

J. W. Hudgins · Steven G. Ralph · Vincent R. Franceschi  
Jörg Bohlmann

## Ethylene in induced conifer defense: cDNA cloning, protein expression, and cellular and subcellular localization of 1-aminocyclopropane-1-carboxylate oxidase in resin duct and phenolic parenchyma cells

Received: 13 February 2006 / Accepted: 20 March 2006 / Published online: 17 May 2006  
© Springer-Verlag 2006

**Abstract** Members of the Pinaceae family have complex chemical defense strategies. Conifer defenses associated with specialized cell types of the bark involve constitutive and inducible accumulation of phenolic compounds in polyphenolic phloem parenchyma cells and oleoresin terpenoids in resin ducts. These defenses can protect trees against insect herbivory and fungal colonization. The phytohormone ethylene has been shown to induce the same anatomical and cellular defense responses that occur following insect feeding, mechanical wounding, or fungal inoculation in Douglas fir (*Pseudotsuga menziesii*) stems (Hudgins and Franceschi in *Plant Physiol* 135:2134–2149, 2004). However, very little is known about the genes involved in ethylene formation in conifer defense or about the temporal and spatial patterns of their protein expression. The enzyme 1-aminocyclopropane-1-carboxylate oxidase (ACO) catalyzes the final step in ethylene biosynthesis. We cloned full-length and near full-length ACO cDNAs from three conifer species, Sitka spruce (*Picea sitchensis*), white spruce (*P. glauca*), and Douglas fir, each with high similarity to

*Arabidopsis thaliana* ACO proteins. Using an *Arabidopsis* anti-ACO antibody we determined that ACO is constitutively expressed in Douglas fir stem tissues and is up-regulated by mechanical wounding, consistent with the wound-induced increase of ethylene levels. Immunolocalization showed cytosolic ACO is predominantly present in specialized cell types of the wound-induced bark, specifically in epithelial cells of terpenoid-producing cortical resin ducts, in polyphenolic phloem parenchyma cells, and in ray parenchyma cells.

**Keywords** ACC oxidase · Douglas fir · Hormone · Immunolocalization · Insect · *Picea*

### Introduction

In North America, Europe, and much of Asia, conifers of the Pinaceae family are the most economically important forest tree species and, due to their extensive range and long life times, are central to many ecosystems. Members of the Pinaceae, such as species of spruce, pines, or Douglas fir, are subject to infestations from numerous species of fungal pathogens and insects. Although conifers are resistant to most generalist herbivores, some specialized insects such as certain bark beetles (Scolytidae) and weevils (Curculionidae) along with their associated fungal pathogens can cause substantial damage to individual trees or entire conifer forests (Alfaro et al. 2002; Raffa et al. 2005). Conifers have evolved effective anatomical and chemical defense mechanisms that limit the damage from herbivores or pathogens (Phillips and Croteau 1999; Trapp and Croteau 2001; Huber et al. 2004; Franceschi et al. 2005; Martin and Bohlmann 2005). The conifer bark tissues provide the first barrier against insects and pathogens. Mechanical defenses (e.g., lignified periderm, sclereids, fibers, calcium oxalate crystals) and chemical defenses (e.g., phenolics and terpenoid oleoresins) collectively form an effective

J.W. Hudgins and Steven G. Ralph contributed equally to this work.

J. W. Hudgins · S. G. Ralph · J. Bohlmann (✉)  
Michael Smith Laboratories, University of British Columbia,  
321-2185 East Mall, Vancouver, BC, Canada V6T 1Z4  
E-mail: bohlmann@interchange.ubc.ca  
Tel.: +1-604-8220282  
Fax: +1-604-8222114

J. Bohlmann  
Department of Botany, University of British Columbia,  
Vancouver, BC, Canada V6T 1Z4

J. Bohlmann  
Department of Forest Sciences, University of British Columbia,  
Vancouver, BC, Canada V6T 1Z4

J. W. Hudgins · V. R. Franceschi  
School of Biological Sciences, Washington State University,  
Pullman, WA 99164-4236, USA

defense system in conifer bark (Hudgins et al. 2003b; Franceschi et al. 2005).

In response to stem-boring insects, fungal pathogens, or wounding, conifer bark tissues respond with induced activation of specialized cell types, which include polyphenolic phloem parenchyma (PP) cells and epithelial cells lining the inner surface of axial and radial resin ducts. Induced PP cells accumulate large quantities of phenolics (Brignolas et al. 1995; Lieutier et al. 1996; Bois and Lieutier 1997; Franceschi et al. 1998, 2000; Kusumoto and Suzuki 2003). Polyphenolics are common and abundant defensive chemicals in conifers (Pan and Lundgren 1996; Viiri et al. 2001) and bark from all conifer species accumulate these compounds in axial PP cells (Franceschi et al. 1998; Krekling et al. 2000; Hudgins et al. 2003a, 2004). At the molecular level only a few studies have been conducted to identify genes induced under biotic stress with a potential role in PP cell-based defenses (Nagy et al. 2004; Ralph et al. 2006a, b).

Terpenoid oleoresins accumulate in large quantities in resin ducts as a chemical defense system common in the Pinaceae (Langenheim 2003). Stem damage leads to the activation of constitutive cortical resin ducts and de novo formation of xylem traumatic resin ducts (TD) from the cambial zone (Alfaro 1995; Franceschi et al. 1998, 2000; Tomlin et al. 1998; Nagy et al. 2000; Hudgins et al. 2003a; Byun-McKay et al. 2003) resulting in increased resin formation and resin accumulation (Martin et al. 2002; Miller et al. 2005). Cortical resin ducts and xylem resin ducts are connected in a three-dimensional system via radial ducts and ray parenchyma cells. The molecular biology, chemistry, and biochemistry of terpenoid resins associated with conifer resin ducts have been the subject of much research and of several recent reviews (Bohlmann and Croteau 1999; Phillips and Croteau 1999; Trapp and Croteau 2001; Huber et al. 2004; Martin et al. 2004; Schmidt et al. 2005; Martin and Bohlmann 2005; Keeling and Bohlmann 2006). Importantly, conifers containing these induced resin duct and PP cell defenses can have systemic and acquired resistance (Christiansen et al. 1999; Krokene et al. 1999, 2003; Bonello et al. 2001; Bonello and Blodgett 2003).

While a considerable amount of information exists on composition and biosynthesis of conifer chemical defenses, in particular terpenoid defenses, little is known about the endogenous signaling of induced cell activation of PP cells and resin ducts in conifer bark following wounding, insect attack, or pathogen inoculation. Recent studies using exogenously applied methyl jasmonate (MJ) (Franceschi et al. 2002; Martin et al. 2002; Fäldt et al. 2003; Hudgins et al. 2003b, 2004; Huber et al. 2005; Miller et al. 2005) and ethylene (Hudgins and Franceschi 2004) have demonstrated that these compounds can act as efficient elicitors of conifer cellular defense responses. Similar to the effect of insect attack or fungal inoculation, PP cell activation and TD development can be strongly elicited with exogenous treatments of MJ in several conifer species, including species of spruce and Douglas fir. In Douglas fir, exogenously applied ethylene also induces PP

cells and TDs (Hudgins and Franceschi 2004). At the molecular level, insect feeding by white pine weevils induces accumulation of transcripts of the octadecanoid and ethylene pathways in Sitka spruce (Miller et al. 2005; Ralph et al. 2006b). In Douglas fir, both wounding and MJ application were found to induce ethylene evolution in stems and up-regulate expression of 1-aminocyclopropane-1-carboxylate oxidase (ACO) protein, while parallel treatments with the ethylene action inhibitor 1-methylcyclopropene reduced MJ- or wound-induced accumulation of phenolics and resin and decreased TD development (Hudgins and Franceschi 2004). Together, these studies suggest that both octadecanoids and ethylene are involved in inducible conifer chemical and cellular defenses, with ethylene acting downstream of octadecanoids (Hudgins and Franceschi 2004).

Ethylene has important functions in numerous plant processes that include cell differentiation, growth, development, and response to environmental stress (Yang and Hoffman 1984; Abeles et al. 1992; Kende 1993; Bleecker and Kende 2000). Ethylene is generated from 1-aminocyclopropane-1-carboxylic acid (ACC), which is synthesized by ACC synthase (ACS). ACC is subsequently converted to ethylene by ACO (Dong et al. 1992). In angiosperms, various types of abiotic and biotic stresses differentially up-regulate ACS and ACO (Kende 1993; Bleecker and Kende 2000) and both enzymes potentially control and regulate ethylene production. ACO is a ferrous-dependent non-heme oxygenase encoded by small gene families in angiosperm species. Conflicting studies have indicated different subcellular locations for ACO in angiosperms (Reinhardt et al. 1994; Rombaldi et al. 1994; Ramassamy et al. 1998; Chung et al. 2002). Therefore it was of interest to determine the subcellular location of ACO in a gymnosperm, building on previous tissue localization work in Douglas fir (Hudgins and Franceschi 2004).

Following the recent findings of ethylene as a signaling compound in inducible phenolic and terpenoid defenses in conifers (Hudgins and Franceschi 2004), we report here the cloning and sequence characterization of gymnosperm ACO from three members of the Pinaceae family, Sitka spruce, white spruce, and Douglas fir, and new results that confirm wound-induced expression and cellular location of ACO in specialized cells of wound-induced Douglas fir bark tissue.

