Title:

Molecular cloning and heterologous expression of novel glucosyltransferases with broad substrate specificity that were induced by salicylic acid and auxin from tobacco cultured cells

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Abbreviations: GTase, glucosyltransferase; CGTase, UDP-glucose: hydroxycoumarin 7-*O*-glucosyltransferase; SA: salicylic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; rNTGT1: recombinant enzyme of *NtGT1* gene

Enzymes

UDP-glucose: glucosyltransferase (EC 2.4.1. -)

UDP-glucose: flavone 7-*O*-beta-glucosyltransferase (EC 2.4.1.81)

UDP-glucose: flavonol-3-*O*-glucosyltransferase (EC 2.4.1.91)

UDP-glucose: 2-coumarate *O*-beta-glucosyltransferase (EC 2.4.1.114)

UDP-glucose: scopoletin glucosyltransferase (EC 2.4.1.128)

Running Title

GTase with broad specificity from tobacco

Summary

Scopoletin is one of the phytoalexins in tobacco. Cells of the T-13 cell line (*Nicotiana tabacum* L. Bright Yellow) accumulate a large amount of scopoletin, 7-hydroxy-6-methoxycoumarin, as a glucoconjugate, scopolin, in vacuoles. We report here the molecular cloning of glucosyltransferases that can catalyze the glucosylation of many kinds of secondary metabolites including scopoletin. Two cDNAs encoding glucosyltransferase (*NtGT1a* and *NtGT1b*) were isolated from a cDNA library derived from the tobacco T-13 cell line by screening with heterologous cDNAs as a probe. The deduced amino acid sequences of *NtGT1a* and *NtGT1b* exhibited 92% identity with each other, about 20-50% identities with other reported glucosyltransferases. Heterologous expression of these genes in *Escherichia coli* showed that the recombinant enzymes had glucosylation activity against both flavonoids and coumarins. They also strongly reacted with 2-naphthol as a substrate. These recombinant enzymes can utilize UDP-glucose as the sugar donor, but they can also utilize UDP-xylose as a weak donor. RNA blot analysis showed that these genes are induced by salicylic acid and auxin, but the time course of the expression was different. The result is similar to the changes in scopoletin glucosylation activity in these tobacco cells after addition of these plant growth regulators. These results might suggest that one of the roles of these genes products are scopoletin glucosylation, in response to salicylic acid and/or auxin, together with the other glucosyltransferases in tobacco cell.

Key words: *Nicotiana tabacum* L. cv Bright Yellow; glucosyltransferase; phenylpropanoid; naphthol; 2,4-dichlorophenoxyacetic acid; salicylic acid.

## Introduction

In higher plants, secondary metabolites are often converted to their glycoconjugates, which are then accumulated and compartmentalized in vacuoles. These glucosylation reactions are catalyzed by glucosyltransferases (GTases). Several roles have been postulated for the glucosylation of various low-molecular -mass compounds in plants, as follows: 1) Glucosylation allows solubilization of the compounds in water [1]; 2) it acts to detoxify harmful metabolites or environmental compounds, such as herbicides [2]; and 3) it can regulate the action of functional compounds, such as the plant hormones, IAA and cytokinins [3,4]. The first gene that encodes an enzyme for glucosylation in plant was isolated from maize in an analysis of the *bronze* locus that encodes *UDP-glucose: flavonol glucosyltransferase* [5]. Subsequently, related genes for enzymes that mainly catalyze the glucosylation of flavonoids in the biosynthesis of anthocyanin were cloned from many plant species using the *flavonol glucosyltransferase* gene from maize as a probe (for review, [6]). Recently, several GTases that catalyze mainly the hydroxyl group or carboxyl group of 'non-flavonoid' compounds were also reported including description on the procedure of enzyme purification and subsequent cloning from the partial amino acid sequence [3,7-9]. The "inducible" *GTase* genes have been isolated from several plant species by mRNA differential display methods; some of those were induced by salicylic acid [10], or methyl-jasmonate [11], some of those were found in the anthocyanin-producing tissue [12], or ripening fruit [13]. Most of them are highly conserved in the C-terminus, named PSPG box (plant secondary product glucosyltransferase signature) [14], forming a super gene family. The genome project of *Arabidopsis* showed that about 100 GTase genes exist in a plant species; however, the functions of those genes have not been

investigated. Further studies are required to clarify the relationships between structures and functions of GTases and to understand their roles in plants.

Coumarins are common secondary metabolites that have been found in many botanical families [15]. They are reported to function in the protection of plants, having, for example, antimicrobial activity, the ability to deter feeding by insects, an autoinhibitory effect on germination, and a shielding effect against ultraviolet irradiation [15]. Most coumarins in higher plants exist as glucoconjugates, and appear to be accumulated in vacuoles [15-17]. Scopolin, a 7-*O*-glucoconjugate of scopoletin, which is a coumarin derivative, is accumulated in tobacco cells (T-13 cell line). Addition of methyl jasmonate or kinetin to the T-13 cell cultures stimulates the biosynthesis of scopoletin [18] but not glucosylation activity [19]. On the other hand, it was observed that addition of synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and salicylic acid (SA) induced scopoletin glucosylation activity in the tobacco cells [19-21].

In our previous study, a UDP-glucose: hydroxycoumarin 7-*O*-glucosyltransferase (CGTase) that converts scopoletin to scopolin was purified from the tobacco cells and characterized [20]. The enzyme showed a broad substrate specificity including other coumarins and flavonoids. In the purification steps, it was found that there are many GTases catalyzing scopoletin glucosylation in the tobacco cells. These enzymes also showed the potency to catalyze glucosylation of flavonoids. In this work, in order to clarify the glucosylation of secondary products in tobacco cells, cDNAs that encode GTases were isolated from tobacco cells and expressed in *E. coli*. Their properties and expression pattern were also investigated. The roles of these enzymes in the tobacco cells were discussed.

## Materials and methods

### Culture of plant cells.

Cells of T-13 habituated callus of *Nicotiana tabacum* L. cv. Bright Yellow [21] were used in this study. These were maintained on the hormone-free Murashige and Skoog (MS) medium as described previously [20,21].

### Reagents

Kaempferol -7-*O*-glucoside used as a standard was kindly donated by Dr K. Yoshitama (Kumamoto University). Scopolin was synthesized as described previously [20]. Isoscopoletin, daphnetin, 3-hydroxycoumarin, and kaempferol -3-*O*-glucoside were obtained from Extrasynthèse (Genay, France). The standards of 4-methyl umbelliferone-glycosides were obtained from Sigma. 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.

### cDNA library construction

Total RNA was extracted form 6-day-old T-13 cell culture by the phenol-SDS method [22]. Then, poly-$(A)^+$ RNA was purified from the total RNA using Oligotex dT-30 Super (Takara Shuzo, Kyoto, Japan). The cDNA was synthesized from 1 μg of poly-$(A)^+$ RNA using a ZAP cDNA synthesis kit (Stratagene). The cDNA was ligated with *Eco* RI adapters and cloned into uni-ZAP (Stratagene) and the resulting constructs were packaged *in vitro* using the Gigapack Gold (Stratagene). The resulting primary library contained 1.3 X $10^6$ plaque forming units.

