Effects of overexpression of endogenous phenylalanine ammonia-lyase (PALrs1) on accumulation of salidroside in *Rhodiola sachalinensis*


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INTRODUCTION

*Rhodiola sachalinensis* A. Bor is in the Crassulaceae and related to *Rhodiola rosea* L. (golden root), a perennial herbaceous plant that has been used in China as an adaptogenic herbal medicine for more than 800 years. Recently, phytochemical extracts of *Rhodiola* species have been the source of important commercial preparations widely used throughout Europe, Asia, and the USA, with biological activities including antiallergenic and anti-inflammatory effects, enhanced mental alertness, and a variety of other therapeutic applications (Tolonen et al. 2003; Yousef et al. 2006). It is well established that salidroside is the main bioactive component in the root of salidroside, a novel effective adaptogenic drug extracted from the medicinal plant *Rhodiola sachalinensis* A. Bor, can be derived from phenylalanine or tyrosine. Due to the scarcity of *R. sachalinensis* and its low yield of salidroside, there is great interest in enhancing production of salidroside by the plant. In this study, a cDNA clone encoding phenylalanine ammonia-lyase (PAL) was isolated from *R. sachalinensis* using rapid amplification of cDNA ends. The resulting cDNA was designated PALrs1. It is 2407-bp long and encodes 710 deduced amino acid residues. Southern blot analysis of genomic DNA indicated that the PAL gene family is composed of three to five genes in the *R. sachalinensis* genome. Northern blot analysis revealed that transcripts of PALrs1 were present in calli, leaves and stems, but expression in roots was very low. The PALrs1 under the 35S promoter with double-enhancer sequences from CaMV-Ω and TMV-Ω fragments was transferred into *R. sachalinensis* via *Agrobacterium tumefaciens*. PCR and PCR–Southern blot confirmed that the PALrs1 gene had been integrated into the genome of transgenic plants. Northern blot analysis revealed that the PALrs1 gene had been expressed at the transcriptional level. High-performance liquid chromatography indicated that overexpression of the PALrs1 gene resulted in a 3.3-fold increase in p-coumaric acid content, as expected. In contrast, levels of tyrosol and salidroside were 4.7-fold and 7.7-fold, respectively, lower in PALrs1 transgenic plants than in controls. Furthermore, overexpression of the PALrs1 gene resulted in a 2.6-fold decrease in tyrosine content. These data suggest that overexpression of the PALrs1 gene and accumulation of p-coumaric acid did not facilitate tyrosol biosynthesis; tyrosol, as a phenylethanoid derivative, is not derived from phenylalanine; and reduced availability of tyrosine most likely resulted in a large reduction in tyrosol biosynthesis and accumulation of salidroside.

ABSTRACT

Keywords

Overexpression; phenylalanine ammonia-lyase (PAL); *Rhodiola sachalinensis*; salidroside; tyrosol.

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R. sachalinensis, and it is reported to have many pharmacological uses, such as treating anoxia, microwave radiation and fatigue and slowing the ageing process (Saratikov 1968).

Salidroside is a tyrosol 8-O-β-D-glucoside that is synthesized by the glucosylation of tyrosol, catalyzed by UDP-glucosyltransferase (Ma et al. 2007; Xu et al. 1998; Fig. 1). As the storage form of tyrosol in plants, salidroside has also been identified in other plants such as Linaria japonica (Otsuka 1993), birch (Shen et al. 1999) and olive trees (Maestroduran et al. 1994). However, although it is well known that tyrosol is a shikimate pathway-derived secondary metabolite, the biosynthetic pathway of tyrosol and its regulation are less well understood.

In the literature, there are two different views on the salidroside biosynthetic pathway. One is that tyrosol is presumably produced from a p-coumaric acid precursor by a decarboxylase, which is mainly derived from the phenylalanine (Li et al. 2005; Xu & Su 1997; Fig. 1A); the other view is that the precursor of tyrosol may be tyramine, which is synthesized from tyrosine (Landtag et al. 2002; Ellis 1983; Fig. 1B).

