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Characterization of *Arabidopsis thaliana* Pinoresinol Reductase, a New Type of Enzyme Involved in Lignan Biosynthesis*

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A lignan, lariciresinol, was isolated from *Arabidopsis thaliana*, the most widely used model plant in plant bioscience sectors, for the first time. In the *A. thaliana* genome database, there are two genes (At1g32100 and At4g13660) that are annotated as pinoresinol/lariciresinol reductase (PLR). The recombinant AtPLRs showed strict substrate preference toward pinoresinol but only weak or no activity toward lariciresinol, which is in sharp contrast to conventional PLRs of other plants that can reduce both pinoresinol and lariciresinol efficiently to lariciresinol and secoisolariciresinol, respectively. Therefore, we renamed AtPLRs as *A. thaliana* pinoresinol reductases (AtPrRs). The recombinant AtPrR2 encoded by At4g13660 reduced only (−)-pinoresinol to (−)-lariciresinol and not (+)-pinoresinol in the presence of NADPH. This enantiomeric selectivity accords with that of other PLRs of other plants so far reported, which can reduce one of the enantiomers selectively, whatever the preferential enantiomer. In sharp contrast, AtPrR1 encoded by At1g32100 reduced both (+)- and (−)-pinoresinols to (+)- and (−)-lariciresinols efficiently with comparative k_{cat}/K_m values. Analysis of lignans and spatiotemporal expression of *AtPrR1* and *AtPrR2* in their functionally deficient *A. thaliana* mutants and wild type indicated that both genes are involved in lariciresinol biosynthesis. In addition, the analysis of the enantiomeric compositions of lariciresinol isolated from the mutants and wild type showed that PrRs together with a dirigent protein(s) are involved in the enantiomeric control in lignan biosynthesis. Furthermore, it was demonstrated conclusively for the first time that differential expression of PrR isoforms that have distinct selectivities of substrate enantiomers can determine enantiomeric compositions of the product, lariciresinol.

Lignans are phenylpropanoid dimers in which the monomers are linked by the central carbon (C8) atoms (1, 2) and are distributed widely in the plant kingdom (1, 3). Biosynthesis of the compounds has been receiving widespread interest, because they have various important features. First, biosynthetic reactions of lignans involve unique stereochemical properties. For example, the initial step of lignan biosynthesis is an enantioselective radical coupling reaction (23), which is of great interest in the field of bioorganic chemistry and may help to provide a model for biomimetic chemistry. Second, lignans have various clinically important biological activities. Some lignans are used in medicines and nutritional supplements, such as the podophyllotoxin-derived semisynthetic lignans used in cancer therapies (4) and sesamin used in health and nutrition (5). Mechanisms for the control of the gene expression of their biosynthesis are helpful for their biotechnological production. Third, lignans and related compounds, such as norlignans, are often biosynthesized and deposited in significant amounts in the heartwood region of trees as a metabolic event of heartwood formation. Because the metabolic event is specific to trees, biosynthesis of lignans and norlignans can be a clue in helping to elucidate heartwood formation mechanisms.

Lignans can be classified into three categories, lignans with 9(9′)-oxygen, lignans without 9(9′)-oxygen, and dicarboxylic acid lignans (1). Of the three categories, the study of the biosynthesis of lignans with 9(9′)-oxygen is the most advanced (1, 6, 23). This type of lignan is formed by the enantioselective dimerization of two coniferyl alcohol units with the aid of dirigent protein (DP)[2] to give rise to pinoresinol (furofuran). Pinoresinol is then reduced by pinoresinol/lariciresinol reductase (PLR) via lariciresinol (furan) to secoisolariciresinol (dibenzylbutane), which is in turn oxidized to afford matairesinol (dibenzylbutyrolactone) by secoisolariciresinol dehydrogenase (SIRD) (Fig. 1). The conversion from coniferyl alcohol to matairesinol is believed to be the general biosynthetic pathway for lignans with 9(9′)-oxygen (1, 6, 23).

Although DP is an asymmetric inducer, its enantiomeric control is not strong enough to give rise to optically pure pinoresinol. Thus, the enantiomeric composition of pinoresinol from various plant species varies largely (1, 6, 7). In contrast, dibenzylbutyrolactone lignans, such as matairesinol, are optically pure (1, 6, 7). These facts unequivocally indicate that not only was the first step mediated by DP, but the subsequent metabolic steps catalyzed by PLR and SIRD were also involved in determining the enantiomeric composition of the lignans (8). It was also suggested that a variation in the enantiomeric composition of the upstream lignans (lariciresinol, secoisolariciresinol, and matairesinol) among different plant species may be ascribed, at least in part, to the selectivities of PLR and SIRD

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[2] The abbreviations used are: DP, dirigent protein; PLR, pinoresinol/lariciresinol reductase; SIRD, secoisolariciresinol dehydrogenase; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; RT, reverse transcription; MeOH, methyl alcohol; AcOEt, ethyl acetate; qRT, quantitative real time; RT, reverse transcription; PrR, pinoresinol reductase; e.e., enantiomer excess.

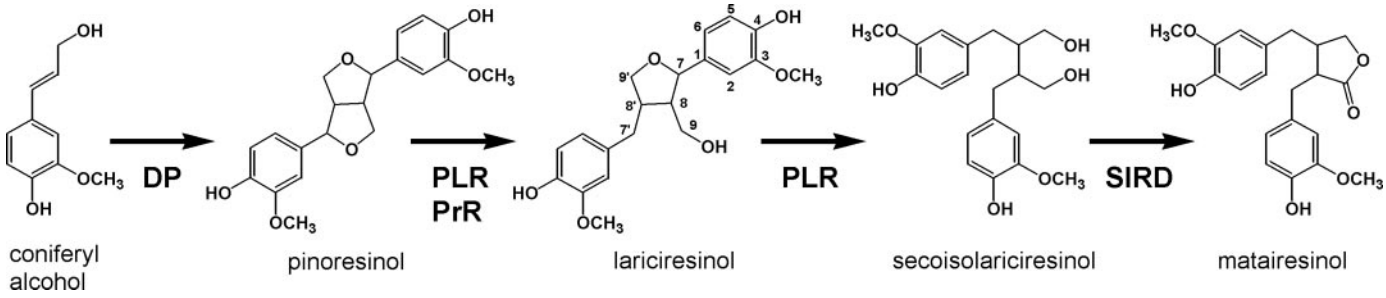

FIGURE 1. **The lignan biosynthetic pathway.**

isoforms in terms of substrate enantiomers as well as their spatiotemporal expression patterns (8). However, this view has not yet been proven to be related to gene expression.

