Characterization of glycosyltransferases responsible for salidroside biosynthesis in Rhodiola sachalinensis

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A B S T R A C T
Salidroside, the 8-O-β-D-glucoside of tyrosol, is a novel adaptogenic drug extracted from the medicinal plant Rhodiola sachalinensis A. Bor. Due to the scarcity of R. sachalinensis and its low yield of salidroside, there is great interest in enhancing production of salidroside by biotechnological manipulations. In this study, two putative UDP-glycosyltransferase (UGT) cDNAs, UGT72B14 and UGT74R1, were isolated from roots and cultured cells of methyl jasmonate (MeJA)-treated R. sachalinensis, respectively. The level of sequence identity between their deduced amino acid sequences was ca. 20%. RNA gel–blot analysis established that UGT72B14 transcripts were more abundant in roots, and UGT74R1 was highly expressed in the calli, but not in roots. Functional analysis indicated that recombinant UGT72B14 had the highest level of activity for salidroside production, and that the catalytic efficiency (Vmax/Km) of UGT72B14 was 620% higher than that of UGT74R1. The salidroside contents of the UGT72B14 and UGT74R1 transgenic hairy root lines of R. sachalinensis were also ~420% and ~50% higher than the controls, respectively. UGT72B14 transcripts were mainly detected in roots, and UGT72B14 had the highest level of activity for salidroside production in vitro and in vivo.

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1. Introduction

Glycosylation reactions, like oxidations, reductions, hydroxylations and methylation, are often indicated for secondary metabolic reactions in plant cells (Banthorpe, 1994). The glycosyltransferase (GT) multigene family is composed of 92 families numbered according to sequence similarity, signature motif, stereochemistry of the glucoside linkage formed, and target specificity (Cantarel et al., 2009; http://www.cazy.org/GlycosylTransferases.html). The GT family 1 contains UDP-glycosyltransferases (UGTs) (Li et al., 2001; Mackenzie et al., 1997; Paquette et al., 2003; Ross et al., 2001), which have been identified in plants, animals, fungi, bacteria and viruses (Campbell et al., 1997; http://www.unisa.edu.au/pharm_medsci/Gluc_trans/table21.htm). The plant GT family 1 in plants is encoded by 120 UGT genes in Arabidopsis thaliana, and 165 have been identified in Medicago truncatula (Achnine et al., 2005; Paquette et al., 2003). Many properties of aglycones are regulated by glucosylation. Secondary metabolite glucosides generally have reduced chemical reactivity and enhanced water solubility and can be responsible for either storage or transport (Li et al., 2001). Glycosylation of biologically active compounds has attracted considerable attention because it often improves the effect of a drug and its pharmacokinetic parameters (Kren and Martinкова, 2001).

Rhodiola spp. grow 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 (Fu et al., 2009). Rhodiola rosea L. (golden root), historically used as an adaptogen in Russia, northern Europe, and in China (Tolonen et al., 2003; Yousef et al., 2006), and Rhodiola sachalinensis A. Bor has been widely used in traditional Chinese medicine (Ma et al., 2007; Wu et al., 2003; Xu et al., 1998). Recently, the phytochemical extracts of Rhodiola spp. have been a source of important commercial preparations used throughout Europe, Asia and the USA, with biological activity that includes anti-allergenic and anti-inflammatory effects and enhanced mental alertness, and had 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 of Rhodiola spp., including R. rosea and R. sachalinensis, and is found mainly in roots. Salidroside, a tyrosol 8-O-β-D-glucoside 3 (Fig. 1), synthesized by...
2.2. Isolation and sequence analysis of UGT72B14 and UGT74R1 cDNAs

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 adaptogenic effects, such as treating anoxia, microwave radiation and fatigue and slowing the aging process (Mao et al., 2010; Ouyang et al., 2010).

There is considerable interest in the pathway of salidroside synthesis and identification of targets for biotechnological manipulation of its product accumulation. In our recent work (Ma et al., 2007), the putative UGT cDNA UGT73B6 was isolated from calli of *R. sachalinensis*. Its over-expression induced a twofold increase in salidroside content compared with that of untransformed controls in transgenic *R. sachalinensis*. These data suggested that UGT73B6 can regulate conversion of the tyrosol aglycone 1 to salidroside 3 in *R. sachalinensis*; however, in that study UGT73B6 expression was barely detectable in leaves under normal growth conditions, although a basal level of salidroside 3 remained in the leaves. This result indicated that other UGTs active toward tyrosol 1 might exist in *R. sachalinensis*. In this study, in order to elucidate the molecular pathways of salidroside 3 biosynthesis, two UGT clones were isolated from the roots and cultured cells of methyl jasmonate (MeJA)-treated *R. sachalinensis*. Our intention was to combine the catalytic specificity of these UGTs and UGT73B6 (Ma et al., 2007) in vitro in order to change the level of salidroside 3 in vivo by over-expression of the above UGTs.