The shikimate pathway is initiated by condensation of phosphoenolpyruvate and erythrose 4-phosphate (E-4-P), followed by several reactions to yield arogenate, after which the pathway bifurcates to produce phenylalanine and tyrosine (Yao et al. 1995; Fig. 1). Phenylalanine and tyrosine are important precursors of many secondary metabolites.
metabolites in higher plants, and enzymes [such as phenylalanine ammonia-lyase (PAL) and tyrosine decarboxylase (TyrDC)] responsible for diverting these essential primary metabolites into secondary metabolic pathways typically have key regulatory functions (Facchinetti et al. 2000). Plants synthesize a wide variety of natural products from phenylalanine, based on the phenylpropanoid skeleton. Biosynthesis of phenylpropanoid products is initiated by the deamination of phenylalanine to cinnamic acid, and then continued by the hydroxylation of cinnamic acid to p-coumaric acid. PAL catalyzes the deamination of phenylalanine, which is the first committed step in regulation of the overall flux into the phenylpropanoid pathway (Bate et al. 1994; Fig. 1).

There is considerable interest in the regulation of salidroside biosynthesis, both to explain and clarify the biosynthetic pathway of tyrosol and also for the identification of targets for biotechnological manipulation of product accumulation. However, the biosynthetic pathway of tyrosol and its regulation are less well understood. A summary of the results of related studies revealed that there are two different possibilities for the tyrosol biosynthetic pathway (Fig. 1). In order to elucidate the molecular pathways needed for tyrosol biosynthesis, herein we report the cloning and expression pattern of a cDNA encoding a PAL from R. sachalinensis. More specifically, in the present study, we analyzed whether overexpression of endogenous PALrs1 in R. sachalinensis leads to higher levels of p-coumaric acid and salidroside. As expected, overexpression of the PALrs1 gene resulted in a 3.3-fold increase of p-coumaric acid; however, the levels of tyrosine, tyrosol and salidroside were 2.6-fold, 4.7-fold and 7.7-fold lower, respectively, in the transgenic plants than in controls. Overexpression of the PALrs1 gene and accumulation of p-coumaric acid did not facilitate tyrosol biosynthesis. The data presented in this report provide in vivo evidence that tyrosol is not derived from phenylalanine; and that reduced availability of tyrosine may be responsible for the reduction in accumulation of salidroside.

MATERIALS AND METHODS

Plant materials and culture conditions

Seeds of Rhodiola sachalinensis were collected from Changbai Mountain, Jilin Province, China. The seeds were surface-sterilized for 20 min with 0.1% mercuric chloride. The seeds were then washed thoroughly with sterile distilled water, and germinated on solid MS medium (Murashige & Skoog 1962) supplemented with 3.0% sucrose and 0.75% agar, pH 5.8, before addition of agar. After approximately 30 days of culture, the middle or upper leaves of R. sachalinensis were used as explants. The leaves were cut into 0.5 x 0.5-cm segments and transferred to callus-inducing medium (MS basal medium supplemented with 1.5 mg l-1 6-BA, 0.15 mg l-1 NAA, and 0.5 mg l-1 2,4-D) to induce callus. Further subculturing was carried out every 3–4 weeks on callus subculturing medium (MS basal medium supplemented with 1.5 mg l-1 6-BA, 0.15 mg l-1 NAA). All plants and cultures were kept at 24 ± 1 °C, with a photoperiod of 16 h light and 8 h dark in an environmental chamber with a light intensity of 150 µE m-2 s-1 provided by cool white fluorescent lamps during the light period.

