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ORIGINAL PAPER

A tyrosine decarboxylase catalyzes the initial reaction of the salidroside biosynthesis pathway in *Rhodiola sachalinensis*

Ji-Xing Zhang · Lan-Qing Ma · Han-Song Yu · Hong Zhang · Hao-Tian Wang · Yun-Fei Qin · Guang-Lu Shi · You-Nian Wang

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Abstract Salidroside, the 8-*O*-*β*-D-glucoside of tyrosol, is the main bioactive component of *Rhodiola* species and is found mainly in the plant roots. It is well known that glucosylation of tyrosol is the final step in the biosynthesis of salidroside; however, the biosynthetic pathway of tyrosol and its regulation are less well understood. A summary of the results of related studies revealed that the precursor of tyrosol might be tyramine, which is synthesized from tyrosine. In this study, a cDNA clone encoding tyrosine decarboxylase (TyrDC) was isolated from *Rhodiola sachalinensis* A. Bor using rapid amplification of cDNA ends. The resulting cDNA was designated *RsTyrDC*. RNA gel–blot analysis revealed that the predominant sites of expression in plants are the roots and high levels of transcripts are also found in callus tissue culture. Functional analysis revealed that tyrosine was best substrate of recombinant RsTyrDC. The over-expression of the sense-*RsTyrDC* resulted in a marked increase of tyrosol and salidroside content, but the levels of tyrosol and salidroside were 274 and 412%, respectively, lower in the antisense-*RsTyrDC* transformed lines than those in the controls. The data presented here provide in vitro and in vivo evidence that the *RsTyrDC* can regulate the tyrosol and salidroside biosynthesis, and the *RsTyrDC* is most likely to have an important function in the initial reaction of the salidroside biosynthesis pathway in *R. sachalinensis*.

Keywords Over-expression · *Rhodiola sachalinensis* · Salidroside · Tyrosine decarboxylase (TyrDC) · Tyrosol

Abbreviations

HPLC	High-pressure liquid chromatography
IPTG	Isopropyl thio-*β*-D-galactopyranoside
LC-ESIMS	Liquid chromatography electron spray ionization mass spectrometry
PLP	Pyridoxal 1-phosphate
TyrDC	Tyrosine decarboxylase

Introduction

Rhodiola spp. grows at high altitude and in cold regions of the northern hemisphere. The flora of China contains about 90 species, with 55 in China and 16 endemic there (Fu et al. 2009). Recently, phytochemical extracts of *Rhodiola* spp. have been the source of important commercial

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Ji-Xing Zhang, Lan-Qing Ma contributed equally to this work.

Gene: *RsTyrDC* (GenBank accession no. DQ471943).

J.-X. Zhang
College of Life Science, Inner Mongolia University for Nationalities, 028043 Tongliao, People's Republic of China

L.-Q. Ma · H. Zhang · Y.-F. Qin · G.-L. Shi (✉) · Y.-N. Wang (✉)
Key Laboratory of Urban Agriculture (North) of Ministry of Agriculture People's Republic of China, Beijing University of Agriculture, 102206 Beijing, People's Republic of China
e-mail: glshi@bac.edu.cn

Y.-N. Wang
e-mail: lqma@bac.edu.cn

H.-S. Yu
Food Science and Engineering College, Jilin Agricultural University, 130118 Changchun, People's Republic of China

H.-T. Wang
School of Pharmaceutical Sciences, Jilin University, 130012 Changchun, People's Republic of China

preparations used throughout Europe, Asia and the USA (Tolonen et al. 2003; Yousef et al. 2006). It is well established that salidroside is the main bioactive component of *Rhodiola* spp., and is found mainly in the roots. Salidroside, a tyrosol 8-*O*-*β*-D-glucoside (Fig. 1) that is synthesized by the UGT-catalyzed glucosylation of tyrosol (Ma et al. 2007; Xu et al. 1998), has recently attracted increased attention because of its important role in the adaptogenic effect, such as treating anoxia, microwave radiation and fatigue and slowing the aging process (Mao et al. 2010; Ouyang et al. 2010).

It is well known that glucosylation of tyrosol is the final step in the biosynthesis of salidroside; the storage form of tyrosol in plants (Fig. 1); however, the biosynthetic pathway of tyrosol and its regulation are less well understood. The results of related studies revealed that there are two different possibilities for the tyrosol biosynthetic pathway.

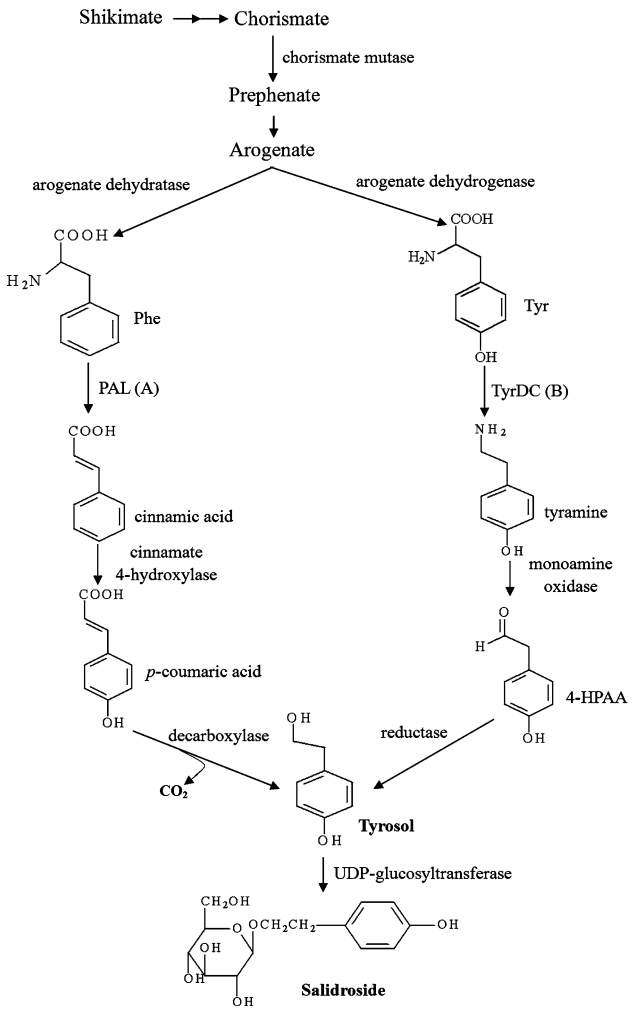

Fig. 1 Proposed biosynthetic pathway of salidroside formation (Ma et al. 2008). Phe, phenylalanine; Tyr, tyrosine; PAL, phenylalanine ammonialyase; TyrDC, tyrosine decarboxylase; 4-HPAA, 4-hydroxyphenylacetaldehyde

