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Pinoresinol–lariciresinol reductases with different stereospecificity from *Linum album* and *Linum usitatissimum*

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Abstract

Recently it was found that cell cultures and plants of *Linum* species contain lignans of various chemical structures. The stereochemistry of these compounds differ among species. Cell cultures of *L. album* accumulate (−)-podophyllotoxin together with pure (−)-secoisolariciresinol. The presence of both enantiomers of the precursor pinoresinol indicates that in *L. album* cell cultures the reactions from pinoresinol to secoisolariciresinol are the first steps determining enantiospecificity in biosynthesis of podophyllotoxin. Seeds of *L. usitatissimum* contain almost enantiomerically pure (+)-secoisolariciresinoldiglucosid derived from (+)-secoisolariciresinol. A cell culture of this species contains a mixture of both enantiomers of pinoresinol and pure (+)-secoisolariciresinol. In order to get more insight into the mechanism of (−)- and (+)-secoisolariciresinol biosynthesis, respectively, we isolated a cDNA encoding pinoresinol–lariciresinol reductase (PLR) from *L. album*. The heterologously expressed PLR-La1 converts only (+)-pinoresinol into (−)-secoisolariciresinol. In contrast, the heterologously expressed PLR from *L. usitatissimum* converts only (−)-pinoresinol to (+)-secoisolariciresinol confirming the results from others. Comparison of all available PLR protein sequences resulted in a few amino acids which may be responsible for the action of the PLRs with respect to the different enantioselectivity. A mutagenesis approach could not confirm this hypothesis. Aspects about the evolution of PLRs are discussed.

Keywords: Linum album; Linum usitatissimum; Linaceae; Evolution; Stereochemistry; Lignans; Podophyllotoxin; Secoisolariciresinol; Pinoresinol–lariciresinol reductase

1. Introduction

Lignan biosynthesis was studied intensively during the last 15 years. Thus, in 1990 Umezawa et al. published the first example for the formation of an optically pure lignan from the achiral precursor coniferyl alcohol (**1**) with cell-free extracts from *Forsythia intermedia*. Davin et al. (1990) could show that already the coupling of two molecules of **1** gave enantiomerically pure (+)-pinoresinol (**2**) (Fig. 1). They isolated and cloned a cDNA encoding a "dirigent" protein which is involved in this coupling without any catalytic activity by itself (Davin et al., 1997; Gang et al., 1999a; Davin and Lewis, 2003). **2** is reduced via lariciresinol (**3**) to secoisolariciresinol (**4**) by the pinoresinol–lariciresinol reductase (PLR). Dinkova-Kostova et al. (1996) cloned a cDNA encoding this enzyme from *F. intermedia* (*PLR-Fi1*). The heterologously expressed protein is specific for (+)-**2** and forms enantiomerically pure (+)-**3** and (−)-**4**, the same enantiomers as in *Forsythia* spec. plants (Fig.

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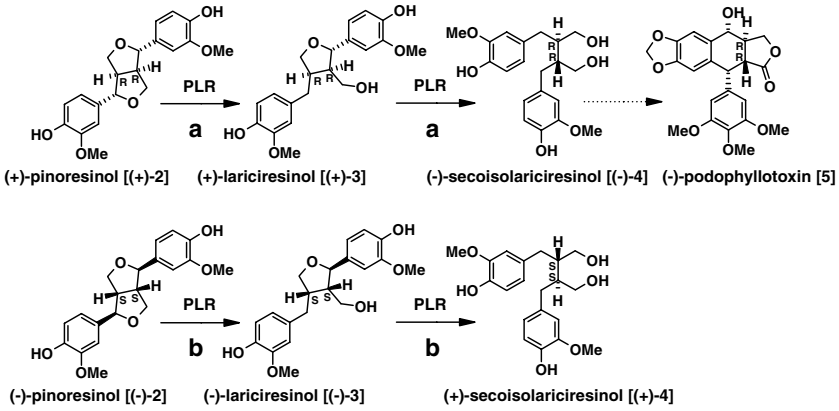

Fig. 1. Comparison of the reductions catalysed by PLRs from *Linum album* (PLR-La1), *Forsythia intermedia* (PLR-Fi1) or *Thuja plicata* 2 (PLR-Tp2) (a) and from *Linum usitatissimum* (PLR-Lu1) or *Thuja plicata* 1 (PLR-Tp1) (b).

1). The presence of cDNAs corresponding to two stereochemically distinct PLRs in a single plant species, *Thuja plicata*, has been demonstrated by Fujita et al. (1999). Four cDNAs with high homologies to the PLR from *F. intermedia* were grouped two (1 and 3) by two (2 and 4). Heterologously expressed PLR-Tp1 reduces (+)-**2** to (−)-**4**, PLR-Tp2 reduces (−)-**2** to (+)-**4**. The authors did not determine the relationship between the expression of the different PLRs and the enantiomeric composition of lignans in *T. plicata*. However, in other plant species not only the sign of optical rotation of particular lignans but also the enantiomeric composition can vary (Umezawa et al., 1997). The stereochemistry of lignans can also vary in different parts of a plant. Petioles of *Arctium lappa* accumulate mainly (+)-**4**, whereas seeds of the same plant species accumulate predominantly lignans with opposite stereochemistry (i.e., (−)-matairesinol, (−)-arctigenin and (−)-**4**) (Suzuki et al., 2002). Enzyme preparations of petioles catalyze the formation of mainly the (+)-enantiomers of **2**–**4**, extracts from seeds catalyze the formation of an enantiomeric excess of the lignans with opposite optical rotation. The authors explain these results by the presence of two PLR isoforms in *A. lappa* which show different enantioselectivity and are expressed differentially. Unfortunately, no PLR cDNAs were cloned from *A. lappa* to prove this assumption. Seeds of *Linum usitatissimum* contain almost (97–99%) pure [(+)-secoisolariciresinoldiglucoside derived from] (+)-**4**, the opposite enantiomer of the one found in *Forsythia* (Fig. 1) (Ford et al., 2001; Sicilia et al., 2003). A heterologously expressed cDNA encoding PLR-Lu converts (−)-**2** into (−)-**3** and seems to be inhibited by (+)-**2** (Davin and Lewis, 2003). No detailed data are shown, a manuscript is in preparation (Teoh et al.). In summary, only a few PLRs have been cloned and their function proven by heterologous expression so far. Only in one case, *F. intermedia*, one can find detailed descriptions of a cDNA encoding PLR which was cloned, heterogously expressed and the function of the protein discussed in comparison to the accumulation and enantiomeric composition of the lignans in the plant. The isolation of PLR encoding sequences from a cell suspension culture of *Linum album* which accumulates podophyllotoxin (**5**) (Fuss, 2003) and of a cell culture from *L. usitatissimum* is reported. The PLRs were expressed in *Escherichia coli* and their function characterized with respect to their enantiospecificity. The enantiospecificity of the proteins is compared with the enantiomeric composition of lignans found in the cell cultures of this plant species. Aspects of the evolutionary origin of different enantiospecificity of PLRs are discussed.