Isolation of the PALrs1 cDNA

Total RNA was extracted from 3-week-old calli with Trizol® reagent (Invitrogen™; Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. The 3’-RACE first-strand cDNA was synthesized using the oligo dT-adaptor primer (CCAGTGAGCAGAGTGACGAGGACCTGAGCTCAAGGCTTTTTTTTTTTTTTTT) and synthesized using an ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA). Degenerate primers were designed on the basis of an alignment of amino acid sequences deduced from plant PAL genes as follows: RPLP1: 5’-GA(G/A)GC(T/A)GC(T/C)/AAT(A/T)CATGGAC-3’ and RPLP2: 5’-GG(G/A/C)CC(T/A)/C(A/T)(A/G)AT(C/T/C/G)AT(T/C/G) CG-3’ (Fig. 2). The cDNA template was amplified by PCR with RPLP1 and dT-adaptor as primers. A 1-µl aliquot of the first PCR product was used as template and amplified with RPLP2 and dT-adaptor as primers. PCR using the primer pair RPLP2 and dT-adaptor gave only one 1132-bp fragment whose deduced amino acid sequence exhibited approximately 80% identity with some previously cloned PALs. To obtain the full-length gene, 5′-RACE was performed using three gene-specific primers, designed according to partial sequences obtained through 3′-RACE. 5′-RACE cDNA synthesis was performed with a 5′-RACE System (version 2.0; Invitrogen Life Technologies) according to the manufacturer’s instructions. Databank searches were conducted through the BLAST program. The multiple sequence alignment was analyzed with the DNAMAN software package (version 4.0; Lynnon Biosoft, Quebec, Canada). A phylogenetic tree was constructed with the neighbor-joining algorithm using the DNAMAN software package, based on calculations from pairwise amino acid sequence distances for protein analyses derived from the multiple alignment formats. The dataset was subjected to 1000 bootstrap replications.

Southern and Northern blot analyses

Genomic DNA was isolated from Rhodiola sachalinensis calli using a modified CTAB method (Porebski et al. 1997). Southern blot analysis was done using the standard method (Sambrook et al. 1989): 10 µg genomic DNA of R. sachalinensis were digested with HindIII and KpnI (TaKaRa), resolved on 0.7% agarose gel, and then blotted onto a Hybond-N™ nylon membrane (Amersham). Hybridizations were performed for 16 h at 65 °C with the full-length PALrs1 cDNA probe labeled with digoxigenin-11-dUTP (DIG DNA Labeling and Detection Kit; Roche
stems and roots using Trizol (Invitrogen). For Northern
total RNA was prepared from the cultured calli, leaves,
Hybond N+ nylon membrane (Amersham). Hybridization
a formamide-containing agarose gel and blotted onto a
Protocol supplied by Roche.
luminescence detection was carried out according
time with MS liquid medium, and the
R. sachalinensis transformation. The leaves were immersed in 30 ml
A. tumefaciens; after 5–7 min, the explants were taken
hygromycin B was used to select the transfor-
medium + 0.1 mg
transformation. The leaves were immersed in 30 ml
Agrobacterium suspension was used for
agarose gel. Blots were washed twice with 2
chemiluminescence detection were as described above for DNA
gel blotting analysis.

Construction of plant expression vector
A fragment containing the open-reading frame (ORF) was
PCR-amplified using the primers RPLP-L (5'-ATATGGA
TTCCCTCTCACATTCGCAAGC-3') and RPLP-R (5'-
ATATGGTACCAGATGATGGGTAGGGGAGC-3'),
which the restriction enzyme sites BamHI (RPLP-L) and
Pml (RPLP-R) were introduced (underlined). The fragment including
PALrs1 cDNA digested with BamHI-
KpnI was subcloned into the BamHI-KpnI sites of the binary
vector pBrin713 driven by the 35S promoter with double-
enhancer sequences from the cauliflower mosaic virus
(CaMV) and tobacco mosaic virus (TMV) Ω-fragments.
The hygromycin gene encoding phosphotransferase was
used as the selection marker gene. Recombinant plasmids
(pRBinPALrs1) were identified by restriction analysis of
purified plasmid DNA and used for sequencing, and then
transformed into Agrobacterium tumefaciens (EHA105) by
the liquid nitrogen freeze–thawing method. A. tumefaciens
harboring the recombinant vector pBinnPALrs1 and an
empty vector (without insert) were used for R. sachalinensis
transformation.

