Evolution of Plant Defense Mechanisms

RELATIONSHIPS OF PHENYLCOUMARAN BENZYLIC ETHER REDUCTASES TO PINORESINOL-LARICIRESINOL AND ISOFLAVONE REDUCTASES

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Pinoresinol-lariciresinol and isoflavone reductase classes are phylogenetically related, as is a third, the so-called “isoflavone reductase homologs.” This study establishes the first known catalytic function for the latter, as being able to engender the NADPH-dependent reduction of phenylcoumaran benzylic ethers. Accordingly, all three reductase classes are involved in the biosynthesis of important and related phenylpropanoid-derived plant defense compounds. In this investigation, the phenylcoumaran benzylic ether reductase from the gymnosperm, Pinus taeda, was cloned, with the recombinant protein heterologously expressed in Escherichia coli. The purified enzyme reduces the benzylic ether functionalities of both dehydrodiconiferyl alcohol and dihydrodehydrodiconiferyl alcohol, with a higher affinity for the former, as measured by apparent \( K_m \) and \( V_{\max} \) values and observed kinetic \( ^{3}H\)-isotope effects. It abstracts the 4R-hydride of the required NADPH cofactor in a manner analogous to that of the pinoresinol-lariciresinol reductases and isoflavone reductases. A similar catalytic function was observed for the corresponding recombinant reductase whose gene was cloned from the angiosperm, Populus trichocarpa. Interestingly, both pinoresinol-lariciresinol reductases and isoflavone reductases catalyze enantiomeric conversions, whereas the phenylcoumaran benzylic ether reductase only shows regiospecific discrimination. A possible evolutionary relationship among the three reductase classes is proposed, based on the supposition that phenylcoumaran benzylic ether reductases represent the progenitors of pinoresinol-lariciresinol and isoflavone reductases.

The biosynthesis of various plant defense 8−8′-linked lignans and isoflavonoids has been shown to involve a class of closely related enzymes, the pinoresinol-lariciresinol reductases (PLRs)1 (1−4, 12) and isoflavone reductases (IFRs) (5−7).

Based on their high gene sequence homology, these enzymes appear to be phylogenetically linked (8). They also share comparable physiological roles for their products, in addition to having presumed similar catalytic modes of action, as shown in Fig. 1, A and B.

In recent years, a relatively large number of reductases with significant homology to both PLRs and IFRs (so-called IFR “homologs”) have also been discovered (8), mainly through screening for genes induced by fungal elicitors (9) or in relation to other defensive responses to “stress” (10). To date, at least 19 such “homologs” have been reported in both woody and non-woody angiosperms and gymnosperms, in plants as diverse as Arabidopsis thaliana (11), birch (Betula pendula).2 Forsythia intermedia (8), maize (Zea mays) (10), pear (Pyrus communis),3 poplar (Populus trichocarpa),4 tobacco (Nicotiana tabacum) (9), and western red cedar (Thuja plicata) (12). Their roles in planta, however, have not been established; of those examined for enzymatic activity, none were able to catalyze either the reduction of isoflavones (9) or the reduction of pinoresinol-lariciresinol-type (8−8′-linked) lignans (see below). Thus, their biochemical function has been unknown.

Lignans and isoflavonoids play important roles in plant defense. For example, their constitutive deposition significantly helps confer durability, longevity, and resistance to the heartwoods of many tree species against wood-rotting fungi. In this regard, western red cedar (T. plicata) heartwood formation results via deposition of ~20% of 8−8′-linked lignans, such as plicatic acid 7 (15−23) (see Fig. 2), which help permit this species to grow to well over 60 meters tall and live for longer than 3000 years. The heartwoods of many Pinaceae species, such as loblolly pine (Pinus taeda), also contain significant levels of 8−5′-linked (phenylcoumaran) lignans such as dehydrodiconiferyl alcohol 5 (see Fig. 1C), dihydrodehydrodiconiferyl alcohol 8 (Fig. 2), and related metabolites. These and their higher molecular weight (non-lignin) derivatives are laid down in the heartwood as protective postlignification infusions (4). Additionally, many tropical legume tree species contain significant levels of pterocarpan isoflavonoids, such as pterocarp 9 in Pterocarpus indicus, thereby allowing these species to thrive in an environment highly conducive to fungal growth. Remarkably, almost nothing is known about the biochemistry of alcohol; DDT, dithiothreitol; DDC, dihydrodehydrodiconiferyl alcohol; IDDC, isohydrodehydrodiconiferyl alcohol; TDDC, tetrahydrodehydrodiconiferyl alcohol; MES, 4-morpholineethanesulfonic acid; Bis-Tris propane, 1,3-bis[(tris(hydroxymethyl)methylamino)propane].

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of heartwood formation (4), despite the fact that it is responsible for ~90% of all woody biomass.

Lignans and isoflavonoids can also reputedly act as phytoalexins (8, 24), in both woody and non-woody species, being formed de novo in response to fungal attack. For example, the 8–8′-linked lignan, (−)-matairesinol 10, is produced in *Picea abies* in response to attack from *Fomes annosus* (25), whereas the pterocarpan isoflavonoid, (−)-medicarpin 11, accumulates in alfalfa (*Medicago sativa*) when exposed to *Colletotrichum lindemuthianum* (26).

Lignan formation is not limited to significant deposition in heartwood and tissues attacked by fungi. For example, the 8–5′-9′-linked lignans, dehydrodiconiferyl alcohol triacetate 12 and tetrahydrodehydrodiconiferyl alcohol tetraacetate 13, accumulate in small amounts in the leaves of *Cryptomeria japonica* (27). Moreover, dehydrodiconiferyl alcohol (DDC) 5 and its 7′-8′ (allylic bond)-reduced derivative, dihydrodehydrodiconiferyl alcohol 8 (DDDC), appear to be ubiquitous throughout the plant kingdom, being found in plants as diverse as loblolly pine (*P. taeda*) (28), tobacco (29), ferns (30), and hornworts (31), i.e. spanning all major groups of vascular plants and suggesting the involvement of a universal defense system. Interestingly, the 8–5′-linked lignans can also co-occur in many species with their (presumed) phenylcoumaran benzylic ether-reduced counterparts. As shown in Fig. 3, this includes examples such as dihydrodehydrodiconiferyl alcohol triacetate 12 with tetrahydrodehydrodiconiferyl alcohol tetraacetate 13 in *C. japonica* (27) and hancinone 14 with isodihydrofutoquinol A 15 in *Piper*...
In contrast to the 8–8' linked lignans and the isoflavonoids (1, 5, 8, 24), however, there has been no explicit description of the biochemical pathways and enzymes involved in either the formation or subsequent metabolism of 8–5' linked lignans.