2. Results and discussion

2.1. Time course of glucosyltransferase activity in MeJA-treated *R. sachalinensis*

Considering the fact that MeJA can rapidly and markedly induce expression of various UGTs and secondary metabolites in some plants and cell cultures (Kaminaga et al., 2003, 2004), and salidroside 3 exists mainly in the roots of *Rhodiola* spp. (Xu et al., 1998; Yousef et al., 2006), roots and cultured cells of MeJA-treated *R. sachalinensis* were used for isolation of novel UGT cDNAs. The time course of the glycosyltransferase reaction was investigated to determine the optimum culture period for extraction of total RNA for isolation of novel UGT cDNAs (Fig. 2) and the enzymatic activity of the glycosyltransferase was measured using tyrosol 1 and UDP-glucose 2 as substrates (Fig. 1). It was found that after treatment with MeJA, the glycosyltransferase activity reached a maximum at 14 days in *R. sachalinensis* roots and at day 10 in *R. sachalinensis* cultured cells. Total RNA was extracted from roots on day 12 and from cultured cells on day 8 to examine transcription before the synthesis of proteins.

2.2. Isolation and sequence analysis of UGT72B14 and UGT74R1 cDNAs

Because the molecular skeleton of tyrosol 1 (2-[(4-hydroxyphenyl)ethanol]) is somewhat similar to hydroquinone and salicylic acid, we chose several plant UGTs that act in vitro on the small molecules referred to above to design nested primers. Using the RACE (rapid amplification of cDNA ends) method, two putative UGT cDNAs were isolated from roots and cultured cells of MeJA-treated *R. sachalinensis*. They were named UGT72B14 (GenBank Accession No. EU567325) from the roots and UGT74R1 (GenBank Accession No. EF508689) from the cultured cells, based on nomenclature proposed by Mackenzie et al. (1997).

The full length of the UGT72B14 cDNA is 1671 bp; it contains an ORF of 1422 bp, a 53 bp 5′-untranslated region (5′-UTR) and a 97 bp 3′-untranslated region (3′-UTR), including a 20 nt poly(A) tail. Analysis of the UGT72B14 cDNA sequence predicted a polypeptide of 473 amino acids with a calculated molecular mass of 51.5 kDa and a predicted isoelectric point (pI) of 6.36 (data not shown). The nucleotide sequence of UGT74R1 is 1547 bp long with an ORF of 1362 bp, a 40 bp 5′-UTR and a 145 bp 3′-UTR, including a 19 nt poly(A) tail. UGT74R1 cDNA encodes 453 deduced amino acid residues with a calculated molecular mass of 51.1 kDa and a predicted pI of 5.4 (data not shown). There was ~20% sequence identity between UGT72B14 and UGT74R1. In the deduced sequence, UGT72B14 and UGT74R1 shares a conserved UDP-binding domain of 44 amino acid residues located in the C-terminal region (PSPG-box; Hundle et al., 1992). Within this PSPG-box, the peptide sequence of HCGWNS, that has been detected in 95% of all glycosyltransferases (Vogt and Jones, 2000), was located at position 363 for UGT72B14 and position 346 for UGT74R1 (data not shown). Analyses of the sequence of UGT72B14 included ~61% identity with *Rauvolfia serpentina* arbutin synthase (AS; data not shown). UGT74R1 was also ~48% identical with a salicylate-induced glucosyltransferase of *Nicotiana tabacum* (Lee and Raskin, 1999; data not shown).

2.3. Characterization of UGT72B14, UGT74R1 and UGT73B6

The functional diversity and the promiscuity of the substrate specificity of the plant-specific UGTs are such that most UGTs have been shown to exhibit broad substrate specificity in vitro (Taguchi et al., 2001). To examine the catalytic function of UGT72B14, UGT74R1 and UGT73B6 (Ma et al., 2007), the three genes were expressed heterologously in *Escherichia coli* with an additional hexahistidine tag at the C terminus. During SDS–PAGE, the purified enzymes migrated as single bands at the position expected for a protein with a molecular mass of ~52 kDa in Fig. 3. The enzyme activity of the purified UGT72B14, UGT74R1 and UGT73B6 was...
measured using five aglycones and UDP-glucose, with products identified by high-pressure liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS), respectively. HPLC showed that recombinant UGT77B14, UGT74R1 and UGT73B6 yielded salidroside 3 as a single product from tyrosol 1 and UDP-glucose 2 (Figs. 1 and 4). The relative activity of recombinant UGT77B14, UGT74R1 and UGT73B6 was examined for various phenolic compounds (Table 1). This result showed that recombinant UGT77B14, UGT74R1 and UGT73B6 exhibited the highest relative rates towards tyrosol 1, salicylic acid and p-coumaric acid, respectively (Table 1). For catalytic ability ($V_{\text{max}}/K_m$), UGT77B14 exhibited the highest level of activity for salidroside 3 production, which was ~620% and ~170% higher than that of UGT74R1 and UGT73B6, respectively (Table 2).