Genetic transformation and regeneration of R. sachalinensis
Rhodiola sachalinensis transformation was performed essentially according to the method described by Ma et al.
(2007). For transformation experiments, young leaves of
4-week-old aseptic seedlings were used as explants. For
inoculation, A. tumefaciens was grown overnight at 28 °C,
under shaking, in liquid LB medium supplemented with
100 mg l−1 rifampicin, 50 mg l−1 streptomycin and
50 mg l−1 kanamycin. A 400-μl aliquot of the overnight
cultures was subcultured in 50 ml fresh LB medium
(without antibiotics) until the OD600 reached 0.6–0.8.
The subculture was pelleted by centrifugation and resus-
pended three times with MS liquid medium, and the
bacterial suspension was used for R. sachalinensis
transformation. The leaves were immersed in 30 ml
A. tumefaciens; after 5–7 min, the explants were taken
out, blotted with sterile filter paper and co-cultured on
solid MS medium in the dark at 25 °C for 5 day. After
co-cultivation, the explanted leaves were transferred to
solid callus-inducing medium (see above) to induce calli
growth. Hygromycin B was used to select the transfor-
mament cultures because of the high sensitivity of R. sachali-
linensis to hygromycin B (data not shown). The explanted
leaves were first cultured on medium without hygromycin
B for 10 day and then transferred onto medium with
10 mg l−1 hygromycin B for the first cycle selection. One
two weeks later, a few of the transformed calli were
brown and black. Most calli could grow normally, but
their growth rate was lower than in non-selection condi-
tions. Three to four weeks later, the calli were transferred
onto medium with 20 mg l−1 hygromycin B and culturing
was continued for 3–4 weeks. The observed results for
non-transformed calli, as controls, showed that they were
dark-brown and dead. Soon after, the resistant
calli were put onto medium with hygromycin B 30 mg l−1
for third cycle selection. Using the above selection pro-
cesses, a large number of morphologically normal and
well-grown putative transgenic calli were obtained. Calli
of bright green color after resistance selection were trans-
ferred to solid shoot-inducing medium (MS medium
+1.5 mg l−1 6-BA +0.05 mg l−1 NAA +0.15 mg l−1
GA₃) to induce shoots. Cefotaxime (cef) was used to eliminate
surplus Agrobacterium. The explants were transferred
to fresh medium every week during the first month; after
which, a subculture was made every 3 weeks. Cefotaxime
was reduced to 100 mg l−1 after 15 weeks and completely
omitted after 21 weeks. Six months later, the shoots were
transferred into a root-inducing medium (½ strength MS
medium + 0.1 mg l−1 NAA + 5.0 mg l−1
hygromycin B). The transgenic plants were subcultured in half-strength
MS medium for 45 day, and then transferred into med-
ium including sand and vermiculite (1:1). One month
later, plants were transplanted into pots.

Fig. 2. Amino acid sequence comparison of seven phenylalanine ammonia-lyase of plant origin. Multiple sequence alignment was calculated with the DNAMAN package. Black shading shows amino acid identities and gray shading shows amino acid similarity. Underlining indicates the PALrs1 cDNA fragment as a probe for Northern blot analysis. Arrows indicate the primer sites used in this study for 3' RACE. The abbreviations for species and GenBank accession numbers are: PALrs1 R.s. (Rhodiola sachalinensis, AY879309); PAL1 P.h. (Petunia x hybrida, AY705976); PAL N.t. (Nicotiana tabacum, X78269); PAL5 S.i. (Solanum lycopersicum, A44133); PAL1 A.t. (Arabidopsis thaliana, NM_129260.2); RlPAL2 R.i (Rubus idaeus, AF237955), and PAL P.t. (Populus trichocarpa x Populus deltoides, L11747.1).
Molecular characterization of transgenic *R. sachalinensis*

**PCR and PCR–Southern blot analysis**

Putatively transformed plants were initially analyzed by PCR assays. The genomic DNA of hygromycin B-resistant plants was isolated using sodium dodecyl sulfate (SDS) extraction methods (Lin et al. 2001). To avoid disturbance of the endogenous gene in *R. sachalinensis*, two specific primers were used in the PCR: the first was a sense primer 35S-UP (5′-TGATATCTCTCAGTGTAAGGGATG-3′) corresponding to the sequence of the CaMV35S promoter, and the other was RPLP-R (see above). The primers for empty vector transgenic plants used in the PCR were 35S-UP (see above) and GUS-R: 5′-GTGCCGATTCACCTGTCAC-3′. The amplified fragments were electrophoresed on 0.8% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham). After which, PCR–Southern blot analysis was carried out using the digoxigenin-11-dUTP-labeled full-length PALrs1 cDNA as probe.