Screening and sequencing of glucosyltransferase genes

The cDNA library was screened using a heterologous probe. A fragment of *Perilla* glucosyltransferase gene (3R4) [12] containing a region highly conserved among glucosyltransferases (PSPG box, [14]) was prepared by PCR using primers 5'-CGCCGCATGGCCAACACAGC -3', and 5'- TGGCTTCTCTGGCCAAAGTC - 3'. The purified PCR fragment was then labeled with [$^{32}$P]-dCTP by random labeling (Amersham Pharmacia Biotech), and used as a probe to screen 120,000 plaque forming units of the cDNA. The hybridization conditions were as follows: 6 X SSC, 5 X Denhaldt's solution, 0.1% SDS, 25% formamide, 0.1mg/mL sonicated salmon sperm DNA, 42 ºC [23]. Forty positive clones were isolated by three rounds of plating and hybridization, and then the cDNA inserts were isolated according to the manufacture's instruction (Stratagene). The cDNAs were sequenced partially from the direction of 3'-UTR using a DNA sequencer (Model 373A, Applied Biosystems); five clones that carrying the conserved PSPG box [14] were subcloned and sequenced using Thermo Seqenase (Amersham Pharmacia Biotech) and a DNA sequencer (DSQ-2000L, Shimadzu, Kyoto, Japan).

Expression of recombinant NTGT1a and 1b in *E. coli*

The coding region of *NtGT1a* and *1b* was amplified by PCR using the following primers: NtGT1a-fw2 5'- CGGGATCCAAATGAAGACAACAGAGT-3' and NtGT1a-rv 5'- CCGCTCGAGACTGATGATAGTACTG -3', and NtGT1b-fw2 5'-CGGGATCCAAATGAAGACAGCAGAGT -3' and NtGT1b-rv 5'-CCGCTCGAGCATTTCTGCAAGAGGC -3', respectively, and was then subcloned

into the *Bam*HI / *Xho*I site of the pET28c(+) vector (Novagen, Madison, WI, U. S. A.). The plasmid was used to transform *E. coli* BL21 (DE3) (Novagen). The transformed *E. coli* was cultured at 20 ºC for 1 day. Isopropyl β-D- thiogalactopyranoside (200 μM) was added to the culture that was then further incubated for 5 hr. The crude enzyme was extracted according to the manufacture's instruction using 50mM Tris-HCl (pH 7.5) containing 5 mM 2-mercaptoethanol as an extraction buffer. The recombinant enzyme was purified using nickel-equilibrated iminodiacetic acid-Sepharose 6B (Amersham Pharmacia Biotech) according to the manufacture's instruction.

Properties of the recombinant enzymes (rNTGT1a and 1b)

For the determination of the properties, affinity-purified enzymes were used. Glucosyltransferase assay was performed as described previously [20]. The generated products were detected by HPLC (LC10Avp system: Shimadzu, Kyoto, Japan). The optimum pH was determined using scopoletin as a substrate. To determine the substrate specificity, each reaction mixture (10 μL) contained a recombinant enzyme, 0.5 mM UDP- [$^{14}$C] glucose (740 MBq mmole$^{-1}$; American Radiolabeled Chemicals), 0.5 mM substrate, 0.01% BSA, 5 mM 2-mercaptoethanol, and 50 mM Tris-HCl (pH 7.5). After incubation for 90 min, the reaction was stopped by addition of methanol (10 μL). An aliquot was spotted on a silica-gel plate (Kieselguhr 60 $F_{254}$; Merck), and the plate was developed with a mixture of ethyl acetate, methanol and water (20:4:3, v/v) that had been acidified with one drop of acetic acid. The plate was exposed to an imaging plate (Fuji Film, Japan) for autoradiography. The intensity of each radioactive spot was estimated using an image analyzer (STORM 860 system, Amersham Pharmacia Biotech). For the specificity to UDP-sugars, 0.5 mM of 4-methyl umbelliferone was

used as a substrate. UDP-sugars, such as UDP-glucose, UDP-glucuronic acid, UDP-galactose, and UDP-xylose were used as sugar donors at 2mM. For determination of Km values of the substrates, their concentrations were varied from 2.5 to 50 μM for kaempferol, 5 to 500 μM for 4-methylumbelliferone, 10 to 2000 μM for scopoletin, with the UDP-glucose concentration maintained at 2 mM. For determination of Km values of UDP-sugars, their concentrations were varied between 20 and 2000 μM, with the concentration of 4-methylumbelliferone was maintained at 500 μM. The reaction was performed for 5 min, or 60 min (for UDP-xylose), and the reaction mixture was then subjected to HPLC.

HPLC conditions

HPLC was performed using an ODS column (4.6 mm i.d. x 150 mm: LUNA 5u C18 (2), Phenomenex, Torrance, CA, U. S.A.). For detection of coumarins, the column was eluted with solvent A (0.1 N $KH_2PO_4$ with 40% methanol) at a flow rate of 1 mL/min at 40 $^{o}$C. Scopolin and 4-methylumbelliferyl-glycosides were detected using a fluorescence detector (RF-10AXL; Shimadzu) with excitation and emission at 350 nm and 430 nm, and 320 nm and 376 nm, respectively. For detection of flavonoids, the column was eluted in a linear gradient using 20% to 50% solvent B (acetonitrile with 1% acetic acid) in solvent C (1% acetic acid) for 12 min., followed by 50% solvent B in C for 5 min. at a flow rate of 1 mL/min at 40 $^{o}$C. Flavonoids were detected by monitoring at 365 nm using a diode array detector (SPD-M10Avp, Shimadzu).

Plant hormone-induced and organ-specific expression of *NtGT1*

Total RNA was extracted from T-13 cells treated with 2,4-D (5 μM), SA (50 μM), or methyl jasmonate (25 μM) by the phenol-SDS method [22]. Total RNA was separated by electrophoresis on 1.2% agarose and 0.66% formaldehyde gel, and separated RNA fragments were blotted on a nylon membrane (Gene screen plus, NEN). The *Eco*RI / *Xba*I fragment of *NtGT1a* (901 bp) was labeled with [$^{32}$P] dCTP by the random labeling method, and was used as a probe. Hybridization was performed in 6 X SSC, 1% SDS, 5 X Denhaldt's solution, and 50% formamide. The membrane was washed in 1 X SSC and 0.1% SDS at 42 ºC [23]. The hybridization signals were detected using an imaging analyzer as described above. To detect the organ-specific expression, total RNA was extracted from the flower, leaf, stem, and root of tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-4) plant by the phenol-SDS method, and then subjected to RNA blot analysis as described above.

DNA blot analysis

Genomic DNA was extracted from T-13 cultured cells by crude nuclear pellet methods [24]. DNA (25 μg) digested with appropriate restriction enzymes was separated by electrophoresis on 1.0% agarose gel, and separated DNA fragments were then blotted on a nylon membrane (Gene screen plus, NEN) and subjected to DNA blot analysis following the same method as that described in 'RNA blot analysis'.

