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Original Paper

Molecular and functional characterization of novel furofuran-class lignan glucosyltransferases from *Forsythia*

Eiichiro Ono[1,*], Hyun Jung Kim[2], Jun Murata[2], Kinuyo Morimoto[2], Atsushi Okazawa[3], Akio Kobayashi[3], Toshiaki Umezawa[4], Honoo Satake[2]

[1] Core Research Group, R&D Planning Division, Suntory Holdings Ltd., Suntory Research Center, Mishima, Osaka 618-8503, Japan; [2] Suntory Institute for Bioorganic Research, Mishima, Osaka, 618-8503 Japan; [3] Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Osaka 565-0871, Japan; [4] Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan

* E-mail: Eiichiro_Ono@suntory.co.jp Tel: +81-75-962-2244 Fax: +81-75-962-3791

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Abstract Lignan is a large class of plant secondary metabolites, which has long attracted pharmacological interest because of its anti-tumor and estrogenic activities. *Forsythia* plants are known to produce a wide variety of lignans, such as (−)-matairesinol, (−)-secoisolariciresinol, (+)-pinoresinol, and (+)-phillygenin. The majority of such lignans are accumulated in glucoside forms. However, their glucosylation mechanisms largely remain to be elucidated. Here we describe the sequence, enzymatic activities, and gene expression profiles of UDP-sugar dependent-glycosyltransferases (UGT) from *Forsythia koreana* through a reverse-genetic approach. A *Forsythia* UGT, UGT71A18 protein, expressed in *E. coli*, preferentially glucosylated furofuran-class lignans, including (+)-pinoreisnol, (+)-epipinoreisnol, and (+)-phylligenin. Moroeover, the recombinant UGT71A18 exhibited specificity to UDP-glucose as a glycosyl donor. Gene expression analysis revealed that *UGT71A18* is expressed predominantly in leaves and the suspension cell culture of *F. koreana*, and that the *UGT71A18* transcript is upregulated in the transgenic cell culture expressing the RNAi construct of the *pinoresinol lariciresinol reductase* (PLR) gene, compared to non-transformants. These results are consistent with the remarkable elevation of pinoresinol glucosides in the *PLR*-RNAi lines. Collectively, the present data strongly suggests that UGT71A18, in part, is responsible for glucosylation of furofuran-class lignans, including (+)-pinoresinol and/or structurally related lignans *in vivo*.

Key words: *Forsythia*, glucosylation, lignan, secondary metabolism, UGT.

Lignan is a major class of secondary metabolites in plants, and are known for their beneficial biological effects such as their antioxidative, anti-bacterial, anti-fungal, and anti-viral properties (Ayres and Loike 1990; Apers et al. 2003). These findings provoked the idea that lignans are involved in plant defense response and function as preformed phytoalexins. For example, constitutive deposition of lignans in heartwood is believed to confer durability, longevity and resistance against wood-rotting fungi (Gang et al. 1999). Lignans occur in various plants, including *Thymelaeaceae, Asteraceae, Pedaliaceae* and *Oleaceae* families. However, their physiological functions remain largely unknown.

Forsythia koreana and its related species, which belong to the *Oleaceae* family, possess a considerable amount of lignans (Tokar and Klimek 2004; Umezawa 2003). In *Forsythia* plants, pinoresinol, epipinoresinol, phillygenin, matairesinol, arctigenin and their respective glycosides (pinoresinol 4-*O*-glucoside, epipinoresinol 4-*O*-glucoside, phillyrin, matairesinoside, arctiin) are accumulated almost throughout the entire plant body, as well as in cell suspension cultures (Guo et al. 2007; Kitagawa et al. 1984; Kitagawa et al. 1988; Nishibe et al. 1988; Piao et al. 2008; Rahman et al. 1986; Rahman et al. 1990a, 1990b, 1990c; Schmitt and Petersen 2002a; 2002b; Tokar and Klimek 2004). The biosynthetic pathway of *Forsythia* major lignan aglycones has been well characterized (Figure 1). (+)-Pinoresinol is synthesized by stereo-specific radical coupling of two coniferyl alcohols in the presence of dirigent protein (DIR), and then is converted to (−)-secoisolariciresinol

Abbreviations: DIR, dirigent protein; OMT, *O*-methyltransferases; PIP, Pinoresinol-lariciresinol/Isoflavone/Phenylcoumaran benzylic ether reductase; PLR, pinoresinol lariciresinol reductase; PSS, (+)-piperitol/sesamin synthase; SIRD, secosisolariciresinol dehydrogenase; UGT, UDP-sugar dependent glycosyltransferase; UDP, uridine diphosphate

Footnotes: UGT72B13 (Accession AB524715), UGT88A10 (AB524716), UGT71A17 (AB524717), UGT71A18 (AB524718), UGT71A19 (AB524719), UGT71A21 (AB524720)

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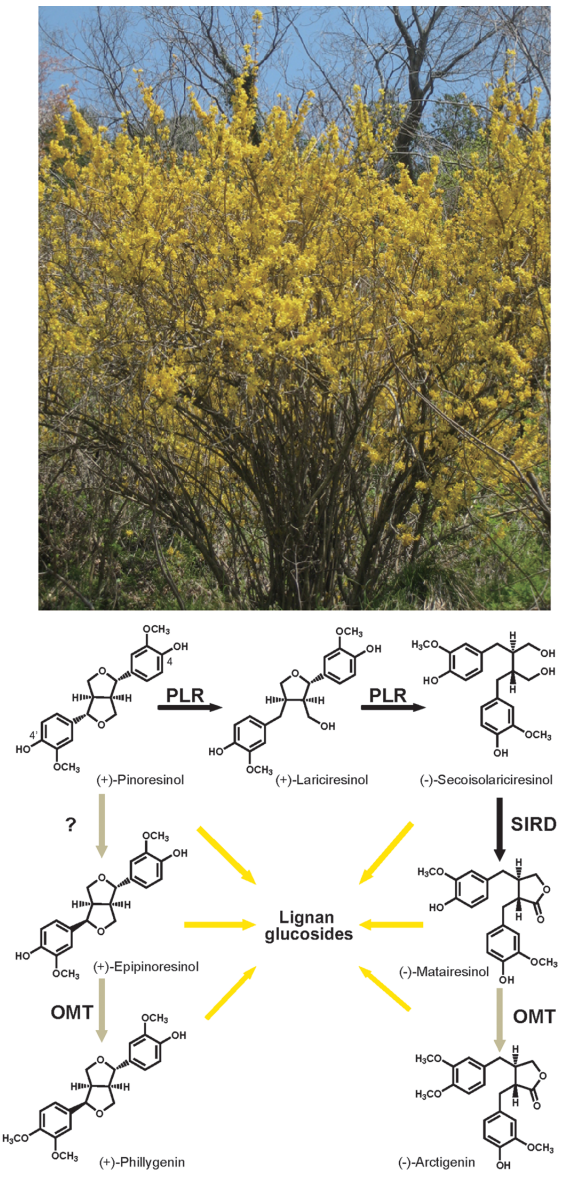

