Molecular Characterization of the Phenylalanine Ammonia-Lyase from Ephedra sinica

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The cDNAs (Espals) encoding phenylalanine ammonia-lyase (PAL) were cloned from Ephedra sinica by reverse transcription polymerase chain reaction (RT-PCR) using degenerate primers and by 5′ and 3′-rapid amplification of cDNA ends (RACE). 2166 bp of the open reading frame (ORF) encoded 722 amino acids; sequence analyses of Espal clones suggested that at least four isoforms of ESPAL (EsPAL1, 2, 3, 4) existed, with nine amino acids substitution in their sequences. Phylogenetic analysis of ESPAL and PALS from other plant species revealed that ESPAL and Pinus PALS formed a gymnosperm-type PALS subfamily. The recombinant EsPAL1 to 4 functionally catalyzed a PAL reaction and their \( K_{\text{m}} \), \( V_{\text{max}} \), \( K_{\text{cat}} \) and \( K_{\text{cat}}/K_{\text{m}} \) values did not show significant differences. Semi-quantitative RT-PCR analysis indicated that the expression of Espal genes in the roots was higher than in the plant’s aerial parts. In addition, the activity of PAL in the roots was also higher than in the aerial parts. These results suggest that Espal genes are expressed in the whole plant but are dominant in the roots rather than in the aerial parts.

Key words Ephedra sinica; ephedrine alkaloid; biosynthesis; gymnosperm-type phenylalanine ammonia-lyase

Plants produce various chemical compounds, which include more than 200000 metabolites.1) The secondary metabolites isolated from medicinal plants have been used for the treatment of disease and the maintenance and improvement of health in man and animals. In the Far East, medicinal plants have been used as the sources of “traditional Chinese medicine”, or “Kampo medicines” in Japan, because they have pharmacological effects due to the various biologically active metabolites they contain. The Ephedra plant, or ‘Ma Huang’ as it is known in traditional Chinese medicine, is one of the oldest medicinal plants known to mankind.2) Ephedra species are indigenous to regions of Asia, North, Central and South America and Europe and more than 45 species exist. Several Ephedra species contain ephedrine alkaloids as their principal metabolites (Fig. 1), which are primarily localized in the plant’s aerial parts. Naturally occurring ephedrine alkaloids are three pairs of diastereomeric compounds, which are (−)-ephedrine and (＋)-pseudoephedrine, (−)-N-methyl-ephedrine and (＋)-N-methylpseudoephedrine, and (−)-norpseudoephedrine and (＋)-norpseudoephedrine. Several Ephedra species (e.g. E. sinica, E. intermedia and E. equisetina) contain varying amounts of these alkaloids;3) the ephedrine alkaloid concentration in the aerial parts of Ephedra plants ranges from 0.02 to 3.4%.4) The major isomer is (−)-ephedrine, which was first isolated by Dr. Nagayoshi Nagai in 1887. It is well known that (−)-ephedrine is pharmacologically a sympathomimetic agonist at both \( \alpha \) and \( \beta \)-adrenergic receptors, resulting in enhanced cardiac rate and contractility, peripheral vasoconstriction, bronchodilation and central nervous system stimulation.5) In addition, Ephedra plants have been used as sources of ephedrine alkaloids in dietary supplements, e.g. in stimulants and weight-loss aids. It should be noted that their misuse and abuse have caused serious adverse medical events, including several deaths.6)