---

## Materials and methods

### Plant materials and treatments

Three-year-old Douglas fir saplings from a commercial nursery (Lawyer Nursery, Olympia, WA, USA) were grown in a Washington State University-Pullman greenhouse. They were approximately 0.5 m in height, with a diameter of 1–1.5 cm at the bottom internode. Saplings were obtained in February 2004 and potted in 2 l con-

tainers and allowed to acclimate at 16°C day/10°C night temperatures for 2 weeks without supplemental lighting, then grown at 23°C day/18°C night temperatures under a 14 h light/10 h dark regime with supplemental lighting for 3 months prior to treatments. They received 500 ml of water daily and were fertilized weekly [Scotts Brand, Peters Fertilizer (20:20:20 N:P:K)]. For ACO protein expression analysis and localization, 30 saplings were selected from which 15 were wounded through the bark and into the sapwood with an 18-gauge hypodermic needle, and 15 were left untreated as controls. For wounded-treated saplings, the bottom and middle internodes received 10 wounds in a spiral pattern around the stem to mimic multiple attacks by stem-boring insects. Tissue was collected from three wounded and three control saplings at 10, 24, 48, 72, and 96 h after treatment with five replicate-treated saplings for each sample time. Bark tissue including cambium was separated from xylem, immediately frozen in liquid N<sub>2</sub>, and stored at -80°C for protein extraction and western blot analysis. Tissue from the same saplings was also placed in fixative and prepared for immunocytochemistry. For analysis of ethylene production, 18 Douglas fir saplings obtained from the same nursery at the same time were grown along with the saplings described above. Of these 18 saplings, 9 were wounded with a 1 mm cork borer along a 6 cm length on the bottom internode of the stem, and 9 were left unwounded. Intact stem sections (6 cm long), representing wounded and control tissues from both treatments, were collected at 6, 24, and 48 h at the treated internodes, with three replicate-treated saplings from each sample time. To obtain ACO cDNA clones from Douglas fir, we utilized three tissue sources: (1) bark tissue (including phloem and cambium) of a 2-year-old tree growing outdoors under natural conditions at the University of British Columbia (UBC) that was harvested in June 2002, 24 h after being cut horizontally at 5 mm intervals on opposite sides along the entire length of the stem; (2) green shoot tips harvested in July 2002 from lateral branches of a 20-year-old tree grown outdoors under natural conditions at the UBC South Campus farm; and (3) mature phloem harvested from the stem of the same tree.

#### Isolation of spruce and Douglas fir ACO full-length cDNA clones

A TBLASTN search of the Treenomix spruce expressed sequence tag (EST) and full-length cDNA (FLcDNA) database (Ralph et al. 2006b), containing 147,146 ESTs derived from 3'-end sequencing, was performed using ACO sequences from both tomato (*Lycopersicon esculentum*) and *Arabidopsis thaliana* to identify 20 putative spruce ACO ESTs. The CAP3 sequence assembly software (Huang and Madan 1999) was used to group ESTs into singletons and contigs (40 bp overlap, 95% identity). ACO clones in the pBluescript II SK (+) vector were identified in our cDNA library glycerol stocks, insert sizes were determined using PCR with -21M13 forward

(5'-TGTAACACGACGGCCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') primers, and selected cDNA inserts were sequenced from both ends using the same primers. In this manner, two unique spruce ACO FLcDNAs were obtained. To capture cDNA clones representing ACO from Douglas fir, total RNA was isolated, quantified, and checked for integrity and purity by spectrophotometer, agarose gel, and reverse transcription with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with an oligo d(T)<sub>18</sub> primer and αP<sup>32</sup> dGTP (Amersham-Pharmacia Biotech, Buckinghamshire, UK) incorporation according to Kolosova et al. (2004). Prior to reverse transcription, 5 μg of total RNA per tissue source was treated with DNase I (Invitrogen) digestion according to the manufacturer's instructions to remove genomic DNA. Primers were designed at the open reading frame (ORF) termini based on the spruce FLcDNA sequences *PgACO* and *PsACO* (DF\_F1 5'-GTTTGTGTGAAGGATTTCTGTG-3'; DF\_R2 5'-TCAGTACTGCAAGGCCGTCAC-3') and used to perform reverse transcription PCR with Superscript II reverse transcriptase (Invitrogen), an oligo d(T)<sub>18</sub> primer, and SuperTaq Plus polymerase (Ambion, Austin, TX, USA) according to the manufacturer's instructions. All three tissue sources gave a single PCR product of ca. 1.5 kbp. The PCR product from wounded bark tissue was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then sequenced using primers DF\_F1 and DF\_R2, as well as additional primers designed to the internal sequence (DF-Int1 5'-CAGA GACTGCTCGAATTAC-3'; DF-Int2 5'-GACGA GGTCTGGTCTTGAAC-3'). Other primer combinations designed within the untranslated regions (UTRs) and ORF provided slightly shorter PCR products of identical sequence (data not shown).

#### Sequence and phylogenetic analyses

Predictions for pI and molecular mass were made using the entire ORF and the pI/MW tool at ExPasy ([http://www.expasy.org/tools/pi\\_tool.html](http://www.expasy.org/tools/pi_tool.html)). Amino acid alignments of selected gymnosperm and angiosperm ACO proteins and Fe(II)-dependent dioxygenases (Prescott and John 1996) were made with ClustalW (<http://www.ebi.ac.uk/clustalw/>) and Boxshade (<http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html>). The sequence alignments were then manually adjusted prior to maximum likelihood analysis using Phyml v2.4.1 (Guindon and Gascuel 2003) with the JTT (Jones et al. 1992) amino acid substitution matrix. The proportion of invariant sites and the alpha shape parameter were estimated by Phyml. Trees were generated using BIONJ (Gascuel 1997), a modified neighbor-joining algorithm. SEQBOOT of the Phylip v3.62 package (Felsenstein 1993; <http://evolution.genetics.washington.edu/phylip.html>) was used to generate 100 bootstrap replicates, which were then analyzed using Phyml and the previously estimated parameters. CON-SENSE, also from Phylip, was used to create a consensus

tree. Treeview (Page 1996) was used to visualize all trees. Bootstrap values above 50% were added to the maximum likelihood tree generated from the original data set. The subcellular localization of ACO proteins was predicted using the TargetP v1.01 (Emanuelsson et al. 2000; <http://www.cbs.dtu.dk/services/TargetP/>) and PSORT (Nakai and Horton 1999; <http://psort.nibb.ac.jp/>) software programs.

#### Protein extraction and western immunoblot analysis

Total protein extractions from the 3-year-old saplings were conducted as described by Martin et al. (2002), with the addition of acid-washed sand and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 0.5 mM) and Complete Tablets (Roche, Nutley, NJ, USA). Total protein concentrations were determined using a Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal masses (15 µg) of protein were loaded in each lane and the samples were subjected to SDS-PAGE using 4% (w/v) polyacrylamide stacking gel and 12% (w/v) polyacrylamide separating gel. Proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated for 1 h with TBST + BSA [Tris-Buffered Saline-Tween; 10 mM Tris, 100 mM NaCl, 0.1% (v/v) Tween 20 + 1% (w/v) BSA, pH 7.2] and then with a 1:500 dilution of an affinity-purified polyclonal anti-*Arabidopsis* ACO (aN-19, sc-12781, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h at room temperature. The ACO antibody was raised against an *Arabidopsis* synthetic peptide (15 amino acids) near the N-terminal of the protein. Following incubation with the primary antibody, membranes were rinsed with TBST + BSA (4 × 15 min) and then treated for 1 h at room temperature with rabbit anti-goat alkaline phosphatase-tagged secondary antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:20,000 in TBST + BSA. Following the secondary antibody, membranes were rinsed and equilibrated in reaction buffer (100 mM Tris, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, pH 9.5) for 10 min, and the antibody was detected with BCIP/NBT (Roche Diagnostics, Indianapolis, IN, USA).

#### Ethylene analysis

The stem sections were weighed and placed individually in 10 ml glass vials with a rubber septum stopper. Following three incubation periods of 1, 2, and 3 h at 22°C for each sapling, 0.5 cm<sup>3</sup> gas samples were drawn from the head space with a tuberculin syringe and injected into a Hewlett Packard 5890 GC-MS (HP, Palo Alto, CA, USA) with a J&W Scientific CARBONPLT Column (30 m × 0.53 mm; Folsom, CA, USA) and a flame ionization detector, and analyzed as previously described (Hudgins and Franceschi 2004). Means and standard errors were calculated based on three replicate runs from three saplings at time points following wounding using *t* tests with unequal variances and constant *P* values (< 0.05).

#### Microscopy and immunocytochemistry

Stem samples from the 3-year-old saplings were collected and directly placed in fixative solution [2% (v/v) paraformaldehyde and 1.25% (v/v) glutaraldehyde buffered in 50 mM L-piperazine-*N,N'*-bis (2-ethane sulfonic) acid, pH 7.2]. Samples were subsequently cut into 2.5 mm<sup>2</sup> blocks and allowed to fix for 24 h at 4°C. Blocks were sub-sampled and rinsed with the same buffer solution, dehydrated with an ethanol series, and infiltrated with L. R. White acrylic resin (London Resin Company Ltd, Reading, UK). Samples were polymerized in fresh resin at 55°C for 12 h.