Herein, we demonstrate that *Arabidopsis thaliana* PLRs showed strict substrate preference toward pinoresinol but only weak or no activity toward lariciresinol, unlike conventional PLRs that can reduce both pinoresinol and lariciresinol efficiently. Therefore, we will refer to AtPLRs as *A. thaliana* pinoresinol reductases (AtPrRs). It was also demonstrated that the enantiomeric composition of lariciresinol in *A. thaliana* can be controlled by the differential expression of two *AtPrR*s by using biochemical characterization of their recombinant enzymes and spatiotemporal expression analysis of the genes.

EXPERIMENTAL PROCEDURES

Instruments and Chromatography—^{1}H NMR spectra were obtained with a JNM-LA400MK FT-NMR system (JEOL). Chemical shifts and coupling constants (*J*) were expressed as δ values and in Hz, respectively. Gas chromatography-mass spectrometry (GC-MS) measurement of enzyme assay products was performed on a JMS-DX303HF mass spectrometer (JEOL) equipped with a Hewlett-Packard 5890J gas chromatograph and a JMA-DA5000 mass data system (electron impact mode, 70 eV; gas chromatographic column, Shimadzu HiCap CBP-10 M25-025 (5 m × 0.22 mm); temperature, 40 °C at $t = 0$–2 min and then to 230 °C at 30 °C min^{-1}; carrier gas, helium; splitless injection). Quantifications of lariciresinol in the roots of *A. thaliana* wild type and mutants were done with a GC-MS QP5050A mass spectrometer (Shimadzu) equipped with a Shimadzu GC-17A gas chromatograph (electron impact mode, 70 eV; gas chromatographic column, Shimadzu HiCap CBP-10 M25-025 (20 m × 0.22 mm); temperature, 40 °C at $t = 0$–2 min and then to 230 °C at 30 °C min^{-1}; carrier gas, helium; splitless injection).

Reverse-phase high performance liquid chromatography (HPLC) of lariciresinol and pinoresinol and chiral HPLC analyses of pinoresinol were done as previously described (9). Chiral liquid chromatography-mass spectrometry (LC-MS) of lariciresinol was done as follows. The LC-MS system consisted of a Shimadzu LC-10AD HPLC series liquid chromatograph and a Shimadzu LC-MS-2010A single quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization interface and a Q-array-Octapole-Quadrupole mass analyzer. Shimadzu LCMS Solution Version 3.0 was used for data acquisition and processing. LC separation was achieved using a Chiralcel OC column (250 × 2.0 mm, Daicel Chemical Co.) maintained at 40 °C. The mobile phase used for the analysis consisted of EtOH/*n*-hexane (80:20). The mobile phase was delivered at a flow rate of 0.13 ml/min. The following MS parameters were selected: curved desolvation line temperature, 200 °C; block temperature, 200 °C; probe temperature, 400 °C; and probe voltage, −4.0 kV. Curved desolvation line voltage and Q-array voltage were according to default values by autotuning. Nitrogen served as the nebulizer gas (flow rate, 2.5 liters/min) and curtain gas (pressure, 0.02 megapascals). The MS acquisition was operated in selected ion monitoring mode for negative ions at a dwell time of 1.5 s. The molecular ion $[M - H]^-$ of lariciresinol at *m*/*z* 359 and $[M - H]^-$ of (±)-[9,9,9′,9′-$^2H_4$]lariciresinol as an internal standard at *m*/*z* 363 were monitored.

Silica gel column chromatography employed Kieselgel 60 (230–400 mesh; Merck). Silica gel TLC employed Kieselgel 60 F254 (20 × 20 cm, 0.25 mm; Merck).

Chemicals—(±)-Pinoresinols (10), (±)-[9,9,9′,9′-$^2H_4$]pinoresinols (9), and (±)-[9,9,9′,9′-$^2H_4$]lariciresinols (9) were prepared previously. (+)-Pinoresinol and (−)-pinoresinol were obtained by chiral HPLC separation of (±)-pinoresinols.

Plant Material—Seeds of wild-type and mutants of *A. thaliana* (ecotype Columbia) were surface-sterilized with 5% (v/v) NaOCl and sown on a 0.8% (w/v) agar solidified medium supplemented with Murashige and Skoog salt, 1% (w/v) sucrose, 0.5 g/liter 2-morpholinoethanesulfonic acid (pH 5.8), 100 mg/liter *myo*-inositol, 1 mg/liter thiamine hydrochloride, and 0.5 mg/liter nicotinic acid. Seeds on the medium in rectangular plastic plates were incubated at 4 °C for 2–3 days and then placed at 22 °C under a 16-h light/8-h dark regime for about 4 weeks. The plates were placed vertically, so that the roots grew down along the surface of the solid medium.

The seeds of the mutant lines (SALK_018045 and SALK_058467 for At1g32100 and SALK_123621 for At4g13660) in the Salk Institute Genomic Analysis Laboratory T-DNA insertion collection were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus). Verification of the *A. thaliana* T-DNA mutants was done according to Kai *et al.* (11). Briefly, genomic DNA was extracted from leaves, according to the method reported by Liu *et al.* (12). T-DNA insertions were confirmed by DNA amplification with the left T-DNA border-specific primer LBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′) and the gene-specific primers as follows: AtPrR1-R (5′-GTCGACCTCGAGTCAGAGGAACATTCTTAAGTAATC-3′) for SALK_018045, AtPrR1-F (5′-GGA-

TCCCATATGGGAGAGAGCAAACGCACCGAG-3′) for SALK_058467, and AtPrR2-F (5′-CGTTTCTTACCATCAGAATTTG-3′) for SALK_123621. The absence of the wild-type amplification product with the gene-specific primers, AtPrR1-F and AtPrR1-R for SALK_018045 and SALK_058467, and AtPrR2-F and AtPrR2-R (5′-TCTAGACTCGAGTTATACGAAAATTTTCAAATATTCATCC-3′) for SALK_123621, confirmed the homozygous nature of the mutants. The positions of the T-DNA insertion were confirmed by the sequencing of PCR products amplified with the T-DNA-specific primer LBb1 and the gene-specific primers.

