

Title:

Exogenously added naphthols induce three glucosyltransferases, and are accumulated as glucosides in tobacco cells.

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Abstract

Plants detoxify and accumulate several compounds as glucosides. In this work, detoxification of the exogenously added harmful compound naphthol in tobacco cells (*Nicotiana tabacum* L. Bright Yellow) was studied. When 250 μ M of 1-naphthol or 2-naphthol was added to the tobacco cells, most of the naphthol was accumulated in the cell as glucosides and in further modified forms. The glucosylation activities against naphthols were increased in proportion to the concentration of naphthols in the culture medium. Addition of 1 mM naphthols caused cell death. Three glucosyltransferase genes, namely *NtGT1a*, *NtGT1b* and *NtGT3* were isolated and characterized. The recombinant enzymes encoded by these genes showed glucosylation activity against naphthols and other phenolic compounds. It was also shown that these genes were induced following the addition of naphthols to the tobacco cells. These results suggest that naphthols are metabolized by glucosyltransferases whose production is inducible by naphthol itself.

Key words: *Nicotiana tabacum* L. cv Bright Yellow; glucosyltransferase; naphthol;

xenobiotics

Abbreviations: GTase, glycosyltransferase; RT, reverse transcription; rNTGT, recombinant enzyme of *NtGT*; SA, salicylic acid.

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1. Introduction

In higher plants, secondary metabolites are often converted to their glycoconjugates, which are then accumulated and compartmentalized in vacuoles [1-3]. These glycosylation reactions are catalyzed by glycosyltransferases (GTase). Several roles have been postulated for the glycosylation of various low-molecular-mass compounds in plants, as follows: 1)

Glucosylation allows solubilization of the compounds in water [4], 2) It serves in the detoxification of harmful metabolites or environmental compounds, such as herbicides [5,6], 3) It regulates the action of functional compounds, such as plant hormones, indole acetic acid and cytokinins [7,8], 4) It stabilizes the labile compounds [9].

Recently, many genes encoding GTases were reported. Some of these GTases have strict substrate specificity [7,8,10,11], the others have broad substrate specificity [12-14]. Some are induced by stresses and/or plant hormones [12-17], some are expressed in

anthocyanin-forming plants [11], in petals [18] or during ripening [19]. These results indicated the importance and the variety of roles of glycosylation. GTase are highly conserved in the C-terminus (PROSITE)[20][21], and form a gene superfamily. The *Arabidopsis* genome project showed that one plant species has almost one hundred putative GTase genes [22], however, the exact functions of most of them are not determined, and many orphan GTase genes remain to be studied. Further studies are required to clarify the relationships between the structures and functions of GTases and to understand their roles in plants.

Detoxification is one of the plausible roles of glycosylation in plants, because glucuronosyltransferases are well known as detoxifying enzymes in mammals (see review [23,24]). GTase activity for the exogenous compound have been found from many plant species [25] and GTase genes whose products showed the activity for the exogenous substrate *in vitro* were found in many cases (see review [9]). However, GTase genes whose products are actually catalyzing the detoxification in plants have been investigated in a few cases. One of them is a report of maize GTases that work on the detoxification of phytotoxin, benzoxazinoids [26].

In our previous study, GTase genes, *NtGT1a* and *NtGT1b*, whose products showed broad substrate specificity were isolated from tobacco [14]. Recombinant enzymes of these genes

showed glucosylation activity against exogenous substrates including the harmful compound naphthol. We report here the accumulation of glucoconjugate compounds of exogenously added naphthols as model compounds for xenobiotics, and suggest some GTases roles on the detoxification of naphthols or related compounds. Its effect on the GTase genes expression and glucosylation activities are also studied.

2. Materials and methods

2.1 *Culture of plant cells*

Cells of T-13 habituated callus of *Nicotiana tabacum* L. cv. Bright Yellow were used and maintained as described previously [27, 28].

2.2 *Reagents*

Kaempferol -3-*O*-glucoside was obtained from Extrasynthèse (Genay, France). All other chemicals and solvents were obtained from Sigma, Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Tokyo Kasei Industries (Tokyo, Japan) unless otherwise specified.

2.3 Synthesis of the glucoside standards

1-naphthol-*O*-glucoside, 2-naphthol-*O*-glucoside, and 7-hydroxyflavonol-*O*-glucoside was synthesized from 1-naphthol, 2-naphthol, or 7-hydroxyflavonol and acetobromoglucose [2].

The synthesis of 3-hydroxyflavone-*O*-glucoside was performed enzymatically using the recombinant *NtGT3* and *NtGT1a* enzymes and 3-hydroxyflavone and UDP-glucose as substrates. The entire synthesized compound was checked by a mass spectrum.

2.4 Metabolism of naphthols in the tobacco cell

Seven-day old tobacco cells (tube-culture or plate culture [29]) were treated with 50 to 1000 μ M of 1- naphthol or 2-naphthol (0.2 % dimethylsulfoxide). The incorporation of the naphthols were estimated by monitoring the concentration of naphthols in the culture medium in time course. After the treatment of naphthols for 20 hr, the cells were harvested and part of them were subjected to extract with methanol. The extract was hydrolyzed by addition of 0.2 vol. of 10 M NaOH at 60 °C for 3 hours. The samples were acidified by addition of 2 M HCl, and were then treated with β -glucosidase from almond (Oriental Yeast, Tokyo, Japan) at 30 °C for 2 hours. All the samples were analyzed by HPLC.

The samples for enzyme assay were extracted from the residual cells with Tris-HCl (pH

7.5) containing 5 mM β -mercaptoethanol as described [28]. GTase reactions were performed for 60min at 30 °C using 200 μ M 1- or 2- naphthol and 500 μ M UDP-glucose as substrates, then the generated glucoside was determined by HPLC. Cell viability was checked by the staining with fluorescein diacetate as described [29,30].

