Plant Biotechnology 26, 285–292 (2009)

Original Paper

Identification of an inducible glucosyltransferase from *Phytolacca americana* L. cells that are capable of glucosylating capsaicin

Akio Noguchi[1], Satoshi Kunikane[1], Hiroaki Homma[1], Wenhai Liu[1], Takashi Sekiya[1], Miho Hosoya[1], Soonil Kwon[2], Shingo Ohiwa[2], Hisashi Katsuragi[3], Tokuzo Nishino[1], Seiji Takahashi[1], Hiroki Hamada[2], Toru Nakayama[1,*]

[1] Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Sendai, Miyagi 980-8579, Japan; [2] Department of Life Science, Faculty of Science, Okayama University of Science, Okayama 700-0005, Japan; [3] Sunny Health Co. Ltd., Chuo-ku, Tokyo 103-0028, Japan

* E-mail: nakayama@seika.che.tohoku.ac.jp Tel & Fax: +81-22-795-7270

Received December 22, 2008; accepted February 16, 2009 (Edited by K. Yazaki)

Abstract Cell suspension cultures of *Phytolacca americana* L. (pokeweed) are capable of glycosylating capsaicinoids that have several biomedical applications. To identify the capsaicinoid glucosyltransferase involved in this biotransformation, we isolated three full-length cDNAs (*PaGTs*) encoding homologs of plant secondary product glycosyltransferases from cultured cells of *P. americana* L. These glycosyltransferase cDNAs were heterologously expressed in *Escherichia coli* cells and the expressed products were functionally characterized. Although all of these glycosyltransferases displayed broad glucosyl-acceptor specificities toward phenolics, capsaicinoid glucosyltransferase activity was found only for one of the cloned enzymes, PaGT3. Phylogenetic analysis showed that PaGT3 is the most closely related to betanidin 5-*O*-glucosyltransferase from *Dorotheanthus bellidiformis*, and in fact, it displayed a weak betanidin 5-*O*-glucosyltransferase activity. Transcription analyses showed that the expression of *PaGT3* in *P. americana* L. was strongly induced by exposure of the cells to capsaicin (0.65 mM). These results show that PaGT3 should be, at least in part, responsible for the capsaicinoid glucosyltransferase activity of this plant.

Key words: Capsaicin, glycosyltransferase, *Phytolacca americana* L., UDP-glucose.

Glycosylation is a powerful method for the structural and functional modification of bioactive compounds—it enhances solubility, physicochemical stability, biological half-life, membrane permeability, intestinal absorption, and improves taste (Vogt and Jones 2000). This is well-illustrated by modification of capsaicinoids by glycosylation.

Capsaicinoids are a series of branched or straight-chain alkylvanillylamides produced by plants of the *Capsicum* species and may be involved in the defense mechanism of this plant species (Siegler 1998). Capsaicinoids are important sources of foods, spices, and medicines. Capsaicin, *N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-(*E*)-6-nonenamide, is the most pungent principle compound among naturally-occurring capsaicinoids. Capsaicinoids show several bioactivities of medical interest. For example, these compounds reduce adipose tissue weight and serum triacylglycerol concentrations in rats by enhancing energy expenditure due to elevated β-adrenergic activity (Kawada et al. 1986; Henry and Emery 1986). Capsaicinoids also display analgesic effects and have been used to treat a variety of painful conditions affecting the periphery, such as rheumatoid arthritis and diabetic neuropathy (Caterina et al. 1997; Dray 1992). Moreover, capsaicinoids are reported to display anti-genotoxic, anti-mutagenic, and anti-carcinogenic effects (Surh and Lee 1995; Surh et al. 1998; Park et al. 1998). However, extensive use of capsaicinoids as food additives and medicines is limited, mainly because they are irritants, producing a burning sensation in the mouth, skin, and mucous membranes. In addition, capsaicinoids are only poorly absorbed after oral administration due to their poor water-solubility.

Thus far, cell suspension cultures of several plant species, such as those of *Coffea arabica* (Kometani et al. 1993), *Phytolacca americana* (Hamada et al. 2003), and *Catharanthus roseus* (Shimoda et al. 2007), have been shown to be capable of glycosylating capsaicinoids. In

Abbreviations: CapGT, capsaicinoid glucosyltransferase; GT, glycosyltransferase; PaGT, *Phytolacca americana* L. glucosyltransferase; PSPG, plant secondary product glycosyltransferase; RT-PCR, reverse-transcription polymerase-chain reaction

This article can be found at http://www.jspcmb.jp/

these systems, glycosyl donor molecules (UDP-sugars) can be reproduced in the cultured cells, so that the addition of exogenous glycosyl donors to the reaction mixture is not required. The resulting glycoconjugates of capsaicinoids are water-soluble and, interestingly, have essentially no pungency (Kometani et al. 1993). Thus, these easily ingested glycoconjugates may be promising pro-drug and weight-loss formulations. However, the enzyme(s) involved in the biotransformation of capsaicinoids by glycosylation *in planta* remain to be identified.

The purpose of the present study was to identify the cDNA of the enzyme that is responsible for glucosylation of capsaicinoids in the cells of *P. americana* L. (pokeweed, a Caryophyllales plant) (Hamada et al. 2003). Glycosyltransferase cDNAs (*PaGTs*) were isolated from the *P. americana* L. cells and heterologously expressed in *Escherichia coli* cells. A comparison of the substrate specificities of the expressed products allowed us to identify PaGT3 as a strong candidate for an enzyme responsible for the capsaicinoid glucosyltransferase (CapGT) activity. Transcription analyses show that *PaGT3* is induced upon the addition of capsaicin.

Materials and methods

Plant materials and chemicals

The callus tissues from *P. americana* L. (Hamada et al. 2003) were obtained by subculturing on Murashige & Skoog's (MS) agar medium (Murashige and Skoog 1962) containing 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid at 25°C in the light. To produce the suspension cultures, callus tissue (20 g) that had been cultured in the light for 4 weeks each were transferred to an Erlenmeyer flask (300 ml in size) containing 100 ml MS liquid medium, and 20 mg of capsaicin dissolved in ethanol was added to the flasks. The callus tissues were cultured at 25°C with shaking in the light. At time intervals, the cells were collected by filtration and stored at −80°C until use.

