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Short Communication

Glucosyltransferase activity of *Arabidopsis* UGT71C1 towards pinoresinol and lariciresinol

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Abstract Lignans are a class of phenylpropanoids that are widely distributed in the plant kingdom and some lignans are known to be present as glycosides. In the model plant *Arabidopsis thaliana*, pinoresinol and lariciresinol as well as their glucosides are found to be accumulated in the roots, but the enzymes involved in the glucosylation of lignans remain to be characterized. UGT71C1 showed activity towards several phenylpropanoids in previous studies, although its activity towards lignans has not been investigated. In the present study, the involvement of UGT71C1 in lignan glucosylation was examined. Quantification of lignans in a T-DNA knockout line of the *UGT71C1* gene, *ugt71c1*, by an ultra performance liquid chromatography-tandem mass spectrometry showed that the content of pinoresinol glucoside decreased in parallel with an increase of pinoresinol at the corresponding degree. Two major peaks corresponding to lariciresinol glucosides were detected in the mass chromatogram of the extract from the wild type and one of the peaks decreased in the *ugt71c1* line suggesting that the amount of lariciersinol glucoside also decreased in the mutant. UGT71C1 expressed in *Escherichia coli* showed glucosyltransferase activity towards pinoresinol and lariciresinol. The present results suggest that UGT71C1 is involved in lignan glucosylation in *A. thaliana*.

Key words: *Arabidopsis thaliana*, lariciresinol, lignan, pinoresinol, uridine diphosphate glucose:lignan glucosyltransferase.

Lignans are a class of specialized metabolites that are widely distributed in the plant kingdom and are produced by oxidative dimerization of two phenylpropanoids through a C8–C8′ linkage (Umezawa 2003). Some lignans are used as precursors for the synthesis of medicines and dietary supplements because of their diverse bioactivities (Saleem et al. 2005). Despite their wide distribution and extensive use, their physiological roles in plants remain unclear. Similar to other specialized metabolites, lignans may play an important role in defense against biological stressors such as pathogens and pests (Dixon et al. 2002).

The lignan biosynthetic pathway starting with pinoresinol has been extensively studied, especially in the lignan-rich *Forsythia* spp. (Davin and Lewis 2003; Suzuki and Umezawa 2007). Pinoresinol is formed by the enantioselective dimerization of two coniferyl alcohol molecules with the assistance of dirigent proteins (DPs) (Davin et al. 1997). Pinoresinol is sequentially reduced by pinoresinol/lariciresinol reductases to yield secoisolariciresinol (Dinkova-Kostova et al. 1996). In *Arabidopsis thaliana*, lariciresinol glucosides are accumulated in the roots as major lignans because pinoresinol reductases (PrRs) in this plant species show weak or no activity towards lariciresinol (Figure 1) (Nakatsubo et al. 2008). In a previous study from our group, quantification of lignans in *A. thaliana* showed that the content of pinoresinol glucoside was more than twice that of its aglycone, pinoresinol (Okazawa et al. 2011). These results suggested that most lignans in *A. thaliana* exist as glucosides. However, the enzymes involved in the glucosylation of lignans have not been characterized in *A. thaliana*.

Glycosylation of plant specialized metabolites is generally catalyzed by the Family 1 uridine diphosphate

Abbreviations: DP, dirigent protein; EIC, extracted ion chromatogram; PrR, pinoresinol reductase; MR, mutual rank; MRM, multiple reaction monitoring; UDP, uridine diphosphate; UGT, uridine diphosphate glycosyltransferase; UPLC-MS/MS, ultra performance liquid chromatography-tandem mass spectrometry; WT, wild type.