2.5 Screening and sequencing of glucosyltransferase genes

A cDNA library of tobacco (T-13 callus) was constructed previously [14]. The conserved region of glucosyltransferases was used to design a degenerated primer (UDP-F1: 5'-GCTCTAGACKCATTGYGGNTGGAAYTC-3'). The fragments containing parts of GTase-genes were amplified from the cDNA library (2.0 X 10⁶ plaque forming units) with UDP-F1 and the vector sequence (M13 primer M4, Takara Shuzo, Kusatsu, Japan) using *Ex Taq* (Takara Shuzo) under the condition of 95 °C 5min, 35 cycles of 95 °C 30 sec., 50 °C 1 min., and 72 °C 2 min., followed by 72 °C 7 min. The amplified PCR fragment was cloned into the *Xba*I / *Xho*I site of the pBluescript SK⁺ vector (Stratagene, La Jolla, CA.). The fragments containing full-length *NtGT3* were amplified from the cDNA library with *NtGT3*-rv (5'- CCGCTCGAGGATGGCCATTTAACGC -3') and the vector sequence (M13 primer RV, Takara Shuzo) as described above, then the amplified PCR fragment was cloned

into the *Bam*HI / *Xho*I site of the pBluescript SK⁺. To avoid PCR errors, the library was screened again using 794-bp *Xba*I fragment of *NtGT3* labeled with [³²P]-dCTP by random labeling (Amersham Pharmacia Biotech), as a probe. The hybridization conditions were as follows: 6 X SSC, 5 X Denhardt's solution, 0.1% SDS, 50% formamide, 0.1mg/ml sonicated salmon sperm DNA, 42 °C according to the method of Sambrock *et al.* [31]. The cDNA inserts of the clones selected by three rounds of plating and hybridization were isolated according to the manufacture's instruction (Stratagene). The cDNAs were sequenced using DNA sequencers (DSQ-2000L, Shimadzu, Kyoto, Japan and Genetic Analyzer 310, Applied Biosystems, CA).

2.6 Expression of recombinant *NtGT* in *E. coli*

The coding region of *NtGT3* was amplified by PCR with Pyrobest DNA polymerase (Takara Shuzo) using the following primers: *NtGT3*-fw 5'-CGGGATCCCATATGAAAGAAACCAAGAAA -3' and *NtGT3*-rv, respectively, and was then subcloned into the *Nde*I / *Xho*I site of the pET28c (+) vector (Novagen, Madison, WI). The plasmid was used to transform *E. coli* BL21 (DE3) (Novagen). The expression of the recombinant enzyme was performed essentially as described for *NtGT1* [14].

2.7 Properties of the recombinant NtGT enzymes

GTase assay was performed as described previously [14,28]. The generated products were detected by HPLC (LC10Avp system: Shimadzu). The optimum pH was determined using scopoletin as a substrate. Substrate specificity was determined as described previously [14,28]. For the determination of K_m values of the substrates, their concentrations were varied from 0.5 or 2.5 to 50 or 200 μM for flavonoids, 5 to 200 μM for naphthols, 20 to 2000 μM for scopoletin, with UDP-glucose concentration maintained at 1 mM. For determination of K_m values of UDP-glucose, its concentration was varied between 5 and 2000 μM , with 4-methylumbelliferone concentration was maintained at 500 μM . The reaction was performed for 5 min, stopped by addition of methanol, and subjected to HPLC.

2.8 HPLC conditions

HPLC was performed using an ODS column (4.6 mm i.d. x 150 mm: LUNA 5u C18 (2), Phenomenex, Torrance, CA) with LC10Avp system (Shimadzu). The condition for coumarins was described previously [14]. For separation of naphthols, the column was eluted with solvent A (0.1 N KH_2PO_4) / methanol = 6/4 or 1/1, at a flow rate of 1 ml /min at 40 °C. For

separation of kaempferol and 7-hydroxyflavone, the column was eluted in a linear gradient using 20 % to 50 % solvent B (acetonitrile with 0.5 % acetic acid) in solvent C (0.5% acetic acid) for 12 min., followed by 50 % solvent B in C for 5-10 min. at a flow rate of 1 ml /min at 40 °C. For separation of 3-hydroxyflavone, the column was eluted in a linear gradient using 30 % to 50 % solvent B in C for 10 min., followed by 70 % solvent B in C for 5-10 min. at a flow rate of 1 ml /min at 40 °C. Kaempferol-glucoside, 3-hydroxyflavone-glucoside, and 7-hydroxyflavone-glucoside were detected by monitoring at 350 nm, 310 nm, and 310 nm, respectively, using a diode array detector (SPD-M10Avp, Shimadzu). Naphthol-glucosides were detected using a fluorescence detector (RF-10AXL; Shimadzu) with excitation and emission at 285 nm and 350 nm (for the enzyme assay) or SPD-M10Avp (270nm; for the cell extracts).