From a biochemical perspective, enzymatic reduction of the benzylic ether of 8–5' linked lignans, such as DDC 5 and DDDC 8, might involve an analogous process to that envisaged for reduction of pinoresinol 1 (Fig. 1A) and isoflavonoids, such as 2'-hydroxypseudobaptigenin 3 (Fig. 1B), e.g. through formation and reduction of conjugated enone intermediates, as shown in Fig. 1C, or through reduction of some other enzyme-bound molecular species. Accordingly, we deduced that the PLR and IFR “homologs” may, in fact, be phenylcoumaran benzylic ether reductases (PCBERs). In order to test this hypothesis, and since suspension culture cells of the gymnosperm, loblolly pine, contain DDC 5, DDDC 8 (28), and related metabolites, we first constructed a cDNA library from RNA isolated from P. taeda suspension culture cells growing in 2,4-dichlorophenoxyacetic acid-containing medium (34). Next, when the F. intermedia pinoresinol-lariciresinol reductase cDNA was used as a probe, a “homolog” cDNA from loblolly pine was isolated, with its recombinant protein subsequently expressed in Escherichia coli. This protein, in the presence of NADPH, reduced the benzylic ether functionalities of both DDC 5 and DDDC 8. Another IFR “homolog” from the angiosperm, poplar (P. trichocarpa), when similarly expressed and tested for PCBER activity, also effected the same conversion. These findings thus identified a catalytic function for this class of enzymes as being capable of engendering phenylcoumaran benzylic ether reduction. This finding thus provided evidence that the so-called IFR “homologs” are indeed phylogenetically linked to PLR and IFR and demonstrated further the evolution and conservation of the phenylpropanoid pathway throughout the plant kingdom (8).

In contrast to the enantiospecific PLR and IFR, however, the phenylcoumaran benzylic ether reductases only displayed regiochemical specificities, since both (+)- and (-)-enantiomers of DDC 5 were effectively reduced.

**Experimental Procedures**

**Plant Materials**—P. taeda cell suspension cultures were maintained as described previously (34), in Brown and Lawrence medium containing 2,4-dichlorophenoxyacetic acid. Cells were harvested by filtration 7 days after transfer to fresh media, frozen in liquid nitrogen, and stored at −80 °C. *Populus deltoides × P. trichocarpa*, clone 064 (Afocel, France) and *P. trichocarpa* cv. Trichobel were grown in the Institute of Forestry and Game Management nursery (Geraardsbergen, Belgium).

**General Methods**—All molecular biological techniques, unless expressly described otherwise below, were performed according to standard methods (35, 36). Protein purification procedures using a fast protein liquid chromatography (Amersham Pharmacia Biotech) system were carried out at 4 °C, whereas those employing the BioCad (PerSeptive Biosystems) system were conducted at ambient temperature. All chromatographic eluents were monitored at 280 nm. Protein concentrations were determined with the Bio-Rad protein determination kit. Polycrylamide gel electrophoresis used gradient (4–15%) linear gradient; Bio-Rad) gels under denaturing and reducing conditions in the Lasemmi buffer system (37), followed by visualization of the proteins by silver staining (38).

**Other Materials**—All solvents and chemicals used were reagent or high performance liquid chromatography (HPLC) grade. Taq thermostable DNA polymerase, competent NovaBlue cells, and radiolabeled nucleotide ([α-32P]dCTP) were purchased from Promega, Novagen, and NEN Life Science Products, respectively. The pCRII TA cloning kit was from Invitrogen. Restriction endonuclease NdeI was from New England Biolabs.

Oligonucleotide primers for polymerase chain reaction (PCR) and sequencing were obtained from Life Technologies, Inc. Genecreen II® kits for PCR fragment purification were from BIO 101 Inc., with the gel-purified DNA concentrations determined by comparison with a low DNA mass ladder (Life Technologies, Inc.) in 1.3% agarose gels.

**Instrumentation**—A Lambda 6 UV-visible spectrophotometer (Perkin-Elmer) was used for recording all RNA and DNA determinations at A260, and a Tephron II thermocycler (Thermolyne) was employed for all PCR amplifications. The Wizard® Plus SV Miniprep DNA Purification System (Promega) was used to purify plasmid DNA for sequencing using an Applied Biosystems model 373A automated sequencer. All HPLC separations were performed on either a Millennium™ (Waters Inc.) or Alliance™ (Waters Inc.) instrument. Amino-terminal protein sequencing was carried out as described previously (1). Matrix-assisted laser desorption ionization-time of flight mass spectrometry was performed on a VG 7070 at the Laboratory for Bioanalysis and Biotechnology at Washington State University.

**P. taeda cDNA Library Synthesis**—Total RNA (100 μg/g fresh weight) was obtained using the method of Dong and Dunstan (39) from frozen loblolly pine (P. taeda) cells grown as described above. A P. taeda cDNA library was constructed using 5 μg of purified poly(A)+ mRNA (Oligotex-dT24 suspension; Qiagen) with the ZAP-cDNA® synthesis kit, the Uni ZAP™ XR vector, and the Gigapack® II Gold packaging extract (Stratagene), with a titer of 1×109 plaque-forming units (PFU). NcoI and NdeI digestion yielded a total of 106 plaque-forming units/ml; 120 ml total) was used for screening (1).

**DNA Probe Synthesis**—The 5'-end of the previously isolated pinoresinol-lariciresinol reductase cDNA (PLR-F1) was used to construct a probe, as described previously (1), to screen the P. taeda cDNA library for similar/homologous genes.

**Library Screening**—600,000 plaque-forming units of the P. taeda amplified cDNA library were screened for genes homologous to PLR-F1. This was done exactly as described by Dinkova-Kostova et al. (1), with the exception that hybridization temperatures were reduced to 45 °C. 20 positive plaques were purified through two more rounds of screening.

**In Vivo Excision and Sequencing of Putative Reductase cDNA-containing Phagenoids**—Purified cDNA clones were rescued from the phage following Stratagene’s in vivo excision protocol. Both strands of several different cDNAs that coded for genes homologous to PLR-F1...
were completely sequenced using overlapping sequencing primers. Sequence Analysis—DNA and amino acid sequence analyses were performed using the Unix-based GCG Wisconsin Package (40, 41) and the Expasy Web Wide Web molecular biology server (42).

Expression in Escherichia coli as a Fusion Protein—The open reading frame of the putative reductase from *P. taeda* was in frame with the β-galactosidase gene complementation particle in pBluescript. Thus, its recombinant fusion protein was expressed in NovaBlue cells as described previously (1) and then assayed for both phenylcoumaran benzylic ether reductase and pinosynol-lariciresinol reductase activities, as described below.

