Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 67 (2006) 1765–1780
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Pinus taeda phenylpropenal double-bond reductase: Purification, cDNA cloning, heterologous expression in *Escherichia coli*, and subcellular localization in *P. taeda* ☆

Hiroyuki Kasahara, Ying Jiao, Diana L. Bedgar, Sung-Jin Kim, Ann M. Patten, Zhi-Qiang Xia, Laurence B. Davin, Norman G. Lewis *

Institute of Biological Chemistry, Washington State University, Pullman, WA 99164-6340, United States

Received 3 June 2006; received in revised form 3 July 2006
Available online 14 August 2006

Dedicated to Rodney Croteau in honor of his 60th birthday.

Abstract

A phenylpropenal double-bond reductase (PPDBR) was obtained from cell suspension cultures of loblolly pine (*Pinus taeda* L.). Following trypsin digestion and amino acid sequencing, the cDNA encoding this protein was subsequently cloned, with the functional recombinant protein expressed in *Escherichia coli* and characterized. PPDBR readily converted both dehydrodiconiferyl and coniferyl aldehydes into dihydrodehydrodiconiferyl and dihydroconiferyl aldehydes, when NADPH was added as cofactor. However, it was unable to reduce directly either the double bond of dehydrodiconiferyl or coniferyl alcohols in the presence of NADPH. During this reductive step, the corresponding 4-*proR* hydrogen was abstracted from [4*R*-^{3}H]-NADPH during hydride transfer. This is thus the first report of a double-bond reductase involved in phenylpropanoid metabolism, and which is presumed to be involved in plant defense. *In situ* mRNA hybridization indicated that the PPDBR transcripts in *P. taeda* stem sections were localized to the vascular cambium, as well as to radial and axial parenchyma cell types.

Additionally, using *P. taeda* cell suspension culture crude protein extracts, dehydrodiconiferyl and coniferyl alcohols could be dehydrogenated to afford dehydrodiconiferyl and coniferyl aldehydes. Furthermore, these same extracts were able to convert dihydrodehydrodiconiferyl and dihydroconiferyl aldehydes into the corresponding alcohols. Taken together, these results indicate that in the crude extracts dehydrodiconiferyl and coniferyl alcohols can be converted to dihydrodehydrodiconiferyl and dihydroconiferyl alcohols through a three-step process, i.e. by initial phenylpropenol oxidation, then sequential PPDBR and phenylpropanal reductions, respectively.

Keywords: *Pinus taeda*; Pinaceae; Loblolly pine; Phenylpropenal double bond reductase; Lignans; Lignins; Dihydroconiferyl aldehyde; Dihydrodehydrodiconiferyl aldehyde

1. Introduction

The lignans are an ubiquitous class of phenylpropanoid metabolites present in vascular plants, ranging in size from dimers to oligomers. They are conveniently classified on the basis of their structural interunit linkages, e.g. 8–8′, 8–*O*–4′, 8–5′, etc. (Lewis and Davin, 1999). Their primary function in plants appears to be in defense (e.g. as biocides, antioxidants, etc.) (Ayres and Loike, 1990; Lewis and Davin, 1999; Davin and Lewis, 2005). In certain families,

Abbreviations: CAD, cinnamyl alcohol dehydrogenase; DDC, dehydrodiconiferyl alcohol; DDCAL, dehydrodiconiferyl aldehyde; DDDC, dihydrodehydrodiconiferyl alcohol; DDDCAL, dihydrodehydrodiconiferyl aldehyde; ENR, enoyl acyl carrier protein reductase; ER, enoate reductase; GST, glutathione *S*-transferase; IDDDC, isodihydrodehydrodiconiferyl alcohol; IPTG, isopropyl β-D-thiogalactoside; PCBER, phenylcoumaran benzylic ether reductase; PLR, pinoresinol/lariciresinol reductase; PPDBR, phenylpropenal double-bond reductase; SDH, secoisolariciresinol dehydrogenase; TDDC, tetrahydrodehydrodiconiferyl alcohol.

☆ Data deposition: The sequence reported in this paper has been deposited in the GenBank database (Accession No. DQ829775).

* Corresponding author. Tel.: +1 509 335 8382; fax: +1 509 335 8206.
E-mail address: lewisn@wsu.edu (N.G. Lewis).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.07.001

such as the Pinaceae, they also typically help determine the characteristic color and properties of heartwood tissue. For example, over the last two decades, a range of dimeric and oligomeric lignans have been reported in the Pinaceae (Sakakibara et al., 1987; Barrero et al., 1993, 1996; Kawamura et al., 1997).

Recently, the biosynthetic pathways to the 8–8′ linked lignans (see Fig. 1) have been defined at the metabolic, enzymatic, and molecular levels (Chu et al., 1993; Ozawa et al., 1993; Dinkova-Kostova et al., 1996; Davin et al., 1997; Fujita et al., 1999; Gang et al., 1999a; Xia et al., 2000, 2001; Halls and Lewis, 2002; Kim et al., 2002; Min et al., 2003; Halls et al., 2004; Moinuddin et al., 2006). This demonstrated the existence of two distinct pathways using the monolignol, coniferyl alcohol (**1**), namely either directly into the various non-structural lignans or partitioned for polymerization into the lignins (Gang et al., 1999a; Burlat et al., 2001). For the 8–8′ linked lignans, a biosynthetic pathway (Fig. 1) from *E*-coniferyl alcohol (**1**) through stereoselective coupling to give (+)-pinoresinol (**2a**) has been established (Davin et al., 1997; Gang et al., 1999a; Halls and Lewis, 2002; Halls et al., 2004), with the latter being enantiospecifically metabolized into various bioactive compounds (Ozawa et al., 1993; Dinkova-Kostova et al., 1996; Fujita et al., 1999; Xia et al., 2000, 2001). These, depending upon the plant species, can include formation of molecules such as secoisolariciresinol (**4**), matairesinol (**5**), α-conidendrin (**6**), plicatic acid (**7**), podophyllotoxin (**8**) and their (oligomeric) congeners (Lewis and Davin, 1999). That is, pinoresinol (**2**) is a common precursor of the 8–8′ lignans and can be metabolized into a very broad range of natural products.

In contrast to the 8–8′ linked lignans, the biosynthetic pathways (metabolic steps, enzymes and genes involved) to the structurally diverse 8–5′ linked lignans are only now yielding to inquiry. The initial coupling step leads to dehydrodiconiferyl alcohol (**9**, DDC) which can, depending upon the species, be found in either racemic (Cutillo et al., 2003) and/or optically active (Yoshikawa et al., 2003) forms. Some of these enantiomerically pure metabolites have relatively low optical rotations, e.g. (+)- and (−)-dihydrodehydrodiconiferyl alcohols (**10a** and **10b**, $[\alpha]_D^{25} = +5.1$ and −9.18, respectively) (see Section 4) (Lewis and Davin, 1999).

Loblolly pine (*Pinus taeda* L.) is a popular and commercially important softwood species, and has been used as a model plant for studies on lignin biosynthesis and monolignol regulation (Eberhardt et al., 1993; Nose et al., 1995; Anterola et al., 1999, 2002). Moreover, its cell suspension

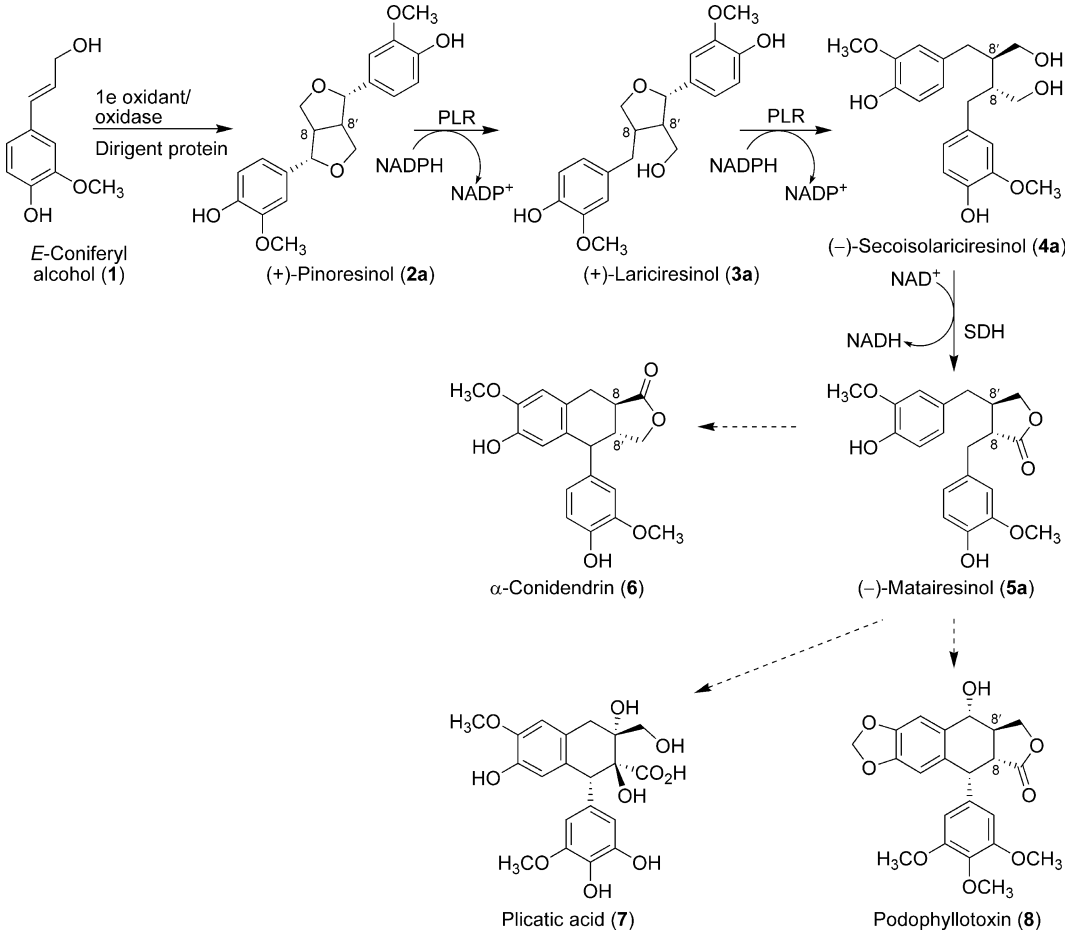


Fig. 1. Biosynthetic pathway to (−)-matairesinol (**5a**) and proposed conversions to α-conidendrin (**6**), plicatic acid (**7**) and podophyllotoxin (**8**).

cultures accumulate various lignans, including those that are 8–5′ linked such as dehydrodiconiferyl alcohol (**9a/b**), when placed in a solution containing 8% sucrose and 20 mM KI (Nose et al., 1995).

In the Pinaceae, the 8–5′ linked lignans typically have also undergone further post-coupling metabolism, e.g. to afford (±)-dihydrodehydrodiconiferyl alcohols (DDDC, **10a/b**) described above (Nose et al., 1995; Pan and Lundgren, 1995) and/or the related demethylated product, cedrusin (**11**) (Agrawal et al., 1980; Nose et al., 1995). During "normal" growth and development, such substances are synthesized in different tissues and organs as part of the characteristic heartwood oligomeric lignans; they can also accumulate in cell suspension cultures (e.g. *P. taeda*). Previously, we had reported that a provisionally annotated phenylcoumaran benzylic ether reductase (PCBER), cloned from the cDNA of loblolly pine and obtained in functional recombinant form, regiospecifically cleaves the 7–*O*–4′ benzylic ether linkages of both (±)-DDC **9a/b** and (±)-DDDC **10a/b** (Fig. 2) to give (±)-isodihydrodehydrodiconiferyl (IDDDC, **23a/b**) and (±)-tetrahydrodehydrodiconiferyl (TDDC, **24a/b**) alcohols (Gang et al., 1999b). These findings were in agreement with our view that DDC (**9a/b**) is the common precursor of the 8–5′ linked lignans commonly found in plants.