2.9 RT-PCR analysis

Total RNA from T-13 cells treated with 1- or 2-naphthol (500 μ M) for 9 hours, salicylic acid (SA, 50 μ M) for 4 hours, and dimethylsulfoxide (solvent control, final concentration, 0.2%) for 0 or 9 hours was extracted according to the method of Shirzadegan *et al.* [32]. These RNA samples (0.5 μ g each) were used for cDNA synthesis by reverse-transcription (RT) in a 20 μ l

reaction mixture using TrueScript II Reverse Transcriptase RNase H⁻ system (Sawady Technology, Tokyo, Japan) with oligo-dT primer at 50 °C for 60 minutes. A reaction without reverse transcriptase was used as a control. One micro littler of cDNA sample from RT reaction was used for PCR reaction using *Ex Taq* (Takara Shuzo) under the condition of 95 °C 5 min, 25 cycles of 95 °C 30 sec., 50 °C 1 min (58 °C for *NtGT1a*), and 72 °C 2 min., followed by 72 °C 7 min with the following primers: NtGT3-716 5'- GTTTCGAGAAACCAAAGGT -3' and NtGT3-1562n 5'- GGGATGGCCATTTAACGC -3' for 847 bp fragment of *NtGT3*; NtGT1-670 5'- TCAGGGAGACGAAAGGA -3', and NtGT1a-1533n 5'- AGACTGATGATAGTACTGG -3' for 864 bp fragment of *NtGT1a*; NtGT1-670 and NtGT1b-1579n 5'- CTGCATTTCTGCAAGAGG -3' for 902 bp fragment of *NtGT1b*; Act-fw 5'- GATTGGAATGGAAGCTG -3' and Act-rv 5'- CCTCCAATCCAAACACT -3' for 231 bp fragment of consensus region from tobacco actin (GenBank accession numbers, X63603, U60489, U60491, AF154640). PCR product was analyzed by electrophoresis in 1.5 % agarose gel.

3. Results

3.1 *Metabolism of naphthols in tobacco cells*

To investigate the naphthol metabolism in tobacco, 1- naphthol or 2-naphthol was applied to the liquid-cultured tobacco cells. The naphthols acted as toxic to the tobacco cells; addition of 1 mM 1-naphthol or 2-naphthol caused cell death without color change; addition of 500 μ M 1-naphthol or 2- naphthol caused cell damage with color change to gray or light brown respectively, but the cells survived after 20-hour incubation (confirmed by the fluorescein diacetate staining [30]). Naphthols (250 μ M) were added to the tobacco cells and the residual amounts in the medium were monitored (Fig 1). The amount of 1-naphthol or 2-naphthol in the culture medium decreased to about 20 % after 18 hours of addition, and 80 % of it seemed to be incorporated into the cells. When naphthol (250 μ M)-treated cells were transferred into a new medium after 20-hour incubation, they grew as well as control cells (data not shown), suggesting that the added naphthol was detoxified in the tobacco cells. To confirm these findings, methanol extracts from the 2-naphthol-treated and untreated cells were analyzed by HPLC (Fig. 2). By comparison between them, a major peak and some minor peaks specific to 2-naphthol treated cells were detected (Fig. 2A-b, c). Since the spectra of these peaks were similar to that of 2-naphthol (Fig. 2B), they appear to be the peaks of metabolites of

2-naphthol. To confirm these compounds, the extract was treated with 2 M NaOH. A new compound that was considered a derivative from the main peak compound was generated by alkaline hydrolysis and was corresponding to 2-naphthol-glucoside (Fig. 2A-d). This compound was digested to 2-naphthol by means of subsequent treatment of β -glucosidase (Fig. 2A-e). The similar results were obtained in the case of 1-naphthol (data not shown). These results showed that naphthols added to the culture were incorporated into the tobacco cells, glucosylated, modified to some alkaline-labile conjugates, and then accumulated.

Glucosylation activities in the naphthol-added tobacco cells were tested (Fig. 3). Tobacco cells are able to glucosylate 1-naphthol or 2-naphthol under normal culture conditions. The activity increased in proportion to the concentration of naphthols added to the culture medium, and reached to 2 to 3-fold after 20-hour incubation. Either 1-naphthol or 2-naphthol increased the glucosylation activity against both substrates, 1-naphthol and 2-naphthol.

3.2 Isolation and sequence analysis of the glucosyltransferase cDNA

In the previous study, we have isolated and characterized two cDNA clones from tobacco cells [14]. The recombinant enzymes of the cDNAs showed glucosylation activities against flavonoids and hydroxycoumarins. Interestingly, the enzymes also showed the activity against

naphthols, which are not natural components of plants. To investigate these naphthol glucosylation activities in tobacco further, we attempted to clone some more cDNAs encoding GTases employing the PCR technique. The PCR reaction was performed with a degenerated primer, which was designed from the conserved region of GTases, using the tobacco cDNA library as a template. Forty clones were obtained and classified into four types: three of them corresponded to the genes previously reported, namely *NtGT1a*, *NtGt1b* [14] and *IS5a* [15], respectively. The other clone was named *NtGT3*. The full-length cDNA of *NtGT3* was amplified by PCR, followed by screening of the cDNA library with the 794-bp *Xba*I fragment of *NtGT3*. Three full-length cDNAs were obtained, whose sequences were the same as the PCR-amplified cDNAs; the longest clone with the 5' and 3'-UTR sequences was selected for further analysis. *NtGT3* (1698 bp, GenBank accession number AB072918) encodes a protein (NTGT3) of 482 amino acid residues with a calculated molecular mass of 54.2 kDa. The blastp search (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed the highest identity of about 60 % for NTGT1a and NTGT1b (The identity between NTGT1a and NTGT1b is 92%). NTGT3 also showed 30-50 % identity with the amino acid sequences deduced from *UGT71Bs* of *Arabidopsis* [22] and *CGT1* of cassava [21], whose exact functions were not reported. The alignment of the deduced amino acid sequences of these genes is shown in Fig

4. There are many highly conserved sequences other than the C-terminus conserved region among UDP-glycosyltransferase (PROSITE [20]). No significant identity of amino acid sequence was found between NTGT3 and mammal glucuronosyltransferases except for the C-terminus conserved region.

DNA blot analysis using genomic DNA extracted from T-13 cells showed that there are two copies of *NtGT3* (data not shown). This could be due to the amphidiploidy of *N. tabacum*.