Results

Isolation and sequence analysis of the glucosyltransferase cDNA

In our previous study, a UDP-glucose: hydroxycoumarin 7-*O*-glucosyltransferase (CGTase) that converts scopoletin to scopolin was purified from T-13 tobacco cells and

characterized [20]. The N-terminus amino acid sequence of the enzyme suggests that the enzyme is a homologue of the salicylate-inducible GTase (IEGT)[10]. The properties of CGTase suggest that this enzyme is different from a scopoletin glucosyltransferase previously purified from tobacco [20,21]. Moreover, it was found that there are many GTase isozymes catalyzing scopoletin glucosylation in tobacco cells along the purification steps of CGTase. These enzymes also catalyze other phenylpropanoid compounds. In order to clarify the relationships between the GTases and metabolism of such compounds in tobacco cells, a cDNA library was screened using an *IS5a* fragment, a cDNA of IEGT, as the probe at first. However, we could obtain only the *IS5a* and *IS10a* fragments, so we changed the probe to a fragment of *Perilla* glucosyltransferase gene (3R4) [12] containing a region highly conserved among glucosyltransferases (PSPG box, [14]). Forty positive clones were sequenced partially from the direction of 3'-UTR. Then five clones that contained the conserved PSPG box [14] were subcloned and sequenced. Three clones encoded the same gene, named *NtGT1a*, the other two clones were named *NtGT1b* and *NtGT2*. The nucleotide and the deduced amino acid sequences of *NtGT1a* are shown in Fig. 1. *NtGT1a* (1577 bp, Genbank AB052557) contains an open reading frame of 1437 bp that encodes a protein (NTGT1a) of 478 amino acid residues. *NtGT1b* (1691 bp, Genbank AB052558) also contains an open reading frame of 1440 bp that encodes a protein (NTGT1b) of 479 amino acid residues. The calculated molecular masses of NTGT1*a* and 1b were 53.5 kDa and 53.3 kDa, respectively. The degree of the identity between the two enzymes was 92.1%. These proteins showed about 50 % identity with a glycosyltransferase from cassava (*CGT1*), but its function was not investigated [14]. An alignment between the deduced amino acid sequence of the glucosyltransferases

isolated from tobacco was shown in Fig. 1. The percent identities with them are 23-28 %. The amino acid residues that are conserved in plant GTases are also shown in Fig. 1. The region between 348 and 391 amino acid residues of NTGT1a (underlined) was called the PSPG box, a highly conserved region among plant secondary product glucosyltransferases [14]. This region was proposed as a signature of UDP-glycosyltransferases by PROSITE [25]. In addition to this region, the positions 266-281 are also highly conserved. Several conserved amino acid sequences may reflect the important structural or functional domains of GTases. Similar to other GTase, NTGT1a and 1b also start from a hydrophobic region in the N-terminus. This may suggest that these enzymes are loosely membrane-associated with this region [9] and belong to the 'type II' class of the eucaryotic membrane-bound GTases proposed by Narimatsu [26].

Properties of recombinant enzymes expressed in *E. coli*

To clarify the properties of NTGT1a and 1b, their cDNAs were subcloned in pET28c(+) vector and introduced into *E.coli* BL21 (DE3). When the transformant was cultured at 20 ºC, a part of the recombinant protein was expressed as a soluble protein. The extract was further purified and concentrated using a nickel affinity. The purified enzyme fraction included some minor bands, which corresponded to the bands obtained from the affinity-adsorbed extract from BL21 (DE3) harboring the pET28c(+) vector (data not shown). This fraction had no glucosyltransferase activity; therefore the affinity-purified fractions of the recombinant enzymes (rNTGT1a and rNTGT1b) were used for the experiments. When the pH value was changed between 5.5 and 9.0, the maximum enzyme reaction was observed at pH 7.2-7.5. The rNTGT1a utilized

scopoletin, esculetin and kaempferol similar to CGTase and its isozymes. To investigate the specificity further, several phenolic compounds were used as the substrate of the reaction with UDP-$^{14}$C-glucose. The glucosylated products were developed by TLC (Fig. 2), and then the relative amounts of the products were estimated from the density of spots on the autoradiogram (Fig. 3). The structures of the compounds tested are also shown in Fig. 3. The reaction of rNTGT1b was almost the same results as those of rNTGT1a (data not shown). Flavonoids were catalyzed at the 3-, 6- or 7- hydroxyl group, but weakly at the 4'- hydroxyl groups, and not at the 5-hydroxyl group, when the reaction was performed using single-position hydroxylated flavonoid (Fig. 2). However, only the peak corresponding to 3-*O*-glucoside was observed and no other major peak corresponding to the glucoside standard was observed when the product of rNTGT1a-kaempferol reaction was analyzed by HPLC (data not shown). The result indicates that rNTGT1a reacted predominantly with the 3-hydroxyl group of kaempferol, although it has the potential to react with the other hydroxyl groups. The enzyme activity was weak against quercetin, which has a di-hydroxylated B-ling structure, compared to that against kaempferol, suggesting the stereochemical inhibition of the reaction with the hydroxyl groups. Coumarins were catalyzed at the 3-, 6-, or 7-hydroxyl group, but less at the 4-hydroxyl group (Fig. 2). The enzyme reaction with coumarins that have the 6- or 7-hydroxyl group was also compared. The enzyme activities against the compounds that have a methoxy group next to the hydroxyl group were weaker than those against the other compounds (Fig.3). rNTGT1a also reacted with 2-hydroxycoumaric acid and caffeic acid weakly, but not with other hydroxycinnamates or salicylic acid (Fig. 3).

To determine the sugar donor specificity of the recombinant enzymes, four UDP-sugars were reacted with the enzyme using 4-methylumbelliferone as a sugar acceptor. UDP-glucose was the best donor; however, the enzymes also utilized UDP-xylose as a weak donor (Fig. 4). UDP-galactose and UDP-glucuronic acid were not utilized. Kinetic parameters of the rNTGT1a were determined with UDP-glucose or UDP-xylose as the sugar donor, and scopoletin, 4-methylumbelliferone or kaempferol as the sugar acceptor. The kinetic parameters of rNTGT1a are shown in Table 1. These results also suggest that rNTGT1a has a broad substrate specificity.

Plant hormone-induced and organ-specific expression of *NtGT1*

To investigate the expression of *NtGT1s*, total RNA was extracted from T-13 tobacco cells treated with 2,4-D (5 μM), SA (50 μM), and methyl jasmonate (25 μM). These plant hormones are known to affect scopoletin biosynthesis or metabolism in tobacco cells at these concentrations [17-20]. The total RNA (8 μg) was subjected to RNA blot analysis. Because the identity between *NtGT1a* and *1b* is very high, it was difficult to distinguish one from the other. A 901-bp *Eco*RI / *Xba*I fragment of *NtGT1a* was used as a probe. This probe hybridized with both *NtGT1a* and *1b*, but not with *NtGT2* or the*IS5a* gene under the conditions performed (data not shown). The result is shown in Fig. 5. These genes are very weakly expressed in normal culture of T-13 cells. The treatment with 2,4-D and SA induced the expression of these genes with different time course; in contrast, methyl jasmonate is not effective at the concentration that induced scopoletin biosynthesis. To investigate further, total RNA was extracted from the flower, leaf, stem, and root of a mature tobacco plant (Bright Yellow-4). The RNA blot analysis showed no signals (data not shown).

DNA blot analysis using genomic DNA extracted from T-13 cells (Fig. 6) showed that there are two copies of *NtGT1* gene that correspond to *NtGT1a* and *1b* in tobacco cells.

## Discussion

In general, GTases that use secondary metabolites as substrates are minor constituent proteins in plant cells [27]. Although many GTases were isolated from several plant species and characterized, including those we have previously reported, their roles in the metabolism of secondary products were not sufficiently clarified. The genome-project of *Arabidopsis* revealed many GTase homologues in this plant, suggesting that there are many kinds of GTase in each plant. Considering the importance of glycosylation on the metabolism or accumulation of secondary products, the isolation of each gene of GTase and the functional analysis of each enzyme are required.