In the primary step of the ephedrine alkaloid biosynthesis, \( \text{L-Phe} \) is deaminated by action of phenylalanine ammonia-lyase (PAL) (E.C. 4.3.1.5) to form trans-cinnamic acid and ammonia (Fig. 1). Since PAL was first described by Koukol and Conn,7) a number of PALS have been characterized by enzyme purification, cDNA cloning, gene expression analysis, studies on a mechanism of catalysis, studies on enzyme kinetics, three-dimensional structural analysis and physiological studies.8) It is also known that PAL catalyzes the primary step of the phenylpropanoid biosynthetic pathway for production of secondary metabolites such as flavonoids, flavonols, anthocyanins, condensed tannins, isoflavones, iso-flavanones, lignin, sinapate esters, coumarins, chlorogenic acid and salicylic acid.9) It is supposed that PAL is the first and the key enzyme for flux from primary metabolism into the phenylpropanoid pathway, supported by the correlation studies. Examples of these include: a quantitative study of the relationship between PAL levels and phenylpropanoid accumulation in transgenic tobacco;10) a quantitative analysis of phenylpropanoid accumulation in transgenic tobacco overexpressing the bean \( \text{pal2} \) gene;11) a study on the reduction of lignin content and alteration of its composition using transgenic tobacco in which the \( \text{pal} \) gene is down-regulated.12) PAL may also play an important role as the first catalytic enzyme in ephedrine alkaloid biosynthesis. The biochemical study of Ephedra PAL has been carried out using E. distachya cultures which accumulated \( \text{p-coumaroyl} \) amino acids by elicitor treatment.13) In ephedrine alkaloid biosynthesis, trans-cinnamic acid produced from a PAL reaction is converted into benzoic acid or benzoyl-CoA by \( \beta \)-oxidation, branching off from the common phenylpropanoid pathway (Fig. 1). These \( \text{C}_6\text{C}_1 \) units or their alternatives react to pyruvic acid, which is a donor of the \( \text{C}_3 \) unit; through this condensation, the basic \( \text{C}_6\text{C}_3 \) skeleton of ephedrine alkaloids is biosynthesized.14) This \( \text{C}_6\text{C}_3 \) intermediate is biosynthesized through a specific and unique biosynthetic pathway in Ephedra species. Although this process has been studied extensively, the molecular biological characterization of ephedrine alkaloid biosynthesis remains incomplete.
This is the first report that the cDNAs encoding PAL involved in ephedrine alkaloid biosynthesis were cloned from \textit{E. sinica} and \textit{Ephedra} PALS were molecularly characterized. In this study, the four cDNAs, Espal\textit{I} to \textit{4}, were cloned and their functional analyses were performed using recombinant proteins. The expression analysis of \textit{Espal} genes by semi-quantitative RT-PCR and the measurement of PAL activity in both the aerial parts and the roots of \textit{E. sinica} were performed. In addition, the phylogenetic relationship between gymnosperm-type PALS including ES-PAL and PALS from other plant species was discussed. Among the gymnosperm-type \textit{pal} genes, the \textit{pal} gene from \textit{Pinus taeda} was studied in the transcriptional profiling of the genes involved in the phenylpropanoid pathway. This study laid the groundwork for molecular and biochemical characterization of both the ephedrine alkaloid biosynthesis and the gymnosperm type-PAL.

**MATERIALS AND METHODS**

**Plant Material** The seeds of \textit{E. sinica} were germinated in moistened vermiculite in daylight in a greenhouse at ca. 25 °C. \textit{E. sinica} was grown until the 4th joint of the plant’s aerial parts was generated.

**Chemicals** \textit{l}-Phe, \textit{l}-Tyr, \textit{trans}-cinnamic acid and \textit{p}-coumaric acid were purchased from Wako Pure Chemical Industries (Japan). All other chemicals and reagents were of the highest grade available.

**Molecular Cloning of \textit{Espals} from \textit{E. sinica}**

Total RNA was extracted from \textit{E. sinica} using a RNeasy Plant Mini Kit (QIAGEN, Germany). First-strand cDNA was synthesized using AMV Reverse Transcriptase XL with an Oligo dT-3sites Adaptor Primer (TaKaRa Bio, Japan). In order to clone \textit{Espals} by RT-PCR, the degenerate primers were designed from highly homologous regions among the \textit{pal} genes from other plant species. The first PCR reaction was performed using the forward primer of \textit{Espal}-F-D, 5’-GAYCTBGTYCBBYTVCYTA-3’, and the reverse primer of 3sites Adaptor Primer (TaKaRa Bio) which was complementary to the region synthesized by an Oligo dT-3sites Adaptor Primer (TaKaRa Bio). Nested PCR was performed using the forward primer of Espal-F-D and the reverse primer of Espal-R-D, 5’-AGYTCNG-WRAAYTGVGCRAACAT-3’. The amplified fragments were cloned into pBluescript II SK(−) (Stratagene, U.S.A.). As a result, a 674 bp DNA fragment which is highly homologous to the \textit{pal} genes from other plant species was subcloned. 3’ and 5’-RACE were then performed to analyze the 3’ and 5’-terminal sequences using a 3’-Full RACE Core Set and a 5’-Full RACE Core Set (TaKaRa Bio) respectively. The specific primers for 3’ and 5’-RACE were designed from the nucleotide sequence of the subcloned 674 bp-fragment. With the reverse primer of 3sites Adaptor Primer (TaKaRa Bio), 3’-RACE was performed using the forward primers of Espal-3F-1, 5’-TGACACATCTATGATCGATGTCG-3’, and Espal-3F-N, 5’-CTATCGGTGTCCTCATGGATAATC-3’, for the 1st and nested PCR respectively. In 5’-RACE, reverse transcription from the total RNA was performed using the 5’-end-phosphorylated primer, 5’-TGCTTGAGACGGTG-3’. Subsequently, the 1st PCR was performed using the forward primer of Espal-5F-1, 5’-CTGTTGACCGTG-3’, and the reverse primer of Espal-5R-1, 5’-CTGACCGTGACTGGC-3’, on the 1st and nested PCR respectively. In 5’-RACE, reverse transcription from the total RNA was performed using the 5’-end-phosphorylated primer, 5’-TGCTTGAGACGGTG-3’. Subsequently, the 1st PCR was performed using the forward primer of Espal-5F-1, 5’-CCATGTTCCTTGAA-GTCAATCGCC-3’, and the reverse primer of Espal-5R-1, 5’-CCTGGGGTGTCAATTCGAAG-3’. Finally, end-to-end PCR was performed using the forward primer of Espal-F-E, 5’-CGGAATTCTCATGTGTCGGAGCCAGAGATGGCGCAG-3’, and the reverse primer of Espal-R-E, 5’-CCGCTCGAG-GAGATCGATCAACCGGTATTGAG-3’, in order to clone the ORF regions of \textit{Espals}. The DDBJ/EMBL/GenBank accession numbers of \textit{Espal}1, 2, 3 and 4 are AB300199, AB300200, AB300201 and AB300202, respectively.