Transfer of the Loblolly Pine (P. taeda) Phenylcoumaran Benzylic Ether Reductase cDNA into pSBETa—The overexpression plasmid, pSBETa (43), which contains the Ndel restriction site for native expression, was used. Since the putative reductase from *P. taeda* had no internal Ndel sites, two primers were designed to introduce Ndel sites at the start methionine (primer PT-ATG-Ndel, TTC AGG CAC ATT GGA AGC AGG AGC AGG ATA CTC) and in the 3′-end untranslated region (primer PT-Rev-Ndel, I, TGT CGA ATA CAT ATG AAA GGC). These two primers (5 pmol each) were used in five PCRs with 10 ng of the cDNA encoding the putative reductase being eluted in fractions 33–43. These fractions were then pooled, concentrated to 100% Buffer A in 30 ml. 4-ml fractions were collected, with the desired protein being eluted in fractions 33–43. These fractions were then pooled, concentrated to 5 mg/ml in a Centricron 10 micro concentrator (Amicon, Inc.), desalted back into Buffer A over PD-10 columns, and assayed for activity.

Affinity Column (Affi-Blue Gel) Purification—A 1.6-cm diameter, 11.5-cm-long (23-mL bed volume) Affi-Blue gel column (Bio-Rad) column was equilibrated in Buffer B. The desired 40–70% fraction (15 ml, 513 mg) was then applied to the column. After washing with 300 ml of Buffer A (1 ml/min), the reductase was eluted by running a linear gradient from 0 to 100% Buffer B (Buffer A plus 5 M NaCl) in 30 ml, holding at 100% Buffer B for 60 ml and returning from 100% Buffer B to 100% Buffer A in 30 ml. 4-ml fractions were collected, with the reductase being eluted in fractions 33–43. These fractions were then pooled, concentrated to ~5 mg/ml in a Centricron 10 micro concentrator (Amicon, Inc.), desalted over PD-10 columns, and assayed for activity.

Cation Exchange Chromatography—The resulting enzyme solution (118 mg) was next applied to a POROS 20 QE Perfusion cation exchange column, equilibrated in Buffer C (50 mM Bis-Tris propane, pH 6.8, 5 mM DTT). The desired protein did not bind to the column, whereas the majority of the contaminating *E. coli* proteins remained bound under these conditions.

Affinity Column (Affi-Blue Gel) Purification—A 1.6-cm diameter, 11.5-cm-long (23-mL bed volume) Affi-Blue gel column (Bio-Rad) column was equilibrated in Buffer B. The desired 40–70% fraction (15 ml, 513 mg) was then applied to the column. After washing with 300 ml of Buffer A (1 ml/min), the reductase was eluted by running a linear gradient from 0 to 100% Buffer B (Buffer A plus 5 M NaCl) in 30 ml, holding at 100% Buffer B for 60 ml and returning from 100% Buffer B to 100% Buffer A in 30 ml. 4-ml fractions were collected, with the reductase being eluted in fractions 33–43. These fractions were then pooled, concentrated to ~5 mg/ml in a Centricron 10 micro concentrator (Amicon, Inc.), desalted over PD-10 columns, and assayed for activity.

Anion Exchange Chromatography—The resulting enzyme solution (118 mg) was next applied to a POROS 20 QE Perfusion anion exchange column, equilibrated in Buffer C (50 mM Bis-Tris propane, pH 6.8, 5 mM DTT). The desired protein did not bind to the column, whereas the majority of the contaminating *E. coli* proteins remained bound. 36 mg of purified reductase protein were thus obtained, this being apparently homogeneous, as revealed by SDS-polyacrylamide gel electrophoresis with visualization by silver staining (see Fig. 6 and “Results and Discussion”).

Cloning of *P. trichocarpa* cv. Trichobel Benzylic Ether Reductase—Wood of *P. deltoides × P. trichocarpa*, clone 604, was sampled in May 1992 by removal of a 2-year-old branch from a 20-year-old tree and separating the bark from the wood. Wood pieces were immediately frozen in liquid nitrogen and stored at −70 °C. These were subsequently used for two-dimensional gel electrophoresis and subsequent microsequencing of the most abundant protein in the gels.6 For the sampling of *P. trichocarpa* cv. Trichobel, 3-year-old trees were harvested, with the bark again separated, and the selected woody tissues immediately frozen in liquid nitrogen and stored at −70 °C. Woody tissue (~10 cm long) was obtained from the stem 10 cm above ground level. A root sample was also harvested at −5 cm below ground level. Peptide sequences obtained from the most abundant protein in two-dimensional gel electrophoresis and subsequent microsequencing of the most abundant protein in the gels sampled from *P. deltoides × P. trichocarpa*, clone 604, were as follows: 1) TGYIGK, 2) PTFALV, 3) GVTLLHGDV, 4) FPSEFGM, 5) VFPTETK, and 6) TTVEEYLDQFV. The amino acid sequence of the two internal peptides (numbers 3 and 4 above) and one C-terminal peptide (number 6) were next used to design nucleic acid amplification primers: PT primer 2 (GIG GTI ACI (CTT/TI ATI CA/C/T) GGI GA/GT) GT; reverse PT primer 5 (CAT ICC G/A/AA (C/T) GGA GA/GAA (G/A/AA); and reverse PT primer 9 (TA

5 K. Vander Mijnbrugge, G. Baw, M. Van Montagu, and W. Boerjan, manuscript in preparation.

6 K. Vander Mijnbrugge, G. Baw, M. Van Montagu, and W. Boerjan, manuscript in preparation.
Expression of the Poplar (P. trichocarpa) Reductase in E. coli—To assess whether other IFR “homolog” proteins were also able to reduce DDC or DDDC, the angiosperm IFR “homolog” reductase from P. trichocarpa, PCBER-Pop1, was analyzed for reductase activity. A pGEX-2T-based plasmid, containing the cDNA in frame with Schistosoma japonicum glutathione S-transferase, was constructed according to the manufacturer’s instructions (Amersham Pharmacia Biotech). This expression plasmid was then transformed into NovaBlue E. coli cells, which were then grown, induced for protein production as described above for the pSBETa-based system, except that carbencillin (50 μg/ml) was used as antibiotic instead of kanamycin; and finally pelleted (3000 × g, 25 min) for storage at −80 °C. After cell lysis in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM K2HPO4, 1.8 mM KH2PO4), containing 5 mM DTT, the protein was purified over glutathione-Sepharose 4B resin according to Pharmacia’s instructions. The N-terminal glutathione S-transferase fusion was then removed from 50 mg of purified protein by cleavage at 4 °C for 16 h with 500 units of thrombin (Amersham Pharmacia Biotech), with the resulting protein tested for both phenylcoumaran benzylic ether reductase and PLR activities, as described below.