2.4. Over-expression of UGT77B14, UGT74R1 and UGT73B6 in the transgenic hairy roots of R. sachalinensis

Transformed root (hairy root) cultures have been shown to be good for studies of many secondary metabolites. In this investigation, genetic transformation and induction of the hairy roots of R. sachalinensis were established. A total of 37 hygromycin B-resistant lines of UGT77B14, UGT74R1 and UGT73B6 and three independent empty vector-transformed lines were obtained and identified using PCR, PCR–Southern blot (data not shown) and RNA gel–blot analysis (Fig. 5). Molecular analysis and HPLC indicated that, as expected, over-expression of UGT77B14, UGT74R1 and UGT73B6 in transgenic hairy roots of R. sachalinensis resulted in accumulation of salidroside 3 (Fig. 6). The salidroside 3 content (mg g$^{-1}$ dry wt) of the UGT77B14, UGT74R1 and UGT73B6 transgenic lines was ~19.81, 5.72 and 8.76, respectively, which was ~420%, ~50% and ~130% higher than that in the empty vector-transformed controls (Fig. 6). In our recent work, the results for transgenic R. sachalinensis indicated that over-expression of the UGT77B36 gene results in a salidroside content that was ~100% and ~140% higher compared to that of the untransformed controls in the transgenic plants and transgenic calli, respectively (Ma et al., 2007). The transformed root (hairy root) culture system had the highest level of ability to produce salidroside 3. The salidroside 3 content of the UGT77B36 transgenic hairy root lines was ~8.76 mg g$^{-1}$ dry wt, which was ~312% and ~117% higher than that of UGT73B6 transgenic plants and transgenic calli systems, respectively.

2.5. Phylogenetic analysis

The UGT genes discussed in this work belong to the GT family 1 and are predicted to the GT-B fold (the basic structure observed for GT families). They are envisaged to act via an inverting mechanism (Cantarel et al., 2009; http://www.cazy.org/GlycosylTransferases.html). Crystal-based 3D structures of four plant UGTs were published recently, and despite a low level of sequence conservation, the UGTs show highly conserved secondary and tertiary structures: these plant UGT crystal structures established detailed interactions between the enzyme and its donor, substrate as well as the roles of the UGT signature motif in substrate recognition and the catalytic activity of the enzyme (Osmani et al., 2009). The molecular phylogenetic tree, based on a multiple sequence alignment of 40 plant UGTs retrieved from the database, showed that R. sachalinensis UGT77B14, R. serpentina AS and A. thaliana UGT77B1 and UGT72E2 belong to group E (Fig. 7). It was reported that R. serpentina AS is a UDP-glucose-dependent hydroquinone: O-glucosyltransferase (Heffner et al., 2002). A. thaliana UGT77B1 displayed activity toward 3-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid and 3,4-dihydroxybenzoic acid (Lim et al., 2002) and R. sachalinensis UGT77B14 showed the highest level of activity toward tyrosol 1 (4-hydroxyphenylethanol) in this study (Tables 1 and 2). R. sachalinensis UGT77B14, N. tabacam SAGT, A. thaliana UGT74F1 and UGT74F2 belong to group L (Fig. 7). An earlier report indicated that recombinant SAGT is a UDP-glucose:salicylic acid glucosyltransferase (Lee and Raskin, 1999). A. thaliana UGT74F1 and UGT74F2 are active toward 2-hydroxybenzoic acid, suggesting they are the Arabidopsis salicylic acid glucosyltransferases (Lim et al., 2002). Indeed, the relative activity showed recombinant UGT74R1 had the highest specificity towards salicylic acid (Table 1). As mentioned above, R. sachalinensis UGT73B6 is classified into group D together with N. tabacam TOGT1, Solanum lycopersicum TWI1 and A. thaliana UGT73B3 and UGT73B4 (Fig. 7). Fraissinet-Tachet et al. (1998) reported that recombinant TOGT proteins produced in E. coli had very high glucosyltransferase activity towards p-coumaric acid and caffeic acid. Like the TOGT, R. sachalinensis UGT73B6 was active toward p-coumaric acid (Table 1).