2. Results and discussion

2.1. Lignans in cell suspension cultures of *L. usitatissimum* and *L. album*

L. usitatissimum seeds contain almost enantiomerically pure (+)-secoisolariciresinoldiglucosid derived from (+)-**4** (Ford et al., 2001; Sicilia et al., 2003). The enantiomeric composition of **2** and **4** isolated from cell suspension cultures of this species was determined by chiral column HPLC (Fig. 2). Since **2** was a mixture of both enantiomers and **4** consists of only the (+)-enantiomer, the enantiomeric purity of the lignans is reached in the reactions catalysed by the PLR in *L. usitatissimum* cell cultures.

L. album cell suspension cultures accumulate as main lignans podophyllotoxin (**5**) and/or 6-methoxypodophyllotoxin. **2**, **3**, matairesinol, deoxypodophyllotoxin, β-peltatin and α-peltatin could be detected in smaller amounts (Smollny et al., 1998; Seidel et al., 2002; Fuss, 2003). The specific optical rotation $[\alpha]_D^{20}$ of **5** isolated from the suspension culture was determined as −95° and found to be in agreement with the literature value of $[\alpha]_D^{14}$ − 101° for **5** from *Podophyllum* species (Buckingham et al., 2004). Therefore, **5** in *L. album* suspension cultures consists of the (−)-enantiomer (Fig. 1). Since