Chemical Synthesis of (+)-Dehydrodiconiferyl Alcohol (5)—To a solution of E-coniferyl alcohol 16 (400 mg, 2.2 mmol) in acetone (15 ml) was added FeCl3·6H2O (1.5 g, 5.5 mmol) in H2O (35 ml), with the resulting suspension stirred for 10 min at ambient temperature and then extracted with ethyl acetate (3 × 50 ml). The ethyl acetate solubles were combined, washed with H2O (3 × 30 ml), evaporated to dryness in vacuo, and reconstituted in chloroform (10 ml). This was then applied to a silica gel column (3 × 20 cm) eluted with a gradient solvent system from chloroform to chloroform/acetone (3:2). Fractions containing the desired product were combined and evaporated to dryness in vacuo, recrystallization of which from chloroform/acetonitrile (3:2) yielded (5 μg); 1H NMR (300 MHz, acetone-d6, J = 7 Hz, 6.5 and 7 Hz, C9 H, 3.76–3.91 (2H, overlapping, C9 H), 6.25 (1H, dt, J = 17 and 6 Hz, C9 H), 6.52 (1H, d, J = 17 Hz, C9 H), 6.81 (1H, d, J = 6 Hz, C9 H), 6.89 (1H, dd, J = 9 and 2 Hz, C9 H), 6.96 (1H, d, C9 H), 7.04 (1H, d, J = 2 Hz, C9 H), 7.62 (1H, s, Ar-CH); for EIMS, see Fig. 7D. The (+) and (−)-enantiomers of DDC 5 were

![FIG. 5. Amino acid sequence alignment of the P. taeda and poplar PCBER, similar isoflavone reductase “homologs”, PLRs, and IFRs.](image-url)
acetic acid (2 \times 500 \mu l), with the ethyl acetate solubles combined and evaporated to dryness in vacuo. The resulting residue was redissolved in acetonitrile/H2O (1:9) and applied to a Symmetry Shield column for HPLC analysis and purification. The fractions containing the desired compound were collected and dried in vacuo to give isodihydrodiconiferyl alcohol (TDDC) \( \delta (300 \text{ MHz}, \text{CDCl}_3) = 2.95 (2H, d, J = 7 \text{ Hz}, \text{C}_8 \text{H}_2), 3.47 (1H, m, \text{C}_9 \text{H}), 3.81 (3H, s, \text{OMe}), 3.85 (2H, overlapping, \text{C}_9 \text{H}_2), 3.91 (3H, s, \text{OMe}), 4.30 (2H, d, J = 7 \text{ Hz}, \text{C}_9 \text{H}), 5.47 (1H, s, \text{Ar-OH}), 6.04 (1H, s, \text{Ar-OH}), 6.20 (1H, d, J = 7 \text{ and } 14 \text{ Hz}, \text{C}_9 \text{H}), 6.52 (1H, d, J = 14 \text{ Hz}, \text{C}_9 \text{H}), 6.65-6.83 (5H, Ar-H); for EIMS, see Fig. 7E.

**Ratios of Enantiomers of (-)-Tetrahydrodehydrodiconiferyl Alcohol and Analogs 6, 8, and 17**—Separation of dehydrodiconiferyl alcohol 5, dihydrodehydrodiconiferyl alcohol 8, isodihydrodiconiferyl alcohol 6, and tetrahydrodehydrodiconiferyl alcohol 17 was accomplished using a reversed-phase column (Symmetry Shield RP-18, 3.9 \times 150 \text{ mm}, Waters) eluted with an acetonitrile, 3\% acetic acid in H2O (1:9), this being held for 5 min following sample introduction. A linear gradient from 3\% acetic acid in H2O was applied over 30 min, followed by a linear gradient to 100\% acetonitrile over 25 min.

**Chiral Column HPLC Separation of the (+) - and (-) -Enantiomers of Dehydrodiconiferyl Alcohol and Analogs 5 and 6**—Chiral HPLC separations of (+) - and (-) -DDDC 5, (+) - and (-) -DDDC 8, (+) - and (-) -TDDC 6, and (+) - and (-) -TDDC 17 were accomplished with detection at 280 nm using a Diatex Chiralcel OF \( (250 \times 4.6\text{ -mm inner diameter}) \) column. The following isocratic solvent systems were employed: for (+) - and (-) -DDC 5, hexane/2-propanol (1:1) at a flow rate of 0.2 ml/min, with elutions at 16.3 and 19.7 ml, respectively; for (+) - and (-) -DDDC 8, hexane/2-propanol (1:1) at a flow rate of 0.4 ml/min, with elutions at 10.4 and 13.7 ml, respectively; and for (+) - and (-) -TDDC 6 hexane/2-propanol (3:2) at a flow rate of 0.2 ml/min, with elutions at 19.0 and 21.1 ml, respectively.

**Radiochemical Assays for Dehydrodiconiferyl Alcohol Reductase Activity**—Each 150-\mu l assay consisted of 19 m\& MES/Bis-Tris propane, pH 6.5, 20 \mu l of the protein solution at the corresponding stage of purity, 5 m\& DTT, 2.5 m\& (\( \geq 9\))-dehydrodiconiferyl alcohols 8, and 5 m\& NADPH (14.2 m\&/m\& for preparation as described above). After a 6-h incubation at 22 °C, the assay mixture was extracted with ethyl acetate \( (2 \times 500 \mu l) \). The ethyl acetate-soluble fraction was evaporated to dryness in vacuo and then reconstituted in 100 \mu l of acetonitrile, 3\% acetic acid in H2O (1:9) and subjected to reversed-phase HPLC as described above, with both UV and radiochemical detection. 1-ml fractions were collected, with an aliquot (100 \mu l) of each removed for scintillation counting to determine the level of incorporation of \(^3\)H into the assay products. Controls using denatured enzyme (5 min, 100 °C) or no (\( \geq 9\))-dehydrodiconiferyl alcohol 8 substrates were also performed.

**Nonradioactive Assays for Dehydrodiconiferyl Alcohol Reductase Activity**—Each 150-\mu l assay consisted of 22 m\& MES/Bis-Tris propane, pH 6.5, 20 \mu l of the protein solution at the corresponding stage of purity, 5 m\& DTT, 2 m\& (\( \geq 9\))-dehydrodiconiferyl alcohols 5, and 4 m\& NADPH. After a 3-h incubation at 30 °C, the assay mixture was boiled for 3 min and centrifuged (17,000 \times g, 3 min), with an aliquot (125 \mu l) subjected to reversed-phase HPLC, after 16.6 m\& of acetonitrile was added. Quantification of formation of isodihydrodiconiferyl alcohol 6 was measured using a standard curve. Controls using denatured enzyme (5 min, 100 °C) or no NADPH cofactor were also performed.

