, 3-glutanases, defensins, and a variety of other classic pathogenesis-related protein genes (Ohme-Takagi and Shinshi, 1995; Penninckx et al., 1996). Exogenously supplied ethylene has been found to enhance the accumulation of proteins associated with defense reactions (Chalutz, 1973; Liang et al., 1989), and ethylene has been shown to induce basic PR gene expression in tobacco (Ohme-Takagi and Shinshi, 1995). Basic PRs are intracellular proteins associated with viral, fungal, and bacterial infections and are strongly elicited by ethylene (Kitajima and Sato, 1999).

The signaling pathways of jasmonate (JA), another common PGR, and ethylene can interact with each other to coordinate expression of defense-related PR genes. For example, both the JA

and ethylene pathways work concomitantly to induce the antifungal peptide, PDF1.2, which is active against *A. brassicicola* (Penninckx et al., 1996; Penninckx et al., 1998).

Mechanical stress

Mechanical stress refers to abiotic factors such as wind, rain, vibration, hail, that strain the physical structure of a plant and can, as a consequence, cause acute damage to the plant (wounding). Ethylene biosynthesis is often stimulated by wounding. In most cases, the enhanced ethylene production comes from the induction of ACS activity (Kende, 1993; Watanabe and Sakai, 1998). For example, transcript levels for a particular ACC synthase are strongly induced in tomato fruits by mechanical stress (wounding) (Olson et al., 1991). Wounding can also induce ACC oxidase (Li et al., 1992; Barry et al., 1996).

In *Arabidopsis*, both ethylene after 12 hours of treatment (Fujimoto et al., 2000) and wounding (Delessert et al., 2004) strongly induce the ERF (ethylene-responsive element binding factors) 4 and ERF5 genes. Ethylene also appears to be involved in the wounding-induction of the ZmPox2 gene, which encodes peroxidase in maize (de Obeso et al., 2003). Kim et al. (2003) proposed a model for stress-induced responses in which the perception of increased ethylene production by ethylene receptors triggers downstream response of the SIPK (salicylic acid-induced protein kinase) signaling cascade and activation of ERFs and a subset of defense genes.

As noted with respect to pathogen-induced ethylene responses, ethylene has been suggested to act concomitantly with jasmonates to induce expression of various stress-related genes (Xu et al., 1994; O'Donnell et al., 1996; Penninckx et al., 1998). In tomato, tobacco, melon, and *Arabidopsis*, ethylene and JA have been shown to act together to regulate the expression of defense genes (including proteinase inhibitors, PRs and PDF1.2, respectively) during wounding response or pathogen infection (Xu et al., 1994; O'Donnell et al., 1996; Penninckx et al., 1998).

Similarly, in lima bean leaves, ethylene and JA act synergistically to induce the expression of ethylene biosynthesis genes and PR proteins (Arimura et al., 2002).

Ethylene is also involved in responses to other environmental stimuli, such as drought, cold, gravity, bending, and certain nutrient deficiencies. For instance, ethylene is involved in regulating expression of antifreeze proteins in winter rye leaves in response to cold and drought conditions (Yu et al., 2001). As for the role of ethylene for regulating responses to bending stress, and its impact on vascular tissue development in trees, this is the focus of this dissertation, and will be discussed separately.

III. Ethylene biosynthesis genes

The ethylene biosynthesis pathway in higher plants is now well established (for review, see Bleecker and Kende, 2000). The amino acid, methionine, is the metabolic precursor for ethylene, and the first step towards ethylene is the conversion of methionine to S-adenosylmethionine (SAM/AdoMet), a common carbon metabolism (C1) donor in eukaryotic cells, by the enzyme, SAM synthetase (Schaller and Kieber, 2002). In the first committed step in the ethylene biosynthetic pathway, SAM is converted to ACC by ACC synthase, and this is the rate-limiting step in the ethylene biosynthesis pathway (Sato and Theologis, 1989). The final step in the pathway is the conversion of ACC to ethylene through the activity of ACC oxidase (Zarembinski and Theologis, 1993; Schaller and Kieber, 2002; Wang et al., 2002). The general pathway is shown in Figure 1.1.

SAM synthetase

SAM (S-adenosyl-L-methionine) synthetase (EC 2.5.1.6) catalyzes the nucleophilic substitution reaction between methionine and ATP to yield S-adenosylmethionine (Adomet), which was first described by Cantoni in 1953. SAM is an intermediate for a large number of

biological processes. It acts as a methyl group donor in numerous highly specific transmethylations involving various kinds of acceptor macromolecules, including proteins, lipids, polysaccharides, and nucleic acids (Peleman et al., 1989b). In plants, SAM functions as a precursor in the biosynthesis of ethylene (Yang and Hoffman, 1984) and also serves, after decarboxylation, as a propylamine group donor for the polyamines (Goodwin and Mercer, 1983). SAM synthetase is a cytosolic enzyme (Koshiishi et al., 2001). It has been proposed to play a role during drought-stress-induced betaine biosynthesis (Hanson et al., 1995) and may participate in reactions that enhance the ability of seedlings to survive prolonged drought stress (Mayne et al., 1996). Recent studies in poplar have shown SAM synthetase to be highly abundant in vascular fibers at late stages of development, when the cells are undergoing apoptosis, as well as in cambial and tension wood-forming tissues (Moreau et al., 2005). This suggests that this enzyme is an important metabolite for processes in vascular tissues experiencing these particular developmental processes. Genomic and cDNA clones for SAM synthetase genes have been isolated from a wide variety of plant species, including *Arabidopsis thaliana* (Peleman et al., 1989), carnation (Woodson et al., 1992), petunia (Izhaki et al., 1995), rice (Van Breusegem et al., 1994), tomato (Espareto et al., 1994), parsley (Kawalleck et al., 1992), poplar (Schröder et al., 1997), and *Pinus concorta* (Lindroth et al., 2001). Several SAM synthetase homologs have been found in the EST database of white spruce (Pavy et al., 2005) and loblolly pine (http://ccgb.umn.edu/biodata).

The first crystal structure for a SAM synthetase was resolved in 1996 (Takusagawa et al., 1996). All known SAM synthetases contain a conserved GHPD amino acid motif containing the main catalytic residue (His-14 in the *E. coli* enzyme) (Graham et al., 2000), and the mechanism of reaction is thought to rely on the conserved His-14 residue bonding to ATP. Simultaneously, a

change in the ribose ring conformation from C4'-*exo* to C3'-*endo* occurs, which allows the S-atom of Met to make a nucleophilic attack on the C5'-carbon' of ribose to form SAM (Komoto et al., 2004). The tertiary folding pattern of SAM synthetase is unusual, and possibly unique; with each polypeptide chain carrying subdomains that assume a β-α-β-β-α-β secondary structure module that folds up into a wedge-like shape. A polypeptide contains three tandemly repeated modules, so that the complete SAM synthetase protein folds to look like a three-slice cream pie with a topping of β-sheets. The active, dimeric form of the enzyme then appears to consist of two pies, with β-layers facing each other, and two SAM molecules may be bound between the sheets of this dimer. In the *E. coli* enzyme, each monomer contributes important amino acid residues for SAM binding (Komoto et al., 2004). In particular, residues Asp163, Arg229, Phe230 on one subunit and Ser99 on the other subunit bind adenosine directly, while additional amino acid residues on both subunits interact with adenosine via water-mediated hydrogen bonding network. Methionine is bound directly by Gln98 and Asp238 residues on one subunit and the Glu55 residue on the other, and likewise participates in additional water-mediated hydrogen bonding networks (Komoto et al., 2004).

SAM synthetases share considerable sequence similarity throughout all phylogenetic kingdoms. Based on their degree of amino acid sequence similarity, they have been grouped into two different monophyletic groups (Kotb and Geller, 1993; Schröder et al., 1997). In plant species studied to date, genes encoding SAM synthetase have been reported to belong to small gene families whose members share highly conserved sequence within species (Peleman et al., 1989; Espartero et al., 1994; Schröder et al., 1997; Shen et al., 2002; Sanchez-Aguayo et al., 2004). In *Arabidopsis*, four SAM synthetase genes have been identified, while three sequences encoding complete proteins, as well as two sequences encoding truncated proteins have been

found in rice (http://www.gramene.org). Two SAM synthetase genes have been described from *Pinus contorta* (Lindroth et al., 2001), but seven distinct SAM synthetase genes were identified through clustering of sequences from the white spruce EST database analysis (Pavy et al., 2005). The large collection of EST sequences available for *Pinus taeda* contains at least 16 consensus sequences for SAM synthetases (http://ccgb.umn.edu/biodata), which suggests that these enzymes may actually comprise a relatively large gene family in conifers and other gymnosperm species (Pavy et al., 2005).

Because SAM is an essential substance for living cells as methyl group donor and as a precursor of ethylene, polyamines and nicotianamine, SAM synthetase genes are considered housekeeping genes (Tabor and Tabor 1984, Moffatt and Weretilnyk, 2001). Many housekeeping genes are more or less constitutively expressed, but in several different systems levels of SAM synthetase transcripts have been shown to respond to developmental and external conditions. For example, expression of two SAM synthetase genes in pea (*Pisum sativum*) is developmentally regulated (Gómez- Gómez and Carrasco, 1996). In *Arabidopsis*, transcription of SAM synthetase is regulated in a tissue-specific pattern and by stage of development (Peleman et al., 1989). A SAM synthetase (SAM1) gene of *A. thaliana* is expressed primarily in the vascular tissue (Peleman et al., 1989a), and the level of expression of this gene in stems and roots is 10 to 20 times higher than in leaves or flowers (Peleman et al., 1989b). Expression analyses of *Arabidopsis* SAM synthetase gene promoters driving β-glucuronidase reporter gene expression suggested the enzymes to be most highly expressed in lignifying tissues (Peleman et al., 1989a; Peleman et al., 1989b). In rice, a similar pattern and degree of expression has been found in leaves and roots for the SAM synthetase, *sam* (van Breusegem et al., 1994). Differences between

these two species in expression levels for SAM synthetases might, to some extent, reflect proportional difference in the amount of lignified vascular tissue in their organs.

Reports also suggest that SAM synthetases are also involved in root formation in some important way, and there might be a regulated, highly conserved expression pattern of SAM synthetase expression in root formation. PtSAMS1 gene, which encodes a S-adenosylmethionine synthetase in *P.contorta*, was preferentially expressed in roots and exhibited a specific expression pattern in the meristem at the onset of adventitious root development (Lindroth et al., 2001).

SAM synthetases also respond to a variety of external stimuli. For example, SAM synthetase transcript levels were shown to be regulated by fungal elicitors (Gowri et al., 1991; Kawalleck et al., 1992), drought stress (van Breusegem et al., 1994), salt stress (Espartero et al., 1994; Schröder et al., 1997), and ethylene (Gómez- Gómez and Carrasco, 1996; Whittaker et al., 1997). Lima bean SAM synthetase expression was strongly induced in leaves (detached and intact) in response to artificial wounding, infection by *T. urticae,* or any one of seven major volatiles released during *T. urticae* infestation (Arimura et al., 2002).

Overexpression and suppression of SAM synthetase genes can cause abnormal phenotypes in transgenic tobacco (Boerjan et al., 1994). It has been proposed that induction of SAM synthetase under stress conditions is required to meet a demand for increased lignin biosynthesis, or for the modification of other cell wall constituents, such as isoflavonoids and proteins (Whittaker et al., 1997). Espartero et al. (1994) discussed the involvement of SAM in relation to cell wall biosynthesis and modification. including induced lignification. In elicitor-treated alfalfa cells, SAM synthetase is co-induced with caffeic acid 3-O-methyltransferase (COMT), which catalyzes a key enzymatic step in lignin biosynthesis. The expression pattern of SAM synthetase

is similar to that of COMT at various developmental stages in different organs of alfalfa (Gowri et al., 1991).

ACC synthase

ACC (1-aminocyclopropane-1-carboxylate) synthase (EC 4.4.1.14) catalyzes the conversion of S-adenosyl-L-methionine to ACC (Fig. 1.1). This process requires pyridoxal 5'-phosphate (PLP) as an essential cofactor to facilitate the deprotonation of the C-1 carbon, which induces the intramolecular, nucleophilic displacement of methylthioadenosine (MTA) and release of ACC. The MTA enters a separate cycle to be recycled to methionine. ACC synthase is of low abundance enzyme of high lability, and it has been estimated that the level of ACC synthase in ripening tomato pericarp tissue constitutes less than 0.0001% of the total soluble protein (Bleecker et al., 1996).

ACC synthase was first purified from tomato pericarp tissue (Bleecker et al. 1986). That enzyme had a molecular mass of 50kD as determined by SDS-PAGE, and its crystal structure was solved by Capitani et al. (1999). As a member of α-family of pyridoxal-5'-phosphate (PLP) dependent enzymes, it shares moderate sequence similarity with aspartate aminotransferase (AATase) and tyrosine aminotransferase (TATase) (Christen and Metzler, 1985; Alexander et al., 1994). ACC synthase also shares remarkable similarity in the secondary structure with AATase (Ford et al., 1980). In its active form, ACC synthase is a homodimeric protein (White et al., 1994). Each monomer has two distinct domains, similar to AATase, and the active site is localized in a cleft between the subunit domains and along the monomer face involved in dimerization (Capitani et al., 1999). The larger subunit domain is composed of a central sheet of seven β-strands connected by 9 α-helices that pack on both sides of the central sheet (Mcphalen et al., 1992a). The smaller domain consists of a 4-stranded antiparallel sheet and 2-stranded

parallel sheet with five α-helices coordinated around them (Capitani et al. 1999). Sequence alignment demonstrated that ACC synthases share 11 invariant residues with AATases, including four conserved residues present in all aminotransferases (Huang et al., 1991). The principal structural differences between ACC synthases and AATases are the presence of an additional helix (helix-4) in AATases and the presence of an additional helix (helix-2') in ACC synthases (Capitani et al. 1999). To date, four crystal structures for apple (*Malus domestica*) ACC synthase (Capitani et al., 1999; Capitani et al., 2002; Capitani et al., 2003; Capitani et al., 2005) and one of tomato (*Lycopersicum esculentus*) ACC synthase have been solved (Huai et al., 2001). Based on the tomato crystal structure, a catalytic mechanism was proposed (Huai et al., 2001). According to the model, Arg412 holds SAM near the internal aldimine of the PLP cofactor while Tyr240 removes a proton from Lys278 to form an external aldimine. Tyr152 then breaks the C-γ-S bond of SAM via nucleophilic attack and, while Arg157 and Arg412 hold SAM in the correct conformation, MTA is released. A covalent intermediate is presumed to form between Tyr152 and the C-γ of SAM, which is then converted to ACC-aldimine. Finally, Lys278 attacks the C4' of PLP to release ACC. Except the γ elimination in step 3, which appears to be unique to the ACC synthase enzyme, this mechanism is similar to those proposed for other PLP enzymes.

Since ACC synthase mediates the rate-limiting step in ethylene biosynthesis (Sato and Theologis, 1989), regulation of ACC synthase genes has been extensively studied. ACC synthases are typically encoded by a highly divergent multigene family, and the various family members are differentially expressed throughout development or in response to such varied stimuli as germination, leaf senescence, flower abscission, fruit ripening, wounding, flooding, exposure to ozone, touch, hormones, and pathogen attack (Biro and Jaffe, 1984; Yang and Hoffman, 1984; Mattoo and Suttle, 1991; Abeles et al., 1992; Nakatsuka et al., 1998; Peck and

Kende, 1998; Moeder et al., 2002; Raghavan et al., 2006). There are 12 ACC synthase genes in *Arabidopsis*, many of which are regulated at the transcriptional level. One *Arabidopsis* ACC synthase, ACS2, is transcribed in young leaves, but transcription is switched off when the leaves mature (Rodrigues-Pousada et al., 1993) Another family member, ACS4, can be induced by auxins (Abel et al., 1995), while ACS5 is regulated by cytokinins, which appear to stabilize the enzyme (Chae et al., 2003). The ACS6 transcript is induced by ozone, wounding, auxins, and ethylene (Vahala et al., 1998; Tian et al., 2002).

ACC synthase gene promoters from a variety of plant species have shown tissue-specific, inducible or developmentally regulated expression patterns (Lincoln et al., 1993; Rodrigues-Pousada et al., 1993; Gil et al., 1994; Abel et al., 1995; Yi et al., 1999; Yoon et al., 1999; Tsuchisaka and Theologis, 2004). Promoters for the known *Arabidopsis* ACC synthase genes paired with various reporter genes demonstrated differing responses to IAA, wounding, and other treatments, suggesting that the different family members serve in different capacities (Tsuchisaka and Theologis, 2004b). Several of the promoters demonstrated overlapping expression patterns. Possible reasons proposed for this large degree of overlap included: 1) heterodimerization of the ACC synthase enzyme serves as a mechanism for regulating the enzyme; or 2) heterodimers previously identified through *E. coli* complementation studies (Tsuchisaka and Theologis, 2004a) actually exist and function in *planta*. Posttranscriptional regulation has been shown to be an important aspect in the control of ACS expression (Nakajima et al., 1990; Spanu et al., 1994; Oetiker et al., 1997; Vogel et al., 1998; Woeste et al., 1999; Chae et al., 2003). Phosphorylation, for example, is involved in the regulation of ACS activity (Spanu et al., 1994; Liang et al., 1996), and phosphorylation at the C-terminus of the tomato ACC synthase, LE-ACS2, was shown to regulate the enzyme (Tatsuki and Mori, 2001).

An interesting observation is that ACC synthase probes have been shown to hybridize to a pair of transcripts in a wide variety of tissues from different plant species, and that the paired transcripts increase in response to different stimuli. Suspension-cultured tomato cells treated with fungal elicitors accumulated two transcripts (1.6 kb and 1.9 kb) that hybridized with a gene-specific probe for Le-ACS2 (Spanu et al., 1993), and during this response, the 1.6-kb transcript appeared first. The Le-ACS2 probe also hybridized with two transcripts in roots from flooded tomato plants (Olson et al., 1995), as well as in wounded tomato fruit (Li et al., 1992). Organ-specific production of paired transcripts has also been reported in carnation flowers where probes for two different ACC synthase genes, Dc-ACS1 (CARACC3) and Dc-ACS2 (CARAS1), each hybridized with two transcripts in ovaries and styles, but hybridized with only one transcript in petals (ten Have and Woltering, 1997). A probe for a winter squash ACC synthase gene, Cm-ACS2, hybridized to two transcripts in auxin-treated hypocotyls (Nakagawa et al., 1991). In pea, a single gene encoding ACC synthase, Ps-ACS1, produces two different transcripts, apparently through usage of alternate promoters (Peck and Kende, 1998). Four ACC synthase cDNA isoforms were found to arise from a single gene in hybrid papaya. This was explained as a case of alternative splicing, and it was proposed that the various gene products functioned to modulate or fine-tune ACC synthase activity relevant to fruit ripening (Hidalgo et al., 2005).

ACC Oxidase

ACC oxidase or ethylene-forming enzyme (EFE) (EC 1.14.11.4) catalyzes the final step in the ethylene biosynthetic pathway. It belongs to the family of 2-oxoglutarate-dependent dioxygenases (2-ODDs) (Prescott, 1993). Like other enzymes in this family, ACC oxidase requires ferrous iron and a reducing agent (usually ascorbate) for activity *in vitro* (Smith, 1992). However, unlike other members in the family, which use 2-oxoglutarate and molecular oxygen

as co-substrates, ACC oxidase does not use 2-oxoglutarate (Prescott, 1993). ACC oxidase is, however, completely dependent on the presence of CO_2 for activity (Dong et al., 1992).

First isolation of this enzyme was achieved through molecular, rather than biochemical methods. Hamilton et al. (1991) identified the function of an ACC oxidase gene by analyzing the effect of an antisense version of the gene on ethylene production. At almost the same time, *Xenipus lavis* oocytes injected with a cDNA cloned from ripening tomato fruit, pTOM13, were shown to contain EFE activity (Spanu et al., 1991). Correct stoichiometry of the reaction,

ACC + O_2 + ascorbate, CO_2, Fe^{2+} →C_2H_4 + CO_2 + HCN + dehydroascorbate + H_2O

was first described by Dong et al. (1992),.

Two models have been proposed for the catalytic mechanism of ACC oxidase. The first model proposed that the ascorbate association with the Fe(II) ion activates a bound O_2 to yield a high-valency iron-*oxo* species that oxidizes ACC to release ethylene (Zhang et al., 1997). More recently, it has been suggested that the role of Fe(II) is to bind ACC and O_2 simultaneously, and then promote electron transfer, which oxidizes ACC to ethylene (Lay et al., 1996; Rocklin et al., 1999; Zhou et al., 2002; Zhang et al., 2004).

Structural homology modeling of the apple ACC oxidase suggests the protein to contain a compact jelly-roll motif consisting of eight α-helices and 12 β-strands, similar in structure to isopenicillin N synthase, which is another member of 2-ODD enzyme superfamily (Seo et al. 2004). A highly conserved, facial triad motif (His-X-Asp-X-His) forms the Fe^{2+}-binding pocket, and the predicted binding site lies in a group of antiparallel β-strands that forms a cleft in the core of the enzyme (Seo et al. 2004). Modeling suggests that the ascorbate cofactor forms hydrogen bonds with the Arg244 and Ser246 residues of the enzyme, as well as with the Fe^{2+} ion (Seo et al. 2004). The conserved residues of the Fe^{2+}-binding motif were located at positions His177, His234, and Asp179, and these residues, along with Val159 and Thr157, form the core

of the active residues identified in the model proposed for the ACC oxidase enzyme (Seo et al. 2004).

Because ACC synthase levels are low in tissues that do not produce significant amounts of ethylene, and because rapid induction of ACC synthase is typically required before ethylene levels rise in response to stimuli, this enzyme is most often considered the rate-limiting step in ethylene biosynthesis and the major regulatory step for the induction of ethylene (Yang and Hoffman, 1984; Kende, 1993). By contrast, ACC oxidase activity is frequently thought to be constitutively present in most vegetative tissues. However, a more active role for ACC oxidase activity in regulating ethylene biosynthesis is becoming more clear. A rise in ACC oxidase activity was shown to precede ACC synthase activity in preclimatic fruit responding to ethylene, suggesting that ACC oxidase plays a regulatory role in ethylene production in these tissues (Liu et al., 1985). Similarly, patterns of differential expression for ACC oxidase transcripts in various tissues and at different developmental stages further suggest regulatory roles for ACC oxidase (Tang et al., 1994; Nakatsuka et al., 1997; Liu et al., 1997; Yu et al., 1998; Kim et al., 1998; Raz and Ecker, 1999; Yang et al., 2003). That said, some ACC oxidases are expressed more or less constitutively (Liu et al. 1997, Kim et al. 1998, Yu et al. 1998).

ACC oxidases are generally encoded by small gene families (Holdsworth et al., 1988), and different family members often show differential regulation in response to a variety of developmental or external stimuli (Prescott and John, 1996). There are, for example, three ACC oxidase genes in tomato, and ACO1 and ACO3 are involved in the senescence of leaves, flowers and fruit. ACO1 also responds to wounding (Barry et al., 1996). Even though the three ACC oxidase genes are all expressed during flower development, each one was shown to have a temporally distinct pattern of accumulation (Barry et al., 1996). ACC oxidase gene promoters

have been isolated from a variety of species, including tomato (Blume and Grierson, 1997), melon (Lasserre et al., 1997), apple (Atkinson et al., 1998), and peach (Moon and Callahan, 2004). The promoter sequences isolated so far have shown a high degree of similarity in nucleotide sequence within the same species, even though the promoters have demonstrable patterns of differential regulation. For example, in melon, two ACC oxidase gene promoters are differentially activated in response to pathogen attack. CM-ACO1 was preferentially induced in response to stress response, such as inoculation with the bacterium, *Ralstonia solanacearum*, while CM-ACO3 expression was associated with senescence (Lasserre et al., 1997).

Information on the subcellular location of ACC oxidase is conflicting. Various reports place the enzyme in the cell wall/apoplast (Rombaldi et al., 1994; Gomez-Jimenez et al., 2001), the external face of the plasma membrane (Ramassamy et al., 1998), the cytosol (Chung et al., 2002), or the cytoplasm and nucleus (Hudgins and Franceschi, 2004). Nuclear localization of ACC oxidase was suggested to be related to regulation of transcription by ethylene (Hudgins and Franceschi, 2004).

In conifers, ACC oxidase activity has been characterized in stems (Plomion et al., 2000) and needles (Ievinsh and Tillberg, 1995). ACC oxidase appears to be constitutively expressed at moderate levels in stems, but wounding and application of exogenous methyljasmonate can induce large increases in levels of this enzyme (Hudgins and Franceschi, 2004). ACC oxidase has also been linked to reaction wood production in gymnosperm and angiosperm trees. An ACC oxidase was found to be up-regulated in compression wood samples from maritime pine (*Pinus pinaster*) (Ploimon et al., 2000), and ACC oxidase transcripts identified by cDNA-AFLP analyses showed clear qualitative differences between compression wood and opposite wood at day 8 and day 20 post bending treatment (Provost et al., 2003). Likewise, ACC oxidase

displayed increased expression on the tension wood side of *Populus tremula* x *P.tremuloides* stems (Andersson-Gunneras et al. 2003). While the latter study found that ACC levels were increased in tension wood (upper side), they were significantly higher in the opposite wood, leading to the postulation that ACC oxidase expression was the determinant for asymmetric ethylene production in gravistimulated *Populus* stems.