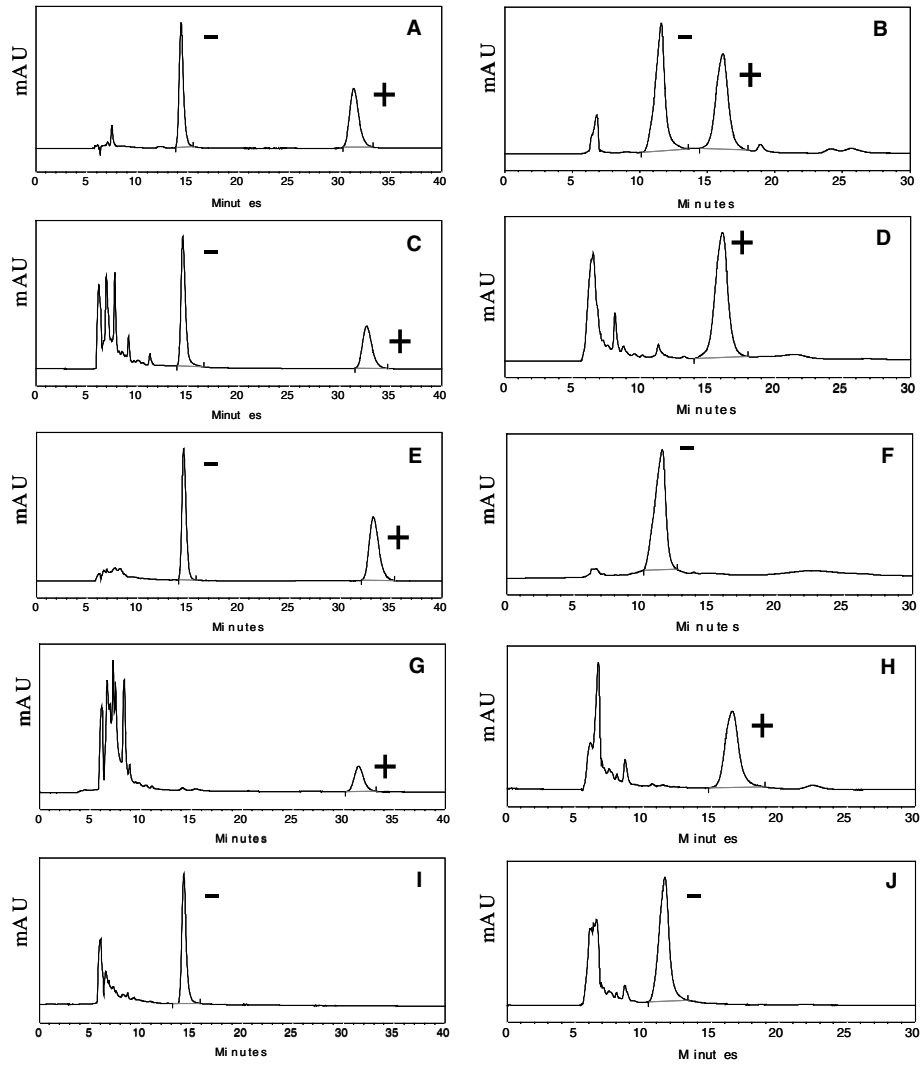

Fig. 2. Chiral column HPLC analysis of A: racemic pinoresinol (**2**) [ratio of (+):(−) = 50:50]; B: racemic secoisolariciresinol (**4**) [ratio of (+):(−) = 50:50]; C: **2** isolated from extracts of *L. usitatissimum* cell cultures [ratio of (+):(−) = 40:60]; D: **4** isolated from extracts of *L. usitatissimum* cell cultures [100% (+)]; E: **2** isolated from extracts of *L. album* cell cultures [ratio of (+):(−) = 52:48]; F: **4** isolated from extracts of *L. album* cell cultures [100% (−)]; G: **2** obtained following reduction of racemic **2** by *L. usitatissimum* PLR [100% (+)]; H: **4** obtained following reduction of racemic **2** by *L. usitatissimum* PLR [100% (+)]; I: **2** obtained following reduction of racemic **2** by *L. album* PLR [100% (−)]; J: **4** obtained following reduction of racemic **2** by *L. album* PLR [100% (−)].

2 from suspension cultures consists of a mixture of both enantiomers and **4** of the cultures consists of only the (−)-enantiomer, the enantiomeric purity of the lignans is reached in the reactions catalysed by the PLR in *L. album* cell cultures like in *L. usitatissimum* cell culture (Fig. 2). But, as expected, in the *L. album* cell culture **4** shows the opposite enantiomeric composition to **4** from *L. usitatissimum* cell cultures (Fig. 1).

2.2. Cloning of PLR encoding sequences from L. album and L. usitatissimum

We cloned a full length cDNA with 1482 bp encoding PLR from *L. album*, *PLR-La1* (Accession No. AJ849358). The translational start of the ORF is at nucleotide 105, the TAG stop at nucleotide 1083 encoding a polypeptide of 326 amino acids (Fig. 3). At the amino acid level the PLR-La1 shows highest similarities to the PLR of *F. intermedia* (75% identity and 85% similarity). Based on the high gene sequence similarity PLRs are phylogenetically linked to isoflavone reductases (IFRs) and phenylcoumaran benzylic ether reductases (PCBERs) (Gang et al., 1999a,b). PLR-La1 shows lower similarities to these enzymes (e.g., IFR of *Betula pendula*, 47% identity and 63% similarity and PCBER of *Pinus taeda*, 47% identity and 66% similarity) indicating that it really functions as a PLR. Next, genomic DNA was digested to completion with either *Eco*RI,

