Title: Molecular cloning, characterization, and downregulation of an acyltransferase that catalyzes the malonylation of flavonoid and naphthol glucosides in tobacco cells.

Authors: Goro Taguchi,[1,]* Yoshihiro Shitchi,[1,2] Sakiko Shirasawa,[1,2] Hirobumi Yamamoto,[3] and Nobuaki Hayashida[1]

Addresses: [1]Division of Gene Research, Department of Life Sciences, Research Center for Human and Environmental Sciences, Shinshu University, 3-15-1 Tokida, Ueda 386-8567, Japan; [2]Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda 386-8567, Japan; [3]Plant Regulation Research Center, Faculty of Life Sciences, Toyo University, 1-1-1 Izumino, Itakura-cho 374-0193 Ora-gun, Gunma, Japan.

For correspondence: Goro Taguchi, Division of Gene Research, Department of Life Sciences, Research Center for Human and Environmental Sciences, Shinshu University, 3-15-1 Tokida, Ueda 386-8567, Japan. Tel: +81-268-21-5800, Fax: +81-268-21-5810 E-mail address: gtagtag@giptc.shinshu-u.ac.jp

E-mail addresses: Hirobumi Yamamoto, yamamoto@itakura.toyo.ac.jp; Nobuaki Hayashida, nobuaki@giptc.shinshu-u.ac.jp

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Summary

Tobacco cells (*Nicotiana tabacum* L. Bright Yellow T-13) exposed to harmful naphthols accumulate them as glucosylated and further modified compounds [Taguchi, G., Nakamura, M., Hayashida, N., Okazaki, M., (2003) *Plant Sci*. **164**, 231-240]. In this study, we identified the accumulated compounds to be 6'-*O*-malonylated glucosides of naphthols. Cells treated with various phenolic compounds accumulated the flavonoids mainly as malonylglucosides. To clarify the function of this malonylation in tobacco, we isolated the cDNA encoding a malonyltransferase (*NtMaT1*) from a cDNA library derived from tobacco cells. The heterologous expression of the gene in *Escherichia coli* revealed that the recombinant enzyme had malonyltransferase activity against several phenolic glucosides, such as flavonoid 7-*O*-glucosides, flavonoid 3-*O*-glucosides and naphthol glucosides. The substrate preference of the enzyme was similar to that of the tobacco cell extract. Malonylation activity in the transgenic cells markedly decreased with the suppression of the expression of *NtMaT1* mRNA in tobacco BY-2 cells by RNA interference. The compounds administered to the transgenic cells were accumulated in the cells as glucosides or other modified compounds in place of malonylglucosides. These results showed that NtMaT1 is the main catalyst of malonylation on glucosides of xenobiotic flavonoids and naphthols in tobacco plants.

Introduction

To cope with harmful low-molecular-mass compounds including endobiotics (e.g., secondary metabolites) and xenobiotics, plants modify them, such as by hydroxylation, glutathione conjugation, glycosylation, malonylation, and sulfonylation (Sandermann 1992, Krenz *et al.* 1996). These modifications are considered comparable to those occurring in the animal liver. Thus they are considered a plant's defense mechanism (Sandermann, 1994). Glucosylation is a common reaction in plants, which changes the solubility and reactivity of aglycons (Jones and Vogt, 2001). Glucosyltransferase genes whose products showed activity for exogenous substrates *in vitro* are present in many plants (Jones and Vogt, 2001), and glucosyltransferase activity that converts exogenous compounds to their glucosides has been found in the cell cultures of many plant species (Pflungmacher and Sandermann, 1998).

We have been investigating the accumulation systems for endo- and xenobiotic phenolic compounds using tobacco cells as a model for the chemical defense metabolism in plants. Many phenolic compounds are accumulated as glucosides in tobacco cells. We isolated glucosyltransferase genes from tobacco and characterized them as broad-substrate glucosyltransferases, which catalyze the glucosylation of flavonoids, coumarins, hydroxycinnamates, and naphthols (Taguchi *et al.*, 2001a, 2003a). The reactions of these enzymes are considered as good models of detoxification systems, because particular glucosyltransferase genes are induced after the addition of the harmful compound naphthol to tobacco cells, and proteins encoded by these genes have glucosylation activity against naphthol (Taguchi *et al.*, 2003a).

We previously reported the existence of water-soluble metabolites formed from naphthol glucoside, which are the main metabolites of naphthol, in naphthol-treated

tobacco cells, but have not reported their structures yet (Taguchi *et al*., 2003a).

In this study, we isolated and determined these metabolites as malonyl esters of naphthol glucosides. Moreover, in tobacco cells, exogenous flavonoid glucosides were also accumulated in the malonylated form. Thus, the malonylation reaction is considered as one of the detoxification systems in tobacco cells. The malonyltransferase genes associated with detoxification have not yet been identified in plants. We constructed degenerated primers from the conserved region of acyltransferases and attempted to isolate the malonyltransferase gene from a tobacco cell cDNA library by PCR-based cloning. We isolated a cDNA encoding a malonyltransferase, and we expressed the recombinant enzyme in *Escherichia coli* cells. The roles of the enzyme in phenolic-compound metabolism were also investigated by the silencing of the malonyltransferase in the cell.

Results

Metabolites of exogenously administered phenolic compounds in tobacco T-13 cells

We isolated the metabolites of 1-naphthol and 2-naphthol (compounds 1 and 2, respectively) from naphthol-treated tobacco cells using preparative HPLC. In the positive fast atomic bombardment-mass spectrometry (FAB-MS), compound 1 exhibited [M+H]$^+$ at *m/z* 393, which is higher than that of 1-naphthol 1-*O*-β-D-glucoside by *m/z* 86. Three singlets at δ 42.8, 169.6, and 168.0 in the ^{13}C NMR spectrum of compound 1 as well as two doublets at δ 3.81 and 3.76 in its ^{1}H NMR spectrum indicate that this metabolite contains a malonyl group. The chemical shift of C'-6 (δ 65.5) is lower than that of 1-naphthol glucoside (δ 62.5) by 3.0 ppm, indicating that the malonyl

group is attached at C-6 of the glucose through an ester bond. A characteristic downfield shift of H-6'a (δ 5.16) and H-6'b (δ 4.86) compared with the corresponding signals in 1-naphthol glucoside (δ 4.53 and 4.29, respectively) was also observed. Therefore, compound 1 was characterized as 1-naphthol 1-*O*-(6-*O*-malonyl-β-D-glucopyranoside) (Figure 1A). Similarly, compound 2 was characterized as 2-naphthol 2-*O*-(6-*O*-malonyl-β-D-glucopyranoside) by ^{1}H and ^{13}C-NMR and FAB-MS analyses.

To study this conjugation of phenolic compounds, we treated the tobacco T-13 cells with several flavonoids or coumarins (Figure 1B), some of which are naturally occurring in tobacco cells (Snook *et al*., 1986). The methanol extracts of the cells were analyzed by HPLC and liquid chromatography in combination with mass spectrometry (LC-MS).

The metabolites of kaempferol showed a main peak (b) and a minor peak on HPLC (Figure 2A). The main peak exhibited [M+H]⁺ at *m/z* 535, which is higher than that of kaempferol glucoside by *m/z* 86, suggesting that the main metabolite of kaempferol is malonylglucoside as in the case of naphthols. The minor peak (a) was identified as kaempferol 3-*O*-glucoside by the direct comparison with an authentic sample. Similarly, the majority of 3-hydroxyflavone or 7-hydroxyflavone was accumulated as a malonylglucoside (> 90% of the metabolite in amount), and quercetin was accumulated as malonylglucosides with further methylation (data not shown). On the other hand, the majority of coumarins tested were accumulated as simple glucosides (The result for 4-methylumbelliferone is shown in Figure 2A, peak c).

Naphthol treatment of tobacco plants

Three-week-old tobacco plants (Bright Yellow-4) grown in MS medium were treated with 2-naphthol for 24 h and the accumulated compound was analyzed by HPLC. Some of the incorporated naphthol was found as a malonylglucoside (Figure 2B, peak e) suggesting that this metabolism is not specific to cultured cells. There was another peak in the naphthol-treated plants, which was not found in T-13 cells treated with 2-naphthol (Figure 2B, peak f). The UV spectrum of the compound (λ_{max}: 226 nm, 272 nm, 310 nm and 323 nm) corresponded to that of 2-naphthol malonylglucoside and 2-naphthol glucoside, suggesting that the compound is one of the major metabolites of 2-naphthol in tobacco plants, although the structure was not determined in the present study.