**Phylogenetic Analysis**

Phylogenetic analysis was carried out using CLUSTAL W packaged in DNASIS Pro version 2.08 (HITACHI Software, Japan).

**Expression and Purification of Recombinant Proteins**

\textit{EcoRI} and \textit{XhoI} sites were created on the 5’ and 3’-ends of the \textit{Espal} by PCR using the primers of Espal-F-E and Espal-R-E respectively. (See the section of “Molecular Cloning of \textit{Espals} from \textit{E. sinica}” in Materials and Methods.) The engineered cDNA fragment was inserted into the \textit{EcoRI}-\textit{XhoI} site of pGEX-6P-1 (GE Healthcare, U.K.), resulting in a recombinant protein product with an N-terminal glutathione \textit{S}-transferase (GST) protein tag with the correct orientation. Escherichia coli BL21 cells harboring pGEX-EsPAL1 to 4 and an empty pGEX vector were grown at 30 to 37 °C in 2× YT-ampicillin (100 \(\mu\)g/ml) liquid medium to an approximately O.D.\textsubscript{600} = 0.6. A solution of 0.2 \text{mm} isopropyl \(\beta\)-D-thio-
galactoside was then added and E. coli cells were cultured at 20 °C for inducible production of the recombinant protein. The following processes were done at 4 °C. The cells were suspended in 1× phosphate buffered saline [140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄ (pH 7.3)] and disrupted by sonication. After the addition of Triton X-100 at 1% concentration, the samples were centrifuged at 12000g for 10 min. The recombinant proteins were purified from the supernatant of crude protein extracts. For purification of the recombinant proteins, a GSTrap HP column (GE Healthcare) whose resin has glutathione as a ligand resulting in affinity to GST was used under control by an ÄKTAPrime Plus System (GE Healthcare). The GST-tags were removed from the GST fusion recombinant proteins by in-column digestion using PreScission Protease (GE Healthcare).

**Protein Extraction from E. sinica** Protein extraction was performed at 4 °C. Crude protein extracts were separately extracted from both the aerial parts and the roots of E. sinica. The plant samples were suspended and homogenized in 3 mL g⁻¹ (fresh weight of plants) of the extraction buffer [200 mM bis-tris propane/HCl (pH 8.5), 1 mM dithiothreitol (DTT) and 0.5 mM ethylenediaminetetraacetic acid (EDTA)] with 0.1 g g⁻¹ (fresh weight of plants) polyvinylpolypyrrolidone. The sample solution was centrifuged at 10000g for 10 min and the supernatant was desalted using a PD-10 column (GE Healthcare).