Figure 1. *Lignan biosynthetic pathways in Forsythia.* A *Forsythia* plant in bloom (Upper). Lignan biosynthetic pathways (Lower). Gray arrows indicate that the enzymes catalyzing the step have not been determined yet. Yellow arrows indicate the possible glucosylating steps in *Forsythia*. PLR, pinoresinol/lariciresinol reductase; SIRD, secosisolariciresinol dehydrogenase; OMT, putative *O*-methyltransferase.

via (+)-lariciresinol by pinoresinol/lariciresinol reductase (PLR), a member of the Pinoresinol-lariciresinol/Isoflavone/Phenylcoumaran benzylic ether reductase (PIP) family, in an enantio-selective manner (Davin et al. 1997; Dinkova-Kostova et al. 1996; Katayama et al. 1992). (−)-Secoisolariciresinol is further converted to (−)-matairesinol by secoisolariciresinol dehydrogenase (SIRD) (Davin and Lewis 2003; Umezawa et al. 1991; Xia et al. 2001). Moreover, a monomethylated epipinoresinol, (+)-phillygein, and a monomethylated matairesinol, (−)-arctigenin are also present, suggesting that *O*-methyltransferases (OMT) are involved in the lignan biosynthesis in *Forsythia* plants (Ozawa et al. 1993). Notably, a majority of these lignans is glycosylated at their phenolic hydroxyl groups (Kitagawa et al. 1984; Nishibe et al. 1988; Rahman et al. 1986, 1990b, 1990c; Tokar and Klimek 2004). Pinoresinol and matairesinol, the two major lignans in the cell suspension culture of *F. intermedia and F. koreana* respectively, were shown to be predominantly present as glucoside forms (Kim et al. 2009; Schmitt and Petersen 2002a).

Diverse lignan glycosides with various different glycoylation patterns have been observed in nature (Ayres and Loike 1990). Glycosylation is generally catalyzed by a superfamily of enzymes, the uridine diphosphate (UDP)-sugar dependent glycosyltransferases (UGT). UGT transfers a sugar moiety from an activated donor (UDP-sugar) to an accepting substrate. However, molecular basis for the glycosylation of lignans was not well understood until we recently identified UGT71A9 as the first lignan UGT from *Sesamum indicum* (Noguchi et al. 2008). UGT71A9 specifically glucosylates a furofuran-class of lignan, (+)-sesaminol. In addition, the glucosylating activity of an anti-tumor lignan, podophyllotoxin, was also shown to be dependent on UDP-glucose in *Linum nodiflorum* suspension cell cultures (Berim et al. 2008). These findings suggest that UGTs play crucial roles in lignan glycosylation, although very few lignan UGTs have been thus far identified.

In this study, we present the molecular and functional characterization of novel *Forsythia* UGTs. In particular, the results herein not only provide evidence that UGT71A18 of *Forsythia* is a novel enzyme responsible for glucosylation of (+)-pinoreisnol, (+)-epipinoreisnol, and (+)-phillygenin *in vivo*, but also pave the way to metabolic engineering of lignan biosynthesis .

Materials and methods

Plant and chemical materials

Forsythia koreana was originally grown at the Research Institute of Sustainable Humanosphere, Kyoto University, and transferred to the greenhouse facility of the Suntory Co. Ltd. Cell suspension culture was prepared from the callus and maintained as previously described (Kim et al. 2009). Lignans used for enzymatic assays were prepared as previously described (Kim et al. 2009; Noguchi et al. 2008).

Molecular cloning of Forsythia UGT genes

An RNeasy Plant Mini Kit (QIAGEN) was used to extract total RNA from leaves and flowers of *Forsythia koreana*. Poly A(+) RNA was obtained from total RNA using an oligotex-MAG mRNA purification kit (TaKaRa Bio, Shiga, Japan). A cDNA library was constructed with 5 μg of poly A(+) RNA by means of a ZAP Express cDNA Synthesis Kit and ZAP Express cDNA Gigapack3 Gold Cloning Kit (Stratagene, CA). This

library had a titer of 2.4×10^6 pfu ml^{-1}. The *Sesamum indicum* (+)-sesaminol glucosyltransferase gene (*UGT71A9*, Accession AB293960) was used for the screening probes (Noguchi et al. 2008), and the full length of *UGT71A9* gene was DIG-labeled by PCR using the following primers (UGT71A9-Fw: 5′-ATG TCG GCG GAC CAA AAA TTA ACC A and UGT71A9-Rv: 5′-TCA AGA AAT GTT ATT CAC GAC ATT) according to the procedure previously described in Ono et al. (2006). Approximately 400,000 pfu of the cDNA library was screened with the DIG-labeled UGT71A9 probe. The screening and detection of positive clones were performed with a DIG-DNA labeling & detection kit (Roche, Mannheim, Germany). Positive clones were detected under low stringency hybridization conditions, as described previously (Yonekura-Sakakibara et al. 2000). After the second screening, positive clones were excised into the pBK-CMV plasmid (Stratagene, CA), and the nucleotide sequences were determined. Sequencing reactions were conducted with a BigDye-terminator ver.3.1 cycle sequencing kit (Applied Biosystems, CA). Subsequently, the sequencing reaction mixtures were analyzed in a 3100 Genetic Analyzer (Applied Biosystems, CA).

