Loblolly pine abietadienol/abietadienal oxidase PtAO (CYP720B1) is a multifunctional, multisubstrate cytochrome P450 monooxygenase

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Edited by May R. Berenbaum, University of Illinois at Urbana–Champaign, Urbana, IL, and approved April 14, 2005 (received for review February 1, 2005)

Cytochrome P450 monooxygenases (P450s) are important enzymes for generating some of the enormous structural diversity of plant terpenoid secondary metabolites. In conifers, P450s are involved in the formation of a suite of diterpene resin acids (DRAs). Despite their important role in constitutive and induced oleoresin defense, a P450 gene of DRA formation has not yet been identified. By using phylogenetic cluster analysis of P450-like ESTs from loblolly pine (Pinus taeda), functional cDNA screening in yeast (Saccharomyces cerevisiae), and in vitro enzyme characterization, we cloned and identified a multifunctional and multisubstrate cytochrome P450 enzyme, CYP720B1 [abietadienol/abietadienal oxidase (PtAO)]. PtAO catalyzes an array of consecutive oxidation steps with several different diterpene alcohols and aldehydes in loblolly pine DRA biosynthesis. Recombinant PtAO oxidized the respective carbon 18 of abietadienol, abietadienal, levopimaradienol, isopimara-7,15-dienol, isopimara-7,15-dienal, dehydroabietadienol, and dehydroabietadienal with apparent Michaelis–Menten (K_m) values of 0.5–5.3 μM. PtAO expressed in yeast also catalyzed in vivo oxidation of abietadienol to abietic acid, but with activity much lower than with abietadienol or abietadienal. Consistent with a role of DRAs in conifer defense, PtAO transcript levels increased upon simulated insect attack using methyl jasmonate treatment of loblolly pine. The multisubstrate, multifunctional P450 diterpene oxidase PtAO, in concert with expression of a family of single- and multiproduct diterpene synthases, allows for formation of a diverse suite of DRA defense metabolites in long-lived conifers.

Diterpene resin acids (DRAs) (Fig. 1A) are important defense compounds of conifers against potential herbivores and pathogens, such as bark beetles and their associated fungi (1–3). DRAs are formed and sequestered as major components of complex oleoresin blends in resin ducts, resin blisters, or resin cells in stems, needles, and roots of most conifers. Biosynthesis of DRAs involves formation of geranylgeranyl diphosphate (GGDP), cyclization of GGDP to a series of diterpene olefins by diTPSs, and three subsequent oxidations of multiple diterpene alcohol intermediates in DRA biosynthesis in loblolly pine. This paper was submitted directly (Track II) to the PNAS office.

Materials and Methods

Materials. Seedlings of loblolly pine and Sitka spruce (Picea sitchensis) were grown to 2-year-old trees as described in ref. 14. Yeast strain YPH499 (MATa, ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, and leu2-Δ1) and yeast dual expression vectors (pESC-Leu and pESC-His) were from Stratagene. Diterpene substrates were prepared from the corresponding DRAs (Helix Biotech, Surrey, BC, Canada) as described in Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

Isolation of P450 and diTPS Full-Length cDNAs and Yeast GGDP Synthase. RNA was isolated from sapling stems harvested 0, 3, and 7 d after spraying with 400 μM MJ in 0.05% Tween 20, and first-strand cDNA was synthesized as described in refs. 14, 15, and 16. Loblolly pine P450 cDNA sequences were identified by a BLAST search (17) of plant ESTs (www.plantgdb.org) by using the first P450 gene of each subfamily in the A. thaliana P450

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abietadienal (reactions B and C) yield the unstable abietadiene-diol, which converts to abietylene (6d), palustric acid; 3d, levopimaric acid; 4d, neoabietic acid; 5d, dehydroabietic acid; 6d, isopimaric acid (isopimar-8 (14),15-dienoic acid); 7d, sandaracopimaric acid; 8d, pimamic acid (pimara-8 (14),15-dienoic acid). (B) Pathway scheme for the formation of abietic acid from GGDP modified from ref. 7. The diTPS LAS catalyzes cyclization of GGDP to abietadiene (1a) (reaction A). Two sequential oxidations (reactions B and C) yield the unstable abietadiene-diol, which converts to abietadienal (1c) by nonenzymatic reaction D. A third oxidation (reaction E) yields the final DRA product, abietic acid.