To confirm the absence of functional transcripts, reverse transcription (RT)-PCR was carried out using gene-specific primers as follows: AtPrR1-F and AtPrR1-R2 (5′-CATCCATGCGTTTGTACTTGACGTC-3′) for the SALK_018045 and SALK_058467, and AtPrR2-F2 (5′-GAATTCCATATGAAAGAGACTAATTTTGGCGAG-3′) and AtPrR2-R for the SALK_123621. Nucleotide sequences of the primers of *Actin2* (At3g18780) amplification were as follows: act2-F (5′-GTGAAGGCTGGATTTGCAGGA-3′) and act2-R (5′-AACCACCGATCCAGACACTGT-3′).

A double mutant was obtained by crossing single mutants of SALK_058467 and SALK_123621 and screening the F2 population by PCR using the primers described above.

Isolation of Lignans—Freeze-dried roots of *A. thaliana* (dry weight 2.3070 g) were disintegrated with scissors and then extracted with 45 ml of hot methyl alcohol (MeOH). The MeOH extract was treated with β-glucosidase (from almonds (Sigma); 218 units in 43.5 ml of NaOAc buffer at pH 5.0) for 24 h at 37 °C. The reaction mixture was extracted three times with 43.5 ml of ethyl acetate (AcOEt), and the solvent was evaporated off. The AcOEt extracts (21.5 mg) obtained were purified by a combination of silica gel column chromatography and reverse-phase HPLC to afford pure lariciresinol. Lariciresinol: ^{1}H NMR: δ_H (C_6D_6): 2.23–2.31 (1*H*, m, C_8H), 2.44 (1*H*, dd, *J* 10.6, *J* 13.3, $C_{7'}$H), 2.64–2.73 (1*H*, m, $C_{8'}$H), 2.83 (1*H*, dd, *J* 4.9, *J* 13.6, $C_{7'}$H), 3.15 (3*H*, s, OCH_3), 3.21 (3*H*, s, OCH_3), 3.44–3.58 (2*H*, m, C_9H × 2), 3.85 (1*H*, dd, *J* 6.8, *J* 8.2, $C_{9'}$H), 4.10 (1*H*, dd, *J* 6.5, *J* 8.4, $C_{9'}$H), 4.90 (1*H*, d, *J* 6.0, C_7H), 6.49 (1*H*, d, *J* 1.7, aromatic H), 6.60 (1*H*, dd, *J* 1.7, *J* 8.0. aromatic H), 6.86 (1*H*, dd, *J* 1.7, *J* 8.0, aromatic H), 6.97 (1*H*, d, *J* 1.7, aromatic H), 7.02 (1*H*, d, *J* 8.0, aromatic H), and 7.11 (1*H*, d, *J* 8.0, aromatic H).

In separate experiments, roots of the mutants (SALK_018045, dry weight 34.1 mg; SALK_058467, dry weight 75.6 mg; and SALK_123621, dry weight 70.2 mg) were freeze-dried, disintegrated with scissors, and then extracted with hot MeOH and treated with β-glucosidase as described above. The extracts obtained were purified by a combination of TLC and reverse-phase HPLC to afford pure lariciresinol.

Lariciresinol contents in roots of wild-type and mutants were determined by GC-MS as previously reported (13). Briefly, freeze-dried roots were disintegrated with scissors and then extracted with hot MeOH. A total of 5 mg of the MeOH extract containing 5 μg of (±)-[9,9,9′,9′-$^2H_4$]lariciresinols as internal standards was treated with β-glucosidase (7.4 units in 0.5 ml of NaOAc buffer at pH 5.0) for 24 h at 37 °C. The reaction mixture was extracted with AcOEt (0.5 ml × 3), and the solvent was evaporated off. The AcOEt extracts obtained were analyzed by GC-MS.

The enantiomeric compositions of lariciresinol isolated from wild type and the mutants were determined by chiral LC-MS analysis using deuterium-labeled (±)-[9,9,9′,9′-$^2H_4$]lariciresinols as internal standards.

Isolation of pinoresinol from the double mutant (SALK_058467 × SALK_123621) was carried out as for lariciresinol. Then the pinoresinol obtained was submitted to chiral LC-MS analysis to determine its enantiomeric composition as follows. Pinoresinol isolated from the double mutant was mixed with (±)-[9,9,9′,9′-$^2H_4$]pinoresinols and submitted for chiral HPLC separation. The fractions corresponding to the (+)- and (−)-enantiomers were recovered individually and subjected to GC-MS analysis after trimethylsilylation. The relative amounts of (+)-pinoresinol and (−)-pinoresinol were determined by comparison of the molecular ion peak intensities with those of the corresponding deuterium labeled standards.

The pinoresinol content in the double mutant was determined by GC-MS using (±)-[9,9,9′,9′-$^2H_4$]pinoresinols as the internal standards as in the lariciresinol quantification.

Preparation of Recombinant Enzymes Encoded by At1g32100 and At4g13660—The plasmid containing the At1g32100 open reading frame was obtained from the *Arabidopsis* Biological Resource Center (Ohio State University). The At4g13660 open reading frame was cloned by PCR using *A. thaliana* cDNA prepared from their roots, Primestar HS DNA polymerase (TAKARA), and the gene-specific primers: AtPrR2-CF (5′-TCATATGAAAGAGACTAATTTTGG-3′) and AtPrR2-CR (5′-TGCGGCCGCTACGAAAATTTTCAAATATTC-3′). The authenticity of the cloned At4g13660 open reading frame was confirmed by sequencing. The open reading frames of At1g32100 and At4g13660 were subcloned individually into an *Escherichia coli* expression vector, pET23 (Novagen). The At1g32100 and At4g13660 constructs were transformed into *E. coli* BL21 (DE3) (Novagen). The induction and expression of recombinant enzymes were conducted according to Li *et al.* (14). After harvesting by centrifugation (2000 × *g* for 10 min), the cell pellet was processed for affinity purification using the His·Bind Resin affinity purification system (Novagen) according to the manufacturer's protocol. Protein concentrations were determined using the Bradford method (15) with bovine serum albumin as a standard protein.