3.3 Comparison of the enzymatic properties of recombinant enzymes expressed in *E. coli*

To compare the properties of NTGT1 and NTGT3, the cDNA of *NtGT3* was subcloned in the pET28c (+) vector and introduced into *E. coli* BL21 (DE3). The optimum pH of the recombinant enzyme of *NtGT3* (rNTGT3) was 7.2 to 7.5. The enzyme utilized many kinds of phenolic compounds such as coumarins (3-hydroxycoumarin, esculetin, umbelliferone, 4-methylumbelliferone, daphnetin, scopoletin, isoscapoletin, and daphnetin-7-*O*-methyl ether), flavonoids (kaempferol, quercetin, 3-hydroxyflavone, 6-hydroxyflavone and 7-hydroxyflavone), naphthols (1-naphthol and 2-naphthol) and *o*-hydroxycinnamic acid, whereas it did not catalyze 5-hydroxyflavone, 4-hydroxycoumarin, *p*-hydroxycinnamic acid and SA (data not shown). The enzyme utilize UDP-glucose as the best sugar donor (K_m value:

42.3 μM); however, the enzyme also utilized UDP-xylose as a weak donor (data not shown). These results are similar to those of the recombinant enzyme of *NtGT1a* (rNTGT1a [14]). To compare the reaction of rNTGT1a and rNTGT3, enzymatic parameters for major substrates (Fig 5) were determined using Lineweaver-Burk plot (Table I). The enzymatic parameters of rNTGT3 differed from rNTGT1 in the following points: 1) rNTGT1a showed stronger activity (larger k_{cat}/K_m value) against 2-naphthol than against 1-naphthol, although the K_m values towards both compounds are almost the same. On the other hand, rNTGT3 showed almost the same activity against these two types of naphthols; 2) rNTGT1a showed stronger activity against 3-hydroxyflavone than against kaempferol, whereas rNTGT3 showed similar activity toward both compounds.

3.4 Expression of *NtGTs* mRNA in the naphthol-treated tobacco cells

To study the relationship between the *NtGT* genes and the naphthol-inducible expression of glucosylation activity, RT-PCR analysis was performed with the specific primers of *NtGT1a*, *NtGT1b* and *NtGT3*, allowing distinguishing the three *NtGT* genes. The transcription of *NtGT1a*, *NtGT1b* and *NtGT3* were induced in tobacco cells by addition of 1- naphthol (500 μM , 9 hours), 2-naphthol (500 μM , 9 hours) or SA (50 μM , 4 hours) (Fig. 6). One main band

and a faint band were observed in the panel of *NtGT1a*, even when the PCR was performed under strict temperature conditions. These two bands were cloned respectively. The DNA sequences showed that the main band is the specific to *NtGT1a* mRNA but not another faint one.

SA induced the expression of these genes stronger than naphthols. The time course of *NtGTs* induction with SA is also different from that with naphthols; SA induced these genes after 4 hours of the addition, in contrast naphthols induced them after 9 to 20 hours of the addition (data not shown).

4. Discussion

In general, GTases that use secondary metabolites as substrates, are minor constituent proteins in plant cells [33]. Although many GTases were isolated from several plant species and characterized, their roles in the metabolism of low-molecular-mass compounds were not sufficiently clarified. The *Arabidopsis* genome project revealed many GTase homologues [22,34], suggesting that there are many kinds of GTases in each plant. Moreover, the genome project also revealed that many gene sets that had been considered to exist in some limited plant species, i.e., homologues of berberine-bridging enzyme or those of tropinone reductase

also exist in *Arabidopsis* [34]. These findings suggest that plants have maintained their potential to metabolize compounds that are now absent in their system throughout their evolution.

In this study, naphthols were used as model compounds of xenobiotic metabolism because the structure is very simple and is resemble to skeletons of many toxic compounds. Naphthols were incorporated into the tobacco cells (Fig. 1) and were accumulated in the form of glucosylated and subsequently modified compounds (Fig. 2). It is reported that glucoside conjugates of low-molecular-mass compounds are often accumulated as alkaline-labile malonylated compounds [5,35] or transported as glutathionylated compounds [36]. The modification of 2-naphthol-glucoside may be malonylation or glutathionylation, because the compound was also alkaline-labile.

Naphthol glucosylation activity was increased depending on the concentration of naphthols in the culture (Fig. 3). GTase genes (*NtGT1a*, *NtGT1b*, and *NtGT3*) were cloned and characterized in our works. The deduced amino acid sequences of these genes were similar. Comparing the enzymatic parameters of the enzymes, rNTGT1 showed specificity against the 3- hydroxyl group of flavonoids or the 2- hydroxyl groups of naphthol; in contrast, rNTGT3 showed a broader specificity (Table 1). These *NtGTs* were induced in response to the addition

of naphthols (Fig. 6). These results suggested that NTGT1 and NTGT3 catalyze the glucosylation of naphthols *in vivo* as *in vitro*. These enzymes also seem to work on other structure-related compounds and detoxify them as glucoconjugates. There are some GTases similar to *NtGTs* reported in several plant species (Fig. 4), suggesting that these GTase would work on the glucosylation of naphthol-like structure compounds in plants.

In mammals, the substrate specificity of GTase which work on the detoxification are quite broad; these enzymes act on both endobiotics and xenobiotics [22]. The reactivity of rNTGT1 and rNTGT3 (flavonoids, hydroxycoumarins, naphthol) were comparable to that of one of the detoxification enzymes UGT2B15 in mammals, which reacted on steroids, flavonoids, naphthols, and hydroxycoumarins [37], though the amino acid sequence is not similar. The physical properties of glucosides and glucuronides are quite different, so that the transport system of glycoside may be different in mammals and plants. But this similarity on the substrate utilization of both enzymes is interesting to consider the development of the detoxification systems. Comparison of the stereochemical structure of these enzymes in mammals and in plants could give a key for these questions.