Fig. 1. Structures of selected diterpenoids and scheme of DRA biosynthesis. (A) Basic carbon skeletons for abietadiene and its positional double-bond isomers (1a–8a). Most diterpenoids accumulate in conifers as DRAs (1d–8d): 1d, abietic acid [abieta-7 (8),13 (14)-dienoic acid]; 2d, palustric acid; 3d, levopimaric acid; 4d, neoabietic acid; 5d, dehydroabietic acid; 6d, isopimaric acid [isopimar-8 (14),15-dienoic acid]; 7d, sandaracopimaric acid; 8d, pimamic acid (pimara-8 (14),15-dienoic acid). (B) Pathway scheme for the formation of abietic acid from GGDP modified from ref. 7. The diTPS LAS catalyzes cyclization of GGDP to abietadiene (1a) (reaction A). Two sequential oxidations (reactions B and C) yield the unstable abietadiene-diol, which converts to abietadienal (1c) by nonenzymatic reaction D. A third oxidation (reaction E) yields the final DRA product, abietic acid.

database (www.p450.kvl.dk) as search sequences. The 5′-end sequences of four unique P450 cDNAs of unknown function (CYP720B1, CYP720B2, CYP750A1, and CYP750C1) were recovered by RACE (Ambion, Austin, TX). ATG start sites were determined by alignment with known plant P450s and according to sequence context characteristic for higher plant start codons (18). PCR of full-length cDNAs was performed over 35 cycles with an annealing temperature of 55°C, Pfu polymerase (Stratagene), and gene-specific oligonucleotide primers (see Table 2, which is published as supporting information on the PNAS web site) (CYP720B1, primers 1 and 2; CYP720B2, primers 3 and 4; CYP750A1, primers 5 and 6; CYP750C1, primers 7 and 8). Primers 9 and 10 were used to PCR-amplify lobolly pine levopimaradiene/abietadiene synthase (LAS) cDNA PaLAS, which was identified in the EST database by using Norway spruce (Picea abies, Pa) PaLAS as a search sequence (6). Two Sitka spruce P450 cDNAs (CYP716B1 and CYP716B2) were isolated by similarity-based PCRs of partial cDNAs followed by RACE as detailed in Supporting Materials and Methods. S. cerevisiae GGDP synthase (ScGs) (19) was amplified from YPH499 genomic DNA (20) by using primers 11 and 12. Full-length cDNA clones were ligated into TOPO blunt-end vector (Invitrogen), and inserts were completely sequenced.

Constructs for P450 Expression in Yeast. Using primer combinations 13/14, 15/16, 17/18, 19/20, 21/22, and 21/23 (Table 2), SpeI-compatible restriction sites were introduced to the 5′ and 3′ ends of the six P450 cDNA clones. PCR-amplified cDNAs were digested with SpeI, XbaI, or NheI and individually ligated into the SpeI cloning site of pESC-Leu::CPR (cytochrome P450 reductase) vector, resulting in expression constructs containing conifer P450 cDNAs tagged by FLAG epitope and poplar CPR (21). Expression of P450s in yeast was assessed by immunoblot analysis by using anti-FLAG antibody (Stratagene) as described in ref. 21. For kinetic analysis, nontagged PtAO was generated in pESC-Leu::CPR vector using primers 13/24 by NheI digestion. A truncated version of the PaLAS cDNA (6) starting at D67 was amplified by using primer pair 25/26, digested with XbaI, and cloned into the SpeI site of pESC-His, resulting in pESC-His::PaLAS. The ORF of yeast GGDP synthase was amplified by using primer pair 27/28, digested with XhoI, and cloned into the SalI site of pESC-His::PaLAS, resulting in pESC-His::PaLAS/ScGs.