The purpose of this particular investigation was to establish further the biochemical nature of the putative phenylpropanoid double-bond reductive step in the Pinaceae which had been preliminarily described in 2001 (see Kasahara et al., 2004). Note, however, that such phenylpropanoid double-bond reductions are not restricted to the 8–5′ linked lignans. For example, representatives of the Pinaceae can form a variety of different phenylpropanoid monomers (**12**–**18**) (Kraus and Spiteller, 1997) and oligomers possessing reduced side-chains (e.g. such as the dimers **19** and **20** in *Pinus massoniana* (Lundgren et al., 1985) and *Picea abies* (Pan and Lundgren, 1995), respectively). Moreover, formation of such substances (e.g. **9**, **11**, **12**–**18**) can also be increased during aphid attack (e.g. *Adelges abietis* in *Picea glauca*) in accordance with their roles in plant defense (Kraus and Spiteller, 1997). Interestingly, in a *P. taeda* multiple mutant, including that of a cinnamyl alcohol dehydrogenase (CAD) mutation, soluble (reportedly lignin-like) preparations contained small amounts of substructures with saturated side-chains (Ralph et al., 1997).

This contribution thus addresses the purification of a phenylpropenal double-bond reductase (PPDBR) from loblolly pine (*P. taeda*) crude extracts of cell suspension cultures, its cDNA cloning and expression in functional form in *Escherichia coli*, as well as determination of its subcellular location in *P. taeda* stem tissue.

2. Results and discussion

2.1. Extraction and partial purification of target protein

As indicated above, *P. taeda* cell suspension cultures, when placed in 8% sucrose and 20 mM KI solution, accumulate metabolites, such as dehydrodiconiferyl alcohol (DDC, **9**) and dihydrodehydrodiconiferyl alcohol (DDDC, **10**). Since DDC (**9**) was potentially a direct precursor of DDDC (**10**), we initially prepared crude enzyme extracts from *P. taeda* cell suspension cultures and assayed them for the conversion of DDC (**9**) to DDDC (**10**), as well as the corresponding aldehydes **21** and **22**. In this regard, it was established that both **9** and **21** underwent reduction to afford **10** and **22**, in the presence of NADPH.

We therefore investigated whether the 4 *pro-R* or 4 *pro-S* hydrogen of the NADPH nicotinamide ring was abstracted during hydride transfer. Both [4*R*-^{3}H] and

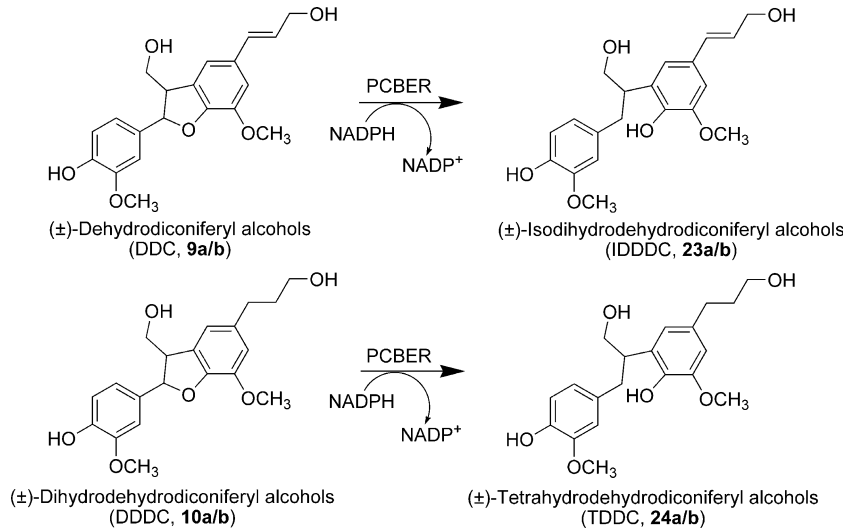
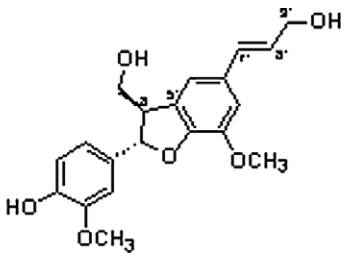
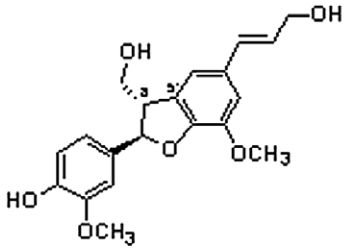
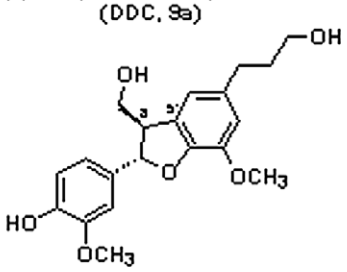
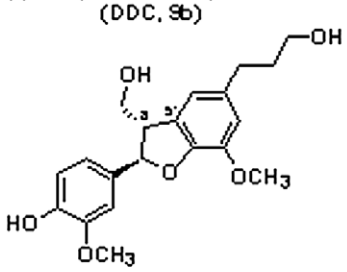


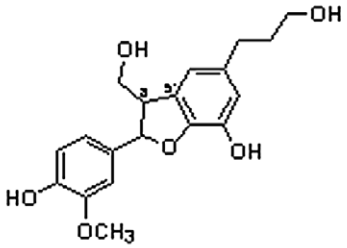
Fig. 2. Biosynthetic pathway to isodihydrodehydrodiconiferyl (IDDDC, **23a/b**) and tetrahydrodehydrodiconiferyl (TDDC, **24a/b**) alcohols.

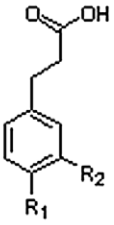

(+)-Dehydrodiconiferyl alcohol
(DDC, 9a)

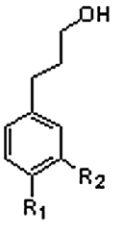

(–)-Dehydrodiconiferyl alcohol
(DDC, 9b)

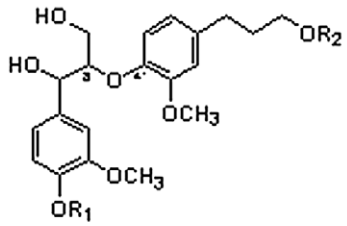

(+)-Dihydrodehydrodiconiferyl alcohol
(DDDC, 10a)

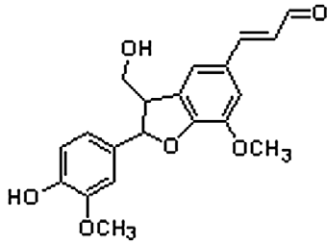

(–)-Dihydrodehydrodiconiferyl alcohol
(DDDC, 10b)

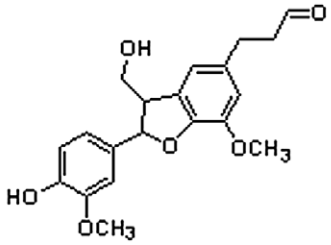

Cedrusin (11)


R_1 = OH, R_2 = H, Dihydro *p*-coumaric acid (12)
R_1 = OGlc, R_2 = H, Dihydro *p*-coumaric acid glucoside (13)
R_1 = OH, R_2 = OCH_3, Dihydroferulic acid (14)
R_1 = OGlc, R_2 = OCH_3, Dihydroferulic acid glucoside (15)


R_1 = OH, R_2 = OCH_3, Dihydroconiferyl alcohol (16)
R_1 = OGlc, R_2 = H, Dihydro *p*-coumaryl alcohol glucoside (17)
R_1 = OGlc, R_2 = OCH_3, Dihydroconiferyl alcohol glucoside (18)


(19): R_1 = H, R_2 = β-D-Glc
(20): R_1 = β-D-Xyl, R_2 = H


(±)-Dehydrodiconiferyl aldehydes
(DDCAL, 21a/b)


(±)-Dihydrodehydrodiconiferyl aldehydes
(DDDCAL, 22a/b)

[4*S*-^{3}H]NADPH were thus enzymatically synthesized as previously described (Chu et al., 1993). As summarized in Table 1 and Fig. 3(c), DDDC (**10**) was only formed (1.32 pkat mg^{-1} protein) from DDC (**9**), for example, when [4*R*-^{3}H]- NADPH was employed as cofactor. This specificity for hydride transfer is comparable to that observed with pinoresinol/lariciresinol reductases (PLR) isolated from *Forsythia intermedia* (Dinkova-Kostova

Table 1
Stereospecificity of hydride transfer from [4*S*-^{3}H] or [4*R*-^{3}H]NADPH when incubated with PPDBR and DDC (**9**)

Cofactors	PPDBR activity[a] (pkat mg^{-1} protein)
[4*R*-^{3}H]NADPH	1.32
[4*S*-^{3}H]NADPH	0.00
None	0.00

[a] Assays were carried out with the native *P. taeda* protein and (±)-DDC (**9**) as substrates.

et al., 1996) and western red cedar (Fujita et al., 1999), as well as the PCBER from *P. taeda* (Gang et al., 1999b); that is, all are type A reductases.

For protein purification purposes, DDC (**9**)/dehydrodiconiferyl aldehyde (DDCAL, **21**) reduction leading to DDDC (**10**)/dihydrodehydrodiconiferyl aldehyde (DDDCAL, **22**) formation was conveniently monitored throughout PPDBR purification using [4*R*-^{3}H]NADPH as cofactor. In this regard, we achieved partial purification of the putative reductase using a four-step protocol that included $(NH_4)_2SO_4$ precipitation and three successive chromatographic steps (Table 2). After the final ADP-agarose chromatographic step for an overall ~4200-fold purification, the resulting protein fraction was subjected to SDS–PAGE to afford three dominant proteins of approximately 38, 39 and 42 kDa. The three proteins, separated in this manner, were then subjected to amino acid sequence analysis.

At this purification level, chiral column HPLC analysis, for example, revealed that both (+)- and (−)-enantiomers of dihydrodehydrodiconiferyl alcohol (**10a** and **10b**) could be formed in equal amounts (Fig. 4), i.e. both (+)- and (−)-dehydrodiconiferyl alcohols (**9a** and **9b**) were regiospecifically reduced, in a manner comparable to the regiospecific phenylcoumaran benzylic ether reductase (PCBER) from *P. taeda* (Gang et al., 1999b). These studies did not, however, establish whether DDC (**9**) was being converted directly into DDDC (**10**), or whether it proceeded through an intermediate form, such as the α,β unsaturated aldehyde (**21**).

2.2. Cloning of the cDNA encoding 38.7 kDa protein (PPDBR)

The three predominant protein bands separated by SDS–PAGE were next individually sectioned, digested with trypsin, and analyzed by microcapillary reversed-phase HPLC nano-electrospray tandem mass spectrometry (see Section 4). The sequences of peptides obtained for the 3 proteins were identified by searching available databases with the program Sequest (Eng et al., 1994) and others developed at the Harvard Microchemistry Facility (Chittum et al., 1998). Analysis of each peptide sequence obtained indicated that the peptide sequences of the 43 kDa protein corresponded to *P. taeda* CAD (MacKay et al., 1995), whereas that of the 38 kDa protein was identical to a fructose-bisphosphate aldolase from *Zea mays* (Dennis et al., 1988). On the other hand, three peptide sequences, ELILVAYANEGPVTDSHLNIR, DGSSGDVAVQNLWISVDPYLR, and ESDDGLYLPSFPLNQAIR, obtained from the ~39 kDa protein, were present in the amino acid sequence of a cDNA clone, 7C5A, which was obtained from an EST library of *P. taeda* immature xylem tissue (Allona et al., 1998); the enzymatic properties of the putative protein from this cDNA, however, had not been characterized. To address the catalytic properties of the ~39 kDa protein, its full length cDNA clone was first obtained by a PCR-based approach using specific oligonucleotides designed on the basis of the EST sequence (Kasahara et al., 2004). As shown in Fig. 5, the cDNA encodes a 351-amino acid protein, corresponding to 38.7 kDa.