Naringenin, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, salicylic acid, salicyl alcohol, hydroquinone, *trans-p*-coumaric acid were purchased from Nacalai Tesque (Kyoto, Japan). Kaempferol and quercetin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Apigenin was purchased from Funakoshi (Tokyo, Japan). Genistein and daidzein were products of Fujicco (Kobe, Japan). Capsaicin, 8-nordihydrocapsaicin, UDP-glucose, and UDP-galactose were purchased from Sigma (St. Louis, MO, USA). Aureusidin (Nakayama et al. 2001) and cyanidin (Noguchi et al. 2007) were obtained as described previously. Betanidin was prepared by treatment of betanin (red beet powder; Mitsubishi Kagaku Foods, Tokyo, Japan) with β-glucosidase (from almond; Sigma) followed by reversed-phase HPLC on a *J*'sphere ODS-M80 column (4.6×150 mm, YMC, Kyoto, Japan). All other chemicals were of analytical grade.

cDNA cloning

Poly(A)$^+$ RNA was isolated from *P. americana* L. cells harvested 3 days after capsaicin addition (see above) using the Straight A's™ mRNA Isolation System (Novagen, Madison, WI, USA). The cDNA was synthesized from 5 μg of poly(A)$^+$ RNA using the ZAP-cDNA™ synthesis kit (Stratagene, La Jolla, CA, USA). The cDNA was ligated with *Eco*RI adapters and inserted into the Uni-ZAP XR vector (Stratagene). The resulting constructs were packaged using the Gigapack III Gold packaging extract (Stratagene). The resulting primary library contained 200,000 plaque-forming units.

Based on the amino acid sequence that is highly conserved among plant secondary product glycosyltransferases (PSPGs), two degenerate PCR primers were designed (PSPGT1, 5′-TT(C/T)ITIACICA(C/T)TG(C/T)GGITGGAA-3′; PSPGT2, 5′-TG(C/T)GGITGGAA(C/T)TCI(A/G)(C/T)I(C/T)TIGA-3′). Total RNA was prepared from *P. americana* L. cells harvested 3 days after the addition of capsaicin using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RT-PCR was performed using the QIAGEN OneStep RT-PCR Kit (Qiagen) with PSPGT1 and oligo dT primers, using total RNA as the template. The thermal cycling sequence was as follows: the RT-PCR mixture was incubated at 50°C for 30 min for reverse transcription; DNA polymerase was activated and reverse transcriptase was inactivated by elevating the temperature to 95°C for 15 min; 30 cycles of PCR (one cycle consists of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min); and, a final incubation at 72°C for 10 min. The RT-PCR product was used as a template for nested PCR using PSPGT2 and oligo dT primers. Thermal cycling conditions used in the nested PCR were as follows: 94°C for 2 min, followed by 30 cycles of PCR (one cycle consists of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min), and 72°C for 10 min. The amplified fragments, approximately 500 bp in length, were cloned into TOPO-pCR2.1 (Invitrogen, Carlsbad, California, USA) and sequenced using a Dye-Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA) with a CEQ 2000 DNA analysis system (Beckman Coulter). The cloned fragments were DIG-labeled using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Basel, Switzerland) and were used as probes to screen the cDNA library. Hybridization was performed at 37°C for 16 h in 5×SSC containing 0.02% (w/v) sodium dodecyl sulfate, 0.1% (w/v) N-lauroylsarcosine, 2% (w/v) blocking reagent (Roche Molecular Biochemicals), and 30% (v/v) formamide. The filters were washed twice in 0.1×SSC and 0.1% (w/v) sodium dodecyl sulfate at 60°C for 15 min. The DIG-DNA Labeling and Detection Kit (Roche Molecular Biochemicals) was used to detect DIG-labeled DNA. The cDNAs of positive clones were rescued in the pBluescript SK-phagemid following the *in vivo* excision protocol and then sequenced. For the clone (*PaGT3*) lacking a translation initiation codon, the 5′-fragment was obtained using a 5′ rapid amplification of cDNA ends (5′RACE) system (Invitrogen) with the primers 5′-ATGTTGAACTTGGCTG-3′, 5′-TGCAG-AGTCGGTCGCCCATG-3′ and 5′-CCATGGGAAGAACAT-ATCCGCC-3′, and with the total RNA prepared from *P. americana* L. cells.

Heterologous expression of PaGTs in E. coli cells

The *PaGT1*-coding sequence of cDNA was amplified by PCR using the primers, 5′-AGATAACATATGAGAAAAACAGA-

GCTGG-3′ and 5′-TGTACGGGATCCGGCTTAGGTTGAG-3′. These primers correspond to the 5′- and 3′-ends of the open reading frame and include *Nde*I and *Bam*HI sites (underlined), respectively. The amplified fragment was digested with NdeI and *Bam*HI and then ligated into a pET-15b vector (Novagen), yielding pET-15b-PaGT1, which was used to transform *E. coli* BL21(DE3) cells. The coding sequences of *PaGT2* and *PaGT3* were amplified by PCR using the primers, 5′-GGTCTCC-CATGGAAATGGAAGCACC-3′ and 5′-AGTTGGGGTCTC-GGATCCTTAGCTTTTGC-3′, for *PaGT2* cDNA, and 5′-CCA-AGTGGTCTCGGATCCATGGGTGC-3′ and 5′-TAGAGAGG-TCTCGGATCCCTAAGCATGATAAC-3′, for *PaGT3* cDNA. These primers correspond to the 5′- and 3′-ends of the open reading frame and include *Bsa*I sites (underlined). To produce *Nco*I (shown with wavy line) and *Bam*HI (double-underlined) ends in *PaGT2* cDNA and two *Bam*HI ends (double-underlined) in *PaGT3* cDNA, the amplified fragments were digested with *Bsa*I. The resulting fragments were ligated into an *Nco*I/*Bam*HI-digested pET-32a vector (Novagen) and a *Bam*HI-digested pQE-30 vector (Qiagen), yielding pET-32a-PaGT2 and pQE-30-PaGT3, which were used to transform *E. coli* BL21(DE3) and *E. coli* XL1-Blue cells, respectively.