Enzyme Assay of Recombinant Enzymes—PLR activity was assayed according to Fujita *et al.* (16) with slight modifications. The standard assay mixture (125 μl) consisted of Tris-HCl buffer (50 mM, pH 7.5), recombinant enzyme (4.9–10.4 μg), 400 μM pinoresinol, and 0.8 mM NADPH. The reaction was performed at 30 °C for 1, 2, and 4 h. The reaction was stopped by the addition of 500 μl of AcOEt containing internal standards, (±)-[9,9,9′,9′-$^2H_4$]lariciresinols. The AcOEt extracts were dried and subjected to GC-MS analysis. For determination of the enantiomeric composition of enzyme assay products, separate enzyme assays were conducted as described above but scaled up proportionately 3–10 times with 4 h of incubation. Lariciresinol fractions of these enzyme assay products were collected by reverse-phase HPLC, freeze dried, and analyzed by chiral LC-MS.

For determination of V_{max} and K_m values, (+)-pinoresinol (0.64–6.4 μM) and (−)-pinoresinol (0.43–14.1 μM) were used as substrates. Protein (1.5 or 1.6 μg), buffer, and substrate were preincubated for 2 min at 30 °C prior to assay initiation by adding NADPH. The reaction was performed at 30 °C for 5 or 20 min. V_{max} and K_m values were determined from Lineweaver-Burk plots, and k_{cat} was determined by dividing V_{max} by the enzyme concentration.

Quantitative Real Time (qRT)-PCR Analysis of At1g32100 and At4g13660—Total RNAs were extracted from the root and stem of wild-type *A. thaliana* using the RNeasy Plant minikit (Qiagen). An aliquot of the RNA extract was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and random hexamers, according to the manufacturer's protocol. The qRT-PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems) in a total reaction volume of 25 μl containing 1 μl of cDNAs, 200 nM gene-specific primers, and 12.5 μl of 2× SYBR Green Universal Master Mix (Applied Biosystems), with a preliminary step of 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR primers used were as follows: At1g32100, forward primer, 5′-AGAGCAAACGCACCGAGAA-3′, reverse primer, 5′-CCCTTACTATCCTCTTCCCTATGTATCC-3′; At4g13660, forward primer, 5′-GAAAGAGACTAATTTTGGCGAGAAA-3′, reverse primer, 5′-CAATCCTCCTCCCCAATGATC-3′. To check the specificity of the annealing of the oligonucleotides, the dissociation kinetics was performed by the machine at the end of the experiment. For quantification, standard curves were generated using each respective recombinant plasmid containing the full-length sequences. The 18 S ribosomal RNA was analyzed as an internal standard to normalize the transcript abundance in each sample. For the analysis of 18 S ribosomal RNA, the reaction volume was 25 μl containing 1 μl of cDNAs, 900 nM gene-specific primers, 250 nM TaqMan probe, and 25 μl of 2× TaqMan Universal Master Mix (Applied Biosystems), with a preliminary step of 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Phylogenetic Analysis of At1g32100 and At4g13660—Amino acid sequence alignment and the neighbor-joining tree were generated using the ClustalX program (17). The resulting dendrograms were visualized using the TREEVIEW program (18).

RESULTS

Analysis of Lignans in A. thaliana—GC-MS analysis of the MeOH extracts from the wild-type *A. thaliana* root indicated the presence of lariciresinol, whereas the other lignans, such as pinoresinol, secoisolariciresinol, and matairesinol, were not detected. On the other hand, none of these lignans were detected in the aerial part. Lariciresinol was isolated from the root, and its identity was confirmed by comparison of the ^{1}H NMR spectrum with those of the authentic sample. Chiral LC-MS analysis showed that the enantiomeric composition of isolated lariciresinol was 88% e.e. in favor of the (−)-enantiomer (Fig. 2*B*).

Expression Analysis of At1g32100 and At4g13660—Two genes (At1g32100 and At4g13660) annotated as PLR were identified by searching the *A. thaliana* database (GenBank™ accession number AY065214 for At1g32100 and BT002882 for

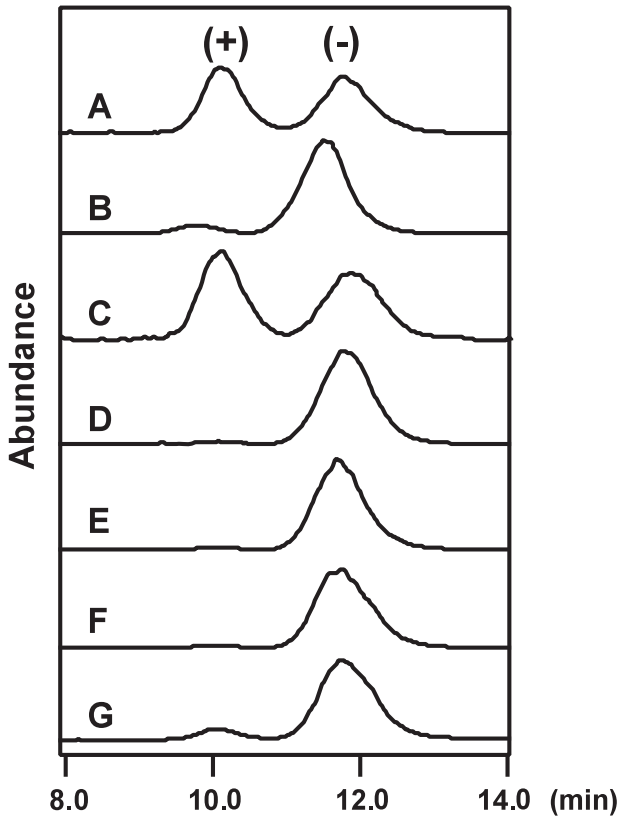

FIGURE 2. **Chiral LC-MS chromatograms of lariciresinol (*B–G*) (*m/z* 359) and (±)-[9,9,9′,9′-$^2H_4$]lariciresinols (*A*) (*m/z* 363).** *A*, racemic (±)-[9,9,9′,9′-$^2H_4$]lariciresinols. *B*, lariciresinol isolated from the root of *A. thaliana* wild-type. *C* and *D*, lariciresinol formed from the incubation of racemic pinoresinol with AtPrR1 and AtPrR2, respectively. *E–G*, lariciresinol isolated from the root of *A. thaliana atprr1-1*, *atprr1-2*, and *atprr2* mutants, respectively. Enantiomeric compositions are summarized in Table 4.