Preparation of recombinant F. koreana UGT71A proteins

The open reading frame of the *UGT71A18* and *UGT71A17* genes was amplified from each pBK-CMV plasmid containing *UGT71A18* and *UGT71A17* cDNA, respectively, using the following primer set (NdeI-FkUGT71A-Fw: 5′-CAC CCA TAT GGC AGA AAC AAA GAA ATC AGA and BglII-FkUGT71A-Rv: 5′-AGA TCT TTA ATC CGT CAT TGG AAT GTT AT), and was subcloned into a pENTR-Directional-TOPO vector (Invitrogen, CA) and sequenced to confirm the absence of PCR errors. The plasmid of the *UGT71A18* or *UGT71A17* cDNA was digested using NdeI and BglII. The resulting DNA fragment was ligated with a pET-15b vector (Novagen, Darmstadt, Germany) that had previously been digested with NdeI and BamHI. The resultant plasmid was transformed into *Escherichia coli* BL21 (DE3). The transformant cells were prepared as previously described in Noguchi et al. 2008. The recombinant *E. coli* cells were harvested by centrifugation (7,000×*g*, 15 min), washed with distilled water, and resuspended in buffer A (20 mM sodium Pi (pH 7.4), containing 14 mM 2-mercaptoethanol and 0.5 M NaCl) along with 20 mM imidazole. The cells were sonicated on ice by five cycles of ultrasonication (where one cycle corresponds to 10 kHz for 1 min followed by an interval of 1 min). The cell debris was removed by centrifugation (7,000×*g*, 15 min). To the supernatant solution, polyethyleneimine was slowly added to a final concentration of 0.12% (v/v). The mixture was placed at 4°C for 30 min, and this was followed by centrifugation (7,000×*g*, 15 min). The supernatant was applied to a HisTrapTM HP column (1 ml, GE Healthcare Bio-Science, UK) that had been equilibrated with buffer A containing 20 mM imidazole. The column was washed with buffer A containing 20 mM imidazole, and the enzyme was eluted with buffer A containing 200 mM and 500 mM imidazole. The active column-bound fractions were concentrated and desalted using VIVASPIN 30,000 MWCO (VIVASCIENCE, Hannover, Germany), followed by substitution with buffer B (20 mM potasium Pi (pH 8.0), containing 14 mM 2-mercaptoethanol).

Enzyme assays

The standard assay mixture (50 μl) consisted of a 200 μM sugar acceptor (lignans), a 2 mM sugar donor (UDP-glucose, UDP-galactose, and UDP-glucuronic acid), a 100 mM potassium phosphate buffer (pH 8.0), and enzymes. After a 10-min pre-incubation of the mixture without the enzyme at 30°C, the reaction was initiated by the addition of the enzyme. After incubation at 30°C for 60 min, the reaction was terminated by the addition of 50 μl of MeCN containing 0.5% (v/v) trifluoroacetic acid (TFA). To determine the initial velocity of the recombinant UGT71A18 enzyme, the assays were carried out under the standard assay with various substrate concentrations (0.014–1 mM for pinoresinol, 0.01–3 mM for UDP-glucose). The apparent *K*m value for the glucosyl donor and acceptor substrate in the presence of a saturating concentration of the counter substrate were determined by fitting the initial velocity data to the Michaelis-Menten equation using non-linear regression analysis (Leatherbarrow 1990; Segel 1975). The range of pH from 6.5–8.5 was tested for estimation of the optimal pH.

LC-MS

The reverse-phase HPLC procedure was performed with an LC-2010A HT system with a SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan) on the Develosil column (Develosil) C30-UG-5 at 40°C (4.6 mm I.D.×150 mm, NOMURA CHEMICAL, Aichi, Japan). Each sample was eluted with a linear gradient of 5–100% solvent B [90% MeCN containing 0.1% (v/v) TFA] in solvent A [H_2O containing 0.1% (v/v) TFA] for 20 min at a flow rate of 1 ml min^{-1}, and then was further eluted with 90% solvent B for 5 min. Lignans were monitored by UV absorption at 230 and 280 nm.

LC-TOF-MS analysis of the enzyme reaction mixtures was carried out using an electrospray ionization ion-trap time-of-flight mass spectrometry (ESI IT-TOF MS) instrument with LC-20AD HPLC (Shimadzu, Kyoto, Japan) on a Develosil C30-UG-3 column (2.0 mm×150 mm, Nomura Chemical, Aichi, Japan) in a ternary solvent system comprised of solvent A [H_2O containing 0.1% HCOOH], and solvent B [MeCN containing 0.1% HCOOH]. The sample was eluted using a linear gradient of 5 to 100% of solvent B in solvent A for 20 min at a flow rate of 0.2 ml min^{-1}, and then with 100% of solvent B for 5 min. The mass spectrometer scanned from *m*/*z* 200–1000. The interface voltage was 4.5 kV in positive ion mode and −3.5 kV in negative ion mode. (+)-Pinoresinol monoglucoside was detected in a negative mode to be a deprotonated molecule $[M-H]^-$ at *m*/*z* 519.1639 (exact mass: 520.1945) in the reaction mixture of UDP-glucose and (+)-pinoresinol with the recombinant UGT71A18 protein.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed on cDNAs prepared from each organ. The following specific primer sets (UGT71A17-Fw: 5′-TAG CGG ATC AAC CAA CTA AAC and UGT71A17-Rv: 5′-TCT TGC CAT ACC GAG GAA CAT for UGT71A17 (Accession AB524717), UGT71A18-Fw: 5′-TAG CAG ATC AAC CCA GTA AAT and UGT71A18-Rv: 5′-TCT TGC CAT ACT GAC

GAA TGG for UGT71A18 (Accession AB524718), PLR-Fw: 5′-ATG GGA AAA AGC AAA GTT TTG ATC ATT GG and PLR-Rv: 5′-CAC GTA ACG CTT GAG GTA CTC TTC CAC for *Forsythia* PLR(Accession AAC49608), and rRNA-Fw: 5′-GAA ACC TGC AAA GCA GA and rRNA-Rv: 5′-CTG ACC TGG GGT CGC TGT CGA for *Forsythia* rRNA (Accession AJ236041)) were used to amplify DNA with ExTaq DNA polymerase (TaKaRaBio, Shiga, Japan) for 25–35 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 1 min (GeneAmp 2400, PerkinElmer). Each PCR product was separated (in) on 0.8% agarose gel and visualized by ethidium bromide staining.