IV. Ethylene in conifers

Wood formation in gymnosperms

Wood, one of the most important renewable resources, is formed through the successive addition of secondary xylem tissue from differentiation of the vascular cambium in woody stems. Wood is composed of both non-conducting and conducting elements implicated in the long distance transport of water and nutrients, as well as physical support of the photosynthetic structures, in trees. In conifers, wood is comprised of two main cell types -- tracheids and ray parenchyma. Wood formation, also called xylogenesis, includes a succession of four major steps: cell division, cell expansion, secondary cell wall thickening, and programmed cell death (for reviews, see Lachaud et al., 1999; Plomion et al., 2001). As natural resources go, wood is a highly variable raw material. Genetic factors influence the activity of the vascular cambium and the differentiation of newly divided cells, ultimately influencing the bulk properties of wood, and as a consequence, the properties of materials manufactured from wood (Cornelius, 1984; Zobel and Van Buijtenen, 1989; Pot et al., 2002). Other factors influencing the wood and its properties include aging, seasonal effects, nutritional status, and gravitational effects. So-called juvenile and mature woods are formed from the vascular cambium by trees at different ages. Seasonal effects impact the formation of early-wood and late-wood, as well as the timing for transition from early-wood to late-wood production. Large changes to the orientation of tree stems and

branches stimulate the formation of reaction woods, such as compression wood on the underside of conifer stems and branches.

Compression Wood

Reaction woods are formed in woody species by the displacement of stems and branches by wind or other mechanical stresses. This response is unilateral and enables the tree to resist physical strains in wood, forcing the stem or branch back toward its original orientation in space (Timell, 1986). In gymnosperms, the reaction wood is called compression wood, and it is formed on the lower side of displaced stems. Compression wood is characterized by short, rounded tracheids that have thickened walls with increased lignin content and increased microfibril angles (Timell, 1986). The formation of compression wood is often accompanied by a stimulation of cambial cell division, while cell division on the opposite side of the stem is inhibited. Detailed understanding of the molecular and biochemical factors governing formation of compression wood is still lacking, but the process appears to be related to the gravitropic responses observed in many other plant species. These gravitropic responses typically involve changes in intrinsic growth direction, as well as redistribution of phytohormones, particularly ethylene and auxin (Timell, 1986; Sundberg et al., 1994; Little and Eklund, 1999). Jaffe (1980) suggested the following hypothesis with respect to gravitropic responses in bean plants:

> "Mechanical stimuli trigger a membrane alteration that results in a burst of ethylene biosynthesis. The release of ethylene in some manner blocks the basipetal flow of auxin. Accumulated auxin enhances cambial activity and xylem production and, at the same time, stimulates further ethylene biosynthesis."

This hypothesis accounts for the interaction between ethylene and auxin during mechanical stimuli, which seems to apply to describe the process of compression wood formation.

Ethylene and wood formation

Although wood quality has a genetic component, it is also influenced strongly by the environment (Lee 1999). 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, 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 (Tuomainen et al., 1997). Increased levels of gibberellins result in production of more tracheids of larger size (Eriksson et al., 2000). Under the influence of ethylene, poplar cambial tissues produced more parenchyma, shorter fibres and shorter vessels than untreated controls (Junghans et al., 2004).

Focusing on ethylene, application to conifer stems of ethrel, an ethylene-forming compound, resulted in increased radial growth (Barker, 1979). It was shown in different conifer species that ethylene evolution from stems was significantly higher when the cambium is growing than when it was dormant (Eklund, 1990, 1993b; Ingemarsson et al., 1991b). There was positive correlation between stem diameter in *Pinus taeda* L. and ethylene evolution per unit cambial surface area in seedlings subject to mechanical stress, and a negative correlation between the stem diameter and ethylene evolution per unit fresh weight in unstressed seedlings (Telewski, 1990). By applying IAA and ethrel to stems, Eklund and Little (1995) found that cambial activation was associated with a rise in ethylene evolution. They concluded that, while ethylene did not directly stimulate cambial activity and tracheid production (as did IAA), it could stimulate production of tracheids when elevated levels caused accumulation of IAA. Kalev and Aloni (1999) made longitudinal cuts in the hypocotyls of *Pinus pinea*, and applied ethylene (via ethrel application) to the separated pieces of hypocotyl. They reported 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, and that ethylene induced the conversion of parenchyma cells to tracheids on the cut hypocotyl surface in the absence of auxin. These results led to the postulation that ethylene is involved in formation of rays. Given these conflicted results, the specific role of ethylene in cambial tissue differentiation remains uncertain.

Besides its apparent role in cambial growth, several studies have shown that ethylene can play a major role in the control of xylem differentiation. Thus, ethylene is required for xylem differentiation in *Lactuca* (Miller et al., 1984). However, ethylene-insensitive genotypes of *Arabidopsis* and other plant species appear to develop perfectly normal vascular tissues, which strongly suggests that ethylene is not needed for normal xylem development (Moreau et al., 2005). Ethylene in loblolly pine has been shown to stimulate production of arabinogalactan-proteins, which are thought to be important participants in xylogenesis (No and Loopstra 2000). By inducing the activity of enzymes involved in lignification and by affecting polysaccharide deposition during cell wall formation (for review, see Eklund and Tiltu, 1999), ethylene can play an important role in xylem differentiation. Ethylene is also presumed to be involved in regulation of xylem cell death, and this is supported by the activation of several ethylene-related transcripts in the late-maturing xylem fibers of both *Arabidopsis* and *Populus* (Moreau et al., 2005).

Besides its production in cambial tissues, ethylene is also produced in the transition zone between heartwood and sapwood (Nelson 1978), it has been implicated as a major signal governing the transition between heartwood and sapwood metabolism (Shain and Hillis 1973). Evidence for ethylene serving as a causative agent for heartwood formation was obtained by

Nilsson et al. (2002). In their experiment, holes bored into *Pinus sylvestris* trunks were treated with ethylene, carbon dioxide, or 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, and fresh gas injections were made regularly over six 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 show 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 the ethylene application contained resin acids, pinosylvin, and pinosylvin monomethyl ether, which are classic indicators of heartwood formation in pines (Nilsson et al. 2002). Therefore, it seems clear that ethylene promoted heartwood formation.

Ethylene and Compression wood

Physical stresses, such as bending and shaking, can result in compression wood formation, and these same stresses have been shown to stimulate ethylene production in several conifer species (Telewski and Jaffe, 1986; Telewski, 1990). Ethylene evolution was found to be greatest from the lower sides of tilted stems of *Abies balsamea*, where compression wood formed, than from the upper sides (Little and Eklund, 1999). Savidge et al. (1983) demonstrated elevated levels of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), in compression wood tissues. However, other studies have provided conflicting results about the relationship between ethylene and compression wood formation. For example, ethylene evolution was found to be similar from the upper and lower sides of tilted *P. densiflora* stems (Yamamoto and Kozlowski, 1987a). Application of ethrel, an ethylene-releasing compound, to vertical stems did not induce compression wood formation in a variety of species, including *Pinus taeda* (Telewski et al., 1983), *P. densiflora* (Yamamoto and Kozlowski, 1987a), and *A.*

balsamea (Eklund and Little, 1996). Thus, a well defined role for ethylene in compression wood formation remains to be determined. Recent molecular evidence has again strengthened arguments for a role for ethylene in compression wood formation. An ACC oxidase was found among the up-regulated proteins in compression wood tissues of maritime pine (Plomion et al., 2000), and ACC oxidase transcripts identified by cDNA-AFLP showed quantitative differences between compression wood and opposite wood of maritime pine (Provost et al., 2003). Du et al. (2003) showed that ethylene evolution was significantly increased on the compression wood side of *Metasequoia glyptostroboides* when the trees were tilted at 45^{o} 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). Interactions between ethylene and IAA in the regulation of compression wood formation were demonstrated by Little and Eklund (1999). So the close relationship between accelerated ethylene evolution and compression wood formation may yet turn out to be an auxin-related phenomenon, as IAA and ethylene could regulate each other's activities and levels (Abeles et al., 1992). Ethylene may act to modulate compression wood formation by changing tissue sensitivity to auxin or by interacting with the auxin signal transduction pathways. Ethylene may also act through its own signal transduction networks to alter the expression of various target genes whose products may drive compression wood formation (for review, see Krieber, 1997). So, in the end, the evidence points to a role for ethylene in compression wood formation. More research will be needed to better clarify its mechanism of action.

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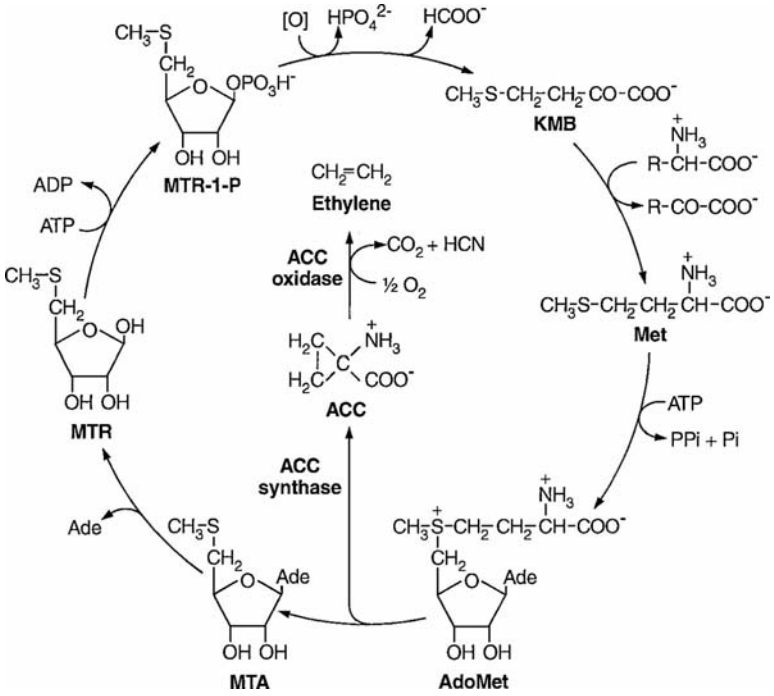
Figure 1.1. The ethylene biosynthetic pathway and the methionine cycle. ACC, 1-aminocyclopropane-1-carboxylic acid; Ade, adenine; AdoMet, S-adenosyl-L-methionine. KMB, 2-keto-4-methylthiobutyric acid; Met, L-methionine; MTA, 5'-methylthioadenosine; MTR, 5'-methylthioribose; MTR-1-P, 5'-methylthioribose-1-phosphate. Adapted from Bleeker and Kende (2000).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Met
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PPi + Pi
NH3
CH3-S-CH2-CH2-CH-COO-
Ade
OH OH
AdoMet
ACC synthase
ACC
H2C
NH3
H2C
COO-
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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF ACC OXIDASE GENES FROM LOBLOLLY PINE (*PINUS TAEDA* L.) [*]

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Abstract

1-Aminocyclopropane-1-carboxylate (ACC) oxidase, a member of the Fe-ascorbate oxidase enzyme family, catalyzes the final reaction in the ethylene biosynthetic pathway, converting ACC into ethylene. A cDNA clone encoding a putative ACC oxidase, PtACO1, was isolated from cDNA library produced using mRNA from the lignifying xylem in trunk wood of loblolly pine (*Pinus taeda*). The cDNA clone comprised an open reading frame of 1461 bp encoding a protein of 333 amino acids. Using PCR amplification techniques, a genomic clone corresponding to the PtACO1 was isolated and its sequence showed three introns with GT/AG boundaries defining the splice junctions. The deduced amino acid sequence of PtACO1 shares 70% identity with a gene from European white birch (*Betula pendula*). Using the cDNA sequence of PtACO1 in a BLAST analysis of pine root ESTs, several homologs were identified. Quantitative PCR showed that the PTACO1 transcript was expressed in a regulated fashion in various pine tissues.

Keywords: ACC oxidase, ethylene biosynthesis, EST.

Introduction

Ethylene, widely known as the ripening hormone, has been linked to such plant growth and development processes as germination, senescence, and abscission of leaves and flowers, as well as fruit development and ripening (Smalle and Van Der Straeten, 1997; Bleeker and Kende, 2000). Although direct evidence for the involvement of ethylene in wood formation is limited, studies clearly show that ethylene can inhibit stem elongation in woody plants, just as it does in many herbaceous species (Morgan and Drew, 1997), and ethylene has been shown to play a role in tree stem responses to mechanical shaking (Telewski, 1990). Given numerous observations correlating ethylene with development of plant form (Dolan, 1997), an examination of the genes that regulate ethylene biosynthesis and study of their expression should provide a better

understanding of whether products of the ethylene biosynthetic pathway are directly involved in wood formation.

Ethylene is synthesized from S-adenosyl methionine via 1-aminocyclopropane-1-carboxylic acid (ACC) by the sequential action of ACC synthase and ACC oxidase (Yang and Hoffman, 1984). Levels of ACC are normally low in vegetative tissues, thereby limiting rates of ethylene production. An increase in ethylene biosynthesis typically requires an initial induction of ACC synthase, the highly regulated enzyme that is generally considered the primary regulator of the pathway (Jackubowicz 2002; Chae and Keiber 2005). This commonly results in a burst of ethylene because of constitutive low-level expression of ACC oxidase in most tissues and positive feedback effects of ethylene on ACC oxidase (Peck and Kende, 1995; Petruzzelli et al., 2000).

ACC oxidase, the actual ethylene-forming enzyme (sometimes referred to in older literature as EFE (Mattoo et al., 1982), converts ACC to ethylene. The enzyme requires Fe^{2+} and CO_2 as cofactors, and uses O_2 and ascorbate as cosubstrates (McGarvey and Christofferson 1992). ACC oxidase is usually encoded by gene families whose members are highly homologous (Barry et al., 1996). ACC oxidase gene sequences reported in GenBank include several isolated from tree species, including peach (*Prunus persica*), apple (*Malus domestica*), pear (*Pyrus pyrifolia*), papaya (*Carica papaya*), mango (*Mangifera indica*), various *Citrus* species, birch (*Betula pendula*), beech (*Fagus sylvatica*), hybrid poplar (*Populus tremula* x *Populus tremuloides*), and white spruce (*Picea glauca*) (Ruperti, et al., 2001, Ross, et al., 1992, Andersson-Gunneras, et al., 2003, Lopez-Gomez, et al., 2004). Although it is well established that ethylene is produced during wood formation (Little and Pharis, 1995), the ethylene biosynthetic genes, ACC synthase and ACC oxidase, have not been cloned and characterized from economically important, wood-

producing tree species, such as loblolly pine (*Pinus taeda*). However, proteomics work has shown that an ACC oxidase protein is upregulated during compression wood formation in maritime pine (*Pinus pinaster*) (Plomion et al., 2000). Among the expressed sequence tag (EST) sequences recovered in a recent loblolly pine gene discovery project (http://www.fungen.org/Projects/Pine/Pine.htm), several putative ACC oxidase genes were recovered from cDNA libraries made using mRNAs from root tissues.

In this report, we describe the cloning and characterization of a full-length ACC oxidase cDNA from lignifying xylem tissues of loblolly pine (*P. taeda*), as well as its comparison with the related EST sequences. Expression profiles for this ACC oxidase gene are also reported for different tissues in pine seedlings.

Materials and Methods

RNA and DNA extraction from pine tissues

Messenger RNA was isolated from lignifying xylem scraped from the trunk of a 15-year old feral loblolly pine as described elsewhere (Lorenz and Dean 2002). A cDNA library was prepared from this mRNA according to the protocol in Lorenz et al. (2006). Pine tissue samples, including roots, candles (the elongating stems and shoot tips at the ends of branches prior to needle expansion), primary flush needles, secondary flush needles, photosynthetic bark, and xylem, collected in the field and flash-frozen in liquid nitrogen, were transported to the laboratory on dry ice for storage at -80°C until use. Tissues were ground using a liquid nitrogen cooled freezer mill to ensure complete breakdown of the tissue prior to extracting RNA by the method of Chang et al. (1993).

Genomic DNA was extracted from suspension-cultured cells of loblolly pine (Line L797-11 provided by Dr. S.A. Merkle) using the modified method described by Lassner et al. (1988).

DNA was purified by repeated extraction (2-4 times) with phenol:chloroform, and the final concentration of DNA was determined by fluorescence measurements of PicoGreen dye binding (Singer et al., 1997).

Gene cloning by polymerase chain reaction (PCR) amplification

Single-stranded cDNA was synthesized using Superscript (II) reverse transcriptase (GibcoBRL, Bethesda, MD). Total RNA (2 μg) from trunk wood lignifying xylem in 10 μl water was incubated at 70°C for 10 minutes with the oligo-dT adapter primer provided in the 3'RACE (rapid amplification of cDNA ends) kit (Roche Applied Science, Indianapolis, IN) and subsequent reaction was performed according to the protocol provided with the kit.

The initial primers for PCR-based cloning were designed using the Oligo-4 software program (National Biosciences Inc., Plymouth, MN) to analyze a 790 bp EST sequence from loblolly pine xylem that was recognized as encoding a partial putative ACC oxidase (GenBank accession AA556710). A degenerate forward primer, ACO58FD, (5'CC(A/C/G/T)GT(A/C/G/T)AT(A/C/T)GA(C/T)ATG(A/G)A(A/G)AA(A/G)3') (see Fig. 2.1 for the detailed location) was designed to match the most conserved stretch of sequence identified by aligning the deduced amino acid sequence from the EST with those of known ACC oxidase genes from other tree species (Fig. 2.2). A pair of nested degenerate reverse primers, ACO342RD (5'(A/G)AA(A/C/G/T)GT(A/C/G/T)(C/G)(A/T)(C/T)TCCCA(A/G)TC3'), and ACO495RD (5'(C/T)TT(A/C/G/T)A(A/G)(A/G)TA(A/C/G/T)CC(C/T)TT(C/T)TC3'), were designed using the same strategy. The reaction mixture (25 μl total volume) contained 2 units Promega Taq DNA polymerase (Promega, Madison, MI), 250μM dNTPs, 2mM Mg^{2+}, 200nM primers, and cDNA synthesized from 100ng RNA. Gradient PCR was used in two rounds of amplification with the nested primers to amplify the target sequence from the cDNA template

pool. The PCR cycling conditions used a single denaturation step of 95°C for 2 min, followed by 35 to 40 cycles of 94°C for 45s, 58° to 62°C gradient ramp over 45s, and 72°C for 1.5 min. Amplification reactions ended with an incubation step at 72°C for 7 min followed by a hold at 4°C. Amplimers were subcloned into the TOPO TA cloning vector following instructions provided by the kit manufacturer (Invitrogen, Carlsbad, CA), and DNA sequencing of fragments was performed using PRISM BigDye reagent kits and an ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA). Multiple independent clones were sequenced in order to minimize errors introduced by the Taq polymerase. DNA sequences were analyzed using the Sequence Analysis package of the GCG software suite (Wisconsin Package, Version 10.1, Genetic Computer Group, Madison, WI), and used to design gene-specific oligonucleotide primers.

The 5' and 3' ends of the cDNA were captured using RACE kits and following the manufacturer's instructions (Roche Applied Science). To amplify the 5' end, the kit AP primer and gene-specific primers, ACO851R (5'ATGCTCTTGCATTTCCCATTGGTCAT3') and ACO168R (5'CGAGAAGAGCGTGAGGTATT3'), were used for nested PCR under the same cycling conditions described above, except that the annealing temperature was fixed at 58°C (no gradient). To amplify the 3' end, gene-specific primers, ACO831F (5'AATGGGAAATGCAAGAGCAT3') and ACO428F (5'ATGAAACTTATGACACAGTG3'), were used with the AP primer, and cycling was performed under the same conditions described for 5' RACE. Amplimers were subcloned into the TOPO TA vector and sequenced as described above. For the full-length cDNA sequence, primers designed at the 5' and 3' UTR, which are ACO42F (5'CTGTTTTTCTGCTTTTCTGT3') and ACO1114R (5'CCAGAACCAGTTGAAAGTGA3') respectively, are used to amplify the cDNA synthesized

from candles using 1xExpand High Fidelity PCR system PCR buffer (without $MgCL_2$), 2mM Mg^{2+}, 0.5mMdNTPs, 280nM primers, 2 units of Expand High Fidelity PCR system (Roche, Indianapolis, IN). The cycling parameters for the amplification reactions were similar to the gradient PCR cycling except the annealing temperature which was 56°C in this reaction. The PCR product were subcloned in to TOPO TA vector and sequenced as described above. The full-length cDNA was designated PtACO1.

For the isolation of genomic DNA encompassing the full-length ACC oxidase transcribed sequence, the same primers designed for the cDNA isolation were used with a hot-start (Kellogg et al., 1994) touchdown PCR reaction (Don et al., 1991). The primers used to amplify the genomic DNA were ACO42F (5'CTGTTTTTCTGCTTTTCTGT3'), ACO342R (5'AAGAAGCCCGTTTCCCAGTC3'), ACO125F (5'GAGGGAAGTGACGATGGCTA3'), ACO775R (5'TTCGACGTCGAACCAAGTGC3'), and ACO1114R (5'CCAGAACCAGTTGAAAGTGA3') in case that a certain primer covers the region where intron inserts. Genomic DNA template (25 ng) was added to a reaction mixture that contained 1xExpand High Fidelity PCR system PCR buffer (without $MgCL_2$), 2mM Mg^{2+}, 0.5mMdNTPs, 280nM primers, 2 units of Expand High Fidelity PCR system (Roche, Indianapolis, IN). The cycling parameters for the amplification reactions were as follows: 1) denaturation stage, 100°C to 87°C over 5 min and hold until polymerase addition; 2) annealing stage, two cycles of 97°C for 1 min, 65°C for 1 min, and 72°C for 2 min before dropping the annealing temperature 1°C every second cycle until the "touchdown" temperature of 55°C was reached, after which 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min were run; 3) extension stage, 5 min at 72°C. The resultant amplimers were subcloned, sequenced, and analyzed as described above.

Multiple sequence alignments and phylogenetic tree generation

A preliminary amino acid sequence alignment of putative loblolly pine ACC oxidases with GenBank sequences for several tree ACC oxidases was generated using PILEUP in the GCG software suite (Genetics Computer Group). A more extensive multiple sequence alignment was generated using the ClustalW module of the Sequence Analysis software package (Genetics Computer Group). 2-Oxoglutarate-dependent dioxygenease (2ODD) sequences in the *Arabidopsis* genome were recovered through a BLAST search using the *Arabidopsis* ACC oxidase gene, At1g62380. Sequences having expected values $\leq 4.5 \times 10^{-7}$ were used along with the putative loblolly pine ACC oxidase sequence in the ClustalW analysis. The resultant output (msf) file was imported into GENEDOC (Nicholas et al., 1997) for manual adjustment and exported in PHY file format. The PHY file was used to run the protein sequence parsimony (ProtPars), the protein maximum likelihood (ProML), and the protein maximum likelihood with molecular clock (ProMLK) executable files in PHYLIP (Felsenstein, 1993). The output files from these programs were further edited in TreeView (Page, 1996) to generate phylogenentic trees. ACC oxidases from different species were collected from http://www.ncbi.nih.gov using "ACC oxidase" to search the protein database. The 158 protein sequences, together with PtACO1 and RTNACL1_6_A03, were used to run clustalW at http://www.ebi.ac.uk/clustalw/. and the resultant phylogeny tree was used for phylogeny study.

Quantitative PCR

Quantitative PCR (qPCR) was used to study ACC oxidase gene expression in different loblolly pine tissues (Heid et al., 1996). Gene-specific primers for loblolly pine ACC oxidase and 18s rRNA were designed using Beacon Designer, v 2.0 (PremierBiosoft, Palo Alto, CA). The respective primer pairs were PTACO1 (ACO2F 5'-GCA GAA TGG CAA TGA AAA TGA C-

3') and PTACO121R (5'-CTC TCA TAG CCT CGA ATC GC-3'), as well as 18srRNA-F (5'-GAC GGA CCA CTG CGA AA-3') and 18srRNA-R (5'-CCC TGG TCG GCA TCG-3'). Both primer sets were tested against both cDNAs and plasmid clones to make sure that no secondary PCR products or primer dimers were produced. Reactions were conducted using DyNAmo SYBR Green qPCR mastermix (MJ Research, Watertown, MA) and an iCycler (BioRad, Hercules, CA), following the manufacturer's recommendations. Sample made up of five trees in the field were measured three times for ACC oxidase gene expression levels and twice for the 18s rRNA gene. A standard curve based on known quantities of plasmid containing the loblolly pine ACC oxidase cDNA was determined for each experiment. Cycling conditions for qPCR were as follows: 95°C for 10min followed by 50 cycles of 95°C for 1sec and 55°C for 1min. Template cDNA for all samples was made using 1µg of DNase-treated total RNA and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The first strand RT reaction was diluted two-fold and 3.5µl of the diluted cDNA product was used for each qPCR reaction. The Cycle Threshold (Ct) value was assigned manually as the point where the R^2-value for the standard curve was highest.