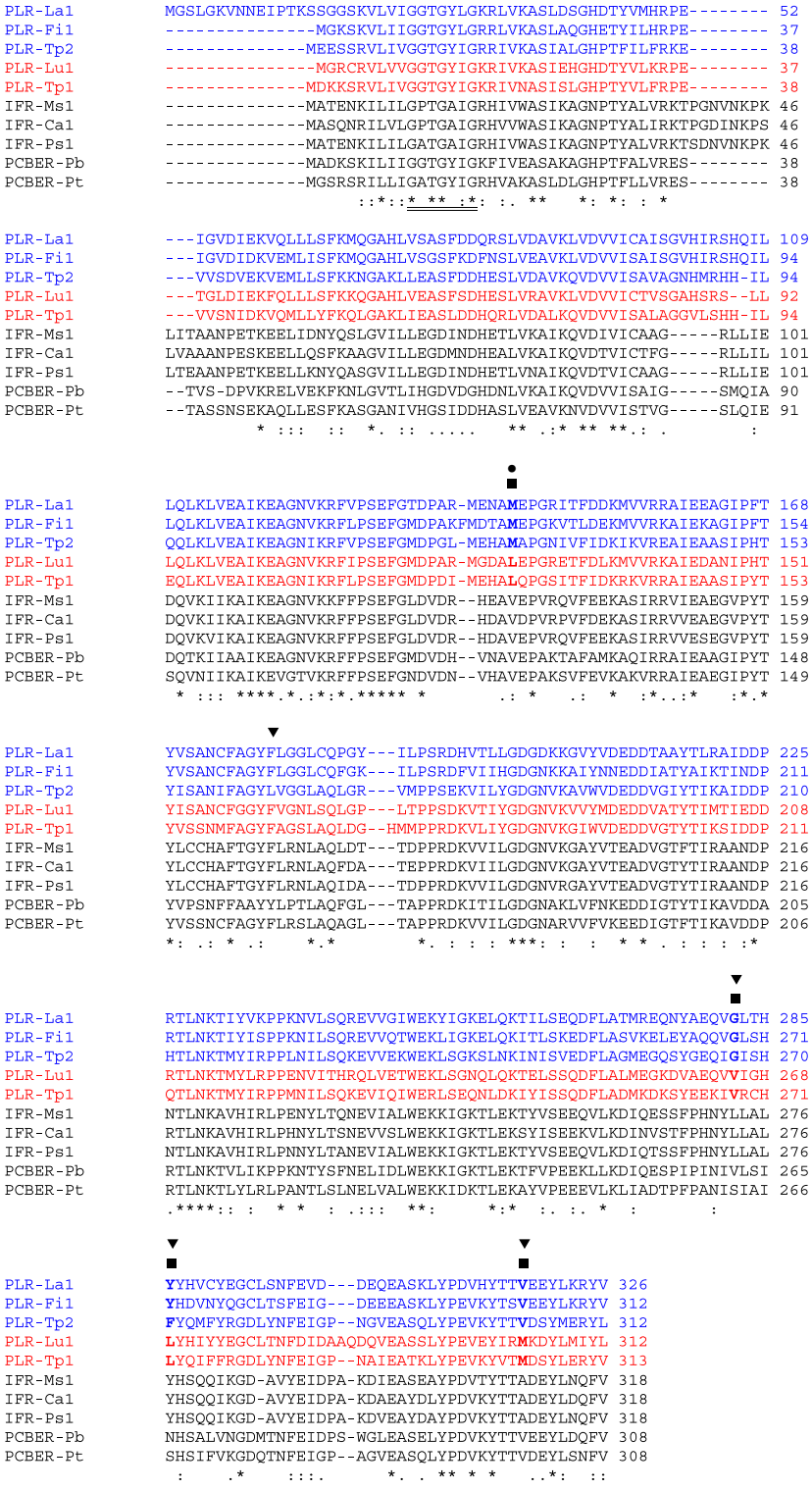

Fig. 3. Amino acid sequence alignment between PLRs with specificity to form (−)-**4** (blue) from *Linum album* (PLR-La1, Accession No. AJ849358), *Forsythia intermedia* (PLR-Fi1, Accession No. U81158) and *Thuja plicata* (PLR-Tp2, Accession No. AF242504) and PLRs with specificity to form (+)-**4** (red) from *Linum usitatissimum* (PLR-Lu1, Accession No. AJ849359) and *Thuja plicata* (PLR-Tp1, Accession No. AF242503) and (black) IFRs from *Medicago sativa* (IFR-Ms1, Accession No. U17436), *Cicer arietum* (IFR-Ca1, Accession No. Q00016) and *Pisum sativum* (IFR-Ps1, Accession No. S72472) and PCBERs from *Populus trichocarpa* (PCBER-Pb, Accession No. AJ132262) and *Pinus taeda* (PCBER-Pt, Accession No. AF242490). The conserved sequence "GxxGxxG" of the NADPH binding domain is double underlined. Black squares and bold letters indicate amino acid positions with differently conserved amino acids in PLRs with different stereochemistry. Black dots indicate amino acid positions which are located only near to the catalytic center of PLRs according to Min et al. (2003). Black triangles indicate amino acid position which are discussed to be involved in stereospecificity by Min et al. (2003).

*Eco*RV, *Hind*III, *Xba*I or *Bgl*II, which do not cut in the cDNA, and subjected to Southern blot analysis (Fig. 4). The ORF of *PLR-La1* was used as probe. For each restriction digest one strong band, for digestion with *Eco*RI two bands were observed indicating that *PLR-La1* is a single copy gene in the *L. album* genome. Screening the databases for sequences encoding PLR from *L. usitatissimum* yielded one ORF (Accession No. AX191955, Lewis et al., 2001). Whether this sequence was expressed to show that PLR-Lu converts (−)-**2** to (−)-**3** is not described (Davin and Lewis, 2003). Amplification of this ORF with gene specific primers and cDNA of *L. usitatissimum* cell suspension cells as template gave an ORF (PLR-Lu1, Accession No. AJ849359) which encodes a protein of 312 amino acids. PLR-Lu1 shows thirteen nucleotide exchanges some responsible for four amino acid substitutions in comparison to PLR-Lu. The amino acid sequence of PLR-Lu1 shows high similarities to the PLR of *L. album* (66% identity and 75% similarity). It shows lower similarities to IFRs (e.g., *B. pendula*, 43% identity and 63% similarity) and PCBERs (e.g., *P. taeda*, 47% identity and 66% similarity). Genomic DNA of the *L. usitatissumum* cell suspension was digested to completion with either *Eco*RV, *Eco*RI, *Xba*I and *Bgl*II which do not cut in the cDNA and *Hind*III which cuts app. 80 bp before the 3′-end of the cDNA (Fig. 4). The ORF of *PLR-Lu1* was used as probe. For each restriction digest at least three bands were observed indicating that *PLR-Lu1* belongs to a small gene family in the genome of *L. usitatissimum*. Since both enantiomers of **4** are detectable in the seeds of this species the genome should contain at least two PLR genes encoding PLRs of opposite stereospecificity.

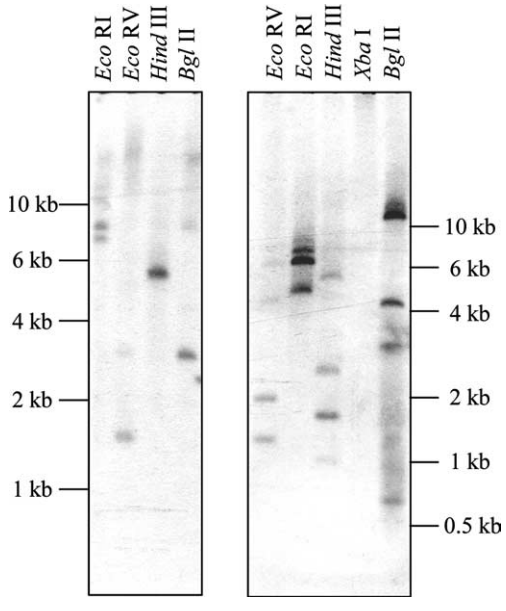

Fig. 4. Southern hybridisation of *L. album* and *L. usitatissimum* genomic DNA. DNA was digested with *Eco*RI, *Eco*RV, *Hind*III, *Bgl*II and only gDNA of *L. usitatissimum* with *Xba*I. The resulting membranes were probed with the open reading frames of *PLR-La1* (A) and *PLR-Lu1* (B), respectively.