Results and discussion

Molecular cloning of Forsythia UGTs

To identify *Forsythia* lignan UGTs, we initially prepared crude enzyme fractions from the mature leaves and a leaf-derived suspension of cells of *F. koreana*, and tested their glucosylating activity toward (+)-pinoresinol, (+)-phillygenin, and (−)-arctigenin. After the incubation of the reaction mixtures of the crude enzymes with these lignans, new products were observed (Figure 2). These were not produced in the absence of UDP-glucose, strongly suggesting that UGT family enzymes are involved in the glucosylation of these lignans *in vivo*.

Based on the structural similarity to the *Sesamum* lignan UGT, we screened a *Forsythia koreana* cDNA

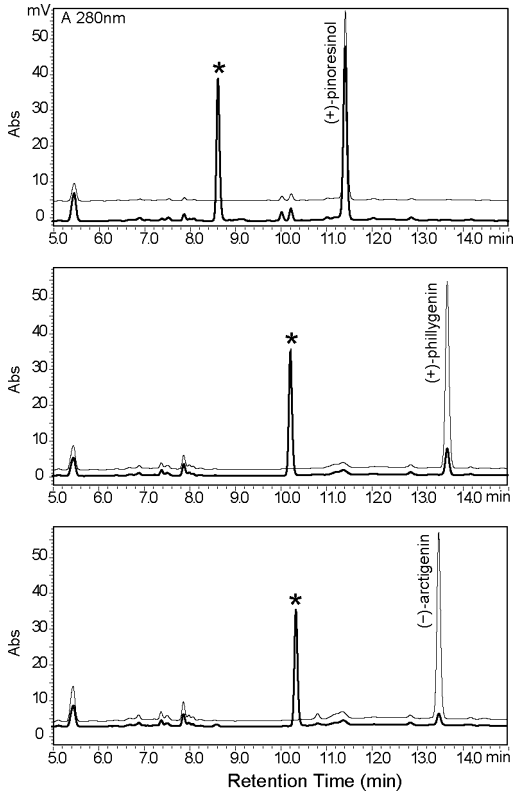

Figure 2. *Lignan glucosylating activity in* Forsythia *crude enzymes*. The HPLC profiles of reaction of the crude enzyme extracts from *Forsythia* suspension cell cultures with (+)-pinoresinol (top), with (+)-phillygenin (middle), and with (−)-arctigenin (bottom). Similar glucosylating activity was observed in the crude enzyme prepared from leaves (data not shown). Each chromatogram indicates absorption at 280 nm. Thick and thin lines indicate the reaction in the presence and absence of UDP-gucose. Asterisk indicates each glucosylated lignan.