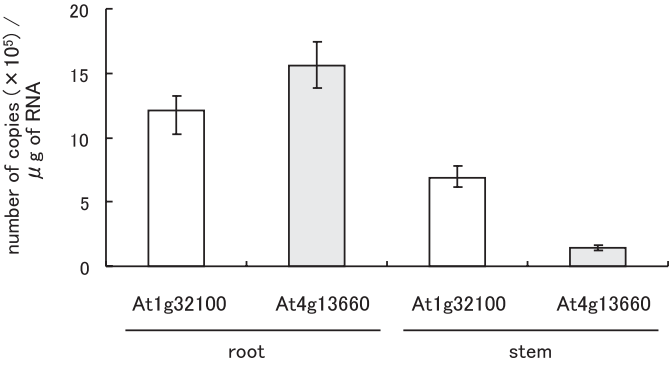

FIGURE 3. **The expressions of At1g32100 and At4g13660 genes in root and stem of *A. thaliana*.** Transcript levels are represented as absolute values after being normalized to the 18 S ribosomal RNA levels. Quantification of the cDNA levels of each gene was performed for each of the three replicates of cDNA prepared.

At4g13660). qRT-PCR analysis (Fig. 3) indicated that both genes exhibited similar transcript levels in root, which were higher than those in stem (At1g32100, 2 times; At4g13660, 8 times). This result was in accordance with AtGenExpress data (19).

Substrate Specificity of AtPrR1 and AtPrR2—To carry out the biochemical characterization of the enzymes encoded by At1g32100 and At4g13660, the recombinant enzymes prepared using *E. coli* were tested *in vitro* for their activity toward

TABLE 1
Relative activities of purified recombinant AtPrR1 and AtPrR2 toward racemic pinoresinols and lariciresinols

Enzyme[a]	Substrate	Relative activity
		nmol min^{-1} mg^{-1} protein
AtPrR1	(±)-Pinoresinols	35.5
	(±)-Lariciresinols	1.1
AtPrR2	(±)-Pinoresinols	5.9
	(±)-Lariciresinols	ND[b]

[a] AtPrR1 and AtPrR2, recombinant enzyme encoded by At1g32100 and At4g13660, respectively.
[b] Not detected.

TABLE 2
Kinetic properties of purified recombinant AtPrR1 and AtPrR2

Enzyme	Substrate	K_m	k_{cat}/K_m
		μM	μM^{-1} min^{-1}
AtPrR1	(+)-Pinoresinol	1.6	0.77
	(−)-Pinoresinol	7.3	1.14
AtPrR2	(−)-Pinoresinol	12.6	0.07

pinoresinol and lariciresinol (Table 1). First, using (±)-pinoresinols, they were found to reduce pinoresinol efficiently to lariciresinol. Interestingly, however, both enzymes, unlike conventional PLRs, did not reduce (±)-lariciresinols to secoisolariciresinol within 2 h of incubation time. Longer incubation times of up to 4 h gave only trace amounts of secoisolariciresinol (data not shown). Hence, (±)-lariciresinols were incubated individually with both recombinant enzymes, and it was determined that the activity of the enzyme encoded by At1g32100 toward lariciresinol was 35 times lower than that of pinoresinol (Table 1). On the other hand, the enzyme encoded by At4g13660 could not reduce lariciresinol to secoisolariciresinol even after 1 h of incubation (Table 1). These results clearly demonstrated that these enzymes showed strict substrate specificity toward pinoresinol and only weak or no activity toward lariciresinol. This is in sharp contrast to conventional PLRs that can reduce both pinoresinol and lariciresinol efficiently giving rise to secoisolariciresinol. Thus, the enzymes encoded by At1g32100 and At4g13660, which were annotated as AtPLRs, constitute a new type of PLR in terms of substrate specificity. Therefore, we will refer to this PLR as pinoresinol reductase (PrR): *AtPrR1* for At1g32100 and *AtPrR2* for At4g13660.

Next, we tested the enantiomeric selectivity of the substrate, pinoresinol, in the AtPrR-catalyzed reactions. Chiral LC-MS analysis showed that the enantiomeric composition of lariciresinol formed following incubation of (±)-pinoresinols with AtPrR1 was 6% e.e. in favor of the (+)-enantiomer (Fig. 2*C* and Table 4). On the other hand, AtPrR2 gave (−)-lariciresinol (96% e.e.) under the same assay conditions (Fig. 2*D* and Table 4). Next, an individual incubation of (+)- and (−)-pinoresinols with AtPrR1 formed efficiently (+)- and (−)-lariciresinols, respectively; on the other hand, AtPrR2 reduced only (−)-pinoresinol and not (+)-pinoresinol.

Enzyme Reaction Kinetics of Recombinant AtPrR1 and AtPrR2—Kinetic analysis of AtPrR1 and AtPrR2 using both enantiomers of pinoresinol as substrates was conducted (Table 2). K_m values of AtPrR1 were 1.6 μM for (+)-pinoresinol and 7.3 μM for (−)-pinoresinol. On the other hand, the K_m value of AtPrR2 for (−)-pinoresinol was 12.6 μM. In addition, k_{cat}/K_m values of AtPrR1 were 0.77 min^{-1} μM^{-1} for (+)-pinoresinol and 1.14 min^{-1} μM^{-1} for (−)-pinoresinol, whereas the value of AtPrR2 for (−)-pinoresinol was 0.07 min^{-1} μM^{-1}. As for AtPrR1, there was no significant difference in activities between (+)- and (−)-pinoresinols with regard to k_{cat}/K_m values. In contrast, the k_{cat}/K_m value of AtPrR2 for (−)-pinoresinol was 16 times lower than that of AtPrR1 for (−)-pinoresinol.