2.3. Functional expression of L. album and L. usitatissimum PLRs in E. coli

The ORFs of *PLR-La1* and *PLR-Lu1* were cloned in an expression vector for a heterologous expression as N-terminal His-fusion proteins in *E. coli*. The formation of **3** as well as **4** by using racemic **2** as substrate was observed for both fusion proteins. No activity was observed in extracts from cells containing the expression vector lacking an insert. To determine the enantiospecificity of the two PLRs racemic **2** was used as substrate and the assay carried out at optimal reaction conditions (30 °C, pH 7.1) for 4 h. The remaining **2** and the formed **4** in the assay with the PLR-La1 fusion protein consist of the pure (−)-enantiomers. After incubation with PLR-Lu1 fusion protein the (+)-enantiomers of **2** and **4** were detected indicating the same enantiospecificity as previously observed for PLR-Lu (Davin and Lewis, 2003). PLR of *L. album* shows the opposite enantiospecificity than PLR from *L. usitatissimum*. PLR-La1 converts (+)-**3** into **4**, but, as expected, (+)-**3** it is not a substrate for PLR-Lu1, confirming the opposite enantiospecificity of these two PLRs. Different protein concentrations (0, 0.3, 1, 2–20 μg in steps of 2 μg) of PLR-La1 and 20 min incubation with racemic **2** as substrate yielded **2**–**4** (Fig. 5). Protein concentrations as low as 6 μg/100 μl assay only formed **3** while the amount of **2** decreased until half of **2** was used. Then, no more **2** was converted and the concentration of **4** increased, while the amount of **3** decreased. Similar results were observed for depletion of **2** and product formation after 0, 5, 10, 20, 30, 50 and 90 min (data not shown). These results lead to an interesting reaction mechanism for PLR-

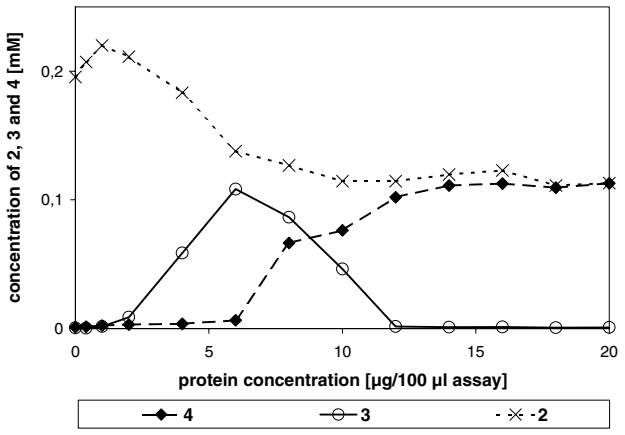

Fig. 5. Turnover of pinoresinol (**2**) and formation of lariciresinol (**3**) and secoisolariciresinol (**4**), following incubation of racemic **2** with different amounts of heterologously expressed and purified PLR from *L. album*.

La1: First **3** is formed from (+)-**2** until no (+)-**2** is left. Then (−)-**4** can be formed from **3**. In the case of *F. intermedia* formation of **4** was only retarded and occurred already when (+)-**2** was still in the reaction mixture (Katajama et al., 1993). PLRs from *T. plicata* were shown to produce the same ratios of **3** and **4** from **2** during the whole time course of 0 to 240 min (Fujita et al., 1999). Thus, PLR-La1 works already enantiospecifically in the first reaction step from **2** to **3**, since with higher protein concentrations and with prolonged reaction times we could never monitor the depletion of more than 50% of the racemic **2**, **3** was completely converted to enantiomerically pure (−)-**4**, and the remaining **2** was enantiomerically pure (−)-**2**. *F. intermedia* PLR (Katajama et al., 1993) reduced **2**–**3** with high enantiospecific preference (>99:1), the step from **3** to **4** was strictly enantiospecific in favor for the (−)-**4**. PLRs from *T. plicata* work only strictly enantiospecifically at the level of formation of **4** from **3** (Fujita et al., 1999).

2.4. Sequence homology comparison

PLRs, IFRs and PCBERs form a class of reductases with significant homology (Gang et al., 1999b). In all sequences the NADPH binding motif "GxxGxxG" at the amino terminus is conserved (Fig. 3). Insertions of amino acids in comparison to PCBERs may be specific for the other classes of reductases, namely in IFRs, position 38–48, and in PLRs, position 84–87. Recently, the crystal structures of PLR-Tp1 and PCBER-Pt were elucidated and the structure of IFR-Ms was modelled (Min et al., 2003). Min et al. found that the PLR specific insert is involved in building the substrate binding pocket supporting the importance of that sequence in PLR specific activity. In four positions in PLRs which produce only (−)-**4** (e.g., PLR-Tp2, PLR-La1) one amino acid is highly conserved whereas in PLRs which produce only (+)-**4** (e.g., PLR-Tp1, PLR-Lu1) another amino acid is conserved. These amino acids are: Met^{128}, Gly^{267}, Phe^{271} and Val^{304} in PLR-Tp2 instead of Leu^{128}, Val^{265}, Leu^{269} and Met^{304} in PLR-Tp1. In PLT-Tp1 Val^{265} and Leu^{269} are in the binding pocket for the substrate and also suggested by Min et al. as involved in enantiospecificity. Leu^{128} and Met^{304} are at the borders of the pocket. Additionally, Min et al. suggested the position Phe^{164} as possibly involved in enantiospecificity, but in our sequence comparisons also PLRs with the opposite specificity have a Phe at the comparable position. PLR-La1 was altered by site-directed mutagenesis to change its enantiospecificity. Primers were designed such that Gly^{282} was changed to Val and Tyr^{286} to Leu. In a second mutant these amino acids were changed, but in addition, Met^{143} was changed to Leu and Val^{318} to Met (Fig. 3). The heterologously expressed proteins show in first experiments the same enantiospecificity as wild type PLR-La1 indicating that the mutated positions are not sufficient to determine enantiospecificity.