One possibility is that tyrosol is produced from a *p*-coumaric acid precursor, which is derived mainly from phenylalanine (Li et al. 2005; Xu and Su 1997) (Fig. 1a). The second possibility is that the precursor of tyrosol might be tyramine, which is synthesized from tyrosine (Ellis 1983; Landtag et al. 2002) (Fig. 1b). Our recent work demonstrated that over-expression of the endogenous phenylalanine ammonia-lyase gene (*PALrs1*) and accumulation of *p*-coumaric acid did not facilitate tyrosol biosynthesis. In contrast, levels of tyrosol and salidroside were 470 and 770% lower, respectively, in *PALrs1* transgenic plants than those in the controls. Furthermore, over-expression of the *PALrs1* gene resulted in a 260% decrease in tyrosine content. These data suggest that the reduced availability of tyrosine most likely resulted in a large reduction of tyrosol biosynthesis and accumulation of salidroside. Tyrosol, a phenylethanoid derivative that is not derived from the phenylpropanoid pathway, is most likely to originate from the alkaloids pathway (Fig. 1); (Ma et al. 2008).

Tyrosine is an important precursor of many secondary metabolites in higher plants, and tyrosine decarboxylase (TyrDC, EC 4.1.1.25), which is responsible for diverting these essential primary metabolites into secondary metabolic pathways, typically has key regulatory functions in the control of end-product biosynthesis (Facchini et al. 2000). Here, we report the cloning and expression pattern of a cDNA encoding *TyrDC* from *R. sachalinensis* in an attempt to elucidate the molecular pathways needed for tyrosol biosynthesis in *Rhodiola* plants. More specifically, in the present study, the biochemical assays of recombinant RsTyrDC and the effects of sense and antisense over-expression of endogenous *RsTyrDC* in *R. sachalinensis* on tyrosol synthesis were investigated. The data presented here provide in vitro and in vivo evidence that the *RsTyrDC* is most likely to have an important function in the initial reaction of the salidroside biosynthesis pathway in *R. sachalinensis*.

Materials and methods

Plant material

Whole plants

Seeds of *R. sachalinensis* were collected from Changbai Mountain, Jilin Province, China. The seeds were surface-sterilized by immersion in 0.1% (w/v) mercuric chloride for 20 min, washed thoroughly with sterile distilled water and then allowed to germinate on solid MS medium, pH 5.8 (Murashige and Skoog 1962) supplemented with 3.0% (w/v) sucrose before the addition of agar to a final concentration of 0.75% (w/v). After ~30 days of culture, the

middle or upper leaves of *R. sachalinensis* were used as explants.

Callus cultures

The middle or upper leaves of whole plants were cut into 0.5 cm × 0.5 cm pieces and transferred to callus-inducing medium (MS basal medium supplemented with 1.5 mg l^{-1} 6-BA (6-benzylaminopurine), 0.15 mg l^{-1} NAA (α-naphthlcetic acid) and 0.5 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid)) to induce callus formation. Further subculturing was done every 3–4 weeks on callus subculturing medium (callus-inducing medium without 2,4-D). All plants and cultures were kept at 24 (±1)°C, with a photoperiod of 16 h light/8 h dark in an environmental chamber with a light intensity of 150 μE m^{-1} s^{-1} provided by cool white fluorescent lamps. The *R. sachalinensis* plants were rooted on MS medium and grew on peat soil in a greenhouse with alternating 16 h light (5000 lx) at 27°C and 8 h dark at 20°C.

Chemicals

Salidroside was purchased from Tauto Biotech (Shanghai China). Tyrosol, L-tyrosine, L-dopa, L-tryptophan, L-phenylalanine, tyramine, dopamine, tryptamine, phenethylamine and pyridoxal 1-phosphate were purchased from Sigma-Aldrich (China).

PCR-based cloning

Total RNA was prepared from 3 weeks old calli of *R. sachalinensis* using a Total RNA Isolation Kit (Autolabtech, Beijing, China). Reverse transcription was done at 42°C with the ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA) and the oligo(dT)-adaptor primer: PdT, CCAGTGAGCAGAGTGACGAGGACTC GAGCTCAAGCTTTTTTTTTTTTTTTT. The degenerate primer was designed for 3′-rapid amplification of cDNA ends (RACE) on the basis of the consensus-degenerate hybrid oligonucleotide primers (CODEHOP) strategy by the block maker program (http://www.blocks.fhcrc.org/blocks/blockmkr/make_blocks.html) as follows: PRsTDC, 5′-GAGTTCCAGCACTACCTCGAyggnrtngar-3′ where *n* is any nucleotide, *r* is A or G and *y* is C or T (Fig. 2). The DNA fragment was amplified using high-fidelity PrimeSTAR® HS DNA Polymerase (TaKaRa, Dalian, China). Denaturation at 95°C for 4 min, then 30 cycles at 94°C for 30 s, 55°C for 40 s, 72°C for 120 s and a final extension step at 72°C for 7 min. The gel-purified PCR product was ligated into pMD18-T Vector (TaKaRa) and sequenced.

The full-length cDNA sequence was obtained by 5′-RACE with a SMART™ RACE cDNA Amplification Kit (CLONTECH Life Technologies) and specific primers based on the cDNA sequences obtained: TGSP1, 5′-CGC TAGAGATTGGATTAGG-3′ and TGSP2, 5′-CACATGA AGCAGCAATCGAG-3′. The PCR conditions were the same as described above for the 3′-RACE.