2.3. Expression of the recombinant 38.7 kDa protein (PPDBR)

To produce the recombinant 38.7 kDa protein in *E. coli*, we first employed the pSBET vector, which was previously successfully used for expression of the native PCBER (Gang et al., 1999b). The cDNA clone corresponding to the 38.7 kDa protein was then transferred to the pSBET vector by the Sticky-end PCR method (Zeng, 1998), with the pSBET construct subsequently transformed into competent BL21RIL cells. Production of the putative PPDBR protein (~39 kDa by SDS–PAGE) was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37 °C. However, its availability as a soluble protein was very minor, as the vast majority was present in inclusion body form. Modifications of the induction conditions, e.g. at low temperature and/or low IPTG concentration were all unsuccessful.

We, therefore, next attempted expression of this protein as a glutathione *S*-transferase (GST) fusion, using the pGEX-4T-1 expression vector. Thus, the cDNA clone corresponding to the 38.7 kDa protein was transferred to the pGEX-4T-1 vector as described in Section 4. In this way, the GST-fused protein was successfully expressed, being approximately 64 kDa on SDS–PAGE. It was, however, only present as inclusion bodies when induction by IPTG (1 mM) was carried out at 37 °C, whereas small amounts of soluble GST-fused protein could be obtained when induction was performed at 20 °C. Under these conditions, the GST-fused protein was purified over glutathione-Sepharose 4B (GS-4B) resin, with the N-terminal GST fusion subsequently removed by thrombin cleavage to afford the requisite recombinant ~39 kDa protein. The latter was then purified to apparent homogeneity using a Mono Q HR5/5 column (see Section 4). An amino acid sequence analysis gave a N-terminal sequence of GSMEQRVPNR, which verified that the 39 kDa protein included the two extra amino acids, Gly and Ser, at the N-terminal end of the native protein; these additions resulted from the design of the vector for thrombin cleavage.

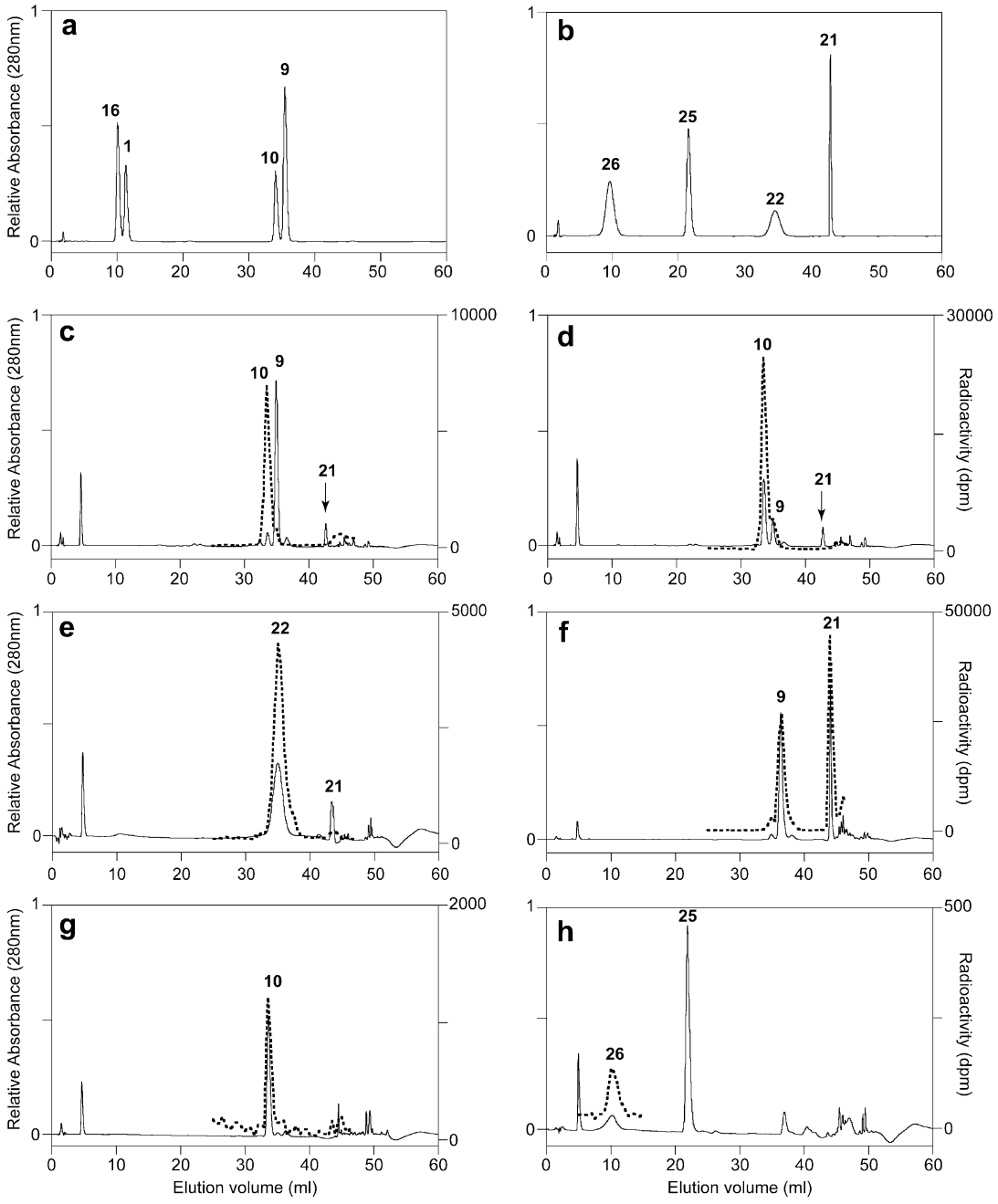


Fig. 3. Reversed-phase HPLC analyses of double bond reductions catalyzed by PPDBR. (a) Standards (alcohols): dihydroconiferyl alcohol (**16**), coniferyl alcohol (**1**), dihydrodehydrodiconiferyl alcohol (DDDC, **10**), dehydrodiconiferyl alcohol (DDC, **9**). (b) Standard (aldehydes): dihydroconiferyl aldehyde (**26**), coniferyl aldehyde (**25**), dihydrodehydrodiconiferyl aldehyde (DDDCAL, **22**), dehydrodiconiferyl aldehyde (DDCAL, **21**). (c) Enzymatic reduction of DDC (**9**) to [^{3}H]-DDDC (**10**) by *P. taeda* protein extracts in presence of [4*R*-^{3}H]NADPH. (d) Enzymatic reduction of DDC (**9**) to [^{3}H]-DDDC (**10**) by *P. taeda* protein extracts in presence of [4*R*-^{3}H]NADPH and recombinant PPDBR. (e) Enzymatic reduction of DDCAL (**21**) to [^{3}H]-DDDCAL (**22**) by recombinant PPDBR in presence of [4*R*-^{3}H]NADPH. (f) Enzymatic oxidation of [9,9′-^{3}H]-DDC (**9**) to [9-^{3}H]-DDCAL (**21**) by *P. taeda* crude extracts in presence of NADPH. (g) Enzymatic reduction of DDDCAL (**22**) to [^{3}H]-DDDC (**10**) by *P. taeda* crude extracts in the presence of [4*R*-^{3}H]NADPH. (h) Enzymatic reduction of coniferyl aldehyde (**25**) to [^{3}H]-dihydroconiferyl aldehyde (**26**) by recombinant PPDBR in presence of [4*R*-^{3}H]NADPH. HPLC separations (for elution conditions see Section 4) monitored both at 280 nm (*solid lines*) and by scintillation counting of collected fractions (*dashed lines*).

2.4. Catalytic activity of the recombinant ~39 kDa protein (PPDBR)

We next investigated whether the conversion of DDC (**9**) into DDDC (**10**) occurred directly, or whether the corresponding aldehyde **21** served as the true substrate. However, with DDC (**9**) as a potential substrate for the recombinant ~39 kDa protein in the presence of NADPH, no catalytic activity was detected. On the other hand, when the putative reductase was added to the crude *P. taeda* protein extract in the presence of NADPH, the conversion of DDC (**9**) to DDDC (**10**) was apparently enhanced

Table 2
Purification of PPDBR from *P. taeda* cell suspension cultures

Purification step	Protein (mg)	Total activity (nmol)	Specific activity (nmol mg^{-1})	Purification factor (-fold)
Ammonium sulfate	2454	14,212	5.8	1
Phenyl-Sepharose	37	34,779	940	160
Mono Q HR5/5	0.5	1973	3946	680
ADP-agarose	0.01	243	24,300	4190

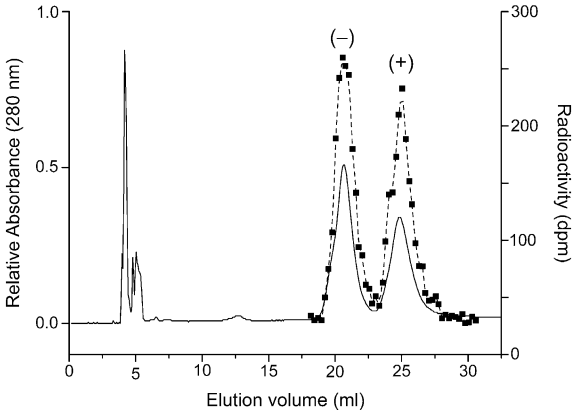


Fig. 4. Chiral column HPLC separation of (+)- and (−)-dihydrodehydrodiconiferyl alcohols (**10a** and **10b**) formed upon incubation of purified *P. taeda* PPDBR with dehydrodiconiferyl alcohols (**9a** and **9b**) in presence of [4*R*-^{3}H]NADPH. For elution conditions, see Section 4.

(Fig. 3(d)). This indicated that the DDC (**9**) conversion required other factor(s) which were present in the crude extract. Among the possibilities envisaged, we had considered that other enzyme(s) in the crude extract, e.g. CAD, could oxidize DDC (**9**) to dehydrodiconiferyl aldehyde (DDCAL, **21**), with this subsequently undergoing double bond reduction to afford dihydrodehydrodiconiferyl aldehyde (DDDCAL, **22**) via the action of PPDBR, with subsequent aldehydic reduction to give DDDC (**10**), as shown in Fig. 6(a).

This possibility was considered since it was known that the bacterial enoate reductase converts allyl alcohols to saturated propanols in concert with an alcohol dehydrogenase (Bader et al., 1981). In potential agreement with this, it was found that when DDC (**9**) was incubated with *P. taeda* crude extracts, a small peak of an unidentified compound was detected by HPLC analysis at an elution volume of ~42.5 ml (shown with arrow in Fig. 3(c) and (d)). LC–MS analysis of this peak gave an $[M]^+$ at *m/z* 356, this corresponding to the $[M]^+$ of DDCAL (**21**) previously isolated from another plant species, *Balanophora japonica* M. (Haruna et al., 1982). We thus considered that CAD, which was co-eluting with the ~39 kDa protein of interest in the active fractions of *P. taeda*, could convert DDC (**9**) to DDCAL (**21**), and thus form the true substrate for the PPDBR.

