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Original Paper

Molecular cloning and characterization of coumarin glucosyltransferase in hairy roots of *Pharbitis nil* (*Ipomoea nil*)

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Abstract The hairy roots induced from *Pharbitis nil* (*Ipomoea nil*) by infection of the plant pathogen *Agrobacterium rhizogenes* produced and released the phytoalexin of umbelliferone into the culture medium by treatment with copper sulfate. However, this umbelliferone was immediately recovered in the hairy roots as its glucoside, skimmin, after a few hours. To analyze the details of these phenomena, we searched for glucosyltransferase (GTase) genes related to skimmin production and isolated two full-length cDNAs, named *PNgt1* and *PNgt2*. They contain the plant secondary product glucosyltransferase (PSPG) box that is a common signature of plant GTases. Phylogenetic analyses of *PNgt1* and *PNgt2* indicated that they encode a broad substrate-specific GTase and an unclassified GTase, respectively. An enzyme assay of the recombinant PNGT1 protein showed a high glucosylation rate against coumarin and benzaldehyde derivatives, especially umbelliferone. The expression level of *PNgt1* after treatment with umbelliferone showed a correlation with skimmin production. Moreover, we found the expression levels of *PNgt1* were altered by the plant hormones salicylic acid and methyl jasmonate. These results suggest the importance of plant GTases in phytoalexin production and plant defense mechanisms.

Key words: Glucosyltransferase, hairy root, *Pharbitis nil* (*Ipomoea nil*), phytoalexin, umbelliferone.

Hairy roots induced from plant tissues infected by the plant pathogen *Agrobacterium rhizogenes* tend to exhibit a high proliferation rate. They are also capable of secondary metabolite biosynthesis due to the presence of differentiated cells. Thus, the hairy roots are very convenient plant culture cells for the production of useful biologically active compounds and the biotransformation of exogenously supplied organic compounds to useful biological products. There have been many reports on the production of biologically active compounds by hairy roots (Banerjee et al. 2012; Zhou et al. 2011). We reported the high yield production of tropane alkaloids by *Hyoscyamus niger* hairy roots (Uchida et al. 1993), the production of novel sesquiterpenoids having vetispirane skeletons as phytoalexins by *Hyoscyamus albus* hairy roots treated with cupper sulfate ($CuSO_4$) and methyl jasmonate (MeJA) (Kuroyanagi et al. 1998), and the production of a coumarin-type phytoalexin by *Pharbitis nil* (*Ipomoea nil*) hairy roots (PNHR) treated with $CuSO_4$ (Yaoya et al. 2004). We also reported a highly expressed gene from *H. niger* hairy roots and its function in enhancing lateral root formation. This report also showed the experimental convenience of hairy roots as a basic plant model system (Mikami et al. 1999).

Glucosylation, controlled by glucosyltransferase (GTase), is a major modification of plant secondary metabolites. This reaction plays numerous roles such as the stabilization, detoxification, solubilization, and regulation of biologically active levels of metabolites (Gachon et al. 2005). Genome analyses of several major plants, such as *Arabidopsis thaliana* and *Oryza sativa*, revealed the existence of hundreds of GTase in one plant species (Yonekura-Sakakibara and Hanada 2011). Phylogenetic studies on these GTases revealed they were subdivided depending on the substrate specificity (Vogt and Jones 2000). However, the functions of most

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have not been determined, and the relationship between their structures and functions is also incomplete. Therefore, further studies of plant GTases are required to understand their roles in plants. In our previous study, PNHR showed the ability to glucosylate several types of low molecular phenolic compounds, such as coumarin, benzaldehyde-type compounds, and flavonoid compounds (Kanho et al. 2004; Kanho et al. 2005). These results suggested a strong GTase activity and high expression of the GTase gene in the PNHR.

Phytoalexin plays a key role in the plant defense system against several harmful microbes and herbivores (Ahuja et al. 2012; Ali and Agrawal 2012). Umbelliferone has been reported as a phytoalexin of *Platanus acerifolia* and is phytotoxic (Afek et al. 1999). In our previous study, we found that stress caused PNHR to release umbelliferone into the medium, and this compound was rapidly taken up by PNHR as skimmin (Yaoya et al. 2004). Skimmin is a glucoside of umbelliferone, and it has shown very weak toxicity against PNHR (Yaoya et al. 2004). A similar detoxification method or defense system has been reported in *A. thaliana* and *Nicotiana tabacum* (Bowles et al. 2005; Taguchi et al. 2003a). In these reports, this detoxification system was controlled by a GTase. However, the GTase related to detoxification, defense and phytoalexin production has only been investigated in a few plants. Interestingly, the expression levels of GTases related to detoxification are altered by MeJA and salicylic acid (SA) (Taguchi et al. 2001; Taguchi et al. 2003a; Taguchi et al. 2003b). These plant hormones are closely related to the activation or inactivation of plant defense systems (Pieterse et al. 2009), and the results suggest that GTases will play key roles in the plant defense system. Thus, the study of GTases related to phytoalexin production is important to understand their relationship to plant defense systems.