For confocal immunolocalization microscopy, cross-sections (1 µm thick) were cut with a diamond knife on a Sorvall MT 5000 Ultramicrotome and dried from a drop of water onto gelatin-coated slides. Sections were first blocked for non-specific binding of antibodies by incubation for 1 h in TBST + BSA [10 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween 20 + 1% (w/v) BSA, pH 7.2] and 0.05% (w/v) polyvinyl pyrrolidone (PVP, 10,000 MW). Primary antibody incubations were with anti-ACO polyclonal antibody (see above) diluted 1:75. The sections were then rinsed with TBST + BSA (4 × 15 min). For the secondary antibody, sections were incubated for 1 h at room temperature with rabbit anti-goat gold-tagged secondary antibody diluted 1:100 in TBST + BSA. After washing, the sections were silver enhanced (Amersham Silver Enhancement Kit, Amersham-Pharmacia Biotech) and counter-stained with 0.5% (w/v) aq. Safranin O. The sections were cover slipped with immersion oil as the mounting media and imaged with a confocal microscope (BioRad 1024, Hercules, CA, USA), using a combination of reflected and transmitted imaging. Cross-sections (100 nm thick) for transmission electron microscopy were collected on Formvar-coated nickel grids. Immunocytochemical conditions were carried out as described for confocal microscopy and sections post-stained with a 3:1 (v/v) solution of 4% (w/v) uranyl acetate and 1% (w/v) potassium permanganate. Images were collected using a JEOL JEM-1200 EX TEM (JEOL USA, Inc., Peabody, MA, USA) with AnalySIS imaging software (Soft Imaging Systems, Lakewood, CO, USA).

---

## Results

#### cDNA cloning of ACO genes from Sitka spruce, white spruce, and Douglas fir

In order to obtain sequence information for ACO genes from conifers we first screened the spruce EST and FLcDNA databases developed in the Treenomix project (Ralph et al. 2006b). These databases have been developed with an emphasis on induced tissues enriched for expression of conifer defense genes. A TBLASTN search of the spruce EST database was performed using ACO sequences from both tomato

and *Arabidopsis*, which led to the identification of 20 putative spruce ACO ESTs. A comparison of sequences obtained by complete cDNA insert sequencing indicated these ESTs represent two unique FLcDNAs, *PsACO* and *PgACO*, of 1,505 and 1,451 bp in length, respectively (Fig. 1a and Table 1). The translated proteins are predicted to capture the entire ORF based on overall similarity to angiosperm ACO proteins, and the presence of a starting methionine with stop codons upstream within the 5' UTR. *PsACO* and *PgACO* were identified from Sitka spruce clone FB3-425 and white spruce clone PG29, respectively, and both FLcDNAs encode the same predicted 324 amino acid protein with a pI value of 4.97 and molecular mass of 36.5 kDa (Table 1). The 5' UTR of *PsACO* is 95 bp while that of *PgACO* is only 39 bp and both FLcDNAs possess a 3' UTR of 422 bp, excluding the polyA tail. At the nucleotide level these FLcDNAs share 99.8% identity within their ORFs, with slightly lower identity (98.1%) within the 3' UTR, suggesting these FLcDNAs likely represent species-specific alleles of the same ACO gene. Interestingly, all of the remaining 18 putative ACO EST sequences we identified in the spruce sequence database represent truncated forms of these two FLcDNAs. Considering the large number of spruce ESTs available within the Treenomix collection, the majority of which were obtained from normalized cDNA libraries (Ralph et al. 2006b), this suggests there may be only a single ACO gene expressed in Sitka spruce and white spruce, in contrast to four ACO genes identified in tomato and five in *Arabidopsis*.

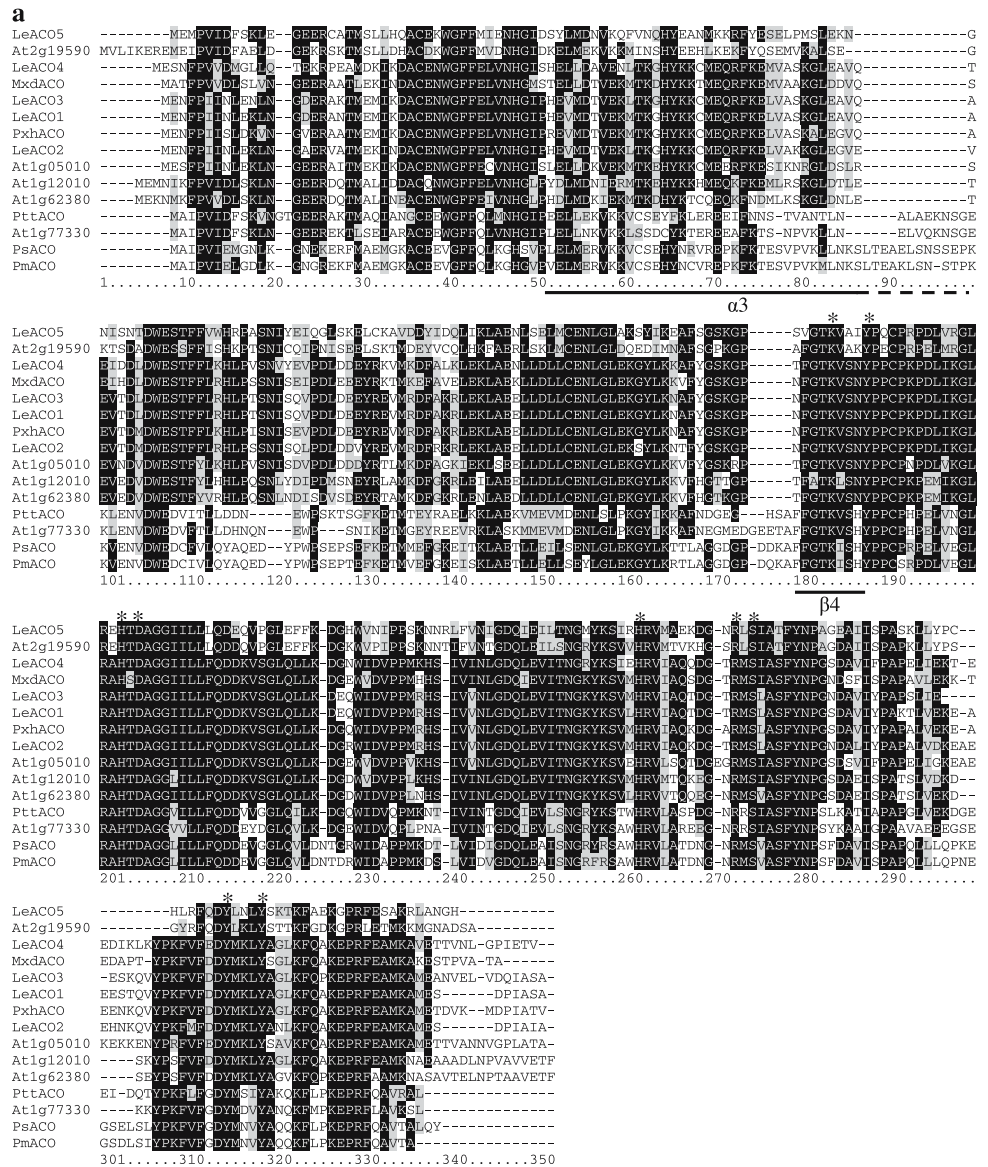
In order to obtain an ACO cDNA sequence from Douglas fir we next designed sets of primers both within the UTR regions and at the ORF termini based upon the *PsACO* and *PgACO* sequences and performed reverse transcription PCR using three tissue sources: (1) wounded bark (including phloem and cambium), (2) green shoot tips, and (3) phloem. One primer combination produced a single PCR product of ca. 1.5 kbp from all three tissue sources. Purification and sequencing of the PCR product derived from the bark RNA template provided a cDNA (*PmACO*) of 989 bp, which when translated encodes a 320 amino acid protein with a predicted pI value of 5.05 and molecular mass of 35.8 kDa (Table 1 and Fig. 1a). Pairwise sequence alignments of the deduced amino acid sequences indicate the putative starting methionine of *PmACO* overlaps with that of *PsACO* and *PgACO*; however, the 3'-end of *PmACO* is three amino acids shorter than *PsACO* and *PgACO* and the cDNA does not contain a stop codon, suggesting we have captured a near FLcDNA representing ACO from Douglas fir. The spruce and Douglas fir ACO proteins share 90.1% amino acid identity (Table 2) and 92.7–93.0% nucleotide identity. Using the TargetP v1.01 and PSORT subcellular localization software, we predict that both the spruce and Douglas fir ACO proteins are localized within the cytoplasm.

