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Cloning and characterization of glucosyltransferase cDNA from *Eucalyptus perriniana* cultured cells

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Abstract The *Eucalyptus perriniana* cultured cells are widely used to biotransform a variety of compounds. The glucosyltransferase activity of a crude protein extract of *E. perriniana* cultured cells was maximized when cell growth was in the pre-logarithmic to logarithmic phase. We cloned a cDNA encoding glucosyltransferase (EPGT) from *E. perriniana* cultured cells by RT-PCR using a degenerated primer and RACE-PCR. The cDNA contained an open reading frame encoding 467 amino acids with a calculated molecular mass of 51.6 kDa. The consensus sequence of the plant glucosyltransferases was included in the deduced amino acid sequence. The amino acid sequence of EPGT showed a high identity to glucosyltransferases from tobacco and petunia. The recombinant EPGT was expressed in *Escherichia coli* and its substrate specificity was examined using UDP-[U-^{14}C] glucose. Cinnamic acid was the best sugar acceptor in the compounds tested.

Key words: *Eucalyptus perriniana*, glucosyltransferase, glycosyltransferase.

Glycosylation is one of the secondary metabolic reactions in plant cells as well as oxidation, reduction, hydroxylation and methylation (Banthorpe, 1994). This reaction is catalyzed by glycosyltransferases (EC 2.4.x.x). Glycosyltransferases ubiquitously exist in almost all organisms, and are widely investigated because of their importance in biological activities (Ross et al. 2001; Mackenzie et al. 1997). The roles of plant glycosyltransferases have also been reported, e.g., the stabilization of pigments, the regulation of plant growth factors, and the increase in the aglycone solubility (Jones and Vogt, 2001). Recently, the glycosylation of biologically active compounds has drawn considerable attention because glycosylation often improves the effect of the drug and pharmacokinetic parameters (Kren and Martinkova 2001). For instance, it was shown that the glycoside of morphine has less side effects without reducing the analgesic effects of the morphine itself (Carrupt et al. 1991).

Biotransformation is one of the effective methods to obtain glycosylated compounds (Stöckigt et al. 1995). In general biotransformations, a single compound is converted to many products via several reactions. It is difficult to control these reactions by changing the reaction conditions or the culture conditions. Choosing the species of plant cells could be a solution of this problem. In some cases, it was reported that the reaction products of a compound varied between plant cells (Ushiyama and Furuya 1989; Orihara et al. 1991). The cultured cells of *Eucalyptus perriniana* have been used to biotransform a wide range of external compounds to glucosides and/or hydroxides (Table 1). Although glycosylation was the main reaction in the *E. perriniana* cultured cells, hydroxylated aglycones were also obtained in many cases. The enzyme fractions of the *E. perriniana* cells have been employed to produce glucosides of β-thujaplicin (Nakajima et al. 1997), kojic acid and daidzein (Nakajima et al. 2001). We report here the cDNA cloning and heterologous expression of a glucosyltransferase from *E. perriniana* cultured cells to characterize the glycosyltransferases in these cells. A full-length cDNA clone (EPGT) was obtained by a combination of RT-PCR using a degenerated primer and RACE-PCR. It was shown that recombinant EPGT expressed in *Escherichia coli* used a wide range compounds as an aglycone.

Abbreviations: EPGT, *Eucalyptus perriniana* glucosyltransferase; rEPGT, recombinant EPGT; PSPG, plant secondary product glucosyltransferase consensus sequence; UDP, uridine diphosphate.

Table 1. Biotransformation of compounds by *E. perriniana* cultured cells.

Compound	Type of reaction	Reference
(*RS*)-tropic acid	glycosylation	Ushiyama and Furuya 1989
(−)-menthol	glycosylation, hydroxylation	Furuya et al. 1989
18*β*-glycyrrhetinic acid	glycosylation, hydroxylation	Orihara and Furuya 1990
steviol	glycosylation	Orihara et al. 1991
isoeugenol eugenol	glycosylation	Orihara et al. 1992
(−)-borneol	glycosylation, hydroxylation	Orihara and Furuya 1993
caryophyllene oxide	glycosylation, hydroxylation, epoxidation, isomerization, ring opening	Orihara et al. 1994
1,8-cineole	glycosylation, hydroxylation	Orihara and Furuya, 1994a
(+)-camphor	glycosylation, hydroxylation, ring opening	Orihara et al. 1994
(+)-fenchone (−)-fenchone	glycosylation, hydroxylation	Orihara and Furuya 1994b
β-thujaplicin	glycosylation	Furuya et al. 1997
p-aminozenzoic acid	glycosylation	Furuya et al. 1998
bisphenol A	glycosylation, hydroxylation	Hamada et al. 2002