Yeast in Vivo Assays. Yeast transformation, growth media, and culture conditions were as described in refs. 21, 22, 23, and 24. To screen engineered yeast strains for oxidative transformation of diterpenoid substrates, abietadiene (in pentane), abietadienol (in ethanol), or abietadienol (in ethanol) was added to induced yeast cultures to final concentrations of 100 μM. For analysis of de novo diterpenoid formation in engineered yeast strains, cells were grown first in 25 ml of 2% dextrose and Leu/His dropout selective medium for 12 h, and then in 25 ml of 2% galactose and Leu/His dropout medium for 20–36 h. Yeast cells were pelleted, and medium was extracted twice with 20 ml of pentane or 20 ml of diethyl ether. Pooled extracts were concentrated under N2 to 300 μl and methylated as described in ref. 3. Organic solvents were then completely evaporated under N2, and dried compounds were dissolved in 50 μl of diethyl ether. Diterpenoids were analyzed by GC–MS by using a 6890 GC-MSD system (70 eV, Agilent Technologies, Palo Alto, CA) with a DB1 capillary column (0.25 mm × 0.25 μm × 30 m, J & W Scientific, Folsom, CA) (14). Injections of 2-μl samples were splitless at 220°C with a column flow of 1 ml of He/min. The GC oven temperature was programmed to increase at 3°C/min from 100°C to 300°C (for analyses shown in Fig. 2), at 5°C/min from 100°C to 320°C (for analyses shown in Fig. 3), or at 7.5°C/min from 40°C to 320°C (for analyses shown in Fig. 4).

In Vitro Assays. Yeast cell culture, induction, and microsomal preparation for P450 enzyme assays were as described in refs. 21, 22, and 23. All enzyme assays for kinetic analyses were conducted by using a single microsomal batch from a 1.5-liter culture. For kinetic analyses, enzyme reactions were initiated by adding 100 μl of 1 mM NADPH to 0.9 ml of phosphate buffer (pH 7.6) containing diterpenoid substrate at 0.2–40 μM, 2 mM glucose 6-phosphate, 0.5 units of glucose 6-phosphate dehydrogenase, and 100 μg of microsomal protein. Methyl stearate (3.3 μM) was used as quantitative internal standard. After 10 min of incubation at 30°C, reactions were stopped by adding 10 μl of 8 M H2SO4, and extracted twice with 1 ml of diethyl ether. Extracts were concentrated under N2 to 200 μl, followed by methylation (3). Dried samples were dissolved in 30 μl of hexane. Five-microliter samples were used for GC-flame ionization detection on an Agilent 6890 GC system by using an AT 1000 capillary column (0.25 mm × 0.25 μm × 30 m, Alltech Associates) with 1°C/min increments from 180°C to 220°C. Each assay was repeated at least three times, and kinetic constants were determined by nonlinear regression to the Michaelis–Menten equation with the EXCEL template ANOMENA (Microsoft) (25).

Northern Blot Analysis. Three lobolly pine trees of 70- to 90-cm height were used for tissue profiling of PAO (CYP720B1) and PaLAS expression. Root tissues were collected from the tips (5 cm) of secondary roots. In elicitor experiments, four trees were used per treatment. Trees were sprayed with 300 ml of MJ or methyl salicylate at 400 μM in 0.05% Tween 20 or with 300 ml of 1 mM ethephen in water. Ethephen-treated trees were
covered with plastic bags for 6 h. Tissues were harvested 48 h after treatment. Total RNA was isolated as described in ref. 15. Northern blot analysis was performed with pooled RNA from the four trees as described in ref. 16 by using full-length PtLAS and PaAO cDNA probes.

**Results**

**P450 Candidate Gene Discovery.** To mine conifer genomes for candidate P450s of DRA biosynthesis, we initially searched the loblolly pine EST database and performed homology-based PCR screening of Sitka spruce cDNAs. Characterization of pine and spruce candidate P450s focused on those with similarity to P450s of known functions in terpenoid metabolism in other species. We recovered full-length cDNAs of four P450s from pine (CYP720B1, CYP720B2, CYP750A1, and CYP704C1) and two different P450s from Sitka spruce (CYP716B1 and CYP716B2). Deduced amino acid sequences of all six cDNAs showed the characteristic P450 N-terminal membrane-anchoring domain, heme-binding domain, and absolutely conserved cysteine amino acid (see Fig. 6, which is published as supporting information on the PNAS web site). CYP750A1 and CYP704C1 exhibited highest sequence similarity with members of the CYP75/92 (40–43% identity) and CYP704 (47–54% identity) subfamilies, respectively. The former cluster also contains several P450s of known functions in monoterpene, sesquiterpene, or diterpene metabolism.