2.6. Expression of UGT77B14 and UGT74R1 in R. sachalinensis

RNA gel–blot analysis indicated R. sachalinensis UGT77B14 (Fig. 8) and UGT73B6 (Ma et al., 2007) are highly expressed in roots, but no transcript of UGT74R1 was detected in the roots (data not shown). The facts that UGT77B14 transcripts exist mainly in the roots and UGT72B14 exhibited the highest level of activity for salidroside 3 production in vitro and in vivo suggests that it has an important function. Compared to UGT74R1, UGT73B6 showed greater activity for salidroside 3 production, and this isozyme was also highly expressed in roots; therefore, it is tempting to speculate that UGT73B6 can also contribute to salidroside 3 synthesis. However, the findings that UGT74R1 expression was barely detectable in the roots and tyrosol 1 was not the best substrate tested for UGT74R1, implied a lack of correlation between levels of UGT74R1 transcriptional expression and the accumulation of salidroside 3.

3. Concluding remarks

In this study, the effect on salidroside 3 biosynthesis of over-expression of two endogenous UDP-glycosyltransferases UGT77B14 and UGT74R1, and our previously reported UGT73B6 (Ma et al., 2007), was investigated in transgenic hairy root lines of R. sachalinensis. UGT77B14 transcripts were mainly detected in roots and UGT72B14 exhibited the highest level of activity.
for salidroside production in vitro and in vivo suggested that UGT72B14 has an important function. Compared to UGT74R1, UGT73B6 showed a higher level of activity for salidroside production, and this isozyme is highly expressed in the roots, so it

Fig. 4. HPLC elution profiles of enzyme reaction products of UGTs. Enzyme reaction products of (A) recombinant UGT72B14; (B) recombinant UGT74R1; (C) recombinant UGT73B6; HPLC separation conditions are described in Section 4.
is very likely that UGT73B6 can contribute to salidroside synthesis. Compared to the UGT73B6 transgenic plants and transgenic calli systems (Ma et al., 2007), the transformed root (hairy root) culture system exhibited the highest level of salidroside 3 production.

Table 1
Substrate specificity of recombinant UGT72B14, UGT74R1 and UGT73B6 from Rhodiola sachalinensis.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Aglycone</th>
<th>UGT72B14</th>
<th>UGT74R1</th>
<th>UGT73B6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;m&lt;/sub&gt; (µM)</td>
<td>V&lt;sub&gt;max&lt;/sub&gt; (pkat mg&lt;sup&gt;–1&lt;/sup&gt;)</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>Tyrosol 1</td>
<td>4.7 ± 0.35</td>
<td>57.8 ± 3.2</td>
<td>12.3</td>
<td></td>
</tr>
<tr>
<td>Hydroquinone</td>
<td>172.4 ± 14.1</td>
<td>293.1 ± 14</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>54.3 ± 4.9</td>
<td>249.8 ± 13</td>
<td>4.6</td>
<td></td>
</tr>
</tbody>
</table>

Fugation at 15,000 x g, (20 ml, pH 7.5) containing 1 mM dithiothreitol (DTT). After centrifugation at 15,000 x g, (20 ml, pH 7.5) containing 1 mM dithiothreitol (DTT). After centrifugation at 15,000 x g, (20 ml, pH 7.5) containing 1 mM dithiothreitol (DTT).

Table 2
Kinetic parameters of recombinant UGT72B14, UGT74R1 and UGT73B6 toward tyrosol 1 substrate. Results represent means of three independent replicates ± SD.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (µM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (pkat mg&lt;sup&gt;–1&lt;/sup&gt;)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT72B14</td>
<td>4.7 ± 0.35</td>
<td>57.8 ± 3.2</td>
<td>12.3</td>
</tr>
<tr>
<td>UGT74R1</td>
<td>172.4 ± 14.1</td>
<td>293.1 ± 14</td>
<td>1.7</td>
</tr>
<tr>
<td>UGT73B6</td>
<td>54.3 ± 4.9</td>
<td>249.8 ± 13</td>
<td>4.6</td>
</tr>
</tbody>
</table>

4. Experimental

4.1. Chemicals

Salidroside 3 was purchased from Tauto Biotech (Shanghai, China). Tyrosol, hydroquinone, salicylic acid, benzoic acid, p-coumaric acid and UDP-glucose 2 were purchased from Sigma–Aldrich (China).