In this study, we reported the molecular cloning of GTase genes, *PNgt1* (*Pharbitis nil* glucosyltrasferase 1) and *PNgt2* (*Pharbitis nil* glucosyltransferase 2), from PNHR and the characterization of the glucosylation ability of the recombinant PNGT1 protein against several compounds. The results indicated that recombinant PNGT1 has a strong glucosylation activity against coumarin derivatives, especially umbelliferone. The expression of *PNgt1* in PNHR treated with umbelliferone, MeJA and SA is also discussed.

Materials and methods

Chemicals

Umbelliferone was purchased from Nacalai Tesque (Kyoto, Japan); esculetin and scopoletin were purchased from Tokyo Chemical Industry (Tokyo, Japan); 3-hydroxy-flavone, 3,6-dihydrixy-flavone and 3,7-dihydroxy-flavone were purchased from Sigma-Aldrich (St. Louis, MO, USA); Glucosides of umbelliferone, esculetin, scopoletin, vanillyl alcohol, vanillin, 3-hydroxyflavone, 3,6-dihydroxyflavone and 3,7-dihydroxyflavone were obtained by glucosylation of their aglycones by PNHR as previously reported (Kanho et al. 2004; Kanho et al. 2005).

Cloning of GTase cDNA from PNHR

PNHR were obtained from young *P. nil* (*I. nil*) plants germinated under microbial free conditions by infection with *A. rhizogenes* MAFF 03-1755 as described in previous papers (Yaoya et al. 2004). Total RNA from PNHR after 8 h of umbelliferone treatment was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized using Superscript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and the oligo$(dT)_{20}$ primer according to the manufacturer's instructions. Degenerate primers were designed according to conserved regions among known GTases in *Ipomoea batatas*, *Ipomoea purpurea*, *N. tabacum*, *Scutellsaria bicalensis*, *A. thaliana* and *Perilla frutescens*. Details of sequence design and primer positions are shown in Figure 2 and Table S1. The cDNA fragment was amplified by *Ex-Taq* (TaKaRa, Kyoto, Japan) with degenerate primers under the following conditions: denaturation for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and 72°C for 5 min.

Total RNA from PNHR after 8 h of umbelliferone treatment was used for rapid amplification of cDNA ends (RACE) library construction. The RACE library was synthesized using the Superscript III First-Strand Synthesis System (Invitrogen) and the PNGT1-5′ R primer, the PNGT2-5′ R primer or the oligo$(dT)_{20}$ primer according to the manufacturer's instructions. A new poly (A)-tail at the 5′ end of the sequence was added with terminal deoxynucleotidyl transferase (Invitrogen) for 5′ RACE. The cDNA fragments were amplified by Phusion High-Fidelity DNA Polymerase (Finzyme, Espoo, Finland) with gene-specific primers, the oligo$(dT)_{20}$ primer and the RACE library under the following conditions: denaturation for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and 72°C for 5 min.

The PCR products were electrophoresed on 1.5% agarose gels, and resolved fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN). Purified fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA) following a standard protocol. The cloned fragments were sequenced by the dideoxynucleotide chain termination method using a Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an ABI Prism 310 genetic analyzer. The sequences of *PNgt1* (accession number AB757750) and *PNgt2* (accession number AB757751) were deposited in DDBJ. A list of primers used for these experiments is shown in Table 1.

Phylogenetic analysis and sequence comparison

Amino acid sequences of known GTases were obtained from the NCBI proteins database (http://www.ncbi.nlm.nih.gov/

Table 1. PCR primers.