**Enzyme Characterization—**Optimum temperature and pH were determined using standard (nonradioactive) assay conditions. The Michaelis-Menten constant \( K_m \) was not determined, except that the buffer concentration was increased to 19 m\& and the protein following the Afib-Blue column chromatographic step was used (30 m\&). For temperature optimum, incubations were performed at constant temperature (7.0 °C with varying temperature (6–58 °C); for pH optimum, incubations were performed at constant temperature (30 °C) with varying pH (5.5–9.5). Initial velocity kinetics were analyzed by assaying the protein activity under standard (nonradioactive) conditions at pH 7.0, 7.4, and 8.0.
but with 11 different concentrations of lignan substrate (0.167–2.5 mM) and at 22 °C for 6 h, while holding the NADPH concentration constant at 5 mM. To determine whether the 4R- or the 4S-hydride of the NADPH cofactor was utilized in the reduction catalyzed by the enzyme, radiochemical assays were performed as described above with specifically labeled [4R-3H]NADPH (1.67 mM, 10.7 MBq/mmol) or [4S-3H]NADPH (1.67 mM, 9.8 MBq/mmol) (1) and analyzed for radiochemical incorporation into the isodihydrodehydrodiconiferyl alcohol product.

Chiral HPLC Analysis of IDDDC 6 Generated by Action of Phenylcoumaran Benzylic Ether Reductase—Assays using DDC 5 as substrate were performed as above. The remaining substrate 5 and resulting product IDDDC 6 were separated by Symmetry Shield HPLC, collected individually, dried in vacuo, redissolved in methanol, and subjected to chiral HPLC analysis as described above. Both (+) and (−)-enantiomers of substrate DDC 5 were detected in equal amount as were both enantiomers of product IDDDC 6.

Radiochemical Assays for Pinoresinol-Lariciresinol Reductase Activity—Pinoresinol reductase and lariciresinol reductase activities were evaluated by monitoring formation of 3H-labeled product as described elsewhere (12).

Nonradioactive Assays for Pinoresinol-Lariciresinol Reductase Activity—Pinoresinol and lariciresinol reductase activities were further evaluated in assays performed as described in Ref. 12 with the following exceptions: total volume was 150 μl; 4 mM NADPH and 2 mM (±)-

FIG. 7. Reversed-phase HPLC and liquid chromatography-mass spectrometry analyses of the reductions catalyzed by the benzylic ether reductase, PCBER-Pt1. A–C, assays were carried out in the presence of [4R-3H]NADPH, and HPLC separations were monitored both at 280 nm (solid lines) and by scintillation counting of collected fractions (dashed lines). A, standards. 17, TDDC; 6, IDDC; 8, DDDC; 5, DDC. B, HPLC chromatogram for enzymatic reduction of DDC 8 to [3H]IDDDC 6. C, HPLC chromatogram for enzymatic reduction of DDC 8 to [3H]TDDC 17. D–F, mass spectra of DDC 5, and enzymatically generated 6 and 17 from unlabeled substrates. D, DDC 5 (substrate); E, IDDDC 6 (product of DDC 5 reduction by the phenylcoumaran benzylic ether reductase); F, TDDC 17 (product of DDDC 8 reduction by the phenylcoumaran benzylic ether reductase).
RESULTS AND DISCUSSION

In recent years, a relatively large family of three classes of reductases (8) has been identified from diverse members of the angiosperms and gymnosperms, with each correlated to functions associated with plant defense. Two of these classes were demonstrated to play significant roles in the biosynthesis of important plant defense phenylpropanoid-derived compounds, based on known catalytic function, i.e. the PLRs and the IFRs (8). Members of the third class, however, had no known function but were described as IFR/PLR “homologs,” since they share a significant level of homology, with ∼60–70% similarity at the amino acid level to IFRs and PLRs. All three classes possess NADPH binding sites and appear to be cytosolic enzymes, since they lack secretory pathway signal sequences. Additionally, they may be regulated in a comparable manner, not only at the transcriptional level (24) but also at the enzymatic level by protein phosphorylation, as evidenced by several potential phosphorylation sites that are conserved among all of the members (1, 8).

Pinoresinol-lariciresinol reductases and isoflavone reductases have been characterized at the enzymatic level and show comparable modes of catalytic action. Both abstract the 4R-hydride from the NADPH cofactor during catalysis (1, 2, 5–8, 45–47), and each has region- and enantiospecific toward its substrate (1, 2, 12, 45–47); i.e. for all of the IFRs identified to date, only the (−)-enantiomers of their products are formed, e.g. (−)-sofophorol 4 from 2′-hydroxypseudoapigenin 3 in pea (Pisum sativum; see Fig. 1B). On the other hand, the PLRs can exist in two distinct forms, e.g. in western red cedar (T. plicata), where one form reduces the (+)-enantiomer of pinoresinol 1 and lariciresinol 2, whereas another acts on the (−)-enantiomer (12). One question that has not been resolved, however, is whether the molecular species undergoing reduction is the conjugated enone, as shown in Fig. 1, A and B, or whether it is some other form of the molecule.

The PLR/IFR “homologs,” on the other hand, had no known catalytic function, since of those examined, none were able to catalyze either the reduction of isoflavonoids (9) or 8′–8′′-linked lignans (see below), and no other role has been forthcoming. However, their formation is inducible, both in cell culture and in planta, by factors such as sulfur starvation (oxidative stress) (10), fungal elicitors (9), and UV irradiation (48), indicative of a general plant defense role.

DCC 5, DDDC 8, and analogs thereof are 8′–5′-linked (phenylcoumaran) lignans that appear to be ubiquitous in the plant kingdom, being found in a wide array of plant families, such as, for example, the Asteraceae (49), Pinaceae (50), and Urticaceae (51). Although they were originally proposed to be formed either as intermediates in lignification (52–54) and/or in their glycoside forms as plant growth regulators (52, 55, 56), no consideration was ever given, from a biochemical perspective, to their further metabolism.