To investigate whether such a process was in effect, DDCAL (**21**) and DDDCAL (**22**) were next synthesized. This was achieved by allyl alcohol oxidation of DDC (**9**) using pyridinium dichromate in DMF to give DDCAL (**21**), whereas DDDCAL (**22**) was obtained by non-selective alcohol oxidation of DDDC (**10**) by pyridine sulfur trioxide. HPLC analysis of the synthetic DDCAL (**21**)

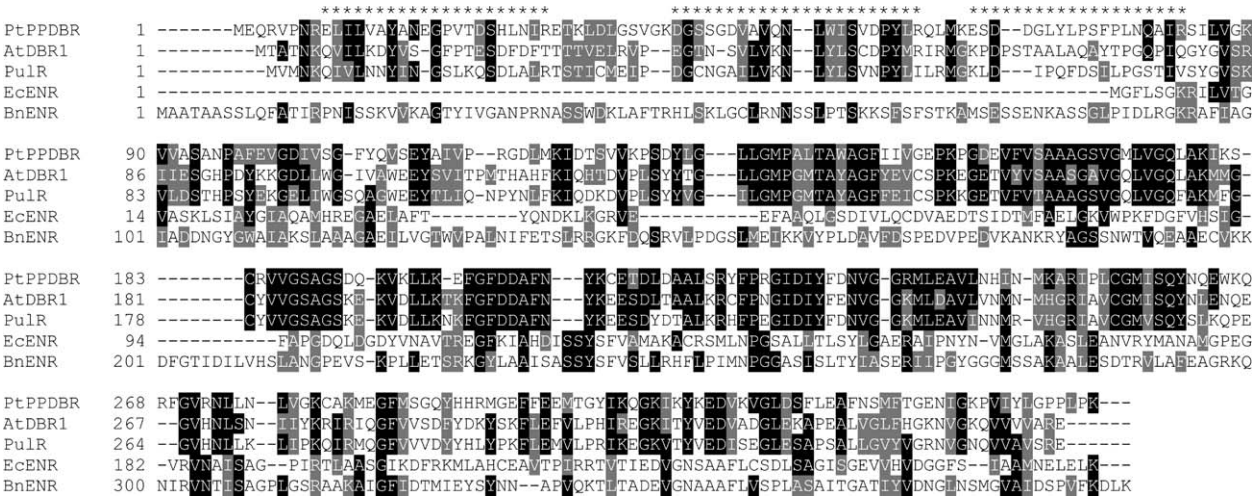


Fig. 5. Alignment of deduced amino acid sequences of the cDNA encoding *P. taeda* phenylpropenal double bond reductase (PPDBR, GenBank Accession No. DQ829775) and those of various double bond reductases. Sequence data obtained by microcapillary reversed-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) sequencing are depicted by asterisks (*) for PPDBR. Identical and similar amino acid residues are shown in black and gray, respectively. BnENR: enoyl-[acyl carrier-protein] reductase from *B. napus* (GenBank Accession No. CAC41367), EcENR: enoyl reductase from *E. coli* (GenBank Accession No. AAN80225), PulR: (+)-pulegone reductase from *Mentha* × *piperita* (GenBank Accession No. AAQ75423) and AtDBR1 (At5g16970 from *A. thaliana*).

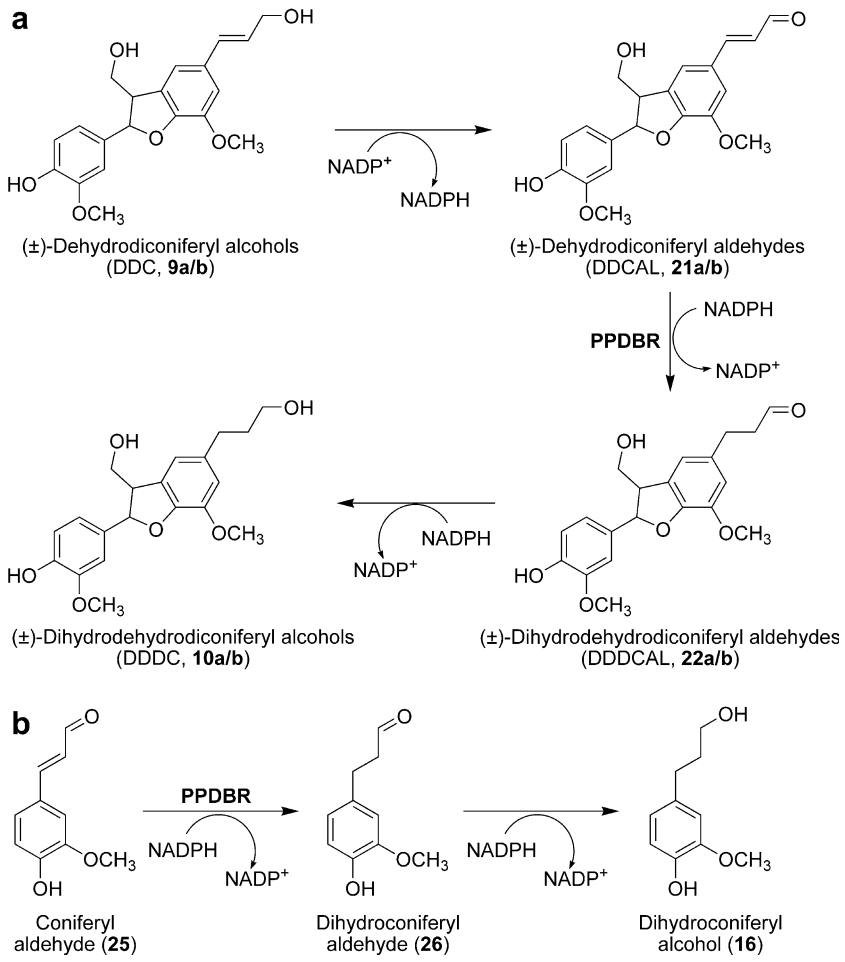


Fig. 6. Proposed biosynthetic pathways to (±)-dihydrodehydrodiconiferyl alcohols (**10a/b**) and dihydroconiferyl alcohol (**16**).

revealed that the retention time (elution volume: 42.5 ml) was identical to that of unidentified compound in Fig. 3(c) and (d) (arrow). LC–MS data of synthetic DDCAL (**21**) also indicated further that the unidentified compound was DDCAL (**21**). Synthetic DDCAL (**21**) was thus next subjected to an *in vitro* enzyme assay and, as anticipated, it was efficiently converted into DDDCAL (**22**) by incubation with the putative reductase in the presence of [4*R*-^{3}H]-NADPH (Fig. 3(e)). The product was identified as DDDCAL (**22**) by comparison of its spectroscopic data with that of synthetic DDDCAL (**22**).

With regard to the presumed oxidation of DDC (**9**) to DDCAL (**21**) by *P. taeda* crude extracts, this was also demonstrated by incubating [9,9′-^{3}H]-DDC (**9**) in the presence of NADPH. As shown in Fig. 3(f), [9,9′-^{3}H]-DDC (**9**) was readily oxidized, with DDCAL (**21**) accumulating following incubation with the crude extract. Identification of DDCAL (**21**) was initially confirmed by coincidence of the retention time with the radioactivity, and then by using natural abundance DDC (**9**) as a substrate, with the structure of the enzymatically generated DDCAL (**21**) confirmed by LC–MS (data not shown). Additionally, synthetic DDDCAL (**22**) was reduced to DDDC (**10**) by incubation with the crude extract in the presence of [4*R*-^{3}H]-NADPH (Fig. 3(g)). Taken together, these findings support the possible DDC (**9**) metabolic pathway as shown in Fig. 6(a). Purification and identification of the other enzyme(s) which catalyze DDC (**9**) oxidation and DDDCAL (**22**) reduction will be the subject of future enquiry.

To account for the observed low, but detectable, PPDBR catalytic activity, some discussion of how DDC (**9**) could possibly be oxidized to DDCAL (**21**) throughout enzyme purification is needed, since NADP, a possible cofactor required for DDC (**9**) oxidation, was not added to the assay system. Among the possibilities envisaged, a small amount of DDC (**9**) conversion could result from auto-oxidation with O_2, together with small amounts of NADP being generated from NADPH oxidation.

Another potential double-bond reduced product, dihydroconiferyl aldehyde (**26**), was also synthesized from dihydroconiferyl alcohol (**16**) by an one-step oxidation using pyridine sulfur trioxide as described in Section 4. Next, coniferyl aldehyde (**25**) was incubated with the recombinant PPDBR in the presence of NADPH, and this too was readily converted into dihydroconiferyl aldehyde (**26**) (Fig. 3(h)); the latter's structure was identified by comparison of its spectroscopic data to that of a synthetic standard

(data not shown). In this reaction, [^{3}H] was also incorporated into **26** from [4*R*-^{3}H]-NADPH, but not [4*S*-^{3}H], by this recombinant enzyme. Since synthetic dihydroconiferyl aldehyde (**26**) was reduced to dihydroconiferyl alcohol (**16**) by the *P. taeda* crude extract in the presence of NADPH (data not shown), the double-bond reduction system of coniferyl aldehyde (**25**) to dihydroconiferyl aldehyde (**26**) was proposed as shown in Fig. 6(b).

2.5. Localization of PPBDR mRNA transcript

The PPDBR mRNA was localized *in situ* to various stem tissues of *P. taeda* by hybridization of 2.0 μg ml^{-1} of specific antisense alkaline-cleaved (~100 bp) riboprobes labeled with digoxygenin (DIG). Using young *P. taeda* stem tissue from just below the apical meristem, PPDBR was visualized as a blue color in the vascular cambium (*vc*), radial parenchyma cells of the xylem (*rc*) and axial parenchyma cells of the cortex (*ap*) as seen in the cross-section shown in Fig. 7(a). The efficiency and specificity of probe binding was demonstrated by application of negative control (sense) riboprobe (2.0 μg ml^{-1}) to a serial section, in which no apparent binding was observed (Fig. 7(b)). Additional analyses, using slightly more mature tissue and 5.0 μg ml^{-1} of DIG-labeled antisense riboprobe, show that PPDBR mRNA occurs in same cell types (*vc*, *rc*, *ap*) as the younger tissue (Fig. 7(c)). The requirement of a 2.5-fold increase in riboprobe concentration for clear visualization in this section indicates that, compared to very young tissue, transcription in older tissues may be decreasing or turning over faster as translation occurs. Localization of PPDBR mRNA within the secondary tissue was confirmed through some of the stem, by applying an identical treatment to a longitudinal section (Fig. 7(e)). Again, efficiency and specificity of binding in this older tissue were confirmed using an equal concentration of control DIG-labeled riboprobe. In the cross-section, the control probe showed little to no binding in the *vc* (Fig. 7(d)). In a longitudinal section, again with 5 μg ml^{-1} of control riboprobe, little to no binding was again observed (Fig. 7(f)), confirming the specificity of the anti-sense probe results. The localization of PPDBR mRNA to cells of the *vc*, *pr*, and *ap* correlate exactly to previous results found for the mRNA of PLR and PCBER, both of which function to produce lignans in *F. intermedia* and *P. taeda*, respectively (Kwon et al., 2001). These results, in conjunction with those, strongly indicate that mRNAs of dihydrophenylpropanoid monomer and lignan synthesis enzymes are transcribed in the vascular cambium and differentiating cells of the radial and axial parenchyma of the developing stem. With maturity, mRNA turnover likely increases in the latter cell types, as these cells serve to transport metabolites to developing heartwood. (No other tissues were, however, examined (e.g., needles) to identify any additional patterns of gene expression.)