Primer	Sequence(5′ to 3′)[a]	Purpose
GT-F	G(C/T)(G/T/C)GT(G/T/C)TA(C/T)(G/C/A)T(G/T)(A/T)(G(C/T)TT(C/T)GG	Degenerate PCR
GT-R	GA(A/G)TTCCA(A/C/T)CC(A/G)CA(A/G)TG(C/T)GT	Degenerate PCR
PNGT1-5′R	CCTATAACTTTTCCGATGCT	5′RACE
PNGT1-3′F	AGTTTCCCGGAGTCTCAAGT	3′RACE
PNGT2-5′R	CTCACTATCTGCCCATTCTC	5′RACE
PNGT2-3′F	TCTCAACACCTTAACAATGG	3′RACE
PNGT1-RT-F	CGAAAGTACAGGGACTGCGTT	RT-PCR for *PNGT1*
PNGT1-RT-R	CAACTCCCATTCCTATGTCCGTTA	RT-PCR for *PNGT1*
act-F	GATGTGGATATCAGGAAGA	RT-PCR for actin
act-R	CTCAGCTTTCGAAATCCACA	RT-PCR for actin
PNGT1-Ex-F	CATATGGAAGAAGCTATAGAGCTG	Heterologous expression
PNGT1-Ex-R	CTCGAGCTATTTGAGGTTCTTCACGA	Heterologous expression

[a] Underline indicate *Nde*I (CATATG) and *Xho*I (CTCGAG) recognition site.

guide/proteins/). These amino acid sequences, along with those of *PNgt1* and *PNgt2*, were aligned with ClustalW in the Molecular Evolutionary Genetic Analysis 4 (MEGA4) program (Tamura et al. 2007). Phylogenetic analyses were carried out using the neighbor-joining method using the MEGA4 program.

Alignment of *PNGT1*, *NtGT1a*, *NtGT1b*, and *FaGT6* was performed by ClustalW in genome net (http://www.genome.jp/tools/clustalw/). The presence of the PSPG box in these proteins was confirmed by InterProScan (Quevillon et al. 2005). A list of accession numbers for sequences used in this study is included in Table S2.

Expression of recombinant PNGT1 in Escherichia coli

A forward primer containing the *Nde*I restriction enzyme site (PNGT1-Ex-F) and a reverse primer containing the *Xho*I restriction enzyme site (PNGT1-Ex-R) were designed at the 5′ and 3′ ends of the full-length ORF of PNGT1, respectively. The amplified fragment was digested with *Nde*I and *Xho*I. The resulting fragment was subcloned into the *Nde*I/*Xho*I sites of the pET-15b expression vector (Novagene, Madison, WI, USA), yielding pPNGT1. *E.coli* BL21 (DE3) pLysS transformed with pPNGT1 was cultured at 25°C for 26 h in Luria–Bertani medium containing 50 μg/ml carbenicillin. As a control, *E.coli* BL21 (DE3) pLysS cells were transformed with an empty pET-15b vector. The expression of PNGT1 was induced for 6 h by adding isopropyl-β-D-thiogalactopyranoside (1 mM) to the culture. The crude enzyme was extracted using xTractor Buffer Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The His-Tag fusion of PNGT1 was affinity-purified using the TALON Purification Kit (Clontech) according to the manufacturer's instructions. The protein concentration of purified PNGT1 was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The presence of recombinant PNGT1 was confirmed by SDS-PAGE. A list of primers used for these experiments is shown in Table 1.

Enzyme assays

Enzyme activity was assayed in a buffer containing 50 mM Tris-HCl (pH 7.5). Standard assays contained 10 μM substrate, 20 μM UDP-glucose and 0.1 mg of recombinant PNGT1. Enzyme assays were performed for 90 min at 30°C. The reaction mixture was extracted with *n*-buthanol (*n*-BuOH). The *n*-BuOH soluble phase was concentrated, and the residue was dissolved in methanol (MeOH) and filtrated. The filtered MeOH solution was analyzed by high performance liquid chromatography (HPLC) using a reversed phase column (Mightysil RP-18, C_{18}, 250×4.6 i.d., 5 μm, Kanto Chemical Ltd., Tokyo, Japan), and CH_3CN-H_2O solvent system and identified by glucopyranosides derived from the aglycones produced from biotransformation with PNHR.

Umbelliferone, methyl jasmonate (MeJA) and salicylic acid (SA) treatments

PNHR were subcultured on Murashige and Skoog (MS) medium for 3 weeks (Murashige and Skoog 1962). Subsequently, umbelliferone (100 μg/ml MS medium) or MeJA (20 μg/ml MS medium) or SA (30 μg/ml MS medium) was added into PNHR culture. Umbelliferone-treated hairy roots were collected after 1, 2, 4, 8, 12, 24 and 48 h. MeJA and SA-treated PNHR were collected after 3, 6, 12, 24 and 48 h. The collected samples were quick-frozen in liquid nitrogen and stored at −80°C.