Results

Cloning of an ACC oxidase gene from loblolly pine

Degenerate primers corresponding to conserved regions of an apparent pine ACC oxidase EST sequence from lignifying xylem tissues were used to amplify products of the expected size from xylem cDNA templates using PCR amplification. The 5' and 3' UTRs were recovered using the RACE approach. The full-length ACC oxidase cDNA was 1461bp in length and appeared to encode a 333-amino-acid protein in addition to having a 77-nucleotide 5' untranslated region and a 382-nucleotide 3' untranslated region (Fig. 2.1).

Genomic DNA extracted from loblolly pine suspension cells was amplified using the nested sets of primers (ACO42F, ACO342R and ACO125F, ACO775R, ACO1114R) to ACC oxidase coding sequences. The PCR products that were recovered spanned the entire coding sequence and three introns of 111bp, 186bp, and 98bp, respectively. All intron and exon boundaries were identified as GT/AGs couplets (Fig. 2.1). The AT content of the three introns was, respectively, 20%, 13.6% and 18.4% greater than that in the flanking exonic regions extending 50 nucleotides upstream and downstream of the splice junction (Table 2.1). These values are in the range expected for plant introns (Joshi, 1987).

Sequence alignment of ACC oxidases from loblolly pine and other trees

BLASTP analysis against sequences in GenBank found that the predicted amino acid sequence encoded by PtACO1 had the highest identity and similarity (52% and 70%, respectively) to an ACC oxidase from European white birch (CAA71738). BLASTN comparison with a loblolly pine EST database (http://www.fungen.org) identified several homologs of PtACO1, including RTNACL1_6_A03 (GenBank CV032104 and CV032179), which differed from PtACO1 by a 12 amino-acid indel and three single amino acid changes. The deduced amino acid sequences of the putative loblolly pine ACC oxidases, PtACO1 and RTNACL1_6_A03, along with ACC oxidases from birch (*Betula pendulas*), poplar (*Populus tremula* x *Populus tremuloides*), white spruce (*Picea glauca*) and apple (*Malus domestica*, Seo et al., 2004) were used to generate a multiple sequence alignment (Fig. 2.2). The alignment highlighted twelve amino acids residues (P^{4}, A^{27}, G^{32}, H^{39}, H^{174}, D^{176}, L^{192}, Q^{193}, G^{215}, H^{231}, R^{241}, S^{243}) that are conserved in all Fe-ascorbate oxidases (Tang et al., 1993; Lin et al., 1997), as well as the HXD…H motif shown to be involved in metal ligation during catalysis by Fe (II)-dependent oxygenase/oxidases (Zhang et al., 1997). Also identified as conserved in the alignment were

residues corresponding to apple Md-ACO1 residues His^{177}, His^{234} and Asp^{179}, which had been shown to be involved in Fe^{2+} binding, as well as residues Arg^{244} and Ser^{246} that were shown to be important for enzyme activity in the apple enzyme (Seo et al., 2004).

Phylogenetic comparison of PtACO1 with 2-oxoglutarate-dependent dioxygenases from Arabidopsis

ACC oxidase is a member of the 2-oxoglutarate-dependent dioxygenase (2ODD) enzyme superfamily (Ververdis and John 1991), and like many other members of this family ACC oxidase requires Fe (II) and ascorbate for activity (Smith et al., 1998). As evidence that PtACO1 encodes an ACC oxidase rather than some other member of the 2ODD family, the deduced sequences of PtACO1 and the RTNACL1_6_A03 were placed in a phylogenetic tree with 101 2ODD sequences identified in the *Arabidopsis* genome. The phylogenetic tree generated using protein maximum likelihood with molecular clock (ProMLK) is shown in Figure 2.3. In this analysis the deduced PtACO1 and RTNACL1_6_A03 were clearly clustered with the five *Arabidopsis* ACC oxidase genes (At1g77330, At1g12010, At1g62380, At1g05010, At2g19590) and, in particular, At1g77330 appeared to be more closely related to the loblolly pine genes than to the other four *Arabidopsis* ACC oxidase genes (Fig. 2.4). The same grouping of sequences was seen after using the parsimony or protein maximum likelihood without molecular clock phylogeny methods (data not shown). The phylogeny of ACC oxidase from different species was shown in Figure 2.4. According to the phylogeny tree, PtACO1 has homolog with ACC oxidase from *Oryza sativa*, *Sorghum bicolor*, *Zea mays*, *Musa acuminata*, *Arabidopsis thaliana*, *Populus tremula x Populus tremuloides*, *Cicer arietinum*.

PtACO1 homologs in the loblolly pine EST dataset

Pine roots subjected to various biotic and abiotic stresses were used to produce about 170,000 ESTs that constitute the richest available source of expressed conifer genes. A BLAST search of this collection using the PtACO1 nucleotide sequence returned 49 sequences with expected values $\leq 4\times10^{-4}$ (Table 2.2). Thirty-two of these 3' and 5' ESTs represented 16 clones with both 5' and 3' ends and 17 represented only the 5' or 3' end of a cDNA sequence. Only the 16 clones were used in further analyses to avoid the possibility of that some of the partial sequences represented 2ODD enzymes other than ACC oxidase. The 16 clones were recovered from the 10 cDNA libraries detailed in Table 2.2. Three clones were identified in the RTDS3 (drought-stressed) library, while two clones were identified in the FLD1 (flood-stressed), RTHG1 (mercury-stressed), STRR1 and STRS1 (both challenged with a fungal pathogen) libraries. Overall, fourteen of the sixteen ACC oxidase clones were recovered from cDNA libraries representing stressed loblolly pine root tissues. Sequence alignment of PtACO1 with five clones that have full-length (or nearly full-length) amino acid sequences (RTNACL1_6_A03, STRR1_11_C02, STRS1_53_C11, RTHG1_1_C09, RTHG1_21_A11, STRS1_27_D10) showed that they are quite similar, with a difference of one or two amino acids (Fig. 2.6).

3'UTR analysis for PTACO1 and related ESTs

Alignment of the 3'UTRs of PtACO1 and the 16 clones recovered in the EST dataset identified three different sequence patterns based on the presence or absence of two small indels of 19 and 9 nucleotides (Fig. 2.5). PtACO1, representing Pattern_1, lacked both of the indel sequences, and this was the most common structure amongst 17 sequences analyzed. RTNACL1_6_A03 and RTHG1_1_C09, containing both indel sequences and were classified as

Pattern_3, while RTDS3_24_F11, RTDS3_1_E07 and STRS1_27_D10 fell into Pattern_2 in which the short, 3'-most indel sequence was missing.

Tissue-specific expression of PtACO1

Levels of PtACO1 expression in loblolly pine roots, candles, xylem, photosynthetic bark, primary needles, secondary needles, and needle collars were examined using qPCR (Fig. 2.7). ACC oxidase transcripts were barely detectable in secondary needles and xylem. Expression was moderate in primary needles and the photosynthetic bark, but expression was found to be highest in roots, candles, and needle collars. Thus, PtACO1 gene expression appeared to be most strongly correlated with tissues undergoing expansive growth.

Discussion

This study reports the first cloning and characterization of an ACC oxidase from loblolly pine. The alignment of the deduced PtACO1 amino acid sequence with ACC oxidase genes from other species showed the encoded protein to contain all of the conserved residues that characterize ACC oxidases (Figs. 2.1, 2.2). In comparison with the well-studied apple ACC oxidase, Md-ACO1 (Seo et al., 2004), PtACO1 retains the conserved cofactor-binding pocket and the substrate-binding pocket, the conserved residues, His^{186}, His^{243}, Asp^{188}, Arg^{253}, Ser^{255}. Md-ACO1 folds into a structural motif having eight α-helices and 12 β-strands (Seo et al., 2004). The sequence alignment shows PtACO1 to retain these general secondary structural characteristics, except in the region of α-helix E, which lies at the surface of the enzyme according to the Md-ACO1 model (Seo et al., 2004).

In the phylogenetic comparison of PtACO1 with members of 2ODD gene family from *Arabidopsis* (Fig. 2.3), PtACO1 clearly clustered with the five *Arabidopsis* ACC oxidase genes. ACC oxidases occur as small gene families in all species studied to date. Comparing DNA

sequence of the EST library clone RTNACL1_6_A03 to the genomic sequence of PtACO1, it was noted that the 12-amino-acid indel fell at the 3' end of the second PtACO1 intron, suggesting that this EST clones could have arisen as a splicing variant of PtACO1. But the difference of three other amino acids between them suggested this clone to be the product of a different ACC oxidase gene (Fig. 2.3).

ACC oxidase genes have previously been shown to be differentially expressed at the tissue level in various plant species (Tang et al., 1994; Pogson et al., 1995; Barry et al., 1996; Liu et al., 1997). The ACO1 and ACO2 genes of potato were strongly expressed in leaves, but were expressed at low levels in the roots and tubers (Nie et al., 2002). PtACO1 transcripts were expressed most strongly in candles, roots, and needle collars, while levels were lowest in mature needles and xylem. This expression pattern suggests that PtACO1 is mainly associated with young, expanding tissues, which may not be surprising since ethylene is known to have a definite role in the control of cell expansion and subsequent determination of size and shape (Abeles, 1973; Kieber et al., 1993). The expression of *Arabidopsis* ACS1 (ACC synthase) was strongly correlated with immature tissues (Rodrigues-Pousada et al., 1993), and young tissues are good sources of auxin, which is a well-known mediator of certain ethylene effects (Rodrigues-Pousada et al., 1993). Testing of whether PtACO1 is affected by auxin levels is suggested from these results as a productive line of investigation.

An ACC oxidase cDNA was identified in a control root library from the EST project, suggesting at least a low level of constitutive expression for the gene in these tissues. ACC oxidase gene expression has frequently been shown to be stimulated by treatment of plant tissues with such agents as ethylene, indol-3-acetic acid, and wounding (Tang et al., 1993; Lasserre et al.; Peck and Kende, 1995). Although this study did not directly examine PtACO1 under various

stress conditions, analysis of the available EST data suggests that the loblolly pine ACC oxidase genes do respond to various experimental stimuli. Thus, drought-stressed loblolly pine root libraries yielded four ACC oxidase clones versus only one from control root libraries (Table 2.2). While these numbers are not sufficient for statistical validity, they are strongly suggestive that in pine ACC oxidase transcripts accumulate in response to drought stress. Drought-responsive ACC oxidase gene expression has also been seen in sunflower (*Helianthus annuus* L.) where drought conditions induced ACC oxidase expression in a drought-tolerant line of sunflower (Ouvrard et al., 1996).

A future study of the regulation of PtACO1 under different external stimuli and under hormonal treatment, particularly under IAA treatment, combined with promoter analysis, may lead to a more detailed view of the molecular patterns governing ethylene production and even the effect of ethylene in loblolly pine.

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Table 2.1. Loblolly pine ACC oxidase splice junction AT content

AT content A or T/ total nt (%)	Intron 1	Intron 2	Intron 3
Intron	78/111 (70%)	135/186 (73%)	68/98 (69%)
Flanking regions*	50/100 (50%)	59/100 (59%)	51/100 (51%)

* Regions extending 50nt upstream and downstream of the splice junction.

Table 2.2. Putative ACC oxidase clones identified in the loblolly pine root EST collections

Library	Total EST number*	EST Clones	3'UTR pattern
FLD1	10415	FLD1_16_F06	Pattern_1
		FLD1_64_H06	Pattern_1
RTDR1	3121	RTDR1_1_F11	Pattern_1
RTDS2	2739	RTDS2_2_C10	Pattern_1
RTDS3	2904	RTDS3_1_E07	Pattern_2
		RTDS3_24_D11	Pattern_1
		RTDS3_24_F11	Pattern_2
RTHG1	2477	RTHG1_1_C09	Pattern_3
		RTHG1_21_A11	Pattern_1
RTMNUT1	3185	RTMNUT1_24_C04	Pattern_1
RTNACL1	5329	RTNACL1_6_A03	Pattern_3
RTWW1	3327	RTWW1_16_C12	Pattern_1
STRR1	9511	STRR1_11_C02	Pattern_1
		STRR1_14_G03	Pattern_1
STRS1	7701	STRS1_27_D10	Pattern_2
		STRS1_53_C11	Pattern_1

* The number represents the sum of 5'EST clones and 3' EST clones. Adapted from Lorenz et al. (2006).

Figure 2.1. Nucleotide and deduced amino acid sequence of the loblolly pine PtACO1 gene. Bold and underlined "ATG" indicates the putative translational start site. An asterisk (*) marks the putative stop codon. Boldface and underlined amino acids (P4, A27, G32, H39, H174, D176, L192, Q193, G215, H231, R241, S243) represent conserved residues necessary for ferrous ion and ascorbate binding among all ACC oxidases. Shadowed boldface fonts indicate the conserved region to which degenerate primers were designed.

Figure 2.2. Amino acid sequence alignment of PtACO1 with ACC oxidase sequences from birch (GB CAA71738), apple (GB Q00985), poplar (GB AAN87846), and spruce (GB AAA85365). Asterisks indicate amino acid residues involved in iron binding and plus signs indicate amino acid residues important for enzyme activity according to Seo et al. (2004). H and S are used to indicate sequences predicted to form α-helix and β-strand secondary structures (Solomon et al., 2000).

Figure 2.3. Phylogenetic tree of 2-oxoglutanate-dependent dioxygenase (2ODD) enzyme superfamily from *Arabidopsis* and their relative relationship to the putative loblolly pine ACC oxidase sequences, PtACO1 and RTNACL1_6_A03.

Figure 2.4. Phylogenetic tree of ACC oxidase from different species.

Figure 2.5. Multiple sequence alignment of PtACO1 with ACC oxidase clones recovered from loblolly pine root EST collection.

Figure 2.6. Different nucleotide sequence structures for 3'UTRs of ACC oxidase clones recovered from loblolly pine root EST collection.

Figure 2.7. PtACO1 transcript levels in different loblolly pine tissues as determined by qPCR.

ATCGAGTCCGCTGTTGGAAGTATACACAGCTTGCCAGTACGCTGTTTTTCTGCTTTTCTGTTTGT
GATTTATCAAAG**ATG**GCAGTC**CCCGTGATTGACATGAAGAAG**ATGTTGAATGGAGAAGAGAGGGA
M A V **P** V I D M K K M L N G E E R E
AGTGACGATGGCTAAGATACAAAATGCCTGCCAAGAATGGGGCTTCTTTCAGgtcattcccccat
V T M A K I Q N **A** C Q E W **G** F F Q
taattcccatcctataatttcattaactcaagtacaatcgttttatttttctgctatattatc
acttcttattgtatgtcatgtgttattatgcagCTTCTGAACCACGGAATACCTCACGCTCTTCT
L L N **H** G I P H A L L
CGACCGAGTGAAGGAGCTGTTCAAGGAACATTACAAAAATTCCATGGACGCAGAATTTCAGAAGT
D R V K E L F K E H Y K N S M D A E F Q K S
CTGAGATTGTAGGGATGCTTGAAAGTGCTGTCTCCCAAGGCAAGAATTTCGGTACTACGAAGATA
E I V G M L E S A V S Q G K N F G T T K I
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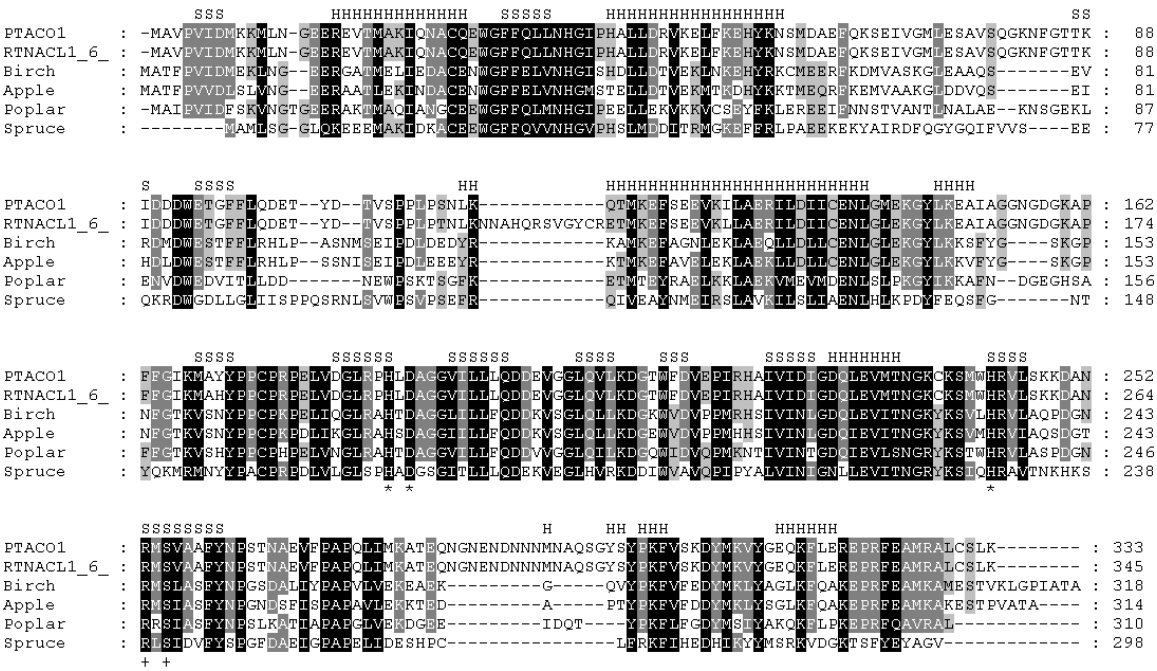

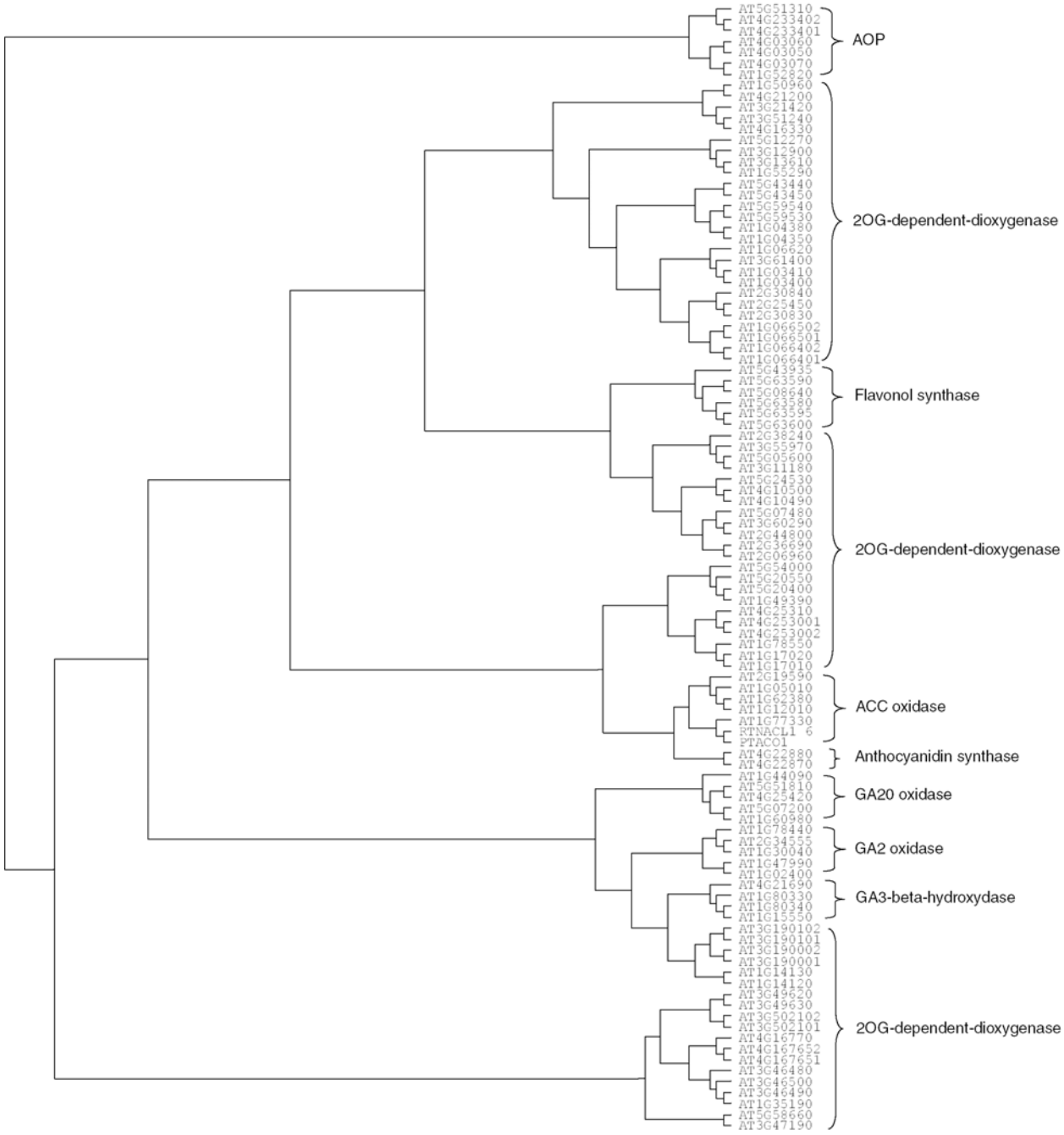
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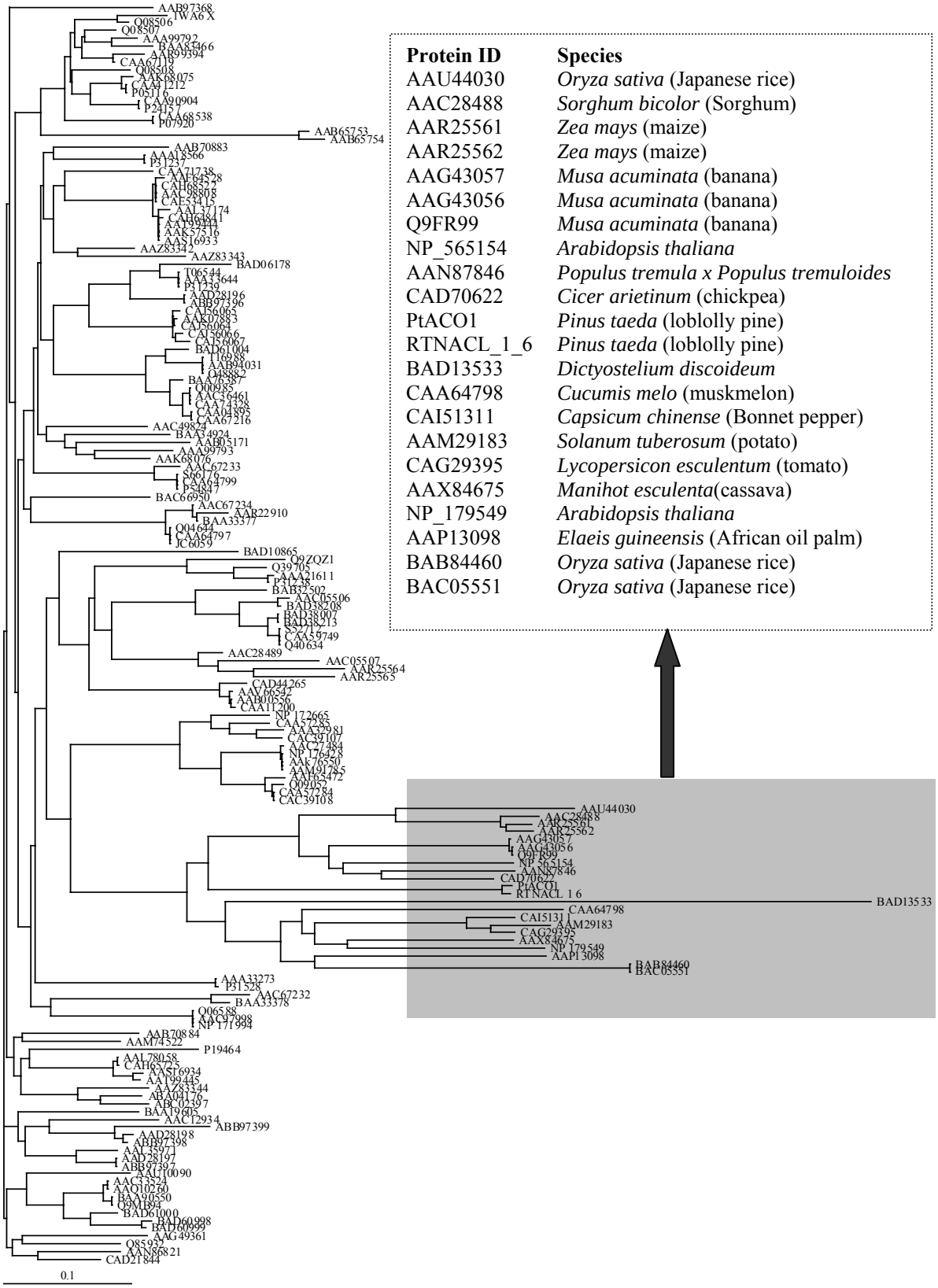
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AAG43057 Musa acuminata (banana)
AAG43056 Musa acuminata (banana)
Q9FR99 Musa acuminata (banana)
NP_565154 Arabidopsis thaliana
AAN87846 Populus tremula x Populus tremuloides
CAD70622 Cicer arietinum (chickpea)
PtACO1 Pinus taeda (loblolly pine)
RTNACL_1_6 Pinus taeda (loblolly pine)
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CAI51311 Capsicum chinense (Bonnet pepper)
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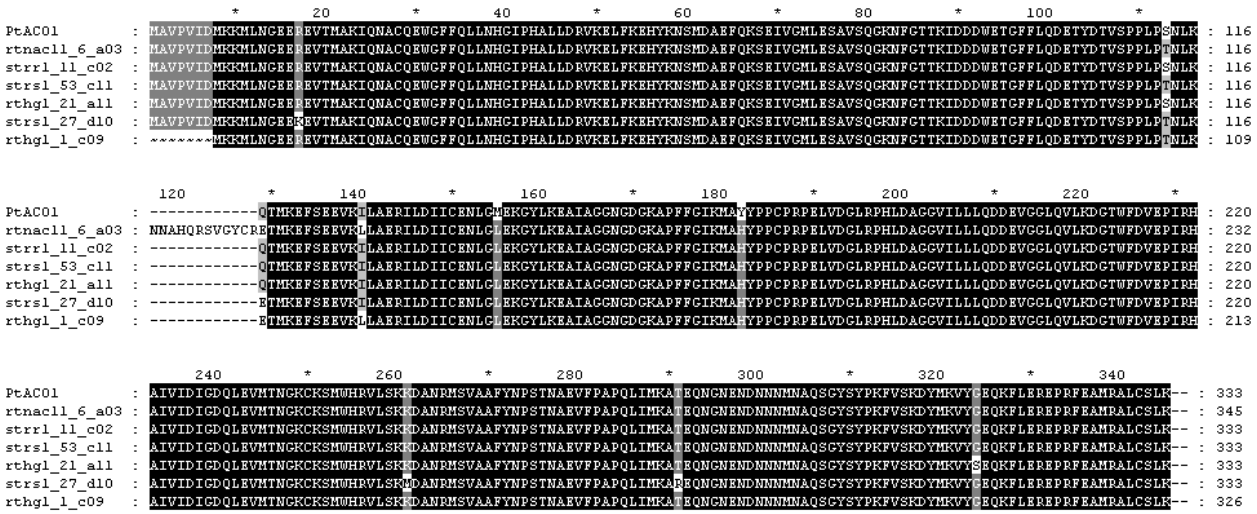
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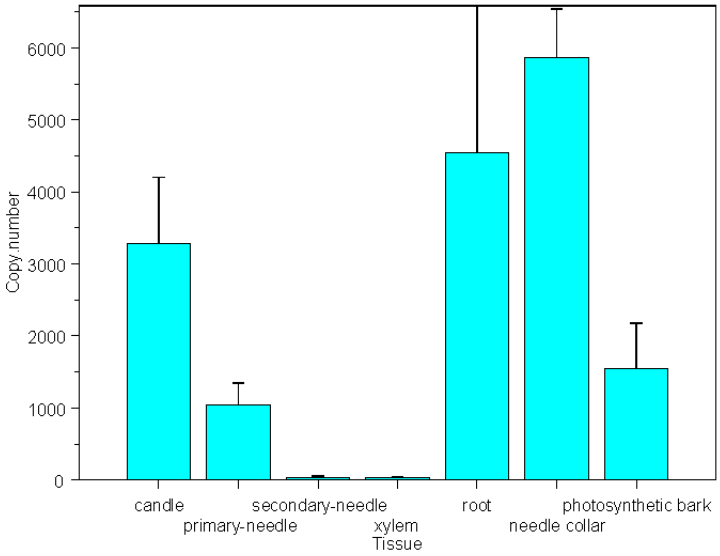
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CHAPTER 3