A possible mechanism for the metabolism of harmful compounds in tobacco is as follows. When the concentrations of naphthols incorporated in the cells are sufficiently high, enzymes,

such as GTases are induced, naphthols are glucosylated by them and further modified, and then the modified compounds would be compartmentalized in vacuoles. The metabolism of naphthols is similar to that of scopoletin, which is an endobiotic compound and considered as a phytoalexin in tobacco. A part of scopoletin is existing in the culture medium of tobacco cell [38]. After treatment of SA and /or auxin to the tobacco cell, scopoletin in the culture medium is incorporated and accumulated in the vacuole after glucosylation [2,29]. There are two distinct aspects between them. 1) The naphthol-glucosides are accumulated in the cells as further modified form, in contrast, scopolin is accumulated as a glucoside; 2) Naphthol incorporation needs no additional signals, in contrast, scopolin incorporation demands SA and /or auxin signals. Frangne *et al.* [3] reported that flavone glucoside uptake into barley vacuoles and *Arabidopsis* vacuoles are different, the former is mediated by H⁺ antiport transporter and the latter is ATP-binding cassette transporter, and proposed that the difference might be responsible for the sequestration of endogenous and xenobiotic compounds in planta. It also seems that xenobiotic naphthols are distinguished from endobiotic scopoletin exactly in tobacco and each compound may be accumulated by different ways. The uptake and the accumulation of glucosides in tobacco need to be further studied. In a previous study, we discussed that the products of *NtGT1a* and *NtGT1b* might play some roles in the

metabolism of scopoletin [14]. The results of this study may suggest that these genes including *NtGT3* are involved in both endo- and/or xeno- biotic metabolism in tobacco.

Acknowledgments

The authors wish to express their thanks to Drs Y. Amano and T. Tsuchida (Shinshu University) for the mass spectrum analysis, Dr H. Yamamoto (Nagasaki University) for the technical advises for HPLC analysis, Dr. H. Nozue for her help on preparing the manuscript.

This work was partially supported by Hokuto Foundation for Bioscience (grant to G.T.).

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Table 1 Kinetic parameters of the recombinant enzyme rNTGT1a and rNTGT3.

The K_m (μM) and k_{cat} (s^{-1}) values were calculated using data from the Lineweaver-Burk plot.

The reaction was performed three times, and mean \pm standard error was presented.

Enzymes	rNTGT1a			rNTGT3		
	K_m	k_{cat}	k_{cat} / K_m	K_m	k_{cat}	k_{cat} / K_m
Substrate	(μM)	(s^{-1})	($\mu\text{M}^{-1} \text{s}^{-1}$)	(μM)	(s^{-1})	($\mu\text{M}^{-1} \text{s}^{-1}$)
Flavonoids						
kaempferol	13.1 \pm 1.6	3.1 \pm 1.3	0.23	3.5 \pm 0.3	7.1 \pm 0.3	2.0
3-hydroxyflavone	1.2 \pm 0.2	8.5 \pm 0.3	7.0	10.3 \pm 0.5	7.9 \pm 0.0	0.77
7-hydroxyflavone	24.2 \pm 5.5	0.36 \pm 0.02	0.01	32.5 \pm 1.2	0.86 \pm 0.01	0.03
Naphthols						
1-naphthol	25.3 \pm 2.9	0.28 \pm 0.06	0.01	51.5 \pm 3.2	4.8 \pm 0.5	0.09
2-naphthol	35.5 \pm 0.3	2.1 \pm 0.6	0.06	68.1 \pm 4.8	4.4 \pm 0.5	0.07
Hydroxycoumarin						
scopoletin	1400 \pm 2.1	0.42 \pm 0.02	0.0003	1000 \pm 55	0.70 \pm 0.09	0.0007

Legends for figures

Figure 1 Decrease of exogenously added naphthols in the culture medium.

Residual amount of naphthol in the medium of tobacco T-13 cells (7-days) after addition of 250 μ M of 1-naphthol (1-NA) or 2-naphthol (2-NA) are shown as percentage of the initial amount of it.

Figure 2 HPLC analysis of methanol extract of tobacco cells treated with 2-naphthol. Tobacco cells were treated with or without naphthols, harvested after 20 hour of the addition, and then an aliquot of the cell was extracted with methanol. HPLC was performed as described in Materials and methods with a solvent of 0.1 N KH_2PO_4 / methanol = 6/4. The eluate was monitored by a photodiode array detector (absorbance at 270 nm) as described in Materials and Methods. Panel A: The results of HPLC analysis. Standard of scopolin, scopoletin, 2-naphthol glucoside and 2-naphthol (a); the methanol extracts from the tobacco cells treated with solvent (dimethylsulfoxide) only (b) or 2-naphthol (c). The methanol extracts from naphthol-treated cells that were further hydrolyzed with 2M NaOH (d) then treated with β -glucosidase (e). Peak identification: 1, scopolin; 2, scopoletin; 3, naphthol-metabolite; 4, 2-naphthol-glucoside (2-NAG); 5, 2-naphthol (2-NA). Panel B: The spectra of the peak 3, 4, and 5 in the panel A.

Figure 3. Glucosyltransferase activity against naphthols in tobacco cells and their changes after treatment with naphthols.

Cells were incubated for 20 hours with 1-naphthol (1-NA) or 2-naphthol (2-NA) at concentrations indicated in the figure, then used for the enzyme assay with 1-naphthol (solid column) or 2-naphthol (empty column) as a substrate.