RNA gel–blot analysis

To study the tissue specificity of *RsTyrDC* expression, total RNA was prepared from young leaves, stems, roots or calli. Total RNA was examined qualitatively and quantitatively by measuring A_{260} and A_{280}. Samples (10 μg) of total RNA were separated by electrophoresis in a formaldehyde-containing 1.2% (w/v) agarose gel in Mops buffer and transferred to a Hybond-N^+ nylon membrane (Amersham) by capillary transfer (Sambrooke and Russell 2001). Hybridization was done using an [α-^{32}P]dCTP-labeled *RsTyrDC* fragment (623 bp) including both the coding and 3′ untranslated regions as probe (Fig. 2). The primers used to amplify the probe were: forward, 5′-CTCGATTGCTGC TTCATGTGG-3′ and reverse, 5′-GATGACAACACAC GCAACCCTA-3′. Probe preparation was done according to the protocol of the Prime-a-Gene Labeling System (Promega). The DNA blot was washed in 2× SSC, 0.1% (w/v) SDS at 65°C for 15 min, then in 0.1× SSC, 0.1% SDS at 65°C for 30 min, and exposed at −75°C for 72 h to Kodak X-ray film (Kodak Rochester, NY, USA).

Heterologous expression in *Escherichia coli* and recombinant enzyme purification

Heterologous expression and purification of recombinant RsTyrDC were as described (Ma et al. 2009a, b). For *RsTyrDC*, the open reading frame (ORF) of the cDNA was amplified using N-terminal and C-terminal PCR primers: forward, 5′-TATAGGTACCATGGGAAGCTTGCCATCT CC-3′ where the *Kpn*I site is underlined; reverse, 5′-TATAGAATTCCTATGGCATGATGCTTCGAGCTG-3′ where the *Eco*RI site is underlined. The amplified DNA fragment from *RsTyrDC* was digested with *Kpn*I/*Eco*RI, and cloned into the *Kpn*I/*Eco*RI site of pET-30a (+) (Novagen, Darmstadt, Germany). The recombinant enzymes contained a His_6 tag at the N-terminus.

After sequencing the ORF on both strands, the recombinant plasmid was introduced into *E. coli* Rosetta-gami™ (DE3) (Novagen) and grown at 37°C with shaking (200 rpm) in 200 ml of Luria–Bertani (LB) medium containing kanamycin (50 μg ml^{-1}) and chloramphenicol (34 μg ml^{-1}). At an A_{600} of 0.6–0.8, isopropyl thio-β-D-galactopyranoside (IPTG) was added to 1.0 mM final concentration and the incubation temperature was reduced to 27°C. After incubation for 7 h, cells were harvested by low-speed centrifugation, suspended in 3 ml of 0.1 M potassium phosphate