LOBLOLLY PINE (*PINUS TAEDA* L.) ACC OXIDASE GENE PROMOTERS DISPLAY DIFFERENTIAL RESPONSES TO BENDING STRESS AND OTHER EXTERNAL STIMULI IN TRANSGENIC *ARABIDOPSIS THALIANA* [*]

[*] Yuan S, Dean JFD to be submitted to Plant Physiology.

Abstract

ACC oxidase, the terminal enzyme in the ethylene biosynthetic pathway, catalyzes conversion of 1-aminocyclopropane-1-carboxylate (ACC) into ethylene, using iron and ascorbate as cofactors, and oxygen as a co-substrate. In this study, the promoter regions of two ACC oxidase genes from loblolly pine (*Pinus taeda* L.), designated pACO1 and pACO2, were recovered by PCR amplification from genomic DNA. Quantitative PCR (qPCR) analyses demonstrated that expression of the PtACO1 gene, which encodes an ACC oxidase from loblolly pine, product was stimulated in loblolly pine seedlings in response to bending and IAA treatments. Transgenic *Arabidopsis* plants harboring genetic constructs from which β-glucuronidase (GUS) expression was driven by the full-length (pACO1-GUS, pACO2-GUS) or truncated (pACO1-1.2-GUS, pACO2-1.2-GUS) loblolly pine ACC oxidase gene promoters displayed different patterns of tissue-specific expression for the various promoter constructs. Both full-length promoter constructs, but not those harboring truncated promoters, responded to IAA and wounding. Both pACO1-GUS and pACO1-1.2-GUS responded to fungal infection of transgenic *Arabidopsis*, while neither the full-length or truncated versions of pACO2 promoter responded to infection. In the inflorescence stalks of transgenic *Arabidopsis*, the full- length pACO1 promoter construct, but not pACO1.1-GUS or either pACO2 construct, responded to bending stress. When flowering transgenic *Arabidopsis* plants were laid horizontally for 48 hours, expression from pACO2-GUS, but not the other constructs, was induced on the underside of shoots undergoing gravitropic reorientation. The expression pattern for the pACO2-GUS construct in transgenic *Arabidopsis* was consistent with what might be expected for a gene promoter involved in the compression wood response in loblolly pine. These results are

discussed with respect to potential functional differences between the two ACC oxidase genes from loblolly pine.

Keywords: ethylene biosynthesis, ACC oxidase gene promoter, compression wood, bending stress, auxin, wounding, fungal infection, conifer, forest tree, wood formation

Introduction

Compression wood is a reaction wood that forms on the underside of branches and leaning stems of conifers in response to shear stresses, and its function is to oppose the pull of gravity (Timell, 1986). Compression wood contains more lignin and less cellulose than normal wood, making it undesirable for many commercial uses (Core et al., 1976). Studies on the formation of compression wood have focused on such aspects as environmental stress (Mi Kwon et al., 2000), effects of plant growth regulators (PGRs) (Little and Lavigne, 2002), and changes in gene expression patterns (Zhang et al., 2000). With respect to studies of PGR effects on compression wood formation, IAA (Little and Lavigne, 2002) and ethylene (Plomion et al., 2000) have been studied most often.

As a PGR, ethylene has been shown to be involved in many aspects of plant growth and development, including seed germination, cell elongation, fruit ripening, senescence, and abscission, as well as stress responses, including wounding, and defense against pathogen attack (Abeles et al., 1992; Klee and Tieman, 2002). Studies clearly indicate that ethylene inhibits stem elongation in woody plants, as it does in many herbaceous species (Morgan and Drew, 1997), and that ethylene is involved in tree responses to stress (Little and Pharis, 1995; Morgan and Drew, 1997). The radial growth of *Pinus taeda* stems was positively correlated with ethylene production in seedlings subjected to mechanical shaking (Telewski, 1990). Ethylene production was greater in tissues from the underside of *Cupressus arizonica* branches, where compression

wood forms, than from the upper side of those same branches (Blake et al., 1996). 1-Aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene, was detected in the cambial tissues from the underside, but not the upper side, of *Pinus contorta* branches (Savidge, 1983), and application of indole acetic acid (IAA) in combination with ACC produced more compression wood than using either PGR alone (Savidge, 1983). Although none of these studies directly demonstrated a cause-and-effect relationship between ethylene production and compression wood formation in conifers, they suggest that ethylene plays a role in wood formation and may govern conifer wood quality as it relates to compression wood.

The precise role that ethylene plays in conifer wood formation has remained a mystery in part because previous manipulative studies have relied heavily on observation of effects resulting from exogenous application of ethylene and ethylene precursors. Molecular genetic analyses of ethylene biosynthetic pathway genes and gene products in conifers, as a prelude to genetic testing of the link between ethylene and compression wood formation, are rare. ACC oxidase, the terminal enzyme in the ethylene biosynthetic pathway, was found among the up-regulated proteins in compression wood from maritime pine (*Pinus pinaster* Ait.) (Plomion et al., 2000). In a follow-up study, ACC oxidase was identified by cDNA-AFLP as being differentially expressed between compression and opposite wood in maritime pine at both eight and twenty days after initiation of treatment (Provost et al., 2003). These observations strengthen the case for ethylene playing a role in compression wood formation.

Regulation of ethylene biosynthesis has been studied intensively in a wide variety of plant species (Chae and Kieber, 2005). Ethylene is synthesized in a two-step biosynthetic pathway that starts with conversion of S-adenosylmethionine to ACC in a reaction catalyzed by ACC synthase. This reaction is thought to be the rate-limiting step in the pathway under most

circumstances. ACC oxidase subsequently converts ACC into ethylene (Yang and Hoffman, 1984). ACC oxidase was for a long time refractive to biochemical characterization, and it was through molecular biological approaches that a cDNA clone, TOM13, from tomato (*Lycopersicon esculentum*) was shown by functional expression in *Saccharomyces cerevisea* (Hamilton et al., 1991) and *Xenopus laevis* oocytes (Spanu et al., 1991) to encode an enzyme harboring ACC oxidase activity. This gene was subsequently used to isolate ACC oxidase genes from numerous other plant species (for review, see Barry et al., 1996). Transcript levels for many of these ACC oxidase genes have been shown to increase greatly upon wounding (Holdsworth et al., 1987; Hamilton et al., 1990; Kim and Yang, 1994; Lasserre et al., 1996; Weterings et al., 2002; Nie et al., 2002; Lopez-Gomez et al., 2004), suggesting besides the established regulatory role of ACC synthase in the pathway, ACC oxidase expression is also important for controlling ethylene production. With respect to woody plant species, ACC oxidase has been characterized mostly from fruit-producing trees, such as apple (*Malus domestica*), pear (*Pyrus pyrifolia*), papaya (*Carica papaya*), kiwi (*Actinidia deliciosa*), banana (*Musa acuminata*), passion fruit (*Passiflora edulis*), and peach (*Prunus persica*). Recent work with an ACC oxidase gene from another woody angiosperm, poplar (*Populus tremula* L. x *P. tremuloides*), showed that ACC oxidase activity, but not ACC availability, was important for control of asymmetric ethylene production within poplar stems when tension wood was induced by gravitational stimulation (Andersson-Gunneras et al., 2003). Considering the differences in characteristics between compression wood and tension wood (the reaction wood formed on the top side of branches and leaning stems of woody angiosperms), it was deemed important to evaluate the ACC oxidase expression pattern during compression wood formation in a gymnosperm species.

In previous work, we cloned an ACC oxidase gene from loblolly pine (PtACO1), and showed that it was differentially expressed in various tissues. In this study, we characterize two ACC oxidase gene promoters, including one that controls expression of PtACO1. Because the only reliable systems for stable transformation of loblolly pine are held in the private sector, these experiments examined transcriptional regulation of the ACC oxidase promoters, pACO1 and pACO2, in transgenic *Arabidopsis* plants. β-Glucuronidase (GUS) activity expressed from ACC oxidase gene promoter-GUS constructs was demonstrated histochemically under the situations of bending stress and PGR treatment as they are thought to relate to compression wood formation. The different promoter-reporter gene constructs also showed differential responses to wounding and pathogens. The possible relationships between compression wood formation, bending stress and the different loblolly pine ACC oxidase promoters are discussed.

Materials and Methods

Plant materials and treatments

Loblolly pine seeds were cut at the tip and stratified in cold water (4°C) for one week prior to planting. Seedlings were maintained under normal greenhouse conditions until treatment and harvest. For bending treatments, candles from 7-month-old seedlings were bent to approximately a 90° angle using string and left until harvest at various timepoints. Five candles (different genotypes) were harvested and pooled for each time point. At harvest, candles were cut from the seedlings, flash-frozen in liquid nitrogen, stripped of all needles, and stored at -80°C until assayed. Only the section of candle stem involved in bending was collected. For the 3-hour bending treatment, additional candles were collected, and tissues from the upper and lower side of the bend were separated using a razor. For IAA treatments, 7-month-old seedlings were cut at the root:shoot junction, and immersed upright in a graduated cylinder containing an aqueous

solution of 1mM IAA and 1% ethanol. Control shoots were immersed in water containing 1% ethanol. Five seedlings were treated and pooled for each time point. Treated stems were frozen and stored as described above.

Arabidopsis thaliana (seeds provided by Dr. C.J. Nairn, University of Georgia) were grown in soil in a growth chamber maintained at 22ºC with a photoperiod of 16h light/8h dark. For the screening of transgenic *Arabidopsis*, seeds were planted in petri dishes on semi-solid MS medium (Invitrogen, Carlsbad, CA) supplemented with 1.5% sucrose and 0.7% PhytoAgar (Invitrogen). For IAA treatments, 20-day-old transgenic *Arabidopsis* plants planted on MS plates were treated by immersion for two hours in 100μM aqueous IAA. For bending treatments, transgenic *Arabidopsis* plants were grown in soil under ambient conditions in the greenhouse from January to March until inflorescence stalks formed. Inflorescence stalks were decapitated 2-4 times to provoke secondary growth in the stalk. Unless otherwise stated, stalks were bent for about 90º for three hours prior to histochemical staining.

RNA extraction and quantitative PCR

RNA was extracted using the method of Chang et al. (1993). For quantitative PCR (qPCR), gene-specific primers were designed using Beacon Designer, v2.0 (Premier Biosoft, Palo Alto CA). The primer sets made to divergent sequences in the 3' UTR of PtACO1 (**ACO2F**, 5'-GCA GAA TGG CAA TGA AAA TGA C-3'; **ACO121R** 5'-CTC TCA TAG CCT CGA ATC GC-3') and 18s rRNA (**18srRNA-F** 5'- GAC GGA CCA CTG CGA AA-3'; **18srRNA-R** 5'-CCC TGG TCG GCA TCG-3') were tested against both cDNAs and plasmid clones to verify that no secondary PCR products or primer dimers were produced. QPCR was conducted using DyNAmo SYBR Green qPCR mastermix (MJ Research, Watertown, MA) in an iCycler (BioRad, Hercule, CA), following the manufacturer's recommendations. All samples were measured in triplicate

for ACC oxidase transcript levels and duplicate for 18s rRNA transcript levels. A standard curve based on known quantities of plasmid containing the loblolly pine ACC oxidase cDNA was determined for each experiment. Cycling conditions for qPCR were as follows: 95°C for 10 min followed by 50 cycles of 95°C for 1sec and 55°C for 1min. Template cDNA for all samples was made using 1 μg of Dnase-treated total RNA and Superscript III (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The first-strand RT reaction was diluted 2 and 3.5µl of cDNA product 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 was highest.

Isolation of the PtACO1 gene promoter

Loblolly pine genomic DNA was isolated from needles ground in liquid nitrogen and purified by centrifugation through a CsCl gradient (Sambrook et al., 1989). Putative promoter regions in genomic DNA upstream of loblolly pine ACC oxidase genes were amplified and isolated using the Universal Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA), following the manufacturer's protocols. Briefly, isolated genomic DNA was digested with restriction endonucleases (DraI, EcoRV, PvuII, and StuI) that have 6-base recognition sites and leave blunt-ends on digested fragments. Adaptor DNA containing the AP1 and AP2 primer-binding sites, synthesized by Intergrated DNA Technologies (Coralville, IA)was linked to both ends of the released DNA fragments by ligation overnight at 16˚C. Gene-specific primers (**PTACO1-74R,** 5'-CCA TCG TCA CTC CCT CTC TTC TCC ATT C -3'; **PTACO1-102R** 5'-CAC TCT TGG CAG GCA TTT TGT ATC TTG G-3'; **PTACO1-342R** 5'-AAG AAG CCC GTT TCC CAG TC-3') were designed using Oligo 4.0s (Molecular Biology Insights, Inc., Cascade, CO). First- and second-round PCR amplifications were performed using nested gene-specific primers with adaptor primers AP1 or AP2 according to the manufacture's

recommendations. For first-round amplifications, 2.5 µl of 10x PCR buffer (Promega, Madison, WI), 2 µl of 25mM $MgCl_2$, 0.5 µl of 10 µM dNTPs, 0.5 µl of 10 µM AP1 primer, 0.5 µl of 10 µM PTACO1-342R primer, 1 unit of Taq polymerase (Promega), 1.5 µl DMSO, 16.2 µl distilled H_2O and 1 µl of one of the four Genome Walker libraries were mixed and subjected to thermocycler conditions according to the manufacture's recommendation. For the second-round amplifications, components and cycling conditions were identical to the first-round, except for use of the AP2 and PTACO1-102R (or PTACO1-74R) primers and 1 µl of the PCR products from the first-round amplification as template. Three fragments having lengths of approximately 1.4kb, 2.4kb and 2.0kb were amplified from second-round amplification of the EcoRV library. An additional fragment having a length of about 1.3 kb was amplified from the PvuII library. All amplimers were cloned into the pCR II-TOPO vector using the TOPO TA vector kit (Invitrogen, Carlsbad, CA).

DNA sequencing

DNA fragments and constructs were sequenced in both directions using an ABI 3700 automated DNA sequencer and Big Dye terminator chemistry (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Plasmid DNA for sequencing was isolated using QIAGEN midiprep kits (QIAGEN, Inc., Valencia, CA). Sequences were compiled and aligned using the Wisconsin Package software suite, v10.1 (Genetics Computer Group, Madison, WI). Sequencing primers for the 2.4kb and 2.0kb promoter fragments were designed using Oligo 4.0. DNA sequence analysis identified the 2.0kb fragment.

Construction of β-glucuronidase (GUS) reporter gene constructs

The two longer amplimers (2008bp and 2375bp, respectively) were chosen to make promoter-reporter constructs. For the shorter fragment, the full-length fragment was amplified

using Expand High Fidelity PCR system (Roche Diagnostics Corp., Indianapolis, IN) using a 5' forward primer (**pACO1-SpeI**, 5'-TCG ACT AGT ATC CAT TGT CCC TGC TAA TTT CCC-3') containing a SpeI recognition site and a 3' reverse primer (**pACO1-PstI**, 5'CAC GAC TGC AGC TTT GAT AAA TCA CAA ACA GAA AAA CAG AAA AAC-3') containing a PstI recognition site. For the longer fragment, a 5' forward primer (**pACO2-SpeI**, 5'-TCG ACT AGT ATC ATT GTC CCT GCT AAT TTC CCA) and a 3' reverse primer (**pACO2-PstI**, 5'-CAC GAC TGC AGC TTT GAT AAA TCA CAA ACA GAA AAG CAG AAA AAT-3') were used for the amplification. Sequence alignment showed that the two putative promoters shared a significant length of sequence identity. A primer matching this region (**pACO-1.2-SpeI**, 5'-TCG ACT AGT CTT CCT TCC CCT TAC TTC TTC GAA GTC-3') was used to amplify shortened versions of the two full-length fragments by pairing with the two previously designed reverse primers (pACO1-PstI and pACO2-PstI). The resultant full-length and truncated amplimers were digested sequentially with SpeI and PstI, and ligated in-frame to the 5' end of a GUS reporter gene in the pUPC5-GUS vector (provided by Dr. C.J. Nairn) as replacements for the ubiquitin-3 promoter contained in that construct. The resultant constructs were verified by restriction digests and DNA sequencing. A positive control construct was prepared by replacing the ubiquitin-3 promoter in pUPC5-GUS with the cauliflower mosaic virus (CaMV) 35S promoter. The five resultant promoter:GUS fusions were subsequently cut with SpeI and AscI, and the released fragments were subcloned into the pZP-NPTII binary vector (courtesy Dr. CJ Nairn). As a negative control, a promoterless GUS gene was cut from pUPC5-GUS using AscI and PstI, and ligated into the pZP-NPTII vector. The six different promoter-GUS constructs resulting from these efforts, designated p-GUS, p35S-GUS, pACO1-GUS, pACO1-1.2-GUS, pACO2-GUS and

pACO2-1.2-GUS, were introduced into the *Agrobacterium tumefaciens* strain, AGL1, by electroporation.

Arabidopsis transformation

Arabidopsis thaliana (L.) Heynh. plants of ecotype Columbia were transformed using the floral-dip method of Clough and Bent (1998) and the *Agrobacterium tumefaciens* strains carrying the six promoter test constructs, p-GUS, p35S-GUS, pACO1-GUS, pACO1-1.2-GUS, pACO2-GUS, pACO2-1.2-GUS. Plants were grown as described previously and fed once a week by spray with Miracle-Gro plant food (Scotts Miracle-Gro, Marysville, OH). Water was withheld from plants once seedpods began turning brown. When plants were fully dried, they were placed in a brown paper bag for 1 week prior to collecting seeds. Seeds were collected by hand-rubbing plants and pods, and repeated filtering through a 0.7-mm mesh sieve (Newark Wire Cloth Co., Newark, NJ) until seeds were reasonably free of debris. Transgenic seedlings were selected on solid MS media containing 100µg/mL kanamycin. Kanamycin-resistant lines were tested by PCR for presence of the appropriate construct using a GUS-specific primer (5'GAT TTC ACG GGT TGG GGT TTC T 3') and primers designed specific for each promoter (pACO1-65f, 5'ACA CTC AAA TTT ACT CTA AAC ACT 3'; pACO2-74f, 5' AGG CTT CTG TGA TTC ATA ATG GTA 3'). The PCR cycling conditions used a single denaturation step of 95°C for 2 min, followed by 40 cycles of 94°C for 45s, 55° for 45s, and 72°C for 1.5 min. Amplification reactions ended with an incubation step at 72°C for 7 min followed by a hold at 4°C. The reaction mixture (25 µl total volume) contained 1 unit Promega Taq DNA polymerase (Promega, Madison, MI), 250µM dNTPs, 2mM Mg^{2+}, 200nM primers, and genomic DNA prepared from transgenic *Arabidopsis*.

Histochemical GUS staining

Staining for β-glucuronidase activity was based on the method of Jefferson et al. (1987). Tissues from transgenic plants at different stages of development were immersed in a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-glucuronide (X-gluc) (Gold Biotechnology, St. Louis, MO), 100 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. Samples were incubated overnight at 37ºC, and to enhance contrast, chlorophyll was removed by washing the stained tissues in three changes of 100% ethanol. Tissues were then left in 70% aq ethanol until observation. Nine independent transgenic lines were analyzed in this manner for each treatment.

Results

Cloning of ACC oxidase gene promoters from loblolly pine

Four different loblolly pine genomic DNA fragments of approximately 2.4kb, 2.0kb, 1.4kb and 1.2kb corresponding to ACC oxidase promoter regions were recovered from the Genome Walker libraries using nested primers corresponding to conserved ACC oxidase gene coding sequences. Once the fragments were sequenced, promoter-specific primers were paired with a series of primers designed against divergent sequences in the three introns of the genomic ACC oxidase clones characterized in previous studies, and subsequent PCR amplification experiments identified the 2.0kb promoter fragment as corresponding to the previously characterized PtACO1 gene. As the two shorter fragments (1.2kb and 1.4kb) were deemed a bit small for reliable analyses of promoter function, the 2.0kb PtACO1 promoter fragment and the 2.4 kb promoter fragment were selected for further analysis.

Sequence comparison of these two putative ACC oxidase gene promoters, designated pACO1 and pACO2, revealed that regions flanking the TATA boxes and parts of the 5'UTRs, as

well as the spacing between the TATA boxes and putative transcription start sites, were remarkably similar, with only 2 two-nucleotide differing between them (Fig. 3.1). With the exception of three prominent insertions that differ these two promoters, the two promoters are nearly identical for the first 1.3kb upstream of the 5' UTRs; however, the sequences differ significantly for the remainder of their sequences. Truncated versions of the two promoters, designated pACO1-1.2 and pACO2-1.2 and containing most of the highly conserved sequences, were isolated in order to study whether or not the insertion sequences played a significant part in regulating gene expression. The 5' ends of these truncated promoters are indicated by the underlined sequence in Figure 3.1.