Materials and methods

Plant material

The callus tissue of *Eucalyptus perriniana* was derived from the young stems of *E. perriniana* in 1980 (Furuya et al. 1987) and maintained on MS medium (Murashige and Skoog 1962) containing 1 mg l^{-1} 6-benzylaminopurine, 30 g l^{-1} sucrose and 9 g l^{-1} agar. To produce the cell-suspension cultures, approximately 5 g of three-week-old callus tissue was transferred into 125 ml of liquid medium in a 500-ml Erlenmeyer flask and cultured at 25°C in the dark at 80 rpm on a reciprocal shaker.

Preparation of crude protein extract from cultured cells

The cultured cells of *E. perriniana* were filtered and washed with distilled water. Two ml of buffer (10 mM 2-mercaptoethanol, 100 mM Tris-HCl, pH7.0) per g fr. wt. was added and homogenated using a glass homogenizer. The cell debris was removed by centrifuging at 20,000 *g* for 10 min. The low-molecular-weight compounds were removed by gel-filtration on a PD-10 column (Amersham Biosciences) equilibrated with the same buffer.

Enzyme assay

The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.5), 1 mM substrate, 2.3 kBq UDP-[U-^{14}C] glucose and 3 μl of enzyme solution in a final volume of 60 μl. The reaction mixture was incubated at 37°C for 30 min. and extracted with 500 μl of water-saturated 1-butanol. Distilled water (100 μl) was added in a 470-μl aliquot of a 1-butanol layer and thoroughly vortexed. The radioactivity in a 400-μl aliquot of the 1-butanol extract was measured using a liquid scintillation counter (Beckman LS6000IC).

Cloning of EPGT cDNA

Standard recombinant DNA manipulation was done according to Sambrook et al. (1989). The total RNA was isolated from the 3-week-cultured cell suspension cultures according to Shirzadegan et al. (1991). A degenerated primer PSPG-1 (5′-TTYGTIACICAYTGYGGITGGAA-3′) derived from the plant secondary product glycosyltransferase consensus sequence (Hughes and Hughes, 1994) was designed for the RT-PCR. The RNA PCR Kit (AMV) version 2.1 (Takara) was employed for the RT-PCR. The PSPG-1 and poly (A)$^{+}$ specific primer supplied with the kit were used for the RT-PCR. The first denaturation was performed at 95°C for 3 min., followed by 35 cycles of 60 s at 95°C, 30 s at 40°C and 90 s at 72°C. The RACE-PCR was carried out to obtain 5′- and 3′- end sequences of the cDNA. The 5′ RACE System for Rapid Amplification of cDNA Ends version 2 (Invitrogen) and RNA PCR Kit (AMV) version 2.1 (Takara) were used for the 5′-RACE and 3′-RACE, respectively, according to the manufacturer’s protocol.

Full-length EPGT cDNA was obtained by PCR with primers corresponding to the 5′- and 3′- end sequences using the same cDNA as the 3′-RACE as a template. Pyrobest DNA polymerase (Takara) was used to avoid any PCR errors.

The PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen) by TA-cloning. The nucleotide sequences were determined using a DSQ-2000L DNA sequencer (Shimadzu). The nucleic acid sequence was deposited in DDBJ.

Heterologous expression in E. coli

The open reading frame of EPGT cDNA was cloned into an *Nde*I site and an *Xho*I site of the pET-15b vector (Novagen). The recombinant EPGT (rEPGT) was expressed in the *E. coli* strain BL21(DE3)pLysE. *E. coli* transformants were cultured in 50 ml of an LB medium with 50 mg l^{-1} carbenicillin at 37°C. Isopropyl 1-thio-*β*-D-galactopyranoside was added to a final concentration of 1 mM when the OD_{600} reached approximately 0.5 and rEPGT was induced for 20 h at 20°C. All subsequent operations were carried out at 4°C. Cells were collected by centrifugation and resuspended in 1 ml of an extraction buffer (50 mM Tris-HCl, pH 8.0, containing 2 mM EDTA). After sonication with an Astrason sonicator, the cell debris was removed by centrifugation. rEPGT was purified with a HiTrap Chelating HP column (1 ml; Amersham Biosciences) according to the manufacturer's protocol.