**Northern blot analysis**

Total RNA was isolated from the leaves of transgenic plants and untransformed wild-type plants. The probe was the same as that described above for RNA gel blot analysis. The RNA (10 μg) was fractionated on a 1.2% formaldehyde–agarose gel. The fractioned RNA was transferred onto a Hybond-N+ nylon membrane (Amersham) by capillary blotting. Hybridization, washing of the membrane and chemiluminescence detection were the same as described above.

**Determination of phenolics and tyrosine**

Determination of phenolics was done essentially according to the method of Yao et al. (1995) and Xu et al. (1998). Plant materials were collected and then dried for 24 h in an oven at 60 °C. The dried plant material was ground to fine powder. 0.1–0.5 g of precisely weighed powder was added to an extraction bottle containing 5–10 ml of 100% methanol and extracted in a supersonic bath for 30 min at room temperature. The homogenate was incubated at 60 °C for another 30 min and then centrifuged for 15 min at 12,000 g. The supernatant was collected, and the pellets were extracted once more with 50% methanol. The supernatants were combined and purified using a 0.45-μm NC filter, then dried in a vacuum and redissolved in 3.0 ml 50% methanol. This preparation was used for quantitation of p-coumaric acid, tyrosine, tyrosol, and salidroside by HPLC. HPLC was performed under the following conditions: 3.9 × 300 mm C18, reverse phase column (Waters, Milford, MA, USA), mobile phase composed of water:methanol:acetonitrile (70:25:5, v/v/v), flow rate of 0.8 ml/min⁻¹, and a wavelength of 275 nm. The injection volume was 10 μl. The amount of tyrosine, tyrosol and salidroside was determined using authentic standards (Sigma). The p-coumaric acid was kindly provided by Dr Liu (Technische Universität Braunschweig, Germany).

**RESULTS**

**Isolation and sequence analysis of the PALrs1 cDNA**

A cDNA clone encoding a PAL was isolated from *Rhodiola sachalinensis* and named PALrs1 (GenBank accession No. AY879309). The nucleotide sequence of PALrs1 is 2407-bp long with an ORF of 2133 bp (positions 124–2256), a 123-bp leader sequence, and a 3′ untranslated region of 133 bp, including an 18 nucleotide poly (A) tail. A putative polyadenylation signal was found 51 nucleotides before the poly (A) tail. The analysis of the PALrs1 cDNA clone predicted a polypeptide of 710 amino acids with a calculated molecular mass of 77.45 kDa and a predicted isoelectric point of 6.5 (data not shown). The deduced *R. sachalinensis* PALs1 protein shared, at the amino acid level, a degree of similarity of about 80% with those deduced from cDNA clones isolated from other dicotyledons (Fig. 2). The amino acid sequence of PALrs1 revealed 82% identity with *Populus trichocarpa* PAL (Subramaniam et al. 1993), 80% identity with *Nicotianatabacum* PAL (Pellegrini et al. 1994), 79% identity with *Arabidopsis thaliana* PAL1 (Coehrane et al. 2004), 78% identity with *Rubus idaeus* RiPAL2 (Kumar & Ellis 2001), 76% identity with *Petunia hybridra* PAL2 (Verdonk et al. 2005), 65% identity with *Oryza sativa* PAL (Minami et al. 1989), and 64% identity with *Zea mays* PAL (Rosler et al. 1997). Phylogenetic analysis of the PAL genes from plants also showed that PALrs1 is located adjacent to *P. trichocarpa* PAL, *A. thaliana* PAL1 and *Vitis vinifera* PAL. Using the *Rhodosporidium toruloides* PAL sequence as an outgroup, the high bootstrap values (100% of the 1000 bootstrap replications) place dicot and monocot PALs in separate monophyletic groups (data not shown).

**Genomic structure of PALrs1 in *R. sachalinensis***

Genomic DNA isolated from calli of *R. sachalinensis* was digested with *HindIII* and *KpnI*. Southern blot analysis performed under the most stringent hybridization conditions using PALrs1 cDNA as a probe revealed three (the blot of the single *KpnI* fragments) to five fragments (the blot of the *HindIII* fragments) (Fig. 3). The number of hybridized fragments obtained with restriction enzymes that do not have a *HindIII* and *KpnI* recognition sequence within the *PALrs1* cDNA indicates that the *R. sachalinensis* PAL gene family is composed of 3–5 genes.