In silico analyses of pACO1 and pACO2

pACO1 and pACO2 were examined for *cis*-regulatory elements using the online search tool, plantCARE (Lescot et al., 2002). Figure 3.2 highlights a number of potential regulatory sequences and promoter-like elements identified in the PtACO1 fragment. A putative TATA box was found at position -35, whereas two CAAT boxes were identified at positions -139 and -148. A typical ethylene responsive element (ERE) (ATTTCAAA) (Itzhaki and Woodson, 1993) was found at position -1932. A motif (TGACG) potentially involved in methyl-jasmonate-responsiveness (Rouster et al., 1997) was identified at position –1298, and a potential auxin response element, the TGA-box (Hagen et al., 1991), occurs at position -385. A GARE-motif, which could indicate gibberillin responsiveness (Pastuglia et al., 1997), was located at position –851. One element responding to environmental condition was identified, HSE (AAAAAATTTC) (Pastuglia et al., 1997) involved in heat shock responsiveness located at –1936. Several potential light-response elements, including Sp1 (Litts et al., 1992), G-box (Rouster et al., 1997), MRE (Feldbrugge et al., 1997), were also identified in the pACO1 promoter, as were three abscisic

acid response (ABRE) elements located at positions –188, -385, and -1116 (Yamaguchi-Shinozaki and Shinozaki, 1993). Despite the large differences in the 5' ends of the pACO1 and pACO2 sequences, the potential cis-acting regulatory elements detected using PlantCARE were quite similar except that three motifs responding to light were only present in pACO1, GAG-motif (Rundle and Zielinski, 1991), AE-box (Conley et al., 1994), GATA-motif. In pACO2, three motifs were different from those found in pACO1, which are box-W1, DRE, and MBS1.

Regulation of PtACO1 expression in loblolly pine

Changes in IAA levels have previously been shown to accompany compression wood formation (Wilson et al., 1989; Funada et al., 1990; Sundberg et al., 1994), and the expression of ethylene biosynthetic genes in many plants responds to auxin (Zhang and O'Neill, 1993; Saito et al., 2005; Steed et al., 2004). The presence of potential auxin response elements (TGA-box) in pACO1 and pACO2 suggested the same might be true for loblolly pine ACC oxidase genes. To test this, loblolly pine seedling stems were immersed in an IAA solution for different lengths of time and the expression of PtACO1 was measured using qPCR (Fig. 3.3). ACC oxidase transcript levels in IAA-treated stems were approximately 3x higher than in control stems at three and six hours post-immersion, but began to drop by eight hours. Immersion alone appeared to stimulate PtACO1 gene expression 4- to 5-fold.

Reaction wood formation occurs in bent tree stems (Timell, 1986), and ACC oxidase expression levels were increased in reaction wood-forming tissues from both angiosperm (Andersson-Gunneras et al., 2003) and gymnosperm (Plomion et al., 2000) trees. PtACO1 transcripts levels were measured in loblolly pine seedling stems that were bent to a 90° angle and left for various lengths of time (Fig. 3.4). Transcripts levels began to increase after about 30 minutes and peaked about three hours after the start of the bending treatment. At their peak,

PtACO1 transcript levels were more than 20-fold increased over basal levels. Even after 24 hours transcript levels were still several-fold increased over basal levels. Dissection of bent loblolly pine stems into compression and opposite wood pools showed that PtACO1 transcripts were about two-fold higher in compression wood than in opposite wood (Fig. 3.5).

Loblolly pine ACC oxidase promoter expression in heterologous systems

To study the gene expression patterns conferred by pACO1 and pACO2, the full-length and truncated versions of these promoters were used to prepare GUS reporter gene constructs. Preliminary experiments in which a Helios Biolistic gun (BioRad, Hercules, CA) was used to introduce the two full-length promoter-reporter constructs into onion epidermal skin cells showed the constructs to be functional in transient expression assays (data not shown). In subsequent experiments, *Arabidopsis* plants were transformed with all four test constructs and two control constructs, and T1 plants were analyzed with respect to their GUS expression patterns. All of the loblolly pine promoter constructs were functional in *Arabidopsis* and revealed distinct staining patterns associated with actively dividing/expanding tissues in various organs of the plant. Figure 3.6 shows the expression patterns for the pACO1-GUS construct. In the 8-day-old seedlings, GUS expression was strong at the apex of the cotyledon (Fig. 3.6a), which is the region of that organ undergoing the most rapid expansion. Other tissues characterized by rapid expansion, including the apical meristem and seedling hypocotyl close to the apical meristem (Fig. 3.6b), as well as root tip and sites of lateral root initiation (Fig. 3.6c,d), also stained strongly for GUS activity. In the 15-day-old seedlings, GUS expression in leaves was concentrated at the apex (Fig. 3.6e), but by the time seedlings reached 22 days of age, GUS staining nearly disappeared from the leaves, although some leaves showed patchy staining suggestive of elicited responses to microbes growing on the leaves (Fig. 3.6f). In flowers, GUS activity was detected

mainly in the sepals (Fig. 3.6g), although some staining was associated with vascular tissues in the stamen as it was with vascular tissues in most other parts of the plant.

The truncated versions of both ACC oxidase promoters were competent to drive GUS expression in *Arabidopsis*, and although they generally mirrored the expression patterns of the respective full-length promoters, some differences were noted. For seedlings transformed with the pACO1-1.2-GUS construct, GUS staining was nearly abolished from the apical meristem and hypocotyl, except at the root:hypocotyl junction (Fig. 3.7b,g). In addition, GUS staining in the roots of these plants was, in general, not as intense as that seen in plants harboring the full-length promoter construct (Fig. 3.7c).

The GUS staining pattern for transgenic *Arabidopsis* harboring the pACO2-GUS construct was distinctly different from that seen for plants containing the pACO1-GUS construct (Fig. 3.8). In the 8-day-old seedlings, pACO2-GUS expression in the cotyledon occurred mainly in the vascular tissue with prominent expression at three foci around the periphery (Fig. 3.8a). Staining in the root was similar to that seen for pACO1-GUS (Fig. 3.8c,d), but with more intense expression throughout the root, in contrast to pACO1-GUS, which had strong expression only in the elongation zone behind the root tip. GUS activity in the apical meristem and hypocotyl was nearly the same for both constructs (Fig. 3.8b), but staining in 15-day-old and 22-day-old leaves (Fig. 3.8e,f), resembles expression in cotelydons with staining primarily was in the vascular tissues and the three growth points for vascular tissue around the leaf periphery. GUS expression was absent from fully expanded leaves (data not shown). The GUS staining pattern for the pACO2-GUS constructs in flowers was distinctly different from that seen for the pACO1-GUS construct. pACO2 conferred expression in the base of the flower and the tip of the stigma, but very weak GUS staining could also be seen in the vascular tissues of the stamen (Fig. 3.8g).

For the truncated pACO2-1.2-GUS, expression was nearly abolished, except in the roots of transgenic *Arabidopsis* where staining was mostly concentrated in the root tip and elongation zone, as well as in the lateral root initiation sites (Fig. 3.9c,f). Visible staining was still apparent in the center of the apical meristem (Fig. 3.9b), and in the veins of 15-day-old leaves (Fig. 3.9d).

ACC oxidase promoter responses to IAA

GUS activity was induced by IAA in *Arabidopsis* plants transformed with ACC oxidase promoter construct pACO1-GUS on the surface of 20-day-old leaves (Fig. 3.10), while for transgenic *Arabidopsis* transformed with construct pACO2-GUS, the GUS expression was upregulated upon immersion of 100μM IAA for 2 hours (Fig. 3.10).

Induction of the pACO1 promoter in response to microbial challenge

As previously noted (Fig. 3.6e,f), irregular GUS staining patterns on the surface of transgenic *Arabidopsis* leaves suggested that pACO1 might be induced in response to microbial challenge. Under typical greenhouse conditions, infection of *Arabidopsis* leaves by fungi of undetermined species is common. Infected leaves from transgenic *Arabidopsis* plants carrying each of the five GUS reporter constructs were stained for GUS activity, and the results showed that the pACO1 promoter and its truncated derivative responded to fungal infection (Fig. 3.11 b,c), while the pACO2 promoters did not (Fig. 3.11 d,e).

ACC oxidase promoter responses to bending stress

An ACC oxidase expressed in *Arabidopsis* roots was previously shown to be repressed in response to both gravitropic and mechanical stimuli (Kimbrough et al., 2004). Given our observations that bending stress induces expression of the ACC oxidase, PtACO1, in loblolly pine, it was unclear how pACO1 and pACO2 would response to bending treatments in transgenic *Arabidopsis*. Inflorescence stalks from all six transgenic lines were bent to a 90° angle for three

hours and then stained for GUS activity. Both of the full-length ACC oxidase promoters, but not their truncated derivatives, showed wound-induced staining on their cut ends, but only the pACO1-GUS construct responded to bending stress (Fig. 3.12). GUS staining in the inflorescence stalks of pACO1-GUS plants was limited to the region that underwent bending, but cross-sections of the bent region did not reveal differential GUS expression on opposite sides of the stalks (data not shown).

To examine the effect of a gravitropic response on these promoters, transgenic plants with well developed inflorescence stalks were placed in a horizontal position for 48 hours, by which point the apical and lateral shoot tips had started to reorient grow upwards. Staining these tissues for GUS activity revealed that pACO2-GUS construct expression was induced on the underside, but not the topside of stems reorienting themselves to gravity (Fig. 3.13). The pACO1-GUS construct showed no response to reorientation.

Discussion

Compression wood is an undesirable material from the standpoint of the forest products industry, although its presence in coniferous trees is almost certainly necessary for their erect growth and particular branching habits. Previous studies have linked compression wood formation with both auxin (Wilson et al., 1989; Little and Lavigne, 2002; Hellgren et al., 2004) and ethylene (Du et al., 2000; Plomion et al., 2000). This study sought to probe the link between ethylene and compression wood more deeply by following the expression of ACC oxidase genes, whose products catalyze the terminal step in the ethylene biosynthetic pathway, under conditions that lead to compression wood formation in loblolly pine. Thus, bending and IAA were demonstrated to independently induce expression of the previously characterized PtACO1 ACC oxidase in the elongating stems (candles) of loblolly pine (Figs. 3.3-3.5).

To more precisely define the genetic elements controlling expression of loblolly pine ACC oxidase genes, putative promoter sequences were cloned from genomic DNA using PCR amplification with nested primers based on conserved coding sequence and adaptor primers developed for the genome walking technique. Because of the high degree of sequence conservation found previously in the 5' end of loblolly pine ACC oxidase cDNAs, it was not immediately apparent which of the four amplified putative promoter sequences corresponded to that for the previously characterized PtACO1 ACC oxidase gene. In fact, efforts to link a specific promoter sequence with PtACO1 using a promoter-specific primer and primers based on sequences from introns 1 and 2 of the PtACO1 gene amplified both the pACO1 and pACO2 promoters, suggesting that a high degree of sequence conservation is retained between introns 1 and 2 of both genes. However, a primer based on PtACO1 intron 3 specifically amplified the promoter subsequently designated as pACO1. Efforts to clone genomic coding sequence and introns for the gene corresponding to pACO2 were so far unsuccessful.

Sequence comparison of the two promoters showed them to be remarkably conserved in the 5'UTR and throughout the region up to approximately 1300bp upstream of the transcriptional start site. Similarly high levels of sequence conservation have been seen previously in the promoters for the three members of the ACC oxidase gene family (LEACO1, LEACO2, LEACO3) from tomato (Blume and Grierson, 1997), as well as the core promoter regions for the four-member ACC oxidase gene family from *Petunia hybrida* (Tang et al., 1993). The highly conserved "core" sequences of the pACO1 and pACO2 loblolly pine promoters were subcloned for comparative expression analyses with the full-length promoters.

Studies of conifer promoter function have languished to some extent due to the lack of efficient transformation systems for most species. Systems for loblolly pine transformation have

been described (Tang et al., 2001, Gould et al., 2002), but they are not rapid or efficient, nor are they sufficiently facile to be easily adopted by any laboratory. Consequently, a decision was made to initially test promoter function of the pACO1 and pACO2 sequences in a heterologous system. ACC oxidase promoters tested in this manner have previously yielded useful information; for example, a tomato promoter in tobacco (Blume and Grierson 1997), an apple promoter in tomato (Atkinson et al., 1998), and a peach promoter in tomato (Moon and Callahan 2004). *A. thaliana* has been used previously to test conifer gene promoter function with results suggesting that many aspects of the gene expression patterns were maintained, even across this wide phylogenetic gap (Avila et al., 2001, Hofig et al., 2003, Liu et al., 2005). Thus, constructs from which GUS reporter gene expression was driven by the pACO1 and pACO2 promoters and their two derivatives comprising the promoter core sequences were used to stably transform *Arabidopsis*. Preliminary tests using onion epidermal cells showed that the promoter constructs were even competent for transient assays in a monocot.

In transgenic *Arabidopsis*, GUS expression driven from pACO1 was found mainly in actively expanding tissues (Fig. 3.6), including the shoot apical meristem, root tips, initiation sites for lateral roots, and the tips of cotyledons and leaves, which is in basic agreement with observations of native PtACO1 gene expression in loblolly pine tissues. We have no direct information about expression of the native gene product expressed from pACO2 in loblolly pine due to an inability to date to design sufficiently specific primers for qPCR analyses.

Although both the pACO1 and pACO2 constructs were expressed more or less constitutively in *Arabidopsis* root tissues (Figs. 3.6c, 3.8c), their expression patterns were distinctly different in the aerial portions of the transgenic plants, with pACO1 specifically expressed in the ends of leaves and cotyledons (Fig. 3.6a,e) and pACO2 leaf expression associated with the vascular

tissues (Fig. 3.8a,e). ACC oxidase genes from other species are also differentially expressed in different tissues. For example, PP-ACO1 from peach was expressed in flowers, fruitlet abscission zones, mesocarp and in young fully expanded leaves, but PP-ACO2 was only detected in fruits during early development (Ruperti et al., 2001). Three ACC oxidase genes from tomato displayed unique patterns of expression in which NG-ACO1 and NG-ACO3 transcripts were predominantly found in roots or in leaves undergoing senescence, whereas the NG-ACO2 mRNA was found mainly in stems (Kim et al., 1998). Cell-specific transcriptional regulation of the ACC oxidase genes during vegetative tissue development, similar to what was seen with our loblolly pine promoter constructs, was described as ontological in the case of the ACC oxidase genes in white clover (Chen and McManus, 2006).

The strong expression of both promoters in meristematic tissues, as well as expression of pACO1 in the ends of leaves and cotyledons and pACO2 in the end points of leaf vascular tissue suggested endogenous auxin as a driver for expression. Auxin and ethylene coordinately regulate several developmental programs in plants (Swarup et al., 2002), and they have been described to interact at the level of ethylene biosynthesis (Abeles et al., 1995; Woeste et al., 1999). The principal auxin, IAA, is synthesized in meristematic regions at the shoot apex and transported to the root tip, but additional auxin synthesis takes place in young developing leaves and roots (Ljung et al., 2002). These were the same sites where expression of pACO1 or pACO2 was strongest in transgenic *Arabidopsis*. ACO2 ACC oxidase transcripts were increased in response to incubation with IAA (0.1mM) of excised hypocotyls segments of mung bean (Yu et al., 1998), and large increases in levels of the OS-ACO2 ACC oxidase transcript were observed in IAA-treated etiolated rice seedlings (Chae et al., 2000). Similarly, we showed that IAA could increase PtACO1 transcripts about 10 fold after a 3-hour treatment with IAA, and GUS expression in

Arabidopsis plants transformed with two constructs (pACO1-GUS, pACO2-GUS) was up-regulated after a 2-hour 0.1mM IAA treatment. Whether the TGA element (Hagen et al., 1991) located at –385 in the pine promoters is the primary element controlling these responses remain to be seen. Auxin is often linked to gravitropism and polar auxin transport is important for proper gravitropic responses (Morita and Tasaka, 2004). The observation that the pACO2 promoter has a gravitropic response (Fig. 3.13) suggests that further experiments to see whether polar auxin transport interacts with pACO2 expression would be a productive line of inquiry.

The role auxin plays in wood formation has been the focal point of numerous studies. For example, IAA was shown to inhibit tracheid differentiation when supplied to the basal ends of *Pinus contorta* cuttings predisposed to differentiate new tracheids (Savidge, 1983). However, the role of IAA in compression wood formation has been more controversial. The held view, based on experiments using exogenous application of IAA, is that high concentrations induce compression wood (Timell, 1986). However, saturating the polar transport system with exogenous IAA did not result in compression wood formation in *Pinus sylvestris* shoots (Sundberg and Little, 1990). Positive relationships between compression wood formation and increased cambial levels of endogenous IAA were described for *Cryptomeria japonica* (Funada et al., 1990) and *Metasequoia glyptostroboides* (Du et al., 2004), but not *Pseudotsuga menziesii* (Wilson et al., 1989) or *Pinus sylvestris* (Hellgren et al., 2004). Compression wood was associated with IAA in that gravimorphic growth inhibition involved reductions in the shoot's capacity to export IAA and hence to mobilize photoassimilates. Reductions in the supply of available photoassimilates resulted in increased cambial sink activity associated with compression wood formation (Little and Lavigne, 2002). Results with the loblolly pine ACC

oxidase promoters were consistent with what might be expected for genes whose products act to link auxin and compression wood formation.

The pACO1 and pACO2 promoters also showed distinctly different responses to bending stress and fungal infection (Figs. 3.11-3.13). pACO1, but not pACO2, was induced by fungal infection, as well as bending stress. A similar pattern of differential response was observed for two ACC oxidase gene promoters from melon where the CM-ACO1 promoter responded preferentially to stress responses, while the CM-ACO3 responded primarily to developmental processes (Lasserre et al., 1997). Considering the similarity of motifs identified in the two loblolly pine ACC oxidase gene promoters using the plantCARE search tool, the significantly different expression patterns for the two promoters suggests the presence of other regulatory elements for which examples have not yet been identified. More detailed promoter deletion experiments will be necessary to better define the nature of these unidentified elements.

With respect to functional differences between "core" promoters, leaf tip, sepal, and pathogen response expression pattern differences between the pACO1-1.2-GUS and pACO2-1.2-GUS constructs (Figs. 3.7, 3.9) suggest that the pertinent elements controlling these patterns reside in the first 1300 bp of sequence upstream of the transcriptional start site, possibly related to the three major insertions sequences identified in the promoters. Sequences beyond the core promoter appear to regulate expression of pACO1 in apical meristems, and the expression of pACO2 in flower stigmas as well as the gravitropic response. Unfortunately, none of these differences in response could be directly assigned to any of the putative regulatory motifs identified in the two promoters using plantCARE. More detailed promoter dissection analyses will be required to specifically identify the critical regulatory motifs.

One of the most exciting observations from our studies was the asymmetric expression pattern for pACO2 in inflorescence stalks undergoing gravitropic response, as it exactly mirrored the pattern seen for compression wood formation in conifers. Interestingly, the *Arabidopsis* ACC oxidase gene, AtACO2, is differentially expressed in outer versus inner hook tissues (Raz and Ecker, 1999). Provost et al. (2003) demonstrated that an ACC oxidase identified in maritime pine using cDNA-AFLP analyses showed clear differences between compression wood and opposite wood expression. Thus, we believe that the gene product expressed *in vivo* from the pACO2 promoter may be particularly important in the process of compression wood formation.

This is the first study of ACC oxidase promoters from a coniferous species, and the results lend further support to the idea that ethylene biosynthesis may play a fundamental role in the formation of compression wood. Heterologous expression of the various loblolly pine ACC oxidase gene promoter constructs in transgenic *Arabidopsis* yielded gene expression patterns consistent with what might be expected for these promoters *in vivo*, which opens their detailed analyses to use of the many genetic tools available for studies of *Arabidopsis* gene regulation. We anticipate that these constructs and transgenic lines will enable us to identify the specific regulatory signals controlling the expression of these genes during the process of compression wood formation. In addition, we anticipate that this work will provide useful information for breeding efforts to alter compression wood formation in commercial conifers.

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Figure 3.1. Sequence alignment of the pACO1 and pACO2 promoters. The underlined nucleotides correspond to the primer used to recover the truncated promoters, pACO1-1.2 and pACO2-1.2. Black shading denotes identical nucleotides, while light gray shading indicates sequence differences. TATA-Box and the transcription starting site and translation start codon are also shown.

Figure 3.2. Nucleotide sequence of the genomic DNA upstream of the loblolly pine ACC oxidase gene, PtACO1. The region containing the promoter and putative *cis*-acting elements controlling expression of the gene, where ► denotes the position of the primer used to amplify the truncated pACO1-1.2 and pACO2-1.2 fragments. Potential *cis*-acting element include LTR, a low temperature responsive element (White et al., 1994); ERE, an ethylene-responsive element (Itzhaki and Woodson, 1993); the TGACG-motif, a MeJA-response element; the TGA-box, part of an auxin responsive element (Hagen et al., 1991); the TCA-element, involved in salicylic acid responsiveness (Pastuglia et al., 1997); the Sp1, light response element (Litts et al., 1992); the G-box, light response element (Rouster et al., 1997); the MRE, light response element (Feldbrugge et al., 1997); ABRE elements, involved in the abscisic acid responsiveness (Yamaguchi-Shinozaki and Shinozaki, 1993); and DRE, an element involved in dehydration, low-temp, and salt stress responses (Yamaguchi-Shinozaki and Shinozaki, 1993).

Figure 3.3. Expression of PtACO1 in loblolly pine seedling stems treated with IAA. Quantities are expressed as ratios of transcript levels as determined by qPCR between treated and untreated stem of 7-month-old seedlings. Green bar - IAA treatment; Pink bar – water.

Figure 3.4. Induction of PtACO1 gene expression in response to bending treatment loblolly pine seedlings. Quantities are expressed as ratios of transcript levels as determined by qPCR between bent and unbent stems of 7-month-old seedlings.

Figure 3.5. Relative levels of PtACO1 expression in tissues on opposing sides of bent loblolly pine seedling stems. Quantities are expressed as ratios of transcript levels as determined by

qPCR between bent and unbent stems of 7-month-old seedlings. Abbreviations represent shoot tips (ST), compression wood (CW), and opposite wood (OW).

Figure 3.6. Histochemical localization of GUS activity in transgenic *Arabidopsis* harboring the pACO1-GUS construct.

Figure 3.7. Histochemical localization of GUS activity in transgenic *Arabidopsis* harboring the pACO1-1.2-GUS construct.

Figure 3.8. Histochemical localization of GUS activity in transgenic *Arabidopsis* harboring the pACO2-GUS construct.

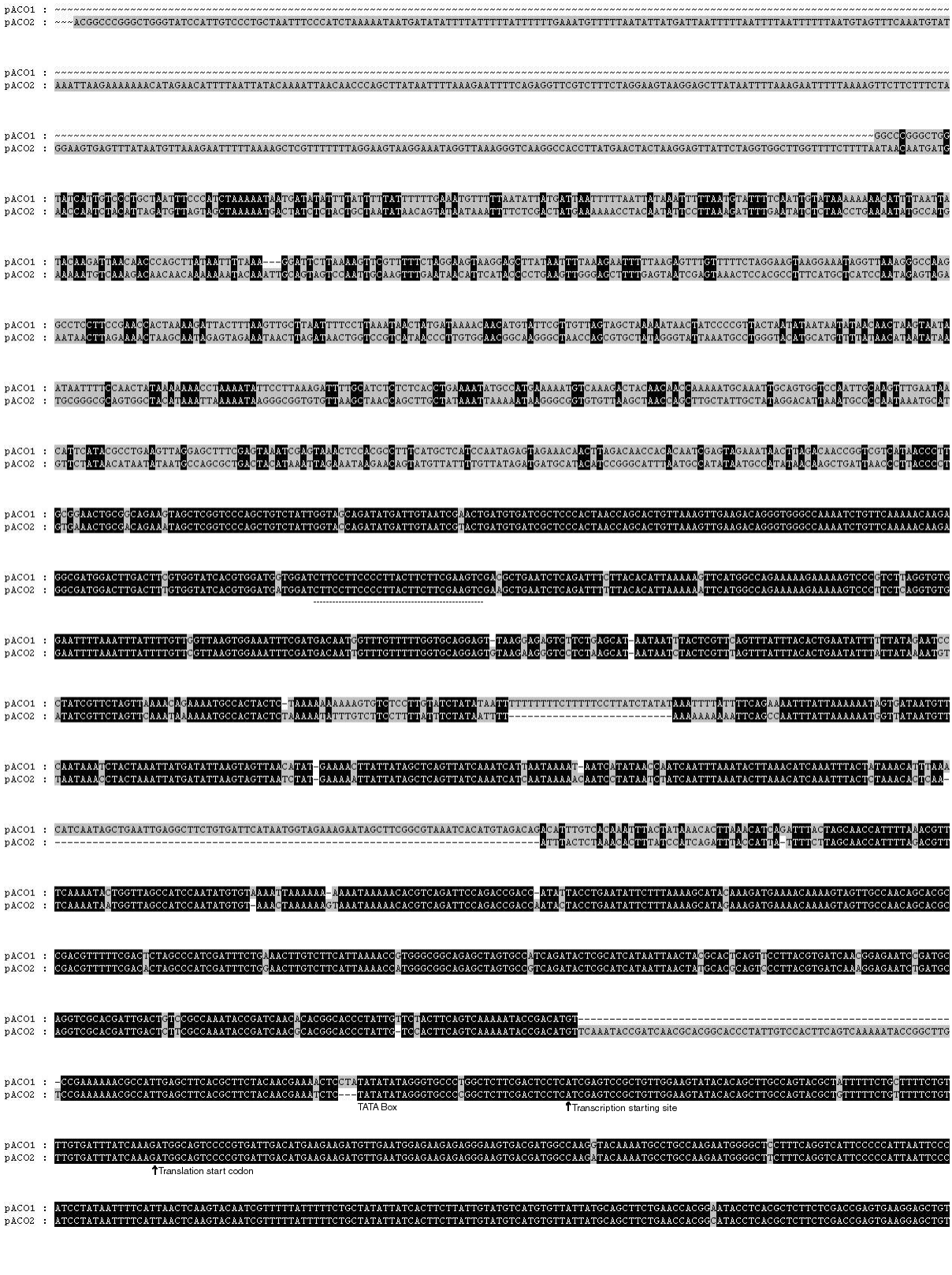
Figure 3.9. Histochemical localization of GUS activity in transgenic *Arabidopsis* harboring the pACO2-1.2-GUS construct.