Transformant cells were grown in Luria-Bertani (LB) broth containing 50 μg/ml ampicillin with shaking at 37°C for 16 h. Ten milliliters of the culture were inoculated into 2000 ml of LB broth and the cells were grown with shaking at 20°C. When optical density at 600 nm of the culture reached 0.5, isopropyl 1-β-D-thiogalactoside was added to the culture at a final concentration of 0.4 mM, followed by cultivation at 20°C for an additional 15 h.

All subsequent methods were performed at 0–4°C. The cells were harvested by centrifugation at 5,000×*g* for 15 min, washed with distilled water, and resuspended in buffer H (20 mM sodium phosphate (pH 7.4), containing 15 mM 2-mercaptoethanol, 10 mM imidazol, and 0.5 M NaCl). The cell suspension was subjected to ultrasonication, and the resulting debris was removed by centrifugation at 5,000×*g* for 15 min. Polyethyleneimine was added at a final concentration of 0.12% (v/v), and the mixture was allowed to stand for 30 min. After centrifugation at 5,000×*g* for 15 min, the supernatant was applied to a HisTrap HP column (1 ml, GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with buffer H. The column was washed with buffer H, and the enzyme was eluted with buffer H containing 200 mM imidazole. The fraction eluted with 200 mM imidazole was concentrated and equilibrated with 20 mM potassium phosphate (pH 7.2), containing 15 mM 2-mercaptoethanol by repeated concentration and dilution using an Amicon Ultra-15 Centrifugal Filter Device (Millipore, Billerica, MA, USA). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the purified enzyme was carried out using a 10% gel according to the method of Laemmli (Laemmli 1970).

Enzyme assays

The standard reaction mixture (100 μl) consisted of 50 μM capsaicin (or other glycosyl acceptor), 100 μM UDP-glucose, 50 mM potassium phosphate (pH 7.2), and enzyme. After a 10-min pre-incubation of the mixture without the enzyme at 30°C, the reaction was started by addition of the enzyme. After incubation at 30°C for 60 min, the reaction was stopped by the addition of 150 μl of 2.5% (v/v) trifluoroacetic acid. The substrates and glucosylated products (except for betacyanin and its glucoside) were separated by reversed-phase HPLC on a COSMOSIL 5C^{18}-MS-II column (4.6×150 mm, Nacalai tesque, Kyoto, Japan) using a linear gradient of 4.5 to 90% (v/v) CH_3CN containing 0.5% (v/v) trifluoroacetic acid in 15 min at a flow rate of 1 ml/min. The compounds were detected at their λ_{max} values using a SPD-10A *VP* UV-visible detector (Shimadzu, Kyoto, Japan). Betanidin and its glucoside were separated using a linear gradient of 4.5 to 27% (v/v) CH_3CN containing 0.5% (v/v) trifluoroacetic acid in 15 min with a detection wavelength of 540 nm.

Quantitative real-time RT-PCR

Total RNA was prepared from the cultured *P. americana* L. cells using the RNeasy Plant Mini Kit (Qiagen). The *PaGT3* transcript in 0.2 μg of total RNA from the cultured *P. americana* L. cells were quantified by quantitative real-time RT-PCR with the *PaGT3*-specific primers, 5′-GATTTAAGTG-CACTGATTGAGG-3′ and 5′-CTAGCACTCAATTTCGATG-G-3′. Real-time RT-PCR was carried out using the LightCycler Quick System model 330 (Roche Diagnotics, Basel, Switzerland) and the QuantiTect SYBR Green RT-PCR Kit (Qiagen). The thermal cycling conditions for *PaGT3* were as follows: 50°C for 30 min; 95°C for 15 min; and, 35 cycles of 94°C for 15 s, 60°C for 20 s, 72°C for 10 s, and 75°C for 5 s. A plasmid encoding the full-length *PaGT3* cDNA was used as a template for calibration. The *PaGT1* and *PaGT2* transcripts were also quantified as described above. PCR primers used were 5′-GA-ACTCAACCTAAGCCAAATTACG-3′ and 5′-GAGGTAACA-ATATGCTCAAGGC-3′ for *PaGT1*, and 5′-CAAAAGCTAAT-AGAAAGATGTACC-3′ and 5′-CCAGATTTGTAAATGAAA-TGACC-3′ for *PaGT2*. Thermal cycling conditions were as follows: 50°C for 30 min; 95°C for 15 min; and, 35 cycles of 94°C for 15 s, 53°C for 30 s, and 72°C for 30 s for *PaGT1*; and 50°C for 30 min; 95°C for 15 min; and, 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s for *PaGT2*. The results are presented as the average±standard error of three independent determinations.

Results and discussion

Cloning of glycosyltransferase cDNAs from P. americana L. cells

We previously reported the one-step production of capsaicin *O*-β-glucopyranoside by *P. americana* L. callus (Hamada et al. 2003). Under the given conditions (see Materials and methods), maximal accumulation of capsaicin *O*-β-glucopyranoside was attained 2 days after addition of capsaicin and then subsequently decreased. The crude extract showed essentially no CapGT activity (0.08 pkatal mg^{-1}) when the callus was grown in medium without added capsaicin. However, CapGT activity was induced upon addition of capsaicin, reaching the maximum (7 pkatal mg^{-1}) 2–3 days after addition of capsaicin and remaining essentially unchanged thereafter. Thus, *P. americana* L. calli, grown as