4.2. Plant materials and treatment with MeJA

Seeds of R. sachalinensis were collected from Changbai Mountain, Jilin Province, China. The seeds were surface-sterilized for 20 min in 0.1% (w/v) mercuric chloride then washed thoroughly with sterile distilled H<sub>2</sub>O and 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 0.75% agar. After ~30 days of culture, the middle or upper leaves of R. sachalinensis were used as explants. The leaves were cut into 0.5 × 0.5 cm pieces and transferred to callus-inducing medium (MS basal medium supplemented with 1.5 mg l<sup>–1</sup> 6-BA, 0.15 mg l<sup>–1</sup> NAA and 0.5 mg l<sup>–1</sup> 2,4-D) to induce callus. Further subculturing was done every 3–4 weeks on callus subculturing medium (MS basal medium supplemented with 1.5 mg l<sup>–1</sup> 6-BA and 0.15 mg l<sup>–1</sup> NAA). The culture procedure was performed under sterile conditions. 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 µmol m<sup>–2</sup> s<sup>–1</sup> provided by cool white fluorescent lamps. R. sachalinensis plants were rooted on MS medium and grew on peat soil in an artificial greenhouse under 8 h dark at 20 °C/16 h light (5000 lx) at 27 °C.

For root treatment, 300 µM MeJA in EtOH–H<sub>2</sub>O (80:20, v/v) was sprayed onto the surface of the experimental set of plants and the control plants were sprayed with only EtOH–H<sub>2</sub>O (80:20, v/v). Treated plants were left in the open air for about 2 h to allow the remaining MeJA solution to evaporate, followed by transfer into artificial greenhouses under the same conditions. R. sachalinensis cell suspension cultures were maintained in MS medium supplemented with 3.0% sucrose, 1.0 mg l<sup>–1</sup> 6-BA and 0.5 mg l<sup>–1</sup> NAA. Cells were cultured at 25 °C in the dark and subcultured at 3–4 weeks intervals. MeJA was dissolved in dimethylsulfoxide and added to the cultures aseptically through membrane filters to a final concentration of 250 µM 3 days after cell inoculation. The cells were collected by vacuum filtration at the defined times, immediately frozen in N<sub>2</sub>, and stored at −75 °C.

4.3. Enzyme extraction

All steps were carried out at 4 °C. Cultured cells or fresh root materials were mixed with Polyclar AT (3 g) (Serva, Heidelberg, Germany) and homogenized in 0.1 M potassium phosphate buffer (20 ml, pH 7.5) containing 1 mM dithiothreitol (DTT). After centrifugation at 15,000g for 20 min, the supernatant was passed through a PD-10 column (Pharmacia, Freiburg, Germany) equilibrated with the same buffer. The supernatant was used as the preparation of crude enzyme.

4.4. Enzyme assays

For enzyme assays of the preparation of crude enzyme, standard (100 µl) assays contained 50 mM Tris–HCl (pH 7.5), 250 µM tyrosol, 2 mM UDP-glucose and a sample of the crude enzyme preparation containing 0.2 mg of protein. These were incubated at 30 °C for 30 min, and the reactions terminated by adding MeOH (200 µl). After centrifugation at 12,000g for 10 min, products were...
subjected to HPLC analysis on a Kromosil C18 reversed phase column (250 mm × 4.6 mm, 5 μm (diameter), Macherey Nagel, Düren, Germany). A Waters Alliance 2695 HPLC system used a Waters 2487 photodiode array detector and was controlled by a computer running Empower software (Waters, Milford, MA, USA). The eluents were H2O (A) and MeOH (B) at a flow rate of 0.6 ml min⁻¹. The following gradients were used: 30% (v/v) B for 5 min, 30–70% B for 20 min, 70–80% B for 2 min, 80–95% B for 3 min, and 95% B for 5 min. Absorbance was measured at a wavelength of 275 nm. A standard solution of reference compounds was used for quantification.

4.5. Isolation of UDP-glycosyltransferase cDNAs

Total RNA was prepared from R. sachalinensis roots and cultured cells were harvested at 12 days and at 8 days after treatment with MeJA 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: RRP: CCAGTGAGCAGAGTGAGGAGACTGAGCTCAGGTTTTTTTTTTTTTT. The degenerate primer was designed for 3′-rapid amplification of cDNA ends (RACE) based on an alignment of amino acid sequences deduced from several
plant UGTs (NP_180734 and NP_566938 of A. thaliana, BAD34401 of Oryza sativa and ABL5472 of Mucura pomifera) acting in vitro on small molecular mass phenolic compounds, and was produced on the basis of the consensus-degenerate hybrid oligonucleotide primers (CODEHOP) strategy by the Block maker program (http://blocks.fhcrc.org/blocks/blockml/make_blocks.html) as follows: block H: 5’-CACGTTGAGTTTTTGTACCT Ayggygntgga-3’ where n is any nucleotide and y is C or T. The DNA fragment was amplified using high-fidelity PrimeSTAR HS DNA Polymerase (TaKaRa, Dalian, China). After denaturation at 95 °C (4 min), 30 cycles at 94 °C for 30 s, 55 °C for 40 s, 72 °C for 60 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.