Figure 3.10. Histochemical localization of GUS activity in transgenic *Arabidopsis* treated with 100μM IAA for 2 hours.

Figure 3.11. Histochemical localization of GUS activity in transgenic *Arabidopsis* infected by an unknown fungus. Leaves from plants transformed with: a) p35S-GUS; b) pACO1-GUS; c) pACO1-1.2-GUS; d) pACO2-GUS; e) pACO2-1.2-GUS, were stained for GUS after fungal infection.

Figure 3.12. Histochemical localization of GUS activity in inflorescence stalks of transgenic *Arabidopsis* after bending treatments.

Figure 3.13. Histochemical localization of GUS activity in transgenic *Arabidopsis* undergoing a gravitropic response. (a) Transgenic plants were kept in a horizontal position for 48 hours, after which shoop tips, lateral shoots and stalks were collected for GUS staining (b) 1. Lateral shoot taken from a plant containing the pACO1-GUS construct; 2. Lateral shoot taken from a plant containing the pACO2-GUS construct.


pACO1 :
pACO2 :
TATA Box
Transcription starting site
Translation start codon

```
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      ERE(-)  HSE(-)
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AGTTGCTTAATTTTCCTTAAATAACTATGATAAAACAACATGTATTCGTTGTTAGTAGCTAAAAA-1619
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   MRE(+)                          GAG-box(-)  G-box(+)
AAACCTAAAATATTCCTTAAAGATTTTGCATCTCTCTCACCTGAAAATATGCCATGAAAAATGTC-1489
AAAGACTACAACAACCAAAAATGCAAATTGCAGTGGTCCAATTGCAAGTTTGAATAACATTCATA-1424
CGCCTGAAGTTAGGAGCTTTCGAGTAAATCGAGTAAACTCCACGCCTTTCATGCTCATCCAATAG-1359
    AE-box(+)                                                  TGACG-motif(-)
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CGAACTGATGTGATCGCTCCCACTAACCAGCACTGTTAAAGTTGAAGACAGGGTGGGCCAAAATC-1164
                                      G-box(+)  or  ABRE(+)
TGTTCAAAAACAAGAGGCGATGGACTTGACTTCGTGGTATCACGTGGATGGTGGATCTTCCTTCC-1099
                                    TC-rich repeat                •
CCTTACTTCTTCGAAGTCGACGCTGAATCTCAGATTTCTTACACATTAAAAAGTTCATGGCCAGA-1034
TCA-element(+)
AAAAGAAAAGTCCCGTCTTAGGTGTGGAATTTTAAATTTATTTTGTTGGTTAAGTGGAAATTTC -969

GATGACAATGGTTTGTTTTTGGTGCAGGAGTTAAGGAGAGTCTTCTGAGCATAATAATTTACTCG -904
                                    GATA-motif       GARE-motif(+)
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                                                              G-box(+)
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GACAGACATTTGTCACAAATTTACTATAAACACTTAAACATCAGATTTACTAGCAACCATTTTAA -449
                                                    TGA-box,ABRE,TGACG-motif(-)
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ATTCCAGACCGACCATATTACCTGAATATTCTTTAAAAGCATACAAAGATGAAAACAAAAGTAGT -319
TGCCAACAGCACGCCGACGTTTTTCGACTCTAGCCCATCGATTTCTGAAACTTGTCTTCATTAAA -254
     Sp1(+)
ACCGTGGGCGGCAGAGCTAGTGCCATCAGATACTCGCATCATAATTAACTACGCACTCAGTTCCT -189
ABRE(+)                                 CAAT-box(-)   CAAT-box(+)
TACGTGATCAACGGAGAATCCGATGCAGGTCGCACGATTGACTGTCCGCCAAATACCGATCAACA -124
                                                    LTR(+)
CACGGCACCCTATTGTTCTACTTCAGTCAAAAATACCGACATGTCCGAAAAAACGCCATTGAGCT -59
                                TATA-box                    Transcription starting site
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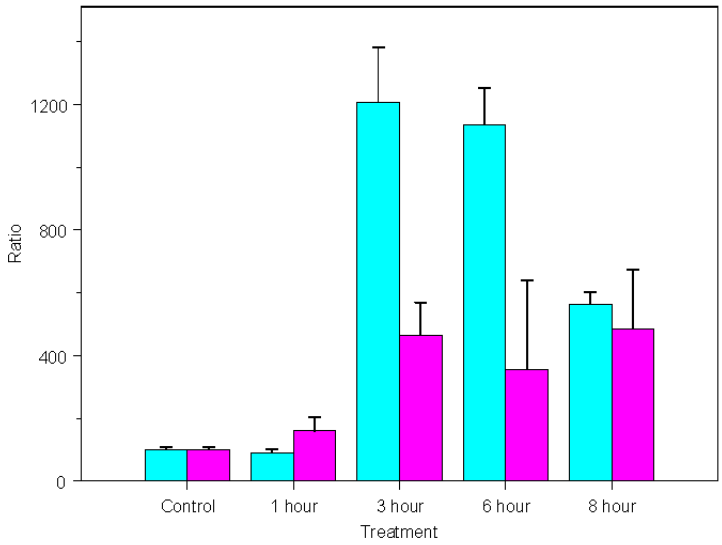
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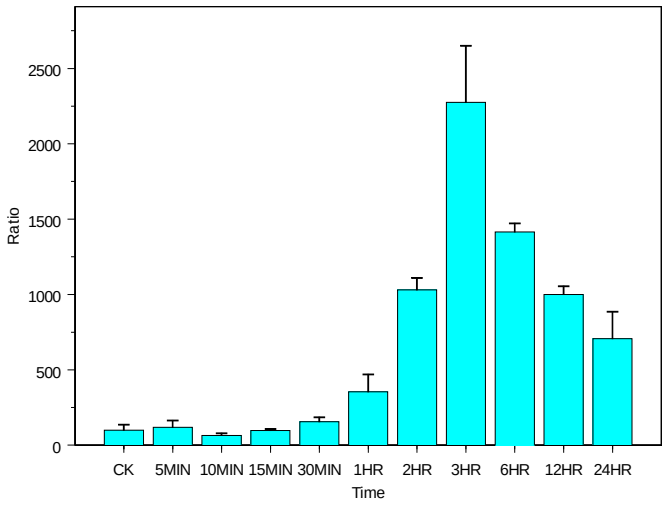
6 hour
8 hour
Treatment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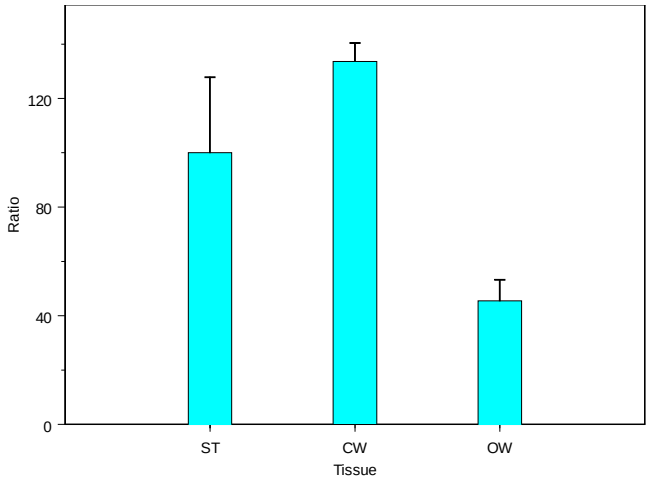
Time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Ratio
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CW
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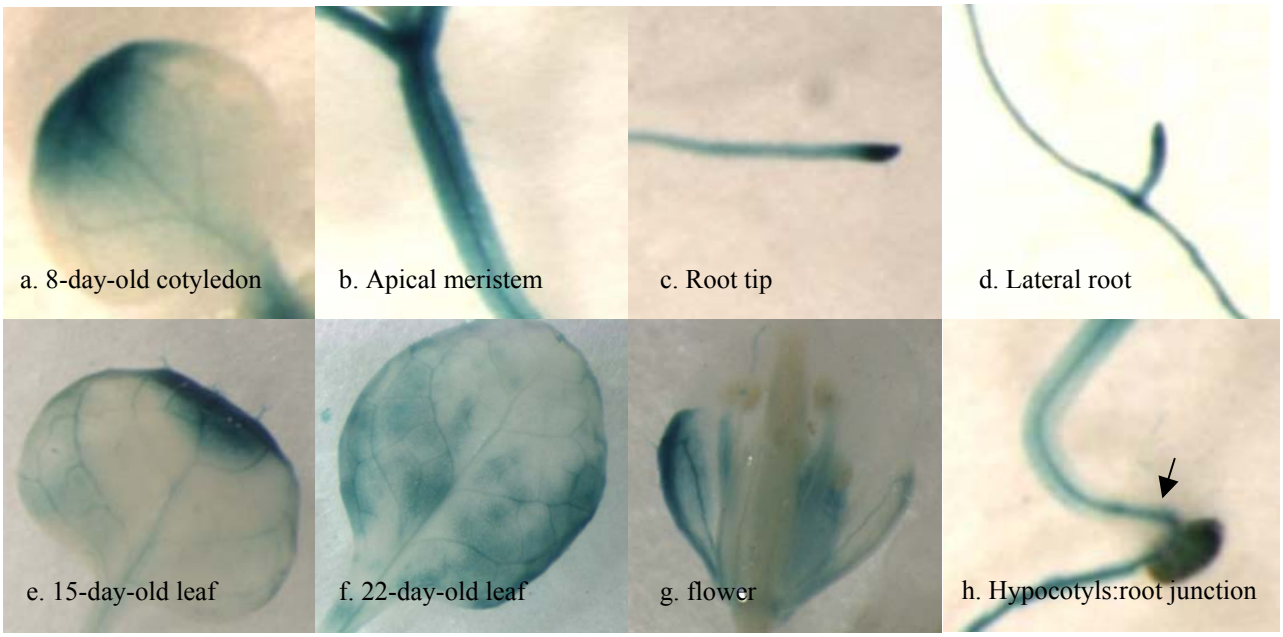
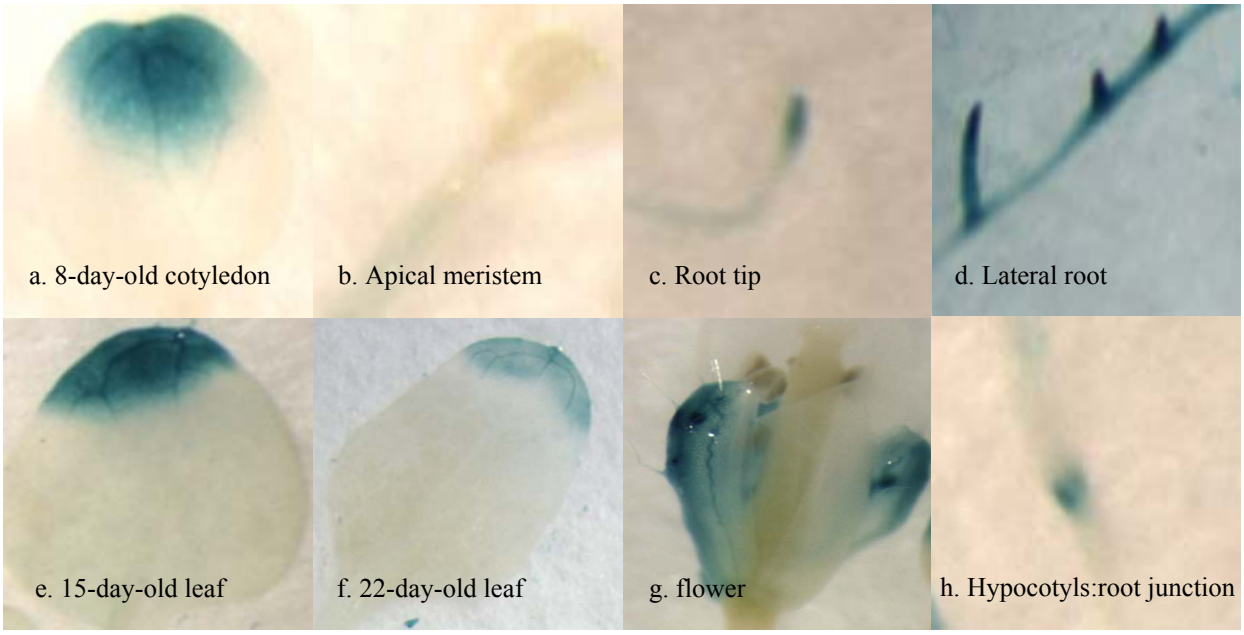
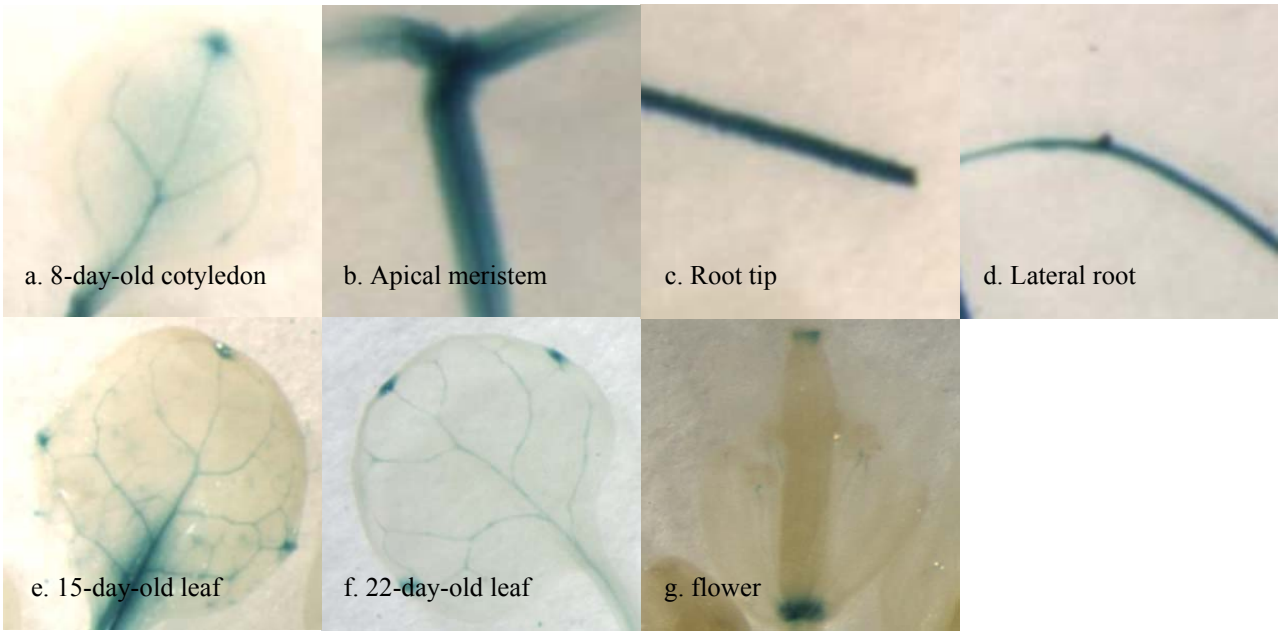

a. 8-day-old cotyledon
b. Apical meristem
c. Root tip
d. Lateral root
e. 15-day-old leaf
f. 22-day-old leaf
g. flower
h. Hypocotyls:root junction


a. 8-day-old cotyledon
b. Apical meristem
c. Root tip
d. Lateral root
e. 15-day-old leaf
f. 22-day-old leaf
g. flower
h. Hypocotyls:root junction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b. Apical meristem
c. Root tip
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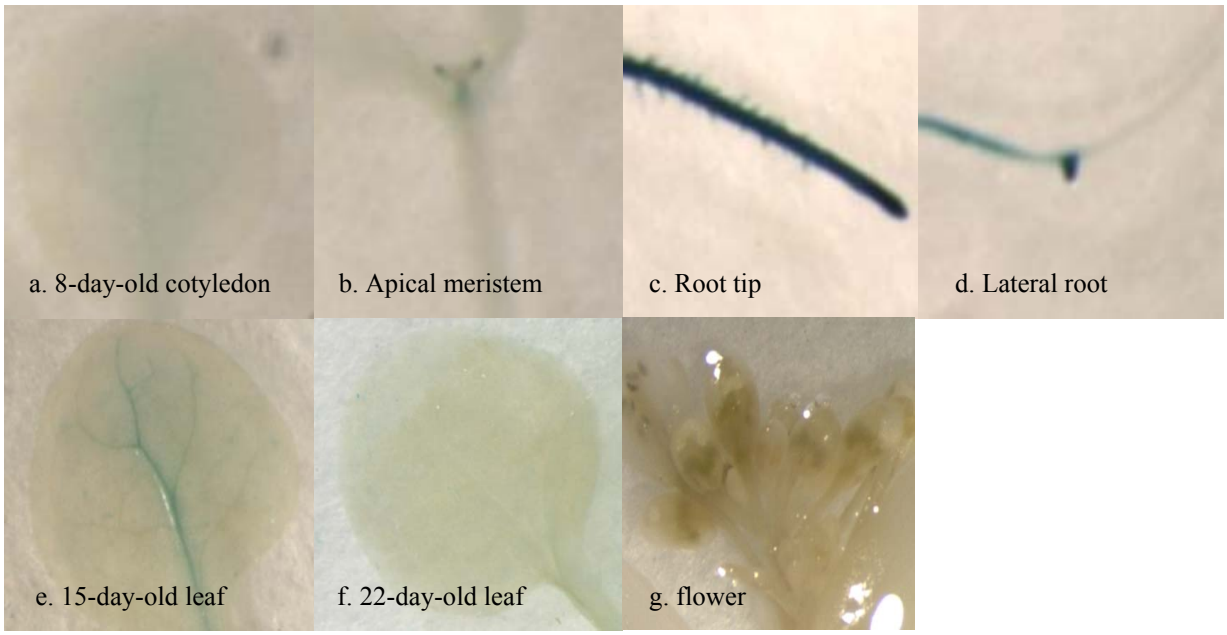
e. 15-day-old leaf
f. 22-day-old leaf
g. flower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c. Root tip
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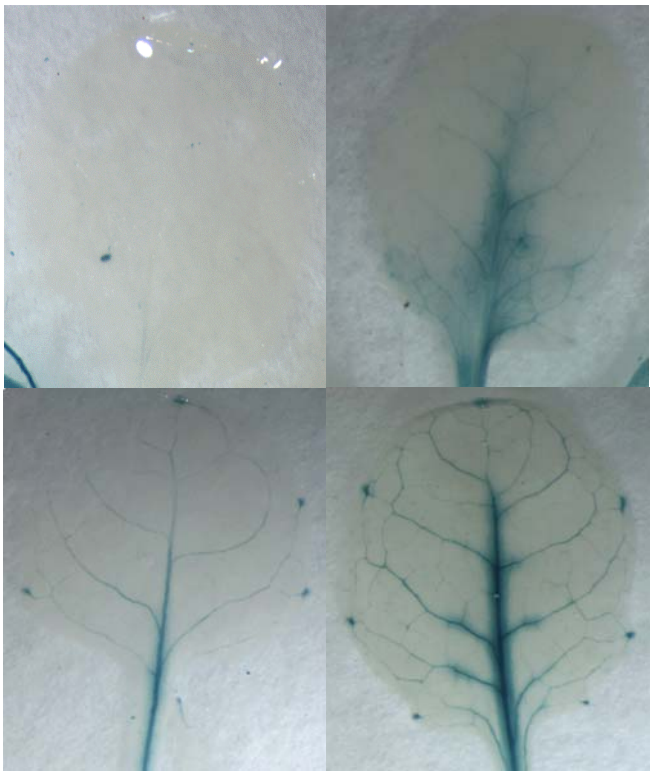
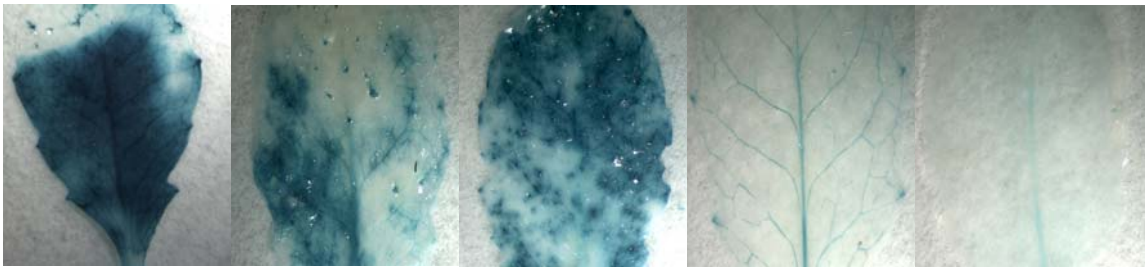
f. 22-day-old leaf
g. flower



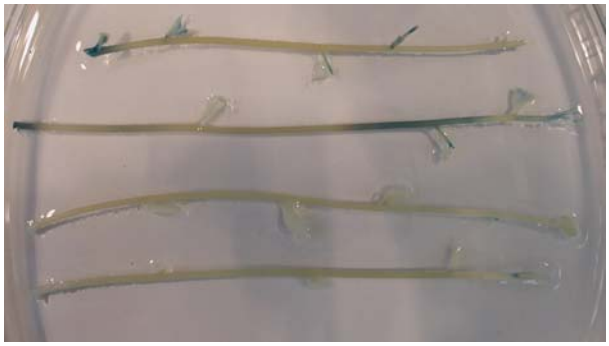
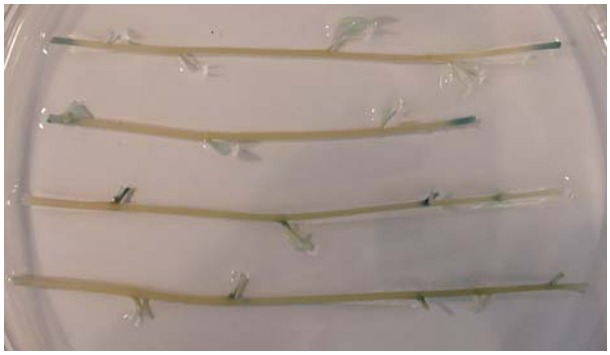
pACO1f

pACO2f

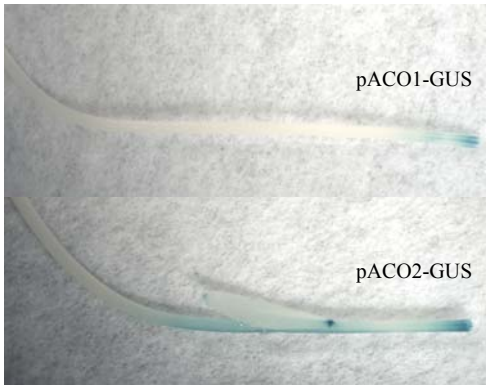
Water 100µM IAA



a b c d e

Bending	promoter
	pACO1
+	
	pACO1-1.2
+	
	pACO2
+	
	pACO2-1.2
+	

a



b

CHAPTER 4

PHYLOGENETIC ANALYSIS OF THE 2-OXOGLUTARATE-DEPENDENT DIOXYGENASE GENE FAMILIES FROM ARABIDOPSIS, RICE, AND POPLAR [*]

[*] Yuan S, Dean JFD to be submitted to Genome Biology.

Introduction

With the completion of genome sequencing for three higher plant species - *Arabidopsis thaliana* (Tabata et al., 2000; Salanoubat et al., 2000), rice (*Oryza sativa*) (Goff et al., 2002) and poplar (*Populus trichocarpa*) (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) - automated gene prediction, gene comparison and gene annotation have become important tools for identifying potential functions of genes in these plant species. Computational gene prediction is a very active area of research in bioinformatics (Mathé et al., 2002), but the reliability of the current gene-finding methods is still limited (Guigó et al., 2000). The primary approaches to gene annotation are based on the recognition of coding-sequence similarities by comparison to protein sequences of known function, and subsequent assignment of various levels of confidence to these predictions using computer programs, such as BLAST (Altschul et al., 1990), PSI-BLAST (Altschul et al., 1997) or Pfam (Sonnhammer et al., 1997). These methods have been used to automatically generate functional annotations for a large fraction of the genes identified in newly sequenced genomes.

Plant 2-oxoglutarate-dependent dioxygenases (2-ODDs) constitute a class of non-heme, iron-containing cytosolic enzymes that utilize a 2-oxoacid as a cosubstrate and a reducing agent, typically ascorbate, to regenerate the reactive iron moiety. The common link for all of the members of this family is that they activate a molecule of dioxygen through enzyme-bound ferrous ions to generate a highly reactive ferryl oxidant. The formation of the ferryl species is linked to the oxidative decarboxylation of 2-oxoglutarate, giving rise to succinate and CO_2, and subsequent hydroxylation of the substrates (Blanchard and Englard, 1983; Hanauske-Abel, 1982). Thus, all members of this family are dependent on ferrous ions, oxygen, and 2-oxoglutarate (or a similar 2-oxoacid) for activity. This widespread class of enzymes has been

implicated in a variety of plant metabolic pathways, including the biosynthesis of certain amino acids, hormones, signaling molecules and a variety of secondary metabolites (Prescott and John, 1996). At the phylogenetic level, plant 2-ODDs comprise an enzyme superfamily with numerous subgroups whose subsets are defined by shared motifs in the encoded proteins. These motifs often comprise the active site of the enzyme and/or binding domains for substrates and cofactors.

ACC oxidase is a member of the 2-ODD superfamily and, as described in a previous chapter, a limited phylogenetic study was used to strengthen the tentative identification of *Pinus taeda* cDNA sequences as encoding ACC oxidases. In this study, more extensive phylogenetic analyses were used to classify and group 345 probable 2-ODD protein sequences identified in the fully sequenced genomes of three higher plant species, *A. thaliana*, rice (*O. sativa*) and poplar (*P. trichocarpa*). The superfamily can be subdivided into five groups and 21 subgroups, with each subgroup having a distinct gene structure and likely protein function.

Materials and Methods

The cDNA sequence of *A. thaliana* ACC oxidase gene, AT1G05010, a member of the 2-ODD family, was used to search the Arabidopsis Genome Initiative (AGI) protein database (http://www.arabidopsis.org/Blast/) using "BLASTX". To exhaustively search the genome related sequences, the expectation value was set to 100 while all other search parameters were left at default values. Returned sequences that were not annotated as "2-OG-dependent Fe(II)" (expected value <0.40) were not included in furthering subsequent alignments. The search returned 111 protein sequences that were used in the alignments.

Hieta and Myllyharju (2002) reported that gene, AT2G43080, encodes a prolyl 4-hydroxylase, a recognized member of the 2-ODD family, but this gene was not among the 111 protein sequences recovered in the initial BLASTX search. The cDNA sequence of AT2G43080

was used to perform a second BLASTX search using the same parameters mentioned previously and 12 additional proteins sequences were recovered and pooled with the sequences returned from the initial search. Thus a total of 122 2-ODD proteins sequences from *A. thaliana* were collected for this phylogenetic study.

Using a similar approach, the cDNA sequence for the rice ACC oxidase gene, OS1A1COX, was used in a TBLASTN search of the *O. sativa* protein database (http://www.ncbi.nlm.nih.gov/BLAST/Genome/plantBlast.shtml). 9 rice P4H sequences were recovered by a BLASTP search (expected value < 3e-22) of the same database using the protein sequence of the *Arabidopsis* P4H gene, AT2G43080. A total of 82 putative 2-ODD protein sequences (expected value < 6e-04) were recovered for rice and included in the phylogenetic analyses.

At the time this work was completed, the *P. trichocarpa* genome sequence was not yet available in a format that could be easily searched using the BLAST-based approaches used for *Arabidopsis* and rice. As an alternative approach, the euKaryotic Orthologous Groups (KOG; Tatusov et al. (2003) browser for the poplar genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) was used to identify the poplar genes already annotated as likely belonging to the 2-ODD family. Under the "Secondary metabolites biosynthesis, transport and catabolism" function category of KOG database, "iron/ascorbate family oxidoreductases" are listed in the group KOG0143, which contains 166 gene models. From that collection only the 128 genes identified as containing the "2OG-Fe(II) oxygenase superfamily" were used for the phylogenetic analyses. Together with the 13 putative poplar P4H genes listed in KOG1591 under the category of "Amino acid transport and metabolism", overall 345 protein sequences from the three plant species were included in the subsequent studies.

Multiple sequence alignment was performed using the ClustalW program in the GCG software suite (Wisconsin Package, Version 10.1, Genetic Computer Group, Madison, WI). The resultant multiple sequence file was imported into GENEDOC (Nicholas et al., 1997) for manual adjustment and exported in PHY file format. The PHY file was used to run the protein maximum likelihood program with molecular clock (ProMLK) executable file in PHYLIP (Felsenstein, 1993). The output files from ProMLK were further edited in TreeView (Page, 1996) to generate phylogenentic trees.

With respect to gene nomenclature used in the final output of this study, for genes from *Arabidopsis*, the AGI gene name was used to identify each gene and the corresponding protein product. For genes having alternative splicing products, an additional numeral was added to the end of the gene name. For example, gene AT5G59540 has two recognized alternative splicing products for which the translated products were designated AT5G595401 and AT5G595402. For 2-ODD genes from rice, the protein product name was used to identify the translated product; for example, XP_470509 and NP_922524. Genes from poplar were identified by the proteinId number and the prefix PT_, such as PT_48812 or PT_51800.

Results and Discussion

2-ODD proteins: classification and functional prediction

Members of the 2-ODD protein superfamily are characterized by a few highly conserved residues that are involved in binding of the ferrous ion in the active site. These residues form a conserved motif, designated the 2-His-1-carboxylate facial triad, typical of non-heme Fe(II) enzymes (Hegg and Que, 1997, Fig. 4.1). Different criteria, including cosubstrate use and substrate specificity, have been used in classification of this protein superfamily, but 2-ODD family members are most often classified into two subclasses according to the cosubstrate. ACC

oxidase, also called ethylene-forming enzyme or EFE (Holdsworth et al., 1987)), gibberellin 20-oxidase (GA 20-ox) (Philips et al., 1995) and isopenicillin N-synthase (INPS) (Ramon et al., 1987) are among those in the family that utilize ascorbate as a cosubstrate, while most other members of the family utilize 2-oxoglutarate instead (French et al., 1989). Mononuclear non-heme Fe (II) enzymes containing a 2-His-1-carboxylate facial triad has been classified into five groups, in which the group of α-Ketoglutarate (KG) enzymes (except 4-hydroxyphenolpyruvate dioxygenase (HPPD)) and the group of other oxidase (including INPS and ACC oxidase) containing a structure motif of jelly fish and a sequence motif of $HXDX_nH$, where n varies from 48 to 153 (Koehntop et al., 2005).

Alignments of the 325 inferred 2-ODD protein sequences from *Arabidopsis*, rice and poplar were analyzed using the maximum likelihood approach to generate the phylogenetic tree depicted in Figure 4.2. The phylogenetic tree is divided into nine distinct branches or groups (I, II, III, IV, V, VI, VII, VIII and IX), and each group comprises one or several subgroups. Twenty-one distinct subgroups can be identified in Figure 4.1, which matches nicely with the results of Prescott and John (1996) who listed 21 different classes of 2-ODD superfamily members from an analysis across plants, animals and fungi. However, several highly divergent proteins could not be assigned to specific groups in our analysis. Thus, perhaps not surprisingly, a more recent analysis of the 2-ODD superfamily spanning multiple kingdoms has identified 25 distinct enzyme classes under the EC 1.14.11 designation, three of which were only designated in 2005 (http://www.brenda.uni-koeln.de/) (Schomburg et al., 2000). This 325 inferred 2-ODD protein sequences contain common sequence motif of $HXDX_nH$, where n varies from 53 to 56, with the exception of P4H family, which has n of about 80. This is also the reason why we could not find

the third conserved amino acid Histidine in Figure 4.1C generated by Weblogo (Crooks et al., 2004).

Tentative functional identifications were assigned to each subgroup in our analysis based on the presence of protein sequences with known functions or activities within the subgroup. Subgroups that did not contain sequences with recognized functions were submitted for BLAST comparisons to the protein database in GenBank. Subgroups were assigned function as "unknown" if the BLAST comparisons of individual sequences within any subgroup did not return a match or return matches that has already been assigned to other groups. Most subgroup designations were decided on the basis of gene products from *Arabidopsis* since this species is the best studied amongst the higher plant species in this analysis. The functional predictions for each subgroup are detailed in Table 4.1.