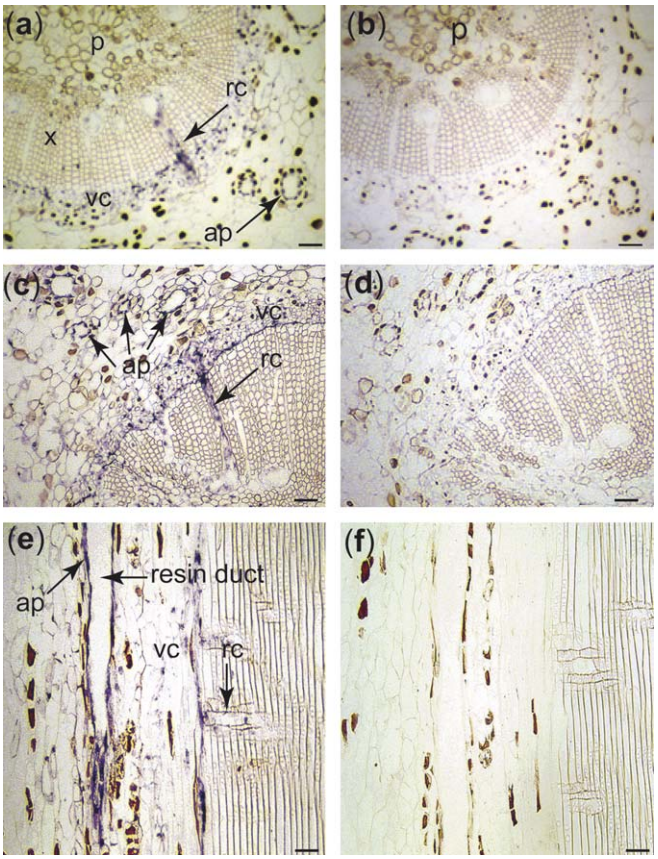


Fig. 7. PPDBR gene expression localization in *P. taeda* stems at different developmental stages. (a, b) Transversal cross-section of meristematic region; (c, d) transversal and (e,f) longitudinal cross-sections of a young stem. Sections were incubated with the antisense probe to PPDBR (a, c and e), as well as with a sense probe for negative controls (b, d and f). The PPDBR digoxigenin-labeled antisense probe was visualized using alkaline phosphatase conjugated anti-digoxigenin antibodies, with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP) as chromophoric substrates giving a purple color. Abbreviations: ap, axial parenchyma cells; p, pith; rc, radial parenchyma cells; vc, vascular cambium.

3. Concluding remarks

To our knowledge, thus far there are four other examples of somewhat comparable double-bond reduction processes reported, these occurring in both plants and bacteria, respectively. The first two were that of the enoyl acyl carrier protein reductase (ENR) from *Brassica napus* (Rafferty et al., 1995) and *E. coli* (Baldock et al., 1998) involved in lipid biosynthesis, as well as an enoate reductase (ER) present in bacteria (*Clostridium* species) (Bader et al., 1981; Rohdich et al., 2001). A third example involves lipid reduction as well, e.g. for the conversion of 4-hydroxy-(2*E*)-nonenal into 4-hydroxy-nonylaldehyde in *Arabidopsis thaliana* (Mano et al., 2002, 2005), although other substrates can also be processed (Mano et al., 2005); the fourth is that of a (+)-pulegone reductase which converts (+)-pulegone into (+)-isomenthone and (−)-menthone, with the latter predominantly being formed (Ringer et al., 2003).

Table 3
Similarity and identity of PtPPDBR to other reductases (for abbreviations see Fig. 5, CtER: *Clostridium tyrobutyricum* (GenBank Accession No. Y09960))

	Similarity (%)/identity (%)					
	PtPPDBR	AtDBR1	PulR	CtER	EcENR	BnENR
PtPPDBR	–	63/43	62/44	19/10	25/11	27/12
AtDBR1	–	–	79/63	19/9	23/12	26/14
PulR	–	–	–	20/10	25/12	26/11
CtER	–	–	–	–	17/9	21/10
EcENR	–	–	–	–	–	35/23
BnENR	–	–	–	–	–	–

Of these, ENR catalyzes the reversible double-bond reduction of enoyl moieties S-linked to the phosphopantetheine group of acyl carrier proteins (ACP), and uses NAD(P)H as a cofactor. By contrast, the enoate reductase (ER), purified from *Clostridium* bacteria, reduces a variety of allyl alcohols (e.g. *n*-butenol) to the corresponding saturated alcohols (e.g. *n*-butanol) via cooperativity with an additional alcohol dehydrogenase (Bader et al., 1981). Interestingly, while all five double-bond reductases (including PPDBR) catalyze similar reactions, the first two (ENR and ER) have little sequence similarity/identity to each other or to any of the other proteins (see Table 3 and Fig. 5) (except *Clostridium*). On the other hand, the (+)-pulegone reductase and the *Arabidopsis* double bond reductase have quite a high level of similarity/identity (79% and 63%), whereas when compared to PtPPDBR their similarity (62–63%) and identity (43–44%) were somewhat lower. Accordingly, we can provisionally suggest that ENR and ER have evolved independently from the other three, albeit with convergence of overall type of biochemical function.

From this particular study, we thus conclude that PPDBR converts α,β-unsaturated aldehydes, such as dehydrodiconiferyl aldehyde **21** and coniferyl aldehyde **25** into the corresponding dihydro derivatives **22** and **26** via a 1,4 addition reaction using NADPH as a hydride donor.

PPDBR is now the second enzyme whose catalytic activity has been identified for the 8–5′ linked lignan biosynthetic pathway, i.e., following that of phenyl coumaran benzylic ether reductase (PCBER). The discovery of these two enzymes strongly supports the view that DDC (**9**) is a common precursor of the lignans of this class, e.g. leading to the defense and heartwood related lignans. Indeed, the subcellular localization of this protein is also in agreement with this viewpoint.

At some point in the future, it will also be instructive to identify which enzyme(s) catalyze DDC (**9**) oxidation, as well as identifying those involved in DDDCAL (**22**) and dihydroconiferyl aldehyde (**26**) reduction in *P. taeda* crude extracts. If CAD is capable of catalyzing both of these dehydrogenation and reduction steps, CAD may have a much wider biological role in phenylpropanoid metabolism than previously contemplated (Lewis et al., 1998, 1999).

4. Experimental

4.1. Instrumentation

^{1}H NMR spectra were obtained on a Varian Mercury-300BB spectrometer with chemical shifts given in δ ppm relative to TMS. HPLC separations were performed on a Millennium™ (Waters Inc.) instrument with a reversed-phase (Symmetry Shield RP_8, 3.9×150 mm, Waters) or chiral (Advanced Separation Technologies Inc., Chirobiotic V, 250×4.6 mm i.d.) columns, whereas electron impact (EI) mass spectra were recorded on a Waters Integrity™ HPLC/MS system at an ionization voltage of 70 eV with a reversed-phase (Symmetry Shield RP_8, 2.5×150 mm). Matrix-assisted laser desorption ionization-time of flight mass spectrometry was performed on a VG 7070 mass spectrometer at the Laboratory for Bioanalysis and Biotechnology at Washington State University. N-terminus amino acid and DNA sequencing were carried out as described previously (Gang et al., 1999b). A Lambda 6 UV/Vis spectrophotometer (Perkin–Elmer) was employed for all protein (Bradford, 1976) and DNA (OD_{260}) determinations. A Temptronic II thermocycler (Thermolyne) was used for all PCR amplifications.

4.2. Materials

All solvents and chemicals used were either reagent or HPLC grade. *Taq* thermostable DNA polymerase was obtained from Gibco BRL Life Technologies, and Deep Vent *Taq* DNA polymerase was purchased from New England Biolabs. Competent Top 10 *E. coli* cells and TOPO TA cloning® kits were obtained from Invitrogen, whereas competent Epicurian Coli® BL21-CodonPlus™(DE3)-RIL *E. coli* (BL21RIL) cells were from Stratagene. Restriction endonucleases *Bam*HI and *Eco*RI were purchased from New England Biolabs. The pGEX-4T-1 plasmid, thrombin, Glutathione Sepharose 4B, Phenyl-Sepharose CL-4B, and Mono Q HR 5/5 were from GE Healthcare, with ADP-agarose (adenosine 2′,5′-diphosphate cross-linked 4% beaded agarose) being obtained from Sigma. Spectra/Por® dialysis membranes (MWCO 3500 and 12–14,000) were purchased from Spectrum Laboratories Inc. Microcon® YM-100 (Millipore) were used to concentrate the gel purified DNA solutions, with DNA concentrations determined by comparison to a low DNA mass ladder (Invitrogen) in 1% agarose gels. Oligonucleotide primers for polymerase chain reaction (PCR) and sequencing were synthesized by Invitrogen.

4.3. Plant materials

Pinus taeda cell suspension cultures were maintained at 25 °C in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) on a platform shaker (Lab-Line Instruments Inc., model No. 3590-3) set at 115 rpm, under continuous illumination (30–40 μmol s^{-1} m^{-2}) provided by two fluores-

cent light bulbs (40 W, Phillips Cool White) as previously described (Nose et al., 1995). The cells, transferred to a sterile aqueous solution containing 8% sucrose and 20 mM KI, were harvested by filtration after 24 h incubation, then frozen in liquid nitrogen and stored at −80 °C until needed.

4.4. General methods

All molecular biological techniques, unless expressly described below, were performed according to standard methods (Sambrook et al., 1989).

4.5. Chemical synthesis

[4*R*-^{3}H]-NADPH and [4*S*-^{3}H]-NADPH were obtained as previously described (Chu et al., 1993) by modification of the procedure by Moran et al. (1984).

E-coniferyl alcohol (**1**) (Xia et al., 2001), (±)-dehydrodiconiferyl alcohols, DDC (**9a/b**) (Gang et al., 1999b), and (±)-dihydrodehydrodiconiferyl alcohols, DDDC (**10a/b**) (Gang et al., 1999b) were synthesized as previously described. Coniferyl aldehyde (**25**) was obtained from Aldrich.

(±)-Dehydrodiconiferyl aldehydes (DDCAL) (**21a/b**): To an ice-cold solution of (±)-dehydrodiconiferyl alcohols (**9a/b**) (180 mg, 0.5 mmol) in dimethyl formamide (5 ml) was added pyridinium dichromate (PDC, 235 mg, 0.6 mmol) (Corey and Schmidt, 1979). After stirring for 5 h at 0 °C, the resulting mixture was next poured into H_2O (30 ml), then extracted with EtOAc (30 ml × 3). The combined EtOAc solubles were washed with saturated NaCl solution, dried (Na_2SO_4), and evaporated to dryness *in vacuo*. The resulting pale yellow extract was then subjected to silica gel preparative TLC, eluted with $CHCl_3$/acetone (4:1), to afford (±)-dehydrodiconiferyl aldehydes (DDCAL, **21a/b**) (25 mg, yield 14%). EIMS (70 eV) *m/z* (rel. int.) 356 $[M]^+$ (40), 338 (50), 323 (35), 151 (40), 137 (100). ^{1}H NMR δ (300 MHz, $CDCl_3$): 3.70 (1H, *m*, C_8H), 3.88 (3H, *s*, OMe), 3.94 (3H, *s*, OMe), 3.96 (2H, overlapped with OMe signal at 3.94, C_9H_2), 5.65 (1H, *d*, $J = 7.9$ Hz, C_7H), 5.70 (1H, *s*, ArOH), 6.61 (1H, *dd*, $J = 17.5$, 8.5 Hz, $C_{8'}H$), 6.90 (3H, overlapping, $C_{2,5,6}H$), 7.05 (1H, *d*, $J = 1.3$ Hz, $C_{2'}H$), 7.13 (1H, *d*, $J = 1.3$ Hz, $C_{6'}H$), 7.40 (1H, *d*, $J = 17.5$ Hz, $C_{7'}H$), 9.65 (1H, *d*, $J = 8.5$ Hz, CHO).