In this study, GTase cDNAs were isolated from tobacco in order to investigate the phenylpropanoid metabolism in tobacco. Four GTase genes from tobacco have been reported, namely the salicylate-inducible genes (*IS5a* and *IS10a*) [10, 30], jasmonate-inducible gene [11], and salicylic acid glucosyltransferase [7]. *NtGT1a* and *1b* showed less homology with these genes. The molecular phylogenic tree based on the deduced amino acid sequence of plant GTase genes is shown in Fig. 7. Based on the utilization of the sugar acceptor by the enzymes encoded by these genes, it appears that these genes can be classified as follows. The flavonoid-3-*O*-GTases from *Perilla* (PfF3GT, AB002818), *Petunia* (PhF3GT, AB027454), *Vitis* (VvF3GT, AF000372), and maize (ZmF3GT, X13502), and a flavonoid-3-*O*-galactosyltransferase from *Petunia* (PhgalT, AF165148) are classified into same group. The anthocyanin-5-*O*-GTases from

*Perilla* (PfA5GT, AB013596), *Petunia* (PhA5GT, AB027455) and *Verbena* (VhA5GT, AB013598) are classified into another group. Some stress-inducible genes, many of which seem to have a potential catalyzing the glucosylation of the7-hydroxyl group of flavonoids, i. e. a salicylic acid-inducible glucosyltransferase from tobacco (NtIS5a, U32644), a baicalein 7-*O*-glucosyltransferase from *Scutellaria* (SbB7GT, AB031274), and a betanidin-5-*O*-glucosyltransferase from *Dorotheanthus* (DbB5GT, Y18871) are in one group. The result indicates that plant GTases can be divided into some subfamilies based on their substrate specificity. *NtGT1s* are belong to a new group that includes *Manihot CGT1* gene, the properties of the gene product was not investigated (MeCGT1, X77459). The subfamily is closed to the products of 'stress-inducible' genes mentioned above and 'zeatin glycosyltransferases (zeatin glucosyltransferase from *Phaseolus* (PlZGT, AF101972) and zeatin xylosyltransferase from *Phaseolus* (PvZXT, AF116858)' including xylosyltransferase [28]. *NtGT1* was induced with plant hormones and showed weak xylosyltransferase activity; this similarity might reflect its evolutional history.

It was reported that products of stress-inducible genes have a tendency to show a broad substrate specificity [8, 9, 29]. A *baicalein 7-O-glucosyltransferase* gene from *Scutellaria* (*SbB7GT*) was induced by wounding, and its product showed activities with several flavonoids and weak activities against coumarin compounds, mainly at the7-hydroxyl group of both compounds [29]. A product of *betanidin-5-O-glucosyltransferase* gene from livingstone daisy (DbB5GT) can catalyze the 4'- or 7-hydroxyl groups of flavonoids and a coumarin derivative esculetin [9]. A homologue of salicylate-inducible gene from tobacco that was also induced with wounding showed a broad GTase activity against coumarin derivatives or salicylic acid

[30]. *NtGT1s* were genes inducible with 2,4-D and /or salicylic acid, and the rNTGT1utilized both coumarins and flavonoids as sugar acceptors similar to other stress-inducible genes. Although rNTGT1 showed strong activity mainly against the 3-hydroxyl group of kaempferol, it was not grouped with other 3-*O*-GTases such as PfF3GT, PhF3GT, VvF3GT, and ZmF3GT. The result suggests that the evolution of these 'stress-inducible' 3-*O*-GTases was independent from other genes encoding 3-*O*-GTase, but was rather concomitant with the 'stress-inducible' 7-*O*-GTases.

In Fig. 8, the structures of flavonoids and coumarins are compared, considering the glucosylation activity of rNTGT1. Their main structures are similar to that of naphthol. Under this supposition, glucosylation was observed mainly at the 2-hydroxyl group of the naphthalene skeleton, which corresponds to the 3-, 6-, or 7-hydroxyl group of flavonoid and the 3-, 6-, or 7-hydroxyl group of coumarin. Indeed, when 1- and 2-naphthol as the sugar acceptors were reacted with rNTGT1 (Fig. 2 & 3), strong activities were observed with both compounds particularly the 2-form (Fig. 3). These results suggest the possibility that *NtGT1s* encode the enzymes that can react with many kinds of naphthalene-like skeleton in tobacco cells.

It was also found that the recombinant enzyme catalyzed the glucosylation of hydroxycinnamates or 4'-hydroxyl group of flavonoids. This is difficult to explain, because these structures do not have the naphthalene-like skeleton (Fig. 8). Further work is needed to solve this problem. GTases that has an activity against naphthol was reported from several insects [31,32] but not from plants. Other researchers may not have been investigated this point because naphthols are hardly found in plant. This potential for the reaction might suggest the roles of the enzyme in detoxification of harmful metabolites or environmental compounds in plant.

RNA blot analysis showed that these genes were induced with salicylic acid and auxin, but the time course of the expression was different. The result is similar to the changes in scopoletin glucosylation activity in tobacco cell after addition of these plant hormones [19, 20]. In the previous work we have also reported that these plant hormones regulate the uptake of scopoletin from the culture medium [19]. In this tobacco cells, scopoletin glucoside is the most abundant phenylpropanoid compound and no flavonoids were detected. The concentration of scopoletin or scopolin in the protoplast of this cell is about 300 nmol / $10^6$ protoplast, which is calculate to be about 2 mM (the average of the diameter of protoplasts is 60 μm) [17]. Though scopoletin is the poor substrate of the products of *NtGT1*, the gene products might play some roles in scopoletin metabolism in response to salicylic acid and/or auxin with other glucosyltranserases in the tobacco cells.

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Table. 1 Kinetic parameters of the recombinant enzyme of *NtGT1a*.

| Substrates | Km (μM) | Vmax (nmol/min/mg) | Vmax/Km |
|---|---|---|---|
| Scopoletin | 1400 | 504 | 0.36 |
| 4MU | 77.1 | 354 | 4.60 |
| Kaempferol | 10.2 | 1190 | 117 |
| UDPG | 37.2 | 298 | 8.1 |
| UDPX | 400 | 11.0 | 0.028 |

Figure legends

Figure 1. Multiple alignment of the deduced amino acid sequences of six glucosyltrasferases from tobacco. Alignment was calculated with clustalX. Black shading shows the identical amino acid at least four sequences. The asterisks and dots above the sequences are perfectly conserved and highly conserved amino acids among 21 plant glycosyltransferase sequences shown in Fig. 7, respectively.
The 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 1991). Abbreviations and Genbank accession nos. are: NtGT1a and NtGT1b, glucosyltransferases with broad substrate specificity (AB052557 and AB052558, this study); NtIS5a and NtIS10a, salicylic acid-inducible glucosyltransferase (U32644 and U32643); NtJIGT, jasmonate-inducible glucosyltransferase (AB000623); NtSAGT, salicylic acid glucosyltransferase (AF190634).

Figure 2. TLC of the $^{14}$C-labeled product after reaction with the recombinant NTGT1a. The substrates used were; 1-naphthol (lane 1), 2-naphthol (lane 2), 3-hydroxycoumarin (lane 3), 4-hydroxycoumarin (lane 4), umbelliferone (lane 5), esculetin (lane 6), isoscopoletin (lane 7), scopoletin (lane 8), kaempferol (lane 9), 3-hydroxyflavone (lane 10), 5-hydroxyflavone (lane 11), 6-hydroxyflavone (lane 12), 7-hydroxyflavone (lane 13), 4’-hydroxyflavanone (lane 14).

Figure 3. Comparison of structures of the phenolic compounds tested as the substrates and the relative preference by the recombinant NTGT1. The enzyme was reacted with

UDP-$^{14}$C-glucose and each compound, and then the glucosylated products were subjected to TLC. The enzyme activity was estimated by autoradiography with an imaging analyzer STORM860 (Amersham Pharmacia Biotech.). The relative amounts of the glucosylated products are indicated as follows. '–', less than 0.2; '±', 0.2-0.5; '+', 0.5-2.0; '++', 2.0-5.0; '+++', more than 5.0 -times product signals, relative to that from scopoletin.