**Assay of Enzymatic Activity** The PAL and tyrosine amonia-lyase (TAL) activities were determined by detection of trans-cinnamic acid produced from L-Phe and p-coumaric acid produced from L-Tyr by enzymatic reactions respectively and the assays performed according to the method described by Cochrane et al.14) The standard reaction mixture consisted of 100 mM bis-tris propane/HCl (pH 8.5), 1 mM DTT, 0.5 mM EDTA, 0.05 to 2.0 mM L-Phe or L-Tyr, and protein. After incubation for 20 to 30 min at 37 °C, the reaction was terminated by addition of acetic acid at a final concentration of 17%. The reaction mixture was centrifuged at 10000g for 10 min and the filtration was performed using a 0.45 μm filter disk. The products from enzymatic reactions using the recombinant EsPAL proteins and the crude protein extracts from E. sinica were analyzed by an ACQUITY ultra performance liquid chromatography (UPLC) system (Waters, U.S.A.) and by an HPLC, respectively. The UPLC and HPLC analyses were performed on an ACQUITY UPLC BEH C18 (2.1×50 mm, 1.7 μm) column (Waters) and on a COSMOSIL 5C18-MS-II (4.6×150 mm, 5 μm) column (Nacalai Tesque, Japan), respectively. The mobile phase consisted of H₂O: acetic acid (97:3, v/v) (mobile phase A) and acetonitrile: acetic acid (97:3, v/v) (mobile phase B) and was pumped at a flow rate of 1.0 mL min⁻¹. In the UPLC analysis, the gradient of mobile phases A and B started at 0% of B for 1.0 min, linearly increased to 50% over 4.0 min, and was then kept at 100% of B for 3.0 min. In the HPLC analysis, the gradient of mobile phases A and B started at 0% of B for 2 min, linearly increased to 100% over 28 min, and was then kept constant for 10 min. trans-Cinnamic acid and p-coumaric acid were monitored at 278 nm and 310 nm respectively.

**Semi-quantitative RT-PCR** The cDNAs from both the aerial parts and the roots were separately synthesized from the same quantity of total RNA using AMV Reverse Transcriptase XL with an Oligo dT-3sites Adaptor Primer (3’-Full RACE Core Set, TaKaRa Bio). PCR was performed using PrimeSTAR HS DNA Polymerase (TaKaRa Bio) using the following primers to amplify the ORF region of *Espals*: the forward primer of Espal-F-RT, 5’-ATGGTTGCGGAGCA-GAGATGGCCGCA-3’, and the reverse primer of Espal-R-RT, 5’-GAAGGGAGGCTGGTCTCTTCCAC-3’. The number of PCR cycles was set at 25 and 23 (94 °C/15 s, 62 °C/5 s, 72 °C/2.5 min). The cDNA encoding RNA polymerase I largest subunit was amplified in the same PCR conditions (25 cycles) using the forward primer, 5’-ATGAATGTC-CACCTTTCCACAGATA-3’, and the reverse primer, 5’-TGATGGTTCTCCTACAGTGAC-3’, designed from the mRNA encoding RNA polymerase I largest subunit from *E. viridis* (DDBJ/EMBL/GenBank accession number AY490543) as a positive control.

**Southern Blot Analysis** Genomic DNA was extracted from *E. sinica* using a DNeasy Plant Mini Kit (QIAGEN). One microgram aliquots of genomic DNA were digested with *EcoRI* and *EcoRV* and separated on a 1.0% agarose gel. After transfer to a Hybond N+ membrane, hybridization was carried out using a Southern-Star chemiluminescent detection system (Applied Biosystems, U.S.A.) with a biotinylated probe which was prepared by PCR using 5’-biotinylated Espal-F-RT and Espal-R-RT primers. (See the section of “Semi-quantitative RT-PCR” in Materials and Methods.) The hybridization signals were detected using a LAS-3000 image analyzer (FUJIFILM, Japan).

**Miscellaneous Techniques** All recombinant DNA technology followed the methods of Sambrook et al.15) DNA sequencing was performed by the dyeex-chain termination method using a reaction reagent of a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3130/3130xl Genetic Analyzer (Applied Biosystems) system. Protein quantitation was performed by the Bradford method.16) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed with the molecular weight markers, a Low Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare) and a High Molecular Weight Calibration Kit for SDS Electrophoresis (GE Healthcare).