Figure 4 Multiple alignment of the deduced amino acid sequences of glucosyltransferases.

Alignment was calculated with Clustalx. Black shading shows the identical amino acid at least among three sequences. The gray-underlined sequence is the region conserved among the plant secondary product glucosyltransferases (PSPG box) proposed by Hughes & Hughes (1994) including the region defined as 'UDP-glycosyltransferase signature' by PROSITE (Bairoch 1992). Abbreviations and Genbank accession nos. are: NtGT1a (AB052557), NtGT1b (AB052558) and NtGT3 (AB072918) from tobacco, CGT1 (X77459) from *Manihot esculenta*, UGT71B1(AB025634) from *Arabidopsis thaliana*.

Figure 5 Structures of the substrates used for the kinetics analysis of rNTGT enzymes

Figure 6 Analysis of the expression level of *NtGTs* mRNA by RT-PCR.

Total RNA was isolated from tobacco T-13 cells treated with 1-naphthol (1NA: 500 μ M, 9 hours), 2-naphthol (2NA: 500 μ M, 9 hours), salicylic acid (SA: 50 μ M, 4 hours), or solvent only (dimethylsulfoxide: final conc. 0.2 %, 0 or 9 hours). The symbol (+) or (-) indicates the presence or absence of reverse transcriptase in the RT reaction. The fragments of *NtGTs* were confirmed by hybridization. An 847-bp cDNA fragment was expected for *NtGT3* mRNA, 864-bp for *NtGT1a* mRNA, 902-bp for *NtGT1b* mRNA, 231-bp for actin mRNA.

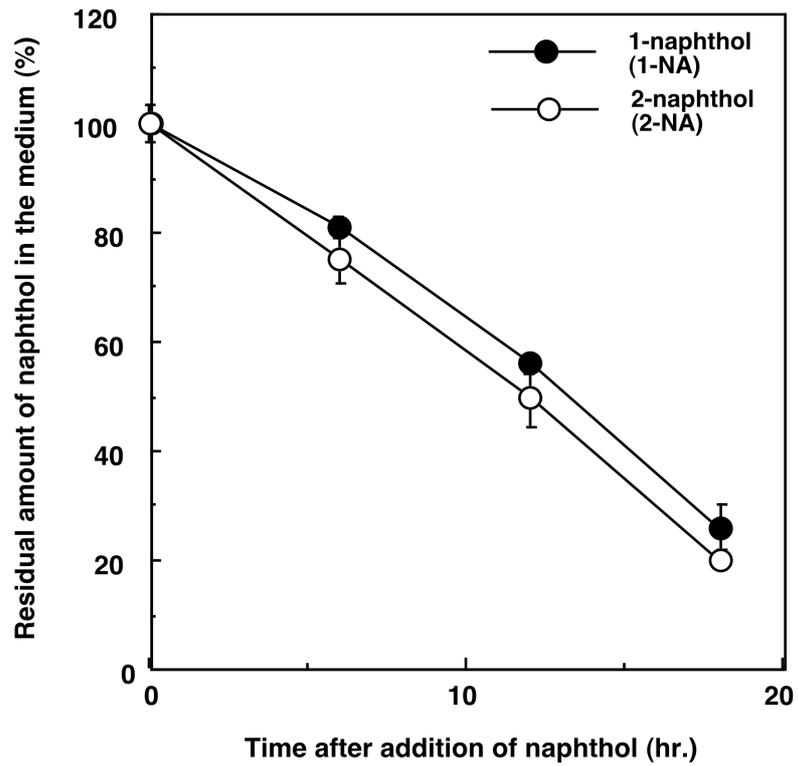


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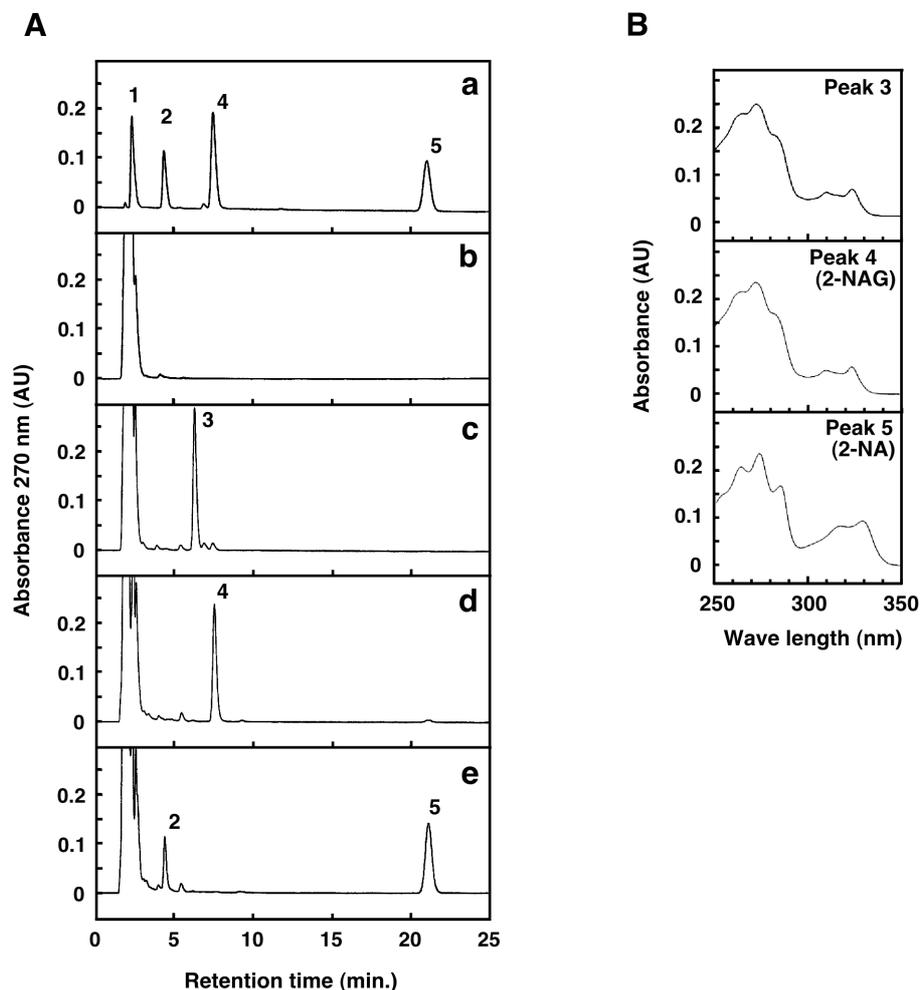


