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

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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 diterpenol and diterpenal intermediates 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 abietadiene 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-product and multiproduct diterpene synthases, allows for formation of a diverse suite of DRA defense metabolites in long-lived conifers.

conifer defense | gibberellic acid | diterpene resin acids | conifer genomics | plant secondary metabolism

Diterpene resin acids (DRAs) (Fig. 1*A*) 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 activity of diterpene synthases (diTPSs), and three subsequent oxidations at carbon 18 (Fig. 1*B*). A small family of single-product or multiproduct diTPSs of DRA biosynthesis has recently been cloned and characterized (4–6). Studies with grand fir (*Abies grandis*) and lodgepole pine (*Pinus contorta*) tissue extracts showed that stepwise oxidation of the diterpene olefin abietadiene (**1a**) to abietic acid (**1d**) can be achieved by membrane-bound cytochrome P450 monooxygenase (P450) and soluble aldehyde dehydrogenase enzyme activities (Fig. 1*B*) (7, 8). The general pathway scheme of oxidation of abietadiene to abietic acid in conifer secondary metabolism resembles that of oxidation of *ent*-kaurene to *ent*-kaurenoic acid in the biosynthesis of gibberellin phytohormones (9–11). A multifunctional P450 responsible for the three-step oxidation of *ent*-kaurene to kaurenoic acid has previously been identified by using genetic approaches in *Arabidopsis thaliana* (10–11). Similarly, a multifunctional P450 also catalyzes the subsequent three-step oxidation from kaurenoic acid to GA_{12} in *Arabidopsis* (12). Cloning and identification of P450s of DRA secondary metabolism have been hampered by difficulties in purifying the corresponding enzymes and by lack of suitable forward genetic tools for conifers. However, large collections of ESTs for loblolly pine (*Pinus taeda*, *Pt*) (13) can enable gene discovery and biochemical identification of candidate P450 cDNAs of terpenoid secondary metabolism in conifers. Here, we describe cloning and functional characterization of abietadienol/abietadienal oxidase (PtAO, CYP720B1), a P450 enzyme that is unusual in that it catalyzes an array of consecutive oxidations of multiple diterpene alcohol and aldehyde intermediates in DRA biosynthesis in loblolly pine. The methyl jasmonate (MJ)-inducible PtAO can account for much of the oxidative diversification of diterpenoid natural product defense compounds in loblolly pine.

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. Diterpenoid 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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Abbreviations: DRA, diterpene resin acid; LAS, levopimaradiene/abietadiene synthase; MJ, methyl jasmonate; *Pt*, *Pinus taeda*; *Pa*, *Picea abies*; PtAO, *Pt* abietadienol/abietadienal oxidase; P450, cytochrome P450 monooxygenase; GGDP, geranylgeranyl diphosphate; diTPS, diterpene synthase; CPR, cytochrome P450 reductase; *ScGS*, *Saccharomyces cerevisiae* GGDP synthase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY779537–AY779543).

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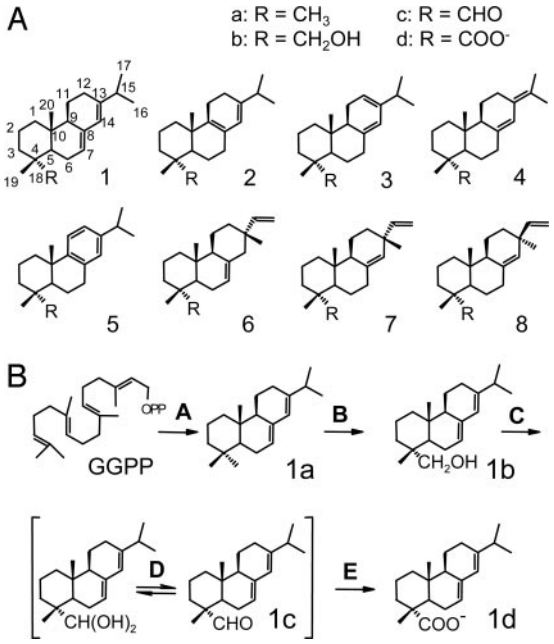

Fig. 1. Structures of selected diterpenoids and scheme of DRA biosynthesis. (*A*) Basic carbon skeletons for abietadiene and its positional double-bond isomers (**1a**–**8a**). Most diterpenoids accumulate in conifers as DRAs (**1d**–**8d**): **1d**, abietic acid [abieta-7 (8),13 (14)-dienoic acid]; **2d**, palustric acid; **3d**, levopimaric acid; **4d**, neoabietic acid; **5d**, dehydroabietic acid; **6d**, isopimaric acid [isopimara-7 (8),15-dienoic acid]; **7d**, sandaracopimaric acid; **8d**, pimaric 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 *CYP704C1*) 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; *CYP704C1*, primers 7 and 8). Primers 9 and 10 were used to PCR-amplify loblolly pine levopimaradiene/abietadiene synthase (LAS) cDNA *PtLAS*, 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 abietadienal (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 N_2 to 300 μl and methylated as described in ref. 3. Organic solvents were then completely evaporated under N_2, 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 H_2SO_4, and extracted twice with 1 ml of diethyl ether. Extracts were concentrated under N_2 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 ANEMONA (Microsoft) (25).