Results

Time course of glucosyltransferase activity and cell growth of cultured cells of E. perriniana

To determine the optimum culture period to extract the total RNA for RT-PCR, the time course of the glucosyltransferase and cell growth were demonstrated (Figure 1). The enzymatic activities of the glucosyltransferase were measured using isoeugenol and UDP-[U-^{14}C] glucose as substrates. It was shown that cell growth reached a maximum on day seventeen and the glucosyltransferase activity reached a maximum on day seven, and then gradually decreased. Considering both the cell growth and glucosyltransferase activity, the total RNA was extracted from the cultured cells on day fourteen.

Cloning, nucleotide sequencing of EPGT cDNA

A degenerated primer PSPG-1 for RT-PCR was designed from the consensus sequence called PSPG box (Figure 2; Hughes and Hughes 1994). The partial EPGT cDNA fragment of approximately 500 bp was obtained by RT-PCR. cDNA fragments of the 5′- and 3′- ends were amplified by RACE-PCR, then the full-length cDNA was cloned by PCR using Pyrobest DNA polymerase that has a proofreading activity that does not introduce a mutation in the sequence. The full length EPGT cDNA was 1,956 bp in length and contained a 1,401 bp open reading frame encoding a polypeptide of 467 amino acids (Figure 3). The calculated molecular mass was 51.6 kDa and the isoelectric point was 4.9.

Heterologous expression of recombinant EPGT in E. coli

To confirm the substrate specificity of EPGT, the open reading frame was subcloned into the expression vector pET15b. rEPGT was expressed in *E. coli* BL21 (DE3) pLysE as a histidin-tagged protein and purified by nickel affinity chromatography. The purified rEPGT was almost homogeneous on Coomasie Brilliant Blue (CBB) stained SDS-PAGE (Figure 4). The enzyme activity of the purified rEPGT was measured using ten aglycones and UDP-[U-^{14}C] glucose. As a comparison, the enzyme activity of a crude protein extract of the *E. perriniana* cultured cells was measured as well (Figure 5). rEPGT showed the highest enzyme activity toward cinnamic acid, while quercetin was the best sugar acceptor for the crude protein extract of the cultured cells.

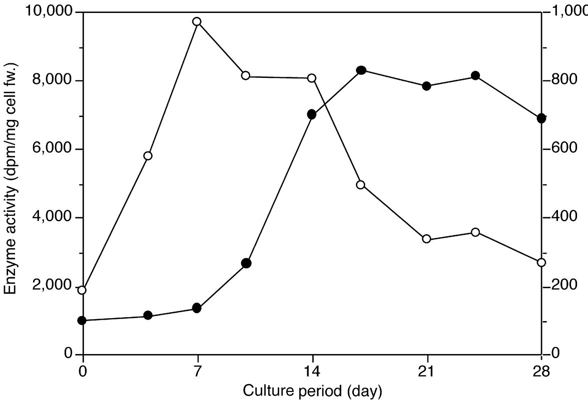

Figure 1. Time course of glucosyltransferase activity of cultured cells of *E. perriniana*. Open and closed circles denote glucosyltransferase activity and cell growth, respectively. Activities were measured using isoeugenol and UDP-[U-^{14}C] glucose as substrates. Cells weight (f.w.) on day zero is shown as 100%.

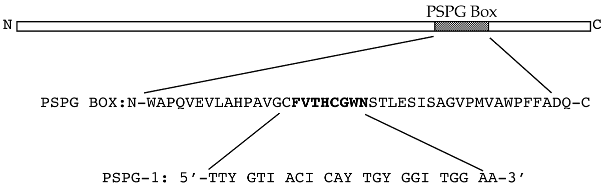

Figure 2. Diagram of the design of the PCR primer PSPG1 used for RT-PCR. A degenerate primer PSPG1 is designed based on the consensus sequence of plant secondary product glucosyltransferase consensus sequence (PSPG box; Hughes and Hughes, 1994). Degeneracy of this primer was eight, and three inosines were included.