**RESULTS AND DISCUSSION**

**Molecular Cloning of Espals from E. sinica** Espals were cloned from *E. sinica* by RT-PCR using degenerate primers, 5’ and 3’-RACE, and end-to-end PCR (Fig. 2). *pal* Genes have high homology among plant species and this facilitate the design of degenerate primers for RT-PCR to clone Espals. As a result of the RT-PCR, a 674 bp nucleotide fragment was subcloned and this sequence had close homology with known plant *pal* genes. Subsequently, 706 to 723 bp from the 5’-end region and 1044 to 1046 bp from 3’-end region were determined by 5’ and 3’-RACE respectively. Finally, 2166 bp of the ORF was determined by end-to-end PCR. The ORF region of *Espal* encoded 722 amino acids and the molecular mass of EsPAL was theoretically 79 kDa. Sequence analyses of twenty *Espal* clones suggested that at least four isofoms of EsPAL, EsPAL1 to 4, existed in *E. sinica*, with nine amino acids substitution among their sequences (Fig. 2).

**Phylogenetic Analysis of EsPAL** The deduced amino
acid sequence of EsP AL was phylogenetically analyzed with PALs from other plant species (Fig. 3). In this analysis, only EsP AL1 was selected from EsP AL1 to 4 because of high homology among them. The most closely related sequence to EsP AL1 was P AL from *Pinus taeda* (a pine tree), with 77% identity of the amino acid sequence (557/719 amino acids). *Ephedra* and *Pinus* species are gymnosperms. *Pinus* PAL has been characterized as an out-group of the PAL family because gymnosperms were considered to be ancestors of angiosperms on the basis of both morphological characteristics and 18S rRNA sequences. It is also known that dicotyledon and monocotyledon PALs independently form each of the subfamilies in the angiosperm-type PAL family. In this study, both EsP AL1 and *Pinus* PAL were phylogenetically separated from the clusters belonging to the angiosperm-type PAL family and formed an independent cluster. This analysis clearly showed that EsP AL was phylogenetically categorized in the gymnosperm-type PAL family. In addition, the result of phylogenetic analysis of plant PALs may suggest that their sequence diversity well agrees with the genetic evolution of the spermatophyte.

**Functional Analyses of Recombinant EsP ALs** To confirm that the isolated cDNA encodes the catalytically-active EsP AL, the functional analysis was carried out using the recombinant protein heterogeneously produced in *E. coli*. The recombinant EsP AL1 to 4 proteins were purified by GST-tag affinity and subsequent cleavage of the tag (Fig. 4). In the in vitro enzymatic assays using purified recombinant EsP AL1 to 4, trans-cinnamic acid produced by the deamination of L-Phe was detected by UPLC analysis (Fig. 5). In addition, no products were formed in the negative control reaction using the protein fraction purified from *E. coli* harboring an empty pGEX vector (Fig. 5). The recombinant PALs had a pH optimum at 8.5. These results indicate that the isolated cDNA actually encodes functional PAL. To determine the kinetic parameters of EsP AL1 to 4, $K_m$, $V_{max}$, $K_{cat}$ and $K_{cat}/K_m$ values were calculated from the results of PAL assays (Table 1). Significant differences in these values were not found among EsP ALs. In a comparative study of four isoforms of PAL from *Arabidopsis thaliana* (AtPAL1, 2, 3 and 4), AtPAL1, 2

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**Fig. 2. Alignment of Four Isoforms of EsPAL**

The nine amino acids (the 85th, 89th, 220th, 233rd, 469th, 597th, 643rd, 661st and 713rd amino acids) substitution was elucidated via alignment of the amino acid sequences of EsPAL1 to 4.

**Fig. 3. Phylogenetic Analysis of EsPAL and PALs from Other Plant Species**

The amino acid sequences of EsPAL1 and PALs from other plant species were phylogenetically analyzed. Their DDBJ/EMBL/GenBank accession numbers were shown in the figure.
and 4 had similar $K_m$ values for l-Phe and displayed similar pH and temperature optima. On the other hand, AtPAL3 was of very low activity and only detectable at high substrate concentrations. In the case of EsPAL isoforms, such kinetic differences between AtPAL1, 2 and 4 and AtPAL3 were not observed. This result might suggest that the amino acids substitution among EsPALS (Fig. 2) was hardly relevant to their kinetic properties in a PAL reaction. In addition, AtPAL1, 2 and 4 have low TAL activity and several monocotyledon PALs also have TAL activity. The PAL from Zea mays, ZmPAL1, was well characterized by functional analysis using recombinant protein, and the TAL activity of ZmPAL1 displayed catalytic efficiency similar to its PAL activity. Although the TAL activity was also confirmed by assays using recombinant EsPALS, $p$-coumaric acid, which is produced by a TAL reaction and is a primary intermediate in the phenylpropanoid pathway, was not detected, at least in this condition.