Full-length cDNA sequence was obtained by 5’-RACE with a SMART™ RACE cDNA Amplification Kit (CLONTECH Life Technologies) according to the manufacturer’s instruction and specific primers based on the obtained cDNA sequences: RGSP1, 5’-CGA-TAATGAGTGTTCTCTGGC-3’ and RGSP2, 5’-CACCACACTCTCCAAATGTC-3’ for plant roots and: CGSP1, 5’-GGACAGTGGGCA CCCCCCATACTGCG-3’ and CGSP2, 5’-GGACAGTGTCACCCCGC-3’ for cultured cells. The PCR program was performed according to the touch-down PCR procedure: denaturation at 95 °C for 5 min, five cycles at 94 °C for 30 s, 70 °C for 30 s and 72 °C for 120 s, then 25 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 120 s, and finally, extension at 72 °C for 9 min.

4.6. RNA gel–blot analysis

Total RNA was isolated from roots, calli (cultured cells), stems and young leaves in order to investigate tissue-specific expression of UGT72B14 and UGT74R1 of R. sachalinensis. 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 an HCHO-containing 1.2% (w/v) agarose gel in MOPS buffer and ligated into pMD18-T Vector (TaKaRa) and sequenced.

4.7. Heterologous expression in E. coli and recombinant enzyme purification

For UGT72B14, the ORF of the cDNA was amplified using PCR primers: sense, 5’-CTACCGCATTGCTGGCAGCCAGG-3’ the Ncol site is underlined and antisense, 5’-CCCAAGCTTATGCTTTACCAGAACCTCTCAC-3’ the HindIII site is underlined. For UGT74R1, the ORF of the cDNA was amplified using N-terminal and C-terminal PCR primers: sense, 5’-CTAGCCATGGGCAAAAGAAATTCTGACTTTTGG-3’ the Ncol site is underlined and antisense, 5’-CCCAAGCTTTAAGGCAACTCTGTGACA-3’ the HindIII site is underlined. After denaturation at 95 °C (4 min), 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 9 min. The amplified DNA fragments from UGT72B14 and UGT74R1 were digested with Ncol/HindIII, and cloned into the Ncol/HindIII site of pET-28b (+) (Novagen, Darmstadt, Germany). The recombinant enzymes contained a His_{6} tag at the C terminus.

For UGT73B6, the ORF of the cDNA was amplified using the PCR primers: sense, 5’-GGAATTCATGTTGCTGCAAACCTGCC-3’ the Ndel site is underlined and antisense, 5’-CCGGATCCATGGACATCTTTCG-3’ the BamHI site is underlined. The amplified DNA was digested with Ndel/BamHI, and cloned into the Ndel/BamHI site of pET-28a (+) (Novagen, Darmstadt, Germany). The recombinant enzyme 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 107 g and 37 °C in Luria–Bertani (LB) medium (200 ml) containing kanamycin (50 µg ml^{-1}) and chloramphenicol (34 µg ml^{-1}). At an A_{600} of 0.6–0.8, 0.6 mM isopropyl thiogalactopyranoside (IPTG) was added and the incubation temperature was reduced to 20 °C. After incubation for 9 h, cells were harvested by low-speed centrifugation, suspended in 0.1 M potassium phosphate (3 ml, pH 7.5), and sonicated on ice for 10 min. The homogenate was centrifuged at 10,000 g for 10 min at 4 °C. 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 mM 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, 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), and the sample was stored at −80 °C. The efficiency of purification was monitored by SDS–PAGE. Protein concentration was determined by the Bradford method with BSA as the standard.

4.8. Enzyme reaction and product analysis

The standard assays (250 µl) containing 50 mM Tris–HCl (pH 7.5), 500 µM phenolic substrate, 2 mM UDP-glucose and 2.0 µg of protein were incubated at 30 °C for 30 min. Reactions were terminated by adding MeOH (250 µl). After centrifugation at 12,000 g for 10 min, the reaction products were analyzed by HPLC as described above. Identity of the products was determined by HPLC co-chromatography with standard compounds and by analysis of their ultra-violet absorption spectra. The amounts of products were determined from standard curves. For determination of kinetic parameters of recombinant UGT72B14, UGT74R1 and UGT73B6, the concentration of the tyrosol 1 substrate was varied from 25 µM to 2 mM, in the presence of 2 mM UDP-glucose. The incubations were carried out at 30 °C for 30 min. The kinetic values were estimated from Lineweaver–Burk plots from duplicate experiments (average of triplicates ± SD).