## Sequence relatedness of conifer and angiosperm ACO proteins

To decipher the evolutionary relationships between conifer (gymnosperm) and angiosperm ACO genes we performed maximum likelihood analysis using 15 ACO protein sequences from spruce, Douglas fir, tomato, *Arabidopsis*, poplar (*Populus tremula* × *tremuloides*), apple (*Malus* × *domestica*), and petunia (*Petunia* × *hybrida*). For further comparison we also included in this analysis proteins from other distinct branches of the plant dioxygenase family, including representatives of flavonol synthases, anthocyanidin synthases, and flavonone 3-hydroxylases from spruce, *Arabidopsis*, apple, and petunia. Multiple protein sequence alignments were performed using the complete ORFs and ClustalW and then manually adjusted. Using the neighbor-joining algorithm we generated a phylogenetic tree (Fig. 2), which suggests ACO proteins are as a group evolutionarily distinct from other groups of the Fe(II)-dependent dioxygenase family. Amino acid identity among the ACO proteins ranges from 35.5% (*Arabidopsis* ACO At2g19590 and *PmACO*) to 93.4% (*LeACO1* and *LeACO3*) (Table 2). As expected, *PsACO* and *PmACO* are most closely related to each other at 90.1% amino acid identity, followed next by 55.5 and 54.3% identity with *PttACO* from poplar, and 55.4 and 52.9% with *Arabidopsis* ACO At1g77330, respectively. Amino acid identity between *PsACO* and *PmACO* and all other angiosperm ACO proteins is 45% or less (Table 2), which is comparable to the lowest identity among the four tomato (45.5%) or five *Arabidopsis* (39.8%) ACO proteins.

For further structural comparison of conifer and angiosperm ACOs, we modeled the spruce and Douglas fir ACO proteins with ACO from petunia, for which a crystal structure is available (Zhang et al. 2004), and ACO from apple, which has previously been modeled on the crystal structure of a related isopenicillin-N-synthase (Seo et al. 2004) and structurally characterized (Rocklin et al. 1999; Zhou et al. 2002; Tierney et al. 2005) (Fig. 1b). The ORF size of petunia (319 amino acids), apple (314 aa) and spruce (324 aa) ACOs are similar, with the only notable differences being an additional 12 amino acid residues in spruce ACO within an extended helix ( $\alpha$ -3; Fig. 1a), identified as a proboscis by Zhang et al. (2004), and an additional four amino acid residues in the predicted  $\beta$ -4 (Fig. 1a) sheet of spruce ACO. However, this latter region of the protein is not within the predicted active site (Zhang et al. 2004). Enzymes of the Fe(II)-dependent dioxygenase family contain a conserved Fe<sup>2+</sup> ligand-binding pocket, referred to as a “2-His-1 carboxylate facial triad.” This site was found in spruce ACO at amino acid positions His-190, His-248, and Asp-192 (Fig. 1b; numbering of amino acid positions according to spruce ACO) and is believed to interact by strong charge-charge interactions (Seo et al. 2004; Zhang et al. 2004). The RXS motif is another common region of the family and binds 2-oxoglutarate (Roach et al. 1995), but has been modeled by Seo et al. (2004) to bind ascorbate

**Fig. 1** Sequence relatedness of gymnosperm and angiosperm ACO proteins. **a** Amino acid sequence alignment of ACO proteins from two conifer species, Sitka spruce (PsACO) and Douglas fir (PmACO), and selected angiosperms generated using ClustalW (blosum matrix, gap open and gap extension penalties of 5 and 0.5, respectively) and Boxshade. Conserved similarity shading is based on 50% identity (*black*) and 50% similarity (*gray*). Amino acid residues designated with *asterisks* have previously been implicated in molecular interactions with substrates in angiosperm ACOs. The *aligned* proteins indicate both PsACO and PmACO contain additional amino acids within an extended helix ( $\alpha$ -3) and the  $\beta$ -4 sheet typical of ACO proteins, as do the *Arabidopsis* (At1g77330) and poplar ACO (PttACO). Nomenclature and GenBank protein accession numbers for all sequences shown in this alignment are provided in Table 2. **b** Schematic model of the spruce and Douglas fir ACO active site modeled based on data for petunia and apple ACO (Rocklin et al. 1999; Zhang et al. 2004; Seo et al. 2004). The conifer ACO model was created with MDL ISIS/Draw 2.5 software (<http://www.mdl.com/index.jsp>)



in ACO (Arg-258-X-Ser-260; Fig. 1b). Other residues highlighted are consistent with the known crystal structure and results from mutagenesis studies. For example,

Lys-171, which projects into the ACO active site (Zhang et al. 2004), has been shown to alter the binding of bicarbonate by kiwi ACO (Lay et al. 1996). Zhang et al. (2004)

**Table 1** Gene name, genotype, protein, and transcript features of gymnosperm ACO genes represented by FLcDNAs (*PsACO*, *PgACO*) and a near FLcDNA (*PmACO*)

Clone ID	Gene name	cDNA source library	Species (genotype)	cDNA length (bp)	ORF length (aa)	Protein pI	Protein MW (kDa)
WS0273_P02	<i>PsACO</i>	Leader tissue subjected to <i>Choristoneura occidentalis</i> (budworm) herbivory	<i>Picea sitchensis</i> (FB3-425)	1,505	324	4.97	36.5
WS00111_I09	<i>PgACO</i>	Young shoots	<i>Picea glauca</i> (PG29)	1,451	324	4.97	36.5
DF_F1R2	<i>PmACO</i>	Bark tissue (with phloem and cambium) harvested 24 h after razor blade wounding	<i>Pseudotsuga menziesii</i> (wild)	989	320	5.05	35.8

**Table 2** Sequence relatedness of plant ACO proteins

	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o
LeACO5 (a)	100														
At2g19590 (b)	63.7	100													
LeACO4 (c)	47.0	43.7	100												
MxdACO (d)	47.6	45.3	75.9	100											
LeACO3 (e)	45.5	44.8	77.3	75.0	100										
LeACO1 (f)	45.9	44.3	76.6	75.6	93.4	100									
PxhACO (g)	46.6	44.5	76.9	76.2	89.4	91.5	100								
LeACO2 (h)	46.4	43.6	73.9	74.8	86.6	88.9	87.8	100							
At1g05010 (i)	44.8	43.2	73.1	71.2	74.0	74.6	74.9	73.4	100						
At1g12010 (j)	45.2	45.2	67.4	67.1	67.5	67.7	68.0	66.9	66.4	100					
At1g62380 (k)	44.2	44.0	65.8	65.8	67.2	67.1	65.8	65.3	65.1	85.0	100				
PttACO (l)	40.9	40.4	45.8	47.1	45.2	46.2	46.8	45.9	44.6	43.5	44.7	100			
At1g77330 (m)	40.9	39.8	45.2	46.8	44.4	44.4	44.1	44.7	44.6	44.2	45.7	71.7	100		
PsACO (n)	37.4	36.3	45.0	43.5	45.3	45.0	44.8	45.3	43.2	44.6	44.3	55.5	55.4	100	
PmACO (o)	36.0	35.5	44.5	43.4	45.4	45.2	44.6	45.2	42.8	43.9	43.6	54.3	52.9	90.1	100

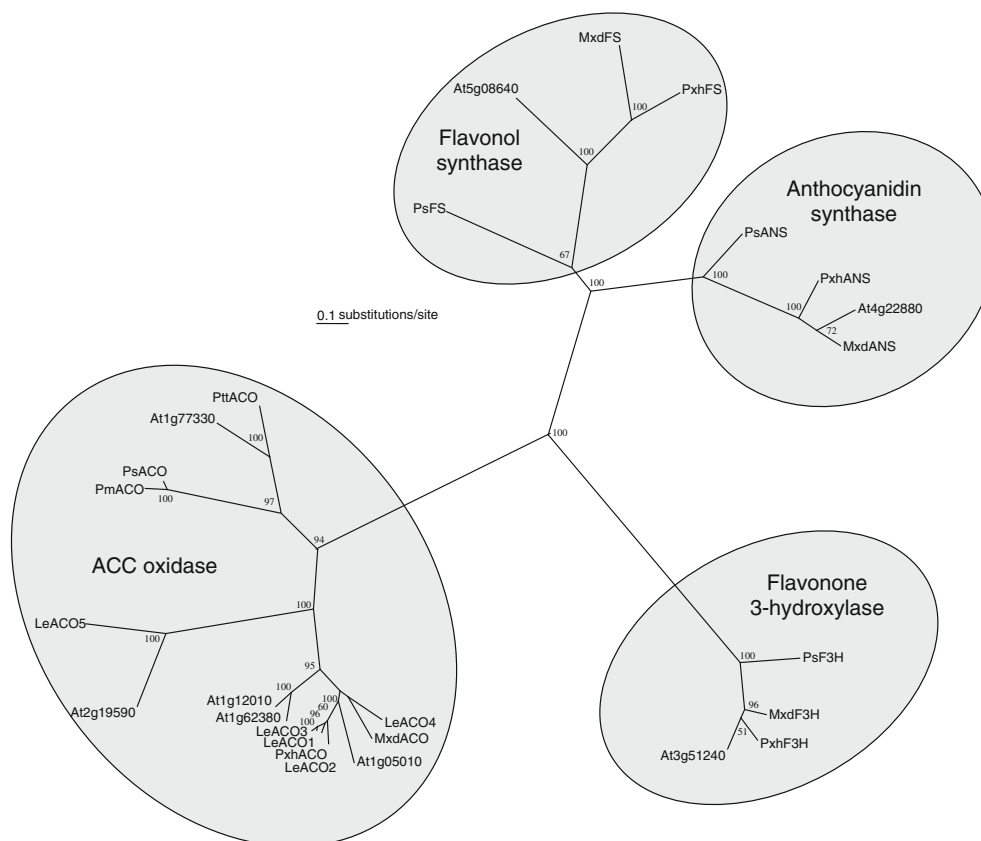
Results from pairwise amino acid sequence comparisons are shown as percent identity. ACO nomenclature and GenBank protein accession numbers are as follows: At1g05010 (NP\_171994), At1g12010 (NP\_172665), At1g62380 (NP\_176428), At1g77330 (NP\_565154), and At2g19590 (NP\_179549), *Arabidopsis thaliana*; LeACO1 (CAA41212), LeACO2 (CAA68538), LeACO3 (CAA90904), LeACO4 (BAA34924), and LeACO5 (CAG29395), *Lycopersicon esculentum* (tomato); MxdACO (Q00985), *Malus × domestica* (apple); PxhACO (Q08506), *Petunia × hybrida* (petunia); PsACO (DQ480740), *Picea sitchensis* (Sitka spruce); PttACO (AAN87846) *Populus tremula × P. tremuloides*; PmACO (DQ480742), *Pseudotsuga menziesii*