**Relationship between PALrs1 expression and accumulation of salidroside**

In order to investigate the tissue-specific expression of PALrs1 of *R. sachalinensis*, total RNA was isolated from roots, calli, stems and leaves. Northern blot analysis was performed using the *PALrs1* cDNA fragment (1703–2213th nucleotide acid sequence) to synthesize the labeled probe; in this region nucleotide homology with other
plant PAL genes was only about 65% (Fig. 2). Northern blot analysis revealed that the accumulation of PALrs1 transcripts was more abundant in calli than in stems and leaves, and very low levels of expression were observed in roots (Fig. 4A). To understand the correlation between PALrs1 expression and salidroside accumulation, the salidroside content of the above materials was determined. The salidroside content of roots and calli was higher than in stems and leaves (Fig. 4B), which indicated a lack of correlation between levels of PALrs1 transcriptional expression and the accumulation of salidroside.

Generation of transgenic R. sachalinensis plants overexpressing PALrs1

To further investigate the role of the PALrs1 gene in the regulation of salidroside accumulation, the plasmid construct pRBinPALrs1 containing the PALrs1 downstream of the 35S promoter with double-enhancer sequences from CaMV and TMV-Ω fragments and empty vector (without insert) were introduced into Rhodiola sachalinensis. For R. sachalinensis, genetic transformation and regeneration was performed essentially according to the method described by Ma et al. (2007). Six PALrs1 transgenic plant lines (TP-B1 to TP-B6) and three independent empty vector-transformed plant lines (EP-B1 to EP-B3) were obtained and identified using PCR, PCR–Southern blot and Northern blot analysis.

Effects of overexpressing PALrs1 on the accumulation of salidroside

DNA was isolated from hygromycin B-resistant plant lines and a non-transformed wild-type plant line, preliminarily identified by means of PCR and PCR–Southern blot. The PCR using relative sequences of the 35S promoter and PALrs1 gene as primers (for the PALrs1-transformed plant lines) and 35S promoter and GUS gene as primers (for the empty vector-transformed plant lines) had a 2.2 kb band (Fig. 5A) and a 500-bp band (data not shown), respectively. The size of the 2.2-kb band was the same as that of pRBinPALrs1 as positive control, and no DNA band was detected in the non-transformed control. PCR–Southern blot analysis confirmed that the 2.2-kb band was indeed a product amplified by the PALrs1 fusion gene (Fig. 5B). The three PCR-positive PALrs1-transformed plant lines (TP-B1, TP-B2 and TP-B5), one empty vector-transformed plant line (EP-B1), and one non-transformed wild-type plant line were selected for Northern blot analysis (Fig. 6). PCR, PCR–Southern blot and Northern blot results indicate that the foreign gene PALrs1 was integrated into the genome of PALrs1 transgenic plants and was overexpressed at transcriptional levels.

In order to detect any changes in the level of phenolic compounds in the leaves of PALrs1 transgenic plants compared to the empty vector-transformed plant and untransformed wild-type plant, HPLC was performed on the above materials of R. sachalinensis. The results indicate that the overexpression of PALrs1 in transgenic...
Accumulation of salidroside in *Rhodiola sachalinensis*

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Fig. 5. Identification of transgenic plants by PCR and PCR–Southern blot analysis for the PALrs1 gene. A: Identification of transgenic plants by PCR. B: Identification of transgenic plants by PCR–Southern blot. The pRBinPALrs1 vector was employed in the PCR with the same primers as the positive control (PCK); DNA isolated from non-transgenic plants was used as a template for the negative control (RCK); TP-B1, TP-B2 and TP-B5: DNA from the leaves of transgenic plant lines.

Fig. 6. Identification of PALrs1 transgenic plants by Northern blot analysis for the PALrs1 gene. 1, 2 and 3: RNA from the leaves of PALrs1 transgenic plant lines TP-B1, TP-B2 and TP-B5; 4: RNA from leaves of the empty vector-transgenic plant line EP-B1. 5: RNA from the leaves of the non-transgenic plant.