![Fig. 2. Diterpene profiles identified by GC–MS in ether extracts of yeast culture medium supplemented with precursors of abietic acid. Abietadiene, abietadienol, or abietadienal were added as substrates for in vivo assays to control or PtAO-transformed yeast cultures. (A–C) Diterpene profiles of CPR-transformed controls. (D–F) Profiles of PtAO/CPR-transformed yeast. Substrates added to the medium are indicated at the top and are marked with asterisks in the GC profile. Time scales are the same in A–F. Insets in A and D are enlarged on the x and y scale. Minor peaks marked by arrowheads in D (Inset [retention time (Rt) = 54.87]) and in C (Rt = 54.87) showed 96% spectral matches with authentic methyl abietate. (G and H) Mass spectra of peak 1 (Rt = 52.98) (G) and peak 2 (Rt = 54.84) (H) of the GC profile shown in E. Retention times and mass spectra for peaks 1 and 2 show excellent matches (99% accurate) to those of authentic standards of abietadienal (Rt = 53.00) and methyl abietate (Rt = 54.86), respectively. The mass spectrum of peak 2 (Rt = 54.86) in F also showed the same spectrum as that of methyl abietate.

![Fig. 3. Total ion chromatograms of products formed in in vitro enzyme assays with microsomal preparations of transformed yeast and diterpene alcohol (A, C, E, and G) and aldehyde (B, D, and F) substrates. Substrates used are indicated (marked by asterisks). Substrates were incubated at 10 μM with 100 μg of microsomal protein for 1 h at 30°C. Dashed lines are chromatograms of extracts from control assays using microsomes from CPR-transformed cells. Solid lines are chromatograms of extracts from enzyme assays with microsomes from PtAO/CPR-transformed yeast. Assay products were identified by GC–MS and are indicated with numbers and letters matching those given with their structures in Fig. 1A. Diterpene acids were monitored in the form of their methyl esters (me). Identification of product peaks was based on the comparison of retention times and mass spectra (detailed in Table 4) with those of authentic standards. Arrows indicate indiscernible enzymatic products from assays using neoabietadienol or neoabietadinal. All chromatograms shown are of identical time scale (33–38 min), except those in E and F (35–40 min).

![Fig. 4. In vivo diterpene formation in genetically engineered yeast. Yeast strains were engineered to express PaLAS, ScGS and PaLAS, ScGS, PaLAS, and CPR; or ScGS, PaLAS, CPR, and PtAO. The last of these is shown with total ion chromatograms of two independently transformed strains. Pentane extracts were analyzed by GC–MS for PaLAS transformants and ScGS/PaLAS transformants. Ether extracts were analyzed for ScGS/PaLAS/CPR transformants and ScGS/PaLAS/CPR/PtAO transformants.
Functional Screening of P450 cDNAs in Recombinant Yeast. For transformation and functional expression in yeast, ORFs of all six P450s cDNAs in C-terminal FLAG-epitope-tagged versions were separately cloned into pESC-Leu::CPR (21). A control yeast strain expressed pESC-Leu::CPR without P450 cDNA. Yeast in vivo assays were used to test for diterpene transformation conducted with all three diterpene intermediates in the biosynthesis of abietic acid (Fig. 1), abietadiene (1a), abietadienol (1b), and abietadienal (1c). Each of 1a, 1b, and 1c was added individually to induced yeast culture, and their transformation was measured by analysis of diterpenes extracted from medium after 20- to 36-h culture period. No substantial conversion of diterpenoids was detected with the control strain (Fig. 2 A–C) except for trace amounts of abietic acid when incubated with abietadienal (Fig. 2 C). All P450-transgenic yeast strains also showed identical diterpene profiles as the control, with the exception of the strain expressing cDNA clone CYP720B1 (Fig. 2 D–F). This strain converted abietadienal to a mixture of abietadienal (peak 1, Fig. 2 E) and abietic acid (peak 2, Fig. 2 E) as identified by GC–MS (Fig. 2 G and H). When abietadienal was used as substrate (Fig. 2 F), this strain gave 20-fold higher amounts of abietic acid than the control strain, demonstrating that oxidation of abietadienal to abietic acid was due to expression of P450 cDNA CYP720B1 (Fig. 2 F). Using abietadienal as substrate, reproducibly, only trace amounts of abietic acid were found, which were not present in controls and accounted for ~2% of the abietadienal substrate (Fig. 2D Inset). Based on diterpene transformation in recombinant yeast cultures, CYP720B1 was identified as PtAO. Expression of P450s was confirmed by immunoblot analysis except for CYP720B2, which was not detectable.