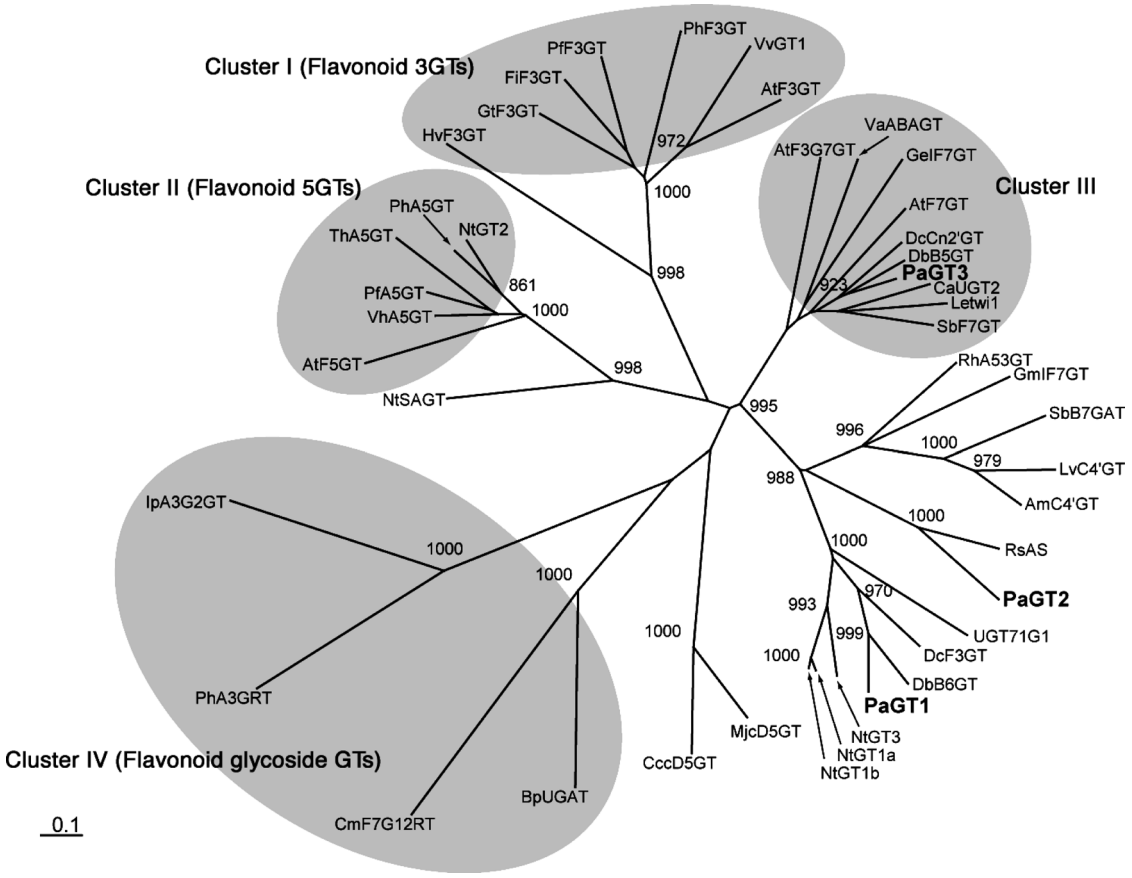


Figure 1. Phylogenetic trees of PSPGs. The sequences used for the alignment were primarily those of PSPGs that have been functionally characterized. The tree was constructed from a ClustalW program multiple alignment using the neighbor-joining method of TreeView (Page 1996). *Bar*=0.1 amino acid substitution/site. *Numbers* indicate bootstrap values >800. Known clusters (flavonoid 3GT, flavonoid 5GT, flavonoid 7GT, and flavonoid glycoside GT) of PSPGs (Vogt and Jones 2000; Sawada et al. 2005; Yonekura-Sakakibara et al. 2007; Noguchi et al. 2007) are shown with *gray circles*. PaGTs are shown in boldface letters. The names and DDBJ/GenBank/EBI accession numbers of PSPGs used for the alignment are as follows: AtF3G7GT, *Arabidopsis thaliana* UDP-glucose:flavonol 3-*O*-glycoside 7-*O*-glucosyltransferase (Q9ZQ95; AGI code, At2g36790); AtF7GT, *A. thaliana* flavonoid 7-*O*-glucosyltransferase (AAL90934; AGI code, At4g34130); AtF5GT, *A. thaliana* flavonoid 5-*O*-glucosyltransferase (AAM91686; AGI code, At4g14090); AtF3GT, *A. thaliana* flavonoid 3-*O*-glucosyltransferase (AAM91139; AGI code, At5g17050); AmC4′GT, *Antirrhinum majus* UDP-glucose: chalcone 4′-*O*-glucosyltransferase (BAE48239); BpUGAT, *Bellis perennis* UDP-glucuronic acid:anthocyanidin-3-*O*-glucoside 2″-*O*-glucuronosyltransferase (AB190262); CaUGT2, *Catharanthus roseus* UDP-glucose:glucosyltransferase 2 (BAD29722); CccD5GT, *C. cristata cyclo*-DOPA 5-*O*-glucosyltransferases (BAD91804); CmF7G12RT, *Citrus maxima* UDP-rhamnose: flavonoid-7-*O*-glycoside 1,2-*O*-rhamnosyltransferase (AAL06646); DbB5GT, *D. bellidiformis* betanidin 5-*O*-glucosyltransferase (CAB56231); DbB6GT, *D. bellidiformis* betanidin 6-*O*-glucosyltransferase (AAL57240); DcCn2′GT, *Dianthus caryophyllus* UDP-glucose:chalcononaringenin 2′-*O*-glucosyltransferase (BAD52006); DcF3GT, *D. caryophyllus* UDP-glucose:flavonol 3-*O*-glucosyltransferase (BAD52004); FiF3GT, *Forsythia intermedia* UDP-glucose: flavonoid 3-*O*-glucosyltransferase (AAD21086); GeIF7GT, *Glycyrrhiza echinata* UDPglucose: isoflavonoid 7-*O*-glucosyltransferase (BAC78438); GmIF7GT, *Glycine max* UDP-glucose:isoflavone 7-*O*-glucosyltransferase (AB292164); GtF3GT, *Gentiana triflora* flavonoid 3-*O*-glucosyltransferase (BAA12737); HvF3GT, *Hordeum vulgare* UDP-glucose:flavonoid 3-*O*-glucosyltransferase (CAA33729); IpA3G2GT, *Ipomoea purpure*a UDP-glucose:anthocyanidin-3-glucoside 2″-*O*-glucosyltransferase (BAD95882); Letwi1, *Lycopersicon esculentum* probable glucosyltransferase *twi1* (X85138); LvC4′GT, *Linaria vulgaris* UDP-glucose:chalcone 4′-*O*-glucosyltransferase (BAE48240); MjcD5GT, *M. jalapa cyclo*-DOPA 5-*O*-glucosyltransferases (BAD91803); NtGT1a, *N. tabacum* glucosyltransferase-1a (BAB60720); NtGT1b, *N. tabacum* glucosyltransferase-1b (BAB60721); NtGT2, *N. tabacum* glucosyltransferase-2 (BAB88935); NtGT3, *N. tabacum* glucosyltransferase-3 (BAB88934); *NtSAGT*, *N. tabacum* UDP-glucose:salicylic acid glucosyltransferase (AAF61647); PfF3GT, *Perilla frutescens* UDP-glucose:flavonoid 3-*O*-glucosyltransferase (BAA19659); PfA5GT, *P. frutescens* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (BAA36421); PhF3GT, *Petunia*×*hybrida* UDP-glucose:anthocyanin 3-*O*-glucosyltransferase (BAA89008); PhA5GT, *P. hybrida* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (BAA89009); PhA3GRT, *P. hybrida* UDP-rhamnose:anthocyanidin-3-*O*-glycoside rhamnosyltransferase (CAA50376); RhA53GT, *Rosa*×*hybrida* UDP-glucose:anthocyanidin 5,3-*O*-glucosyltransferase (BAD99560); RsAS, *Rauvolfia serpentina* arbutin synthase (CAC35167); SbB7GAT, *Scutellaria baicalensis* UDP-glucuronate:baicalein 7-*O*-glucuronosyltransferase (BAC98300); SbF7GT, *S. baicalensis* UDP-glucose:flavonoid 7-*O*-glucosyltransferase (BAA83484); ThA5GT, *Torenia hybrida* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (BAC54093); UGT71G1, *Medicago truncatula* triterpene UDP-glucosyltransferase (AAW56092); VaABAGT, *Vigna angularis* abscisic acid glucosyltransferase (BAB83692); VhA5GT, *Verbena*×*hybrida* UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (BAA36423); VvGT1, *Vitis vinifera* UDP-glucose:flavonoid 3-*O*-glucosyltransferase-1 (AAB81682).