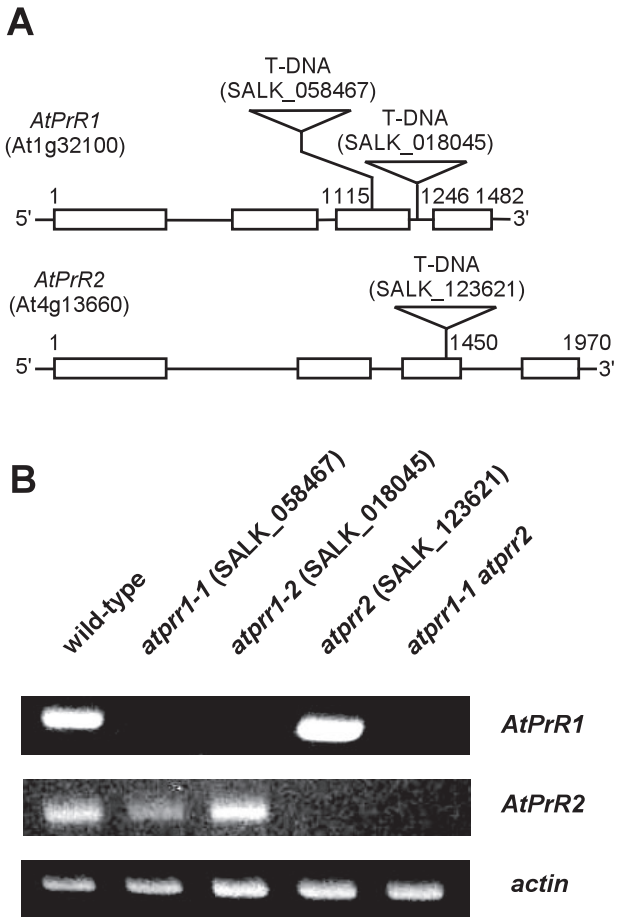

FIGURE 4. **Identification of the *atprr1-1*, *atprr1-2*, *atprr2*, and *atprr1-1 atprr2* mutants.** *A*, the positions of the T-DNA insertion in the SALK_058467, SALK_018045, and SALK_123621 lines. *B*, RT-PCR analysis of *AtPrR1* and *AtPrR2*. RT-PCR was performed with the specific primers for *AtPrR1*, *AtPrR2*, or *actin*.

T-DNA Insertion Mutant Lines of AtPrRs—To confirm the functions of *AtPrR1* and *AtPrR2* in the *A. thaliana* plant, we isolated the functionally deficient mutants of *AtPrR1* and *AtPrR2*. Two T-DNA insertion mutant lines (SALK_058467 (*atprr1-1*) and SALK_018045 (*atprr1-2*)) for *AtPrR1* and one T-DNA insertion mutant line (SALK_123621 (*atprr2*)) for *AtPrR2* were obtained from the *Arabidopsis* Biological Resource Center. After selection of the homozygous T-DNA insertion mutants, the exact position of the T-DNA insertion was determined by sequencing of the PCR products amplified with the gene-specific and T-DNA-specific primers. T-DNA in SALK_058467 (*atprr1–1*) and SALK_123621 (*atprr2*) were found to be inserted in the exon regions of the *AtPrR1* and *AtPrR2* genes (Fig. 4*A*), whereas that in SALK_018045 (*atppr1–2*) was found to be inserted in an intron of the *AtPrR1* gene (Fig. 4*A*). The functional deficiency of *AtPrR1* and *AtPrR2* in the T-DNA tag lines was confirmed by RT-PCR in all three mutants (Fig. 4*B*). In addition, an *atprr1-1 atprr2* double

TABLE 3
Lariciresinol contents of wild type and mutant in the root MeOH extracts of 28-day-old plants
Values show the averages from two experiments. ND, not detected.

A. thaliana line	Lariciresinol contents
	μg mg^{-1} dry weight root
Wild type	0.36
atprr1-1	0.57
atprr1-2	0.64
atprr2	0.56
atprr1-1 atprr2	ND

homozygous mutant was obtained by crossing the single homozygous mutants of *atprr1-1* and *atprr2* and screening the F2 populations by PCR using the primers described above. The functional deficiency of *AtPrR1* and *AtPrR2* in the double homozygous mutant was confirmed by RT-PCR (Fig. 4*B*).

Analysis of Lignans Occurring in T-DNA Insertion Mutant Lines of AtPrRs—Then the lariciresinol contents in the roots of the *atprr1-1*, *atprr1-2*, and *atprr2* single homozygous mutants as well as the *atprr1-1 atprr2* double homozygous mutant were quantified. GC-MS analysis of the MeOH extracts indicated that there was no significant difference between the single mutants and the wild type (Table 3). On the other hand, GC-MS analysis of MeOH extracts of the *atprr1-1 atprr2* double mutant root showed that lariciresinol biosynthesis in the double mutant was inhibited completely (Table 3). This was compensated for by the accumulation of pinoresinol (4.0 μg/mg of dry weight root), which was not observed in the wild type or the single mutants. Therefore, it was confirmed that both *AtPrR1* and *AtPrR2* were involved in lariciresinol biosynthesis.

Enantiomeric Compositions of Lignans Occurring in Arabidopsis Mutants—Enantiomeric compositions of lariciresinol isolated from *atprr1-1* and *atprr1-2* mutants were 94 and 96% e.e. in favor of (−)-lariciresinol (Fig. 2, *E* and *F*, and Table 4), whereas lariciresinol isolated from the *atprr2* mutant exhibited 82% e.e. in favor of the (−)-enantiomer (Fig. 2*G* and Table 4). The enantiomeric composition of the pinoresinol isolated from the *atprr1-1 atprr2* double mutant roots was 74% e.e. in favor of the (−)-enantiomer.

DISCUSSION

The pathway from coniferyl alcohol to matairesinol via pinoresinol, lariciresinol, and secoisolariciresinol (Fig. 1) has been regarded as the general pathway for lignans with 9(9′)-oxygen (1, 6). It was proposed that PLR and SIRD are key enzymes that control the enantiomeric compositions of the lignans in the pathway (8). However, the studies of gene expression levels to examine the working hypothesis have been very difficult, because gene transformation and regeneration systems for plants that are known as lignan producers were very limited, although very recently gene silencing systems for the hairy root lines of the lignan-producing *Linum perenne* have been established (20).

A. thaliana is the most widely used model plant in plant bioscience sectors. Thus, the genome sequence is available, and its transformation and regeneration are very easy. Preliminary GC-MS analysis of MeOH extracts from whole plants of *A. thaliana* showed the presence of trace amounts of lariciresinol (data not shown). However, recently, Kai *et al.* (11) found that coumarins, which belong to another class of phenylpropanoid compounds, were significantly accumulated in the plant roots.

These results encouraged us to seek lignans in the roots, and GC-MS analysis of the *A. thaliana* root MeOH extracts after β-glucosidase treatment showed the presence of significant amounts of lariciresinol (Table 3). Then we isolated lariciresinol from the roots and conclusively identified it by comparing its ^{1}H NMR spectrum with that of an authentic sample. When the root MeOH extracts were analyzed by GC-MS without β-glucosidase treatment, lariciresinol was not detected, indicating that lariciresinol in *A. thaliana* root exists as a glucoside(s). This is the first report on the occurrence of lariciresinol in *A. thaliana*. GC-MS analysis indicated that the lignan was localized in roots like coumarins (11), whereas other typical lignans, such as pinoresinol, secoisolariciresinol, or matairesinol, were detected neither in the roots nor in the aerial parts.