Figure 4. HPLC of enzyme reaction mixtures in the presence of recombinant NTGT1a, 4-methylumbelliferone, and UDP-glucose (A) or UDP-xylose (B). The reaction was performed as described in 'Materials and Methods', and then the reaction was stopped by adding methanol. HPLC was performed as described in 'Materials and Methods'. The eluate was monitored by measuring the fluorescence at ex. 320 nm, em. 376 nm. Peak identification: 1, 4-methylumbelliferyl-glucoside; 2, 4-methylumbelliferyl-xyloside; 3, 4-methylumbelliferone.

Figure 5. RNA blot analysis of tobacco *NtGT1*.

Total RNA was extracted from tobacco T-13 cells that were treated with 2,4-D (5 μM), salicylic acid (SA: 50 μM), and methyl jasmonate (MJ: 25 μM) for 0, 1, 2, 4, 6, 12 and 24 hours. The cells treated with dimethylsulfoxide were used as the control. An 8-μg portion of total RNA was loaded per lane. A 901-bp *Eco* RI-*Xba* I fragment of *NtGT1a* was used as a probe.

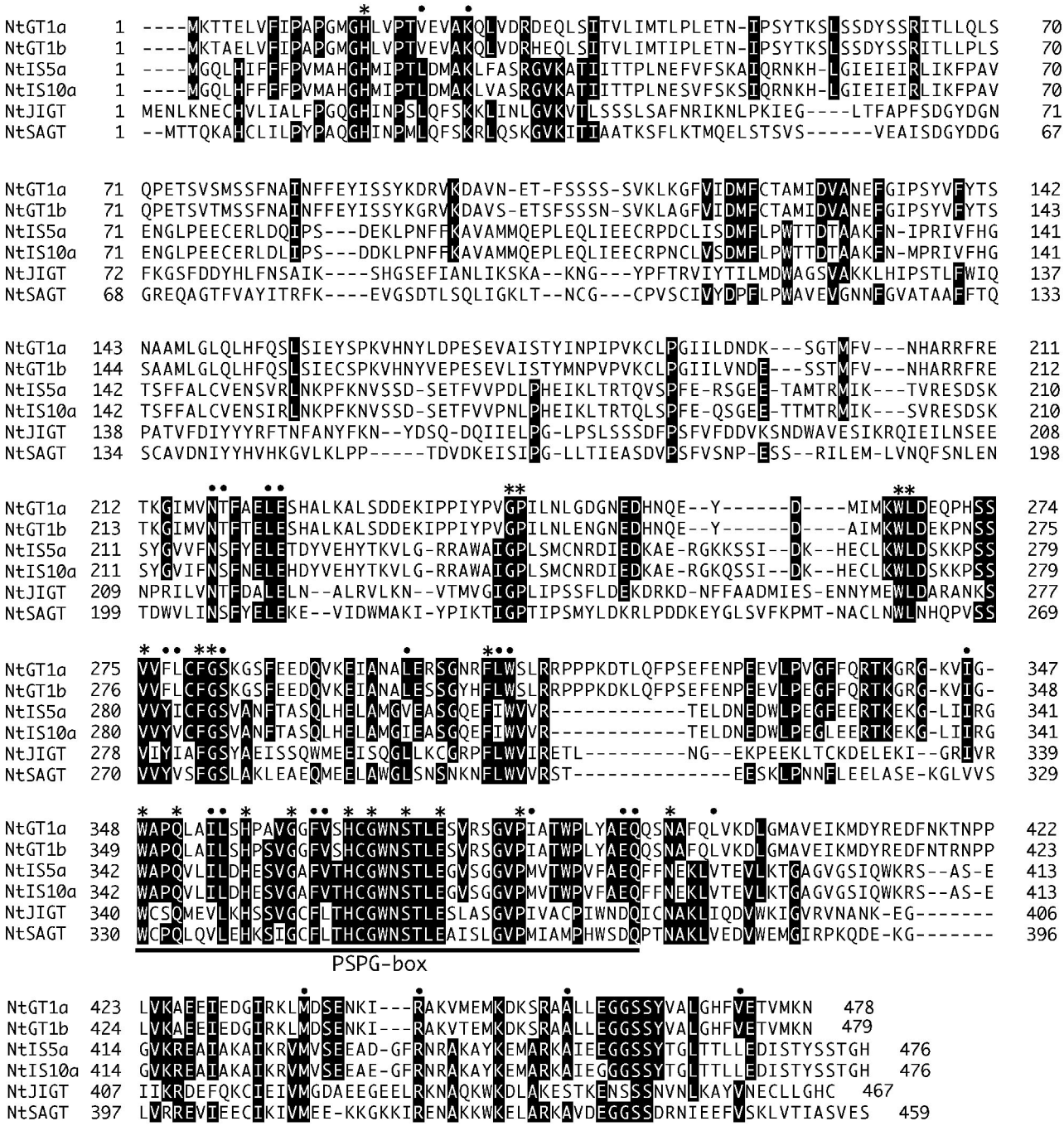
Figure 6. DNA blot analysis of genomic DNA from tobacco. Genomic DNA (25 μg) was digested with *Bam* HI (B), *Eco* RI (E), *Hin*d III (H), *Kpn* I (K), *Xba* I (X),

respectively. A 901-bp *Eco* RI-*Xba* I fragment of *NtGT1a* was used as a probe.

Figure 7. Molecular phylogenic tree based on the deduced amino acid sequence of plant glycosyltransferase. The tree was constructed by the neighbor-joining method. Lengths of lines indicate the relative distance between nodes. Species, abbreviations and Genbank accession nos. are: NtGT1a and NtGT1b, glucosyltransferases from tobacco with broad substrate specificity (AB052557 and AB052558, this study); MeCGT1, *Manihot esculenta* glucosyltransferase1 (X77459); NtIS5a, salicylic acid-inducible glucosyltransferase from tobacco (U32644); SbB7GT, baicalein 7-*O*-glucosyltransferase from *Scutellaria* (AB031274); DbB5GT betanidin-5-*O*-glucosyltransferase from *Dorotheanthus* (Y18871); StSolGT, solanidine glucosyltransferase from potato (U82367); ZmIAAGT, indole-3-acetic acid.glucosyltransferase from maize (L34847); NtSAGT, salicylic acid glucosyltransferase from tobacco (AF190634); NtJIGT, jasmonate-inducible glucosyltransferase from tobacco (AB000623); PfA5GT, PhA5GT, and VhA5GT, anthocyanin-5-*O*-glucosyltransferase from *Perilla* (AB013596), *Petunia* (AB027455) and *Verbena* (AB013598); SbMGT, *p*-hydroxymandelonitrile-*O*-glucosyltransferase from *Sorghum* (AF199453); PfF3GT, PhF3GT, VvF3GT, and ZmF3GT, flavonoid-3-*O*-glucosyltransferases from *Perilla* (AB002818), *Petunia* (AB027454), *Vitis* (AF000372), and maize (X13502); PhgalT, flavonoid-3-*O*-galactosyltransferase from *Petunia* (AF165148); PvZXT, zeatin xylosyltransferase from *Phaseolus* (AF116858); PlZGT, zeatin glucosyltransferase from *Phaseolus* (AF101972).

Figure 8. Comparison the chemical structure of substrates for glucosyltransferase

reaction. The relative amounts of the activity with the compounds are shown same as in fig.3.