Plants resulted in accumulation of p-coumaric acid, as expected. The p-coumaric acid content of the PALrs1 transgenic plant was 3.3-fold higher than in the controls (Fig. 7A). However, the levels of tyrosol and salidroside were 4.7-fold and 7.7-fold lower in two of the PALrs1 transgenic plant lines than in controls (Fig. 7B and C). To investigate further effects of the related tyrosine content caused by the overexpression of the PALrs1 gene, we also tested for the tyrosine content. As shown in Fig. 7D, tyrosine levels were consistently 2.6-fold lower in PALrs1 transgenic plant lines compared with the controls.

**DISCUSSION**

Glucosylation of tyrosol is thought to be the final step in salidroside biosynthesis. However, the biosynthetic pathway of tyrosol and its regulation are less well understood. From a biochemical point of view, tyrosol is a typical phenolic low-molecular weight compound and is likely to originate from the phenylpropanoid pathway (Li et al. 2005; Xu & Su 1997; Fig. 1A). PAL is the first enzyme of the phenylpropanoid pathway. PAL catalyzes the nonoxidative deamination of phenylalanine to yield cinnamic acid, a reaction that is generally considered to represent a key point at which carbon flux into this pathway is controlled.

Manipulation of metabolic pathways is usually achieved by modulating the expression of endogenous enzymes or by introducing new enzyme activities. Here, using the RACE method, we successfully isolated a PAL cDNA clone from the calli of *R. sachalinensis*. The molecular phylogenetic tree based on a multiple sequence alignment of PALs1 and 17 other plant PALs retrieved from the database showed that PALs1 is located adjacent to *P. trichocarpa* PAL, *A. thaliana* PAL1 and *V. vinifera* PAL (data not shown). Southern blot analysis indicated that, in *R. sachalinensis*, PAL is encoded by a small family of three to five genes. PAL genes from other plants, namely parsley, bean, and *Arabidopsis thaliana*, have already been shown to be organized in small families of three to four genes that are expressed differentially during development and in response to different types of environmental stimuli, such as UV irradiation, treatment with a fungal elicitor, or wounding (Pellegrini et al. 1994). In potato, the genomic organization of PAL is particularly complex, since as many as 40 genes per haploid genome have been detected, but similar patterns of PAL expression have been reported (Rumeau et al. 1990; Joos & Hahlbrock 1992).

Northern blot analysis and HPLC results revealed a lack of correlation between the levels of PALrs1 transcriptional expression and the production of salidroside. Molecular analysis and HPLC results for transgenic *R. sachalinensis* indicated that overexpression of the endogenous PALrs1 resulted in a 3.3-fold increase of p-coumaric acid content compared to controls, as expected. Because phenolic compounds rarely accumulate in their free form in plant cells and are often conjugated to sugars, most often glucose (Vogt & Jones 2000), in this experiment we determined the tyrosol and salidroside content at the same time. In contrast, overexpression of the endogenous PALrs1 gene and increased p-coumaric acid content did not result in increased tyrosol and salidroside amounts, and the levels of tyrosol and salidroside were 4.7-fold and 7.7-fold lower in PALrs1 transgenic plants than in empty vector-transformed and untransformed controls. This suggests that tyrosol biosynthesis does not have any direct linkage with p-coumaric acid accumulation and these results demonstrate that it is highly unlikely that p-coumaric acid is a precursor for tyrosol biosynthesis.

Transformation of *R. sachalinensis* with the PALrs1 gene resulted in PALrs1 transgenic plants that contained levels of tyrosine that were 2.6-fold lower than those in empty vector-transformed and untransformed controls. These results suggest that PALrs1 activity has a considerable effect on the content of this amino acid. Tyrosine and phenylalanine are both derived from the shikimate pathway through a branch point controlled by the key enzyme, chorismate mutase (Fig. 1). It is known that...
phenylalanine and tyrosine inhibit their own synthesis through feedback inhibition of chorismate mutase (Yao et al. 1995). Chorismate is converted to prephenate and then to arogenate, after which arogenate is converted either to phenylalanine or tyrosine by arogenate dehydratase or arogenate dehydrogenase (Fig 1). An increase in the cellular concentration of phenylpropanoid metabolites like p-coumaric acid could therefore increase the flux of chorismate through the phenylalanine branch of the pathway, concomitantly decreasing the production of tyrosine. In fact, the concentration of tyrosine was consistently at least 2.6-fold lower in PALrs1 transgenic plants than in the controls (Fig. 7D). Indeed, apart from p-coumaric acid, another possible precursor of tyrosol is tyramine, which is synthesized from tyrosine (Fig. 1B). Tyramine in plants is produced from tyrosine by the enzyme tyrosine