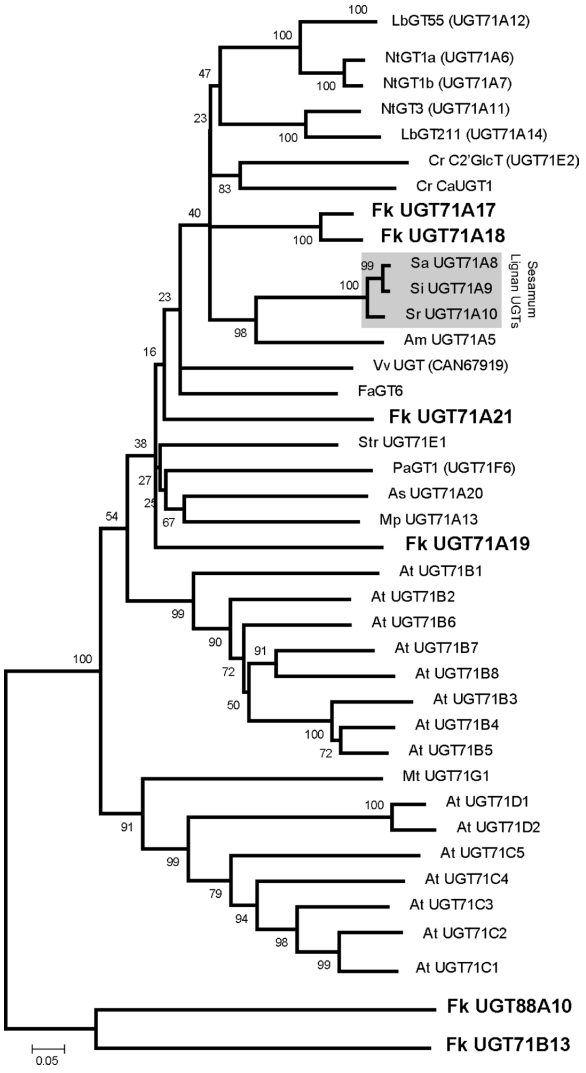

Figure 3. *A phylogenetic tree of* Forsythia *UGTs.* The sequences used for the alignment were primarily those of UGTs that are c lassified as UGT71 family. The tree was constructed from a Clustal W multiple alignment using the neighbor-joining method by MEGA 4 software (Tamura et al. 2007). *Bar*=0.05 amino acid substituteion/site. *Numbers* indicates bootstrap values. *Forsythia koreana* (Fk)UGTs are shown as boldface letters. *Arabidopsis thaliana* (At) UGT71 protein sequences are available at the web site (http://www.p450.kvl.dk/Arab_ugts/table.shtml). *Lycium barbarum* (Lb)GT55/UGT71A12, AB360615; LbGT211/UGT71A14, AB360630; *Nicotiana tabacum* (Nt)GT1a/UGT71A6, AB052557; NtGT1b/UGT71A7, AB052558; AtGT3/UGT71A11, AB072918; *Catharanthus roseus* (Cr)C2'GlcT/UGT71E2, BAF75901; Cr CaUGT1, BAD29721; *Antirrhinum majus* (Am)UGT71A5, AB293962; *Sesamum alatum* (Sa)UGT71A8, AB293959; *Sesamum indicum* (Si)UGT71A9, AB293960; *Sesamum radiatum* (Sa)UGT71A10, B293961; *Maclura pomifera* (Mp)UGT71A13, ABL85473; *Vitis vinifera* (Vv)UGT, CAN67919; *Stevia rebaudiana* (Str)UGT71E1, AY345976; *Medicago truncatula* (Mt)UGT71G1, AAW56092; *Fragaria ananassa* (Fa)GT6, ABB92748; *Phytolacca americana* (Pa)GT1/UGT71F6, AB458516; *Anthriscus sylvestris* (As)UGT71A20, AB524721.

library prepared from leaves using the *S. indicum* (+)-sesaminol 2-*O*-glucosyltransferase, UGT71A9 gene (Noguchi et al. 2008), as a probe. The cDNA library screening resulted in the detection of six novel molecular species of UGT, four of which were classified as UGT71 family enzymes (UGT71A17, UGT71A18, UGT71A19, and UGT71A21), the others as UGT72B13 and UGT88A10 according to the definition of UGT identity (Mackenzie et al. 2005). A phylogenetic tree of UGT71 family proteins with these *Forsythia* UGTs was constructed using the Neighbor-Joining method (Figure 3). UGT71A17 and UGT71A18, two closely related clones, both showed 56% amino acid sequence identity to UGT71A9. UGT71A17 and UGT71A18 encode 469 and 468 amino acids, respectively, and share 90% sequence homology. The high degree of structural similarity of UGT71A17 and UGT71A18 to UGT71A9 allowed us to presume that the two *Forsythia* UGT71A genes encode lignan glucosyltransferases.

Glycosylation activity of Forsythia UGTs

To examine glycosylation activities of UGT71A17 and UGT71A18, the corresponding genes were heterologously expressed as a His-tag fused chimera protein in *Escherichia coli*. The resultant fused proteins were purified with a nickel-affinity column, and their enzymatic activities were evaluated. UGT71A18 reacted with (+)-pinoresinol in the presence of UDP-glucose as a sugar donor, and gave a product identical to the authentic (+)-4-*O*-pinoresinol monoglucoside on HPLC analysis (Figure 4A). The product exhibited a molecular ion at *m/z* 519.1639 $[M-H]^-$, which was perfectly consistent with the mass calculation of (+)-4-*O*-pinoresinol monoglucoside (Figure 4B). These results revealed that UGT71A18 catalyzes mono-glucosylation at the 4-hydroxy group of (+)-pinoresinol. Similarly, the paralog UGT71A17 also exhibited glucosylating activity toward (+)-pinoresinol, but no further biochemical characterization was performed, because its activity was so much lower than that of UGT71A18.