indicated that the side chains corresponding to Tyr-304/300 are pointed toward the metal and may be involved in mediating electron transfer. Tyr-175 is represented in the model as it occurs within the active site face (Zhang et al. 2004). All of the amino acid residues identified in the spruce ACO active site model are also found in the Douglas fir ACO sequence. In summary, comparison of conifer and angiosperm ACO sequences identified conserved active site residues and high overall sequence relatedness of angiosperm and gymnosperm ACO proteins.

#### Wounding induces ACO protein accumulation and increased ethylene levels

Protein expression and cellular localization of ACO has recently been tested in Douglas fir stem tissues using an *Arabidopsis* anti-ACO antibody (Hudgins and Franceschi 2004). The newly available sequences of conifer ACO proteins described here for two species of spruce and Douglas fir support the suitability of an antibody raised against a closely related *Arabidopsis* ACO (Figs. 1,

2) for studies in conifers. The ACO antibody was raised against an *Arabidopsis* synthetic peptide near the N-terminal of the protein (for details, Santa Cruz Biotechnology, SCBT). Amino acid sequence analysis of the Douglas fir ACO described here confirmed the antibody would cross-react with PmACO (JWH, personal communication with SCBT). Sequence analysis indicated that the ACO peptide used for antibody generation does not share homology with the closely related conifer genes from other groups of the dioxygenase family (e.g., PsANS, PsFS, and PsF3H). With this new information, we validated the previous analysis (Hudgins and Franceschi 2004) of ACO expression and determined subcellular tissue localization using an independent set of induced stem tissue samples. In brief, Western immunoblots demonstrated that the anti-ACO antibody cross-reacted with a single band of approximately 35–40 kDa (Fig. 3a) in Douglas fir bark protein extracts, consistent with the predicted MW of the conifer ACO described here. ACO protein was found to be constitutively expressed at low levels in unwounded Douglas fir stems consistent with previous studies by Hudgins and Franceschi (2004). In



**Fig. 2** Phylogenetic analysis of members of the plant ACO and dioxygenase family. Amino acid sequences of 27 proteins were analyzed by maximum likelihood using Phyml. Bootstrap values are only for nodes with greater than 50% support. Maximum likelihood values represent percentages of 100 gamma-corrected replicates (log  $L = -9,729$ ). ACO nomenclature and GenBank protein accession numbers are provided in Table 2. Nomenclature and GenBank accession numbers for flavonol synthase (FS), anthocyanidin synthase (ANS), and flavonone 3-hydroxylase (F3H) proteins are as follows: At5g08640 (NP\_196481), At4g22880 (NP\_194019), and

At3g51240 (NP\_190692), *Arabidopsis thaliana*; MxdFS (AAX89401), MxdANS (BAB92998), and MxdF3H (BAB92997), *Malus × domestica* (apple); PxxFS (CAA80264), PxxANS (P51092), and PxxF3H (AAC49929), *Petunia × hybrida* (petunia). FLcDNA sequences for PsFS, PsANS, and PsF3H were obtained from extended sequencing of *Picea sitchensis* (Sitka spruce) cDNA clones WS0271\_C15 (EST accession DR521499), WS02712\_M02 (EST accession DR522800), and WS02735\_I24 (EST accession DR531482), respectively

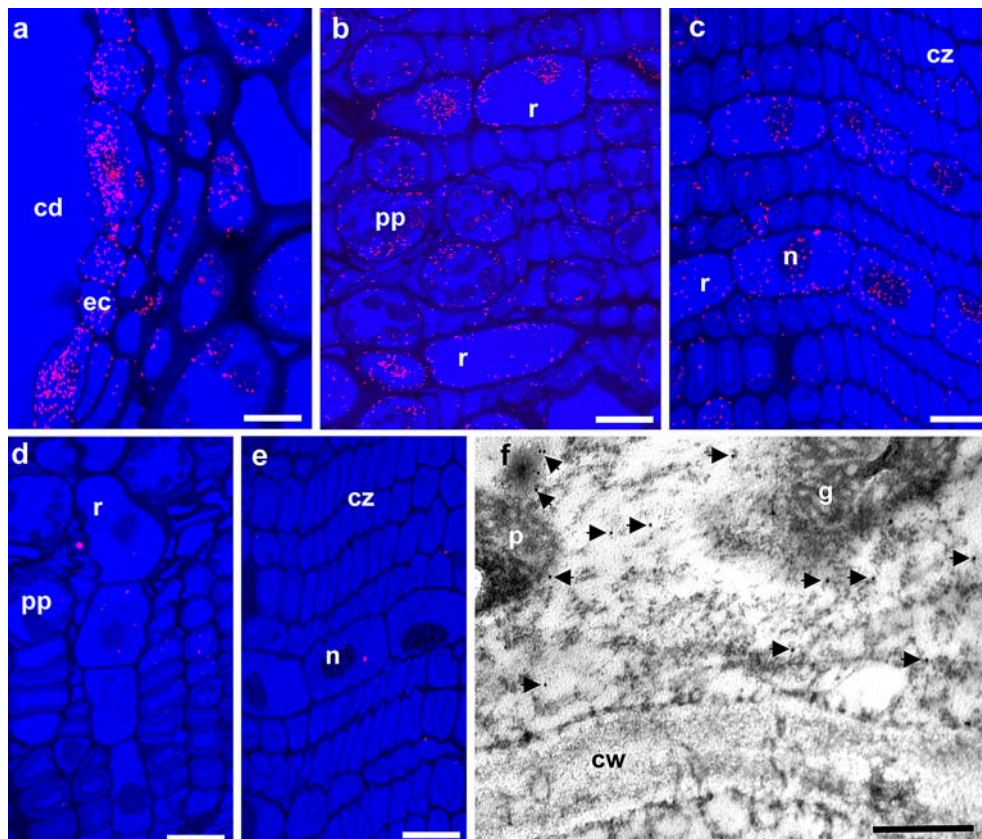
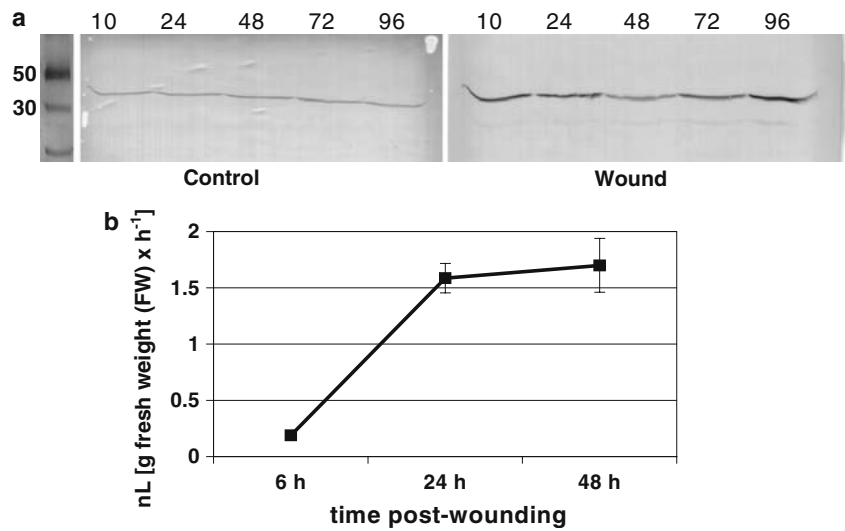
the present study, ACO was found to increase slightly in abundance following wounding and remained higher than constitutive levels at the sampled time points (Fig. 3a). Ethylene measurements were collected to compare with changes in protein levels of ACO. Ethylene could not be detected in unwounded stem sections under the conditions used in this study. However, by 6 h after wounding, ethylene was clearly detectable and increased greatly by 24 h and remained elevated for another 24 h (Fig. 3b). This temporal pattern of induction and the measurable levels of ethylene are consistent with previous observations in an independent sample set described in Hudgins and Franceschi (2004).