(±)-Dehydrodiconiferyl alcohols (DDC) (**9a/b**) and (±)-dihydrodehydrodiconiferyl alcohols (DDDC) (**10a/b**) were synthesized as described in Gang et al. (1999b). The (+)- and (−)-enantiomers of **10**, separated by chiral HPLC (see Section 4.7), had $[\alpha]_D^{25}$ values of +5.1 (MeOH, $c = 0.196$) and −9.18 (MeOH, $c = 0.244$), respectively.

(±)-Dihydrodehydrodiconiferyl aldehydes (DDDCAL) (**22a/b**). To a solution of (±)-dihydrodehydrodiconiferyl alcohol (**10a/b**) (360 mg, 1 mmol) in CH_2Cl_2 (10 ml) was added DMSO (343.8 µl, 4 mmol) and triethylamine (97.7 µl, 1.3 mmol). To this was then added pyridine sulfur trioxide (159 mg, 1 mmol), with the resulting mixture stirred for 30 min at ambient temperature, following which the whole was poured into H_2O (50 ml) (Isobe et al., 1987). The resulting aq. solution was extracted with Et_2O (30 ml × 2) with the organic solubles then washed successively with saturated NH_4Cl and $NaHCO_3$ solutions, H_2O and saturated NaCl solution. The Et_2O solubles were then dried (Na_2SO_4), and the solvent evaporated *in vacuo*. The resulting extract was subjected to silica gel preparative TLC eluted with $CHCl_3$/acetone (4:1) to afford a crude DDDCAL (**22a/b**) preparation, which was subjected to HPLC (see Section 4.7) to obtain pure (±)-dihydrodehydrodiconiferyl aldehydes (**22a/b**) (20 mg, yield 5.5%). EIMS (70 eV) *m/z* (rel. int.) 358 $[M]^+$ (35), 340 (40), 325 (33), 297 (20), 283 (22), 151 (50), 137 (100). ^{1}H NMR δ (300 MHz, $CDCl_3$): 2.74 (2H, *m*, $J = 8.3$, 1.7 Hz, $C_{8'}H$), 2.87 (2H, *t*, $J = 7.6$ Hz, $C_{7'}H$), 3.56 (1H, *q*, 8.3, 6.7 Hz, C_8H), 3.83 (3H, *s*, OMe), 3.84 (3H, *s*, OMe), 3.90 (2H, *d*, $J = 6.67$ Hz, C_9H), 5.50 (1H, *d*, $J = 8.3$ Hz, C_7H), 5.58 (1H, *s*, ArOH), 6.62 (1H, *s*, C_2H), 6.63 (1H, *s*, C_6H), 6.83 (1H, *d*, $J = 8.7$ Hz, C_5H), 6.86 (1H, *dd*, $J = 8.7$, 2 Hz, C_6H), 6.88 (1H, *d*, $J = 2$ Hz, C_2H), 9.79 (1H, *t*, $J = 1.67$ Hz, CHO).

Dihydroconiferyl aldehyde (**26**). To a solution of *E*-coniferyl alcohol (**1**) (720 mg, 4 mmol) in MeOH (10 ml) was added palladium on charcoal (10%, 100 mg), with the resulting solution stirred under H_2 for 4 h at ambient temperature. The catalyst was then removed by filtration, with the filtrate evaporated to dryness *in vacuo*. The resulting residue was dissolved in acetone, subjected to silica gel preparative TLC eluted with $CHCl_3$/acetone (4:1) to afford dihydroconiferyl alcohol (**16**, 650 mg, 90%). To purified **16** (600 mg, 3.3 mmol) in CH_2Cl_2 (5 ml) was added DMSO (3.4 ml, 36.6 mmol), triethylamine (1 ml, 13.2 mmol), and pyridine sulfur trioxide (1.58 mg, 9.9 mmol). The resulting mixture was stirred for 30 min at ambient temperature, following which the whole was poured into H_2O (50 ml). The aqueous solution was extracted with Et_2O (30 ml × 2), with the combined organic solubles washed successively with saturated NH_4Cl and $NaHCO_3$ solutions, H_2O and saturated NaCl solution. The Et_2O solubles were dried (Na_2SO_4), with the solvent evaporated to dryness *in vacuo*. The resulting extract was next subjected to silica gel preparative TLC eluted with $CHCl_3$/acetone (4:1) to afford dihydroconiferyl aldehyde (**26**) (202 mg, yield 27.5%). EIMS (70 eV) *m/z* (rel. int.) 180 $[M]^+$ (50), 137 (100). ^{1}H NMR δ (300 MHz, $CDCl_3$): 2.75 (2H, *m*, C_8H), 2.89 (2H, *m*, C_7H), 3.87 (3H, *s*, OMe), 6.66 (1H, *dd* $J = 8.7$, 2.3 Hz, C_6H), 6.70 (1H, *d*, $J = 2.3$ Hz, C_2H), 6.84 (1H, *d*, $J = 8.7$ Hz, C_5H), 9.81 (1H, *t*, $J = 1.67$ Hz, CHO).

(±)-[9,9′-^{3}H]-dehydrodiconiferyl alcohols (**9a/b**). To [9-^{3}H]-coniferyl alcohol (**1**) (1 mmol in acetone, 7 ml, 1.21 GBq mol^{-1}) (Xia et al., 2001) was added $FeCl_3 \cdot 6H_2O$ (aq. solution, 2.6 mmol, 24 ml), at room temperature. Following stirring for 10 min, the reaction mixture was extracted with Et_2O (30 ml × 3). The Et_2O solubles were combined, this being extracted with H_2O (20 ml), then dried (Na_2SO_4), and evaporated to dryness *in vacuo*. The residue was reconstituted in a minimum amount of CH_2Cl_2

and applied to a silica gel column (15 × 2.5 cm inner diameter) eluted with CH_2Cl_2/Et_2O/MeOH (1:1:0.2) to give (±)-[9,9′-^{3}H]-dehydrodiconiferyl alcohol (**9a/b**) (0.1 mmol, 2.42 GBq mol^{-1}, 20% yield).

4.6. Enzyme assays

Native PPDBR activity was assayed with crude protein extracts as follows using DDC (**9**) for illustrative purposes: Each assay consisted of (±)-DDC (**9**, 12.5 mM in MeOH, 10 μl), the enzyme preparation (50–100 μl) and buffer (50 mM Tris–HCl, pH 7.5) containing DTT (5 mM) in a final volume of 250 μl. The reaction was initiated by addition [4*R*-^{3}H]NADPH (25 mM, 6.7 kBq, 10 μl). Controls were performed using either denatured enzyme (boiled 96 °C, 10 min) or in absence of enzyme. After 3-h (Phenyl-Sepharose step) or 24-h (all other steps) incubation at 30 °C with shaking, the assays were extracted with EtOAc (500 μl) containing DDDC (**10**, 4.75 μg) as radiochemical carrier. After centrifugation (16,000*g*, 2 min), the EtOAc solubles were removed and the extraction procedure was repeated with EtOAc (500 μl) but without addition of radiochemical carrier. The EtOAc solubles were combined with an aliquot (400 μl) removed for determination of its radioactivity using liquid scintillation counting. The remainder of the combined EtOAc solubles was evaporated to dryness *in vacuo*, and then reconstituted in MeOH–H_2O (3:7, 100 μl) with an aliquot (50 μl) subjected to reversed-phase HPLC with both UV (280 nm) and radioactivity detection as described in Section 4.7. Originally, to determine the specificity of hydride transfer, assays were also carried out as described above but with [4*S*-^{3}H]NADPH as cofactor as well. (Comparable assays were carried out with DDCAL **21** to afford DDDCAL **22**.)

Recombinant PPDBR activity was assayed as above in a total volume of 250 μl but with the following modifications: substrates (12.5 mM, 10 μl) were dissolved in DMSO; Tris–HCl buffer (50 mM) was at pH 8.0 with incubation times for 1 h only; the first EtOAc extraction was done without addition of unlabeled products as radiochemical carriers.

4.7. HPLC analyses

Reversed-phase HPLC analyses were carried out on a Symmetry Shield RP_8 column eluted as follows: isocratic solvent system A:B (CH_3CN:3% HOAc in H_2O) (1:9) for the first 5 min, followed by a linear A:B gradient from 1:9 to 1:3 between 5 and 30 min at a flow rate of 1 ml min^{-1} with detection at 280 nm. Fractions (1 ml) were collected, with an aliquot (100 μl) of each removed for scintillation counting. For chiral analysis of the reaction product, enzymatically formed DDDC (**10**) was hand collected after reversed-phase HPLC separation, freeze-dried and reconstituted in MeOH (100 μl) with an aliquot (50 μl) subjected to chiral chromatography and eluates analyzed at 0.3-min intervals for radioactivity content.

Chiral HPLC analyses of (+)- and (−)-dihydrodehydrodiconiferyl alcohols (**10a** and **10b**) were carried out on a Chirobiotic V column eluted with an isocratic MeOH–NH_4NO_3 (1:9) solvent system at a flow rate of 1 ml min^{-1} and detection at 280 nm.

4.8. Extraction and purification of PPDBR

Frozen *P. taeda* cells were ground in a mortar with a pestle with the resulting powder homogenized with PVPP (5% w/w) and Tris–HCl buffer (50 mM, pH 7.5) containing DTT (5 mM) [buffer A]. The homogenate was next centrifuged (10,000*g*, 20 min) with the supernatant fractionated with $(NH_4)_2SO_4$. Proteins precipitating between 20% and 60% saturation were recovered by centrifugation (10,000*g*, 30 min), with the pellet reconstituted in a minimum amount of buffer A.

The crude extracts were applied to a Phenyl-Sepharose column (15 × 1.6 cm) equilibrated in buffer A containing $(NH_4)_2SO_4$ (1 M) at a flow rate of 2 ml min^{-1}, with the column then washed with 60 ml of buffer A. PPDBR was next eluted using a linear gradient of a decreasing $(NH_4)_2SO_4$ concentration (from 1 M to 0 M in 260 ml) with the active fractions so obtained frozen (−20 °C) until needed. Active fractions from the Phenyl-Sepharose column were combined, pooled, concentrated, dialyzed (MWCO: 12–14,000) against buffer A for 2 h, with the resulting dialysate next applied to a Mono Q HR5/5 column equilibrated in buffer A at a flow rate of 1 ml min^{-1}. The column was washed with 15 ml of buffer A, and PPDBR was eluted with a linear NaCl gradient (0–1 M in 80 ml). The active fractions were combined, dialyzed (MWCO: 12–14,000) and next applied to an ADP-agarose column (9.5 × 1.0 cm) previously equilibrated in buffer A containing EDTA (2.5 mM) [buffer B] at a flow rate of 1 ml min^{-1}. The column was then washed with 15 ml of buffer B, with PPDBR eluted using a linear gradient of NaCl in buffer B (0–0.5 M in 80 ml). A typical purification protocol is shown in Table 2.