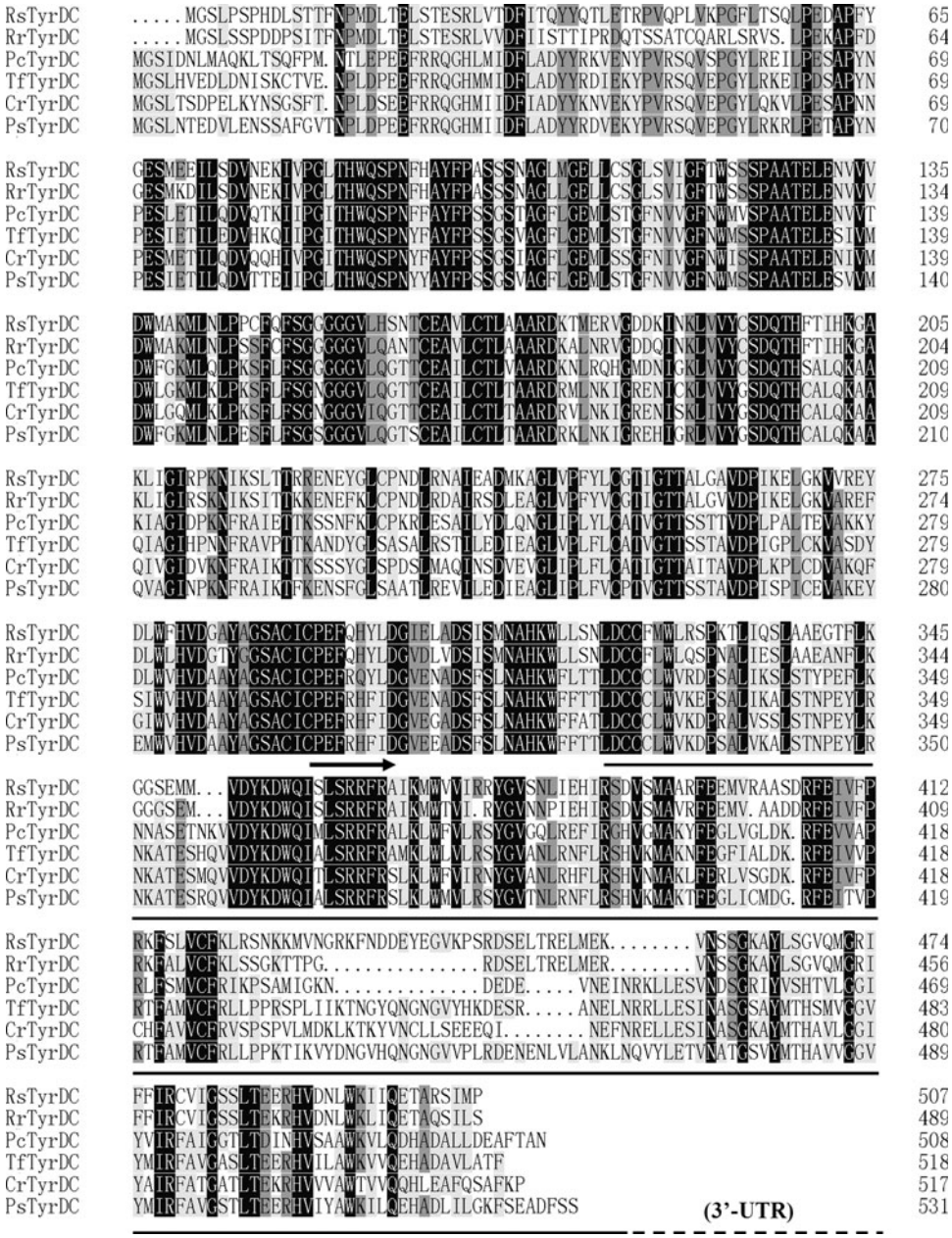

Fig. 2 Amino acid sequence comparison of six tyrosine decarboxylases of plant origin. Multiple sequence alignment was done with the DNAMAN package. *Black shading* shows amino acid identity and *gray shading* shows amino acid similarity. Underlining indicates the *RsTyrDC* cDNA fragment as a probe for RNA gel–blot analysis. *Arrow* indicates the primer (PRsTDC) site used in this study for 3′ RACE. The abbreviations for species and GenBank accession numbers are: RsTyrDC (*Rhodiola sachalinensis*, DQ471943), RrTyrDC (*Rhodiola rosea*, DQ431472), PcTyrDC (*Petroselinum crispum*, M95685), TfTyrDC (*Thalictrum flavum*, AF314150), CrTyrDC (*Citrus reshni*, GQ428145) and PsTyrDC (*Papaver somniferum*, U08598)

(pH 7.5), and sonicated on ice for 10 min. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C, then the supernatant was passed through a column of Ni–NTA His-Bind™ Resin (Novagen) containing Ni^{2+} as an affinity ligand. After washing with 0.1 M potassium phosphate buffer (pH 7.5) containing 0.5 M NaCl and 40 mM imidazole, the recombinant proteins were eluted with 0.1 M potassium phosphate (pH 7.5) containing 400 mM imidazole. For long-term storage at −80°C, the buffer was changed to 0.1 M Tris–HCl (pH 7.5), 10% (v/v) glycerol using PD-10 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The efficiency of purification was monitored by SDS-PAGE. Protein concentration was determined by the Bradford method with BSA as the standard.

Enzyme reaction and product analysis

The 250 µl standard assay volume containing 50 mM Tris–HCl (pH 7.2), 1.0 mM EDTA, 25 µM pyridoxal 1-phosphate (PLP), 0.5 mM aromatic amino acid substrate and 3.0 µg of protein was incubated at 30°C for 60 min, then extracted twice with 250 µl of ethyl acetate and centrifuged at 12,000*g* for 10 min. The residue was dried under vacuum and then dissolved in 50 µl of 50% (v/v) methanol.

Analysis of the enzymatic products was done by high-pressure liquid chromatography (HPLC) on a Kromasil C_{18} reverse phase column (250 mm × 4.6 mm, 5 μm, Macherey–Nagel, Düren, Germany). A Waters Alliance 2695 HPLC system and a Waters 2487 photodiode array detector were controlled by a computer running Empower software (Waters, Milford, MA, USA). The eluent was methanol/water/acetic acid (1:8.95:0.05, by vol.), pH 3.4 at a flow rate of 0.6 ml min^{-1}. Absorbance was measured at a wavelength of 280 nm (A_{280}). The amounts of the products were determined from the standard curves. For determination of kinetic parameters of recombinant RsTyrDC, the concentration of the tyrosine substrate was varied from 10 μM to 3.5 mM. The incubations were carried out at 30°C for 60 min. The kinetic values were estimated of Lineweaver–Burk plots from duplicate experiments (average of triplicates ± SD).

HPLC–mass spectrometry (HPLC–MS) was done with a Shimadzu LC-10ADvp HPLC system coupled to a Shimadzu LCMS-2010A single quadrupole mass spectrometer with an electrospray ionization (ESI) interface (Shimadazu, Kyoto, Japan). Data acquisition and processing were done with Shimadzu LCMS software for LC-ESIMS-2010. HPLC was done under the conditions described above. The optimized MS operating conditions were as follows: all spectra were obtained in positive mode over an *m/z* range of 120–350; drying gas flow, 1.5 l min^{-1}; CDL temperature, 250°C; block temperature, 200°C; and probe voltage +4.5 kV.

Construction of plant sense and antisense expression vectors

To prepare the sense insert, the ORF of the *RsTyrDC* cDNA was amplified using PCR primers: forward, 5′-TATAAGATCTATGGGAAGCTTGCCATCTCC-3′ (pRsTDC-s-up) where the *Bgl*II site is underlined and reverse, 5′-TATAACTAGTGGCATGATGCTTCGAGCTG-3′ (pRsTDC-s-dw) where the *Spe*I site is underlined. To prepare the antisense insert, the ORF of the *RsTyrDC* cDNA was amplified using PCR primers: forward, 5′-TATAAGATCTGGCATGATGCTTCGAGCTG-3′ (pRsTDC-as-up) where the *Bgl*II site is underlined and reverse, 5′-TATAACTAGTATGGGAAGCTTGCCATCTCCTC-3′ (pRsTDC-as-dw) where the *Spe*I site is underlined. The amplified DNA was digested with *Bgl*II/*Spe*I, and cloned into the *Bgl*II/*Spe*I site of binary vector pCAMBIA1301 driven by the CaMV35S promoter. The hygromycin gene encoding phosphotransferase was used as the selection marker. Recombinant plasmids (pCA-s-RsTDC and pCA-as-RsTDC) 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 vectors pCA-s-RsTDC and pCA-as-RsTDC and an empty vector (without insert) were used for *R. sachalinensis* transformation.

Plant transformation

Rhodiola sachalinensis transformation was performed essentially as described (Ma et al. 2007, 2008) but with a small modification. Young leaves of 4-week-old aseptic seedlings were used as explants for transformation experiments. For inoculation, *A. tumefaciens* was grown overnight at 28°C, with 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 sample of the overnight cultures was subcultured in 50 ml of fresh LB medium (without antibiotics) until A_{600} reached 0.6–0.8. The subculture was pelleted by centrifugation and suspended three times with MS liquid medium, and the bacterial suspension was used for *R. sachalinensis* transformation. The leaves were immersed in 30 ml of *A. tumefaciens* growth medium; 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 days. After co-cultivation, the explanted leaves were transferred to solid shoot-inducing medium (MS medium containing 1.5 mg l^{-1} 6-BA, 0.05 mg l^{-1} NAA and 0.15 mg l^{-1} GA3 (gibberellic acid)) to induce shoot growth. Hygromycin B (20 mg l^{-1}) was used to select the transformant cultures because of the high sensitivity of *R. sachalinensis* to hygromycin B (data not shown). Afterward, a subculture was made every 3 weeks. 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. The concentration of cef was reduced to 100 mg l^{-1} after 9 weeks and completely omitted after 15 weeks. Four months later, the shoots were transferred onto a root-inducing medium (half-strength MS medium containing 0.1 mg l^{-1} NAA and 5.0 mg l^{-1} hygromycin B). The transgenic plants were subcultured in half-strength MS medium for 45 days, and then transferred onto medium including sand and vermiculite (1:1). One month later, plants were transplanted into pots.