Having the data confirming the presence of lariciresinol in *A. thaliana* in hand, we next characterized the genes encoding PLR. Two *A. thaliana* genes (At1g32100 and At4g13660), which were annotated as PLR, were identified by searching the *A. thaliana* database. The expression analysis of the two genes using qRT-PCR indicated that both genes exhibited similar transcript levels in root, which were higher than those in stem (Fig. 3). Furthermore, the gene expression profiles matched well with the fact that lariciresinol was localized in the roots of *A. thaliana*, strongly suggesting that the genes were involved in the biosynthesis of lariciresinol.

Next, we carried out the biochemical characterization of recombinant proteins encoded by the genes. The recombinant proteins converted pinoresinol to lariciresinol efficiently (Table 1). On the other hand, the specific activity toward lariciresinol reduction to give rise to secoisolariciresinol was very weak or not detected (Table 1), unlike PLRs of other plants that can reduce both pinoresinol and lariciresinol efficiently (16, 20–22). Therefore, we refer to *A. thaliana* pinoresinol/lariciresinol reductase (AtPLR) as *A. thaliana* pinoresinol reductase (AtPrR). The substrate specificity of AtPrRs can account for why secoisolariciresinol or matairesinol was not detected in *A. thaliana*.

In addition, AtPrR1 encoded by At1g32100 is clearly different from AtPrR2 encoded by At4g13660 with respect to selectivity of substrate enantiomers. AtPrR2 showed strong preference for (−)-pinoresinol. Thus, (−)-lariciresinol with 96% e.e. was formed following incubation of racemic (±)-pinoresinols with the enzyme (Table 4). In addition, when (+)- and (−)-pinoresinols were treated with the enzyme individually, (−)-pinoresinol was reduced to (−)-lariciresinol with a k_{cat}/K_m value of 0.07 μM^{-1} min^{-1} (Table 2), whereas (+)-pinoresinol reduction was negligible. This is in line with the selectivity of substrate enantiomers of PLRs from other plants. *Forsythia intermedia* PLR (PLR-Fi1) (21), *Thuja plicata* PLR2 (PLR-Tp2) (16), and *Linum album* PLR1 (PLR-La1) (22) all catalyzed the selective reduction of (+)-pinoresinol to (+)-lariciresinol and (−)-secoisolariciresinol, whereas *T. plicata* PLR1 (PLR-Tp1) (16), and *Linum usitatissimum* PLR1 (PLR-Lu1) (22) reduced (−)-pinoresinol to (−)-lariciresinol and (+)-secoisolariciresinol (Fig. 5). Recently, Hemmati *et al.* reported that a PLR (PLR-Lp1) from *L. perenne* preferred (+)-pinoresinol in the

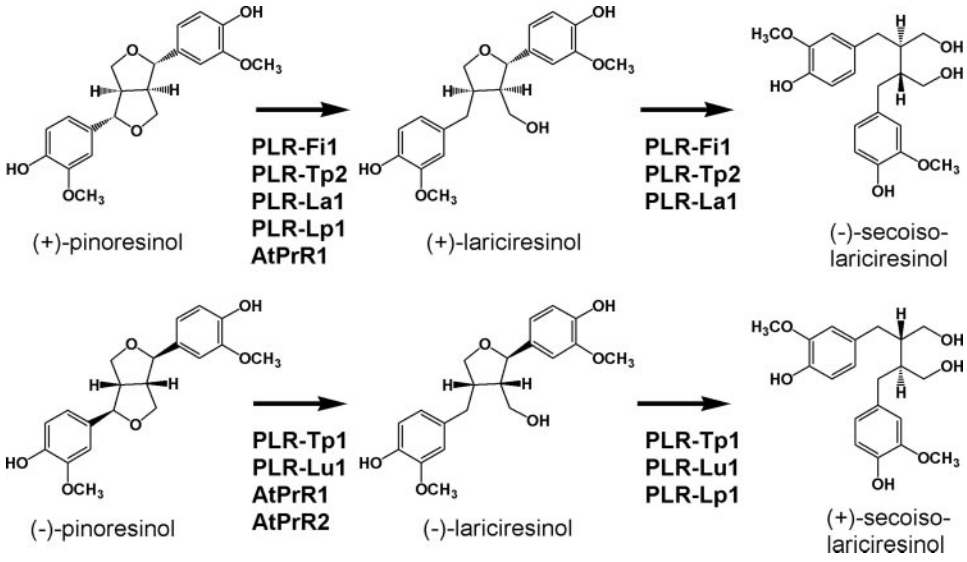

FIGURE 5. **Reactions catalyzed by PLRs from *F. intermedia* (PLR-Fi1), *T. plicata* (PLR-Tp1 and PLR-Tp2), *L. album* (PLR-La1), *L. perenne* (PLR-Lp1), and *L. usitatissimum* (PLR-Lu1) and PrRs from *A. thaliana* (AtPrR1 and AtPrR2).**