Malonylation activity against several phenolic compounds in tobacco cell extract

Our first attempt to purify the malonyltransferase from tobacco T-13 cells was unsuccessful because the activity was unstable during the purification steps. However, since the activity was found to remain stable after DEAE-Sepharose chromatography, we used DEAE-purified and concentrated cell extract for the determination of malonylation activity. We examined the malonylation activity of the extract on several phenolics and their glucosides (structures of aglycons are shown in Figure 1). The substrate preference of the enzyme was as follows: flavonoid 7-*O*-glucosides > flavonoid 3-*O*-glucosides ≥ naphthol glucosides >> coumarin glucosides (Table 1). No activity was detected against their aglycons (data not shown). The peak of the product in the enzyme reaction against naphthol glucoside corresponded to the authentic compound purified from naphthol-treated tobacco cells, suggesting that the position of malonylation is the 6-hydroxyl group of glucose.

Table 1 Malonyl acceptor preference of the malonyltransferase activity in the tobacco cell extract.

Substrates	Relative activity[a]
	%
Flavonoids	
Kaempferol 7-*O*-glucoside	100
Quercetin 7-*O*-glucoside	139 ± 14
7-Hydroxyflavone glucoside	106 ± 16
Kaempferol 3-*O*-glucoside	29 ± 2.2
Quercetin 3-*O*-glucoside	17 ± 1.8
3-Hydroxyflavone glucoside	63 ± 3.3
Naphthols	
1-Naphthol glucoside	14 ± 2.2
2-Naphthol glucoside	21 ± 3.5
Coumarins	
Scopolin	0.4 ± 0.1
Umbelliferone glucoside	0.8 ± 0.1
4-Methylumbelliferone glucoside	3.4 ± 0.8

[a]For the calculation of relative activity against acyl acceptor (100 µM), the activity against kaempferol 7-*O*-glucoside was taken to be 100% in each experiment. The average ± SD of three independent experiments is indicated.

cDNA cloning of malonyltransferase from tobacco cells

For further investigation of the malonyltransferase in tobacco cells, we cloned the cDNAs of malonyltransferases from tobacco T-13 cells. PCR was performed against the cDNA library of the tobacco cells (Taguchi *et al*., 2001a) using primers that were constructed from the consensus sequences of BAHD acyltransferase (St-Pierre and De Luca 2000**)** and vector sequence. Three acyltransferase fragments were obtained, and named *Acyl1, Acyl2* and *Acyl3*. Then a full-length cDNA (*NtMaT1*) corresponding to *Acyl1* and two full-length cDNAs (*NtAcyl3a* and *NtAcyl3b*) corresponding to *Acyl3* were isolated by subsequent PCR and screening. The full-length cDNA of *Acyl2* could not be obtained. *NtMaT1* (1497 bp, Genbank Accession No. AB176525) encodes a protein (NtMaT1) of 453 amino acid residues and the calculated molecular mass of NtMaT1 was 50.8 kDa. Figure 3 shows the multiple alignment of the deduced amino acid sequences of NtMaT1 and other related enzymes. The protein showed about 30-45% identity with quercetin 3-*O*-glucoside malonyltransferases from *Verbena* and *Lamium* (Suzuki *et al*., 2004), anthocyanin-related malonyltransferases (Suzuki *et al*., 2001, 2002) and an anthocyanin hydroxycinnamoyltransferase from *Gentiana* (Fujiwara *et al*., 1998). Several homologous genes are also found in *Arabidopsis* or rice genome, suggesting that these acyltransferases are commonly distributed in higher plants. Besides the conserved region among BAHD acyltransferases, there were many conserved regions among these acyltransferases.

NtAcyl3a and 3b (1447 bp and 1438 bp, Genbank AB176527 and AB176528, respectively) encode proteins of 457 and 453 amino acid residues, respectively. The proteins showed 97% identity with each other, and they showed highest homology (32% identity) with an acetyltransferase involved in the vindoline biosynthesis in

Catharanthus roseus (AF053307, St-Pierre *et al.*, 1998) among acyltransferase genes whose functions were characterized.

Properties of recombinant enzymes expressed in *E. coli*

The cDNAs of *NtMaT1* and *NtAcyl3a* were subcloned in the pET28c(+) vector and introduced into *E. coli* BL21 (DE3) to study the properties of these enzymes. Only the recombinant enzyme of *NtMaT1* (rMaT1) showed malonyltransferase activity against naphthol glucosides. Between pH 5.5 and 9.0, the maximum enzyme reaction of rMaT1 was observed at pH 8.0-8.5 in potassium phosphate buffer. The enzyme was stable below 40 ºC and pH 8.0 for 30 min.

The substrate specificity of the rMaT1 was determined. The enzyme was incubated with several phenolic glucosides as acyl acceptors, using malonyl CoA as a malonyl donor (Table 2). The substrate preference of rMaT1 was as follows: flavonoid 7-*O*-glucosides > flavonoid 3-*O*-glucosides ≥ naphthol glucosides >> coumarin glucosides. The enzyme was inactive on aglycons of these compounds, cyanidin 3-*O*-glucoside, cyanidin 3,5-*O*-diglucoside, salicylic acid glucose ether or quercetin 3-*O*-rhamnoside (data not shown). This was consistent with the malonylation activity in the tobacco cell extract (Table 1), suggesting that NtMaT1 is one of the major catalysts of malonylation activity in tobacco cells.

Table 2 Substrate specificity of recombinant NtMaT1.

Substrates	Relative activity[a]	K_m	V_{max}	k_{cat}	k_{cat}/K_m
	%	μM	nkat mg protein^{-1}	sec^{-1}	sec^{-1} μM^{-1}
Acyl acceptor (against malonyl CoA)					
Flavonoids					
Kaempferol 7-*O*-glucoside	100	26.5 ± 3.7	216 ± 15	11.0 ± 0.8	0.41
Quercetin 7-*O*-glucoside	83± 6.5	nd[c]			
7-Hydroxyflavone glucoside	56± 6.0	nd			
Kaempferol 3-*O*-glucoside	17± 2.8	80.6 ± 2.4	134 ± 5	6.8 ± 0.2	0.08
Quercetin 3-*O*-glucoside	5.5± 2.3	nd			
3-Hydroxyflavone glucoside	40± 5.3	nd			
Naphthols					
1-Naphthol glucoside	6.3± 1.5	nd			
2-Naphthol glucoside	11± 4.0	179 ± 8.9	128 ± 18	6.5 ± 0.9	0.04
Coumarins					
Scopolin	1.2± 0.6	nd			
Umbelliferone glucoside	1.5± 0.4	nd			
4-Methylumbelliferone glucoside	4.1± 1.3	nd			
Acyl donor (against kaempferol 7-*O*-glucoside)					
Malonyl CoA	100	5.5 ± 0.3	159 ± 7	8.0 ± 0.4	1.46
Succinyl CoA	88± 5.6	26.7 ± 1.5	183 ± 4	9.3 ± 0.2	0.35
Methylmalonyl CoA	58± 5.4[b]	nd			
Acetyl CoA	<0.1	nd			

[a]For the calculation of the relative activity, the activity against kaempferol 7-*O*-glucoside (100 μM) and malonyl CoA (100 μM) was taken to be 100%. The average ± SD of three independent experiments is indicated. [b]Sum of two compounds found after the enzyme reaction. [c]nd: not determined.

Then we examined the acyl donor specificity of the recombinant enzyme. Four aliphatic acyl CoAs were tested for the enzyme reaction using kaempferol 7-*O*-glucoside as an acyl acceptor. Malonyl CoA was the best donor, but the enzyme also utilized succinyl CoA and methylmalonyl CoA (Table 2). Acetyl CoA was not utilized. The result was similar to those of studies of some aliphatic acyltransferases, which could utilize other aliphatic CoAs besides natural substrate (Nakayama *et al.*, 2003).