A cladogram of the PLRs, IFRs and PCBERs based on sequence similarity at the amino acid level shows three clusters (Fig. 6). Cluster 1 consists of the two subclusters formed by PCBERs and IFRs. PLRs of *T. plicata* group together in cluster 2. Cluster 3 consists of PLRs from angiosperms: one from *F. intermedia*, the two others from *L. album* and *L. usitatissimum*. Dendograms developed by Fujita et al. (1999) and Gang et al. (1999b) showed three clusters, too. Cluster 1 is formed by PLRs, cluster 2 by IFRs and cluster 3 by PCBERs. PLRs from *T. plicata* with different enantiospecificity group closer together than the angiosperm PLRs which also show within themselves different enantiospecificities. This was already observed by Fujita et al. (1999) having only the published PLR sequences of *Thuja* and *Forsythia* in hand. They concluded: "The clustering of the western red cedar reductases may be due to the facts that these enzymes are from the same species and that most of the differences in the sequences between the various pinoresinol–lariciresinol reductases (PLR-Tp1, PLR-Tp2, PLR-Tp3, PLR-Tp4, PLR-Fi1 and PLRH-La1 from *Lupinus albus*) are not important for substrate enantiospecificity. It is also possible that the higher similarity between the western red cedar reductases may have some significance regarding their ability to reduce both enantiomers of pinoresinol, given that they are less enantiospecific than the *F. intermedia* reductase." The broadened cladogram (Fig. 6)

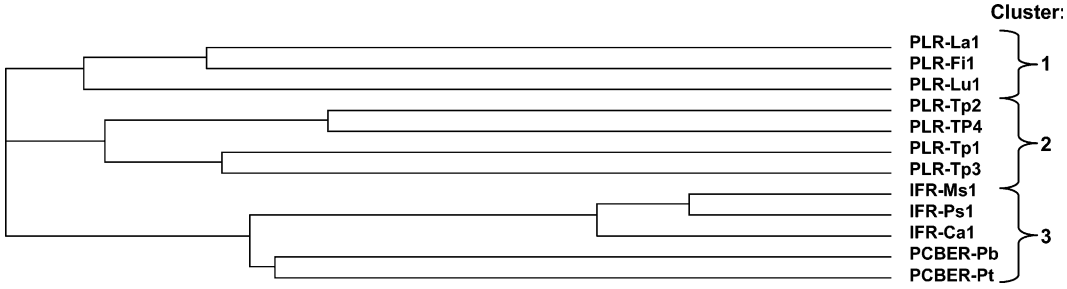

Fig. 6. Cladogram showing relative similarities between PLRs, IFRs and PCBERs from different species (PLR-Tp4, Accession No. AF242506, PLR-Tp3, Accession No. AF242505, for further abbreviations and accession numbers refer to Fig. 3).

implicates that enantiospecificity on different reaction level dos *not* lead to the clustering of *Thuja* PLRs. Enantiospecificity was either developed several times in evolution or already determined very early and then PLRs with already different enantiospecificity evolved further keeping the enantiospecificity and exchanging only amino acids not essential for it.

3. Concluding remarks

Within this study we cloned PLRs from a *L. album* suspension culture, specific for the conversion of (+)-**2** to (−)-**4**. A second PLR specific for the conversion of (−)-**2** to (+)-**4** was cloned from a cell suspension culture of *L. usitatissimum*, which is different from another already cloned PLR from *L. usitatissimum* with the same enantiospecificity (Teoh et al., manuscript in preparation). There is still a PLR from *L. usitatissimum* with opposite enantiospecificity missing because Southern blots indicated the presence of several PLRs in the genome of this species and seeds contain beside (+)-**4** very small amounts of (−)-**4**. In further experiments, we will try to clone this PLR. Comparison between the sequences of all known PLRs as well as IFRs and PCBERs resulted in signatures which occur only in PLRs or IFRs and are therefore discussed as responsible for the different substrate specificities. Four amino acids may be sufficient to determine the enantiospecificity of PLRs. But, PLR-La1 with amino acids of PLR-Lu1 at these positions still shows the enantiospecificity of PLR-La1. A cladogram of PLRs, IFRs and PCBERs shows that these reductases are related to each other and form distinct groups. PLRs with the same enantiospecificity do not group together showing that during evolution either different enantiospecificity evolved several times or was reached at an early stage. Amino acids responsible for the enantiospecificity were kept while other amino acids not important for the specificity could be exchanged. In further experiments we will clone more PLR sequences from other *Linum* species to compare the evolution of PLRs with the evolution of this genus in general.

4. Experimental

4.1. Materials

L. album cell suspension cultures were established and subcultivated according to Smollny et al. (1998). Cell suspension cultures from *L. usitatissimum*, line number PC-1017, were purchased from the DMSZ (Braunschweig, Germany) and maintained in the manner of cultivation of *L. album* suspensions.

All solvents and chemicals were of reagent or HPLC grade unless otherwise specified. **1** was purchased from Sigma. NADPH was from Biomol. Racemic **2** was synthesised according to Xia et al. (2001). (+)-**2** was a gift from N.G. Lewis (Washington State University, USA). (−)-**4** and racemic **4** were gifts from M. Metzler (Universität Karlsruhe, Germany), (+)-**3** was synthesised according to Eklund et al. (2002). The following material was purchased from following companies, as indicated in brackets: RNeasy® Plant Mini, QIAprep Spin Miniprep Kit, PCR Purification Kit and Ni-NTA agarose (Qiagen); Nick translation Kit (Invitrogen); pGEM-T vector system (Promega); 1st strand cDNA synthesis Kit for RT-PCR (AMV), Expand HiFi polymerase and Nylon membranes (Roche); pET15b vector and BL21(DE3)pLysS cells (Novagen); Oligonucleotides (Invitrogen or MWG-Biotech [Germany]); ZAP Express™-cDNA-Synthesis Kit (Stratagene) Sequencing reactions were done at MWG-Biotech (Germany).

4.2. Instrumentation

cDNA synthesis and PCR amplifications used a T-gradient thermal cycler (Biometra). HPLC analyses were carried out by using an HPLC-PDA system from Thermoquest that consists of a degasser, autosampler Spectra Systems AS1000, photodiode array detector Spectra Systems UV6000LP and a pump Spectra Systems P2000. HPLC columns used were Hypersil HyPurity™ Elite C18 (Thermoquest), GROM-Sil 120 ODS-5 ST (Grom Company) and Chiralcel OD-H (Daicel).