ACO is localized to the cytoplasm of cells responsible for inducible defenses

Using immunocytochemical ACO localization with wound-induced Douglas fir bark tissues, we consistently

found the highest abundance of ACO protein in epithelial cells of cortical resin ducts (Fig. 4a), in PP cells (Fig. 4b), and in ray parenchyma (Fig. 4b, c). This is in agreement with previous findings by Hudgins and Franceschi (2004). Non-immune serum did not give appreciable labeling of any cell type (Fig. 4d, e). The epithelial cells of cortical resin ducts (Fig. 4a) usually occur as a single layer of cells that line the inner surface of the extracellular resin duct lumen, which is the predominant site for constitutive and induced resin accumulation. Activated resin duct epithelial cells are believed to be the primary site for cell-specific oleoresin terpenoid formation. Thin-walled PP cells are the proposed site for the accumulation of induced phenolics, which are visible in the form of optically dense droplets (vacuoles) in the PP cell cytoplasm (Fig. 4b), but are absent in the thick-walled sclereids and ray parenchyma cells (Fig. 4b). In both the induced phloem (Fig. 4b) and the cambium zone (Fig. 4c), ACO was found at high levels in ray

**Fig. 3** Wound-induced ACO protein expression and ethylene levels in Douglas fir bark. **a** Western blot analysis of ACO from control or wounding treatments from total protein extracts at 10, 24, 48, 72, and 96 h post-treatment. A total of 15  $\mu$ g protein was loaded per lane, separated by SDS-PAGE, blotted to a nitrocellulose membrane and probed with *Arabidopsis* anti-ACO antibody. Independent repetitions of western blot analysis showed the same protein profiles. **b** Ethylene levels in stems at 6, 24, and 48 h following wounding. In control stem samples, levels of ethylene were below the detection limit of our methods



**Fig. 4** Cellular and subcellular localization of ACO protein in induced bark tissue of Douglas fir sapling stems. **a-e** Immunocytochemical localization of ACO in Douglas fir wound-induced stems by confocal microscopy 24 h after wounding. Reflected/transmitted images are of silver-enhanced gold labeling (pink particles). *cd* cortical duct, *ec* epithelial cell, *pp* polyphenolic parenchyma cell, *r* ray parenchyma cell, *n* nucleus, *cz* cambial zone. Cross-section of wounded tissue shows labeling is abundant in **a** epithelial cells of the phloem and cambial zone, **b** PP cells, and **b** and **c** ray parenchyma cells in the phloem and cambial zone. *Bar* = 40  $\mu$ m. Labeling indicates

ACO in the cytoplasm of epithelial cells, PP cells, and ray parenchyma cells and some possible labeling associated with nuclear regions. **d** Section containing ray parenchyma, PP cells, and **e** cambial zone cells treated with non-immune serum showing label is absent. *Bar* = 40  $\mu$ m. **f** Subcellular localization of ACO by TEM. Cross-section of wounded tissue showing ACO label occurs within cytoplasmic regions of ray parenchyma but not associated with cell walls (*cw*), plastids (*p*), or golgi (*g*). *Bar* = 0.75  $\mu$ m. Examples of labeling are indicated by *arrows*

parenchyma cells that connect the inner and outer conifer stem tissues. In PP and ray parenchyma cells, anti-ACO antibodies also appear to label the nuclear regions in many cells examined; however, this may be a result of the large vacuole of these cell types compressing cytoplasm around the nuclear domains (Fig. 4a–c). At the subcellular level, ACO protein is detectable in the cytoplasm of resin duct epithelial cells. High-resolution transmission electron microscopy (TEM) subcellular localization showed labeling for ACO (Fig. 4f) was associated with the cytoplasm in all cell types, but not with the plasma membrane, cell wall, vacuole, or other organelles, in agreement with previous studies of angiosperm ACOs (Reinhardt et al. 1994; Chung et al. 2002).

## Discussion

With their extremely long life spans of up to hundreds of years and their enormous range of ecological and geographical distribution, members of the Pinaceae are exposed to a vast array of herbivore and pathogen species (Seybold et al. 2000). Genomic hard-wiring and phenotypic plasticity of constitutive and induced chemicals, such as the diverse array of phenolic and terpenoid compounds characteristic of many conifers, appear to be an important component of efficient and long-term conifer defense strategies (Huber et al. 2004). Some of these defenses are rapidly activated by attack from stem-boring pests, such as bark beetles and weevils, along with their symbiotic associated fungi. In recent years, several signaling molecules have surfaced as potential regulators of inducible anatomical and chemical defense systems in conifers. These include the activities of the octadecanoid MJ and ethylene in the induction of phenolic and terpenoid defenses associated with PP cells and resin ducts, respectively (Franceschi et al. 2002; Martin et al. 2002; Hudgins et al. 2003b; Hudgins and Franceschi 2004; Miller et al. 2005; Huber et al. 2005).

In conifers, several endogenous roles have been established for the phytohormone ethylene. Ethylene has been correlated with cambial zone activity leading to xylem (wood) formation and differentiation (Ingemarsson et al. 1991; Eklund and Little 1998; Little and Eklund 1999; Klintborg et al. 2002). Previous studies have established a strong correlation between wounding, endogenous ethylene, and resin accumulation in conifers (Popp et al. 1995; Hudgins and Franceschi 2004). In addition, exogenous ethylene application can increase resin production and the number of resin ducts in several conifer species (Telewski et al. 1983; Yamamoto and Kozlowski 1987; Katoh and Croteau 1997; Hudgins and Franceschi 2004). ACO activity, the final step in ethylene formation, has previously been detected in several conifer species (Plomion et al. 2000; Reynolds and John 2000; Klintborg et al. 2002), but little is known about the corresponding ACO genes, their expression and cellular localization in conifers and the relationship of ACO with ethylene

production and activities of specialized cells for phenolic or terpenoid formation after wounding in stems.

We have identified ACO sequences from conifers by mining of spruce EST and FLcDNA resources. The FLcDNAs *PsACO* and *PgACO* are likely allelic variants of the same conifer ACO. Using the spruce ACO sequences we cloned a near FLcDNA from Douglas fir that is over 90% identical at the amino acid level. Surprisingly, after sequencing nearly 150,000 3'-ESTs from 20 different spruce cDNA libraries representing different tissues, developmental stages, and stress treatments (Ralph et al. 2006b), all other ACO-like cDNAs identified in this database represented partial sequences identical with *PsACO* and *PgACO*. This suggests there may be only one ACO gene expressed in spruce, or that other spruce ACO representatives are considerably divergent in sequence from the three ACO cDNAs reported here and the well-characterized angiosperm ACO genes. Alternatively, other spruce ACO genes may be expressed at very low levels or exhibit tissue- or cell-specific expression and are therefore not captured in our EST collection.

Phylogenetic analysis using angiosperm ACO proteins, as well as representatives from other divisions of the larger plant dioxygenase family (i.e., flavonol synthases, FS; anthocyanidin synthases, ANS; flavonone 3-hydroxylases, F3H), demonstrates that the spruce and Douglas fir sequences reported here belong to the ACO branch of this protein family. Interestingly, for each of the four groups of proteins examined, ACO, FS, ANS, F3H, we found conifer (gymnosperm) and angiosperm representatives grouped within the same clade, suggesting evolutionary separation of these four groups of proteins in a common ancestor of extant conifers and angiosperms. The conifer ACO proteins described here are most closely related to At1g77330 from *Arabidopsis* and PttACO from poplar. The latter has been shown abundantly expressed in wood-forming tissues, with specific up-regulation during secondary wall and tension wood formation (Andersson-Gunneras et al. 2003). Comparisons of the spruce and Douglas fir ACOs with the well-characterized petunia and apple ACOs showed a highly conserved active site structure. Spruce and Douglas fir ACO proteins contain additional residues within the ACO  $\alpha$ -3 and  $\beta$ -4 sheet, which is similarly conserved in the two closely related *Arabidopsis* (At1g77330) and poplar (PttACO) ACO proteins.

In angiosperms, ACO expression is induced in response to various forms of biotic or abiotic stresses and is differentially expressed during plant development (see Kende 1993; Kim and Yang 1994; Kim et al. 1998; Bleeker and Kende 2000). Douglas fir ACO was detected as a single protein band in Western blots. In previous work we demonstrated the protein was rapidly induced with exogenous MJ applications to stems of Douglas fir (Hudgins and Franceschi 2004), but here we show only a weak up-regulation of constitutive ACO in response to mechanical wounding, which more accurately represents stem damage following chewing by a bark-boring insect. The

strong wound-induced evolution of ethylene compared to only moderate ACO protein up-regulation suggests that ACO is not a rate-limiting step in wound-induced ethylene response in the Douglas fir stem tissues tested. With this result and reports of ACS as a rate-limiting step in ethylene production in several angiosperm species (Bleeker and Kende 2000), our future work will test ACS for a role in the regulation of ethylene signaling in the wound-induced defense response in conifers.