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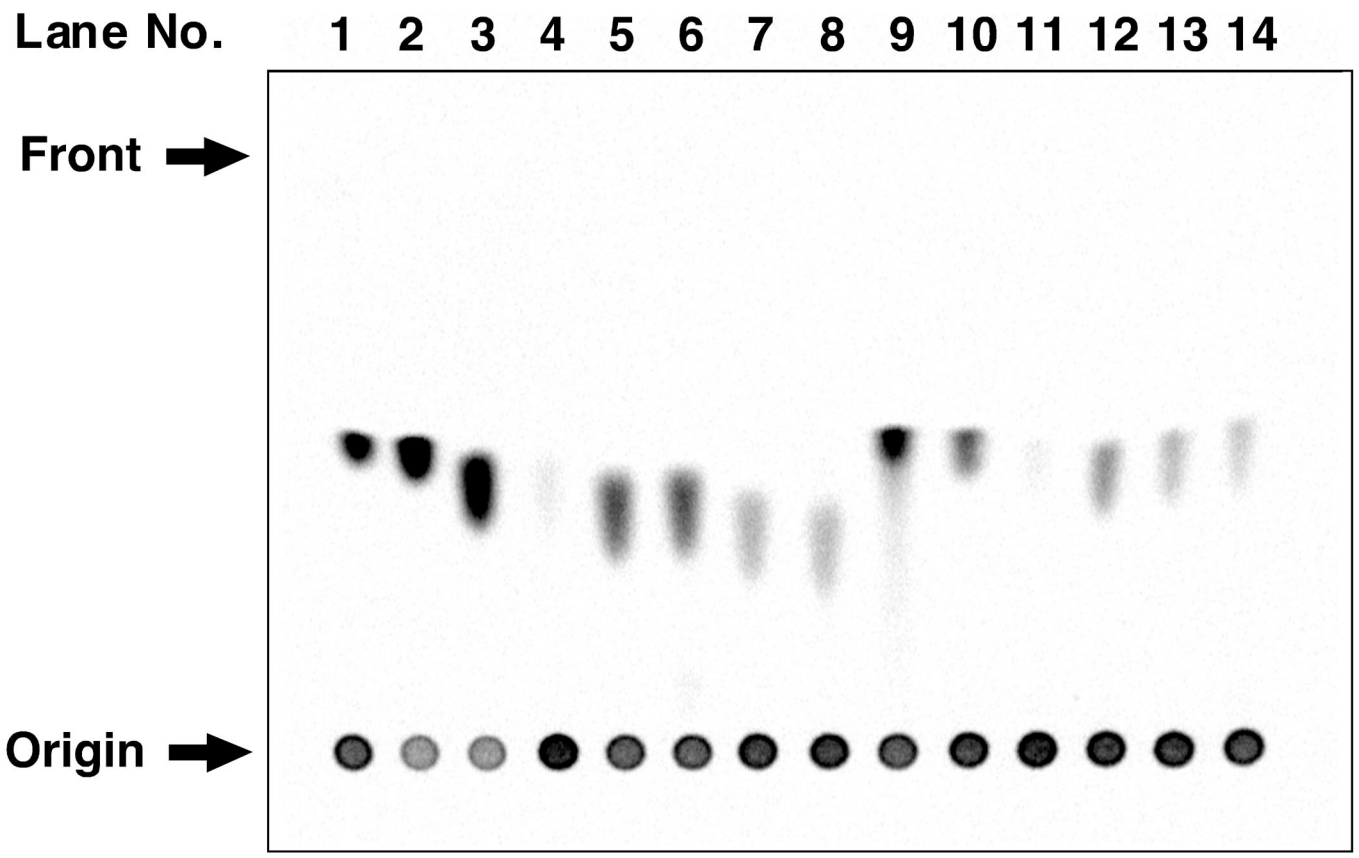


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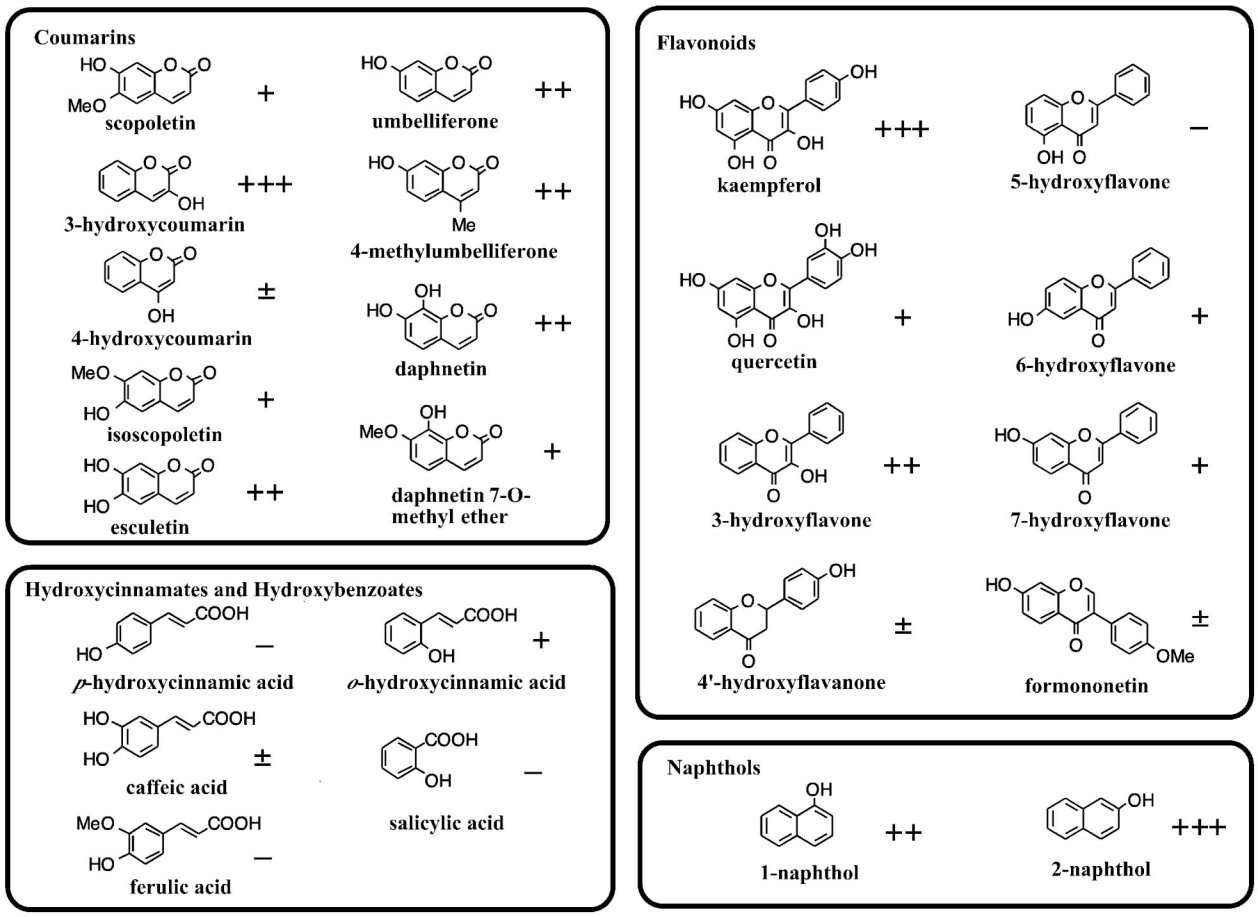