In Vitro Enzyme Activity of PtAO. To verify results from in vivo assays in yeast cultures, non-FLAG-tagged PtAO construct was generated and transformed into yeast, and isolated microsomes were tested in vitro for P450 enzyme activity with abietadienyl, abietadienol, or abietadienal as substrates. Microsomes converted abietadienol to abietadienal and abietadienal to abietic acid (see Fig. 8, which is published as supporting information on the PNAS web site). PtAO activity was strictly dependent on NADPH and was linear for at least 12 min at 30°C, with a pH optimum of 7.5–7.6 (80% activity at ±0.5 pH units). No enzyme activity was found when microsomal preparations were assayed with abietadienyl as substrate at concentrations of up to 200 μM in vitro (data not shown). In extended incubation, trace levels of oxidized diterpene products from diterpene alcohol and aldehyde were detected with microsomes from the control yeast strain. These trace levels were diminished by additional ultracentrifugation and completely abolished by heat treatment, suggesting that some weak diterpene oxidation in controls is due to soluble yeast protein.

Characterization of Recombinant PtAO. Loblolly pine oleoresin contains a mixture of double-bond isomers of abietic acid, including levopimaric acid, neoabietic acid, dehydroabietic acid, and isopimaraic acid (Fig. 1A; see also Table 3, which is published as supporting information on the PNAS web site). To test whether PtAO is specific to the formation of abietadienal and abietic acid, we tested seven additional diterpene alcohols and aldehydes: possible substrates in vivo: levopimaradienal (Fig. 1A, 3b), neoabietadienal (4b), neoabietadienol (4c), dehydroabietadienal (5b), dehydroabietadienol (5c), isopimara-7,15-dienol (6b), and isopimara-7,15-dienal (6c). Levopimaradienal (3c) was unstable and oxidized to 5c in the course of synthesis. It was therefore not used in these assays. Microsome preparations of yeast expressing PtAO converted all of the above substrates, except for 4b and 4c, to the more oxidized diterpenes identified by GC–MS (Fig. 4; see also Table 4, which is published as supporting information on the PNAS web site). Levels of oxidized products formed from 4b and 4c with PtAO did not exceed those of controls lacking PtAO. To verify that PtAO is not active with 4b or 4c, we tested these and all other substrates in vivo in yeast cultures as shown in Fig. 2 by adding mixtures of diterpene alcohols or aldehydes to the CPR/PtAO-expressing yeast. Although the other diterpenoids were rapidly converted to the corresponding aldehydes and acids, 4b and 4c remained unmetabolized in the culture (data not shown). Together, these results from in vivo and in vitro assays showed that PtAO is active with several different diterpene alcohols and aldehydes that are intermediates in DRA biosynthesis in loblolly pine. However, PtAO does not appear to be involved in the formation of neoabietic acid, a major resin acid component in loblolly pine.

We determined basic kinetic properties of PtAO with all seven diterpene substrates for which activities were found. Results are shown in Table 1. In assays with diterpene alcohols as substrate, we monitored for possible turnover of initial diterpene aldehyde products to diterpene acids. Using abietadienol as substrate, abietadienyl accumulated predominantly for the first 10 min, suggesting that abietadienal is not immediately channeled to the formation of abietic acid. This accumulation of abietadienal allowed us to estimate apparent Km values for both diterpene alcohol and aldehyde substrates. In general, PtAO exhibited slightly lower apparent Km values and higher relative Vmax with diterpene aldehydes than diterpene alcohols, thus indicating higher catalytic efficiencies (Vmax/Km) of PtAO with diterpene aldehydes. Similarly low Km values of PtAO in the micromolar or submicromolar range with all diterpene alcohols and aldehydes tested substantiated the result of this study that PtAO is a multifunctional (catalyzing two consecutive oxidation steps) and multisubstrate (using several closely related double-bond diterpene isomers) P450 for DRA biosynthesis in loblolly pine.