Numerous phytochemical reports had, however, identified many plant constituents that appear to be biochemically derived from DCC 5 and/or DDDC 8, e.g. tetrahydrodehydrodiconiferyl alcohol tetraacetate 13 (27) (see Fig. 3) via presumed phenylcoumaran (and allylic bond) reductions and subsequent acylations. Indeed, additional modifications to 8′–5′-linked lignans are also possible, such as attachment of various sugar moieties (50, 57–63) and methylenedioxy bridge formation (e.g. hancinone 14 in P. wightii (32, 33)) and so on. Moreover, a number of these metabolites are found in optically active form (27, 61–63), e.g. hancinone 14 and tetrahydrodehydrodiconiferyl alcohol tetraacetate 13 (Fig. 3), an observation that cannot be explained simply on the basis of random coupling (64).

It could, therefore, be considered that both DCC 5 and DDDC 8 might undergo comparable reductive transformations, analogous to that catalyzed by PLR and IFR, and hence provide an identification of the biochemical role of the so-called “homologs.”

Cloning, Expression, and Purification of PCBER-Pt1—Given the above findings, we investigated whether the “homolog” reductases catalyze reduction of DCC 5 and/or DDDC 8. Of particular interest was whether these reductions, as for their PLR and IFR counterparts, would display any enantiopspecific preference or whether they would only be regiospecific. In the approach undertaken in this study, a λ-phae-based cDNA library was first constructed from RNA isolated from P. taeda cell suspension cultures. These cells had previously been shown to produce DCC 5, DDDC 8 (28), and related lignan metabolites upon treatment with 8% sucrose, 20 mM potassium iodide. This cDNA library was then screened for potential reductases using the 5′-end (∼400 base pairs) of the F. intermedia pinoresinol-lariciresinol reductase (PLR-Fi1) cDNA (1) as a probe (this approach had previously been successful in screening F. intermedia, which resulted in identification of two “homologs” (1, 8)). Accordingly, moderate stringency conditions identified many potentially homologous cDNAs in the P. taeda library, 20 of which were purified through two additional rounds of screening. The pBluescript-based phagemids that carried the cDNAs of interest were excised from the λ-phae and ultimately gave only a single gene with homology to the F. intermedia pinoresinol-lariciresinol reductase from the P. taeda cell suspension culture cDNA library.

Analysis of its cDNA sequence revealed that it had ∼66% similarity and ∼45% identity to PLR from F. intermedia, and thus fell into the category of the IFR/PLR “homologs” (8). As shown in Fig. 4, this P. taeda reductase cDNA encodes a 308-amino acid protein, corresponding to 33.6 kDa. It also appears to be cytosolic, possessing no signal sequences for targeting to the secretory pathway, mitochondria, chloroplasts, or peroxisomes. Additionally, the protein contains the conserved GXX/GXXG consensus sequence, indicative of an NAD(P)/H-binding domain, as well as 12 potential phosphorylation sites (see Fig. 4), most of which are conserved in PLR, IFR, and the IFR “homologs” as well (8). The high similarity between these classes of reductases is further illustrated in Fig. 5, which compares the amino acid sequence of the P. taeda reductase (PCBER-Pt1) with several PLR, IFR, and “homolog” protein sequences. The presumed significance of this is discussed in detail below.

To first obtain catalytically active protein, native expression was performed in E. coli utilizing the overexpression plasmid, pSBETA (43), which contains the pET3a expression cassette, with an NdeI restriction site available for subcloning to yield native expression, as well as an argU gene (for production of the rare AGA-Arg (RNAArg) and kanamycin resistance (for high plasmid stability in liquid cultures). Since the putative reductase from P. taeda had no internal NdeI sites, subcloning into pSBETA was relatively straightforward. Two primers were used in PCRs to introduce NdeI sites at the start methionine (primer PT-ATG-NdeI) and in the 3′-end untranslated region (primer PT-Rev-NdeI) of the putative reductase. The regions of the cDNA sequence used for primer design are underlined in Fig. 4. The resulting PCR fragment was subcloned into a PCR cloning plasmid (pCRII; Invitrogen) and excised by cleavage
with NdeI. The ~1-kilobase pair fragment containing the putative reductase was gel-purified and ligated into pSBETa, which had been previously digested with NdeI. The resulting pSBET construct containing the putative reductase was transformed into competent E. coli cells and purified, and the expression region, containing the desired cDNA, was sequenced completely on both strands to verify that no mutations had been introduced during PCR.

Induction of the reductase in the pSBET overexpression system was accomplished by transforming B834(DE3) expression cells with the pSBET construct and then growing at 37 °C four 1-liter cultures in LB broth supplemented with 50 μg/ml kanamycin until they reached a density of $A_{600}$ ~0.6, at which time they were cooled to ~22 °C and inoculated with isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM. After growth for 21 h at ~20–22 °C, the cells were harvested by centrifugation.

A 12-fold purification to apparent homogeneity of the heterologously expressed native enzyme was accomplished in four chromatographic steps following cell lysis and cellular debris removal by centrifugation (see “Experimental Procedures“ and Table I). The first step employed ammonium sulfate precipitation, where the desired protein precipitated in the 40–70% saturation fraction. This fraction of the protein (513 mg) was desalted into Buffer A and then subjected to consecutive anion (POROS 20 QE) and cation (POROS 20 SP) exchange chromatography. Both chromatographic steps were conducted under conditions (pH 6.8) where the desired protein did not bind, whereas the contaminating E. coli proteins did and were thus removed. Fig. 6 shows an SDS-polyacrylamide gel of the reductase at each step of the purification scheme. After the final purification step, the protein was subjected to SDS-polyacrylamide gel electrophoresis and blotted to a polyvinylidene fluoride membrane, and the amino terminus thereof was sequenced. The amino acid sequence thus obtained matched exactly that deduced from the P. taeda cDNA sequence. The purified protein was also subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry, which showed a molecular mass of 33.6 kDa for the recombinant protein. These results indicated that the target reductase had been expressed.

**Enzymatic Activity Determination**— We next evaluated the P. taeda recombinant reductase for either its ability to convert the phenylcoumaran lignans, DDC 5 and/or DDDC 8, into the benzylic ether-reduced lignans, I-DDDC 6 and TDDC 17, respectively, or to reduce the lignans, pinoresinol 1 and lariciresinol 2.