For on-line HPLC-MS analysis, liquid chromatography was carried out with a Shimadzu LC-10ADvp HPLC system coupled to...
4.9. Construction of plant expression vector

For UGT72B14, the ORF of the cDNA was amplified using PCR primers: sense, 5′-GAAGATCTATGGCTGCTCGGACACA-3′ (pU72B14-up) and antisense, 5′-GGGTAACTCTAATTTTCAAGGCAA GTTCTGTG-3′ (pU74R1-dw) the BstEII site is underlined and antisense, 5′-GGGTAACTTATCTTACGGA CAAAGGAA-3′ (pU74R1-up) the BgII site is underlined and antisense, 5′-GGGTAACTTTAATTTTCAAGGCAA GTTCTGTG-3′ (pU74R1-dw) the BstEII site is underlined. After transformation for 95°C (4 min), 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 9 min. Amplified DNA fragments from UGT72B14 and UGT74R1 were digested with BglII/BstEII, and cloned into the BglII/BstEII site of pCAMBIA1301. For UGT73B6, the ORF of the cDNA was amplified using PCR primers: sense, 5′-GAAGATCTATGGCTGCTCGGACACA-3′ (pU72B14-up) and antisense, 5′-CGGCTAGTTCATTTAAGGCAA GTTCTGTG-3′ (pU73B6-up) the BgII site is underlined and antisense, 5′-CGGCTA GTTCTGTG-3′ (pU73B6-dw) the Nhel site is underlined. The amplified DNA was digested with BgII/Nhel, and cloned into the BgII/Nhel site of binary vector pCAMBIA1301 driven by the CaMV35S promoter. The hygromycin gene encoding phosphotransferase was used as the selection marker. Recombinant plasmids (pCAUGT73B6, pCAUGT72B14 and pCAUGT74R1) were identified by restriction analysis of purified plasmid DNA and used for sequencing, and then transformed into Agrobacterium rhizogenes (ATCC15834) by the Nt freeze–thawing method. A. rhizogenes harboring the recombinant vectors (pCAUGT73B6, pCAUGT72B14 and pCAUGT74R1) and an empty vector (without insert) were used for R. sachalinensis transformation.

4.10. Plant transformation

For R. sachalinensis, genetic transformation and induction of hairy root were done essentially as described (Kim et al., 2007). For transformation experiments, young leaves of 4-week-old aseptic seedlings were used as explants. For inoculation, A. rhizogenes was grown overnight at 28°C with shaking in liquid LB medium supplemented with kanamycin (50 mg l⁻¹). A sample (400-μl) of the overnight cultures was subcultured in fresh LB medium (50 ml) (without antibiotics) until the A₆₀₀ reached 0.6–0.8. The subculture was pelleted by centrifugation and suspended in three volumes of MS liquid medium, and the bacteria-containing suspension was used for R. sachalinensis transformation. Leaves were immersed in the A. rhizogenes suspension (30 ml) described above. After 5–7 min, explants were removed, blotted with sterile filter paper and co-cultured on solid MS medium supplemented with 3% sucrose, 0.8% agar, and 50 μM acetylsyringone at 20°C in the dark for 3 days. After co-cultivation, explants were washed in sterile distilled H₂O and transferred to ½ strength MS medium supplemented with 3% sucrose, 0.8% agar, and 300 mg l⁻¹ of cefotaxime. Afterwards, a subculture was made every 3 weeks; roots longer than 5 cm were excised from the parental tissues and transferred to a selectable medium with 300 mg l⁻¹ of cefotaxime and 20 mg l⁻¹ of hygromycin B (Du chea, Haarlem, Nether land) to screen for putative transformed roots until bacteria had all been killed. Liquid hairy root cultures were established with 2-cm long tips from 1-month-old hairy roots. Five root tips were inoculated in 100 ml Erlenmeyer flasks containing 30 ml of ½ strength MS medium supplemented with 3% sucrose without plant growth regulators. Roots were grown at 100 rpm and 25°C in darkness and subcultured to fresh medium at one week intervals.