Table 1. Kinetic Properties of Recombinant EsPALS

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pkat mg$^{-1}$)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
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<tbody>
<tr>
<td>EsPAL1</td>
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<td>7.45</td>
<td>0.588</td>
<td>3870</td>
</tr>
<tr>
<td>EsPAL2</td>
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<td>5.77</td>
<td>0.457</td>
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<tr>
<td>EsPAL3</td>
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<td>4.58</td>
<td>0.362</td>
<td>3230</td>
</tr>
<tr>
<td>EsPAL4</td>
<td>180</td>
<td>6.53</td>
<td>0.516</td>
<td>2860</td>
</tr>
</tbody>
</table>

Fig. 4. Purification and SDS-PAGE Analysis of Recombinant EsPALS Produced in E. coli

The crude protein extracts from E. coli harboring pGEX-EspAL vectors expressing EspAL1 to 4 and an empty pGEX vector and the purified recombinant proteins were separated by 10% SDS-PAGE. Protein bands were detected by Coomassie Brilliant Blue staining with high and low molecular weight markers (HMW and LMW): lanes 1 and 2, crude protein extracts from E. coli harboring an empty pGEX vector and its purified protein fraction respectively; lane 3, crude protein extracts from E. coli harboring pGEX-EsPAL1; lanes 4 to 7, purified EsPAL1, 2, 3 and 4 respectively. Arrowheads (a) and (b) indicate the positions of purified EsPAL proteins after cleavage of GST-tags and of GST-tags respectively.

Fig. 5. UPLC Analysis of trans-Cinnamic Acid Produced by a PAL Reaction Using Recombinant EsPAL

The results of the PAL reactions, which were carried out with either the purified recombinant EsPAL4 (a) or the protein fraction purified from E. coli harboring an empty pGEX vector (b), are shown. A 0.1 mM standard solution of trans-cinnamic acid (10 µl) was also analyzed (c). The arrowheads indicate the peaks of trans-cinnamic acid.

Fig. 6. Expression Analysis of Espal Genes by Semi-quantitative RT-PCR

The expression patterns of Espal genes in the aerial parts and the roots were analyzed. The results where the number of PCR cycles was set at 25 and 23 were shown. PCR amplifying the cDNA encoding RNA polymerase I largest subunit was performed as a control experiment.

Fig. 7. PAL Activities in the Aerial Parts and the Roots of E. sinica

The relative activities of PAL were measured in the aerial parts (11.5±1.1 pkat/mg protein) and the roots (78.3±8.3 pkat/mg protein). trans-Cinnamic acid produced by a PAL reaction was quantified using HPLC ($n=3$).
may be transferred to the aerial parts in some way. Southern blot analysis indicated that at least 1 copy of a homologous gene was present in the genome of \textit{E. sinica} (data not shown).

CONCLUSION

PAL is thought to be an important catalytic enzyme for secondary metabolism in plants, yeasts and some fungi. PAL is the key enzyme catalyzing the first step in the phenylpropanoid pathway and a PAL reaction introduces primary metabolites into secondary metabolism. In several \textit{Ephedra} species, PAL is also important in the biosynthesis of ephedrine alkaloid and the molecular characterization of \textit{Ephedra} PAL is important to understand the regulation of the primary biosynthetic step. In this study, four \textit{Espal} were cloned from \textit{E. sinica} and molecularly characterized. The functional analyses of EsPALS were carried out using recombinant proteins and it was found that their kinetic properties were almost the same. Expression of \textit{Espal} genes and PAL activity were detected in the whole plant but they were dominant in the roots rather than in the aerial parts. These results suggest that the primary step of ephedrine alkaloid biosynthesis may occur in the whole plant, instead of being restricted to the aerial parts in which ephedrine alkaloids accumulate. This suggests that the molecular characterization of ephedrine alkaloid biosynthesis should not focus only on metabolic events in the aerial parts of \textit{Ephedra} plants. In phylogenetic analysis, EsPAL and Pinus PAL clearly formed a cluster of gymnosperm-type PAL within the plant PALs. This result suggested that the evolution of gymnosperm-type PAL has progressed in a different way, apart from the monocotyledon and dicotyledon-type PAL. This finding also may be applied to the studies on the genetic evolution of plants based on secondary metabolic enzymes. Finally, we hope this study will lead to additional molecular biological and biochemical studies on the ephedrine alkaloid biosynthesis and on the phylogenetically diverse PAL families.

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