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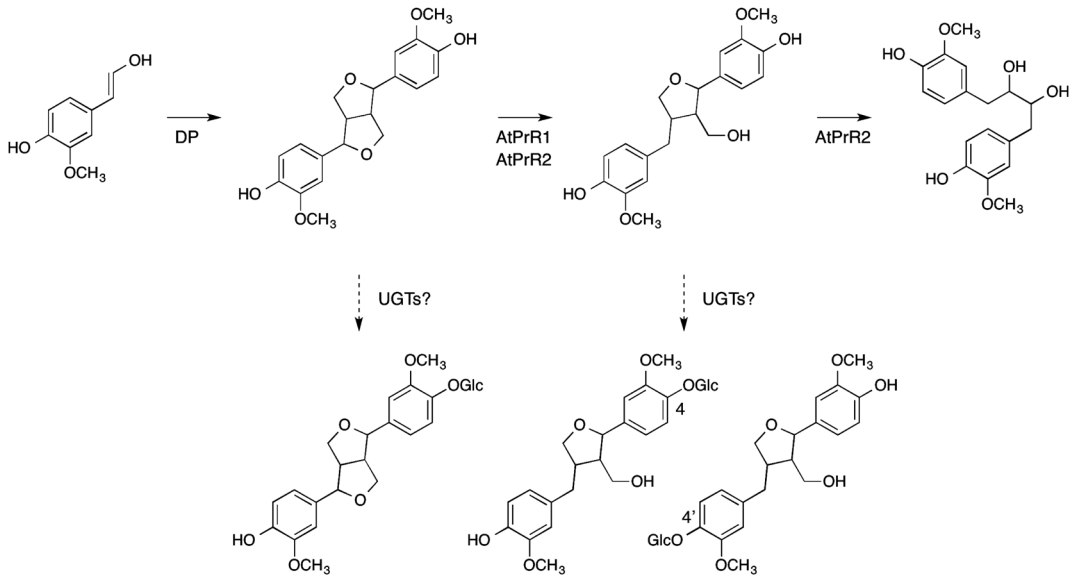

Figure 1. Lignan biosynthetic pathway in *Arabidopsis thaliana*. Pinoresinol is formed by the enantioselective dimerization of two coniferyl alcohol molecules with the assistance of dirigent proteins (DPs). Then, lariciresinol is synthesized by pinoresinol reductases (PrRs). PrR2 has a weak activity towards lariciresinol to produce secoisolariciresinol. These lignans accumulate as glucosides in the roots. There are two glucoside forms of lariciresinol, lariciresinol-4-*O*-*β*-D-glucoside and lariciresinol-4′-*O*-*β*-D-glucoside.

(UDP)-sugar dependent glycosyltransferases (UGTs), and two UGTs in the UGT71A subfamily were characterized to function in glycosylation of lignans. UGT71A9 in sesame (*Sesamum indicum*) catalyzes the glucosylation of (+)-sesaminol (Noguchi et al. 2008) and UGT71A18 in *Forsythia* catalyzes glucosylation of certain furofuran lignans including (+)-pinoresinol in vitro (Ono et al. 2010). Although *A. thaliana* has approximately 120 UGT genes in the genome, it lacks the UGT71A subfamily genes. Instead, there are 15 genes coding for UGT71Bs, UGT71Cs, and UGT71Ds (Ross et al. 2001). Recently, UGT71B6, UGT71B7, and UGT71B8 were shown to be involved in abscisic acid homeostasis (Dong et al. 2014). UGT71Cs and UGT71Ds are thought to be involved in the glucosylation of phenylpropanoids (Lim et al. 2003a; 2003b). Among them UGT71C1 (At2g29750) showed the highest activity towards the model substrates, esculetin and scopoletin (Lim et al. 2003a). UGT71C1 was shown to have glucosyltransferase activity towards phenylpropanoids such as caffeic acid, *o*-coumaric acid, and *p*-coumaric acid, and flavonoids such as quercetin and luteolin (Lim et al. 2003b). These findings suggest that UGT71C1 can transfer glucose to a wide range of phenylpropanoids as a sugar acceptor. However, the activity of UGT71C1 towards lignans, a class of phenylpropanoids, has not been investigated to date. Here we investigated UGT71C1 as a candidate glucosyltransferase involved in lignan glucosylation in *A. thaliana*.