De Novo Diterpenoid Formation in Metabolically Engineered Yeast. The very low levels of abietic acid formed from abietadienyl when added to PtAO-expressing yeast (Fig. 2D) prompted us to further investigate this reaction. It is possible that conversion of abietadienyl by PtAO in in vivo assays or when added to yeast culture medium is limited by poor solubility of the diterpene olefin in the aqueous assay system. We therefore examined potential abietadienyl oxidase activity of PtAO by yet another approach using metabolic engineering of a yeast strain that produces abietadienyl endogenously. For this purpose, ScGS, diTPS LAS (PaLAS), CPR, and PtAO were coexpressed in yeast. We generated a series of yeast strains that were transgenic for PaLAS, PaLAS/ScGS, PaLAS/ScGS/CPR, and PaLAS/ScGS/CPR/PtAO. Diterpene profiles of culture media of transgenic yeast strains are shown in Fig. 4. Abietadienyl was produced with yields of 2–8 μg in 50 ml

Table 1. Kinetic properties of PtAO recombinant enzyme for the oxidation of diterpene alcohol and aldehyde

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Vmax, app.</th>
<th>Km, app.</th>
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<tr>
<td></td>
<td>pmol·min⁻¹·mg⁻¹ of protein</td>
<td>μM</td>
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<tr>
<td>Abietadienol</td>
<td>77 ± 4</td>
<td>0.8 ± 0.3</td>
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<tr>
<td>Dehydroabietadienol</td>
<td>211 ± 25</td>
<td>5.3 ± 2.7</td>
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<tr>
<td>Isopimaradienol</td>
<td>122 ± 13</td>
<td>1.5 ± 0.5</td>
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<td>Levopimaradienol</td>
<td>137 ± 8</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>Abietadienal</td>
<td>181 ± 17</td>
<td>0.5 ± 0.2</td>
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<tr>
<td>Dehydroabietadienal</td>
<td>379 ± 20</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Isopimaradienol</td>
<td>226 ± 15</td>
<td>0.6 ± 0.3</td>
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<tr>
<td>Levopimaradienol</td>
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Data are means ± SD from at least three replicates. ND, not determined.
of culture as a result of coexpression of ScGS and PaLAS, whereas expression of PaLAS alone did not yield detectable levels of abietadiene. The profile of a single diterpene olefin, abietadiene, compared with four different products normally formed by PaLAS (6) can be attributed to the low pH of yeast cultures because it was previously shown that product profiles of this type of diTPS are affected by pH (5). When CPR and PtAO were coexpressed with ScGS and PaLAS, abietadiene remained the dominant diterpene in extracts of yeast culture medium, but low levels of abietic acid were also produced in two independent yeast strains.

Transcript Accumulation of PtAO and PtTAS. Transcripts of PtAO and the diTPS PtTAS were constitutively more abundant in young tissues of loblolly pine, such as flushing buds and green bark tissues, than in older tissues, such as mature needles and bark (Fig. 5A). These results agree with a role of DRAs in constitutive conifer defense. Previous studies with Sitka spruce showed that MJ treatment mimics insect attack on trees (14). In loblolly pine, transcripts hybridizing with PtAO and PtTAS were increased in stem bark and xylem upon MJ treatment as part of the induced resin terpenoid defense (Fig. 5B). Similar constitutive and induced patterns of PtAO and PtTAS expression indicate coordinated regulation of the TPS and P450 genes of DRA biosynthesis in loblolly pine.