4.11. Molecular characterization of transgenic hairy root

4.11.1. PCR and PCR–Southern blot analysis

Putatively transformed hairy roots were initially analyzed by PCR. Genomic DNA of hairy roots was isolated using the SDS extraction method (Lin et al., 2001). To avoid disturbance of the endogenous gene in R. sachalinensis, the PCR using relative sequences of the 35S promoter and R. sachalinensis UGT genes as primers (for the UGT72B14, UGT74R1 and UGT73B6-transformed lines) and 35S promoter and GUS gene as primers (for the empty vector-transformed lines). The first was a sense primer: 35S-UP, 5′-TGATATCTCCACTGACGTAAAGGATGC-3′ corresponding to the sequence of the CaMV35S promoter, and the others were pU73B6-dw, pU72B14-dw and pU74R1-dw (see above). The primers for empty vector transgenic hairy roots used in the PCR were 35S-UP (see above) and: GUS-R, 5′-TGCCGGATTTAGCAGGTCAGC-3′. 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 digoxigenin-11-dUTP-labeled full-length cDNAs as probes.

4.11.2. RNA gel–blot analysis

Total RNA was isolated from leaves of transgenic hairy roots and untransformed wild type plants. The probe was that described above for RNA gel–blot analysis. 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. Hybridization, washing of the membrane and chemiluminescence detection, was as described above.

4.12. Determination of salidroside 3 and tyrosol 1

In order to detect any change in levels of salidroside 3 in transgenic lines compared to empty vector-transformed lines, HPLC analysis was done for the above R. sachalinensis materials. Determination of salidroside 3 and tyrosol 1 was done essentially as described (Ma et al., 2007, 2008). Hairy roots were collected and air-dried for 24 h at 60°C. The dried plant material 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 MeOH 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 12,000g. The supernatant was collected, and the pellets were extracted with MeOH–H₂O (1:1, v/v). The supernatants (7–12 ml) were combined and purified using a 0.45-μm NC filter, then dried in a vacuum and dissolved in MeOH–H₂O (1:1, v/v) 3.0 ml. This preparation was used for quantification of salidroside 3 and tyrosol 1 by HPLC analysis as described above.

4.13. Phylogenetic tree construction

A total of 40 amino acid sequences of plant-specific UDP-glycosyltransferase were aligned with CLUSTALX, version 1.81 (Thompson et al., 1997). The aligned sequences were first subjected to bootstrapping using the program SEQBOOT in the PHYLIP package. Genetic distances within 100 bootstrap replicates were calculated with the PHYLIP program PROTDIST using a Dayhoff’s PAM 100 matrix. The distance matrices were analyzed with the PHYLIP program NEIGHBOR using the neighbor-joining algorithm. The multiple data from the above calculation were analyzed with
the PHYLIP program CONSENSE to obtain the bootstrap values reflecting the consistency of the tree branch pattern. The final tree was viewed using TREEVIEW WIN32 (Page, 1996). The accession numbers used in the analysis are as follows: UGT74R1 (Rhodolia sachalinensis, EF508689), UGT72B14 (Rhodolia sachalinensis, EU567325), UGT73R6 (Rhodolia sachalinensis, AY547304), UGT73G1 (Allium cepa, AY262062), UGT73J1 (Allium cepa, AY263697), ACaGT (Aralia cordata, AB103471), AS (Rauvolfia serpentine, AJ310148), BrSGT1 (Brassica napus, AA983980), UGT92T (Catharanthus roseus, AB159213), SAGT (Nicotiana tabacum AF196034), TOGT1 (Nicotiana tabacum, AF346431), TW1 (Solanum lycopersicum, X85138), UGTc2s (Crocus sativus, AY262037), UGT73A5 (Dorothyanthus bellidiformis, Y18871), 3’GT (Gentiana triflora, AB076697), UGT73F1 (Glycyrrhiza echinata, AB098614), NgTGT1 (Nicotiana tabacum, AB025528), NgTGT2 (Nicotiana tabacum, AB072919), NgTGT3 (Nicotiana tabacum, AB072918), UGT85B1 (Sorghum bicolour, AF199453), UGT74G1 (Stevia rebaudiana, AY345974), UGT85C2 (Stevia rebaudiana, AY345978).

Arabidopsis partial UGTs are as follows: UGT71C1 (AC005496), UGT72B11 (AL161491), UGT72E2 (AB181119), UGT83A3 (AL021961), UGT73B4 (AC006248), UGT73C5 (AC006282), UGT73C6 (AC006282), UGT74B1 (AC002396), UGT74F2 (AC002333), UGT76C1 (AB017060), UGT76C2 (AB005237), UGT78D1 (AC009917), UGT84A1 (AL161541), UGT84A2 (AB019322), UGT84B1 (AC002391), UGT85A1 (AC006551) and UGT89B1 (NM_106048).

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