Figure 2 HPLC analysis of methanol extract of tobacco cells treated with 2-naphthol.

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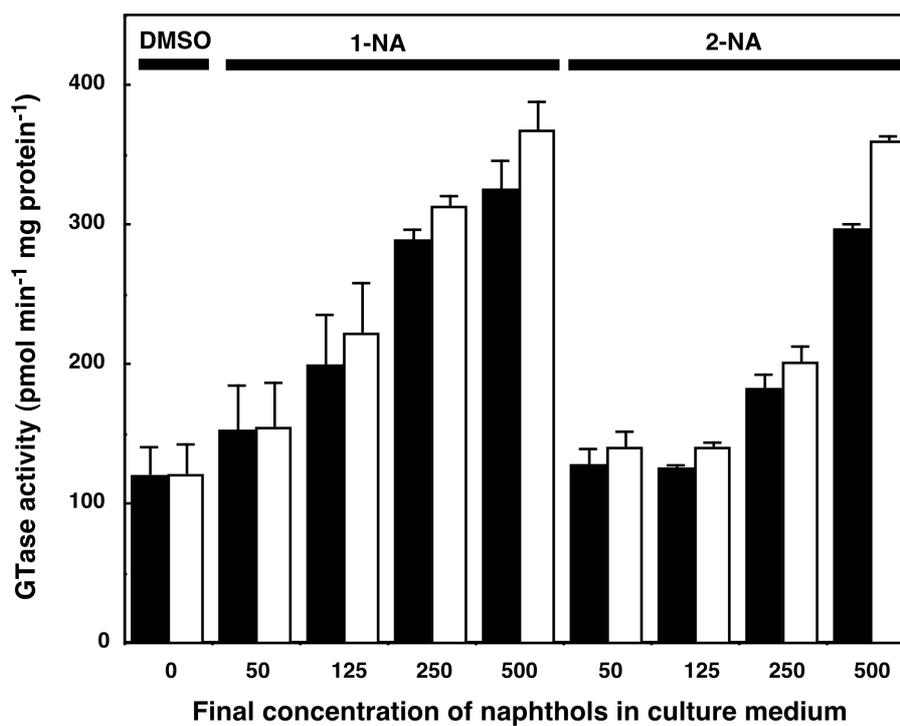


Figure 3. Glucosyltransferase activity against naphthols in tobacco cells and their changes after treatment with naphthols.

Cells were incubated for 20 hours with 1-naphthol (1-NA) or 2-naphthol (2-NA) at concentrations indicated in the figure, then used for the enzyme assay with 1-naphthol (solid column) or 2-naphthol (empty column) as a substrate.