Group I of the 2-ODD phylogeny could be broken into 4 subgroups (Fig. 4.2), in which 2 groups were tentatively identified as likely involved in biosynthesis of flavonoids. Flavonoids, whose biosynthesis in *Arabidopsis* has been reviewed elsewhere (Graham 1998), are important for such diverse functions as determining flower (Scott-Moncrieff, 1936) and fruit colors (Yoshitama et al., 1992), and pollen tube growth (Mo et al., 1992). These compounds are also involved in UV-protection, metabolism and transport of auxin, and they are potent defensive chemicals, or phytoalexins (Stafford, 1990). The 2-ODD dioxygenases that are involved in biosynthesis of flavonoids include flavonone 3-hydroxylase (F3H), flavonol 6-hydroxylase (F6H), flavonol synthase (FLS), flavone synthase I (FSI), and anthocyanidin synthase (ANS).

FLS introduces a double bond between the C-2 and C-3 positions in the C-ring of dihydroflavonol (Holton et al., 1993). Its activity has been detected in a variety of plant tissues, including illuminated parsley cell cultures (Bristch et al., 1981), extracts from *Matthiola incana*

flowers (Spribille and Forkmann, 1984), *Petunia hybrida* flowers (Forkmann et al., 1986) and *Dianthus caryophyllus* flowers (Forkmann, 1991), *Citrus unshiu* (Moriguchi et al., 2002). An FLS gene was first cloned from *Petunia hybrida* (Holton et al., 1993).

FSI catalyzes the conversion of flavonones to flavones by introducing a double bond between the C-2 and C-3 carbons of the C ring, and 2-ODDs catalyzing this activity have so far only been found in parsley and closely related species (Britsch, 1990). However, the parsley gene has been cloned and expressed in yeast (Martens et al., 2001).

The exact *in vivo* role of ANS remains uncertain, and probably varies between plant species. In general, ANS is thought to be responsible for the formation of the colored anthocyanidins from the colorless leucoanthocyanidins, but *in vitro* incubation of purified *Arabidopsis* ANS with 2R,3S,4S-*cis*-leucocyanidin produced the expected cyanidin as only a minor product. The major product of the reaction was quercetin, which arises from a 4-electron oxidation that requires two catalytic cycles (Turnbull et al., 2003). ANS can catalyze the oxidation of other flavonoid substrates, often producing a mixture of products harboring desaturation products that could arise via either oxygenating or non-oxygenating mechanisms (Welford et al., 2005). These studies have characterized ANS as a relatively non-specific dioxygenase, but an ANS gene from *Perilla frutescens* was cloned and its product was characterized as a leucoanthocyanidins (Saito et al., 1999).

F3H, which converts (2S)-flavanones to (2R, 3R)-dihydroflavonols, was first described in crude extracts from *Mantthiola incana* (Spribille and Forkmann, 1984) and parsley cell cultures (Britsch et al., 1981). A gene encoding the enzyme was subsequently cloned from *Petunia hybrida* and functionally expressed as a highly active recombinant enzyme in *Escherichia coli* (Britsch et al., 1992).

F6H, a recently identified flavonol-associated 2-ODD enzyme, catalyzes the 6-hydroxylation of partially methylated flavonols, and is structurally related to F3H. It is present as a single copy gene in the genome of a semi-aquatic weed, *Chrysosplenium americanum*, and a regulatory role in polymethylated flavonol biosynthesis has been suggested for the enzyme (Anzellotti and Ibrahim, 2001).

The existing gene annotations for *Arabidopsis* clearly identify the FLS, ANS subgroups of 2-ODD enzymes. And the subgroups left unidentified might also involved in flavonoid biosynthesis, which deserve to study further.

F3H was not included in Group I in the phylogenic tree. Instead, it diverged quite away from this group and was classified as Group V in our study. The reason why F3H diverges from other 2-ODD members in flavonoid biosynthesis is not clear, but F3H is the most conserved family in the flavonoid 2-ODDs (Fig. 4.3) (The actual number of amino acids in the flavonoid 2-ODDs are shown in Table 4.2). F3H from *Saussurea medurea* was shown to have high similarity to hyosyamine 6-hydroxylase (H6H) (Jin et al., 2005), which is also a member of 2-ODD family. F3H has been shown to be present as a single gene in maize (Deboo et al., 1995), alfafa (Charrier et al., 1995), Arabidopsis (Pelletier and Shirley, 1996) and *Saussurea medusa* (Jin et al., 2005). In our study, F3H in rice is encoded by single gene while in poplar, there are three genes encoding F3H protein.

Putative function of the 3 subgroups in Group II could not be reliably assigned from the available data (Fig. 4.2). Among the potential candidate activities for defining these groups are the E8 protein from tomato, which has previously been described as related to ACC oxidase (Peňarrubia et al., 1992). E8 proteins, which are encoded by a small multigene family in tomato (Kinzer et al., 1990), are regulated by ethylene during tomato fruit ripening and have been shown

to have a negative effect on ethylene biosynthesis (Peňarrubia et al., 1992). Another potential candidate is desacetoxyvindoline 4-hydroxylase (D4H, EC 1.14.11.11), which catalyzes hydroxylation of the C-4 carbon in the indole alkaloid, desacetoxyvindoline. This 2-ODD enzyme has only been studied in *Catharanthus roseus* (De Carolis and De Luca, 1993; Vazquez-Flota and De Luca, 1997), and the inability of cell cultures of *Catharanthus roseus* to produce vindoline was related to lack of expression of the D4H gene (Vazquez-Flota, F., 2002). D4H had high affinity for desacetoxyvindoline, and similar high affinity was observed for another 2-ODD enzyme involved in biosynthesis of a related metabolite, scopolamine (Hashimoto and Yamada, 1987). Our analyses noted that the two introns in the D4H gene were inserted in conserved positions flanking highly conserved amino acid consensus sequences in the genes encoding E8 protein, hyoscyamine-6β-hydroxylase (H6H), and ACC oxidase (Vazquez-Flota et al., 1997). This suggests that the Group II subgroups designated as "Unknowns" might be productively tested for involvement in indole alkaloid biosynthesis.

Group III only consists of the ethylene biosynthetic enzyme, ACC oxidase. ACC oxidase is the final enzyme in the ethylene biosynthesis pathway and is arguably the most studied of all plant 2-ODD superfamily members. Three of the recognized *Arabidopsis* ACC oxidases, ACO1, ACO2, ACO3 can resolve this group easily.

Group IV is possibly the most diverse in this 2-ODD superfamily phylogeny, yet relatively few functional assignments could be made for this group. The subgroup designated Unknown-6 contains XP_476744, a rice gene annotated in GenBank as encoding a putative Ids3 enzyme. Ids3, and the related gene, Ids2, encode 2-ODD enzymes commonly called 2'-deoxymugineic-acid 2'-dioxygenase (EC 1.14.11.24) and mugineic acid 3-dioxygenase (EC 1.14.11.25), respectively. These two enzymes are induced by Fe-deficiency in barley roots (Nakanishi et al.,

1993; Okumura et al., 1994), and are involved in the biosynthesis of the mugineic acid family of phytosiderophores (MAs) used by graminaceous plants to chelate and take up iron from the soil (Takagi, 1984). The IDS2 and IDS3 enzymes are thought to be involved in hydroxylation of MAs. According to the expression patterns of the encoding genes, it is thought that IDS3 hydroxylates the C-2 position of 2'-deoxymugineic acid (DMA) and 3'-epihydroxy-2'-deoxymugineic acid (epiHDMA), while IDS2 hydroxylates the C-3 position of mugineic acid (MA) and DMA (Nakanishi et al., 2000). In the subgroup identified as Unknown-8, the *Arabidopsis* AT1G06650 gene product shared relatively high homology with the maize Bx6 gene product. The Bx6 protein is a 2-ODD enzyme that catalyses introduction of a OCH_3-group at the C-7 position in the benzoxazinone, DIMBOA, a compound that has deters insect feeding on maize (Frey et al., 2003). Interestingly, the Unknown-8 subgroup also contains AT1G06620, which encodes the closest *Arabidopsis* homolog for the tomato (*Lycopersicon esculentum* Mill) LeODD gene product. LeODD is specifically expressed during the cell expansion phase in tomato fruit reaching peak expression about 15 days after flowering (Ohta et al., 2005).

Group VI contains enzymes from the gibberellin biosynthetic pathway - gibberellin 20-oxidase, gibberellin 3β-hydroxylase, and gibberellin 2β-hydroxylase. The gibberellins (GAs) are tetracylic diterpenoid compounds that play important roles in many aspects of plant growth and development, including promotion of cell division and extension, seed germination, stem growth, and fruit set (Crozier, 1983; Hedden and Kamiya 1997). In plants, all GA biosynthetic steps after GA_{53} are catalyzed by dioxygenases (Hedden and Kamiya, 1997; Hedden and Philips, 2000). Last two pathway intermediates, GA_{12} and GA_{53}, are converted by the 2-ODD enzyme, GA 20-oxidase, to GA_9 and GA_{20}, respectively. These compounds can in turn be converted to bioactive GA_4 and GA_1, respectively, through the action of the 2-ODD enzyme, GA 3β-hydroxylase. In

some species, yet another 2-ODD enzyme, GA 2-oxidase, deactivates GAs by 2β-hydroxlation and, in some cases, further oxidation to a ketone function at C-2 position. In this way, GA_9 and GA_{20} can be converted to GA_7 and GA_3, respectively, proceeding through 2, 3-didehydroGA_9 and GA_5 intermediates.

Group VII is made up of 2 subgroups, of which the function is undetermined. According to BLAST search, the *Arabidopsis* 2-ODD sequence most homologous to pumpkin GA 7-oxidase is AT5G05600, which falls into the flavone synthase family in Group II, two other *Arabidopsis* 2-ODDs, AT3G19000 and AT3G19010, are among the *Arabidopsis* proteins next most closely related to GA 7-oxidase. Both AT3G19000 and AT3G19010 fall into the "Unknown-9" subdivision of Group VII, which is next to Group VI containing enzymes involving in GA biosynthesis, suggesting that these gene products might be suitable for testing as GA 7-oxidases. The 2-ODD enzyme, GA 7-oxidase, from pumpkin has been shown to oxidize GA_{12}-aldehyde and GA_{14}-aldehyde to GA_{12} and GA_{14}, respectively (Lange, 1997), but this activity has not been documented in other plant species.

There are three subgroups within the Group VIII 2-ODDs, but a tentative function could only be assigned to one of them. The "AOP" family contains three Arabidopsis genes, APO1, APO2, and APO3, that are involved in the biosynthesis of glucosinolates, which are defense compounds found in many higher plants (Fahey et al., 2001). Through heterologous expression in *E. coli* and correlation of gene expression patterns to glucosinolate composition, AOP2 was shown to direct the conversion of methylsulfinylalkyl glucosinolates to alkenyl glucosinolates, while AOP3 catalyzes the reverse reaction, formation of hydroxyalkyl glucosinolates from methylsulfinylalkyl glucosinolates (Kliebenstein et al., 2001). Although a tentative function cannot yet be assigned to the subgroup identified as Unknown-10, our analysis noted an unusual

gene structure for members of the class with intron number varying from nine to eleven. This seemed quite unusual for the 2-ODD superfamily genes, most of which contained only from one to four introns. .

Group IX contains only members of the prolyl 4-hydroxylase gene family (P4H, EC 1.14.11.2). P4H catalyzes hydroxylation of prolyl residues in specific polypeptides that are incorporated into the collagens of animals and the cell walls of higher plants and algae (Prescott and John, 1996). Although P4Hs have been cloned and characterized from some animal sources (Hopkinson et al., 1994; Annunen et al., 1997), the *Arabidopsis* P4H is the only one so far cloned and characterized from a plant (Hieta and Myllyharju, 2002; Myllyharju, 2003). According to the phylogenetic tree (Fig. 4.2), the P4H family is quite divergent from other groups within the 2-ODD gene family.

Obviously, a great deal of work remains to identify functions for a majority of the 2-ODD enzymes identified in this study, and it is likely that additional 2-ODD superfamily members exist within the *Arabidopsis*, rice and poplar genomes. Despite our initial attempts to exhaustively search these genomes by setting the expected value BLAST search parameter ≤1, we failed to capture the P4H by this method. At least three factors contributed to our difficulties in identifying all 2-ODD genes in these genomes. First, the reliability of current gene prediction methods is limited (Guigó et al., 2000). Next, the BLAST algorithm is limited with respect to which of the two adjunct highest scores will be ignored when the random walk begins to extend the highest hit (Frith et al., 2004). Finally, the 2-ODD gene family is highly diverse and new members are continually being added.

A great variety of 2-ODD enzymes have been identified in animal systems, including gamma-butyrobetaine dioxygenase (EC 1.14.11.1), pyrimidin-deoxynucleoside 2'-dioxygenase

(EC 1.14.11.3), procollagen-lysine 5-dioxygenase (EC 1.14.11.4), thymine dioxygenase (EC 1.14.11.6), procollagen-proline 3-dioxygenase (EC 1.14.11.7), trimethyllysine dioxygenase (EC 1.14.11.8), pyrimidine-deoxynucleoside 1'-dioxygenase (EC 1.14.11.10), aspartate beta-hydroxylase (EC 1.14.11.16), taurine dioxygenase (EC 1.14.11.17), phytanoyl-CoA dioxygenase (EC 1.14.11.18), calvaminate synthase (EC 1.14.11.21), deacetoxycephalosporin-C hydroxylase (EC 1.14.11.26) (Ruetschi et al., 1993; Warn-Cramer et al., 1983; Siegel et al., 1978; Holme, 1975; Myllyla et al., 1981; Vaz et al., 2001; Stubbe, 1985; Groke et al., 1990; Eichhorn et al., 1997; Mihalik et al., 1995; Zhou et al., 2001; Martín et al., 1994). Several of these enzymes are involved in metabolic pathways that should exist in plants, but have not previously been studied outside animal systems. There are also a number of animal proteins derived from 2-ODDs that demonstrate how diverse the functions of these proteins can be. For example, the mammalian transcription factor, hypoxia-inducible factor 1 (HIF-1), has a structure based on the jellyroll-like β-barrel containing the conserved ferrous ion-binding triad of residues that characterizes 2-ODD enzymes (Lee, 2003). Similarly, the DNA repair protein, AlkB, the extracellular matrix protein, leprecan, and the disease-resistance-related protein, EGL-9, define novel families of enzymes whose structures are based on the protein motifs that shared by members of the 2-ODD superfamily (Aravind and Koonin, 2001).

2-ODD gene structure and evolution

The conservation in this family was not restricted to the protein sequences but it also extended to gene structure and organization. The high degree of conservation of intron position among 2-ODDs has been noticed previously (Kanegae et al., 1994). Up to 3 introns at 4 vary position among 2-ODDs have been found in different dioxygenase genes (Prescott and John, 1996). The ACC oxidase family has intron number varied from 1 to 3. The position of these

introns are quite conserved (Fig. 4.4). Comparing the four introns positions of different 2-ODD members, the intron position of ACC oxidase are at position1, 3 and 4, which is thought to be the ancestor of the 2-ODD family (Prescott and John, 1996). The conservativeness of intron position is not uncommnon and has been shown in *adh3* genes in 21 animal species (Gonza`lez-Duarte and Albalat, 2005).