The kinetic parameters of the substrate of rMaT1 were determined using malonyl CoA or succinyl CoA as acyl donors, and kaempferol 7-*O*-glucoside, kaempferol 3-*O*-glucoside or 2-naphthol glucoside as acyl acceptors. K_m values were calculated based on the Lineweaver-Burk plot (Table 2). The results showed that malonyl CoA and kaempferol 7-*O*-glucoside were the most suitable substrate of rMaT1 among the substrates tested.

Plant organ-specific expression of *NtMaT1*

Total RNA was extracted from tobacco T-13 cells, seedling (2-week-old) and several organs of tobacco plant (Bright Yellow -4), and then subjected to RT-PCR analysis (Figure 4). In the cultured cells, *NtMaT1* mRNA was expressed constitutively. In the tobacco plant, *NtMaT1* mRNA was highly expressed in the flower, and was also expressed in other organs.

RNA interference of *NtMaT1* expression in tobacco cells

NtMaT1 transcript accumulation was suppressed by RNA interference (Wesley *et al.*, 2001) to confirm whether NtMaT1 is associated with xenobiotic (naphthol) metabolism

in tobacco cells. The construct yielding the hairpin-RNA of the *NtMaT1* fragment (Figure 5A) was transformed into tobacco BY-2 cells, a standard tobacco cell line (Nagata *et al*., 1992) in which malonyltransferase activity was found as in T-13 cells, because we could not transform the T-13 cells by *Agrobacterium* infection. Twenty-three transformed cell lines were obtained by kanamycin selection. Each cell line was extracted and cell extracts were assayed for malonyltransferase activity against 2-naphthol. Sixteen cell extract samples did not show malonyltransferase activity, and the other seven showed lower activity than wild-type BY-2 cells.

NtMaT1 transcript accumulation in the transformed cell lines was confirmed by RT-PCR analysis. Total RNAs were extracted from six transformed cell lines that showed no malonylation activity and from two transformed cell lines that showed low activity, and then subjected to RT-PCR. The mRNA expression level decreased in proportion to the decrease in malonyltransferase activity (Figure 5B).

The modification of administered phenolic compounds (flavonoid or naphthol) in *NtMaT1*-suppressed and wild-type BY-2 cells was compared. The malonylglucoside of these compounds found in the extract of wild-type BY-2 cells decreased to the trace level in that of *NtMaT1*-suppressed cells. In place of malonylated compounds, simple glucosides (flavonoids), or glucosides and other metabolites (naphthols) were accumulated in the *NtMaT1*-suppressed cells (Figure 5C). These results indicate that NtMaT1 catalyzes the malonylation of phenolic glucosides including xenobiotic naphthols in tobacco cells.

Discussion

Malonylated compounds are often found in plants; for example, flavonoids including anthocyanins are often accumulated in plants as their malonylglucosides (Williams and Harborne, 1994; Strack and Wray, 1994); a final metabolite of chlorophyll NCC-1 is a malonylated compound (Hörtensteiner 1998); *N*-malonic acid conjugates of D-amino acid were found in several plants (Matern *et al.*, 1984); and xenobiotics such as 2,4-dichlorophenoxyacetic acid or dichloroaniline are accumulated as malonylglucosides in plant cells (Schmitt and Sandermann, 1982; Sandermann *et al.* 1991). The roles of malonylation were proposed as follows: the stabilization of labile structures such as isoflavone or anthocyanin (Matern *et al.*, 1983b; Suzuki *et al.*, 2002); the enhancement of the solubility of target compounds in water (Heller and Forkmann, 1994); the detoxification of xenobiotic compounds (Sandermann *et al.* 1991, Sandermann 1994); and the transport of target compounds into the vacuole (Matern *et al.*, 1983b). Recently, genes encoding malonyltransferase that are associated with the biosynthesis of anthocyanins for flower colors have been isolated and characterized from several flowers (Suzuki *et al.*, 2001, 2002), but malonyltransferase genes related to the detoxification of xenobiotics have not been reported yet. In this study, we found that exogenously administered naphthols and flavonoids were accumulated as malonylglucosides in tobacco cells (Figure 2). To investigate the malonylation of phenolic glucosides, we characterized the malonyltransferase in tobacco cells.

The cell extract enzyme reacted with several types of phenolic glucosides (Table 1), suggesting that several malonyltransferases in the cells are involved in the reaction. To identify these compounds, we isolated a malonyltransferase gene *NtMaT1* from the tobacco cell, and expressed the enzyme in *E. coli.* The rMaT1 enzyme catalyzed the

malonylation of several types of phenolic glucosides, with activity similar to that of the tobacco cell extract (Tables 1 and 2). Moreover, the suppression of *NtMaT1* mRNA accumulation by RNA interference caused the disappearance of malonyltransferase activity in tobacco cells (Figure 5). These results showed that the NtMaT1 enzyme is the main catalyst of the malonylation of exogenously administered phenolics in tobacco cells.

NtMaT1 reacted with both 3-*O*-glucoside and 7-*O*-glucoside of flavonoids (Table 2). Matern *et al*. (1983a) reported the purification of flavonoid 3-*O*-glucoside malonyltransferase and flavonoid 7-*O*-glucoside malonyltransferase from parsley cells, and showed strict substrate specificity of these enzymes for the position of glucosylation on the flavonoid skeleton. Suzuki *et al*. (2001) reported that anthocyanin malonyltransferases have very high acyl acceptor specificity, especially for the position of target glucose on the anthocyanin skeleton. Compared with these malonyltransferases, NtMaT1 has a quite broader substrate specificity, which can catalyze many types of glucosides, regardless of the position of glucose conjugation to aglycon.

What is the natural substrate of NtMaT1? The enzymes for xenobiotic metabolism often show activity against endobiotics (Sandermann, 1994). The NtMaT1 enzyme strongly reacted with flavonoid 7-*O*-glucosides and malonyl CoA, and the enzyme transferred the malonyl function to the 6-hydoroxyl group of the glucose moiety of naphthol glucosides. The kinetic parameters of the rMaT1 reaction for kaempferol 7-*O*-glucoside (K_m=26.5 µM, k_{cat}=11.0 sec^{-1}) and malonyl CoA (K_m=5.5 µM, k_{cat}=8.0 sec^{-1}) are comparable to those of the reported acyltransferases such as *Dahlia* Dv3MaT (K_m=46.7 µM, k_{cat}=7.3 sec^{-1} for pelargonidin 3-*O*-glucoside, and K_m=18.8 µM, k_{cat}=7.3 sec^{-1} for malonyl CoA, Suzuki *et al*., 2002) and *Salvia* Ss5MaT (K_m=35.5 µM, k_{cat}=8.7

sec^{-1} for shisonin and K_m=21.9 μM for malonyl CoA, Suzuki *et al.*, 2001). These results suggest that NtMaT1 is a flavonoid 7-*O*-glucoside-6"-*O*-malonyltransferase. However, *NtMaT1* was strongly expressed in the cultured cells where flavonoids were not detected. Since there seemed to be some other natural substrate of the enzyme in the tobacco plant, we could not name the enzyme. Further work will be required to identify the natural substrate of the enzyme.

The modification of exogenously administered phenolic compounds in tobacco cells before their accumulation differed with the compound. Hydroxycoumarins would be accumulated as simple glucosides in the vacuole by a specific transport system (Taguchi *et al.*, 2000). In contrast, naphthols and flavonoids were accumulated as malonylglucosides (Figure 2) perhaps in the vacuole, where malonylated compounds would be accumulated (Matern *et al.*, 1983b). This difference can be explained by the substrate preference of NtMaT1 (Table 2), and it may also be related to the transport systems of these compounds. As malonyl conjugation on the glucosyl moiety causes a negative charge, it may thus correspond to the likewise negatively charged mammalian *O*-glucuronosyl conjugate (Sandermann, 1994). The malonylation of phenolic glucosides in tobacco may be necessary for the efficient transport of these compounds by anionic transporters such as MRP-type ATP-binding cassette transporter, which transport glutathione conjugates, glucuronosyl conjugates or malonyl conjugates (Lu *et al.*, 1998, Liu *et al.*, 2001). At present, we do not know the rule which compounds require further malonylation on the glucosyl moiety for accumulation. Further studies such as membrane transport analysis are required for the clarification of the function of the malonyltransferase on the accumulation of phenolic compounds.