Discussion

The cultured cells of *E. perriniana* possess biotransformation abilities including hydroxylation and glycosylation against a wide range of compounds. In previous investigations, compounds are fed when cell growth reached a maximum, but the time course of the glycosyltransferase activity has not yet been reported. It was shown that the enzyme activity that peaked on day seven to day fourteen corresponds to the pre-logarithmic to logarithmic phase of cell growth (Figure 1). This result shows that the glucosyltransferase in the crude protein extracts may correspond to the primary metabolism such as a cell wall production.

Glycosyltransferase cDNAs, in most cases, were cloned by a screening of the cDNA library using other cDNAs as a probe. In theory, cDNAs that have a high similarity to a probe cDNA will be cloned by this method. To obtain a functionally unique glycosyltransferase cDNA, purification and sequencing the corresponding enzyme are needed, and the cDNA

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   1  GGAAATTCATTTAGTTAAACCGGAGACAAATATAATTTTGTCACTGATTATAAAAATATTCAAAAAGAGTAGGTGAACTCATCATTAAAG    90
  91  ACCAAAAAAAAAAAAGAAACTCATCATTCATCATATTTTAAATTATCTATGACTTGTCCAAGATGAGAAGCAGCGAAGCGTATATGATTA   180
 181  TTATCGTTTTCGCTTCTTCTAACCCTGAGAATAATGAAACTCAACCTCAACACCTTTGAGGAACAACATAGAGTTCCAAATCAAATAATA   270
 271  CTTTTTCACTTAAAACATGCATGTTCGTAATAAAATTCAAAAGCTGCCTTCTCTTATTCGTCATCATTTACGCAAGCCCCTCAAATCCCC   360
 361  AATATAGTATATATAAGGAACAATGAACGCTCTATTCTTGCACCAAAAACTCAAAAAGAAAGAGAGAAAAAAAAAAGGTTATAGCATCAA   450
 451  CCCTAAACCATGGCCCCTCTCAATTATCTTGTAGTAGCATTCCCAGCCCAAGGCCTCATCAACCCAGCCCTCCAAATCGCCAAGCGTCTT   540
   1           M  A  P  L  N  Y  L  V  V  A  F  P  A  Q  G  L  I  N  P  A  L  Q  I  A  K  R  L     27

 541  CTACACGCCGGCGCGCATGTCACCTTCGCCACTGCAGGGTCGGCTTATCGTCGCATGGCCAAGTCCGACCCACCTGAAGGATTGTCCTTC   630
  28  L  H  A  G  A  H  V  T  F  A  T  A  G  S  A  Y  R  R  M  A  K  S  D  P  P  E  G  L  S  F     57

 631  GCTTCCTTCTCCGATGGCTCTGACGAGGGGCTCAAGCCTGGGATCGACTTCAACCAGTACATGGTCGACGTTGAGCGGCTGGGTTCGGAA   720
  58  A  S  F  S  D  G  S  D  E  G  L  K  P  G  I  D  F  N  Q  Y  M  V  D  V  E  R  L  G  S  E     87

 721  ACTTTAAGAGACCTTGTCGTTACCAGCCTTAACGAAGGCCGCAAGTTCGCGTGCATATTTTATACCACAATCATTCCTTGGGTTGCTCAG   810
  88  T  L  R  D  L  V  V  T  S  L  N  E  G  R  K  F  A  C  I  F  Y  T  T  I  I  P  W  V  A  Q    117

 811  GTGGCTCATTCTCTTCAAATCCCCTCAACCCTCATTTGGACTCAGCCTGCTACTCTTCTTGATATATATTACTATTACTTCAATGGTTAT   900
 118  V  A  H  S  L  Q  I  P  S  T  L  I  W  T  Q  P  A  T  L  L  D  I  Y  Y  Y  Y  F  N  G  Y    147

 901  GGTGATATTATCAGAAATCTAGGCAAGGACGATCCTTCGGCGTTGCTTCACTTACCAGGATTGCCACCGCTAACTCCCCCAGACATCCCC   990
 148  G  D  I  I  R  N  L  G  K  D  D  P  S  A  L  L  H  L  P  G  L  P  P  L  T  P  P  D  I  P    177

 991  TCATTTTTCACACCCGATAACCAATATGCTTTTACTCTGCCACTTATGCAAATGCAGTTTGAACTATTCAAGGAAGAAAAGTATCCGAGG  1080
 178  S  F  F  T  P  D  N  Q  Y  A  F  T  L  P  L  M  Q  M  Q  F  E  L  F  K  E  E  K  Y  P  R    207