The *K*m value of UGT71A18 for (+)-pinoresinol and

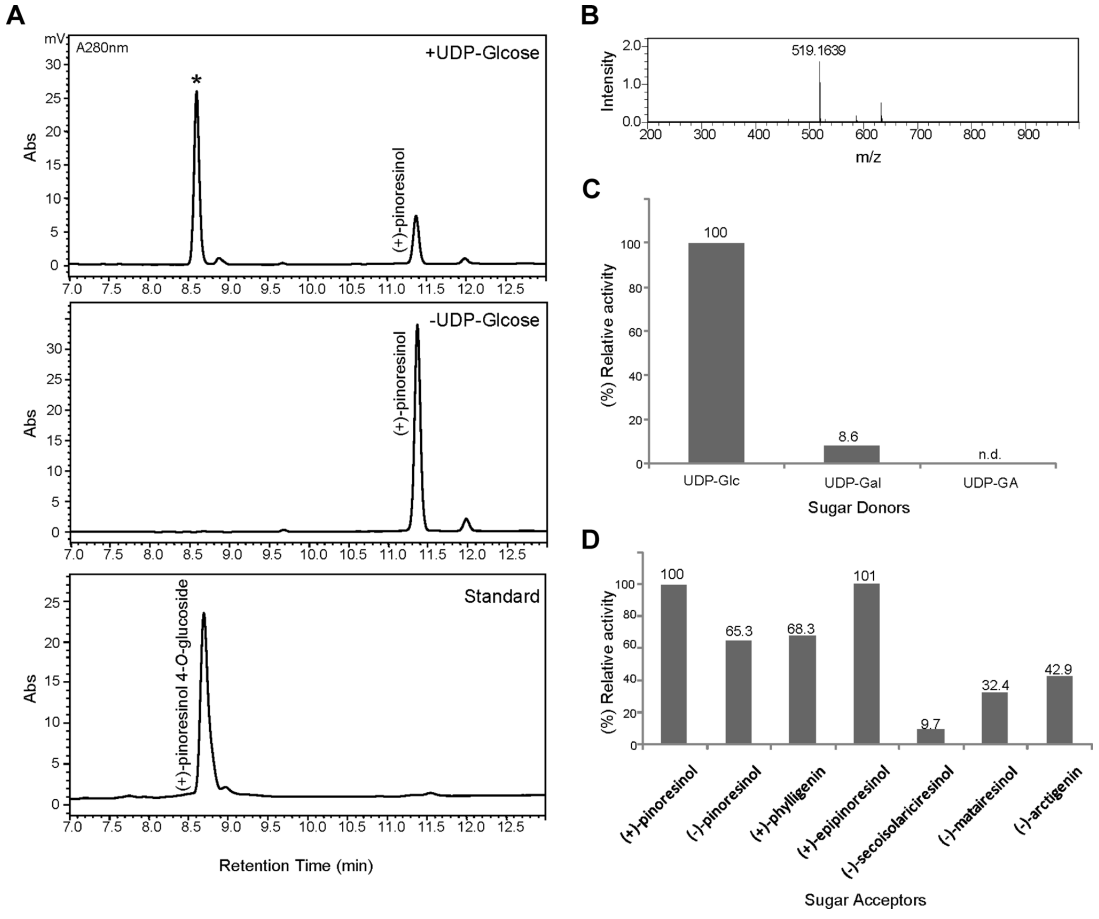

Figure 4. *Biochemical analysis of* Forsythia *UGT71A18.* (A) The HPLC chromatograph of reaction of (+)-pinoresinol with the recombinant UGT71A18 protein in the presence (top) or absence of UDP-glucose (middle). Authentic (+)-pinoresinol 4-*O*-monoglucoside is shown at the bottom. Asterisk indicates the glucosylated (+)-pinoresinol. (B) Mass spectrum of the glucosylated (+)-pinoresinol by UGT71A18. (C) Relative sugar donor specificity of UGT71A18. (+)-Pinoresinol was used as the sugar acceptor. The highest specific activity on UDP-glucose is set as 100%. n.d. indicates "not detected". (D) Sugar acceptor specificity of UGT71A18. Relative activities toward 200 μM solutions of lignans are shown. The glucosylating activity toward (+)-pinoresinol is taken to be 100%.

UDP-glucose was 81.2 ± 54.9 μM and 478.5 ± 16.4 μM, respectively. The optimal pH for the catalysis was determined to be 8.2. Furthermore, UGT71A18 exhibited prominent specificity to UDP-glucose as the sugar donor for the catalysis (Figure 4C), showing that UGT71A18 is a typical UDP-glucose-dependent glucosyltransferase. UGT71A18 exhibited relatively broad sugar acceptor specificity for lignans with a preference for furofuran-class lignans such as (+)-pinoresinol, (+)-epipinoresinol, and (+)-phillygenin, compared with other classes of lignans, including dibenzylbutyrolactone-class (Figure 4D). These results indicate that other UGTs are responsible for the glucosylating activity of non-furofuran class of lignans observed in the *Forsythia* crude enzyme fractions (Figure 2), although the corresponding genes remain to be clarified.

The identification of novel lignan glucosyltransferases from *Forsythia* plants based on the similarity to the *S. indicum* UGT71A9 highlights the structural conservation of lignan UGTs across plant species. Nevertheless, the structural diversity of lignan glycosides strongly suggests that not all lignan UGTs belong to the UGT71 family since flavonoid UGTs form separate phylogenetic clades based on their various regio-specificities (Noguchi et al. 2009). It was previously reported that *Sesamum* UGT71A9 participates in the glucosylation of an intrinsic furofuran lignan, sesaminol, while other UGT71 enzymes are involved in the glucosylation of the flavonoid/triterpene (*Medicago* UGT71G1), a growth inhibitor (*Arabidopsis* UGT71B2/HYR1), exogenous naphthols (*Nicotiana* UGT71A6,7,11), exogenous curcumin (*Catharanthus* UGT71E2), and endogenous phytohormone (*Arabidopsis* UGT71B6) (Kaminaga et al. 2004; Noguchi et al. 2008; Priest et al. 2006; Taguchi et al. 2003; Zhao et al. 2007), so the target compounds of the UGT71 enzymes are evidently structurally diverse (Figure 3). In addition to these reports, the present results support the view that the UGT71 family enzymes possess promiscuous substrate specificity, and some of them have adapted to lignans.

Expression analysis of Forsythia UGTs

Several cell culture systems of *Forsythia* spp. have been shown to accumulate pinoresinol glucoside, but the genes responsible for pinoresinol glucosylating activity have yet to be clarified (Kim et al. 2009; Schmidt and Petersen 2002). To investigate whether UGT71A18 is involved in the glucosylation of pinoresinol in the *Forsythia* cell cultures, expression of the *Forsythia UGT71A* genes was analyzed by RT-PCR. Both the *UGT71A17* and *UGT71A18* genes were shown to be expressed in the leaf, floral bud, and petal where lignan glucosides are accumulated, whereas only the expression of *UGT71A18* was detected in the suspension cell culture (Figure 5A). These results suggest that *UGT71A18*, not *UGT71A17,* plays a major role in the glucosylation of lignans in the *Forsythia* cell suspension cultures, while both of these two genes participate in lignan glucosylation in the leaf, floral bud, and petal of *Forsythia* plants.

We further investigated the gene expression profiles of *UGT71A18* in the transgenic lines overexpressing the *PLR*-RNAi construct (Kim et al. 2009). Interestingly, remarkable up-regulation of the *UGT71A18* gene was observed in the transgenic cell line, compared to the wild type (Figure 5B). Such expression profile is consistent with the previous observation that the *Forsythia PLR*-RNAi cell cultures produce approximately 20-fold higher (+)-pinoresinol glucoside than the wild type (Kim et al. 2009), and also suggests that the *UGT71A18* gene is up-regulated in response to increases in endogenous pinoresinol. Moreover, this result accords with the notion that glycosylation of small molecules is a major plant mechanism underlying detoxification of abnormal intermediates of endogenous metabolites and xenobiotic compounds (Gachon et al. 2005; Lim and Bowles 2004). Together, these data suggest that UGT71A18 serves as a major (+)-pinoresinol glucosyltransferase in the *Forsythia* suspension culture.