In order to do this, the potential substrates and products were first obtained (semisynthetically), i.e. racemic (+)-dehydrodiconiferyl alcohols 5 were synthesized via FeCl$_3$ oxidation of E-coniferyl alcohol 16. DDC 5 was then subjected to catalytic hydrogenation (10% palladium-charcoal, H$_2$, 10 min) to produce the corresponding allylic bond-reduced derivative (+)-DDDC 8 in 79% yield, whose mass and $^{1}$$H$/$^{13}$C NMR spectra revealed that only reduction of the allylic 7'-8'-bond had occurred. In an analogous manner, further reduction (10% palladium-charcoal, H$_2$, 8 h) of DDC 5 produced (+)-IDDDC 17 in 61% yield. Its EIMS gave a molecular ion [M$^+$] at $m/z$ 362 and expected fragments at $m/z$ 208 and 137, whereas its $^1$$H$ NMR spectrum revealed the presence of five methylene groups ($\delta$ 1.79, 2.57, 2.91, 3.57, and 3.81). On the other hand, I-DDDC 6 was obtained semisynthetically, since it was generated by the action of the P. taeda reductase on DDC 8. Its EIMS displayed a molecular ion [M$^+$] at $m/z$ 360, with main fragment ions at $m/z$ 206 and 137. Its $^1$$H$ NMR spectrum displayed characteristic olefinic resonances at $\delta$ 6.20 and 6.52, together with three methylenic functionalities at $\delta$ 2.95, 3.85, and 4.30.

With all potential substrates and products obtained in racemic form, conditions were next identified for their facile reversed-phase HPLC separation. As shown in Fig. 7A, a near base-line separation of all four lignans was obtained (see “Experimental Procedures“ for elution conditions). Additionally, each lignan was subjected to chiral column HPLC analysis using a Daicel Chiralcel OD column, employing hexane/2-propanol isocratic solvent systems (see “Experimental Procedures”), which permitted determination of their enantiomeric compositions.

**Assays for DDC 5 and/or DDDC 8 reductase activities** were next performed (see Fig. 7, B, C, E, F), as described under “Experimental Procedures. “As shown in Fig. 7, B and C, the P. taeda reductase converted both DDC 5 and DDDC 8 into IDDC 6 and TDDC 17, respectively. In each case, identification of the products was determined using HPLC retention volumes, UV and $^1$$H$ NMR spectroscopy, and liquid chromatography-mass spectrometry fragmentation analysis. For illustrative purposes, the liquid chromatography-mass spectrometry spectra of the enzymatic products IDDC 6 and TDDC 17 are shown in Fig. 7, E and F, respectively. Using DDC 5 as a substrate (Fig. 1D), the enzymatically generated IDDC 6 (Fig. 7, B and E) gave a molecular ion [M$^+$] at $m/z$ 360 together with fragments at $m/z$ 342 ($M^+ - H_2O$), 324 ($M^+ - 2 H_2O$), 206 (C-7/C-8 cleavage), 188 (C-7/C-8 cleavage $- H_2O$), and 137 (benzylic fragment following C-7/C-8 cleavage). In an analogous manner, reduction of DDC 8 gave the corresponding TDDC 17 (Fig. 7, C and F), with a molecular ion [M$^+$] at $m/z$ 362 and fragment ions at $m/z$ 344 ($M^+ - H_2O$), 208 (C-7/C-8 cleavage), and 137 (benzylic fragment). Control assays with either DDC 5 or DDDC 8, conducted in the absence of NADPH or with boiled (denatured) enzyme, showed no conversion into IDDC 6 or TDDC 17, respectively. Thus, the so-called IFR/PLR “homologs” were able to function as phenylcoumaran benzylic ether reductases (PCBERs; see Fig. 1C). Fig. 8 shows the proposed biosynthetic pathway, based on these results, to the 8'-5' linked lignans in plants such as P. taeda.

As shown in Table II, further characterization of the P. taeda PCBER-Pt1, using DDC 5 as substrate, gave a pH optimum plateau from 6.4 to 7.0 and a temperature optimum at 49 °C. In addition, it is also a type A reductase, since transfer of the $^3$H from NADPH occurred only with [AR-$^3$H]NADPH and not with [4S-$^3$H]NADPH (Table II). This observation is in agreement with that noted for both PLR (1) and IFR (47).

Initial velocity studies were next carried out in order to determine whether PCBER-Pt1 preferred one substrate over the other (DDC 5 versus DDDC 8). As shown in Table II, the specific activity ($V_{max}$) is 2-fold higher for DDC 5 than for DDDC 8, whereas the $K_m$ for DDC 5 reduction is 3 times lower than that for DDDC 8 reduction, i.e. suggesting a slightly

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| Purification step       | Protein Specific activity | Purification factor |
|-------------------------|--------------------------|---------------------|
| Lysate                  | 1052 mg                   | 11 nmol/h/mg        |
| (NH$_4$)$_2$SO$_4$ precipitation (40-70%) | 513 mg                   | 31 nmol/h/mg        |
| Affi-Blue                | 118 mg                    | 72 nmol/h/mg        |
| Anion exchange (POROS-20 QE) | 59 mg                   | 121 nmol/h/mg       |
| Cation exchange (POROS-20 SP) | 36 mg                  | 134 nmol/h/mg       |

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**Table I**

Purification scheme for the phenylcoumaran benzylic ether reductase from *P. taeda*
higher affinity of the enzyme for the former over the latter. In addition, assays were performed using [4R-\(^3\)H]NADPH as cofactor in order to determine whether PCBER-Pt1 displayed any \(^3\)H kinetic isotope effect (13), with velocities determined both by rate of radiochemical incorporation into the products (\(V_{3H}\)) and by absolute rate of product formation (by comparison of product HPLC peak area with a standard curve, \(V_{3H})\). Interestingly, the kinetic isotope effect (\(V_{1H}/V_{3H}\)) observed is significantly higher for DDC 5 reduction (\(V_{1H}/V_{3H} = 14.4\)) than for DDDC 8 (\(V_{1H}/V_{3H} = 4.0\)); i.e., with DDDC 8, the hydride transfer step no longer contributes to the rate-limiting step of the reaction (13), in contrast to that observed for DDC 5. This is also supported by the increase in \(K_m\) for DDDC 8 reduction, indicating that its binding is less efficient than for DDC 5. These results, therefore, demonstrate that the \(P.\) \(taeda\) PCBER-Pt1 has a higher affinity for DDC 5 as substrate, suggesting that the biosynthesis of molecules such as tetrhydrodehydrodiconiferyl alcohol tetraacetate 13, in \(C.\) \(japonica\), may involve initial formation of the benzyl ether-reduced intermediate prior to allylic bond reduction.

It was next important to ascertain whether the angiosperm “homologs” possessed PCBER activity. Accordingly, we obtained the cDNA encoding an IFR “homolog” from \(P.\) \(trichocarpa\), PCBER-Pop1, in a pGEX-based system (see “Experimental Procedures”), with the cDNA in frame with N-terminal glutathione S-transferase. After expression in \(E.\) \(coli\), purification of the protein over glutathione-Sepharose 4B (see “Experimental Procedures”), and removal of the glutathione S-transferase fusion by thrombin cleavage, the recombinant protein was assayed for PCBER activity. This protein was able to reduce the benzyl ether bond of DDC 5, with a specific activity (53 nmol/mg/h) comparable with the reduction by PCBER-Pt1 of DDDC 8 (see Table II). Thus, the angiosperm IFR “homologs” can function as phenylcoumaran benzyl ether reductases as well.