Molecular characterization of transgenic plant

PCR and PCR–Southern blot analysis

Putatively transformed plants were initially analyzed by PCR. The genomic DNA of hygromycin B-resistant plants was isolated using the SDS extraction method (Sambrooke and Russell 2001). To avoid disturbance of the endogenous

gene in *R. sachalinensis*, two specific primers were used in the PCR. The first was a forward primer: 35 S-UP, 5′-TG ATATCTCCACTGACGTAAGGGATG-3′ corresponding to the sequence of the CaMV35S promoter, and the others were pRsTDC-s-dw and pRsTDC-as-dw (see above). The primers for empty vector transgenic plants used in the PCR were 35 S-UP (see above) and GUS-R, 5′-GTGCGGATT CACCACTTGC-3′. The amplified fragments were subjected to electrophoresis in 0.8% agarose gel and transferred to a Hybond-N^+ nylon membrane (Amersham). PCR–Southern blot analysis was done with [α-^{32}P]dCTP-labeled *RsTyrDC* full-length cDNAs in the sense and antisense orientations as probes.

RNA gel–blot analysis

Total RNA was isolated from the roots of putatively transformed plants. RNA gel–blot analysis was done with [α-^{32}P]dCTP-labeled *RsTyrDC* full-length cDNA as probe. The RNA (10 μg) was fractionated in a formaldehyde-containing 1.2% agarose gel. The fractionated RNA was transferred onto a Hybond-N^+ nylon membrane (Amersham) by capillary blotting and hybridization, washing of the membrane and detection were as described above.

Enzyme extraction and assays

For TyrDC activity analysis of sense-*RsTyrDC* and antisense-*RsTyrDC* transgenic plant lines, the roots (10 g FW) of transformed plant lines and empty vector-transformed lines were mixed with 2.5 g of Polyclar AT (Serva, Heidelberg, Germany) and homogenized in 20 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT). After centrifugation at 15,000*g* for 20 min, the supernatant was passed through a PD-10 column (Pharmacia, Freiburg, Germany) equilibrated with the same buffer. All steps were done at 4°C and the supernatant was used as a crude enzyme preparation.

For enzyme assays of the crude enzyme, the standard 250 μl assay contained 50 mM Tris–HCl (pH 7.2), 1.0 mM EDTA, 25 μM pyridoxal 1-phosphate (PLP), 0.5 mM tyrosine substrate and a sample of the crude enzyme preparation containing 0.2 mg of protein and was incubated at 30°C for 30 min. The reaction was terminated by adding 200 μl of 0.2 M HCl. After centrifugation at 12,000*g* for 10 min, the products were subjected to analysis by HPLC as described above.

Determination of salidroside and tyrosol

The determination of salidroside and tyrosol was done essentially as described (Ma et al. 2007, 2008). Plant material was collected and air-dried for 24 h at 60°C before it was ground to a fine powder and 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 an ultrasonic 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 clarified by passage through a 0.45 μm pore size NC filter, then dried in a vacuum and dissolved in 3.0 ml of 50% methano1. This preparation was used for the quantification of salidroside and tyrosol by HPLC analysis as described above.

Results

Isolation and sequence analysis of *RsTyrDC* cDNA

For 3′-RACE, total RNA was isolated from *R. sachalinensis* calli (cultured cells). PCR with degenerate primer (PRsTDC, Fig. 2) based on conserved amino acid sequences in TyrDCs from other plant species and anchored primer (PdT) gave an 810 bp fragment, whose deduced amino acid sequence exhibited about 60% identity with other plant species TyrDCs. The full-length cDNA sequence was obtained by 5′-RACE and was designated as *RsTyrDC* (GenBank accession no. DQ471943). The nucleotide sequence of *RsTyrDC* is 1,715 bp long with an ORF of 1,542 bp (positions 69–1592), a 68 bp leader sequence, and a 3′ untranslated region (3′-UTR) of 123 bp, including a 19 nucleotide poly(A) tail. The analysis of the *RsTyrDC* cDNA clone predicted a polypeptide of 507 amino acids with a calculated molecular mass of 56.8 kDa and a predicted isoelectric point of 6.2 (data not shown). The deduced amino acid sequence of *R. sachalinensis* RsTyrDC showed 49–79% identity with those of other TyrDCs of plant origin (Fig. 2): 79% identity with *Rhodiola rosea* TyrDC (Gyorgy et al. 2009), 51% with *Petroselinum crispum* TyrDC (Kawalleck et al. 1993), 50% with *Thalictrum flavum* TyrDC (Samanani et al. 2005), 49% with *Citrus reshni* TyrDC (Bartley et al. 2010) and 49% with *Papaver somniferum* TyrDC (Facchini and De Luca 1994).

Expression of *RsTyrDC* in *R. sachalinensis*

Total RNA was isolated from young leaves, stems, roots or calli, in order to investigate the tissue-specific expression of *RsTyrDC* of *R. sachalinensis*. RNA gel–blot analysis was done with an *RsTyrDC* cDNA fragment including both the coding and 3′-UTR regions as probe (Fig. 2). For expression of *RsTyrDC*, RNA gel–blot analysis revealed

that the accumulated *RsTyrDC* transcripts were more abundant in roots, and high levels of transcripts are also found in callus tissue culture. *RsTyrDC* transcripts were present at a low level in young leaves or stems (Fig. 3).

Characterization of RsTyrDC

To examine the catalytic function of RsTyrDC, the gene was expressed in *E. coli* Rosetta-gami™ (DE3) as a histidine-tagged protein and purified by nickel-affinity chromatography. The purified proteins were almost homogeneous as judged by SDS-PAGE and staining with Coomassie brilliant blue (Fig. 4). The enzyme activity of the purified RsTyrDC, which was identified by HPLC and HPLC–MS, was measured using four aromatic amino acid substrates. The relative activity and catalytic ability (V_{max}/K_m) of recombinant RsTyrDC for various aromatic amino acid substrates is given in Table 1. This result showed that tyrosine was best substrate of recombinant RsTyrDC (Table 1).