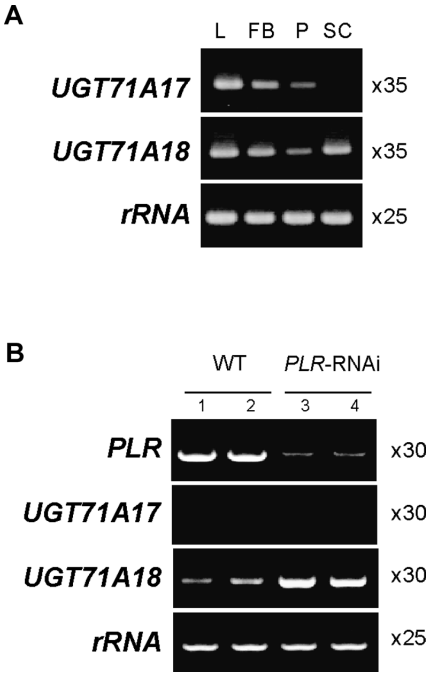

Figure 5. *Expression analysis of* Forsythia *UGT71A17 and UGT71A18.* (A) Expression profiles of *UGT71A17 and UGT71A18* in separate organs and suspension cells. L, leaf; FB, floral bud; P, petal; SC, suspension cells (B) Expression profiles of lignan biosynthetic genes in wild type (WT) and transgenic suspension cells in which *PLR* gene expression is inhibited by RNAi construct. *Numbers* indicate independent cell lines.

Perspective on the application of UGT71A18 to metabolic engineering

We previously identified a cytochrome P450 enzyme gene, *CYP81Q1* from sesame seeds (*Sesamum indicum*). The *CYP81Q1* gene encodes a (+)-piperitol/sesamin synthase (PSS) that catalyzes the sequential conversion of (+)-pinoresinol to (+)-sesamin through (+)-piperitol by forming two methylenedioxy bridges (Ono et al. 2006). During an effort to ectopically produce in *Forsythia* plants the furofuran-class lignan, sesamin originally produced in *Sesamum indicum*, we established *Forsythia* cell cultures co-overexpressing *PLR*-RNAi and *CYP81Q1* constructs, and found that the cell cultures unexpectedly produced large amount of (+)-pinoresinol glucosides, but only a small amount of (+)-sesamin (Kim et al. 2009). These results suggest that the pinoresinol glucosylating activity could be the limiting step for (+)-sesamin production, which is compatible with the fact that CYP81Q1 is unable to catalyze (+)-pinoresinol glucoside as its substrate (Ono et al. 2006). Thus, the inhibition of pinoresinol glucosylation is highly likely to be crucial for efficient production of (+)-sesamin in the *Forsythia* platform.

This study reports the novel lignan glucosylation activity of UGT71 family enzymes, the biochemical properties of which *in vivo* have been mostly unknown. The sequence information and functional characteristics of UGT71A17 and UGT71A18 of *Forsythia* not only partly reveal the lignan glucosylating activity observed *in vivo*, but also serve as promising molecular tools for the metabolic engineering of lignan biosynthesis. Inhibition of the *UGT71A18* gene in combination with the overexpression of *PLR*-RNAi and *CYP81Q1* in the *Forsythia* suspension cell culture platform is expected to improve (+)-sesamin production via a redirection of metabolic flow to the (+)-pinoresinol aglycone which is subjected to the catalysis of CYP81Q1. To this end, generation of transgenic *Forsythia* cell cultures overexpressing the *UGT71A18*-RNAi construct is underway in our laboratory.

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References

Apers S, Vlietinck A, Pieters L (2003) Lignans and neolignans as lead compounds. *Phytochem Rev* 2: 201–217

Ayres DC, Loike JD (1990) *Lignans-chemical, biological, and clinical properties.* Cambridge University Press, Cambridge, UK

Berim A, Ebel R, Schneider B, Petersen M (2008) UDP-glucose : (6-methoxy)podophyllotoxin 7-*O*-glucosyltransferase from suspension cultures of *Linum nodiflorum*. *Phytochemistry* 69: 374–381

Davin LB, Wang HB, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG (1997) Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. *Science* 275: 362–366

Davin LB, Lewis NG (2003) A historical perspective on lignan biosynthesis: monolignol, allylphenol and hydroxycinnamic acid coupling and downstream metabolism. *Phytochem Rev* 2: 257–288

Dinkova-Kostova A, Gang D, Davin LB, Bedgar D, Chu A, Lewis NG (1996) Pinoresinol/Lariciresinol Reductase from *Forsythia intermedia.* *J Biol Chem* 271: 29473–29482

Gachon, CM, Langlois-Meurinne M, Saindrenan P (2005) Plant secondary metabolism glycosyltransferases: the emerging functional analysis. *Trends Plant Sci* 10: 542–549

Gang DR, Kasahara H, Xia ZQ, Vander-Mijnsbrugge, K, Bauw G, Boerjan W, Van Montagu M, Davin LB Lewis NG (1999) Evolution of plant defense mechanisms. Relationships of phenylcoumaran benzylic ether reductases to pinoresinol-lariciresinol and isoflavone reductases. *J Biol Chem* 274: 7516–7527

Guo H, Liu AH, Ye M, Yang M, Guo DA (2007) Characterization of phenolic compounds in the fruits of *Forsythia suspense* by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom* 21: 715–729

Harmatha J, Dinan L (2003) Biological activities of lignans and stilbenoids associated with plant-insect chemical interaction. *Phytochm Rev* 2: 321–330

Hearon WM, MacGregor WS (1995) The naturally occurring lignans. *Chem Rev* 55: 957–1068

Hemmati S, Schmidt TJ, Fuss E (2007) (+)-pinoresinol/(−)-lariciresinol reductase from *Linum perenne* Himmelszelt involved in the biosynthesis of justicidin B. *FEBS Lett* 581: 603–610

Kaminaga Y, Sahin FP, Mizukami H (2004) Molecular cloning and characterization of a glucosyltransferase catalyzing glucosylation of curcumin in cultured *Catharanthus roseus* cells. *FEBS Lett* 567: 197–202

Katayama T, Davin LB, Lewis NG (1992) An extraordinary accumulation of (−)-pinoresinol in cell-free extracts of *Forsythia intermedia*: evidence for enantiospecific reduction of (+)-pinoresinol. *Phytochemistry* 31: 3875–3881