Localization studies with the anti-ACO antibody demonstrated the enzyme was expressed in wound-induced Douglas fir bark tissues with highest abundance in specialized secretory epithelial cells of cortical ducts, PP cells, and ray parenchyma cells in the phloem and cambial zone. Resin duct epithelial and PP cells are known to be responsive to activation by ethylene with respect to defensive chemical synthesis, while no obvious anatomical changes are normally observed in ray parenchyma cells with exogenous ethylene treatments (Hudgins and Franceschi 2004). The presence of ACO in ray parenchyma cells of the phloem and cambial zone in induced Douglas fir stem tissues could have a function in systemic defense response that is not limited to the outermost bark and phloem tissues. Ray parenchyma cells provide radial transport between secondary phloem, cambium, and xylem, and the cambium is primarily supplied by the rays (Lev-Yadun and Aloni 1995). Ray parenchyma cells are also connected to a tangential pathway in the secondary phloem of the stem via the rows of PP cells, which are linked by abundant plasmodesmata in the interconnecting radial walls (Krekling et al. 2000). Signals could be communicated along the radial elements of ray parenchyma and trigger simultaneously specialized cell responses in the phloem, cambial zone, and outer xylem such as induced de novo formation of xylem traumatic resin ducts (Franceschi et al. 2000, 2002; Martin et al. 2002; Hudgins et al. 2003a, 2004). At the subcellular level, labeling for ACO was primarily associated with the cytoplasm and not with other organelles, in agreement with previous localization studies of angiosperm ACO (Reinhardt et al. 1994; Chung et al. 2002). In contrast, Romaldi et al. (1994) and Ramassamy et al. (1998) found ACO from apple fruit localized in cell walls and plasma membranes, respectively.

In future studies we will test the role of ethylene signaling in activation of cells specialized for the formation of defense-related terpenoids and phenolics further, with dissection of other possible up-stream and down-stream signaling events such as ethylene perception in the specialized cells and possible ethylene-dependent activation of kinase-signaling events. While ethylene has been shown to be inducible by exogenous MJ in Douglas fir stems, future work will test the possible interactions of endogenous wound or insect-induced ethylene signaling events in conifer stem tissues.

**Acknowledgments** Confocal and TEM microscopy was done in the Electron Microscopy Center, Washington State University. We thank Sharon Jancsik for technical assistance with cDNA cloning

and RNA isolation. This work was generously supported by grants to JB from Genome Canada, Genome British Columbia, and the province of British Columbia; the Natural Sciences and Engineering Research Council of Canada (NSERC); and with infrastructure funds from the Canadian Foundation for Innovation (CFI) and the British Columbia Knowledge and Development Funds (BCKDF). This paper is dedicated in memory of Dr. Vincent R. Franceschi (1953–2005). Vince was a leading scientist who contributed greatly to plant sciences through his teaching, research, generous collaborative support, and countless other activities. His work on the cell biology of conifer defense has been a great inspiration and provided an important foundation for many current and future studies.

## References

- Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in plant biology, 2nd edn. Academic, San Diego, pp 26–55
- Alfaro RI (1995) An induced defense reaction in white spruce to attack by the white pine weevil (*Pissodes strobi*). *Can J For Res* 25:1725–1730
- Alfaro RI, Borden JH, King JN, Tomlin ES, McIntosh RL, Bohlmann J (2002) Mechanisms of resistance in conifers against shoot infesting insects. In: Wagner MR, Clancy KM, Lieutier F, Paine TD (eds) Mechanisms and deployment of resistance in trees to insects. Kluwer Academic, Dordrecht, pp 101–126
- Andersson-Gunneras S, Hellgren JM, Bjorklund S, Regan S, Moritz T, Sundberg B (2003) Asymmetric expression of a poplar ACC oxidase controls ethylene production during gravitational induction of tension wood. *Plant J* 34:339–349
- Bleeker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. *Annu Rev Cell Dev Biol* 16:1–18
- Bohlmann J, Croteau R (1999) Diversity and variability of terpenoid defenses in conifers: molecular genetics, biochemistry and evolution of the terpene synthase gene family in grand fir (*Abies grandis*). In: Chadwick DJ, Goode JA (eds) Insect plant interactions and induced plant defense. Wiley, West Sussex, pp 132–146
- Bois E, Lieutier F (1997) Phenolic response of Scots pine clones to inoculation with *Leptographium wingfieldii*, a fungus associated with *Tomium piniperda*. *Plant Physiol Biochem* 35:819–825
- Bonello P, Blodgett JT (2003) *Pinus nigra-Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol Mol Plant Pathol* 63:249–261
- Bonello P, Gordon TR, Storer AJ (2001) Systemic induced resistance in Monterey pine. *For Pathol* 31:99–106
- Brignolas F, Lacroix B, Lieutier F, Sauvard D, Drouet A, Claudot A-C, Yart A, Berryman AA, Christiansen E (1995) Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculation with *Ophiostoma polonicum* a bark beetle-associated fungus. *Plant Physiol* 109:821–827
- Byun-McKay SA, Hunter WL, Godard K-A, Wang SW, Martin DM, Bohlmann J, Plant AL (2003) Insect attack and wounding induce traumatic resin duct development and gene expression of (–)-pinene synthase in Sitka spruce. *Plant Physiol* 133:368–378
- Christiansen E, Krokene P, Berryman AA, Franceschi VR, Krekling T, Lieutier F, Lönneborg A, Solheim H (1999) Mechanical injury and fungal infection induce acquired resistance in Norway spruce. *Tree Physiol* 19:399–403
- Chung MC, Chou SJ, Kuang LY, Chang YY, Yang SF (2002) Subcellular localization of 1-aminocyclopropane-1-carboxylic acid oxidase in apple fruit. *Plant Cell Physiol* 43:549–554
- Dong JG, Fernandez-Maculet JC, Yang SF (1992) Purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from apple fruit. *Proc Natl Acad Sci USA* 89:9789–9793
- Eklund L, Little CHA (1998) Ethylene evolution, radial growth and carbohydrate concentrations in *Abies balsamea* shoots ringed with ethrel. *Tree Physiol* 18:383–391
- Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* 300:1005–1016