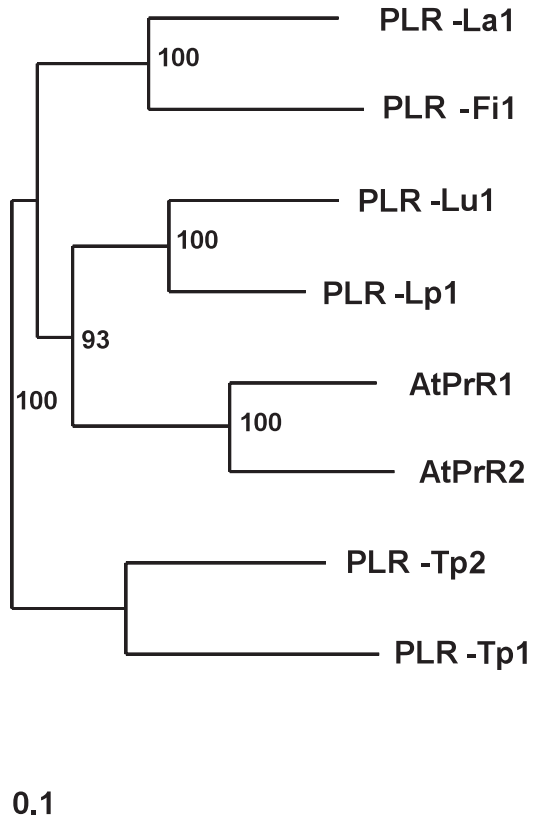

FIGURE 6. **Cladogram of PLRs and PrRs.** *Numbers* at the *forks* are bootstrap values that indicate the percentage values for obtaining this particular branching in 1000 repetitions of the analysis. Only values greater than 60% are shown. Shown are PLRs from *F. intermedia* (PLR-Fi1), *T. plicata* (PLR-Tp1 and PLR-Tp2), *L. album* (PLR-La1), *L. perenne* (PLR-Lp1), and *L. usitatissimum* (PLR-Lu1) and PrRs from *A. thaliana* (AtPrR1 and AtPrR2). The GenBank™ accession numbers are as follows: PLR-La1, AJ849358; PLR-Fi1, U81158; PLR-Lu1, AJ849359; PLR-Lp1, EF050530; PLR-Tp1, AF242503; and PLR-Tp2, AF242504.

first reduction step but (−)-lariciresinol in the second step (20) (Fig. 5). It is noteworthy that (+)-pinoresinol and (−)-lariciresinol have opposite absolute configurations at C8 and C8′. Whatever the preferential substrate enantiomer, these PLRs reduced one of the enantiomers selectively.

In sharp contrast, AtPrR1 reduced racemic (±)-pinoresinols to give rise to almost racemic lariciresinol ((+)-enantiomer, 6% e.e.). In addition, when (+)- and (−)-pinoresinols were treated with the enzyme individually, both were reduced efficiently with comparative k_{cat}/K_m values ((+)-pinoresinol, 0.77 μM^{-1} min^{-1}; (−)-pinoresinol, 1.14 μM^{-1} min^{-1} (Table 2)). Although the amino acid sequences of AtPrR1 show high identity (81%) with that of AtPrR2, the selectivities of substrate enantiomers of AtPrRs are clearly different from each other. Both AtPrR1 and AtPrR2 consist of 317 amino acids, but they have 59 different amino acid residues between them. Therefore, these 59 amino acid residues are probably involved in determining the difference in the enantiomeric selectivity. In addition, phylogenetic analysis indicates that angiosperm PLR genes, *PLR-Fi1*, *PLR-La1*, *PLR-Lu1*, *PLR-Lp1*, *AtPrR1*, and *AtPrR2* fall into a cluster, whereas gymnosperm *PLR-Tp1* and *PLR-Tp2* group into another cluster (Fig. 6), indicating the independence between enantiomeric selectivity and sequence of PLRs, including AtPrRs. Further studies of PLR and PrR structures are required to determine the amino acid residues responsible for their enantiomeric selectivity.

In order to compare the substrate enantiomer selectivity in the *in vitro* experiments with enantiomeric compositions of lariciresinol occurring in *A. thaliana* plants and to examine the roles of *AtPrR1* and *AtPrR2* in the plant, we isolated the T-DNA insertion mutants, *atprr1-1*, *atrprr1-2*, and *atprr2*, respectively, and prepared an *atprr1-1 atprr2* double mutant. GC-MS analysis indicated that the lariciresinol contents in the single mutants, *atprr1-1*, *atprr1-2*, and *atprr2*, were not reduced, but slightly increased compared with the wild-type (Table 3). On the other hand, lariciresinol was not detected in the *atprr1-1 atprr2* double mutant. Instead, significant amounts (4.0 μg/mg of dry weight root) of pinoresinol were detected in the double mutant. These results clearly indicate the functional redundancy of the two *AtPrR* genes.

We next isolated lariciresinol from the mutant lines as well as the wild type and examined their enantiomeric compositions. The enantiomeric composition of lariciresinol occurring in the wild type was 88% e.e. in favor of the (−)-enantiomer (Table 4). On the other hand, lariciresinol isolated from *atprr1-1* and *atprr1-2* mutants showed 94 and 96% e.e. in favor of the (−)-enantiomer, which are higher than that isolated from the wild type (Table 4). This agrees well with the fact that AtPrR1 reduces both (+)- and (−)-pinoresinols almost equally. The deficiency of the isoform was compensated for by the AtPrR2 that reduces preferentially (−)-pinoresinol, giving rise to (−)-lariciresinol, which probably resulted in the increase of the per-

TABLE 4
Enantiomeric compositions of pinoresinol and lariciresinol isolated from *A. thaliana* and formed enzymatically

	Enantiomeric composition	
	Pinoresinol	Lariciresinol
	% e.e.	
***A. thaliana* line**		
Wild type		88 ((−) > (+))
atprr1-1		94 ((−) > (+))
atprr1-2		96 ((−) > (+))
atprr2		82 ((−) > (+))
atprr1-1 atprr2	74 ((−) > (+))	
Enzyme		
AtPrR1		6 ((+) > (−))
AtPrR2		96 ((−) > (+))

centage e.e value in favor of the (−)-enantiomer. On the other hand, lariciresinol from *atprr2* mutant had an 82% e.e. in favor of the (−)-enantiomer, which is lower than that of the wild type (Table 4). Again, this can be accounted for by considering the fact that AtPrR2 reduces (−)-pinoresinol selectively. The deficiency of the isoform was compensated for by AtPrR1 that reduces both (+)- and (−)-pinoresinols almost equally, most probably resulting in the decrease of the percentage e.e value in favor of (−)-enantiomer compared with the wild type (88% e.e.). Although AtPrR1 reduced racemic (±)-pinoresinols to afford almost racemic lariciresinol (6% e.e. in favor of the (+)-enantiomer), the percentage e.e. value of the formed lariciresinol is much higher than racemic. This can be ascribed to the enantioselective pinoresinol formation mediated by the DP. In fact, pinoresinol isolated from the *atprr1-1 atprr2* double mutant had 74% e.e. in favor of the (−)-pinoresinol (Table 4). As long as (−)-pinoresinol with a high percentage e.e. value is supplied, less enantioselective AtPrR1 can produce (−)-lariciresinol with a high percentage e.e. value, as observed in the *atprr2* mutant.

Collectively, our results clearly demonstrate that PrRs together with DP are involved in enantiomeric control in lignan biosynthesis. In addition, differential expression of PrR isoforms that have different selectivity in terms of substrate (pinoresinol) enantiomers can determine the enantiomeric compositions of the product lignan (lariciresinol).

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