RT-PCR analysis

Total RNA from PNHR treated with umbelliferone or MeJA or SA, was extracted using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. First-strand cDNA was synthesized using Superscript III First-Strand Synthesis System (Invitrogen) and the oligo$(dT)_{20}$ primer according to the manufacturer's instructions. The cDNA fragment was amplified by *Ex-Taq* (TaKaRa) with gene-specific primers for *PNgt1* or *actin* under the following conditions: denaturation for 5 min, followed by 25 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and 72°C for 5 min. The resulting 912 bp *PNgt1* fragment or 228 bp *actin* fragment was confirmed by electrophoresis in a 1.5% agarose gel. A list of primers used for these experiments is shown in Table 1.

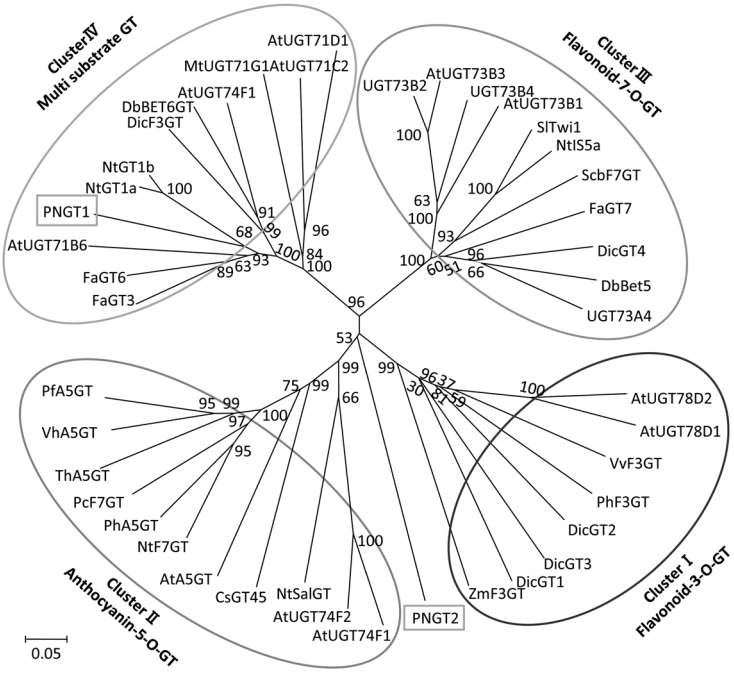

Figure 1. Unrooted phylogenetic tree of GTases. GenBank accession numbers, sources, EC number, and CAZy family for the respective protein sequences are shown in Table S2 (Canterel et al. 2009). The numbers at the nodes are bootstrap values obtained from 1000 replicates and are indicated as percentages. PNGTs are surrounded by a grey border. The horizontal scale shows the number of differences per 50 residues derived from the ClustalW alignment.

Results

Cloning and phylogenetic analysis of GTase from PNHR

To obtain genes of umbelliferone GTase in PNHR, degenerate primers were designed to the conserved regions among randomly selected known GTases in *I. batatas*, *I. purpurea*, *N. tabacum*, *S. bicalensis*, *A. thaliana* and *P. frutescent* (Table S1). In our previous study, umbelliferone was converted to skimmin with high efficiency around 8 h after the addition of umbelliferone to PNHR cultures (Yaoya et al. 2004). Total RNA used as a template in degenerate PCR was obtained after 8 h from PNHR treated with umbelliferone. The resulting single fragment was cloned, and the sequencing of multiple clones identified two different cDNA sequences, *PNgt1* and *PNgt2*, containing the plant secondary product GTase (PSPG) box that is a highly conserved domain of plant GTases. To obtain the full-length cDNAs of these GTases, 5′ or 3′ RACE on *PNgt1* and *PNgt2* was performed. The full-length *PNgt1* cDNA contains an open reading frame of 1452 bp that encodes 483 amino acids (accession number AB757750). The full-length *PNgt2* cDNA contains an open reading frame of 1395 bp that encodes 464 amino acids (accession number AB757751).