Discussion

Despite the importance of complex mixtures of DRAs for resistance of conifers against insects and pathogens (1–3), no progress had been made in the last 10 years (7, 8) toward identification of P450 genes involved in the multiple oxidations leading from diterpene olefins to DRAs. Here, we describe a biochemical, functional genomics approach for successful discovery of a diterpene oxidase, PtAO, in the large family of P450 genes in loblolly pine. Although most P450 enzymes of plant secondary metabolism are highly substrate- and reaction-specific (32), the multifunctional PtAO catalyzes two consecutive oxidations with several similar diterpene alcohols and aldehydes as substrates. Other P450s that catalyze consecutive oxidations in plant terpenoid metabolism include enzymes of gibberellin biosynthesis (11–12) and tobacco 5-epi-aristolochene 1,3-dihydroxylase (33). A few P450s are known to convert multiple terpenoid substrates. For example, limonene hydroxylase functions with both (+)- and (−)-enantiomers of the monoterpene limonene (34), and the diterpene hydroxylase taxadiene 5α-hydroxylase efficiently utilizes two different double-bond isomers of taxadiene (30).

The relatively broad substrate profile of PtAO could have important biological and adaptive functions by generating structural diversity of terpenoid chemical defense in conifers (35). DRA biosynthesis involves initial formation of several different diterpene olefins from GGDP by activity of one or more diTPSs (6). From there, DRA biosynthesis can be viewed as a matrix of three oxidations of each diterpene olefin to the corresponding DRA by means of alcohol and aldehyde intermediates (Fig. 1). The diterpene olefins are products of both single-product and multiproduct diTPSs, which are members of large TPS gene families and which can rapidly evolve new product profiles (6). A P450 enzyme that functions efficiently with multiple diterpene double-bond isomers can convert a dynamic range of diterpenes to the final DRAs. DRAs are then, by mechanisms that are not yet known, secreted and accumulated in extracellular resin ducts or resin blisters. In concert, a few conifer diTPSs and P450s can conceivably account for much of the chemical diversity and plasticity of DRA chemical defense of long-lived conifers. However, additional P450s are required for the formation of an even larger array of DRAs, including neoabiatic acid (Fig. 1, 4d), a major diterpene in loblolly pine. Because levopimaradienol (3b) is efficiently used as substrate by PtAO but the very similar neoabiaticadienol (4d) is not, it appears that substrate affinity of PtAO is determined by relatively subtle structural difference distal to the C18 position, such as different location of a single double bond at C12,13 versus C13,15.

Both oxidation steps catalyzed by PtAO in loblolly pine DRA biosynthesis closely resemble similar oxidations catalyzed by multifunctional angiosperm P450s that yield kaurenoic acid from ent-kaurene in gibberellin biosynthesis (10, 11). These angiosperm P450s catalyze, in addition, the initial oxidation of the ent-kaurene diterpene olefin. Although function of PtAO for the second and third of three oxidation steps in DRA biosynthesis is well supported by several experiments presented in this study, results from testing PtAO for activity with abietadiene in three different systems (in vitro enzyme assays, conversion of substrate added to yeast cultures, and tests for de novo formation in engineered yeast) were less conclusive. Clearly, only very low levels of abietic acid and none of the intermediates were found in both yeast assay systems with abietadiene as substrate, and no conversion of abietadiene was detected in in vitro assays. These findings argue against activity of PtAO in the first of three oxidation steps from abietadiene to abietic acid. Although poor solubility of the diterpene olefin and low pH of yeast culture medium may have confounded these results, it is possible that the first of three oxidation steps in DRA biosynthesis is catalyzed by a separate enzyme. Additional members of the conifer P450 family with high similarity to loblolly PtAO have recently emerged in the spruce EST database (www.treconomix.com), providing additional candidates for enzymes in conifer DRA and potentially in conifer gibberellin biosynthesis. In earlier work with tissue extracts from grand fir and lodgepole pine, it was suggested that the third oxidation step of DRA biosynthesis could be catalyzed by an aldehyde dehydrogenase (7). Although we cannot exclude additional function of an aldehyde dehydrogenase activity in this pathway, submicromolar Km values of PtAO for all diterpene aldehydes tested (Table 1) firmly support a function of this P450 in the final oxidation step of DRA biosynthesis in loblolly pine.

We thank D. Ellis and W. Schuch (both of CellFor, Vancouver) for seedlings, C. Keeling for critical reading of the manuscript, and D. Nelson for assigning official CYP numbers. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Human Frontier Science Program and infrastructure grants from the Canada Foundation for Innovation and the British Columbia Knowledge Development Funds (all to J.B.).