1081  GTACTTGTGAACACTTTCGATGCGCTAGAGCCAGGGCCATTAAAAGCCATTGGCAACGTCACCATGTTTGGGATCGGCCCACTAATTCCT  1170
 208  V  L  V  N  T  F  D  A  L  E  P  G  P  L  K  A  I  G  N  V  T  M  F  G  I  G  P  L  I  P    237

1171  TCTGCATTCTTGGATGGACAAGATCCCTTGGACAAATCCTTCGGAGGCGATCTCTTCCAGGGTTCGAAAGGCTACATCCAGTGGCTAGAC  1260
 238  S  A  F  L  D  G  Q  D  P  L  D  K  S  F  G  G  D  L  F  Q  G  S  K  G  Y  I  Q  W  L  D    267

1261  ACCAAGCCCAAAGGTTCAGTGATTTATGTATCCTTTGGAAGCATCTCCGTGCTATCGAAGGCACAAAAGGAGGAGATGGCACGTGGGTTA  1350
 268  T  K  P  K  G  S  V  I  Y  V  S  F  G  S  I  S  V  L  S  K  A  Q  K  E  E  M  A  R  G  L    297

1351  CTTGGCACCGGCCATCCGTTCTTGTGGGTGATTAGGAAGGACAAAGATGAGGAAGGAGAGGGCGAGCAAGATCATTTGAGCTGTATGGAG  1440
 298  L  G  T  G  H  P  F  L  W  V  I  R  K  D  K  D  E  E  G  E  G  E  Q  D  H  L  S  C  M  E    327

1441  GAATTGGAGCAAAAGGGAATGATAGTTCCATGGTGCTCTCAAGTGGAGGTGCTGTCCCATGCTTCAGTAGGGTGTTTTGTGACTCATAGT  1530
 328  E  L  E  Q  K  G  M  I  V  P  W  C  S  Q  V  E  V  L  S  H  A  S  V  G  C  F  V  T  H  S    357

1531  GGTTGGAACTCAACCTTTGAGAGCTTGGCTTGTGGGGTCCCAATGGTGGCCTTCCCCCAATGGAATGATCAGCTAACCAATGCCATGCTC  1620
 358  G  W  N  S  T  F  E  S  L  A  C  G  V  P  M  V  A  F  P  Q  W  N  D  Q  L  T  N  A  M  L    387

1621  GTTGAGAATGAGTGGAAGGTTGGGGTGAGGGTGAACGTGAATGAGGGAGGCGTAGTTGAAGGAGATGAGATCAAGAGATGCTTGGAATTG  1710
 388  V  E  N  E  W  K  V  G  V  R  V  N  V  N  E  G  G  V  V  E  G  D  E  I  K  R  C  L  E  L    417

1711  GTGGTGGGAGATGGAGAGCAGGGAGAGGAAATTAGAAGGAATGCGAAGAAGTGGAAACATTTGGCAAGAGAAGCTGCTAAAGAAGGTGGG  1800
 418  V  V  G  D  G  E  Q  G  E  E  I  R  R  N  A  K  K  W  K  H  L  A  R  E  A  A  K  E  G  G    447

1801  TCATCGGACAGGAATCTCAAGGCATTCTTGGAGGAGATCCAAATCGAAGCAAATGGTCACTAGAGTACTAGCACTTCACCTGTTATTCCG  1890
 448  S  S  D  R  N  L  K  A  F  L  E  E  I  Q  I  E  A  N  G  H  *                                467

1891  TTGCTTTCGTATTTCTATCTTGTTTGATTTTTACAATATCTGGGAATAAATAAATTCATATTGCTC                        1956
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Figure 3. Nucleotide and amino acid sequences of full length EPGT cDNA. The PSPG-box is shaded.