Experimental procedures

Plant materials

Cells of T-13 habituated calli of *Nicotiana tabacum* L. cv. Bright Yellow were used and maintained as described previously (Taguchi *et al.,* 2001b). Cells of BY-2 calli of *N. tabacum* L. cv. Bright Yellow-2 were maintained as described by Nagata *et al.* (1992). Seeds of *N. tabacum* L. cv. Bright Yellow-4 provided from Leaf Tobacco Research Center, Japan Tobacco Inc. (Oyama, Japan), were sown on solidified MS medium, pH 5.8, 1% sucrose and 0.15% Gellan Gum. For the preparation of plant organs, the seedlings were transplanted to soil and cultured for 4-6 months until flowering. For the naphthol treatment, 2-week-old seedling were transplanted to liquid MS medium and cultured for a week (3-week old plants). The plants were cultured at 25ºC under 14 h light/ 10 h dark conditions.

Reagents

Kaempferol 3-*O*-glucoside was obtained from Extrasynthèse (Genay, France). Other glucosides are stocks of our laboratory (Taguchi *et al*., 2003a, 2003b). All other chemicals and solvents were obtained from Sigma-Aldrich Japan (Tokyo, Japan), Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Tokyo Kasei Industries (Tokyo, Japan) unless otherwise specified.

Determination of naphthol metabolites in tobacco cells

Tobacco cells (7 days in culture) were harvested after a 24 h treatment with 1-naphthol or 2-naphthol (final concentration 200 μM with 0.2% DMSO). The cells were extracted with methanol and the extract was purified by preparative HPLC performed

using an ODS column (7.8 mm i.d. x 300 mm: μBondapak C18, Waters, Milford, MA) with the LC10Avp system (Shimadzu, Kyoto, Japan). The column was eluted with 40% solvent A (methanol containing 0.1% formic acid) in solvent B (0.1% formic acid) for 15 min at a flow rate of 2 ml min^{-1} at 40 ºC, and the eluate was fractionated. The metabolites of 1-naphthol and 2-naphthol were detected at 285 nm and 270 nm, respectively, using a diode array detector (SPD-M10Avp, Shimadzu). The metabolites in water were extracted using ethyl acetate, and then used for the NMR analysis. The ^{1}H and ^{13}C NMR spectra were recorded in pyridine-d5 with a Varian Unity plus 500 spectrometer operating at 500 MHz. MS was recorded on a JEOL JMS DX-303 spectrometer.

1-Naphthol 1-*O*-(6-*O*-malonyl-β-D-glucopyranoside); Positive ion FAB-MS (*m/z*): 393 [M+1]$^+$. 1H NMR (500 MHz, pyridine-d5) δ : 8.64 (1H, d, J = 8.5 Hz), 7.85 (1H, dd, J = 0.7, 8.2 Hz, H-5), 7.72 - 7.68 (1H, *m*, H-3), 7.60 - 7.56 (2H, *m*, H-2, 4), 7.46 (1H, ddd, J = 1.1, 6.9, 8.0 Hz, H-7), 7.37 (1H, ddd, J = 1.1, 6.9, 8.0 Hz, H-6), 5.66 (1H, d, J = 7.8 Hz, H-1') 5.16 (1H, dd, J = 2.1, 11.7 Hz, H-6'a), 4.86 (1H, dd, J = 6.9, 11.7 Hz, H-6'b), 4.47 (1H, dd, J = 7.8, 9.2 Hz, H-2'), 4.35 (1H, dd, J = 8.6, 9.2 Hz, H-3'), 4.31 (1H, ddd, J = 2.1, 6.9, 9.6 Hz, H-5'), 4.20 (1H, dd, J = 8.9, 9.8 Hz H-4'), 3.81, 3.76 (each 1H, d, J = 15.6 Hz, malonyl-CH$_2$). ^{13}C NMR (125 MHz, pyridine-d_5)δ : 169.6, 168.0 (CO), 154.3 (C-1), 135.1 (C-10), 127.9 (C-5), 126.8 (C-6), 126.7 (C-3), 126.7 (C-7), 125.6 (C-9), 122.9 (C-8), 122.3 (C-4), 110.7 (C-2), 103.0 (C-1'), 78.5 (C-3'), 75.5 (C-5'), 75.0 (C-2'), 71.5 (C-4'), 65.5 (C-6'), 42.8 (-CH$_2$-).

2-Naphthol 2-*O*-(6-*O*-malonyl-β-D-glucopyranoside); Positive ion FAB-MS (*m/z*): 393 [M+1]$^+$. ^{1}H NMR (500 MHz, pyridine-d_5)δ: 7.98 (1H, d, J = 8.2 Hz, H-8), 7.88 (1H, d, J = 2.3 Hz, H-1), 7.84 (1H, d, J = 8.9 Hz, H-4), 7.80 (1H, d, J = 8.0 Hz, H-5), 7.51

(1H, *dd*, *J* = 2.3, 8.9 Hz, H-3), 7.45 (1H, *ddd*, *J* = 1.1, 6.9, 8.0 Hz, H-7), 7.35 (1H, *ddd*, *J* = 1.1, 6.9, 8.0 Hz, H-6), 5.69 (1H, *d*, *J* = 7.3 Hz, H-1') 5.19 (1H, *dd*, *J* = 2.1, 11.7 Hz, H-6'), 4.83 (1H, *dd*, *J* = 6.9, 11.7 Hz, H-6'), 4.39 - 4.31 (3H, *m*, H-2', 3' 4'), 4.17 (1H, *ddd*, *J* = 2.1, 6.9, 9.6 Hz, H-5'), 3.82, 3.76 (each 1H, *d*, *J* = 15.3 Hz, malonyl-CH_2). ^{13}C NMR (125 MHz, pyridine-*d$_5$*)*δ*   169.6, 168.0 (CO), 156.4 (C-2), 135.2 (C-9), 130.2 (C-4), 129.8 (C-10), 128.1 (C-5), 127.7 (C-6), 126.8 (C-8), 124.5 (C-6), 119.7 (C-9), 111.7 (C-1), 102.5 (C-1'), 78.4 (C-3'), 75.5 (C-5'), 74.9 (C-2'), 71.4 (C-4'), 65.6 (C-6'), 42.8 (-CH_2-).

Metabolism of phenolics in tobacco cells and tobacco plants

   Tobacco cells (7 days in tube-culture [Taguchi *et al*., 2001b]) or 3-week-old tobacco plants were harvested after a 24 h treatment with various phenolics (final concentration 50 μM with 0.2% DMSO). They were extracted with methanol and concentrated by evaporation for analysis by HPLC. The metabolites were confirmed as malonylated compounds from the mass spectrum by LC-MS.

Malonylation activities in tobacco cells

   Frozen cells (20 g fresh weight) were extracted with 30 ml of buffer A (50 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol) that contained 3% (w/v) polyvinylpolypyrrolidone. All subsequent operations of enzyme purification were carried out at 4 ºC unless otherwise stated. The homogenate was centrifuged for 10 min at 5,000 x g to remove all cell debris. The supernatant was loaded onto a column of DEAE-Sepharose CL-6B (2.2 i.d. x 8 cm; Amersham Bioscience, Tokyo, Japan) equilibrated with buffer A. The column was washed with 50 ml of buffer A, followed

by washing with 50 ml of buffer A containing 50 mM NaCl. Then the absorbed protein

was eluted with 50 ml of buffer A containing 150 mM NaCl. Malonylation activity was

contained in this fraction. The eluate was concentrated and the buffer was replaced with

buffer B (50 mM potassium phosphate, pH 8.0, 5 mM 2-mercaptoethanol) using

CentriconPlus 20 (Millipore, Billerica, MA), the concentrate was used as a crude

enzyme. The reaction mixture (50 µl) contained the enzyme preparation, 100 µM

substrate and 100 µM malonyl CoA in buffer B. Each reaction was initiated by adding

the substrate, and incubated at 30 ºC for 5-60 min. Reactions were terminated by adding

5 µl of 20% (w/v) trichloroacetic acid. The product was confirmed as a malonylated

compound by LC-MS, whose m/z of [M+H$^+$] was higher than that of the substrate by

m/z 86. The amount of the product was calculated from the product peak integral,

assuming that the extinction coefficient of the product (malonylglucoside) was the same

as that of substrate (glucoside) by HPLC. Protein was quantitated as described by

Bradford (1976) using BSA as the standard.