described under Materials and methods, were harvested 3 days after the addition of capsaicin. Total RNA was prepared from the harvested callus and used as a template for RT-PCR with primers designed on the basis of the amino acid sequence that is highly conserved among PSPGs (Vogt and Jones 2000) (for details, see Materials and methods). Three partial cDNA fragments of *ca.* 500 bp were obtained and DIG-labeled. A cDNA library (200,000 clones) prepared from the callus was then screened for the PSPG cDNAs by plaque hybridization using each DIG-labeled cDNA fragments as probes. Three positive clones were obtained, two of which contained full-length cDNAs, termed *PaGT1* and *PaGT2*. The remaining positive clone contained a cDNA that lacked a 5′-coding sequence; the full-length form of the cDNA, termed *PaGT3*, was obtained by means of a 5′RACE system.

The *PaGT1*, *PaGT2*, and *PaGT3* cDNAs encode proteins comprised of 491, 469, and 485 amino acids, respectively (AB458516 for *PaGT1*, AB458515 for *PaGT2*, and AB458517 for *PaGT3*). Sequence identities among these three PSPGs are as follows: 32% between PaGT1 and PaGT2; 27% between PaGT2 and PaGT3; and, 26% between PaGT1 and PaGT3. Phylogenetic analysis showed that these three PSPGs are distantly related to each other (Figure 1). According to the glycosyltransferase nomenclature guidelines (Mackenzie et al. 2005), the systematic names of PaGT1, PaGT2, and PaGT3 are UGT71F6, UGT72B8, and UGT73A11, respectively. PaGT1 and PaGT2 are closely related to betanidin 6-*O*-glucosyltransferase of *Dorotheanthus bellidiformis* (DbB6GT; identity, 68%) (Vogt 2002) and arbutin synthase of *Rauvolfia serpentina* (RsAS, 58%) (Arend et al. 2000), respectively. *PaGT3* is closely related to betanidin 5-*O*-glucosyltransferase of *D. bellidiformis* (DbB5GT, 70%) (Vogt et al. 1999) and belongs to an enzyme cluster (Figure 1) that is characterized by flavonoid 7-*O*-glycosyltransferases, flavonoid 4′-*O*-glycosyltransferases, DbB5GT, and several stress-inducible PSPGs.

PaGT3 displays a CapGT activity

The *PaGT1*, *PaGT2*, and *PaGT3* cDNAs were then heterologously expressed as His_6-tagged proteins. *PaGT3* was expressed by using an *E. coli* XL1-Blue/pQE30 system (see also Materials and methods) and the expressed product was purified to near homogeneity using nickel affinity chromatography (Figure 2). The expression of the *PaGT1* and *PaGT2* cDNAs required the use of host strain-vector systems that were different from that used for *PaGT3* expression (see Materials and methods). The levels of *PaGT1* and *PaGT2* expression were extremely low. Moreover, the expressed PaGT1 and PaGT2 proteins were somewhat unstable during affinity purification. Ultimately, the purities of the PaGT1 and PaGT2 were 12% and 27%, respectively, as estimated from the intensities of bands in SDS-PAGE gels (not shown).

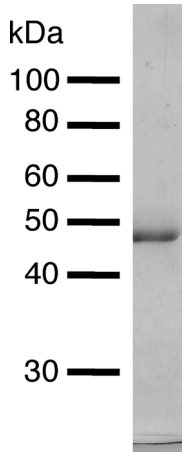
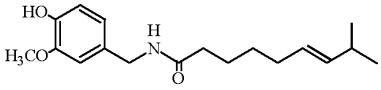
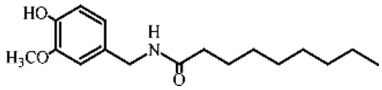
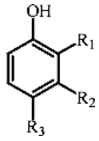
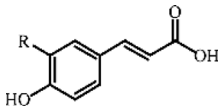
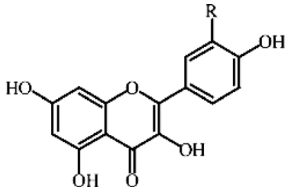
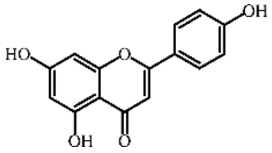


Figure 2. SDS-PAGE analysis of purified PaGT3. Recombinant PaGT3 that was purified from crude extracts of transformant *E. coli* cells were electrophoresed under the conditions described by Laemmli (1970) and stained with Coomassie Brilliant Blue.