NTGT1a	1	---	MKTTEL	LVFIP	AP	GMGHL	VP	TV	VA	KQL	VDR	E	Q	L	S	I	T	V	L	I	M	T	L	P	L	E	T	-	N	I	P	S	Y	T	K	S	L	S	S	D	Y	S	S	R	I	T	L	L	Q	L	S	Q	71																									
NTGT1b	1	---	MKTAEL	LVFIP	AP	GMGHL	VP	TV	VA	KQL	VDR	E	Q	L	S	I	T	V	L	I	M	T	L	P	L	E	T	-	N	I	P	S	Y	T	K	S	L	S	S	D	Y	S	S	R	I	T	L	L	Q	L	S	Q	71																									
NTGT3	1		MKET	KKI	EL	V	F	I	P	S	P	G	I	G	H	L	V	S	T	V	E	M	A	K	L	L	I	A	R	E	Q	L	S	I	T	V	L	I	I	Q	W	P	N	D	K	-	K	L	D	S	Y	I	Q	S	V	A	N	-	F	S	R	L	K	F	I	R	L	P	Q	73								
CGT1	1		-----	-----	-----	M	G	H	L	V	S	A	V	E	T	A	K	L	L	S	R	C	H	S	L	S	I	T	V	L	I	F	N	S	V	V	T	S	K	V	H	N	Y	V	D	S	Q	I	A	S	S	N	R	L	R	F	I	Y	L	P	R	59																
UGT71B1	1	----	MKV	EL	V	F	I	P	S	P	G	V	G	H	I	R	A	T	T	A	L	A	K	L	L	V	A	S	D	N	R	L	S	V	T	L	I	V	I	P	S	R	V	S	D	----	D	A	S	S	V	Y	T	N	S	E	D	R	L	R	Y	I	L	L	P	A	67											
NTGT1a	72		PE	T	S	V	S	M	S	S	F	N	A	I	N	F	F	E	Y	I	S	S	K	D	R	V	K	D	A	V	N	E	T	---	F	S	S	S	S	V	K	L	K	G	F	V	I	D	M	F	C	T	A	M	I	D	V	A	N	E	F	G	I	P	S	Y	V	F	Y	T	S	N	143					
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NTGT3	74		DD	S	---	I	M	Q	L	L	K	S	N	I	F	T	T	F	L	A	S	H	K	P	A	V	R	D	A	V	A	D	I	---	L	K	S	E	S	N	N	T	L	A	G	I	V	I	D	L	F	C	T	S	M	I	D	V	A	N	E	F	L	P	T	Y	V	F	Y	T	S	G	143					
CGT1	60		DE	T	-----	G	-	I	S	S	F	S	L	I	E	K	Q	K	P	H	V	K	S	V	M	K	I	E	F	G	S	S	V	E	S	P	R	L	V	G	F	I	V	D	M	F	C	T	A	M	I	D	V	A	N	E	F	G	V	P	S	Y	I	F	Y	T	S	G	127									
UGT71B1	68		RD	Q	-----	T	-	D	L	V	S	Y	I	D	S	Q	K	P	O	V	R	A	V	S	K	V	A	G	D	V	S	T	R	S	D	S	R	L	A	G	I	V	V	D	M	F	C	T	S	M	I	D	I	A	D	E	F	N	L	S	A	I	I	F	Y	T	S	N	134									
NTGT1a	144		A	A	M	L	G	L	Q	L	H	F	O	S	L	S	I	E	S	P	K	V	H	N	Y	L	D	-	P	E	S	E	V	A	I	S	T	Y	I	N	P	I	P	V	K	C	L	P	G	I	L	D	N	D	K	S	G	T	M	F	V	N	H	A	R	R	F	R	E	T	K	G	I	M	V	217		
NTGT1b	145		A	A	M	L	G	L	Q	L	H	F	O	S	L	S	I	E	C	S	P	K	V	H	N	Y	E	-	P	E	S	E	V	L	I	S	T	Y	M	N	P	V	P	V	K	L	P	G	I	L	V	N	D	E	S	S	T	M	F	V	N	H	A	R	R	F	R	E	T	K	G	I	M	V	218			
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CGT1	128		A	A	F	L	N	F	M	L	H	V	O	K	I	H	D	E	E	N	F	N	P	T	E	F	N	A	-	S	D	G	E	L	Q	V	P	G	L	V	N	S	F	P	S	K	A	M	P	T	A	L	L	S	K	Q	W	F	P	L	L	E	N	-	T	R	R	Y	G	E	A	K	G	V	I	200		
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CGT1	201		N	T	F	E	L	E	S	H	A	I	E	S	F	K	D	-----	P	P	I	Y	P	V	G	P	I	L	D	V	R	S	N	G	R	N	T	N	Q	E	---	I	M	Q	W	L	D	D	Q	P	P	S	S	V	V	F	L	C	F	G	S	N	G	S	F	S	K	264										
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NTGT1a	362		G	F	V	S	H	C	G	W	N	S	T	L	E	S	V	R	S	G	V	P	I	A	T	W	P	L	Y	A	E	Q	Q	S	N	A	F	Q	L	V	K	D	L	G	M	A	V	E	I	K	M	D	Y	R	E	D	F	N	K	T	-	N	P	P	L	V	K	A	E	E	I	E	D	G	I	R	K	435
NTGT1b	363		G	F	V	S	H	C	G	W	N	S	T	L	E	S	V	R	S	G	V	P	I	A	T	W	P	L	Y	A	E	Q	Q	S	N	A	F	Q	L	V	K	D	L	G	M	A	V	E	I	K	M	D	Y	R	E	D	F	N	T	R	-	N	P	P	L	V	K	A										

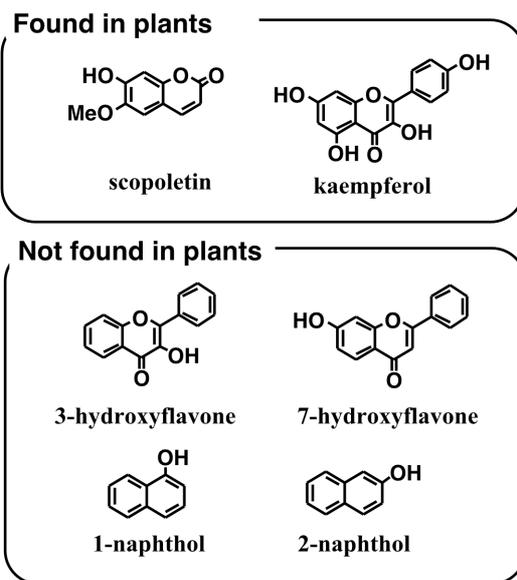


Figure 5 Structures of the substrates used for the kinetics analysis of rNTGT enzymes

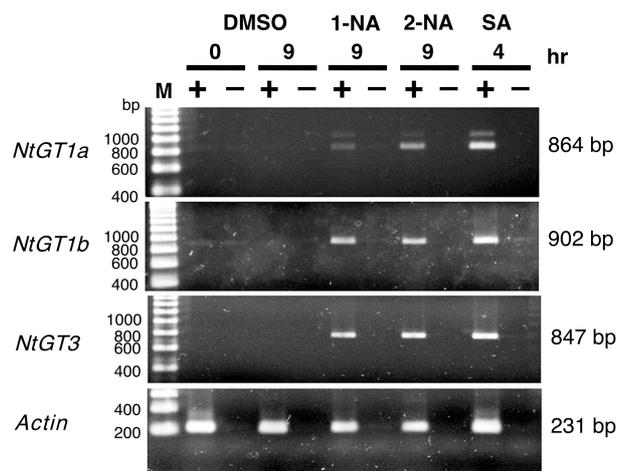


Figure 6 Analysis of the expression level of NtGTs mRNA by RT-PCR.

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