The Isolation and Characterization of ACC Oxidase Genes from Loblolly Pine *(Pinus taeda*)

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ABSTRACT

In all plant species studied so far, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, the enzyme which catalyzes the final step ethylene biosynthesis, is encoded by a multigene family. Expression of the different gene family members is generally regulated at the transcriptional level in various cell types, in tissue at different stages of development, and in response to numerous external stimuli. Elevated ACC levels have been found in the reaction woods of both angiosperms and gymnosperms suggesting that ethylene may play a role in determining the quality changes noted in these wood types. In this study, we report the first isolation and characterization of ACC oxidase genes from loblolly pine. cDNA clones were isolated from auxin-treated shoot tips, trunk wood xylem, and compression wood xylem. The high level of sequence identity in the one Kb coding region and both the 5' and 3' UTRs of the shoot tip and trunk wood cDNAs suggest that these two genes may be allelic. DNA sequence for the compression wood clone is only slightly more divergent in the coding region and 5' UTR, but current data for the 3' UTR suggests that this cDNA derives from a distinct gene. Partial genomic DNA clones suggest that the one Kb coding region is interrupted by three introns. The identity of these putative ACC oxidase genes is being verified by heterologous expression in *E. coli* for subsequent demonstration of the correct enzymatic activity. The use of oligonucleotide primers to conserved domains in the ACC oxidase led to the cloning of partial cDNAs encoding apparent flavanol hydroxylases. The sequences of these other members of the Fe-ascorbate oxidase superfamily will be reported.

INTRODUCTION

Ethylene synthesis in plants is induced both at specific developmental stages and by various external stimuli, including bending, wounding, drought, anaeroblosis, pathogen invasion, or auxin treatment (Morgan and Drew 1997). In higher plants, ethylene (C_2H_4) is produced from methionine via S-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylate (ACC) (Met $\rightarrow$ AdoMet $\rightarrow$ ACC $\rightarrow$ C2H4). The biosynthetic pathway requires three enzymes, AdoMet synthase, ACC synthase and ACC oxidase, respectively. It is generally believed that a low basal level of ACC oxidase is constitutively expressed in most plant tissues, and that expression is generally regulated at the transcriptional level. However, differential expression has been shown for ACC oxidase genes in different plant species, and in response to different environmental factors (Bouquin et al 1997).

ACC oxidase is a member of the Fe(II) - dependent dioxygenase superfamily of enzymes, and it has been shown to be encoded by a gene family in plant species studied to date. In addition to iron, the enzyme requires ascorbate and CO_2 as cofactors (John 1997).

Increased levels of ACC and ethylene have been found in the reaction woods of both angiosperms and gymnosperms (Barry et al 1996, Telewski 1990), suggesting that ethylene may play a role in the formation of reaction woods. However, there have not yet been any studies to demonstrate at the level of gene regulation how ACC oxidase might contribute to variations in wood quality. As an initial step towards developing an understanding of how ACC oxidase might contribute to compression wood formation we have cloned and are characterizing ACC oxidase genes from loblolly pine.

MATERIALS AND METHODS

Three loblolly pine tissues were used in this study -- elongating shoot tips that had been submerged in a 1mM IAA solution for 2 hours prior RNA extraction, and non-lignified xylem from either vertical or bent stems of 7-10 year old loblolly pine trees. Total RNA was isolated from these tissues using the protocol of Alosi (Dendrome web server). Genomic DNA was extracted from suspension-cultured loblolly pine cells, line L797-11 from Dr. S.A. Merkle (UGA), using the CTAB method of Lassner et al. (1988). After extraction, DNA was purified by 2-4 rounds of phenol and chloroform extraction, as needed to ensure reproducible PCR amplification. DNA concentration was determined using measuring fluorescence with *pico*-green dye (Molecular Probes, Bend, OR). First strand cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies, Rockville, MD) and 3' RACE was performed using a kit from Life Technologies. A variety of PCR methods using nested primers, including gradient, hot-start and touch-down PCR, were to recover DNA amplimers. In general, PCR reaction products were cloned into the TA vector without further purification. The initial primers for PCR were designed using sequence from a 790 bp loblolly pine EST encoding an apparent ACC oxidase (GenBank AA556710). In an effort to recover as many ACC oxidase gene family members as possible, the initial PCR primers were chosen from regions of sequence known to be highly conserved in these genes. cDNA libraries from auxin-treated shoot tips, trunk wood and compression wood xylem were screened. Sequence from the recovered PCR products was used to prepare gene-specific primers that spanned the coding region, a full-length ACC oxidase clone was recovered using *Pwo*, a proof-reading DNA polymerase (High-Fidelity PCR System, Roche, Indianapolis, IN).

RESULTS

Isolation of the cDNA clones

Starting with DNA sequence from a loblolly pine EST and an alignment of the twelve most closely related ACC oxidase sequences in GenBank, full-length cDNA clones were isolated from each of the three tissues sampled. The coding sequences of the three clones were nearly identical (>98%) with differences in only 2 of 333 inferred amino acids. Efforts to verify the identity of the genes by testing for ACC oxidase activity in the expressed products are underway.