4.9. Amino acid sequencing

The partially purified PPDBR from the MonoQ HR5/5 column was next subjected to SDS–PAGE (4–15% Ready gel, Bio-Rad) (Laemmli, 1970) to give 3 bands visualized by Coomassie blue staining. Each was excised using a razor blade, with the sections individually washed with CH_3CN–H_2O (1:1, v/v). Amino acid sequence analyses of each were performed at the Harvard Microchemistry Facility by microcapillary reversed-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer. Two of the peptides were a *P. taeda* CAD, and another was identical to a fructose-bisphosphate aldolase from *Z. mays*; these were not examined further. The third (from an ~39 kDa band) gave three peptide sequences (ELILVAYANEG-PVTDSHLNIR, DGSSGDVAVQNLWISVDPYLR, and

ESDDGLYLPSFPLNQAIR) when searching available databases (including EST sequences) (Eng et al., 1994; Chittum et al., 1998). These three amino acid sequences appeared to be a part of an amino acid partial sequence of the cDNA clone 7C5A, accession No. AA556927, of unknown catalytic function, previously obtained from a cDNA library of *P. taeda* immature xylem tissue (Allona et al., 1998).

4.10. Cloning of a cDNA encoding PPDBR

In order to obtain a full length cDNA clone encoding the putative PPDBR from a *P. taeda* cDNA library (Gang et al., 1999b), a reverse primer (PPDBR-IR1, GGAATCCAGCCCATGCA) for PCR amplification was designed on the basis of the cDNA clone 7C5A sequence. Since the cDNAs from the library were cloned in the Uni-ZAP XR insertion vector, of which the multiple cloning site is flanked by T3 and T7 RNA promoters, a T3 forward and the PPDBR-IR1 reverse primers were used to perform PCR with the following conditions: 30 cycles of 1 min at 96 °C, 1 min at 55 °C and 1 min at 72 °C. The resulting PCR product obtained (~590 bp) was then cloned into a pCR®2.1-TOPO (pCR) vector for sequencing, with the resulting pCR construct (PPDBR-NT/pCR), containing the N-terminal region of the putative PPDBR completely sequenced. A N-terminal primer (PPDBR-NT1, AGTGATTGTATGTACAATTGAGG) was next designed, and another PCR was carried out with PPDBR-NT1 and T7 forward primers giving an ~1.4 kb PCR product. This PCR was repeated 3 more times with the PCR products cloned into the pCR vector. Four clones (PPDBR/pCR1–PPDBR/pCR4) were then completely sequenced on both strands in order to verify that no mutations had been introduced during PCR. The full length cDNA clone of the target protein is shown in Fig. 5.

4.11. Transfer of PPDBR into pGEX-4T-1

A pGEX-4T-1 plasmid, containing the cDNA in frame with *Schistosoma japonicum* glutathione *S*-transferase, was employed (Kaelin et al., 1992). The insertion-end, containing the putative PPDBR from *P. taeda*, was prepared by Sticky-end PCR methods (Zeng, 1998). Four primers were designed to introduce a *Bam*HI site at the start methionine (5′-primer 1: P-GATCCATGGAGCAGAGAGTTCCAAACAGAG and 5′-primer 2: CATGGAGCAGAGAGTTC-CAAACAGAG) and an *Eco*RI site at the 3′-end untranslated region (3′-primer 3: CATCCAGAATTTATTTTGGTAGGGG and 3′-primer 4: P-AATTCATCCAGAATTTATTTTGGTAGGGG). The four primers were used for PCR with 10 ng of the PPDBR/pCR1 containing the gene encoding the *P. taeda* putative reductase. Two PCR products (~1.1 kb) were each purified, combined, denatured and re-annealed to give the insertion-end with the desired restriction enzyme sites at both ends. The resulting ~1.1 kb fragments were directly used for ligation with the pGEX-4T-1 plasmid which had previously been digested with *Bam*HI and *Eco*RI. Only the insertion-end which contains the desired restriction enzyme sites was ligated into the pGEX-4T-1 plasmid, with the resulting pGEX-4T-1 construct (PPDBR/pGEX4T1) transformed into competent Top10 cells. The PPDBR/pGEX4T1, containing the putative PPDBR, was purified, and the expression region, containing the desired cDNA, was sequenced completely on both strands.

4.12. Overexpression of P. taeda PPDBR in E. coli

The resulting PPDBR/pGEX4T1 plasmid was transformed into the competent BL21RIL cells for expression. Expression of the putative reductase was achieved by inoculating 1 l of LB broth, supplemented with 50 mg/l carbenicillin, with 4 ml of an overnight grown culture in the same medium. The cells were then allowed to grow at 37 °C with shaking at 250 rpm until a density of $OD_{600} \approx 0.5$ was reached, at which point the temperature was changed to 20 °C. Production of the reductase was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 1 mM final concentration. Cells were allowed to grow for 24 h, and were then harvested by centrifugation (3000*g*, 30 min), with the pellets stored at −80 °C until needed. After cell lysis (using a Virsonic 475 Ultrasonic Cell Disrupter with a microprobe, 30 s × 3) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM K_2HPO_4, 1.8 mM KH_2PO_4) containing 5 mM DTT, the protein was purified over glutathione-Sepharose 4B resin according to the manufacturer's instructions. The N-terminal glutathione *S*-transferase fusion portion of the protein was then removed from the required recombinant reductase by cleavage at 22 °C for 6 h with 250 units of thrombin, followed by glutathione-Sepharose 4B resin purification (280 μg). The recombinant PPDBR (30 μg) was purified to apparent homogeneity by Mono Q HR5/5 column chromatography: the column was washed with 10 ml of buffer A and PPDBR was eluted with a linear NaCl gradient (0–1 M in 180 ml).

4.13. Tissue preparation for in situ hybridization

Young stem tissue (2–5 mm^3) was collected from greenhouse-grown *P. taeda* and fixed in 10% formalin, 5% AcOH, 10% EtOH in H_2O (FAA) overnight at 4 °C. Tissues were then dehydrated in a graded EtOH/FAA series and transferred to a Fisher Histomatic Tissue Processor (model 166A) for infiltration and paraffin embedding. After incubation in paraffin at 62 °C for 7 days to allow complete infiltration, thick serial sections (12 μm) were obtained using a Spencer 820 microtome. Sections were heat-affixed to silane-coated microscope slides (Digene, Beltsville, MD) at 42 °C. Paraffin was removed from the sections by immersion in Hemo-De (Fisher) for 10 min at 25 °C two times, followed by rehydration in a graded H_2O/EtOH series.

4.14. In situ hybridization

The template for the riboprobe was constructed by subcloning a 1.15 kb fragment of the PPDBR cDNA (from bp 21 to bp 1173) into the vector PCRII-TOPO (Invitrogen). Digoxigenin-labeled (DIG) antisense and control (sense) riboprobes were generated using the (SP6/T7) DIG RNA Labeling kit (Boehringer-Mannheim/Roche) per manufacturer instructions. The probes were then fragmented to an average length of 150 bp by alkaline hydrolysis (0.1 M $NaHCO_3$, pH 10.2, at 60 °C, 50 min). Tissue sections were pretreated with protease K (2 μg ml^{-1}) in TE buffer (50 mM Tris–HCl, pH 7.4, 5 mM EDTA) at 37 °C for 30 min. After rinsing with TE buffer, the tissue sections were incubated in hybridization buffer (minus probes) containing: 0.3 M NaCl, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 × Denhardt's, 50% formamide, 0.25 mg ml^{-1} yeast tRNA (Sigma) and 10% dextran sulfate at 25 °C for 2 h. Next, tissue sections were hybridized to the appropriate probes at equal concentration (2 and 5 μg ml^{-1} hybridization buffer) and incubated at 37 °C for 12 h. Post-hybridization washes were conducted with increasing stringency from 4 × SSC to 2 × SSC 1 × SSC and 0.1 × SSC at 25 °C for 30 min each to remove non-specific binding. Hybridized riboprobes were detected using indirect immunolocalization as follows: first, sections were rinsed with TBST (10 mM Tris–HCl, pH 7.5, 10 mM NaCl, 0.3% Tween 20) then blocked with TBST containing 1% BSA, 0.1% PVP and 0.5% whole sheep serum (BBI) at 25 °C for 1 h and finally incubated in sheep-anti-DIG antibody (Roche) at 1:100 in TBST containing 1% BSA at 25 °C for 2 h. Sections, washed in TBST, were then treated with the chromogenic substrates NBT (nitroblue tetrazolium chloride, Roche) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toludine blue, Roche) in Tris (0.1 M, pH 9.5) containing 10 mM NaCl, 50 mM $MgCl_2$, and 0.24 mg ml^{-1} levamisol (Sigma) (Bochenek and Hirsch, 1990). After 30 min at 25 °C, the reaction was stopped by incubating the sections in TE buffer (pH 8.0) for 10 min. Samples were air-dried in the dark and mounted with Permount® (Fisher). Images were recorded on Kodak 64T film using an Olympus camera mounted on Olympus BH-2 light microscope.

Acknowledgements

The authors thank Dr. W.S. Lane at the Harvard Microchemistry Facility for microcapillary reversed-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) sequence analysis. Thanks are extended to Dr. V.R. Franceschi (deceased) and the Washington State University Electron Microscopy Center staff for valuable advice on microscopy techniques and access to facilities. This research was supported in part by the National Science Foundation (MCB-0417291), the United States Department of Energy (DE-FG-0397ER20259), the National Institutes of Health (GM66173), and the G. Thomas and Anita Hargrove Center for Plant Genomic Research, to whom we express our thanks.

References

Agrawal, P.K., Agrawal, S.K., Rastogi, R.P., 1980. A new neolignan and other phenolic constituents from *Cedrus deodara*. Phytochemistry 19, 1260–1261.

Allona, I., Quinn, M., Shoop, E., Swope, K., St Cyr, S., Carlis, J., Riedl, J., Retzel, E., Campbell, M.M., Sederoff, R., Whetten, R.W., 1998. Analysis of xylem formation in pine by cDNA sequencing. Proc. Natl. Acad. Sci. USA 95, 9693–9698.

Anterola, A.M., Jeon, J.-H., Davin, L.B., Lewis, N.G., 2002. Transcriptional control of monolignol biosynthesis in *Pinus taeda*: factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. J. Biol. Chem. 277, 18272–18280.

Anterola, A.M., van Rensburg, H., van Heerden, P.S., Davin, L.B., Lewis, N.G., 1999. Multi-site modulation of flux during monolignol formation in loblolly pine (*Pinus taeda*). Biochem. Biophys. Res. Commun. 261, 652–657.

Ayres, D.C., Loike, J.D., 1990. Lignans: Chemical, Biological and Clinical Properties. Cambridge University Press, Cambridge, United Kingdom.

Bader, J., Kim, M.-A., Simon, H., 1981. The reduction of allyl alcohols by *Clostridium* species is catalyzed by the combined action of alcohol dehydrogenase and enoate reductase. Hoppe-Seyler's Z. Physiol. Chem. 362, 809–820.

Baldock, C., Rafferty, J.B., Stuitje, A.R., Slabas, A.R., Rice, D.W., 1998. The X-ray structure of *Escherichia coli* enoyl reductase with bound NAD^+ at 2.1 Å resolution. J. Mol. Biol. 284, 1529–1546.

Barrero, A.F., Haïdour, A., Dorado, M.M., 1993. Sesquipinsapols A and B: two sesquilignans from *Abies pinsapo*. Nat. Prod. Lett. 2, 255–262.

Barrero, A.F., Haïdour, A., Dorado, M.M., Cuerva, J.M., 1996. Two sesquilignans from the wood of *Abies marocana*. Phytochemistry 41, 605–609.

Bochenek, B., Hirsch, A.M., 1990. In situ hybridization of nodulin mRNAs in root nodules using non-radioactive probes. Plant Mol. Biol. Report 8, 237–248.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burlat, V., Kwon, M., Davin, L.B., Lewis, N.G., 2001. Dirigent proteins and dirigent sites in lignifying tissues. Phytochemistry 57, 883–897.