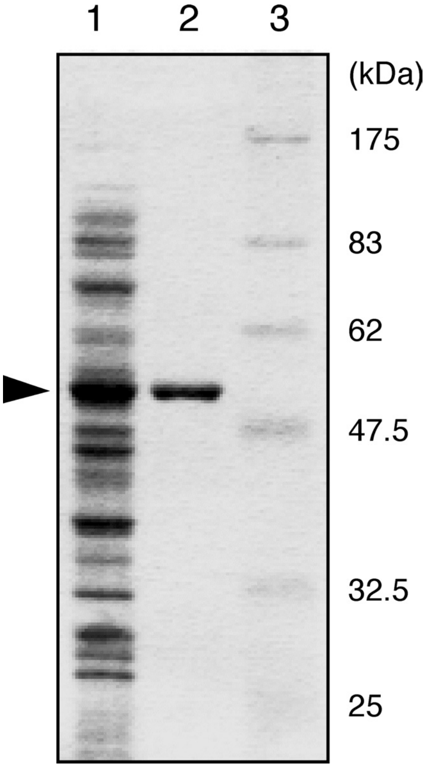

Figure 4. SDS-PAGE of rEPGT proteins. Lane 1, crude protein extract of an induced *E. coli*; lane 2, purified rEPGT protein; lane 3, molecular weight marker proteins. Gel was stained by CBB. The arrowhead shows the rEPGT protein.

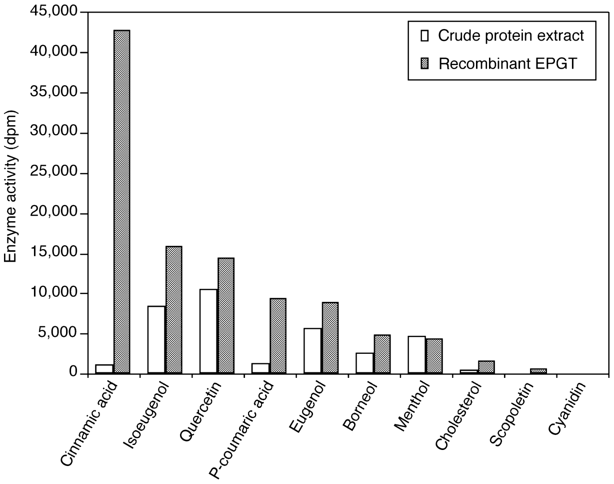

Figure 5. Substrate specificities of rEPGT and a crude protein extract. Activities were measured using UDP-[U-^{14}C] glucose as a sugar donor.

fragment will be cloned by RT-PCR. These are usually very difficult and time-consuming methods. Recently, a RT-PCR method using degenerated primers designed from a consensus sequence of the glycosyltransferases was used to solve these problems (Milkowski et al. 2000; Kramer et al. 2003). Highly conserved amino acids in the PSPG-box (Vogt and Jones 2000) were used to design the degenerated primer PSPG1.

The deduced amino acid sequence of the full-length cDNA has a PSPG-box (Figure 3), and it was predicted to be localized at a cytosol by the PSROT program (Nakai and Horton 1999), and showed high identity with a stress inducible glucosyltransferase from tobacco (52% identity; Taguchi et al. 2003) and an anthocyanin glucosyltransferase from petunia (48% identity;

Yamazaki et al. 2002). It was shown that these two clones show different substrate specificities despite a high identity of the amino acid sequences. Therefore, it was difficult to predict the substrate specificity of EPGT.

rEPGT was expressed in *E. coli* and assayed using ten compounds as an aglycone and UDP-[U-^{14}C] glucose as a sugar donor (Figure 5). Cinnamic acid was the best aglycone for the rEPGT activity and other compounds were also used except cyanidin. This result shows that rEPGT can glucosylate a carboxyl group as well as a hydroxyl group and are compatible with the result in the previous biotransformation experiment using 18*β*-glycyrrhetinic acid. The *E. perriniana* cells glucosylated the carboxyl group at the 30-position rather than the hydroxyl group at the 3-position of 18*β*-glycyrrhetinic acid (Orihara and Furuya 1990). It is fascinating that the activity toward *p*-coumaric acid (*p*-hydroxycinnamic acid) was approximately 22% of the activity toward cinnamic acid. The hydroxyl residue of *p*-coumaric acid might be an obstacle when it gets into the enzymatic reaction area of rEPGT. On the other hand, quercetin was the best for the crude protein extract of cultured cells. The difference in the substrate specificity between rEPGT and a crude protein extract shows that several glucosyltransferases will exist in the *E. perriniana* cultured cells. In this report, only a soluble fraction of the cultured cells was assayed. It has been reported that there is a UDP-glucosyltransferase activity in an insoluble fraction of *E. perriniana* cells (Nakajima et al. 1997, 1999, 2001). Only one UDP-glucosyltransferase cDNA was cloned in this research, but several cDNAs that have different characteristics will be cloned in future studies.

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