HPLC

   HPLC was performed using an ODS column (4.6 mm i.d. x 150 mm: LUNA 5u

C18(2), Phenomenex, Torrance, CA) with an LC10Avp system. For separation of

kaempferol, quercetin, 7-hydroxyflavone and naphthols, we used for elution a linear

gradient of 35% to 50% solvent A in B for 10 min, followed by 50% solvent A in B for

10 min at a flow rate of 1 ml min^{-1} at 40 ºC. For separation of coumarins, 15% to 40%

solvent A in B for 10 min, followed by 40% solvent A in B for 10 min was used. For

separation of 3-hydroxyflavone, 65% solvent A in B for 16 min was used. Glucosides

and malonylated glucosides were detected using a diode array detector (SPD-M10Avp)

by monitoring at 285 nm for 1-naphthol, 270 nm for 2-naphthol, 350 nm for 3-*O*-glucosides of kaempferol and quercetin, 365 nm for 7-*O*-glucosides of kaempferol and quercetin, 340 nm for scopoletin, 320 nm for umbelliferone and 4-methylumbelliferone, and 310 nm for hydroxyflavones, respectively.

LC-MS was performed using an Agilent 1100 LC/MSD Trap VL system (Agilent Technologies, Santa Clara, CA) with an electron-splay ionization probe. Samples were eluted from an ODS column (2.1 mm i.d. x 150 mm: 20RBAX Eclipse XDB-C18, Agilent) using 40% or 50% solvent A in B at a flow rate of 0.2 ml min^{-1}.

Cloning and sequencing of acyltransferase genes

The cDNA library of tobacco (T-13 callus) was constructed previously (Taguchi *et al.*, 2001a). The conserved regions of acyltransferases were compared and the degenerated primers (ACYL1: 5'-ARYTACTTYGGSAAYTG-3' ACYL2: 5'-GATTTTGGATGGGGAAAG-3') were constructed. The fragments containing partial-length acyltransferase genes were amplified from the cDNA library (2.0 X 10^6 plaque-forming units) with ACYL2 and the vector sequence (M13 primer M4, Takara Bio, Kusatsu, Japan) using *Ex Taq* (Takara Bio) under the following conditions: 95 ºC for 5 min, 35 cycles of 95 ºC for 30 sec, 50 ºC for 1 min, and 72 ºC for 1 min, and followed by 72 ºC for 5 min. The amplified PCR fragment was cloned into the pT7Blue vector (Promega, Madison, WI). The fragments containing the full-length *NtMaT1* was amplified from the cDNA library by nested PCR with two sets of primers, NtAcyl1-rva: 5'-TTAACCAGGCTTGCTACAGA-3' and the vector sequence (M13 primer RV, Takara Bio), and NtAcyl1-rvb: 5'-TGCTACAGAAAGCTAATCCCGTG-3' and the vector sequence (T3-pro: 5'-AATTAACCCTCACTAAAGGG-3'). The fragments

containing the full-length *NtAcyl3* was amplified with two sets of primers, NtAcyl3-rva:

5'-CTCAACATCAAAACGAAAGGAC-3' and M13 primer RV, and NtAcyl3-rvb:

5'-GGAAGCAAAATAAACACACAGA-3' and T3-pro. To avoid PCR errors, we

excised the library *in vivo* and transformed into *E. coli* JM109, and 10,000 independent

colonies were screened by colony PCR analysis. The cDNAs were sequenced using a

DNA sequencer (Genetic Analyzer 310, Applied Biosystems, Foster City, CA).

Expression of recombinant NtMaT1 in *E. coli*

The fragment containing the coding region of *NtMaT1* or *NtAcyl3a* was then

subcloned into the *Bam*HI/*Xba*I site of the pET28c(+) expression vector (Merck,

Darmstadt, Germany). The recombinant protein of NtMaT1 was expressed in *E. coli*

BL21 (DE3) and extracted in accordance with the manufacturer's instruction using 100

mM potassium phosphate (pH 8.0) containing 5 mM 2-mercaptoethanol as an extraction

buffer. The recombinant enzyme was purified using nickel-equilibrated iminodiacetic

acid-Sepharose 6B (Amersham Bioscience) in accordance with the manufacturer's

instruction, and then the solution was concentrated and the buffer was replaced with

buffer B as described above.

Enzyme reaction of recombinant NtMaT1

Affinity-purified enzymes were used to determine substrate specificity and enzymatic

parameters. For the determination of the K_m values of the substrates, the concentrations

of kaempferol glucosides were varied from 1 to 50 µM, those of naphthol glucosides

from 20 to 1000 µM, at a fixed malonyl CoA concentration of 100 µM. For the

determination of K_m values, the concentration of malonyl CoA was varied from 0.5 to

100 μM at a fixed kaempferol 7-*O*-glucoside concentration of 50 μM. The enzymatic

reaction was performed in 100 μl of buffer B containing 0.01% BSA, substrates and the

recombinant enzyme. After incubation for 5 min at 30 °C, the reaction was stopped by

adding 10 μl of 1M HCl, and the reaction mixture was subjected to HPLC as described

above.

Detection of *NtMaT1* expression in tobacco by RT-PCR analysis

    Total RNA from tobacco cells (7 days in culture) or tobacco plant was extracted as

described previously (Taguchi *et al.*, 2003a). These total RNA samples (0.5 μg each)

were used for cDNA synthesis by reverse-transcription (RT) in a 20 μl reaction mixture

using RevaTra Ace (TOYOBO, Tokyo, Japan) with an oligo-dT primer at 50 °C for 60

min. One microliter of a cDNA sample from the RT reaction was used for PCR using

Ex Taq under the following conditions: 95 °C for 5 min, 25 cycles of 95 °C for 30 sec,

50 °C for 1 min, and 72 °C for 1 min, and followed by a final extension at 72 °C for 7

min with the primers of NtMaT1-616f 5'- CCGTAATCAAAGACCCTA -3' and

NtMaT1-896r 5'- CATTCCATTCTCGTCGAT -3' for the 281-bp fragment of

NtMaT1, Act-fw 5'- GATTGGAATGGAAGCTG -3' and Act-rv 5'-

CCTCCAATCCAAACACT -3' for the 231-bp fragment of the consensus region from

tobacco actin (Taguchi *et al.*, 2003a). The PCR product was analyzed by electrophoresis

in 1.5% agarose gel.

Construction of a vector for RNA interference and transformation of tobacco BY-2 cells

    Two fragments of *NtMaT1* were amplified by PCR using MaT1-BamHI-fw 5'-

CGGATCCATATGGCATCTGTGATTGA -3' and MaT1-rv 5'-

TTAACCAGGCTTGCTACAGA -3', or MaT1-KpnI-fw 5'-

CGGGTACCATGGCATCTGTGATTGA-3' and MaT1-592n-EcoRI 5'-

GGAATTCAGCGACGTGATGGTTAG -3', then the fragments were cloned into

pBluescript SK$^+$ (Stratagene, La Jolla, CA). The fragments were connected at the *Eco*RI

site, and then cloned into a binary vector pSMAK251 instead of the β-glucuronidase

gene (pSMAK-MaT1-RI, Figure 5A). The vector pSMAK251 was a kind gift from Dr.

H. Ichikawa, National Institute of Agrobiological Sciences, Tsukuba, Japan. The vector

was introduced in *Agrobacterium tumefeciens* LBA4404 (Clontech). The tobacco BY-2

cells were transformed according to An (1987), and then kanamycin (100 mg

l^{-1})-tolerant cells were selected. The transformed cells were cultured in liquid medium

for BY-2 (Nagata *et al*., 1992) containing kanamycin (100 mg l^{-1}), and then used for the

extraction of crude enzyme and RNA.