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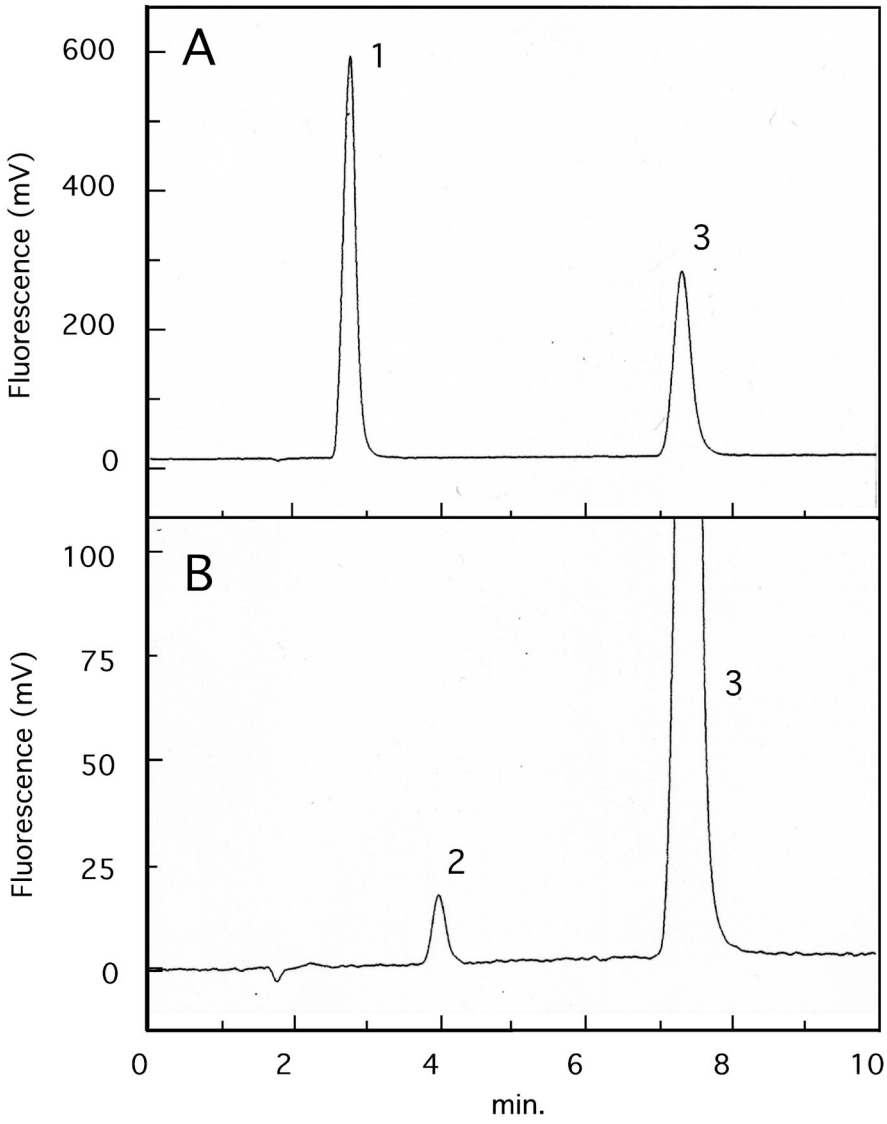


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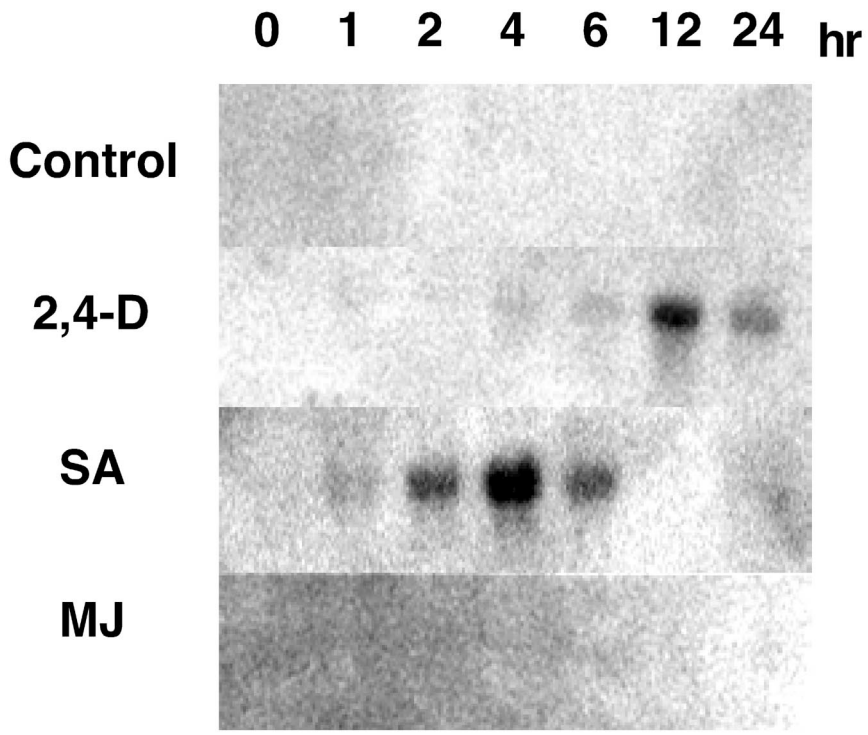


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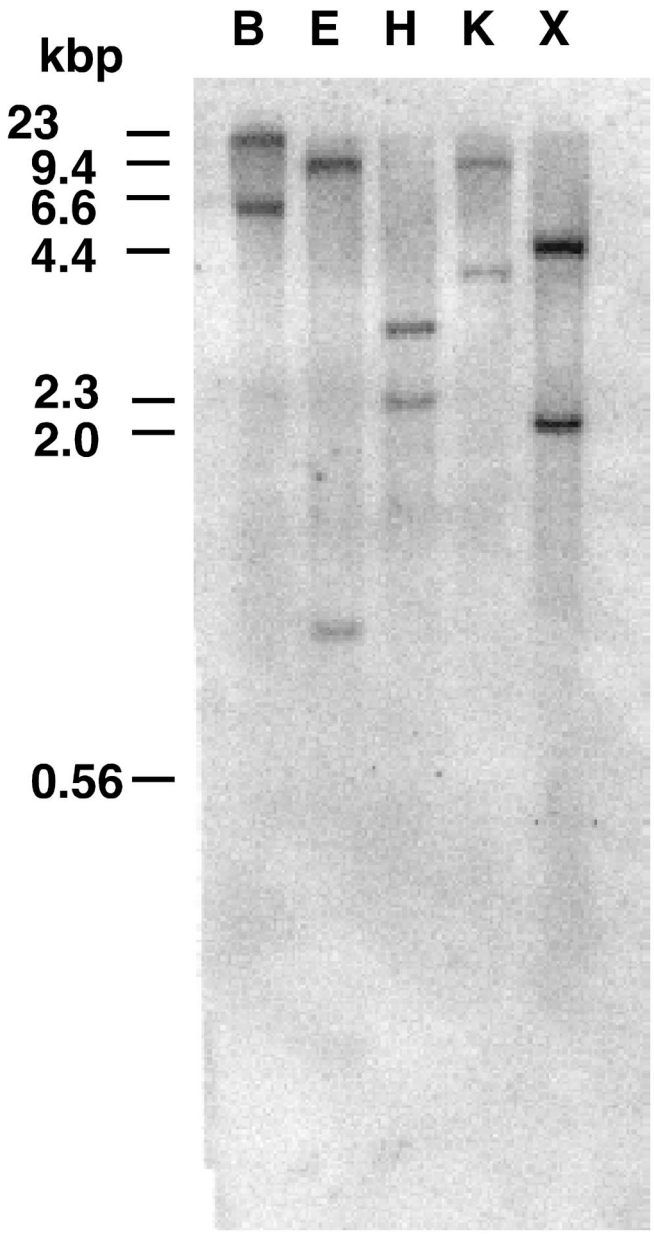