Northern Blot Analysis. Three loblolly pine trees of 70- to 90-cm height were used for tissue profiling of *PtAO* (*CYP720B1*) and *PtLAS* 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 ethephon in water. Ethephon-treated trees were

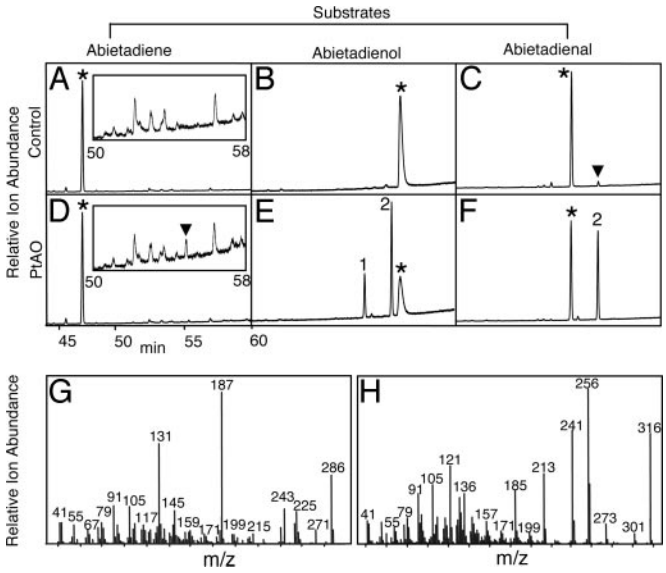

Fig. 2. Diterpene profiles identified by GC–MS in ether extracts of yeast culture medium supplemented with precursors of abietic acid. Abietadiene, abietadieneol, or abietadienal were added as substrates for *in vivo* assays to control or *PtAO*-transformed yeast cultures. (*A–C*) Diterpene profiles 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.

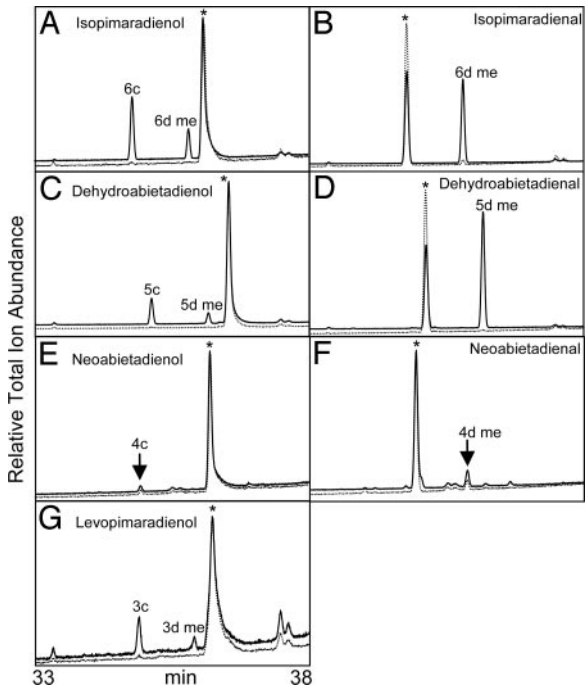

Fig. 3. Total ion chromatograms of products formed in *in vitro* enzyme assays with microsomal preparations of transformed yeast and diterpene alcohol (*A*, *C*, *E*, and *G*) and aldehyde (*B*, *D*, and *F*) substrates. Substrates used are as indicated (marked by asterisks). Substrates were incubated 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. 1*A*. 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).

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 *PtAO* 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). CYP720B1 and CYP720B2 are 65% identical to each other and showed ≈40% amino acid identity to brassinosteroid hydroxylases (*CYP90A1*/*B1*) (26–28). CYP716B1 and CYP716B2 share 95% amino acid identity and have 40–45% identity with the various taxoid hydroxylases (refs. 29 and 30 and references therein). Phylogenetic analysis placed *CYP720B1*/*B2* and *CYP716B1*/*B2* into the CYP85 clan (31) containing several known P450s of diterpenoid (taxol and gibberellin) and triterpernoid (brassinosteroid) metabolism (see Fig. 7, 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.