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Figure legends

Figure 1. Structures of phenolic compounds used in this study.

(A) The reactions of malonyltransferase against 1-naphthol glucoside and 2-naphthol glucoside.

(B) Structures of aglycons of phenolic compounds. 1, 1-naphthol; 2, 2-naphthol; 3, scopoletin; 4, umbelliferone; 5, 4-methylumbelliferone; 6, 3-hydroxyflavone; 7, 7-hydroxyflavone; 8, kaempferol; 9, quercetin.

Figure 2 HPLC of methanol extract of tobacco cells or 3-week-old plants treated with phenolic compounds.

Methanol extracts were prepared from tobacco cells (7 days in culture) treated for 24 h with 50 μM kaempferol, 4-methylumbelliferone, or 2-naphthol, and tobacco 3-week-old plants (grown in MS medium) treated for 24 h with 50 μM 2-naphthol. HPLC was performed in a linear gradient system for kaempferol and naphthol as described in Experimental procedures. The eluate was monitored by a photodiode array detector (absorbance at 350, 320 and 270 nm, for kaempferol, 4-methylumbelliferone and 2-naphthol, respectively) as described in Experimental procedures.

(A) HPLC of methanol extract from T-13 cells treated with kaempferol (A1), 4-methylumbelliferone (A2). Peak identification: a, kaempferol 3-*O*-glucoside; b, kaempferol malonylglucoside; c, 4-methylumbelliferone glucoside; d, 4-methylumbelliferone malonylglucoside.

(B) Methanol extracts from tobacco T-13 cells (B1) or a plant (B2) treated with 2-naphthol. Peak identification: e, 2-naphthol malonylglucoside; f, undefined metabolite of 2-naphthol; g, 2-naphthol glucoside.

Figure 3 Multiple alignment of the deduced amino acid sequence of NtMaT1 and related enzymes.

Alignment was calculated using ClustalX (Jeanmougin *et al*., 1998). Black shading shows the identical amino acids in at least four sequences. Motifs 1-3 are the region conserved among BAHD acyltransferases (St-Pierre and De Luca, 2000, Nakayama *et al*., 2003). Abbreviations and Genbank accession nos. are: NtMaT1, *Nicotiana tabacum* phenolic glucoside-6'-*O*-malonyltransferase with broad substrate specificity (AB176525), Vh3MaT1 and Lp3MaT, quercetin 3-*O*-glucose 6"-*O*-malonyltransferases from *Verbena hybrida* (AY500350) and *Lamium purpureum* (AY500352); SsA5MaT and PfA5MaT, anthocyanin 5-*O*-glucose 6'''-*O*-malonyltransferases from *Salvia splendens* (AF405707) and *Perilla frutescens* (AF405204); GtArT, anthocyanin 5-*O*-glucose 6'''-*O*-hydroxycinnamoyltransferase from *Gentiana triflora* (AB010708); DvA3MaT, anthocyanin 3-*O*-glucose 6"-*O*-malonyltransferase from *Dahlia valiabilis* (AF489108); AtATLP, acyltransferase-like protein At5g39090 from *Arabidopsis thaliana* (AY099805); OsATLP, acyltransferase-like protein from *Oryza sativa* (XM_465684).

Figure 4 Analysis of expression level of *NtMaT1* mRNA by RT-PCR.

   Total RNAs were extracted from a tobacco plant (Bright Yellow-4) and T-13 cells, then subjected to RT-PCR as described in "Experimental Procedures". A 281-bp cDNA fragment was expected for *NtMaT1* mRNA, 231-bp for actin mRNA. Abbreviations are: M, 200 bp marker (Takara Bio), 1, seedlings (2-week-old); 2, stem; 3, leaf; 4, root; 5, flower; 6, flower bud; 7, T-13 cell.
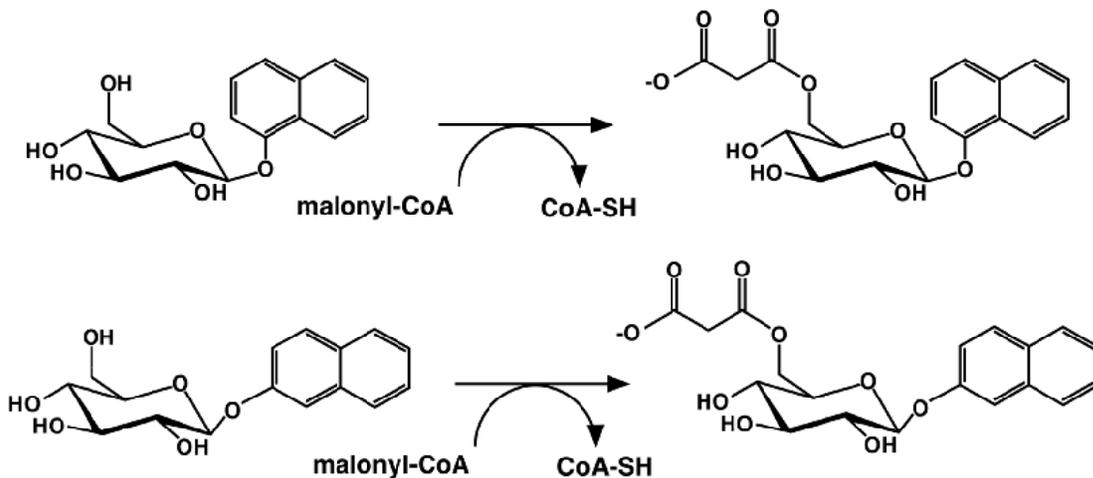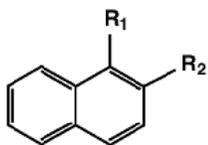
Figure 5 RNA interference of *NtMaT1* expression in tobacco cells.

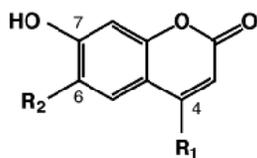(A) Map between left border (LB) and right border (RB) of the plasmid (pSMAK-MaT1-RI) used for transformation. Gray boxes indicate the invert repeat of *NtMaT1* sequence. Abbreviations are: Pnos and Tnos, promoter and terminator of nopaline synthase; NPTII, neomycin phosphotransferase II; P35S, cauliflower mosaic virus 35S promoter; TRbcS, terminator of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit.

 (B) The effect of RNA interference of *NtMaT1*. Accumulation of *NtMaT1* transcript was confirmed by RT-PCR as described in Figure 4 (The cycles of PCR were changed to 28 for *NtMaT1*). Malonyltransferase activity in each transformed cell line is shown relative to that in wild-type (nontransformed) BY-2 cells (7.3 pkat (mg protein)$^{-1}$). 2-Naphthol was used as a substrate. Abbreviations: WT, wild-type BY-2 cells; -, less than 5% activity of WT.