TABLE II

Properties of the phenylcoumaran benzyl ether reductase from \(P.\) \(taeda\)

| Substrate       | DDC 5 | DDDC 8 |
|-----------------|-------|--------|
| \(K_m\) (mM)    | 0.61 ± 0.03 | 1.95 ± 0.10 |
| \(V_{max}\) (nmol/h/mg) | 104.2 ± 10.8 | 55.8 ± 2.7 |
| \(^3\)H-isotope effect with [4R-\(^3\)H]NADPH (\(V_{1H}/V_{3H}\)) | 14.4 | 4.0 |
| Hydride abstraction from [4R-\(^3\)H]NADPH | >99% | ND |
| Hydride abstraction from [4S-\(^3\)H]NADPH | <1% | ND |
| pH optimum      | 6.4–7.0 | ND |
| Temperature optimum (°C) | 49 | ND |

* ND, not determined.

To address the issue of whether PCBER-Pt1 displayed any enantiospecific effect toward its substrates, comparable with both PLR (1, 12) and IFR (45–47), the products of enzymatic conversions of DDC 5 and DDDC 8 were evaluated by chiral HPLC analysis (see “Experimental Procedures”). Unlike the other members of this large family of reductases, however, PCBER-Pt1 showed no apparent enantiospecificity toward either substrate, with racemic products being formed (data not shown).

Studies were also conducted to establish whether pinoresinol 1 or lariciresinol 2 (8–8′-linked lignans) could serve as substrates for PCBERs (see “Experimental Procedures”), using PCBER-Pop1 and PCBER-Pt1 (with the native expressed recombinant enzyme or with the \(\beta\)-galactosidase fusion protein (1)) as before. It was found, however, that with assays utilizing pinoresinol 1 or lariciresinol 2 as substrate and either [4R-\(^3\)H]NADPH or unlabeled NADPH as cofactor, no product formation was observed. This was still the case, even when assays were extended for up to 24 h (i.e. under conditions that would have depleted all available substrate with the \(F.\) \(intermedia\) reductase (PLR-F1) (1)). In addition, as discussed earlier, the IFR “homologs” are apparently unable to reduce isolavonoids (9). Accordingly, the only catalytic function identified to date for both PCBER-Pt1 and PCBER-Pop1 involves that of the first known example of phenylcoumaran benzyl ether cleavage of the 8–5′-linked lignans DDC 5 and DDDC 8. Since recent work with pinoresinol-lariciresinol reductases from western red cedar has demonstrated an apparent \(K_m\) of \(\sim \)0.62 mM for pinoresinol.
ships (at the amino acid sequence level) among PCBER, PLR, and IFR proteins. PLRH, “homolog” of PLR, T. plicata (Cupressaceae) (12); Fi, F. intermedia (Oleaceae) (1); La, L. albus (Fabaceae) (14); Ms, M. sativa (Fabaceae) (5); Ps, P. sativum (Fabaceae) (6); Ca, C. arietinum (Fabaceae) (7); Gn, G. max (Fabaceae); Th, T. heterophylla (Cupressaceae) (D. Gang, M. Fujita, L. B. Davin, and N. G. Lewis, unpublished results); Pt, P. taeda (Pinaceae); Pc, P. communis (Rosaceae); St, S. tuberosum (Solanaceae) (G. J. van Eldik, R. K. Ruiter, M. M. A. van Herpen, J. A. M. Schrauwen, and G. J. Wullems, SwissProt accession number P52578); Bp, B. pendula (Betulaceae); Pop, P. trichocarpa (Salicaceae); At, A. thaliana (Brassicaceae) (11); Nt, N. tabacum (Solanaceae) (9); Zm, Z. mays (Poaceae) (10); Cp, C. paradisi (Rutaceae) (48); cluster 1, PLR proteins; cluster 2, IFR proteins; cluster 3, PCBER proteins.

To compare the relationships between these phenylcoumaran benzyl ether reductases with PLRs, IFRs, and other “homologs,” a dendogram was produced, as shown in Fig. 9, based on sequence similarity at the amino acid level. As can be seen in cluster 1, all of the PLRs group together, along with a “homolog” from Lupinus albus (14), which may also encode a PLR. The IFRs, together with the “homolog” from Glycine max, also form a cluster (cluster 2), suggesting that IFHR-Gm1 may in fact be an isoflavone reductase. Cluster 3, with the exception of IFRH-Cp1, from Citrus × paradisi (48), is an outlier, consisting of two groups, corresponding to those present in the gymnosperms and those in the angiosperms. This, in turn, raises an interesting question about the origin of enantiospecificity among these reductases. Since PCBERs are apparently highly ubiquitous in the plant kingdom, they may have been the progenitor enzymes of both PLR and IFR (with the latter present thus far mainly in the Fabaceae (8)), having been conscripted into these latter biosynthetic pathways, with enantiospecificity evolving later.

**Conclusion**—The enzymes catalyzing phenylcoumaran benzyl ether reductions appear to be ubiquitous in the plant kingdom and were previously known as PLR and IFR “homologs,” based on their high sequence homology to these two classes of related enzymes. The cloning and heterologous expression of the PCBER from P. taeda, together with that from P. trichocarpa (PCBER-Pop1), now identifies the first known catalytic function for this enzyme class. In addition, the region-specific reduction of DDC 5 to IDDC 6 gives the first biochemical proof that 8′,5′-linked lignans are the products of distinct biochemical pathways, unrelated to that involved in lignin biosynthesis. These data provide further evidence against the hypothesis that both phenolic coupling and subsequent metabolism in plants are random, uncontrolled processes, as was once imagined (52, 53).

Having thus identified a catalytic function for these proteins, future studies will determine substrate versatility, the nature of the molecular species that is bound to the enzyme, and the mechanism of the subsequent transformation (i.e. hydride transfer to the quinone methide or some other sequence of reductive events). Additionally, future kinetic and inhibition studies are expected to establish proportions of active enzyme in the recombinant protein preparations. Interestingly, PCBERs effect regio- rather than enantiospecific conversions and may even be the progenitors of both PLRs and IFRs, which became, in contrast, enantiospecific. Moreover, with all three distinct classes of related reductases (PCBER, PLR, and IFR) available, an avenue of comparative mechanistic study is now available, i.e. in order to establish their points of similarity and difference, which permit some (PLR and IFR) to discriminate between substrate enantiomers, whereas others (PCBER) do not.

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