Glycosyl-acceptor specificities of the purified PaGT1, PaGT2, and PaGT3 were examined using a wide variety of phenolics, including capsaicinoids, benzoic acid derivatives, coumarins, flavonoids, and betanidins, using UDP-glucose as the glycosyl donor. All of these glycosyltransferases showed relatively broad acceptor specificities (see Tables 1 for specificity of PaGT3; see Supplementary Table 1S for specificities of PaGT1 and PaGT2). However, PaGT3 was the only enzyme that efficiently glucosylated capsaicin among the PSPGs obtained in this study. Kinetic parameters for the PaGT3-catalyzed glucosyl transfer from UDP-glucose to capsaicin and several other acceptors were determined under steady-state conditions at pH 7.2 and 30°C and are summarized in Table 2. PaGT3 showed the highest activity for flavonoids [*e.g.*, quercetin (a flanvonol), apigenin (a flavone), genistein (an isoflavone), and aureusidin (an aurone)], followed by capsaicin. PaGT3 appeared promiscuous in terms of regiospecificity of glucosyl transfer to flavonoids. For example, quercetin possesses five possible sites of glucosylation, and the reaction of quercetin and UDP-glucose with PaGT3 gave at least four glucosyl transfer products (Supplementary Figure 1S)—all of which were monoglucosides of quercetin (*m*/*z*, 487.35; $[M+Na]^+$), as revealed by mass spectrometric analyses.

It must be noted that the *P. americana* L. callus utilized in this study abundantly produced betanidin 5-*O*-glucopyranoside when grown under light conditions. In addition, PaGT3 is closely related to DbB5GT (see above). DbB5GT may be involved in betacyanin biosynthesis in *D. bellidiformis* cell cultures (Vogt et al. 1997). It is noteworthy, in this regard, that PaGT3

Table 1. Glucosyl-acceptor specificity of PaGT3.

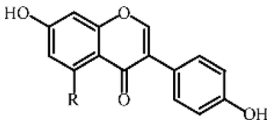
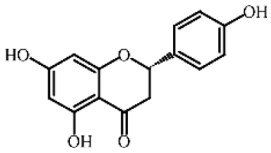
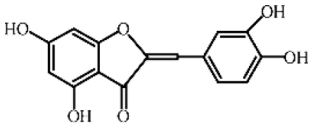
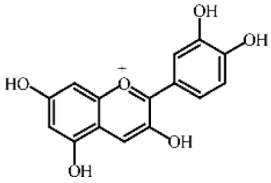
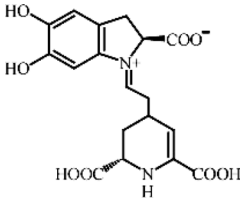
Substrate	Relative activity[a] (%)
	
Capsaicin	88
	
8-Nordihydrocapsaicin	82
	
Salicylic acid (R_1=–COOH, R_2=–H, R_3=–H)	nd
m-Hydroxy benzoic acid (R_1=–H, R_2=–COOH, R_3=–H)	34
p-Hydroxy benzoic acid (R_1=–H, R_2=–H, R_3=–COOH)	nd
Salicyl alchol (R_1=–CH_2OH, R_2=–H, R_3=–H)	4.0
Hydroquinone (R_1=–H, R_2=–H, R_3=–OH)	nd
	
trans-p-Coumaric acid (R=–H)	24
	
Kaempferol (R=–H)	46 (3)
Quercetin (R=–OH)	100 (4)
	
Apigenin	104 (2)

glucsoylated betanidin (Table 1). Reaction of PaGT3 with betanidin and UDP-glucose gave rise to a single transfer product, which was co-eluted with authentic betanidin 5-*O*-glucopyranoside in an analytical reversed-phase HPLC (data not shown), indicating that PaGT3 has betanidin 5-*O*-glucosyltransferase activity. However, it remains to be clarified whether PaGT3 is involved in betacyanin biosynthesis in *P. americana* L.

Glycosyl-donor specificity of PaGT3 was then examined using capsaicin and quercetin as glycosyl acceptors. PaGT3 could not utilize UDP-galactose and UDP-glucuronic acid (relative activity, less than 0.9%); thus, PaGT3 was highly specific for UDP-glucose.

Other enzymatic properties of PaGT3 were also examined. PaGT3 was active over a pH range of 5.5–8.0, with maximal activity at pH 6.5. In addition, PaGT3 was

Table 1. (continued).

Substrate	Relative activity[a] (%)
	
Daidzein (R=–H)	83
Genistein (R=–OH)	100
	
Naringenin	74 (2)
	
Aureusidin	114 (2)
	
Cyanidin	nd
	
Betanidin	23

[a] Assay conditions are described in Materials and methods. When multiple transfer products could be separated under the analytical HPLC conditions employed, the number of the separated peaks is shown in parenthesis. The relative activities were determined from the sum of product peak integrals, assuming that the extinction coefficient of the reaction product(s) was the same as that of the substrate. The activity for quercetin was taken to be 100%. nd, Activity not detected.

Table 2. Apparent kinetic parameters of PaGT3[a].

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)
Capsaicin[b]	145 ± 48	$4.9\times10^{-2}\pm0.1\times10^{-2}$	3.4×10^{-4}
Quercetin[b]	20 ± 1	$6.0\times10^{-2}\pm0.1\times10^{-2}$	3.0×10^{-3}
Apigenin[b]	66 ± 22	$1.4\times10^{-1}\pm2.3\times10^{-1}$	2.1×10^{-3}
Genistein[b]	20 ± 1	$6.2\times10^{-2}\pm0.1\times10^{-2}$	3.1×10^{-3}
UDP-glucose[c]	18 ± 6		
UDP-glucose[d]	150 ± 7		

[a] Activities were determined from the sum of the product peak integrals (see Table 1), assuming that the extinction coefficient (s) of the reaction product(s) was the same as that of the substrate.