4.3. Sequence analysis

DNA and amino acid sequence analyses were performed using the Wisconsin package version of the Genetics Computer Group (GCG, Madison, USA). Comparisons to various PLR, IFR and PCBER sequences were carried out by a NCBI BLAST search. Sequence alignments and preparation of a cladogram were performed by using the ClustalW program on SRS server (EMBL, Heidelberg).

4.4. cDNA library construction and cloning of L. album PLR cDNA

A cDNA library was constructed according to the instruction manual of ZAP Express™-cDNA-Synthesis Kit using four to seven-day-old cell suspension cultures from *L. album* line X4 which produce podophyllotoxin as main lignan (Seidel et al., 2002).

RNA was extracted from flower buds of *F. intermedia* collected in the Botanical Garden of the University of Düsseldorf by using the RNeasy® Plant Mini Kit. RNA was reversely transcribed by AMV cDNA Synthesis Kit using 6 μg for each transcription and oligo-dT for

priming. To amplify a part of the open reading frame of the PLR of *F. intermedia* the following components were used: 2 μl of the cDNA synthesis per 50 μl reaction, 50 pmol of each gene specific primer (PLRFI202F: 5′-CAAGGATTTCAACAGTCTGGTCGAGG-3′ and PLRFI799R: 5′-GGCTTAATCCCACTTGCTGAGCATAC-3′, based on the sequence, Accession No. U81158), 1.5 mM $MgCl_2$, 0.75 mM dNTPs and 1 μl Taq Polymerase. PCR conditions were: 94 °C, 4 min; denaturation 94 °C 1.5 min; annealing 57 °C 2 min; 72 °C 1 min 20 s in 34 cycles; final extension 72 °C 3 min. The PCR product was ligated into pGEM-T, cloned into *E. coli* DH5α and sequenced to confirm its identity. After reamplification as described before but with plasmid DNA as template, the product was purified by gel electrophoresis, [α^{32}P]dATP-labelled (nick translation Kit, Invitrogen) and used to screen app. 400,000 pfu of the *L. album* cDNA library. Hybridisation temperature was 55 °C for the three rounds of screening. cDNA inserts from positive clones were excised and cloned by using the ExAssist helper phage with XLOLR strain as described by the manufacturer. Four clones were selected for sequencing. Three clones contained PLR-like sequences of different length but with identical sequences. The longest clone was fully sequenced. About 50 ng of this plasmid DNA (pEF21K2) served as template to amplify the open reading frame by using HIFI-Taq polymerase according to Roche with 50 pmol of the primers PLR-LA1I105F: 5′-GGAATTCCATATGGGTTCCCTGGGGAAAGTG-3′ and PLR-LA1I1085R: 5′-CCGCTCGAGCTAGACGTAACGCTTTAGATATTCC-3′, introducing a *Nde*I site at the start codon and a *Xho*I site at the stop codon. The resulting PCR product was ligated into pET15b, cloned into *E. coli* DH5α and fully sequenced (pKS2K9).

4.5. Cloning of PLR open reading frame of *L. usitatissimum*

RNA was extracted from a 5 days old cell suspension culture of *L. usitatissimum* by using the RNeasy® Plant Mini Kit. RNA was reversely transcribed by AMV cDNA Synthesis Kit using 5 μg for each transcription and oligo-dT for priming. To amplify the open reading frame of the PLR of *L. usitatissimum* 10 μl of the cDNA synthesis per 100 μl reaction, 100 pmol of each gene specific primer (LUSPLR-ATG-F: 5′-GGAATTCCATATGGGGCGGTGCAGAGTTCT-3′ and LUSPLR-TGA-R: 5′-CGCGGATCCTCAAAGGTAGATCATCAGATAATC-3′, based on the sequence, Accession No. AX191955) which introduce a *Nde*I recognition site at the ATG start codon and a *Bam* HI site at the TGA stop codon, 1.5 mM $MgCl_2$, 0.25 mM dNTPs and 0.75 μl HIFI-Taq Polymerase (Roche) were used. PCR conditions were: 94 °C, 3 min; denaturation 94 °C 30 s; annealing 59 °C 30 s; 72 °C 1 min in 39 cycles; final extension 72 °C 3 min. The PCR product was ligated into pET15b via *Nde*I and *Bam*HI, cloned into *E. coli* DH5α and sequenced (pCH25K2).

4.6. Heterologous expression of PLRs from L. album and L. usitatissimum

Purified plasmid DNA from pKS2K9 and pCH25K2 was transformed into BL21(DE3)pLysS cells. Transformed cells were grown at 37 °C with shaking (200 rpm) to log phase (A_{600} = 0.8–1.0) in LB medium supplemented with 100 μg/ml ampicillin. After growth of the cells at 28 °C for 0.5 h, IPTG (isopropyl β-D-thiogalucopyranoside) was added to a final concentration of 1 mM, and the cells allowed to grow for 5 h at 28 °C. Cells were harvested by centrifugation and frozen at −20 °C. After thawing at room temperature the cells were resuspended in extraction buffer (20 mM Tris/HCl pH 7.2, 2 mM EDTA, 5 mM DTT, 1 mM PMSF and 5 μg leupeptin; 5 ml/200 ml culture) and lysed by sonication (8 × 30 s) (Sonifier B12, Branson Sonic, USA). The supernatant of a centrifugation (13.000 rpm, 60 min) was desalted over a PD10 column according to the instructions of Pharmacia by using the following buffer: 5 mM imidazol, 20 mM Tris pH 8.5, 5 mM mercaptoethanol, 100 mM NaCl. 6 ml of the eluted protein were mixed with 2.5 ml Ni-NTA solution and shaked at 4 °C with 200 rpm for 1 h. The suspension was filled into an empty PD10 column and washed with 8 ml of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazol, pH8.0 [NaOH]). The His-tagged PLR protein was eluted in 7 steps with 0.8 ml elution buffer each (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol, pH 8.0 [NaOH]). Fractions containing His-tagged PLR-La1 or PLR-Lu1 were pooled, desalted by using a PD10 column with extraction buffer (without protease inhibitors) and used for PLR activity assays. All protein concentrations were determined according to Bradford (1976) with BSA as standard.