Kim HJ, Ono E, Morimoto K, Yamagaki T, Okazawa A, Kobayashi A, Satake H (2009) Metabolic engineering of lignan biosynthesis in *Forsythia* cell culture. *Plant Cell Physiol* 50: 2200–2209

Kitagawa S, Hisada S, Nishibe S (1984) Phenolic compounds from *Forsynthia* leaves. *Phytochemistry* 23: 1635–1636

Kitagawa S, Nishibe S, Benecke R, Thieme H (1988) Phenolic compounds from *Forsynthia* leaves. II. *Chem Pharm Bull* 36: 3667–3670

Leatherbarrow RJ (1990) Using linear and non-linear regression to

fit biochemical data. *Trends Biochem Sci* 15: 455–458

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

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

MacRae WD, Towers GHN (1984) Biological activities of lignans. *Phytochemistry* 23: 1207–1220

Nishibe, S, Sakushima A, Kitagawa S, Klimek B, Benecke R, Thieme H (1988) Phenolic compounds from *Forsynthia* leaves (III). On the comparison of constituents between hybrid and parents. *Jpn J Pharmacognosy* (*Shoyakugaku Zasshi*) 42: 324–328 (in Japanese)

Noguchi A, Fukui Y, Iuchi-Okada A, Kakutani S, Satake H, Iwashita T, Nakao M, Umezawa T, Ono E (2008) Sequential glucosylation of a furofuran lignan, (+)-sesaminol, by *Sesamum indicum* UGT71A9 and UGT94D1. *Plant J* 54: 415–427

Noguchi A, Fukui Y, Horikawa M, Fukuchi-Mizutani M, Iuchi-Okada A, Ishiguro M, Kiso Y, Nakayama T, Ono E (2009) Local differentiation of sugar donor specificity of flavonoid 7-*O*-glycosyltransferase in Lamiales. *Plant Cell* 21: 1556–1572

Ono E, Nakai M, Fukui Y, Tomimori N, Fukuchi-Mizutani M, Saito M, Satake H, Tanaka T, Katsuta M, Umezawa T, Tanaka Y (2006b) Formation of two methylenedioxy bridges by a *Sesamum* CYP81Q protein yielding a furofuran lignan, (+)-sesamin. *Proc Natl Acad Sci USA* 103: 10116–10121

Ozawa S, Davin LB, Lewis NG (1993) Formation of (−)-arctigenin in *Forsythia intermedia*. *Phytochemistry* 32: 643–652

Piao XL, Jang MH, Cui J, Piao X (2008) Lignans from the fruits of *Forsythia suspensa*. *Bioorg Med Chem Lett* 18: 1980–1984

Priest DM, Ambrose SJ, Vaistij FE, Elias L, Higgins GS, Ross AR, Abrams SR, Bowles DJ (2006) Use of the glucosyltransferase UGT71B6 to disturb abscisic acid homeostasis in *Arabidopsis thaliana*. *Plant J* 46: 492–502

Rahman MMA, Dewick PM, Jackson DE, Lucas JA (1986) Lignans in *Forsythia* leaves and cell cultures. *J Pharm Pharmacol* 38: 15

Rahman MMA, Dewick PM, Jackson DE, Lucas JA (1990a) Biosynthesis of lignans in *Forsythia intermedia*. *Phytochemistry* 29: 1841–1846

Rahman MMA, Dewick PM, Jackson DE, Lucas JA (1990b) Production of lignans in *Forsythia intermedia* cell cultures. *Phytochemistry* 29: 1861–1866

Rahman MMA, Dewick PM, Jackson DE, Lucas JA (1990c) Lignans of *Forsythia intermedia*. *Phytochemistry* 29: 1971–1980

Schmitt J, Petersen M (2002a) Pinoresinol and matairesinol accumulation in a *Forsythia* × *intermedia* cell suspension culture. *Plant Cell Tissue Organ Cult* 68: 91–98

Schmitt J, Petersen M (2002b) Influence of methyl jasmonate and coniferyl alcohol on pinoresinol and matairesinol accumulation in *Forsythia* × *intermedia* suspension culture. *Plant Cell Rep* 20: 885–889

Segel IH (1975) Enzyme Kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme systems, John Wiley & Sons, New York

Shao H, He X, Achnine L, Blount JW, Dixon RA, Wang X (2005) Crystal structures of a multifunctional triterpene/flavonoid glycosyltransferase *from Medicago truncatula*. *Plant Cell* 17: 3141–3154

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

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596–1599

Tokar M, Klimek B (2004) The content of lignan glycosides in *Forsythia* flowers and leaves. *Acta Pol Pharm* 61: 273–278

Umezawa T (2003) Diversity in lignan biosynthesis. *Phytochem Rev* 2: 371–390

Umezawa T, Davin LB, Lewis NG (1991) Formation of lignans (−)-secoisolariciresinol and (−)-matairesinol with *Forsythia intermedia* cell-free extracts. *J Biol Chem* 266: 10210–10217

Xia ZQ, Costa MA, Pélissier HC, Davin LB, Lewis, NG (2001) Secoisolariciresinol Dehydrogenase Purification, Cloning, and Functional Expression. *J Biol Chem* 276: 12614–12623

Yonekura-Sakakibara K, Tanaka Y, Fukuchi-Mizutani M, Fujiwara H, Fukui Y, Ashikari T, Murakami Y, Yamaguchi M, Kusumi T (2000) Molecular and biochemical characterization of a novel hydroxycinnamoyl-CoA: anthocyanin 3-*O*-glucoside-6″-*O*-acyltransferase from *Perilla frutescens*. *Plant Cell Physiol* 41: 495–502

Zhao Y, Chow TF, Puckrin RS, Alfred SE, Korir AK, Larive CK, Cutler SR (2007) Chemical genetic interrogation of natural variation uncovers a molecule that is glycoactivated. *Nat Chem Biol* 3: 716–721