[b] 100 μM UDP-glucose was used as a glucosyl donor.

[c] 100 μM capsaicin was used as a glucosyl acceptor.

[d] 100 μM quercetin was used as a glucosyl acceptor.

stable over a pH range of 5.0–9.0 (at 20°C for 8 h) and below 40°C (at pH 7.4 for 1 h). Optimal temperature of PaGT3 was 45°C . PaGT3 was completely inhibited by 0.1 mM Fe^{2+}, Fe^{3+}, Hg^{2+} and Zn^{2+} and was partially inhibited by 0.1 mM Ca^{2+}, Cd^{2+}, Co^{2+} and Cu^{2+}. Other metal ions (Mg^{2+}, Mn^{2+} and Sn^{2+}; 0.1 mM), 5 mM EDTA, analogs of glycosyl donors (uridine, UMP, UDP and UTP; 1 mM) and amino acid-modifying reagents (diethylpyrocarbonate and phenylmethanesulfonyl fluoride; 1 mM) had negligible effects on catalytic activity. It is noteworthy that a cysteine residue (Cys371) juxtaposes His370 of PaGT3. This His residue corresponds to His360 of arbutin synthase, which is very important for catalytic activity, (Hefner and Stockigt 2003) and to His350, which participates in binding of UDP-glucose in *Vitis venifera* L. UDP-glucose:flavonoid 3-*O*-glucosyltransferase (VvGT1) (Offen et al. 2006). Therefore, Cys371 may also be located near the active site of PaGT3. Modification of Cys371 with heavy metal ions, such as Fe^{2+} , Fe^{3+} , Hg^{2+} , and Zn^{2+}, may destroy the active site structure and may, at least in part, account for the observed inhibition of PaGT3 activity by these metal ions.

Transcription analyses show that PaGT3 is an inducible enzyme

PaGT3 exhibited a CapGT activity (see above), although capsaicin does not naturally occur in *P. americana* L. cells. Moreover, the crude extract of *P. americana* L. callus showed only negligible CapGT activity when it was grown in medium without added capsaicin. Transcription levels of *PaGT3* were analyzed using quantitative real-time reverse-transcription (RT) PCR with total RNAs extracted from the *P. americana* L. callus as the templates and with specific PCR primers. Figure 3 shows the time course for relative *PaGT3* transcription levels after addition of capsaicin (final concentration, 0.65 mM) to the *P. americana* L. callus cultures, which were grown under the conditions described above. The level of *PaGT3* transcription increased after addition of capsaicin, reaching the maximum after 72 h, consistent with the observed induction of CapGT activity by capsaicin as described above. For comparison, the transcription levels of *PaGT1* and *PaGT2* 48 h after capsaicin addition were 113 ± 14% and 88 ± 6% of the respective levels at 0 h, whereas the corresponding value of *PaGT3* was 452% ± 27%. Thus, induction by capsaicin appears specific for *PaGT3*.

Plant cell cultures as practical sources of inducible glycosyltransferases that modify xenobiotics

All of the results obtained in the present study show that PaGT3 is, at least in part, responsible for the CapGT activity of *P. americana* L. cells. Roles of the family-1

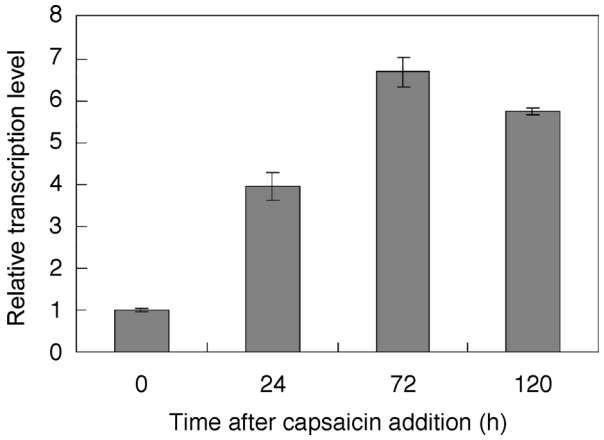


Figure 3. Quantitative real time RT-PCR of *PaGT3*. The relative transcription levels of *PaGT3* in callus tissues collected for analysis 24, 72, and 120 h after capsaicin addition are shown with the value prior to capsaicin addition (0 h) taken to be 1. Average values of four independent determinations of transcription levels are presented with error bars indicating the standard errors.

GTs in plants include biosyntheses of secondary metabolites and detoxification of xenobiotics (Lim and Bowles 2004; Bowles et al. 2005). Glycosylation enhances the solubility of these compounds and allows their storage within vacuoles, thereby maintaining the metabolic homeostasis of host plants (Lim and Bowles 2004; Bowles et al. 2005). Biosynthetic GTs are typified by anthocyanidin GTs, many of which show strict substrate specificity and are expressed in a coordinate fashion with related enzymes (Vogt and Jones 2000; Sawada et al. 2005; Yonekura-Sakakibara et al. 2007; Noguchi et al. 2007). GTs that are involved in detoxification are exemplified by the GTs of *Nicotiana tabacum* (NtGT1a, NtGT1b, and NtGT3) (Taguchi et al. 2001; Taguchi et al. 2003). These enzymes are specifically expressed in response to xenobiotic exposure (*e.g.*, naphthol in the case of *N. tabacum*) and display relatively broad specificities for glycosyl acceptors, which may allow for a wide range of chemical structures. Because prolonged exposure (more than 2 days) of the callus to capsaicin (initial concentration, 0.65 mM) inhibited callus growth (Liu, W., Homma, H., Noguchi, A., and Nakayama, T.; unpublished results), capsaicin should be toxic to *P. americana* L. cells. Thus, although induction of PaGT3 by capsaicin in intact *P. americana* L. plants remains to be examined, PaGT3 likely play a role in detoxification of capsaicin through its solubilization and accumulation in the cultured cells (probably in vacuoles). This role of glycosyltransferases in xenobiotic metabolism explains the fact that cultured plant cells generally serve as practical sources of glycosyltransferases that modify compounds with a wide variety of chemical structures.