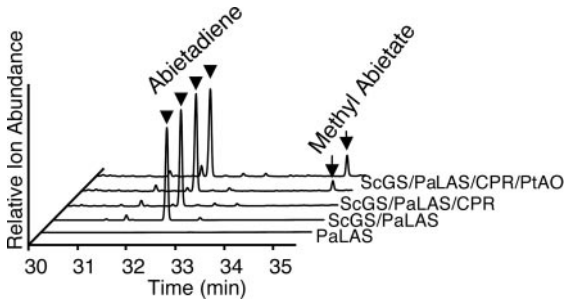

Fig. 4. *In vivo* diterpene formation in genetically engineered yeast. Yeast strains were engineered to express *PaLAS*; *ScGS* and *PaLAS*; *ScGS*, *PaLAS*, and *CPR*; or *ScGS*, *PaLAS*, *CPR*, and *PtAO*. The last of these is shown with total ion chromatograms of two independently transformed strains. 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 to test for diterpene transformation were 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 a 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 abietadienol 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 abietadiene as substrate, reproducibly, only trace amounts of abietic acid were found, which were not present in controls and accounted for ≈2% of the abietadiene substrate (Fig. 2*D 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 abietadiene, abietadienol, or abietadianal 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 abietadiene 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 isopimaric acid (Fig. 1*A*; 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 as possible substrates *in vitro*: levopimaradienol (Fig. 1*A*, **3b**), neoabietadienol (**4b**), neoabietadienal (**4c**), dehydroabietadienol (**5b**), dehydroabietadienal (**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.

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

Substrates	$K_{m,\ app}$, μM	$V_{max,\ app}$, pmol·min^{-1}/mg of protein
Abietadienol	0.8 ± 0.3	77 ± 4
Dehydroabietadienol	5.3 ± 2.7	211 ± 25
Isopimaradienol	1.5 ± 0.5	122 ± 13
Levopimaradienol	1.9 ± 0.4	137 ± 8
Abietadienal	0.5 ± 0.2	181 ± 17
Dehydroabietadienal	0.6 ± 0.2	379 ± 20
Isopimaradienal	0.6 ± 0.3	226 ± 15
Levopimaradienal	ND	ND

Data are means ± SD from at least three replicates. ND, not determined.

We determined basic kinetic properties of PtAO with all seven diterpenoid 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, abietadienal 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 K_m values for both diterpene alcohol and aldehyde substrates. In general, PtAO exhibited slightly lower apparent K_m values and higher relative V_{max} with diterpene aldehydes than diterpene alcohols, thus indicating higher catalytic efficiencies (V_{max}/K_m) of PtAO with diterpene aldehydes. Similarly low K_m 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 abietadiene when added to *PtAO*-expressing yeast (Fig. 2*D*) prompted us to further investigate this reaction. It is possible that conversion of abietadiene by PtAO in *in vitro* 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 abietadiene oxidase activity of PtAO by yet another approach using metabolic engineering of a yeast strain that produces abietadiene 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. Abietadiene was produced with yields of 2–8 μg in 50 ml

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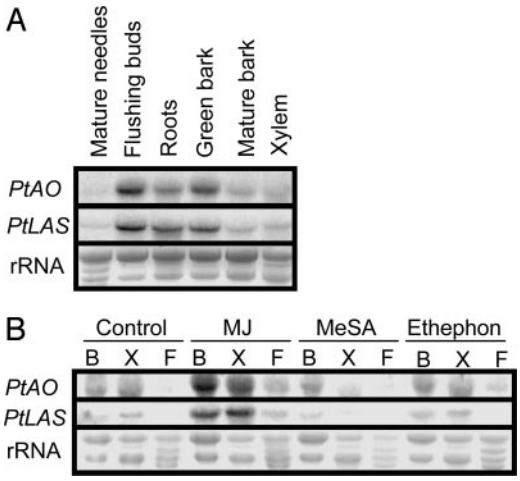

Fig. 5. Constitutive and MJ-induced transcript accumulation of *PtLAS* and *PtAO* in loblolly pine. (*A*) Northern blot analysis of *PtAO* and *PtLAS* transcripts in untreated saplings. (*B*) Northern blot analysis of *PtAO* and *PtLAS* in mature bark (B), xylem (X), and foliage (F) 2 days after treatment with 0.05% Tween 20 (Control), MJ (0.4 mM), methyl salicylate (MeSA, 0.4 mM), or ethephon (1 mM).

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 *PtAS*. Transcripts of *PtAO* and the diTPS *PtLAS* 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. 5*A*). 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 *PtLAS* were increased in stem bark and xylem upon MJ treatment as part of the induced resin terpenoid defense (Fig. 5*B*). Similar constitutive and induced patterns of *PtAO* and *PtLAS* 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 hydoxylase 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 extracelluar 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 neoabietic acid (Fig. 1, **4d**), a major diterpene in loblolly pine. Because levopimaradienol (**3b**) is efficiently used as substrate by PtAO but the very similar neoabietadienoal (**4b**) 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 gene family with high similarity to loblolly *PtAO* have recently emerged in the spruce EST database (www.treenomix.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 K_m 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.

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