4.7. PLR assays and HPLC analysis

The following procedure modified according to Fujita et al. (1999) was used when not otherwise stated. The standard assay mixture (500 μl) consisted of K-Pi buffer (0.1 M, pH 7.1) 300 μg purified His tagged PLR-La1 protein or 150 μg purified His tagged PLR-Lu1 protein, 0.2 mM racemic **2** and 2.5 mM NADPH. Protein, buffer and **2** were pre-incubated for 15 min at 30 °C prior to assay initiation by addition of NADPH. After 30 min, assays were stopped by addition of 500 μl EtOAc and mixing. The assays were extracted three times in total with EtOAc (3 × 500 μl). The combined EtOAc solubles were dried under vacuum. The residue was dissolved in 100 μl MeOH and subjected to HPLC analysis on a Hypersil HyPurity™ Elite C18 column

at a flow rate of 1.1 ml/min with an elution system employing water containing 0.01% (v/v) phosphoric acid (when peaks were collected pure water was used) (A) and acetonitrile (B). The following gradient was used: 20.5% B for the first 35 min., 20.5–65% B in 10 min, hold 65% B for 2 min, 65–20.5% B in 4 min, hold 20.5% B for 4 min. The reaction products **3** and **4** and the remaining **2** were identified by using authentic standards for **2**–**4** for comparison of retention time and UV spectra. At least duplicate assays were conducted with racemic **2** as substrate. For the determination of the enantiomeric composition of **2** and **4** these compounds were collected from reversed phase HPLC, purified by extraction with EtOAc and analysed by chiral column HPLC (Chiracel OD-H) (modified according to Umezawa et al., 1990, 1994). The solvents used were ethanol (A) and *n*-hexane (B) at a flow rate of 0.5 ml/min. For the separation of the enantiomers of **2** a gradient was used: 50–40% B in 45 min. Separation of the enantiomers of **4** was achieved within 35 min with 75% B. HPLC detection wavelength was 280 nm. Identification of the substances was done by comparison of retention time and UV-spectra to authentic standards.

4.8. Lignan extraction and HPLC analysis

Lignan extraction was modified according to Wichers et al. (1990). About 0.2 g of powdered freeze-dried cells were suspended in 2 ml MeOH and incubated in an ultrasonic bath for 2 times 30 s with 30 s break on ice. After addition of 8 ml bidistilled water the pH was adjusted to 5.0 by the addition of diluted *o*-phosphoric acid. After adding 1 mg β-glucosidase (from almonds, $\geqslant$1000 u/mg, Roth, Germany) and incubation at 35 °C for 1 h, the samples were extracted 3 times with 8 ml EtOAc in each step. The combined EtOAc phases were dried and the residue resolved in an appropriate volume of MeOH for HPLC analysis on a Hypersil HyPurity^{TM} Elite C18 column at a flow rate of 1.1 ml/min with an elution system employing water containing 0.01% (v/v) phosphoric acid (when peaks were collected pure water was used) (A) and 100% acetonitrile (B). The following gradient was used: hold 20.5% B for the first 33 min, 20.5–35% B in 17 min, 35–60% B in 10 min, 65–20.5% B in 5 min, hold 20.5% B for 5 min, for the quantification of **2** and **4**. Determination of the enantiomeric composition of **2** and **4** was done as described above. PTOX was collected by HPLC separation on a GROM-Sil 120 ODS-5 ST column with an elution system employing water (A) and 100% acetonitrile (B). The gradient system used was: at 0 min −40% B, flow rate 0.8 ml/min; at 17 min −67% B, flow rate 1 ml/min; at 18 min −40% B, flow rate 1 ml/min; at 24 min −40% B, flow rate 0.8 ml/min. **5** was detected at 290 nm.

4.9. Southern analysis

Genomic DNA was isolated from three-day-old *L. album* or *L. usitatissimum* cell suspension cultures according to Doyle and Doyle (1990), digested with the restriction endonucleases *Eco*RI, *Eco*RV, *Bgl*II, *Hind*III and for gDNA of *L. usitatissimum* also *Xba*I and fractionated on 0.8% agarose gels. Southern alkali blotting, prehybridisation and hybridisation were carried out according to the user manual from Amersham Life Science, Hybond^{TM}-N+; positively charged nylon membrane, Version 2.0 (1992). The last step of washing was done with 0.2× SSPE instead of 0.1 × SSPE. Autoradiography of the blots against X-ray films (Roche, Germany) was performed subsequently at −70 °C with the use of intensifying screens.

4.10. Site-directed mutagenesis

Site directed mutagenesis was performed according to Landt et al. (1990) with purified DNA of pEF21K2 as template. Beside the oligonucleotides LAPLRI105F and LAPLRI1085R the following oligonucleotides were used for mutagenesis and cloning into pET15b as already described above: LAPLR949u960mF (5′-GCAAGTTGTACTGACGCACCTTTATCACGT-3′), LAPLRr1056GinACi (5′-GCGCTCGAGCTAGACG-TAACGCTTTAGATATTCCTCCATGGTGGTGTA-G-3′), LAPLR531mF (5′-GGAAAATGCGTTGGAG-CCAG-3′). Clones with correct sequences were used for expression in *E. coli* and determination of enantiospecificity as described above.

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