 (C) HPLC of methanol extract of transformed (RI-23) and wild-type (WT) BY-2 cells treated with kaempferol or 2-naphthol. Peak identification: a, kaempferol 3-*O*-glucoside; b, kaempferol malonylglucoside; e, 2-naphthol malonylglucoside; g, 2-naphthol glucoside. Arrowheads indicated the 2-naphthol-related undetermined metabolites.
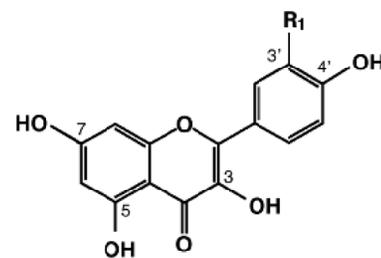
Figure 1. Structures of phenolic compounds used in this study.
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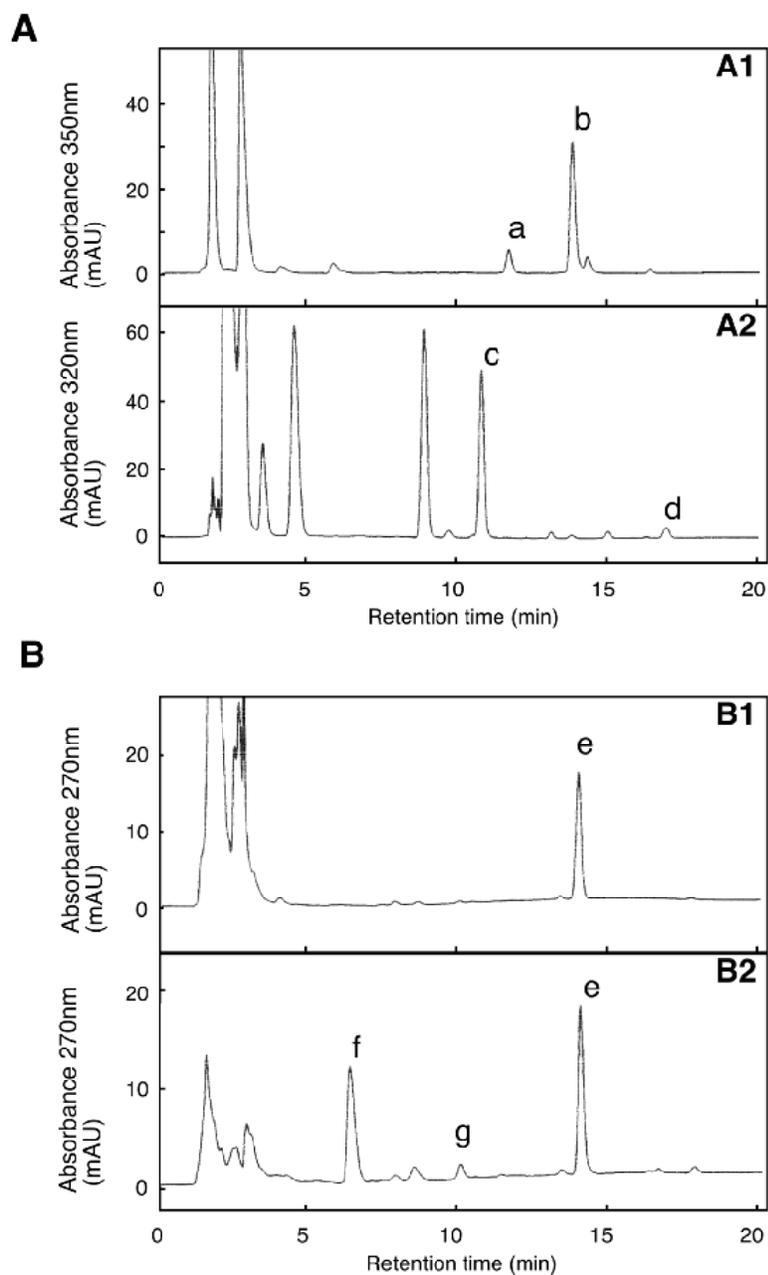8, kaempferol; 9, quercetin.