To classify these GTases in *P. nil* (PNGTs), we performed a phylogenetic analysis using the amino acid sequences of the PNGTs and biochemically characterized plant GTases (Figure 1). The phylogenetic analysis resulted in four clusters that can be generally characterized based on in vitro substrate specificities. Cluster I contains flavonoid-3-*O*-GTase, cluster II contains anthocyanin 5-*O*-GTase, and cluster III contains flavonoid 7-*O*-GTase. *PNgt1* is included in cluster IV, which contains broad substrate-specific GTases, and some of these enzymes reported catalytic activities against coumarin-type compounds. However, *PNgt2* was not included in any cluster. We consulted the CAZy team (Cantarel et al. 2009) and the UGT committee (http://www.flinders.edu.au/medicine/sites/clinical-pharmacology/ugt-homepage.cfm) when classifying *PNgt1* and *PNgt2*. According to this consultation, *PNgt1* and *PNgt2* were classified as UGT71A26 (CAZy family: GT1) and UGT709F1 (CAZy family: GT1), respectively.

We also identified protein motifs and EC number of PNGTs using Pfam search (http://pfam.sanger.ac.uk/). As a result, PNGTs has a PF00201 motif and suggested Glucosyltransferase (EC2.4.1.17) which accepting a wide range of substrates, including phenols, alcohols, amines and fatty acids. *PNgt1* displayed high similarity to a functionally characterized coumarin GTase from *N. tabucum* and *Fragaria*×*ananassa* (Figures 1 and 2). These results suggested that PNGT1 could catalyze glucosylation of coumarin derivatives. Therefore, we studied the substrate specificity of the recombinant PNGT1 against some phenolic derivatives.

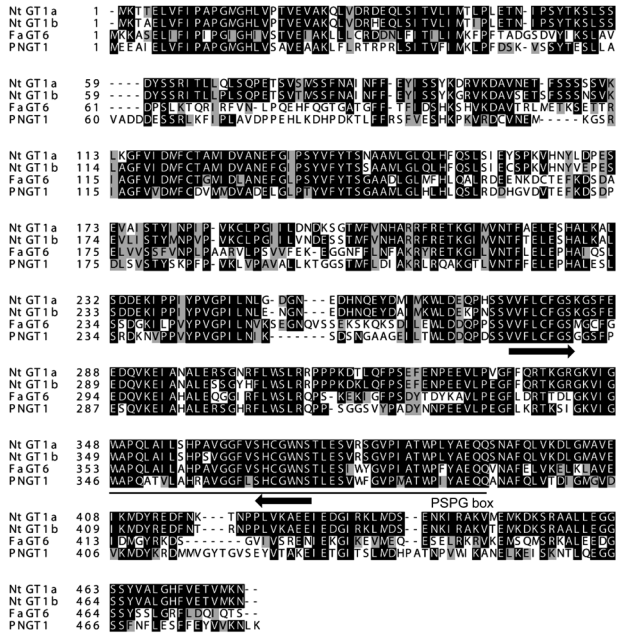

Figure 2. Multiple alignment of amino acid sequences of GTases. GenBank accession numbers and sources for the respective protein sequences are shown in Table S2. Alignment was calculated with ClustalW. The underlined sequence is the conserved plant secondary product glucosyltransferases (PSPG box) region. The arrow indicates degenerate primer positions. Black and gray shadings, done with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html), indicate conserved amino acid residues.

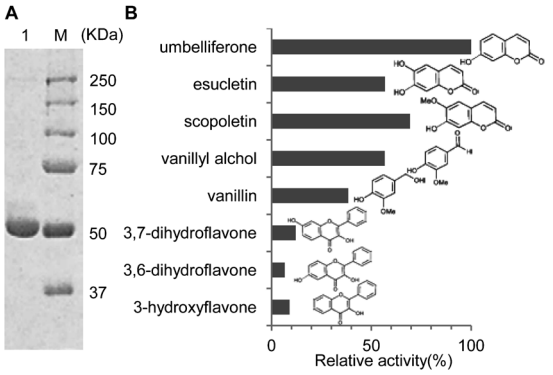

Figure 3. Characterization of glucosylation ability of recombinant PNGT1. (A) Proteins were separated by 10% SDS-PAGE, and visualized with Coomassie Blue staining. Molecular mass is indicated on the right side. Lane 1, purified His-Tag fusion PNGT1; M, molecular mass markers. (B) GTase activity was determined using three flavonoids, two benzaldehydes and three coumarins as the aglycone, and UDP-glucose was used as the sugar donor. The names of experimented chemicals are shown on the left side, and the respective structures of aglycones are shown on the right side. Glucosylation activity against each compound was shown relative to activity against umbelliferone. Data are the means of duplicate assays.