In our study, we also counted the intron number of individual gene. An interesting phenomenon is that the classification of subgroup according to the protein sequence can be related to the number of introns (Table 4.1). For example, in the subgroup of GA 2β-hydroxylase, the intron number varies from 1 to 4, while the number varies from 1 to 2 in GA 3β-hydroxylase. The subgroup labeled as "unknown-10" has more introns than other subgroups, with the number varying from 7 to 12. With a detailed observation about this group, the intron number of most of the genes are between 9 to 11 with the exception of AT4G167652 and AT3G502101, both of which are splicing alternatives. The most divergent family of P4H has the most variable intron number (2~11). This means that the plant 2-ODDs may derive from common ancestor from the genomic point of view.

Phylogenetically, it is evident that homologous genes encoding biochemically related proteins in single pathways are clustered same groups, perhaps as a result of gene duplication and divergence. For example, most of the flavonoid 2-ODDs are clustered together, so are GA 2-ODDs (Fig. 4.2). According to the substrate specificity, flavonol ODDs can cluster into 2 distinct clades, the first consisting of enzymes with a narrow substrate specificity including F3H, FSI and F6H (Anzellotti and Ibrahim, 2004), whereas the second is comprised of FLS and ANS, both possessing broad substrate specificity as has been previously described (Lukacin et al., 2003; Martens et al., 2003; Turnbull et al., 2004). This classification for flavonol 2-ODDs is similar to

the clustering in our result. On the basis of the activity of different members of 2-ODD family found in different kingdoms, Prescott and John (1996) decided the evolutionary order of the members of this family. They suggested, "FSI and F3H were among the earliest 2-ODDs to arise, followed by FLS, and subsequently by enzymes of the GA biosynthetic pathway, and ACO and ANS."

Enzymes in this super family evolve with different pattern among species. For example, in the flavonol synthase family, genes from Arabidopsis appear first followed by gene from rice, then by genes from poplar (Fig. 4.5a). For subgroup GA 2-beta-hydroxylase most genes from different species evolved separately (Fig. 4.5b).

Conclusions

Plant 2-ODDs is made up of diverse enzymes involved basic components, various metabolism, such as the biosynthesis of flavonoids, ethylene, gibberellin, glucotonoid, and many other respect of plant response to environment. In this paper, we annotated most members in this family using phylogeny. By our result, this superfamily could be classified into nine different groups, in which 21 subgroups were identified. Within the 21 subgroups, potential function or activity of 9 subgroups was decided and three other subgroups were discussed about their possible function. Individual family has distinct gene structure and gene family composition. And the evolution of this superfamily, including families within it and enzyme among different species was discussed. With the improvement of gene prediction method and bio-functional study, our knowledge about this superfamily will extend further.

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Table 4.1. Classification and tentative functions for 2-ODDs from *Arabidopsis*, rice and poplar

Group	Enzyme	Intron Number	Gene-ID					
Group I	Flavonol synthase	0-3	AT5G636001	AT5G636002	AT5G63580	AT5G43935	AT5G63595	AT5G08640
			AT5G63590	XP_467968	PT_689203	PT_671897	PT_342436	PT_704457
			PT_690250					
	Anthocyanidin dioxygenase	0-1	NP_918741	PT_682056	PT_548547	AT4G22870	AT4G228801	AT4G228802
	Unknown-1	0-2	PT_727538	NP_910523	AT4G16330			
	Unknown-2	2-3	XP_475566	NP_915344	AT2G38240	PT_653131	PT_688835	PT_351920
			PT_698494	PT_353685	AT3G55970	AT5G05600	AT3G11180	
Group II	Unknown-3	2-4	NP_910588	XP_476310	NP_910582	NP_910590	XP_476311	XP_476309
			NP_910581	PT_740865	PT_351586	PT_578863	PT_98749	PT_106084
			AT5G20550	AT5G54000	AT1G49390	AT5G20400		
	Unknown-4	0-4	PT_558157	XP_470470	NP_908927	NP_922706	NP_922709	NP_922704
			NP_922703	NP_922700	NP_922698	XP_468860	NP_922712	NP_922710
			PT_550094	PT_54771	PT_642190	PT_684065	PT_550348	PT_105969
			AT1G78550	AT1G17020	AT1G17010	AT4G25300	AT4G25300	AT4G25310
	Unknown-5	2-3	NP_914944	PT_54952	AT3G21420			
Group III	ACC oxidase	1-4	NP_917888	PT_561093	AT2G19590	AT1G77330	PT_710897	PT_559288
			AT1G62380	AT1G12010	AT1G05010	PT_704904	PT_568162	PT_573016
			PT_552732	XP_507001	XP_468017			
Group IV	Unknown-6	1-4	XP_476744	PT_97838	PT_581970	PT_559681	PT_55105	PT_551050
			XP_468578	NP_922524	XP_468579	XP_473641	AT5G24530	PT_574636
			PT_569679	PT_56061	PT_550478	AT4G10500	AT4G10490	XP_473642
			PT_57123	PT_65793	PT_585537	PT_83665	PT_65790	PT_582576
			PT_705513	PT_569255	PT_569254	PT_550472	PT_83674	PT_65791
	Unknown-7	1-4	XP_482984	PT_673955	PT_90454	PT_594229	PT_577360	PT_577357
			XP_474264	XP_474265	PT_63322	AT5G07480	AT3G60290	AT2G44800
			PT_698707	AT2G36690	AT2G06960	XP_507337	XP_483774	
	Unknown-8	0-5	PT_51800	AT3G12900	AT5G12270	PT_639660	PT_62715	AT3G13610
			AT1G55290	PT_566450	PT_546523	PT_692157	PT_645172	PT_715135
			PT_342251	PT_80763	PT_56663	XP_474682	PT_569316	PT_55078
			AT5G43450	AT5G43440	AT5G59530	AT5G59540	AT5g59540	AT1G04380
			AT1G04350	AT1G06650	AT1G06650	AT1G06640	AT1G06640	AT2G25450
			AT2G30840	AT2G30830	AT1G06620	AT3G61400	AT1G03410	AT1G03400
			XP_470509	XP_482188	XP_482192	XP_482200	XP_482196	
Group V	Flavonone 3-hydroxylase	1-2	PT_703378	PT_104043	PT_107866	XP_474226	AT3G51240	
Group VI	GA 2-oxidase	1-4	NP_916185	NP_913269	AT1G78440	PT_546400	PT_698991	PT_550316
			AT2G34555	AT1G300401	AT1G300402	XP_475621	NP_908765	AT1G02400
			AT1G47990	PT_57164	PT_348383	PT_723959	PT_353992	
	GA 3-beta-dioxygenase	1-2	NP_916509	AT4G21690	PT_578149	PT_352859	PT_48812	PT_349528
			AT1G80330	AT1G80340	AT1G15550			
	GA 20-oxidase	0-3	AT1G44090	XP_475240	XP_463540	PT_72519	PT_694591	PT_685156
			PT_53144	XP_476745	XP_470479	AT5G51810	AT4G25420	AT5G07200
			AT1G60980	PT_86560	PT_347900	PT_666953	PT_346112	
Group VII	Unknown-9	1-3	XP_480999	PT_79082	PT_581464	AT3G190001	AT3G190002	AT3G190101
			AT3G190102	PT_682489	PT_557330	XP_474083	PT_569976	
	Unknown-10	7-12	NP_914531	PT_91632	AT1G35190	AT3G46500	AT3G46490	AT3G46480
			NP_922401	NP_922400	NP_922402	PT_684983	AT4G16770	AT4G167651
			AT4G167652	AT3G49630	AT3G502101	AT3G502102	AT3G502103	AT3G49620
			PT_686051	XP_450237	XP_507425	XP_506638		
Group VIII	Unknown-11	2-3	XP_472862	AT1G14130	AT1G14120	PT_691928	PT_569214	
	AOP	0-3	AT5G51310	PT_350233	PT_350024	PT_342752	AT4G233401	AT4G233402
			AT4G03060	AT4G030501	AT4G030502	XP_482416	XP_507233	XP_482415
			AT4G03070	PT_548641	AT1G52820	PT_63731	PT_63726	PT_578150
			PT_344700	PT_709592	PT_548643	AT1G52800	AT1G80320	PT_548640
	Unknown-12	1-4	PT_706566	AT5G58660	AT3G47190	XP_473226	PT_80980	PT_354395
			NP_919194	AT1G50960	AT4G21200	PT_568216	PT_555375	PT_85916
			PT_102004	PT_569083	PT_569082			
Group IX	P4H	2-11	AT4G33910	AT2G23096	AT2G43080	AT3G28490	AT3G28480	AT5G18900
			AT3G06300	AT4G35820	AT4G35810	AT2G17720	AT5G66060	AT1G20270
			PT565695	XP_469864	PT_341617	PT_722373	PT_565697	PT_696274
			XP_476973	XP_469991	XP_469992	PT_577550	PT_577548	NP_921460
			XP_550298	PT_356221	XP_472000	PT_684206	NP_922161	PT_593788
			PT_585678	PT_550833	PT_52240	XP_468502		
Ungrouped		1-11	XP_476129	PT_694594	XP_506940	XP_467483	PT_81906	XP_473188
			NP_918132					

Table 4.2. Tentative flavonoid 2-ODDs members with their amino acid length

Enzyme	Gene ID	# of amino acids
Flavonone 3-hydroxylase	PT_703378	363
	PT_104043	152
	PT_107866	201
	XP_474226	377
	AT3G51240	358
Flavonol synthase	AT5G636001	326
	AT5G636002	325
	AT5G63580	250
	AT5G43935	293
	AT5G63595	279
	AT5G08640	336
	AT5G63590	308
	XP_467968	331
	PT_689203	335
	PT_671897	112
	PT_342436	333
	PT_704457	335
	PT_690250	335
Anthocyanidin dioxygenase	NP_918741	375
	PT_682056	362
	PT_548547	361
	AT4G22870	112
	AT4G228801	356
	AT4G228802	356
Unknown-1	PT_727538	232
	NP_910523	352
	AT4G16330	258
Unknown-2	XP_475566	368
	NP_915344	385
	AT2G38240	353
	PT_653131	356
	PT_688835	368
	PT_351920	362
	PT_698494	359
	PT_353685	359
	AT3G55970	363
	AT5G05600	371
	AT3G11180	400

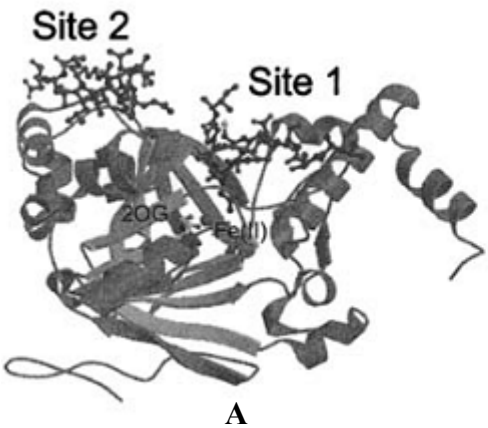
Figure 4.1. The conserved motif of 2-ODD family. (A) Binding of C-terminal transactivate domain (CAD) to factor inhibiting hypoxia inducible factor (FIH). The CAD peptide and 2OG are shown as ball and stick representations and the iron as sphere. (B) The active site of FIH showing both bound substrate and 2OG. Adopted from Hewitson et al. (2003). (C) Partial sequence alignment of 2-ODD family protein from Arabidopsis using Weblogo (http://weblogo.berkeley.edu/logo.cgi). Position 4 and 6 are the conserved amino acids Histidine (H) and Asparic acid (D). The third conserved amino acid Histidine locates at position 60~62.

Figure 4.2. Phylogenetic tree for the 2-ODD proteins from *Arabidopsis*, rice and poplar. Tentative functional assignments for the different enzyme subgroups were abbreviated as follows. Group I: flavanol synthase (FLS), anthocyanidin synthase (ANS), unknown-1, flavone synthase (FSI). Group II: unknown-2, unknown-3. Group III: 1-amino-1-cyclopropanecarboxylate (ACC) oxidase. Group IV: unknown-5, unknown-6, unknown-7. Group V: flavanone 3-hydroxylase (F3H). Group VI: gibberellin (GA) 20-oxidase, GA 3-hydroxylase, GA 2-hydroxylase. Group VII: unknown-8, unknown-9. Group VIII: unknown-10, glucosinolate synthases (AOP), unknown-11. IX: prolyl 4-hydroxylase(P4H). Six genes remained ungrouped on this tree.

Figure 4.3. Summary view of sequence multiple alignment of tentative flavonoid 2-ODDs generated by Genedoc.

Figure 4.4. Genomic organization of ACC oxidase from Arabidopsis, rice and poplar. Gray box represents exon. White line shows the position of intron.

Figure 4.5. The phylogeny of flavonol synthase (a) and GA 2-hydroxylase (b) from Arabidopsis, rice and Poplar.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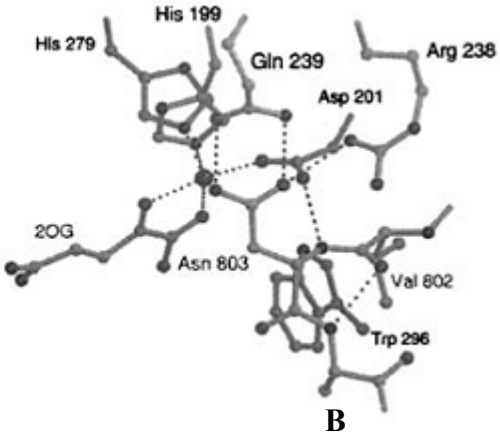
Site 1
2OG
Fe(II)

A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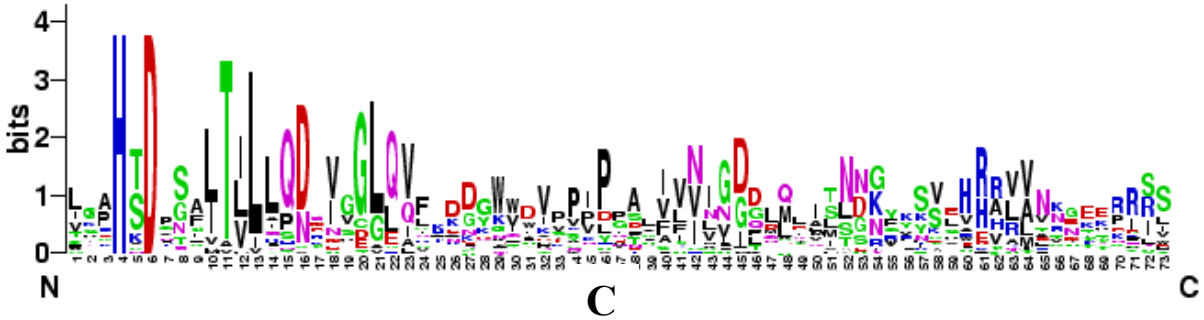
B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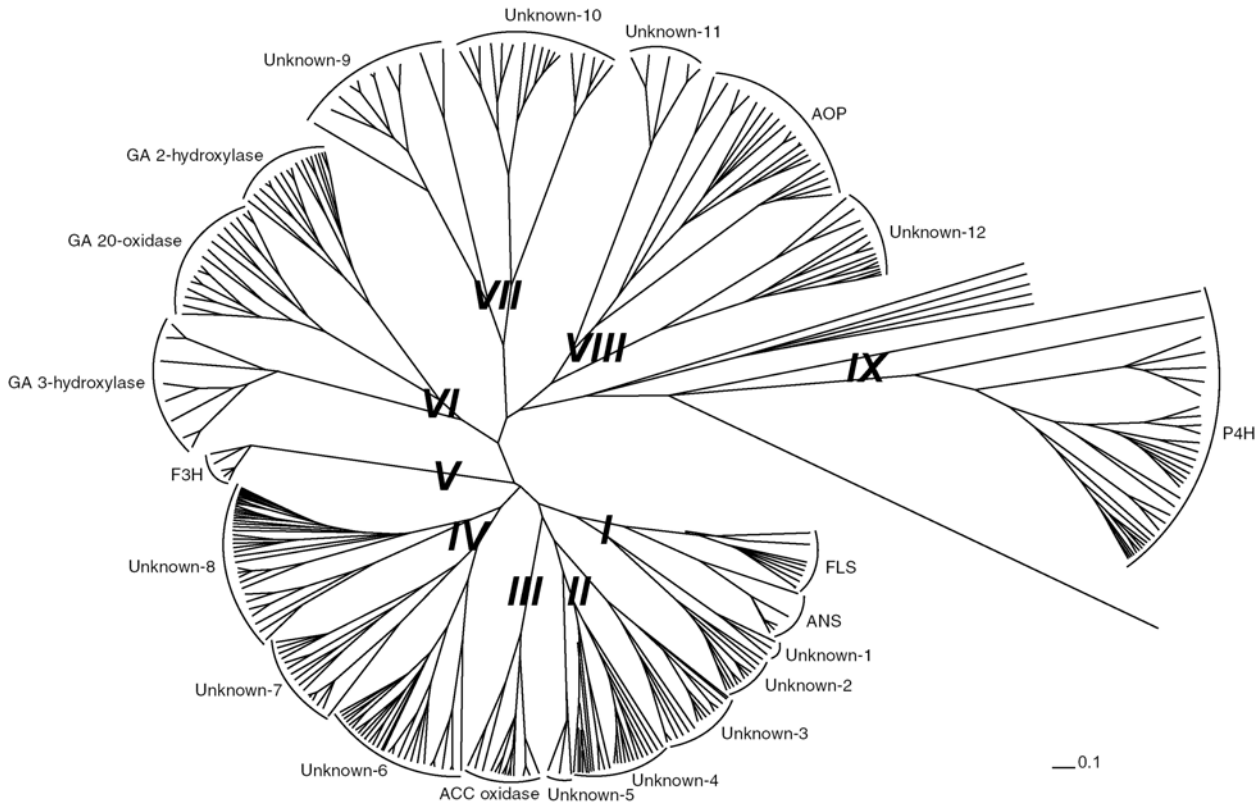
C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F3H
IV
I
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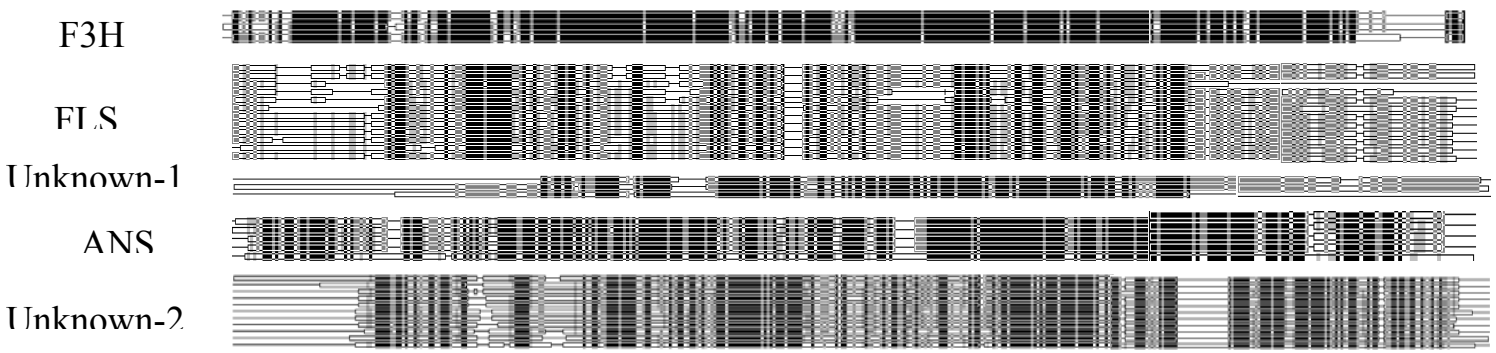
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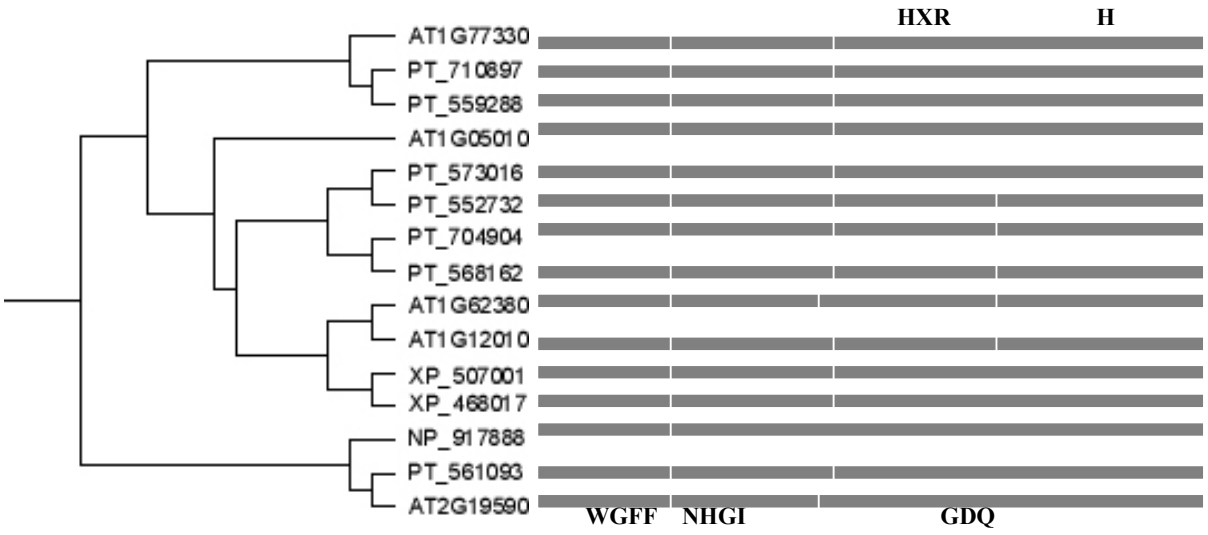
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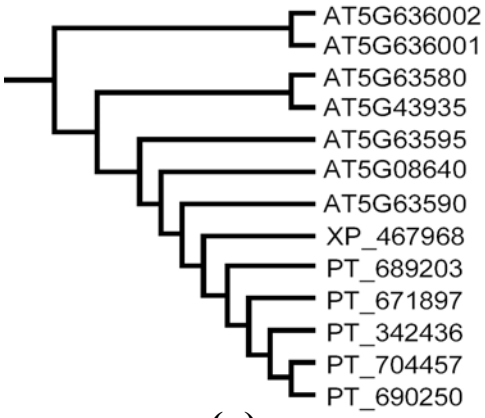
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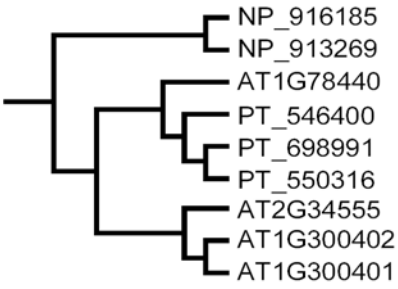
PT_690250

(a)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AT1G78440
PT_546400
PT_698991
PT_550316
AT2G34555
AT1G300402
AT1G300401

(b)

CHAPTER 5

CONCLUSIONS

Ethylene is involved in numerous aspects of plant growth and development, as well as plant responses to the environment. Due to frequent release from plants experiencing various stress conditions, including drought, hypoxia, wind loading and other mechanical stresses, ethylene is often regarded as a "stress hormone". Compression wood is a common wood forming in conifer caused by physical loading stresses. Due to its undesirable characteristics as a raw material, many studies have attempted to identify the regulatory mechanisms governing formation of compression wood. Ethylene is certainly involved in wood formation, and various studies have demonstrated the occurrence of ethylene in compression wood. This study examined a gene from loblolly pine whose product, ACC oxidase, catalyzes a key step in ethylene biosynthesis. Results from this work provide additional support to the idea that ethylene plays an important role in compression wood formation.

1-Aminocyclopropane-1-carboxylate (ACC) oxidase, which catalyzes the final step ethylene biosynthesis, is highly conserved in different plant species at the level of gene structure, i.e. conserved intron number and position, as well as at the level of DNA sequence. In Chapter 4, this conservation of intron number and position was shown for ACC oxidase genes from *Arabidopsis*, rice and poplar, for comparison with the loblolly pine ACC oxidase gene described in Chapter 2. This may underscore the possibility that all ACC oxidase genes in higher plants evolved from the same ancestral gene.

Chapter 2 shows the expression patterns for a loblolly pine ACC oxidase gene recovered as a cDNA and demonstrated that transcripts for the gene are expressed at their higher levels in young, expanding tissues. In Chapter 3, heterologous expression of two different loblolly pine ACC oxidase gene promoters in *Arabidopsis* also demonstrated highest levels of the GUS reporter activity in the tissues undergoing rapid growth, differentiation, and expansion, including

apical meristems, the root tip, sites for lateral root formation, the expansion zone on leaf tip and regions of vascular tissue generation. Numerous studies have shown that auxin is also distributed or involved in these regions, which implies that ACC oxidase is being coordinately regulated by endogenous auxin. This idea is further supported by the results reported in Chapter 2 that IAA can up-regulate PtACO1 transcript levels. Previous studies have also found synergistic effects between ethylene and auxin in plant development and plant responses to environmental conditions.

Even though both loblolly pine ACC oxidase gene promoters drove GUS expression in *Arabidopsis* root tissues, they displayed different patterns of expression in the aerial tissues of *Arabidopsis*. For example, pACO1 was expressed mainly in the expansion zone of young leaf tips, while pACO2 was mainly expressed in leaf vascular tissues, especially in the nodes of vascular tissue development. Differential expression of these two promoters also extended to their responses to external stimuli. The pACO2 promoter did not respond to infection of *Arabidopsis* by a fungus, while the pACO1 did. Similarly, the pACO1 promoter, but not the pACO2 promoter, was induced by physical stresses caused by acute bending of flower stalks. On the other hand, the pACO2 promoter displayed asymmetric expression during a gravitropic response that was completely analogous to what might be expected for a gene involved with compression wood formation in conifer trees.

It remains, however, to identify the *cis*-acting regulatory elements that control the differential responses of these promoters. ACC oxidases typically occur as small gene families in the plant species studied so far. How many ACC oxidase genes exist in loblolly pine and how do the different gene promoters respond to the various conditions encountered in the life history of this woody gymnosperm? This is a topic that deserves further study.

Of course, all these responses were documented in a heterologous system. Although a heterologous expression system that preserves the fidelity of pine gene expression would be greatly welcome, how these gene promoters actually respond in loblolly pine is something we cannot fully determine at this time. No doubt, reliance on a heterologous system would miss numerous details of importance about conifer promoter expression. There are undoubtedly many fantastic mechanisms and patterns of gene expression that remain silent and unknown to our eyes. Think about it – we are dealing with an enormous, perennial organism that stands in one place for decades or centuries, dealing with each new environmental challenge. How different their responses must be from those transient annual grasses dead at the end of each year. With breakthroughs and improvements to micro-propagation and transformation techniques in pine, we can anticipate viewing a more detailed and beautiful picture of how these ancient plants have remained survivors against the challenges of their environment.