Chittum, H.S., Lane, W.S., Carlson, B.A., Roller, P.P., Lung, F.-D.T., Lee, B.J., Hatfield, D.L., 1998. Rabbit β-globin is extended beyond its UGA stop codon by multiple suppressions and translational reading gaps. Biochemistry 37, 10866–10870.

Chu, A., Dinkova, A., Davin, L.B., Bedgar, D.L., Lewis, N.G., 1993. Stereospecificity of (+)-pinoresinol and (+)-lariciresinol reductases from *Forsythia intermedia*. J. Biol. Chem. 268, 27026–27033.

Corey, E.J., Schmidt, G., 1979. Useful procedures for the oxidation of alcohols involving pyridinium dichromate in aprotic media. Tetrahedron Lett. 20, 399–402.

Cutillo, F., D'Abrosca, B., DellaGreca, M., Fiorentino, A., Zarrelli, A., 2003. Lignans and neolignans from *Brassica fruticulosa*: effects on seed germination and plant growth. J. Agric. Food Chem. 51, 6165–6172.

Davin, L.B., Lewis, N.G., 2005. Dirigent phenoxy radical coupling advances and challenges. Curr. Opin. Biotech. 16, 398–406.

Davin, L.B., Wang, H.-B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362–366.

Dennis, E.S., Gerlach, W.L., Walker, J.C., Lavin, M., Peacock, W.J., 1988. Anaerobically regulated aldolase gene of maize. A chimaeric origin? J. Mol. Biol. 202, 759–767.

Dinkova-Kostova, A.T., Gang, D.R., Davin, L.B., Bedgar, D.L., Chu, A., Lewis, N.G., 1996. (+)-Pinoresinol/(+)-lariciresinol reductase from *Forsythia intermedia*: protein purification, cDNA cloning, heterologous expression and comparison to isoflavone reductase. J. Biol. Chem. 271, 29473–29482.

Eberhardt, T.L., Bernards, M.A., He, L., Davin, L.B., Wooten, J.B., Lewis, N.G., 1993. Lignification in cell suspension cultures of *Pinus taeda*. In situ characterization of a gymnosperm lignin. J. Biol. Chem. 268, 21088–21096.

Eng, J.K., McCormack, A.L., Yates III, J.R., 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J. Am. Soc., Mass Spectrom. 5, 976–989.

Fujita, M., Gang, D.R., Davin, L.B., Lewis, N.G., 1999. Recombinant pinoresinol/lariciresinol reductases from western red cedar (*Thuja plicata*) catalyze opposite enantiospecific conversions. J. Biol. Chem. 274, 618–627.

Gang, D.R., Costa, M.A., Fujita, M., Dinkova-Kostova, A.T., Wang, H.-B., Burlat, V., Martin, W., Sarkanen, S., Davin, L.B., Lewis, N.G., 1999a. Regiochemical control of monolignol radical coupling: a new paradigm for lignin and lignan biosynthesis. Chem. Biol. 6, 143–151.

Gang, D.R., Kasahara, H., Xia, Z.-Q., Vander Mijnsbrugge, K., Bauw, G., Boerjan, W., Van Montagu, M., Davin, L.B., Lewis, N.G., 1999b. Evolution of plant defense mechanisms: relationships of phenylcoumaran benzylic ether reductases to pinoresinol–aricresinol and isoflavone reductases. J. Biol. Chem. 274, 7516–7527.

Halls, S.C., Davin, L.B., Kramer, D.M., Lewis, N.G., 2004. Kinetic study of coniferyl alcohol radical binding to the (+)-pinoresinol forming dirigent protein. Biochemistry 43, 2587–2595.

Halls, S.C., Lewis, N.G., 2002. Secondary and quaternary structures of the (+)-pinoresinol forming dirigent protein. Biochemistry 41, 9455–9461.

Haruna, M., Koube, T., Ito, K., Murata, H., 1982. Balanophonin, a new neo-lignan from *Balanophora japonica* Makino. Chem. Pharm. Bull. 30, 1525–1527.

Isobe, M., Ichikawa, Y., Bai, D.L., Masaki, H., Goto, T., 1987. Synthesis of a marine polyether toxin, okadaic acid. IV. Total synthesis. Tetrahedron 43, 4767–4776.

Kaelin Jr., W.G., Krek, W., Sellers, W.R., DeCaprio, J.A., Ajchenbaum, F., Fuchs, C.S., Chittenden, T., Li, Y., Farnham, P.J., Blanar, M.A., Livingston, D.M., Flemington, E.K., 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell 70, 351–364.

Kasahara, H., Davin, L.B., Lewis, N.G., 2004. Patent. Aryl propenal double bond reductase. United States Patent No. 6,703,229. Filed March 27, 2001. Issued March 9, 2004, 30pp.

Kawamura, F., Kawai, S., Ohashi, H., 1997. Sesquilignans and lignans from *Tsuga heterophylla*. Phytochemistry 44, 1351–1357.

Kim, M.K., Jeon, J.-H., Davin, L.B., Lewis, N.G., 2002. Monolignol radical–radical coupling networks in western red cedar and *Arabidopsis* and their evolutionary implications. Phytochemistry 61, 311–322.

Kraus, C., Spiteller, G., 1997. Comparison of phenolic compounds from galls and shoots of *Picea glauca*. Phytochemistry 44, 59–67.

Kwon, M., Davin, L.B., Lewis, N.G., 2001. In situ hybridization and immunolocalization of lignan reductases in woody tissues: implications for heartwood and other forms of vascular tissue preservation. Phytochemistry 57, 899–914.

Laemmli, UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277, 680–685.

Lewis, N.G., Davin, L.B., 1999. Lignans: biosynthesis and function. In: Barton, Sir D.H.R., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 1. Elsevier, London, pp. 639–712.

Lewis, N.G., Davin, L.B., Sarkanen, S., 1998. Lignin and lignan biosynthesis: distinctions and reconciliations. In: Lewis, N.G., Sarkanen, S. (Eds.), Lignin and Lignan Biosynthesis, vol. 697. ACS Symposium Series, Washington, DC, pp. 1–27.

Lewis, N.G., Davin, L.B., Sarkanen, S., 1999. The nature and function of lignins. In: Barton, Sir D.H.R., Nakanishi, K., Meth-Cohn, O. (Eds.), Comprehensive Natural Products Chemistry, vol. 3. Elsevier, London, pp. 617–745.

Lundgren, L.N., Shen, Z., Theander, O., 1985. The constituents of conifer needles. Dilignol glycosides from *Pinus massoniana*. Lamb. Acta Chem. Scand. B39, 241–248.

MacKay, J.J., Liu, W., Whetten, R., Sederoff, R.R., O'Malley, D.M., 1995. Genetic analysis of cinnamyl alcohol dehydrogenase in loblolly pine: single gene inheritance, molecular characterization and evolution. Mol. Gen. Genet. 247, 537–545.

Mano, J.i., Belles-Boix, E., Babiychuk, E., Inzé, D., Torii, Y., Hiraoka, E., Takimoto, K., Slooten, L., Asada, K., Kushnir, S., 2005. Protection against photooxidative injury of tobacco leaves by 2-alkenal reductase. Detoxication of lipid peroxide-derived reactive carbonyls. Plant Physiol. 139, 1773–1783.

Mano, J.i., Torii, Y., Hayashi, S.-i., Takimoto, K., Matsui, K., Nakamura, K., Inzé, D., Babiychuk, E., Kushnir, S., Asada, K., 2002. The NADPH:quinone oxidoreductase P1-ζ-crystallin in *Arabidopsis* catalyzes the α,β-hydrogenation of 2-alkenals: detoxication of the lipid peroxide-derived reactive aldehydes. Plant Cell Physiol. 43, 1445–1455.

Min, T., Kasahara, H., Bedgar, D.L., Youn, B., Lawrence, P.K., Gang, D.R., Halls, S.C., Park, H., Hilsenbeck, J.L., Davin, L.B., Lewis, N.G., Kang, C.H., 2003. Crystal structures of pinoresinol–lariciresinol and phenylcoumaran benzylic ether reductases and their relationship to isoflavone reductases. J. Biol. Chem. 278, 50714–50723.

Moinuddin, S.G.A., Youn, B., Bedgar, D.L., Costa, M.A., Helms, G.L., Kang, C.-H., Davin, L.B., Lewis, N.G., 2006. Secoisolariciresinol dehydrogenase: mode of catalysis and stereospecificity of hydride transfer in *Podophyllum peltatum*. Org. Biomol. Chem. 4, 808–816.

Moran, R.G., Sartori, P., Reich, V., 1984. A rapid and convenient preparation of [4-^{3}H]NADP and stereospecifically tritiated NADP^{3}H. Anal. Biochem. 138, 196–204.

Nose, M., Bernards, M.A., Furlan, M., Zajicek, J., Eberhardt, T.L., Lewis, N.G., 1995. Towards the specification of consecutive steps in macromolecular lignin assembly. Phytochemistry 39, 71–79.

Ozawa, S., Davin, L.B., Lewis, N.G., 1993. Formation of (−)-arctigenin in *Forsythia intermedia*. Phytochemistry 32, 643–652.

Pan, H., Lundgren, L.N., 1995. Phenolic extractives from root bark of *Picea abies*. Phytochemistry 39, 1423–1428.

Rafferty, J.B., Simon, J.W., Baldock, C., Artymiuk, P.J., Baker, P.J., Stuitje, A.R., Slabas, A.R., Rice, D.W., 1995. Common themes in redox chemistry emerge from the X-ray structure of oilseed rape (*Brassica napus*) enoyl acyl carrier protein reductase. Structure 3, 927–938.

Ralph, J., MacKay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W., Sederoff, R.R., 1997. Abnormal lignin in a loblolly pine mutant. Science 277, 235–239.

Ringer, K.L., McConkey, M.E., Davis, E.M., Rushing, G.W., Croteau, R., 2003. Monoterpene double-bond reductases of the (−)-menthol biosynthetic pathway: isolation and characterization of cDNAs encoding (−)-isopiperitenone reductase and (+)-pulegone reductase of peppermint. Arch. Biochem. Biophys. 418, 80–92.

Rohdich, F., Wiese, A., Feicht, R., Simon, H., Bacher, A., 2001. Enoate reductases of *Clostridia*. Cloning, sequencing, and expression. J. Biol. Chem. 276, 5779–5787.

Sakakibara, A., Sasaya, T., Miki, K., Takahashi, K., 1987. Lignans and Brauns' lignans from softwoods. Holzforschung 41, 1–11.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Xia, Z.-Q., Costa, M.A., Pélissier, H.C., Davin, L.B., Lewis, N.G., 2001. Secoisolariciresinol dehydrogenase purification, cloning and functional expression: implications for human health protection. J. Biol. Chem. 276, 12614–12628.

Xia, Z.-Q., Costa, M.A., Procter, J., Davin, L.B., Lewis, N.G., 2000. Dirigent-mediated podophyllotoxin biosynthesis in *Linum flavum* and *Podophyllum peltatum*. Phytochemistry 55, 537–549.

Yoshikawa, M., Morikawa, T., Xu, F., Ando, S., Matsuda, H., 2003. (7*R*,8*S*) and (7*S*,8*R*) 8–5′ linked neolignans from Egyptian herbal medicine *Anastatica hierochuntica* and inhibitory activities of lignans on nitric oxide production. Heterocycles 60, 1787–1792.

Zeng, G., 1998. Sticky-end PCR: new method for subcloning. Biotechniques 25, 206–208.