Characterization of PNGT1 enzymatic activity

To confirm the enzymatic activity of PNGT1, we performed the heterologous expression of PNGT1 in *E. coli*, and purified recombinant PNGT1 (Figure 3a). We also tried to heterologous expression of PNGT2 in *E. coli*. However, we couldn't yield recombinant PNGT2 in this study (data not shown). The enzymatic activity of recombinant PNGT1 was examined with three flavonoids (3-hydroxy flavone, 3,6-dihydroxy flavone, 3,7-dihydroxy flavone), two benzaldehyde derivatives (vanillin, vanillyl alcohol), and three coumarins (umbelliferone, scopoletin, esculetin), using UDP-glucose as the glucosyl donor. The reaction products were identified by comparison with the authentic glucosides by HPLC analysis. PNGT1 showed a high glucosylation ability against the coumarin and benzaldehyde derivatives, and the highest glucosylation ability against umbelliferone. Comparatively, the enzyme showed a lower glucosylation rate against the flavonoids. These results suggested that PNGT1 has substrate specificity for coumarins and the strongest glucosylation ability against umbelliferone (Figure 3b).

Expression of PNgt1 in PNHR treated with umbelliferone

In our previous study, we reported that umbelliferone uptake began at 2 h after its addition to the culture medium, that umbelliferone had completely disappeared from the medium at 12 h after the addition of umbelliferone (100 μg/ml MS medium) to PNHR cultures, and that skimmin content was increased in the hairy roots (Yaoya et al. 2004). To study the relationship between the *PNgt1* expression and glucosylation of umbelliferone, total RNAs were prepared from PNHR under the same conditions as the previous study, and then RT-PCR was performed with specific primers for *PNgt1* (Figure 4a). *PNgt1* was constitutively expressed in the PNHR; however, the expression level of *PNgt1* was enhanced by umbelliferone after 2 h of umbelliferone treatment. The expression returned to basal level after 12 h. This expression pattern was correlated to skimmin production in the umbelliferone-treated PNHR. Therefore, we hypothesize that PNGT1 converts umbelliferone to skimmin in PNHR.

Expression of PNgt1 in PNHR treated with plant hormones

Phytoalexin production is closely related to the stress response mediated by plant hormones such as MeJA and SA (see Discussion). Additionally, there are several reports that plant hormone treatments can alter GTase expression patterns (Taguchi et al. 2001; Taguchi et al. 2003a; Taguchi et al. 2003b). Thus, we hypothesize that the regulation of *PNgt1* expression may be correlated to the plant hormone treatment. To investigate the effects of some plant hormones on the expression of *PNgt1*, total RNA was extracted from PNHR treated with MeJA and SA (Figure 4b). As expected, the expression pattern of *PNgt1* was altered by hormone treatment. The expression of *PNgt1* was suppressed by SA treatment. In the MeJA-treated PNHR, *PNgt1* expression was induced after 6 h

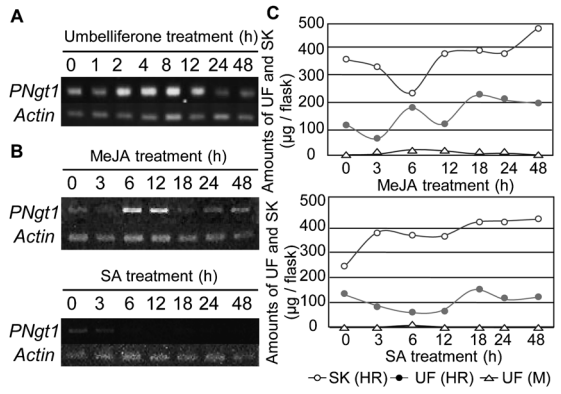

Figure 4. Correlation between *PNgt1* expression and skimmin production in response to umbelliferone (A) and plant hormones (B, C) in the hairy roots of *P. nil*. (A, B) Gene is indicated on the left side. The *actin* gene is shown as an expression control in each experiment. (C) The treatment times of each chemical are shown at the *X*-axis. The amounts of umbelliferone or skimmin in the medium or hairy roots are shown at the *Y*-axis. Detail of quantification methods of umbelliferone and skimmin in medium or hairy root were described previously (Yaoya et al. 2004). Abbreviations are MeJA, methyl jasmonate; SA, salicylic acid; UF, umbelliferone; SK, skimmin; M, medium; HR, hairy root.

of MeJA treatment, and then returned to the basal level after 18 h. To check the correlation between *PNgt1* gene expression and skimmin production, the content of umbelliferone and skimmin in the medium or the hairy roots during the plant hormone treatment was observed (Figure 4c). In this analysis, basal level of skimmin in the hairy roots was slight different. The expression pattern of *PNgt1* was roughly correlated to skimmin production under the plant hormone treatment. Thus, the expression of *PNgt1* may be controlled by plant hormone.