Genetic transformation and regeneration

To further investigate the role of the *RsTyrDC* gene in the regulation of tyrosol and salidroside accumulation, the sense-*RsTyrDC* construct pCA-s-RsTDC, and antisense-*RsTyrDC* construct pCA-as-RsTDC and empty vector (without insert) were introduced into *A. tumefaciens* (EHA105). *Agrobacterium tumefaciens* harboring the recombinant vectors and an empty vector was used for *R. sachalinensis* transformation. For *R. sachalinensis*, genetic transformation and regeneration were essentially as described (Ma et al. 2008, 2007). Nine sense-*RsTyrDC* transgenic plant lines (TRsT-s1–s9), seven antisense-*RsTyrDC* transgenic plant lines (TRsT-as1–as7) with similar phenotypes, and three independent empty vector-transformed plant lines (TRsT-e1–e3) were obtained and identified using PCR, PCR–Southern blot, RNA gel–blot analysis and endogenous TyrDC activity analysis.

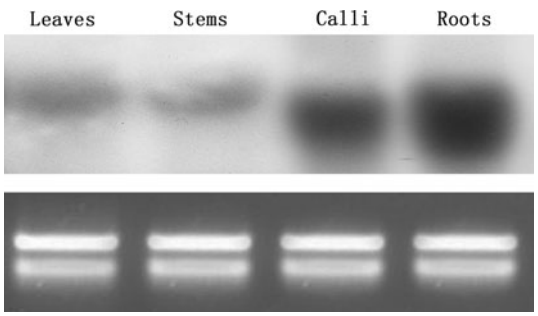

Fig. 3 RNA gel–blot analysis of *RsTyrDC* expression. Total RNA was prepared from leaves, stems, roots or calli. Hybridization was done using an [α-^{32}P]dCTP-labeled *RsTyrDC* fragment including both the coding and 3′ untranslated regions as probe

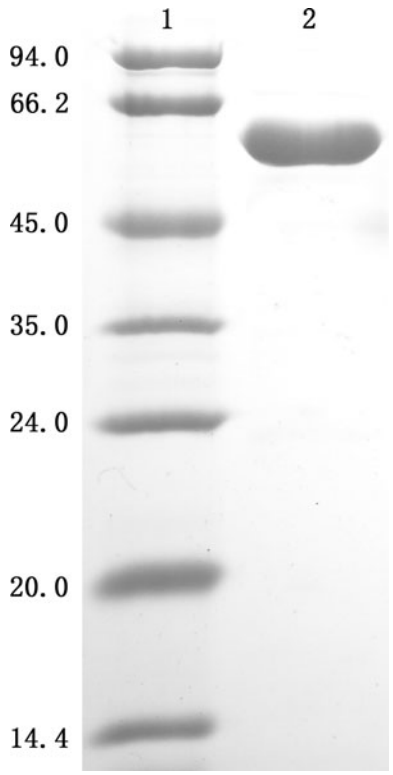

Fig. 4 SDS-PAGE analysis of recombinant RsTyrDC stained with Coomassie brilliant blue. *1*, molecular mass markers with masses indicated in kDa. *2*, recombinant RsTyrDC (3.0 μg) after passage through a PD-10 column

Molecular analysis of transgenic plants

DNA was isolated from transformed plant lines, empty vector-transformed lines and non-transformed wild-type plant line, identified by means of PCR and PCR–Southern blot. The PCR using relevant sequences of the 35 S promoter and *R. sachalinensis RsTyrDC* genes as primers (for the sense-*RsTyrDC* and antisense-*RsTyrDC* transformed lines) and 35 S promoter and GUS gene as primers (for the empty vector-transformed lines) had a 1.6 kb band and a 500 bp band (data not shown), respectively. The size of the 1.6 kb band was the same as that of recombinant vectors (pCA-s-RsTDC and pCA-as-RsTDC) as positive control, and no DNA band was detected in the non-transformed control. PCR–Southern blot analysis confirmed that the 1.6 kb band was indeed a product amplified by the sense-*RsTyrDC* (Fig. 5a) and antisense-*RsTyrDC* fusion genes (Fig. 5b). The PCR–Southern blot results indicated that the sense-*RsTyrDC* and antisense-*RsTyrDC* fusion genes had been integrated into the genome of transgenic plants.

Table 1 Substrate specificity of recombinant RsTyrDC from *R. sachalinensis*

Substrate	Enzyme activity[a] (pkat. mg^{-1} FW) (% of max. each)	K_m (μM)	V_{max} (pkat. mg^{-1})	V_{max}/K_m
L-tyrosine	100	0.9	274.4 ± 15.42	304.9
L-dopa	37	1.7	152.5 ± 7.56	89.7
L-tryptophan	0	–	–	–
L-phenylalanine	3	11.7	50.3 ± 3.23	4.3

[a] Substrate specificity was measured for product formation of the enzyme reaction at optimum pH

The PCR-positive transformed lines and one empty vector-transformed plant line were selected for RNA gel–blot analysis (Fig. 6). For sense-*RsTyrDC* expression, RNA gel blotting indicated that *RsTyrDC* was over-expressed at transcriptional levels in transgenic plants of *R. sachalinensis* (TRsT-s1, TRsT-s3, TRsT-s5 and TRsT-s7, Fig. 6a). The specific TyrDC activity in the *RsTyrDC* transgenic lines was 74 and 127% higher, respectively, than that in the empty vector-transformed controls (Table 2). By contrast, there was an evidence of reduced accumulation of the *RsTyrDC* transcripts in the antisense plants (TRsT-as2, TRsT-as3 and TRsT-as5, Fig. 6b). Decrease of specific TyrDC activity in antisense-*RsTyrDC* transformed lines was noticed. Table 2 gives the results of TyrDC activity assays using tyrosine as substrate. TRsT-as3 and TRsT-as5 contained 36 and 27% of TyrDC activity, respectively, relative to the empty vector-transformed control.