Figure 2 HPLC of methanol extract of tobacco cells or 3-week-old plants treated with phenolic compounds.
Methanol extracts were prepared from tobacco cells (7 days in culture) treated for 24 h with 50 μM kaempferol, 4-methylumbelliferone, or 2-naphthol, and tobacco 3- week-old plants (grown in MS medium) treated for 24 h with 50 μM 2-naphthol. HPLC was performed in a linear gradient system for kaempferol and naphthol as described in Experimental procedures. The eluate was monitored by a photodiode array detector (absorbance at 350, 320 and 270 nm, for kaempferol, 4-methylumbelliferone and 2-naphthol, respectively) as described in Experimental procedures.
(A) HPLC of methanol extract from T-13 cells treated with kaempferol (A1), 4-methylumbelliferone (A2). Peak identification: a, kaempferol 3-O-glucoside; b, kaempferol malonylglucoside; c, 4-methylumbelliferone glucoside; d, 4-methylumbelliferone malonylglucoside.
(B) Methanol extracts from tobacco T-13 cells (B1) or a plant (B2) treated with 2-naphthol. Peak identification: e, 2-naphthol malonylglucoside; f, undefined metabolite of 2-naphthol; g, 2-naphthol glucoside.
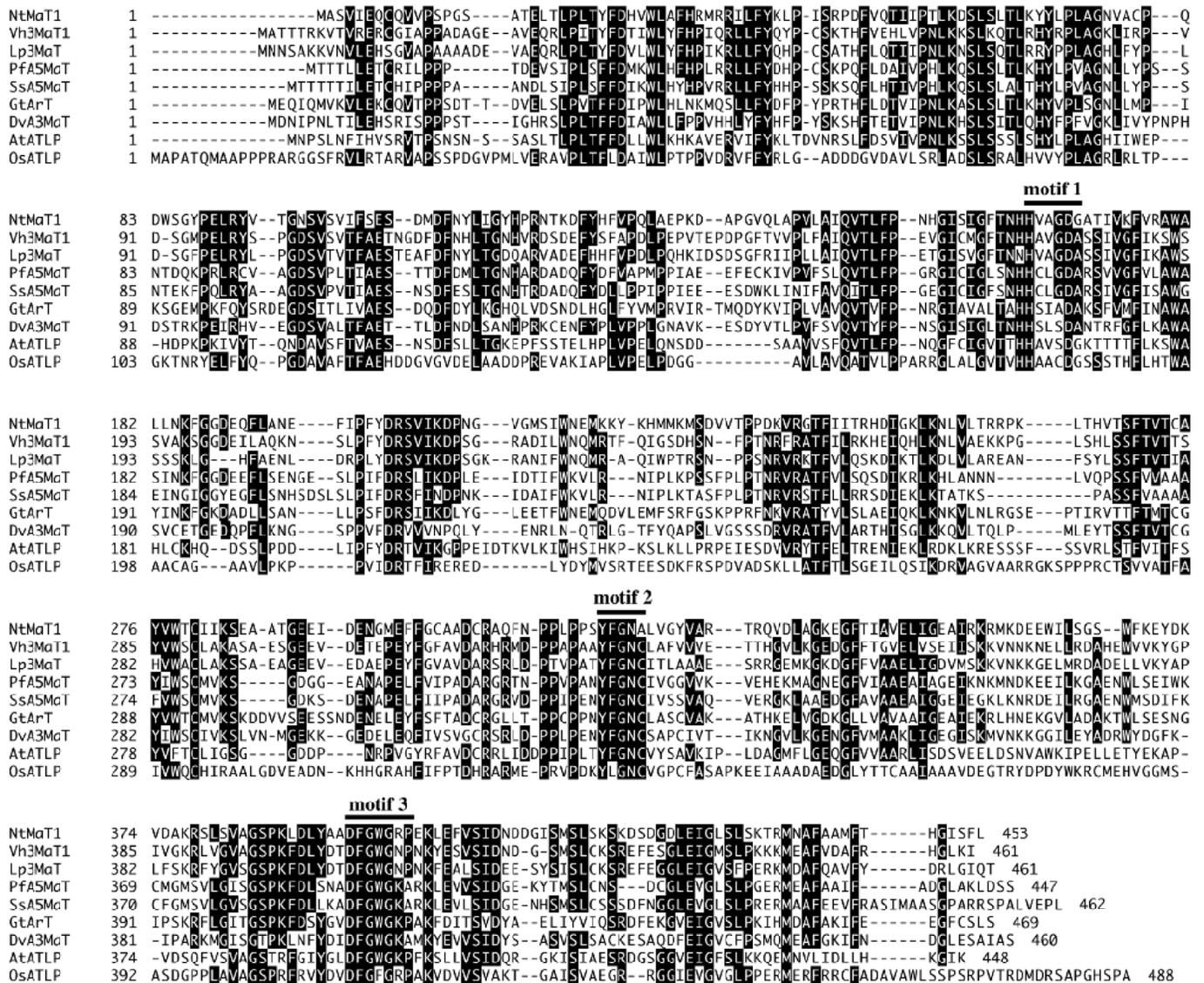
Figure 3 Multiple alignment of the deduced amino acid sequence of NtMaT1 and related enzymes. Alignment was calculated using ClustalX (Jeanmougin et al., 1998). Black shading shows the identical amino acids in at least four sequences. Motifs 1-3 are the region conserved among BAHD acyltransferases (St-Pierre and De Luca, 2000, Nakayama et al., 2003). Abbreviations and Genbank accession nos. are: NtMaT1, Nicotiana tabacum phenolic glucoside-6'-O-malonyltransferase with broad substrate specificity (AB176525), Vh3MaT1 and Lp3MaT, quercetin 3-O-glucose 6"-O-malonyltransferases from Verbena hybrida (AY500350) and Lamium purpureum (AY500352); SsA5MaT and PfA5MaT, anthocyanin 5-O-glucose 6'''-O-malonyltransferases from Salvia splendens (AF405707) and Perilla frutescens (AF405204); GtArT, anthocyanin 5-O-glucose 6'''-O-hydroxycinnamoyltransferase from Gentiana triflora (AB010708); DvA3MaT, anthocyanin 3-O-glucose 6"-O-malonyltransferase from Dahlia valiabilis (AF489108); AtATLP, acyltransferase-like protein At5g39090 from Arabidopsis thaliana (AY099805); OsATLP, acyltransferase-like protein from Oryza sativa (XM_465684).
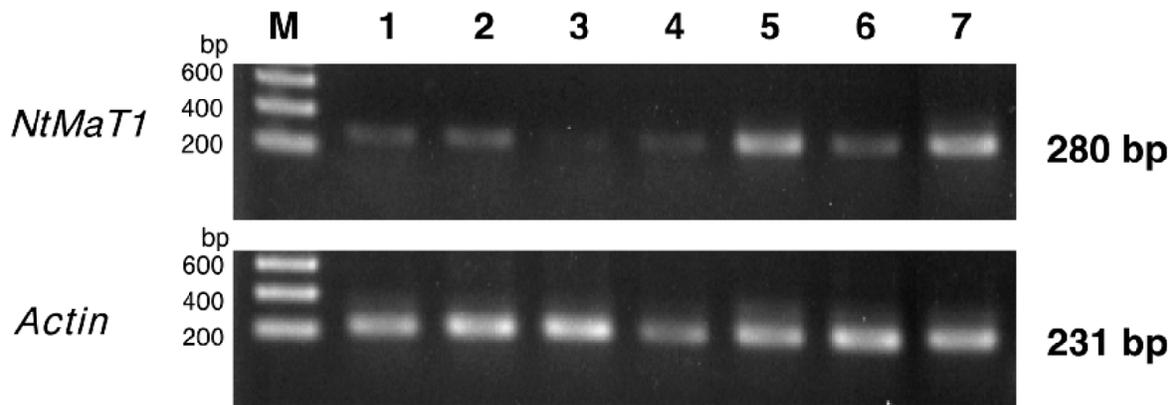
Figure 4 Analysis of expression level of NtMaT1 mRNA by RT-PCR.
Total RNAs were extracted from a tobacco plant (Bright Yellow-4) and T-13 cells, then subjected to RT-PCR as described in "Experimental Procedures". A 281-bp cDNA fragment was expected for NtMaT1 mRNA, 231-bp for actin mRNA. Abbreviations are: M, 200 bp marker (Takara Bio), 1, seedlings (2-week-old); 2, stem; 3, leaf; 4, root; 5, flower; 6, flower bud; 7, T-13 cell.
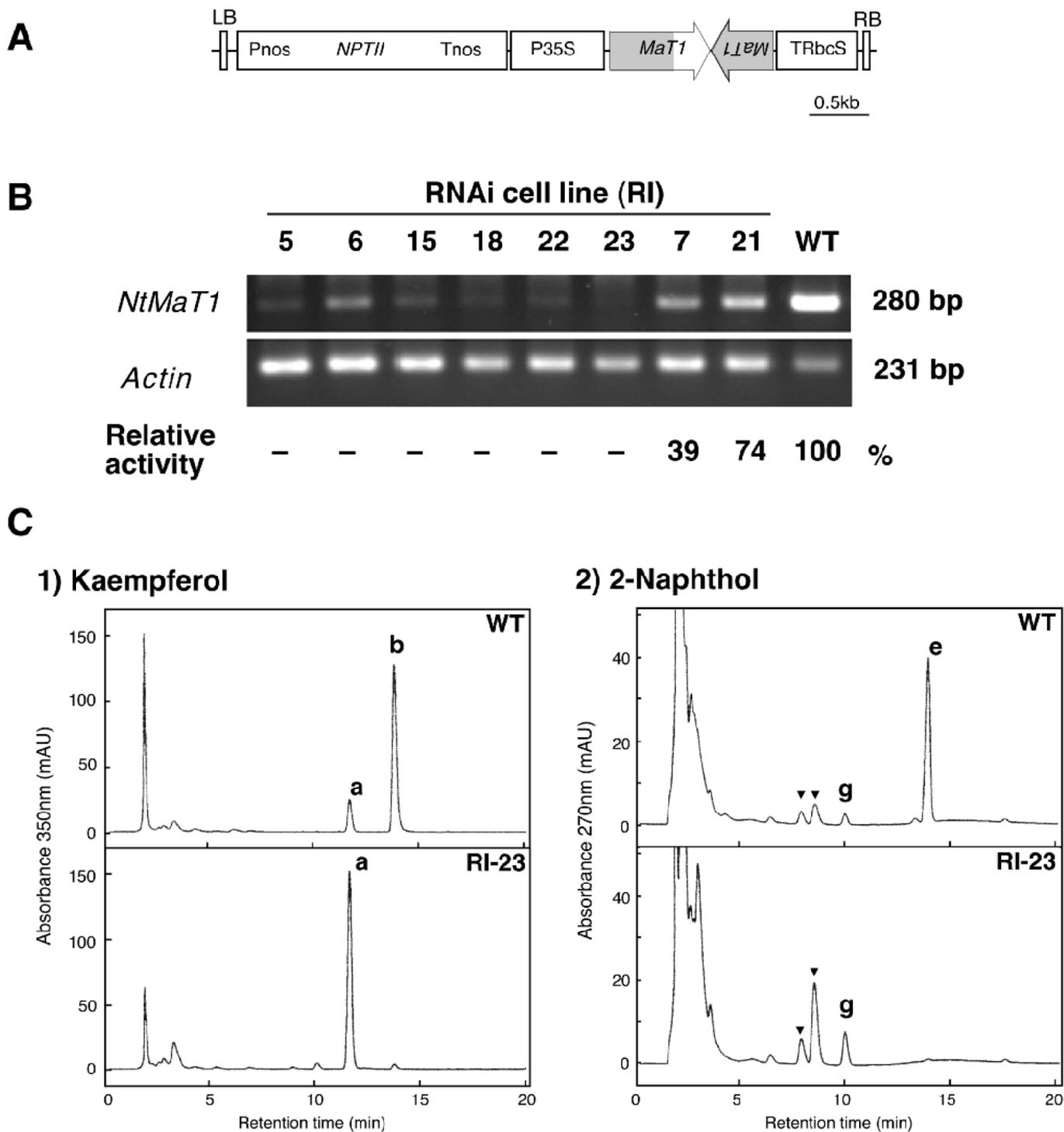
Figure 5 RNA interference of NtMaT1 expression in tobacco cells.
(A) Map between left border (LB) and right border (RB) of the plasmid (pSMAK-MaT1-RI) used for transformation. Gray boxes indicate the invert repeat of NtMaT1 sequence. Abbreviations are: Pnos and Tnos, promoter and terminator of nopaline synthase; NPTII, neomycin phosphotransferase II; P35S, cauliflower mosaic virus 35S promoter; TRbcS, terminator of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit.
(B) The effect of RNA interference of NtMaT1. Accumulation of NtMaT1 transcript was confirmed by RT-PCR as described in Figure 4 (The cycles of PCR were changed to 28 for NtMaT1). Malonyltransferase activity in each transformed cell line is shown relative to that in wild-type (nontransformed) BY-2 cells (7.3 pkat (mg protein)-1).2-Naphthol was used as a substrate. Abbreviations: WT, wild-type BY-2 cells; -, less than 5% activity of WT.
(C) HPLC of methanol extract of transformed (RI-23) and wild-type (WT) BY-2 cells treated with kaempferol or 2-naphthol. Peak identification: a, kaempferol 3-O-glucoside; b, kaempferol malonylglucoside; e, 2-naphthol malonylglucoside; g, 2-naphthol glucoside. Arrowheads indicated the 2-naphthol-related undetermined metabolites.