Discussion

Presently, many kinds of GTases have been isolated from several plant species (Caputi et al. 2012), and their biochemical and physiological characteristics have been studied. However, there have been few reports on the GTases related to the phytoalexin production and plant detoxification systems.

In this study, we isolated two full-length cDNAs of GTases from PNHR by degenerate PCR and RACE experiments. From the deduced amino acid sequence of the PNGTs, they contained the PSPG box sequence motif, which is a common feature of plant GTases. A phylogenetic analysis of these GTases showed that *PNgt1* shared high similarity with the members of the GTases having broad substrate specificity, and *PNgt1* is classified to UGT71A22 by the UGT committee. Interestingly, other UGT71A enzymes are involved in the glucosylation of phenolic compounds. Additionally, amino acid alignments of PNGT1 and GTases that reported having high glucosylation activity against the coumarin derivatives showed a high similarity, suggesting that PNGT1 has a high substrate specificity against coumarin derivatives.

Recombinant PNGT1 exhibits a high glucosylation ability against the coumarin and benzaldehyde derivatives *in vitro*, but a low glucosylation rate against flavonoids. Additionally, recombinant PNGT1 showed the highest glucosylation rate against umbelliferone. In our previous study, we showed that PNHR had an increased ability to glucosylate several low molecular weight compounds, such as the coumarins and benzaldehydes, but it had a decreased glucosylation reaction to flavonoids (Kanho et al. 2004). This result was in accordance with the phylogenetic classification of PNGT1.

We have already shown that at 2 h post-addition exogenously added umbelliferone was taken up, and this umbelliferone had completely disappeared from the medium after 12 h (Yaoya et al. 2004). Additionally, the umbelliferone glucoside accumulated in the PNHR. In agreement with these results, the expression level of *PNgt1* was enhanced after 2 h of umbelliferone treatment, and this activation ceased after 12 h. A similar phenomenon was reported in tobacco cells. GTases (*NtGT1a*, *NtGT1b*, and *NtGT3*) showed substrate specificity against naphthol derivatives, which are toxic to plant cells. The naphthols were converted into their corresponding glucosides by the respective GTase expression (Taguchi et al. 2001; Taguchi et al. 2003a), and the glucosides accumulated in the tobacco cells. Therefore, we suggested that PNGT1 converts toxic umbelliferone into nontoxic skimmin in PNHR.

The expression level of *PNgt1* was also altered by treatment with plant hormones, but the expression pattern differed depending on the plant hormone. In the MeJA-treated cells *PNgt1* showed temporal activation, whereas SA-treated cells were strongly repressed after 3 h. These expression patterns were roughly correlated with skimmin production in PNHR. However, the skimmin content during 3 to 6 h was inconsistent with *PNgt1* expression under the SA treatment. We suggested that one of the causes of this inconsistency is post-transcriptional or post-translational regulations of *PNgt1*. SA and MeJA are key plant signal molecules in the defense or immune systems of plants (Pieterse et al. 2009). The plant hormone-induced scopoletin GTase was shown to be important in plant defense mechanisms in tobacco cells (Chong et al. 2002; Fraissinet-Tachet et al. 1998; Gachon et al. 2004; Horvath and Chua 1996; Matros and Mock 2004). However, the expression patterns of these GTase and *PNgt1* are different. In tobacco cells, scopoletin glucosyltransferase (*TOGT*), which has a strong glucosylation ability against some phenolic compounds, was induced by infection, elicitor, and SA (Fraissinet-Tachet et al. 1998). SA played an

important role in the hypersensitive reaction, and *TOGT* also played a role in the defense mechanism against the tobacco mosaic virus infection (Gachon et al. 2004). In our experiment, *PNgt1* was induced by umbelliferone and MeJA. In the plant defense system, jasmonate, including jasmonic acid and MeJA, is involved in the regulation of biotic and abiotic stress response (Kazan and Manners 2008). Considering the role of GTase in tobacco cells and the jasmonate signaling system, *PNgt1* might be included in the plant defense response. However, hairy roots tend to show abnormal secondary metabolite production because of the function of *rol* genes (Bulgakov et al. 2011). To prove our hypothesis, further experiments using normal plants will be required.