Tyrosol and salidroside content in transgenic plants

No obvious morphological phenotype was detected in the transgenic lines harboring either the sense or antisense construct when compared to the non-transformed controls (data not shown). In order to detect any change in the level of tyrosol and salidroside in the transgenic lines compared to the empty vector-transformed lines, HPLC analysis was done for the above *R. sachalinensis* materials. For sense-*RsTyrDC* expression, the results indicated that the over-expression of *RsTyrDC* in transgenic plants resulted in the accumulation of tyrosol and salidroside. The tyrosol and

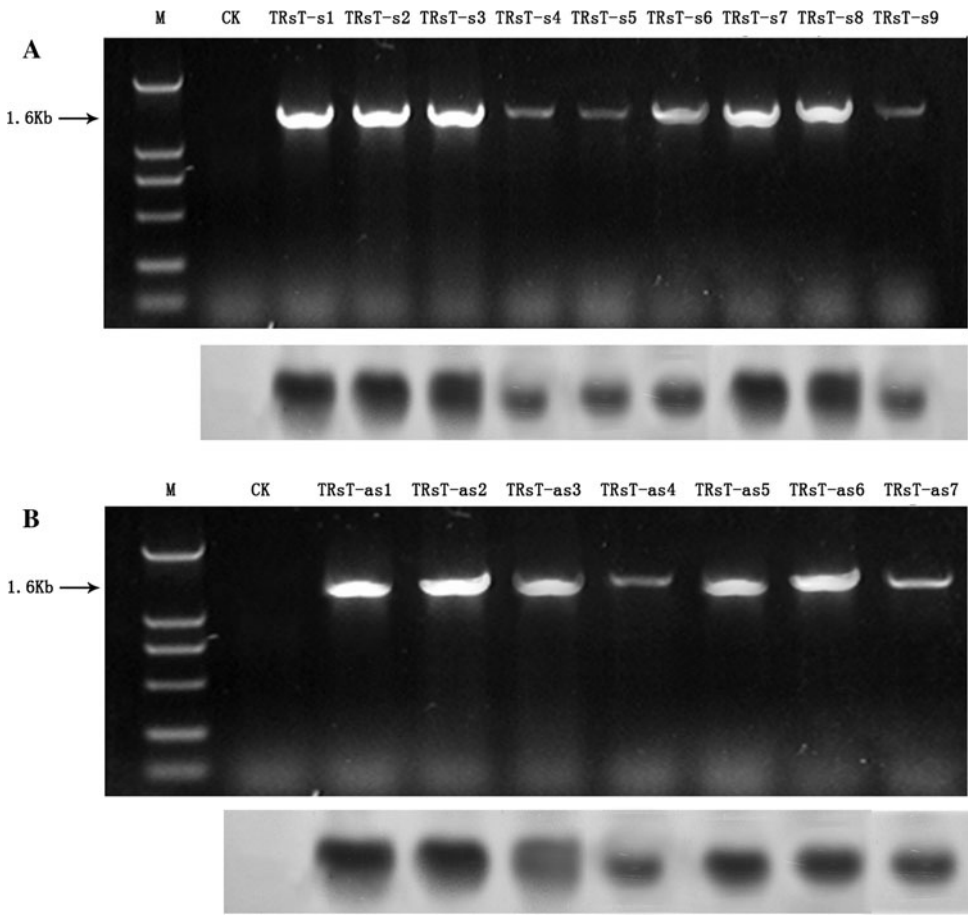

Fig. 5 Identification of sense-*RsTyrDC* transformed plant lines (**a**) and antisense-*RsTyrDC* transformed plant lines (**b**) by PCR and PCR–Southern blot analysis for the *RsTyrDC* gene. CK DNA isolated from empty vector transgenic plants was used as the negative control; TRsT-s1 to TRsT-s9 DNA isolated from the sense-*RsTyrDC* transformed plant lines; TRsT-as1 to TRsT-as7 DNA isolated from the antisense-*RsTyrDC* transformed plant lines

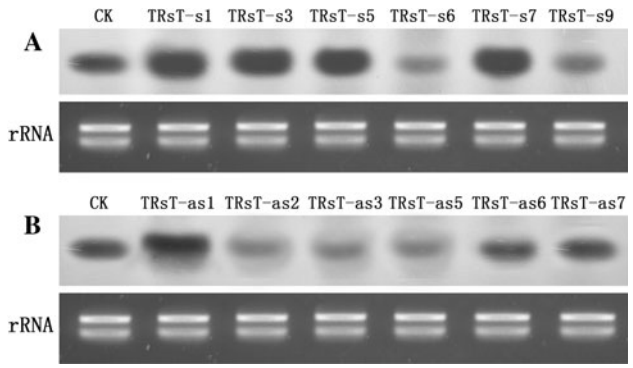

Fig. 6 Identification of sense-*RsTyrDC* transformed plant lines (**a**) and antisense-*RsTyrDC* transformed plant lines (**b**) by RNA gel–blot analysis. CK RNA isolated from empty vector transgenic plants was used as the negative control; TRsT-s1 to TRsT-s9 RNA isolated from the sense-*RsTyrDC* transformed plant lines; TRsT-as1 to TRsT-as7 RNA isolated from the antisense-*RsTyrDC* transformed plant lines

Table 2 TyrDC activity in transformed and control *R. sachalinensis* plants

Line	TyrDC specific activity (pkat. mg^{-1} FW)	WT activity (%)
WT	15.7 ± 1.37	100
TRsT-s3	27.3 ± 1.34	174
TRsT-s7	35.7 ± 2.25	227
TRsT-as3	5.7 ± 0.42	36
TRsT-as5	4.2 ± 0.43	27