3'-RACE was used to recover the 3' untranslated regions (UTRs) corresponding to the ACC oxidase transcripts expressed in shoot tips and trunk wood. Current results suggest that the predominant transcript in compression wood differs in the 3' UTR from the 287 bp UTRs found in the shoot tip and trunk wood transcripts. 5'-RACE analyses revealed that the 87 bp 5' UTR was identical for all of the ACC oxidases.

Primers used for cloning the ACC oxidase cDNAs were used to amplify a 1.6 kb product from genomic DNA, and DNA sequencing identified three introns in the gene (Fig.1).

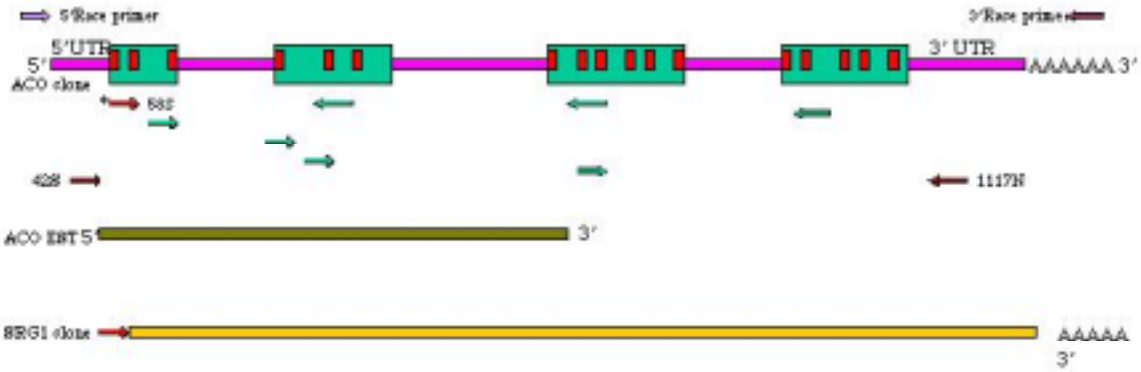

Figure 1. Features of the loblolly pine ACC oxidase gene. Genomic DNA sequence (top) indicated three introns separating 4 exons (large boxes) each containing conserved amino acid sequences indicative of ACC oxidases (small boxes). A variety of oligonucleotide primers (arrows) were used for cloning and sequencing, but the initial primers were based on an EST found in GenBank (middle). The cDNA is represented by the bottom line.

During the initial work to recover cDNA fragments from compression wood using primers to amino acid sequences conserved in ACC oxidases, partial clones corresponding to two other members of the Fe-ascorbate oxidase superfamily were recovered. BLAST analyses indicated that these genes were more closely related to the SRG1 gene family than they were ACC oxidase (Fig. 2).

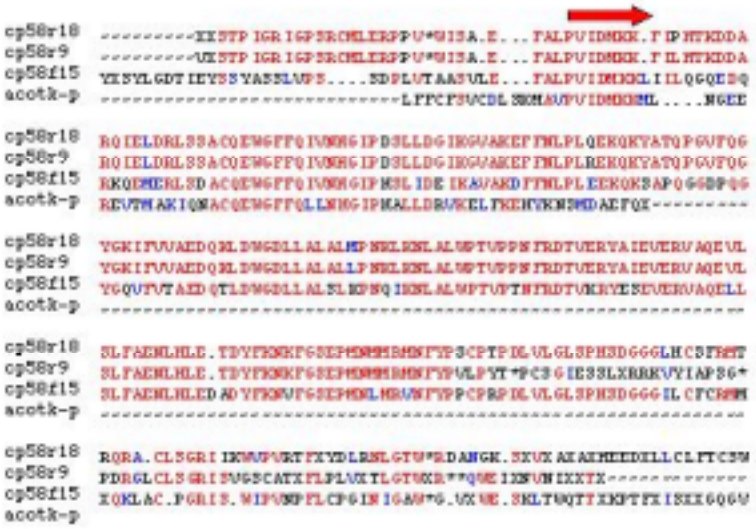

Figure 2. Amino acid alignment of the loblolly pine ACC oxidase with three other putative Fe-ascorbate oxidase partial cDNAs isolated in the course of early 3' RACE experiments. The sequences have been deposited in Genbank under accession numbers BI698178 – BI698180.

DISCUSSION

This is the first report describing putative ACC oxidase genes from pine. Although further work to express and assay the correct activity is needed to conclusively demonstrate this identity, sequence analysis strongly supports the current identification. Whether or not we have truly isolated multiple gene products is less clear as the 5' UTR and coding regions of all three clones are nearly identical. RACE data suggests that differences exist in the 3' UTRs, but we have not yet eliminated the possibility that the compression wood sequence may have arisen via some anomalous PCR event. However, in tomato, the ACC oxidase gene family is comprised of three members which are nearly identical the protein coding regions, but show sequence divergence in the 3' UTRs (Barry et al 1996). The isolation of cDNA fragments encoding other members of the Fe-ascorbate superfamily was not unexpected considering our use of primers for domains that are highly conserved in these enzymes. Most likely the enzymes encoded by these Fe-ascorbate oxidase transcripts are involved in the flavanoid metabolism that imparts the characteristic reddish color to compression wood.

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