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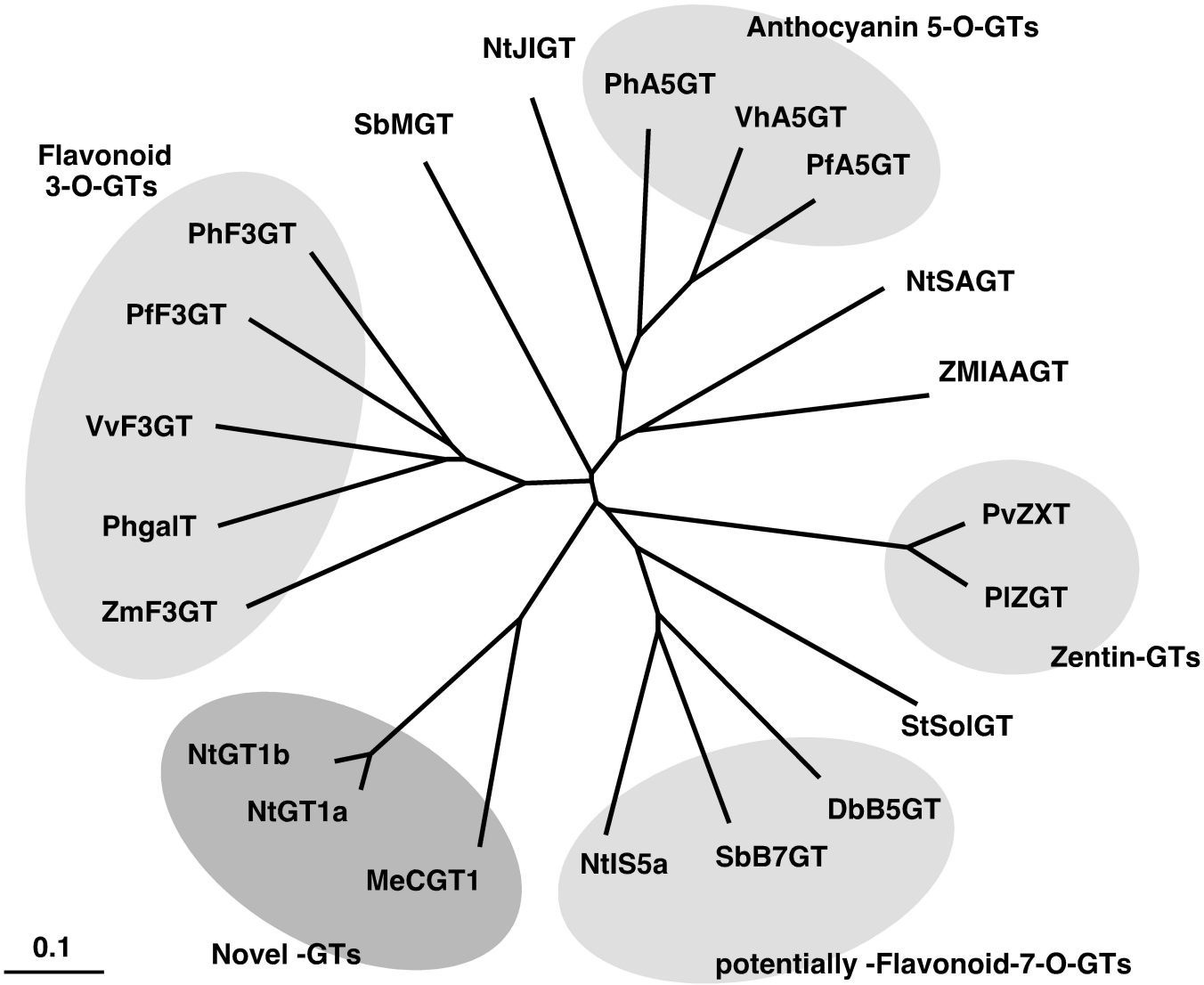


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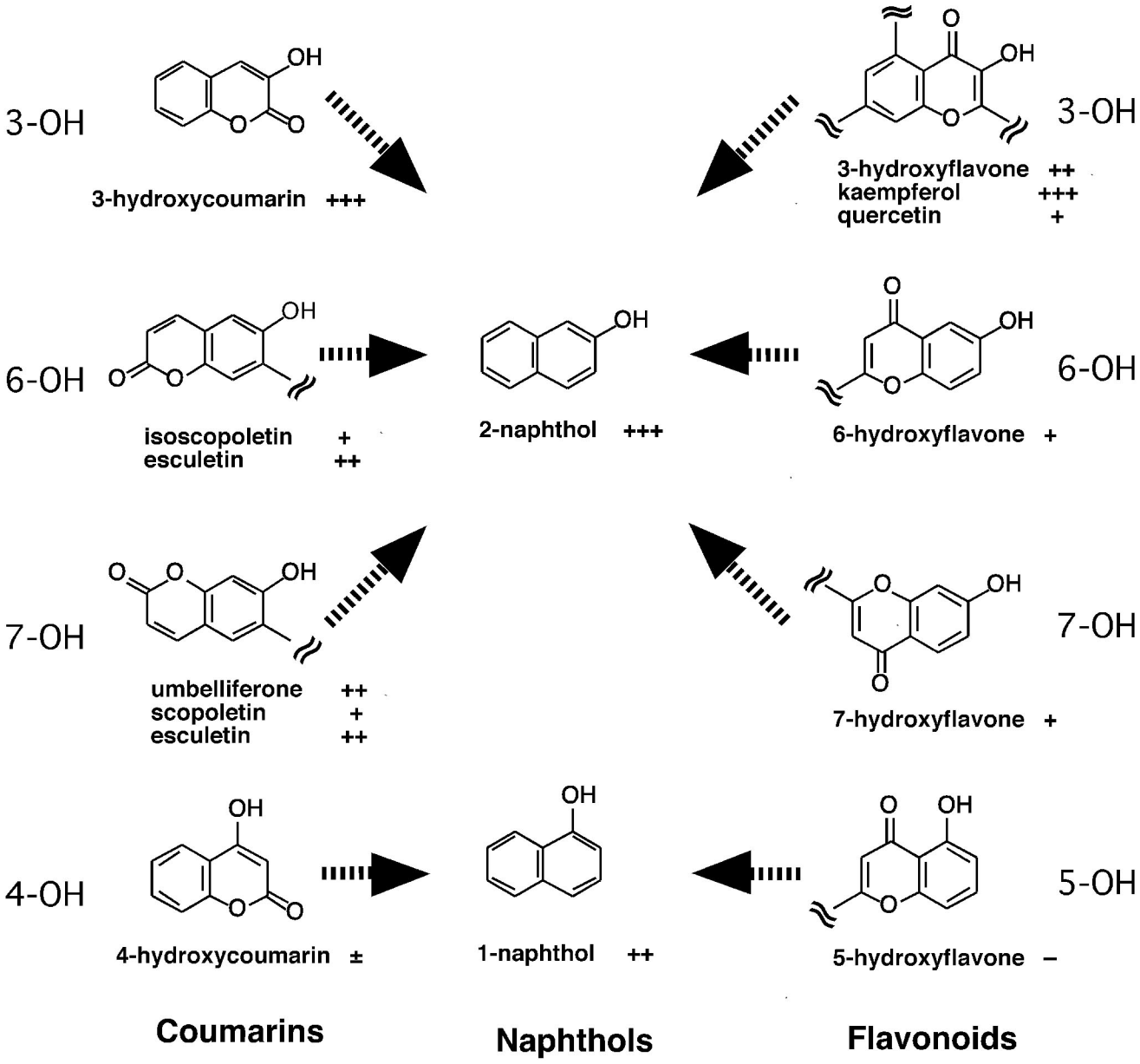


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