In summary, full-length cDNAs named *PNgt1* and *PNgt2* encoding GTase proteins were isolated, and the catalytic activity of recombinant PNGT1 was determined. We also analyzed the correlation between the expression of *PNgt1* and skimmin production in PNHR after treatment with umbelliferone, MeJA and SA. The results suggested PNGT1 has the ability to glucosylate umbelliferone, and that *PNgt1* expression was affected by umbelliferone, MeJA and SA. Our findings illuminate the importance of GTase in the plant defense mechanism related to phytoalexin production.

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Table S1. List of sequence accession numbers of glucosyltransferase genes used for degenerate primer design.

Species	Accession number[a]	Annotation
Scutellaria baicalensis	BAA83484.1	UDP-glucose: flavonoid 7-O-glucosyltransferase
Perilla frutescens	BAA36421.1	UDP-glucose: anthocysnin 5-O-glucosyltransferase
Nicotiana tabacum	AAB36652.1	immediate-early salicylate-induced glucosyltransferase
Ipomoea purpurea	AAB86473.1	UDP glucose: flavonoid 3-O-glucosyltransferase
Ipomoea batatas	BAA90787.1	UDP glucose: flavonoid 3-O-glucosyltransferase
Arabidopsis thaliana	NP_173653.1	AtUGT85A2 (UDP-glucosyl transferase 85A2)

[a] GenBank accession number

Table S2. List of sequence accession numbers of glucosyltransferase genes used for the phylogenetic analysis and alignment of *PNGT1*.

Species	Gene name	Accession number[a]	EC number	CAZy family
Arabidopsis thaliana	AtUGT78D2	NP_197207.1	EC2.4. 1.91	GT1
	AtUGT73B4	NP_179151.2	EC2.4. 1.91	GT1
	AtUGT73B2	AAR01231.1	EC2.4. 1.237	GT1
	AtUGT73B3	NP_567953.1	EC2.4. 1.91	GT1
	AtUGT73B1	NP_567955.1	EC2.4. 1.237	GT1
	AtUGT74F2	NP_181910.1	EC2.4. 1.172	GT1
	AtUGT78D1	NP_180534.1	EC2.4. 1.91	GT1
	AtUGT71B6	NP_188815.2	EC2.4. 1.263	GT1
	AtA5GT	NP_193146.1	EC2.4. 1	GT1
	AtUGT74F1	NP_181912.1	EC2.4. 1.237	GT1
	AtUGT71C2	NP_180535.1	EC2.4. 1	GT1
Nicotiana tabacum	NtF7GT	BAB88935.1	EC2.4. 1	GT1
	NtSalGT	AAF61647.1	EC2.4. 1.172	GT1
	NtIS5a	AAB36653.1	EC2.4. 1	GT1
	NtGT1a	BAB60720.1	EC2.4. 1	GT1
	NtGT1b	BAB60721.1	EC2.4. 1	GT1
Dianthus caryophyllus	DicGT1	BAD52003.1	EC2.4. 1.91	GT1
	DicGT2	BAD52004.1	EC2.4. 1.91	GT1
	DicGT3	BAD52005.1	EC2.4. 1.91	GT1
	DicGT4	BAD52006.1	EC2.4. 1	GT1
Fragalia x ananassa	FaGT3	AAU09444.1	EC2.4. 1.91	GT1
	FaGT6	ABB92748.1	EC2.4. 1.91	GT1
	FaGT7	ABB92749.1	EC2.4. 1.91	GT1
Medicago truncatula	MtUGT71G1	AAW56092.1	EC2.4. 1.115	GT1
Crocus sativus	CsGT45	ACM66950.1	EC2.4. 1	GT1
Zea may	ZmF3GT	P16167.1	EC2.4. 1.91	GT1
Vitis vinifera	VvF3GT	AAB81682.1	EC2.4. 1.91	GT1
Verbena x hybrida	VhA5GT	BAA36423.1	EC2.4. 1.298	GT1
Gentiana triflora	GtF3GT	BAA12737.1	EC2.4. 1.115	GT1
Scutellaria baicalensis	ScbF7GT	BAA83484.1	EC2.4. 1.237	GT1
Pyrus communis	PcF7GT	AAY27090.1	EC2.4. 1.237	GT1
Solanum lycopersicum	SlTwi1	CAA59450.1	EC2.4. 1	GT1
Dorotheanthus bellidiformis	DbBET6GT	AAL57240.1	EC2.4. 1	GT1
Torenia hybrid cultivar	ThA5GT	BAC54093.1	EC2.4. 1	GT1
Perilla frutescens	PfA5GT	BAA36421.1	EC2.4. 1.298	GT1
Petunia x hybrida	PhA5GT	BAA89009.1	EC2.4. 1	GT1

[a] GenBank accession number