Fig. 7. Effects of overexpression of the PALrs1 gene on accumulation of p-coumaric acid (A), tyrosol (B), salidroside (C) and changes in the level of tyrosine (D). 1 and 2: PALrs1 transgenic plant lines TP-B1 and TP-B2; 3: empty vector transgenic plant line EP-B1; 4: non-transgenic plant. Data are the mean ± SD of three experiments.
decarboxylase (TyrDC). An early study indicated that TyrDC supports biosynthesis of the hydroxyphenylethanol glycoside, verbascoside in *Syringa vulgaris* (Ellis 1983). A recent study has demonstrated that overexpression of parsley TyrDC leads to accumulation of a new compound, tyrosol glucoside (salidroside), in potato. However, as an adaptogenic drug, salidroside has been identified in a limited number of plants, such as golden root, *Linaria japonica*, birch and olive trees, but not in potato. Figure 1B outlines the presumed four steps of salidroside biosynthesis in potato (Landtag et al. 2002). In this study, 4-hydroxyphenylacetaldehyde (4-HPAA) is the direct precursor for tyrosol biosynthesis.

Salidroside is a novel effective adaptogenic drug. At present, there is far from enough salidroside on the commercial market because of the relatively low content of salidroside in *R. sachalinensis* (0.5–1.0% dry weight) and because wild *R. sachalinensis* is on the edge of extinction due to over-gathering. Considerable efforts over many years have focused on enhancing the production of salidroside. Chemosynthesis and manipulation of cell and tissue cultures for salidroside production have also been investigated (Zhang et al. 1997; Xu et al. 1998; Wu et al. 2003). In particular, Wu et al. (2003) reported that using a specific combination of culture condition and treatment, salidroside accumulation could reach 57.72 mg g⁻¹ dry weight, which is 5- to 10-fold higher than that detected in field-grown plants. In addition, one of the most reasonable approaches to enhancing the production of salidroside is the use of genetic engineering to regulate enzyme activity of the rate-limiting steps of salidroside biosynthesis. Ma et al. (2007) reported that overexpression of the endogenous UDP-glucosyltransferase (UGT73B6) caused the salidroside content to increase 20-fold compared with that of untransformed controls in the transgenic *R. sachalinensis*; but the lack of tyrosol supply may be a limiting factor for further accumulation of salidroside. However, the biosynthetic pathway of tyrosol and its regulation are less well understood. A consensus of results of related studies shows that there are two different possibilities for the tyrosol biosynthetic pathway. One is that tyrosol is presumably produced from the *p*-coumaric acid precursor by decarboxylase, mainly derived from phenylalanine (Li et al. 2005; Xu & Su 1997; Fig. 1A). The other is that the precursor of tyrosol may be tyramine, which is synthesized from tyrosine (Landtag et al. 2002; Ellis 1983; Fig. 1B). However, the *p*-coumaric acid decarboxylase action has only been described for microorganisms (Cavin et al. 1997); molecular research on genes related to plant *p*-coumaric acid decarboxylase has not been reported and there is no information yet available for plant *p*-coumaric acid decarboxylase. However, the enzymes catalyzing the conversion of tyrosine to 4-HPAA (Fig. 1B) were found in plants (Landtag et al. 2002). The results presented in this paper for the first time provide important *in vivo* evidence that the biosynthesis of tyrosol, a phenylethanoid derivative, is not derived from phenylalanine. The reduced availability of tyrosine most likely resulted in a large reduction in tyrosol biosynthesis and accumulation of salidroside. Based on our data, future investigation of the regulation of tyrosol biosynthesis should focus on a detailed functional analysis of TyrDC genes in *R. sachalinensis*. Recently, a TyrDC clone (GenBank accession No. DQ471943) was isolated from the callus of *R. sachalinensis* in our laboratory. It will be interesting to combine the overexpression and antisense expression of this gene with the consequences of changing the level of tyrosol and salidroside *in vivo*.

REFERENCES


Accumulation of salidroside in *Rhodiola sachalinensis*