- Fäldt J, Martin D, Miller B, Rawat S, Bohlmann J (2003) Traumatic resin defense in Norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. *Plant Mol Biol* 51:119–133
- Felsenstein J (1993) PHYLIP (phylogeny inference package) version 3.62. Distributed by the author. Department of Genetics, University of Washington, Seattle
- Franceschi VR, Kreckling T, Berryman AA, Christiansen E (1998) Specialized phloem parenchyma cells in Norway spruce (Pinaceae) bark are an important site of defense reactions. *Am J Bot* 85:601–615
- Franceschi VR, Krokene P, Kreckling T, Berryman AA, Christiansen E (2000) Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). *Am J Bot* 87:314–326
- Franceschi VR, Kreckling T, Christiansen E (2002) Application of methyl jasmonate on *Picea abies* (Pinaceae) stems induces defense-related responses in phloem and xylem. *Am J Bot* 89:578–586
- Franceschi VR, Krokene P, Christiansen E, Kreckling T (2005) Tansley review: anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol* 167:353–376
- Gascuel O (1997) BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Mol Biol Evol* 14:685–695
- Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704
- Huang X, Madan A (1999) CAP3: a DNA sequence assembly program. *Genome Res* 9:868–877
- Huber DPW, Ralph S, Bohlmann J (2004) Genomic hardwiring and phenotypic plasticity of terpenoid-based defenses in conifers. *J Chem Ecol* 30:2399–2418
- Huber DPW, Philippe RN, Madilao L, Sturrock RN, Bohlmann J (2005) Changes in anatomy and terpene chemistry in roots of Douglas-fir seedlings following treatment with methyl jasmonate. *Tree Physiol* 25:1075–1083
- Hudgins JW, Franceschi VR (2004) Methyl jasmonate-induced ethylene production is responsible for phloem defense responses and reprogramming of conifer stem cambial zone for traumatic resin duct formation. *Plant Physiol* 135:2134–2149
- Hudgins JW, Christiansen E, Franceschi VR (2003a) Methyl jasmonate induces changes mimicking anatomical defenses in diverse members of the Pinaceae. *Tree Physiol* 23:361–371
- Hudgins JW, Kreckling T, Franceschi VR (2003b) Distribution of calcium oxalate crystals in the secondary phloem of conifers: a constitutive defense mechanism? *New Phytol* 159:677–690
- Hudgins JW, Christiansen E, Franceschi VR (2004) Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic treatment. *Tree Physiol* 24:251–264
- Ingemarsson BS, Lundqvist ME, Eliasson L (1991) Seasonal variation in ethylene concentration in the wood of *Pinus sylvestris* L. *Tree Physiol* 8:273–279
- Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. *Comput Appl Biosci* 8:275–282
- Katoh S, Croteau R (1997) Individual variation in constitutive and induced monoterpenes biosynthesis in grand fir. *Phytochemistry* 47:577–582
- Keeling CI, Bohlmann J (2006) Genes, enzymes, and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol* (in press)
- Kende H (1993) Ethylene biosynthesis. *Annu Rev Plant Physiol Plant Mol Biol* 44:283–307
- Kim YS, Yang SF (1994) Structure and expression of cDNAs encoding 1-aminocyclopropane-1-carboxylate oxidase homologs isolated from excised mung bean hypocotyls. *Planta* 194:223–229
- Kim YS, Choi D, Lee MM, Lee SH, Kim WT (1998) Biotic and abiotic stress-related expression of 1-aminocyclopropane-1-carboxylate oxidase gene family in *Nicotiana glutinosa* L. *Plant Cell Physiol* 39:565–573
- Klintborg A, Eklund L, Little CHA (2002) Ethylene metabolism in Scots pine (*Pinus sylvestris*) shoots during the year. *Tree Physiol* 22:59–66
- Kolosova N, Miller B, Ralph S, Ellis BE, Douglas C, Ritland K, Bohlmann J (2004) Isolation of high-quality RNA from gymnosperm and angiosperm trees. *Biotechniques* 36:821–824
- Kreckling T, Franceschi VR, Berryman AA, Christiansen E (2000) The structure and development of polyphenolic parenchyma cells in Norway spruce (*Picea abies*) bark. *Flora* 195:354–369
- Krokene P, Christiansen E, Solheim H, Berryman AA, Franceschi VR (1999) Induced resistance to pathogenic fungi in Norway spruce. *Plant Physiol* 121:565–570
- Krokene P, Solheim H, Kreckling T, Christiansen E (2003) Inducible anatomical defense responses in Norway spruce stems and their possible role in induced resistance. *Tree Physiol* 23:191–197
- Kusumoto D, Suzuki K (2003) Spatial distribution and time-course of polyphenol accumulation as a defense response induced by wounding in the phloem of *Chamaecyparis obtuse*. *New Phytol* 10:1469–1481
- Langenheim JH (2003) Plant resins: chemistry, evolution, ecology, and ethnobotany. Timber Press, Portland
- Lay VJ, Prescott AG, Thomas PG, John P (1996) Heterologous expression and site-directed mutagenesis of the 1-aminocyclopropane-1-carboxylate oxidase from kiwi fruit. *Eur J Biochem* 242:228–234
- Lev-Yadun S, Aloni R (1995) Differentiation of the ray system in woody-plants. *Bot Rev* 61:45–84
- Lieutier F, Sauvard D, Brignolas F, Picron V, Yart A, Bastien C, Jay-Allemand C (1996) Changes in phenolic metabolites of Scots pine phloem induced by *Ophiostoma brunneo-ciliatum*, a bark beetle-associated fungus. *Eur J For Pathol* 26:145–158
- Little CHA, Eklund L (1999) Ethylene in relation to compression wood formation in *Abies balsamea* shoots. *Trees* 13:173–177
- Martin D, Bohlmann J (2005) Molecular biochemistry and genomics of terpenoid defenses in conifers. *Rec Adv Phytochem* 39:29–56
- Martin D, Tholl D, Gershenzon J, Bohlmann J (2002) Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiol* 129:1003–1018
- Martin DM, Fäldt J, Bohlmann J (2004) Functional characterization of nine Norway spruce *TPS* genes and evolution of gymnosperm terpene synthases of the *TPS-d* subfamily. *Plant Physiol* 135:1908–1927
- Miller B, Madilao L, Ralph S, Bohlmann J (2005) Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiol* 137:369–382
- Nagy NE, Franceschi VR, Solheim H, Kreckling T, Christiansen E (2000) Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): anatomy and cytochemical traits. *Am J Bot* 87:302–313
- Nagy NE, Fossdal CG, Krokene P, Kreckling T, Lønneborg A, Solheim H (2004) Induced responses to pathogen infection in Norway spruce phloem: changes in polyphenolic parenchyma cells, chalcone synthase transcript levels and peroxidase activity. *Tree Physiol* 24:505–515
- Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci* 24:34–36
- Page RD (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12:357–358
- Pan HF, Lundgren LN (1996) Phenolics from inner bark of *Pinus sylvestris*. *Phytochemistry* 42:1185–1189
- Phillips MA, Croteau R (1999) Resin-based defenses in conifers. *Trends Plant Sci* 5:184–190
- Plomion C, Pionneau C, Brach J, Costa P, Bailleres H (2000) Compression wood-responsive proteins in developing xylem of maritime pine (*Pinus pinaster* Ait.). *Plant Physiol* 123:959–969
- Popp M, Johnson JD, Lesney M (1995) Changes in ethylene production and monoterpene concentration in slash pine and loblolly

- pine following inoculation with bark beetle vectored fungi. *Tree Physiol* 15:807–812
- Prescott AG, John P (1996) Dioxxygenases: molecular structure and role in plant metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 47:245–271
- Raffa KF, Aukema BH, Erbilgin N, Klepzig KD, Wallin KF (2005) Interactions among conifer terpenoids and bark beetles across multiple levels of scale: an attempt to understand population patterns and physiological processes. *Rec Adv Phytochem* 39:79–118
- Ralph S, Park JY, Bohlmann J, Mansfield SD (2006a) Dirigent proteins in insect-induced conifer defense: discovery, phylogeny and differential expression of a family of DIR and DIR-like genes in spruce. *Plant Mol Biol* 60:21–40
- Ralph S, Yueh H, Friedmann MF, Aeschliman D, Zeznik JA, Nelson CC, Butterfield YSN, Kirkpatrick R, Liu J, Jones SJM, Marra MA, Douglas CJ, Ritland K, Bohlmann J (2006b) Conifer defense against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworm (*Choristoneura occidentalis*) or white pine weevil (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant Cell Environ* (in press)
- Ramassamy S, Olmos E, Bouzayen M, Pech JC, Latche A (1998) 1-Aminocyclopropane-1-carboxylate oxidase of apple fruit is periplasmic. *J Exp Bot* 49:1909–1915
- Reinhardt D, Kende H, Boller T (1994) Subcellular localization of 1-aminocyclopropane-1-carboxylate oxidase in tomato cells. *Planta* 195:142–146
- Reynolds EA, John P (2000) ACC oxidase is found in seedlings of two (Coniferales, Gnetales) of the four gymnosperm orders. *Physiol Plant* 110:38–41
- Roach PL, Clifton IJ, Fueleop V, Harlos K, Barton GJ, Hajdu J, Andersson I, Schofield CJ, Baldwin JE (1995) Crystal structure of isopenicillin-n-synthase is the first from a new structural family of enzymes. *Nature* 375:700–704
- Rocklin AM, Tierney DL, Kofman V, Brunhuber NMW, Hoffman BM, Christoffersen RE, Reich NO, Lipscomb JD, Que L Jr (1999) Role of the nonheme Fe (II) center in the biosynthesis of the plant hormone ethylene. *Proc Natl Acad Sci USA* 96:7905–7909
- Rombaldi C, Lelievre JM, Latche A, Petitprez M, Bouzayen M, Pech JC (1994) Immunocytolocalization of 1-aminocyclopropane-1-carboxylic acid oxidase in tomato and apple fruit. *Planta* 192:453–460
- Schmidt A, Zeneli G, Hietala AM, Fossdal CG, Krokene P, Christiansen E, Gershenzon J (2005) Induced chemical defenses in conifers. *Rec Adv Phytochem* 39:1–28
- Seo YS, Yoo A, Jung J, Sung S-K, Yang DR, Kim WT, Lee W (2004) The active site and substrate-binding mode of 1-aminocyclopropane-1-carboxylate oxidase determined by site-directed mutagenesis and comparative modeling studies. *Biochem J* 38:339–346
- Seybold SJ, Bohlmann J, Raffa KF (2000) Biosynthesis of coniferophagous bark beetle pheromones and conifer isoprenoids: evolutionary perspective and synthesis. *Can J Entomol* 132:697–753
- Telewski FW, Wakefield AH, Jaffe MJ (1983) Computer-assisted image analysis of tissues of ethrel-treated *Pinus taeda* seedlings. *Plant Physiol* 72:177–181
- Tierney DL, Rocklin AM, Lipscomb JD, Que L Jr, Hoffman BM (2005) ENDOR studies of the ligation and structure of the nonheme iron site in ACC oxidase. *J Am Chem Soc* 127:7005–7013
- Tomlin ES, Alfaro RI, Borden JH, He F (1998) Histological response of resistant and susceptible white spruce to simulated white pine weevil damage. *Tree Physiol* 18:21–28
- Trapp S, Croteau R (2001) Defensive resin biosynthesis in conifers. *Annu Rev Plant Physiol Plant Mol Biol* 52:689–724
- Viiiri H, Annala E, Kitunen V, Niemelä P (2001) Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*. *Trees* 15:112–122
- Yamamoto F, Kozłowski TT (1987) Effects of flooding, tilting of stems, and ethrel application on growth, stem anatomy and ethylene production of *Pinus densiflora* seedlings. *J Exp Bot* 38:293–310
- Yang SF, Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. *Annu Rev Plant Physiol* 35:155–189
- Zhang Z, Ren J-S, Clifton IJ, Schofield CJ (2004) Crystal structure and mechanistic implications of 1-aminocyclopropane-1-carboxylic acid oxidase—the ethylene forming enzyme. *Chem Biol* 11:1383–1394
- Zhou J, Rocklin AM, Lipscomb JD, Que L Jr, Solomon EI (2002) Spectroscopic studies of 1-aminocyclopropane-1-carboxylic acid oxidase: molecular mechanism and CO<sub>2</sub> activation in the biosynthesis of ethylene. *J Am Chem Soc* 124:4602–4609