Acknowledgements

We thank Dr. Nobuhiro Sasaki, Tokyo University of Agriculture and Technology, for his critical reading of the manuscript. We are grateful to Prof. Peter Ian Mackenzie (Flinders University School of Medicine) for help with the systematic nomenclature of PaGTs.

References

Arend J, Warzecha H, Stockigt J (2000) Hydroquinone *O*-glucosyltransferase from cultivated Rauvolfia cells: enrichment and partial amino acid sequences. *Phytochemistry* 53: 187–193

Bowles D, Isayenkova J, Lim EK, Poppenberger B (2005) Glycosyltransferases: managers of small molecules. *Curr Opin Plant Biol* 8: 254–263

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389: 816–824

Dray A (1992) Mechanism of action of capsaicin-like molecules on sensory neurons. *Life Sci* 51: 1759–1765

Hamada H, Ohiwa S, Nishida T, Katsuragi H, Takeda T, Hamada H, Nakajima N, Ishihara K (2003) One-step glucosylation of capsaicinoide by cultured cells of *Phytolacca americana*. *Plant Biotechnol* 20: 253–255

Hefner T, Stöckigt J (2003) Probing suggested catalytic domains of glycosyltransferases by site-directed mutagenesis. *Eur J Biochem* 270: 533–538

Henry CJ, Emery B (1986) Effect of spiced food on metabolic rate. *Hum Nutr Clin Nutr* 40: 165–168

Kawada T, Hagihara K, Iwai K (1986) Effects of capsaicin on lipid metabolism in rats fed a high fat diet. *J Nutr* 116: 1272–1278

Kometani T, Tanimoto H, Nishimura T, Kanbara I, Okada S (1993) Glucosylation of capsaicin by cell suspension cultures of *Coffea arabica*. *Biosci Biotechnol Biochem* 57: 2192–2193

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685

Lim EK, Bowles D (2004) A class of plant glycosyltransferases involved in cellular homeostasis. *EMBO J* 23: 2915–2922

Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, Miners JO, Nebert DW (2005) Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenet Genom* 15: 677–685

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Nakayama T, Sato T, Fukui Y, Yonekura-Sakakibara K, Hayashi H, Tanaka Y, Kusumi T, Nishino T (2001) Specificity analysis and mechanism of aurone synthesis catalyzed by aureusidin synthase, a polyphenol oxidase homolog responsible for flower coloration. *FEBS Lett* 499: 107–111

Noguchi A, Saito A, Homma Y, Nakao M, Sasaki N, Nishino T, Takahashi S, Nakayama T (2007) A UDP-glucose:isoflavone 7-*O*-glucosyltransferase from the roots of soybean (*Glycine max*) seedlings. Purification, gene cloning, phylogenetics, and an implication for an alternative strategy of enzyme catalysis. *J Biol Chem* 282: 23581–23590

Offen W, Martinez-Fleites C, Yang M, Lim EK, Davis BG, Tarling CA, Ford CM, Bowles D, Davies GJ (2006) Structure of a flavonoid glucosyltransferase reveals the basis for plant natural product modification. *EMBO J* 25: 1369–1405

Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12: 357–358

Park KK, Chun KS, Yook JI, Surh YJ (1998) Lack of tumor promoting activity of capsaicin, a principal pungent ingredient of red pepper, in mouse skin carcinogenesis. *Anticancer Res* 18: 4201–4205

Sawada S, Suzuki H, Ichimaida F, Yamaguchi MA, Iwashita T, Fukui Y, Hemmi H, Nishino T, Nakayama T (2005) UDP-glucuronic acid: anthocyanin glucuronosyltransferase from red daisy (*Bellis perennis*) flowers. Enzymology and phylogenetics of a novel glucuronosyltransferase involved in flower pigment biosynthesis. *J Biol Chem* 280: 899–906

Shimoda K, Kwon S, Utsuki A, Ohiwa S, Katsuragi H, Yonemoto N, Hamada H, Hamada H (2007) Glycosylation of capsaicin and 8-nordihydrocapsaicin by cultured cells of *Catharanthus roseus*. *Phytochemistry* 68: 1391–1396

Siegler DS (1998) *Plant Secondary Metabolism*. Kuwar Academic Publishers, Massachusetts

Surh YJ, Lee E, Lee JM (1998) Chemoprotective properties of some pungent ingredients present in red pepper and ginger. *Mutat Res* 402: 259–267

Surh YJ, Lee SS (1995) Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. *Life Sci* 56: 1845–1855

Taguchi G, Nakamura M, Hayashida N, Okazaki M (2003) Exogenously added naphthols induce three glucosyltransferases, and are accumulated as glucosides in tobacco cells. *Plant Sci* 164: 231–240

Taguchi G, Yazawa T, Hayashida N, Okazaki M (2001) Molecular cloning and heterologous expression of novel glucosyltransferases from tobacco cultured cells that have broad substrate specificity and are induced by salicylic acid and auxin. *Eur J Biochem* 268: 4086–4094

Vogt T (2002) Substrate specificity and sequence analysis define a polyphyletic origin of betanidin 5- and 6-*O*-glucosyltransferase from Dorotheanthus bellidiformis. *Planta* 214: 492–495

Vogt T, Grimm R, Strack D (1999) Cloning and expression of a cDNA encoding betanidin 5-*O*-glucosyltransferase, a betanidin- and flavonoid-specific enzyme with high homology to inducible glucosyltransferases from the Solanaceae. *Plant J* 19: 509–519

Vogt T, Jones P (2000) Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. *Trends Plant Sci* 5: 380–386

Vogt T, Zimmermann E, Grimm R, Meyer M, Strack D (1997) Are the characteristics of betanidin glucosyltransferases from cell-suspension cultures of Dorotheanthus bellidiformis indicative of their phylogenetic relationship with flavonoid glucosyltransferases? *Planta* 203: 349–361

Yonekura-Sakakibara K, Tohge T, Niida R, Saito K (2007) Identification of a flavonol 7-*O*-rhamnosyltransferase gene determining flavonoid pattern in Arabidopsis by transcriptome coexpression analysis and reverse genetics. *J Biol Chem* 282: 14932–14941