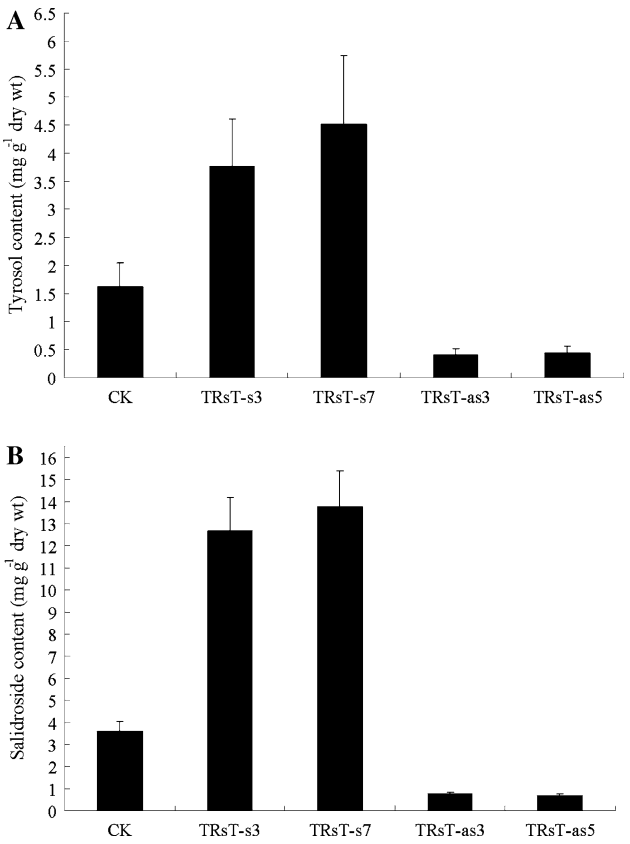

Fig. 7 Effects of the sense and antisense expression of the RsTyrDC on accumulation of tyrosol (**a**) and salidroside (**b**) in transgenic plant lines. CK empty vector transgenic plant line; TRsT-s3 and TRsT-s7 sense-*RsTyrDC* transformed plant lines; TRsT-as3 and TRsT-as5 antisense-*RsTyrDC* transformed plant lines. Results represent means of three independent replicates ± SD

salidroside content of the *RsTyrDC* transgenic lines was 157 and 265% higher, respectively, than that in the empty vector-transformed controls (Fig. 7). *Rhodiola sachalinensis* plants transformed with the antisense construct exhibited dramatic changes in the levels of tyrosol and salidroside compared to empty vector-transformed controls, as shown in Fig. 7. The levels of tyrosol and salidroside were 274 and 412% lower, respectively, in the antisense-*RsTyrDC* transformed lines than the level in the controls (Fig. 7).

Discussion

Due to the scarcity of *Rhodiola* spp. and its low yield of salidroside, there is considerable interest in the regulation of salidroside synthesis, both to explain and to clarify the pathway of salidroside synthesis and for the identification of targets for biotechnological manipulation of product accumulation. Here, using the RACE method, we successfully isolated a *TyrDC* cDNA clone from the calli of *R. sachalinensis*.

Figure 3 shows the differential expression of *RsTyrDC* in *R. sachalinensis*; the predominant sites of expression in plants are the roots and high levels of transcripts are also found in callus tissue culture. The fact that salidroside exists mainly in the roots and calli of *Rhodiola* spp. (Ma et al. 2008, 2007; Xu et al. 1998; Yousef et al. 2006) indicated that there is a good correlation between *RsTyrDC* transcript levels and salidroside accumulation in *Rhodiola* plants. In addition to *RsTyrDC*, similar expression pattern of *TyrDC* was also noticed in *Rhodiola rosea* (Gyorgy et al. 2009). These data most likely suggest that *TyrDC* has a crucial function in the initial reaction of the salidroside biosynthesis pathway.

RsTyrDC from *R. sachalinensis* was expressed heterologously in *E. coli* and its catalytic activity has been investigated. Functional analysis revealed that recombinant RsTyrDC exhibits the highest level of activity toward tyrosine in vitro. In order to further investigate the role of the *RsTyrDC* gene in vivo, the effects of sense and antisense over-expression of endogenous *RsTyrDC* on tyrosol synthesis and salidroside accumulation in *R. sachalinensis*

were investigated. For sense-*RsTyrDC* expression, RNA gel blotting indicated that *RsTyrDC* was over-expressed at transcriptional levels (Fig. 6a). Accordingly, the specific TyrDC activity in the *RsTyrDC* transgenic lines was 74 and 127% higher, respectively, than that in the controls (Table 2). Over-expression of sense-*RsTyrDC* in transgenic plants resulted in the accumulation of tyrosol and salidroside (Fig. 7). By contrast, there was an evidence of reduced accumulation of the endogenous *TyrDC* transcripts of *R. sachalinensis* in the antisense plants (Fig. 6b). Decrease of specific TyrDC activity in antisense-*RsTyrDC* transformed lines was also noticed (Table 2). Accordingly, the levels of tyrosol and salidroside of TRsT-as3 and TRsT-as5 were 274 and 412% lower than that in the controls, respectively (Fig. 7).

Our recent work has indicated that a large reduction in tyrosol biosynthesis and the accumulation of salidroside in transgenic *R. sachalinensis* is most likely to be due to the reduced availability of tyrosine (Ma et al. 2008). Apart from our research results, an earlier study indicated that TyrDC activity supports biosynthesis of verbascoside, the hydroxyphenylethanol glycoside found in *Syringa vulgaris* (Ellis 1983). Interestingly, a recent study has demonstrated that overexpression of parsley *TyrDC* leads to accumulation of a new compound, tyrosol glucoside (salidroside), in potato (Landtag et al. 2002). However, as a main bioactive component of *Rhodiola* plants, the biosynthetic pathway of tyrosol and its regulation are less well understood in *Rhodiola* spp. The data presented in this study for the first time provide in vitro and in vivo evidence that the *RsTyrDC* gene can regulate the tyrosol and salidroside biosynthesis, and the *RsTyrDC* is most likely to have an important function in the initial reaction of the salidroside biosynthesis pathway in *R. sachalinensis*. Summarizing all the above research results, it is tempting to speculate that the biosynthesis of salidroside in *R. sachalinensis* begins with the decarboxylation of tyrosine by TyrDC, which produces tyramine. Through tyramine oxidase, 4-hydroxyphenylacetaldehyde (4-HPAA) is formed, which is then transformed into tyrosol by aryl-alcohol dehydrogenase (Fig. 1b). 4-HPAA is the direct precursor for tyrosol biosynthesis and the enzymes catalyzing the conversion of tyrosine to 4-HPAA (Fig. 1b) have been found in plants (Landtag et al. 2002).

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