Seeds of *Arabidopsis thaliana* L. Col-0 and the T-DNA knockout line of the *UGT71C1* gene (SALK_111765 obtained from Arabidopsis Biological Resource Center), *ugt71c1*, were surface-sterilized with 5% (v/v) sodium hypochlorite containing 0.1% (v/v) Tween 20 and sown in a row on the upper surface of 1/2×Murashige and Skoog medium with 1.5% (w/v) sucrose, 0.3 mg l^{-1} thiamine hydrochloride, and 0.5 mg l^{-1} nicotinic acid solidified with 0.7% agar in a rectangular plate (100×140×10 mm) set perpendicularly. Following vernalization of the seeds at 4°C in the dark for 2 days, seedlings were grown in a growth chamber at 23°C under a 16-h light/8-h dark photoperiod for 3 weeks. Shoots and roots were harvested separately and immediately frozen in liquid nitrogen and stored at −80°C until extraction.

Samples were extracted using a method described previously with minor modifications (Okazawa et al. 2011; Tamura et al. 2014). After freeze-drying, each sample (approximately 15 mg dry weight) was ground with a ball-mill (MM 400; Retsch, Haan, Germany) at 20 Hz for 2 min and mixed with 1 ml of 50% methanol. The mixture was shaken at 60°C at 800 rpm for 1 h and centrifuged at 15,000×*g* for 5 min. An 800-*μ*l aliquot of the supernatant was collected and the residue was re-extracted with 1 ml of 50% methanol. The second extract was centrifuged and 800 *μ*l of the supernatant was combined with the first extract and dried with a centrifugal concentrator (Concentrator 5301; Eppendorf, Hamburg, Germany). The residue was dissolved in 1 ml of 20% acetonitrile, and then passed through a 0.20-*μ*m syringe filter (GL Chromatodisc 4P; GL Sciences, Tokyo, Japan). After filtration, the sample was analyzed by an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

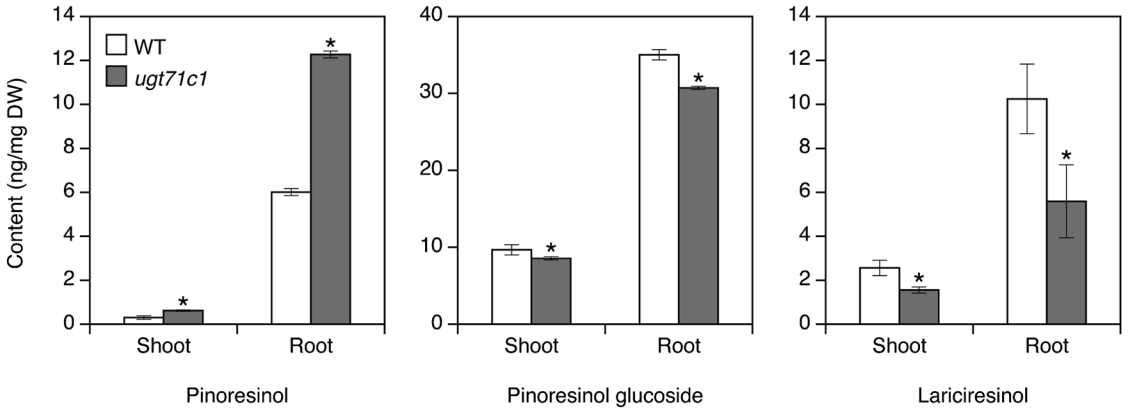

Figure 2. Contents of each lignan in the shoots and the roots of wild type (WT) and *ugt71c1* mutant plants as measured by UPLC-MS/MS. The quantification was performed in triplicate. The values represent the mean±SD. Significant differences between WT and *ugt71c1* samples were determined at $p<0.05$ by Student's *t*-test and are indicated with asterisks.

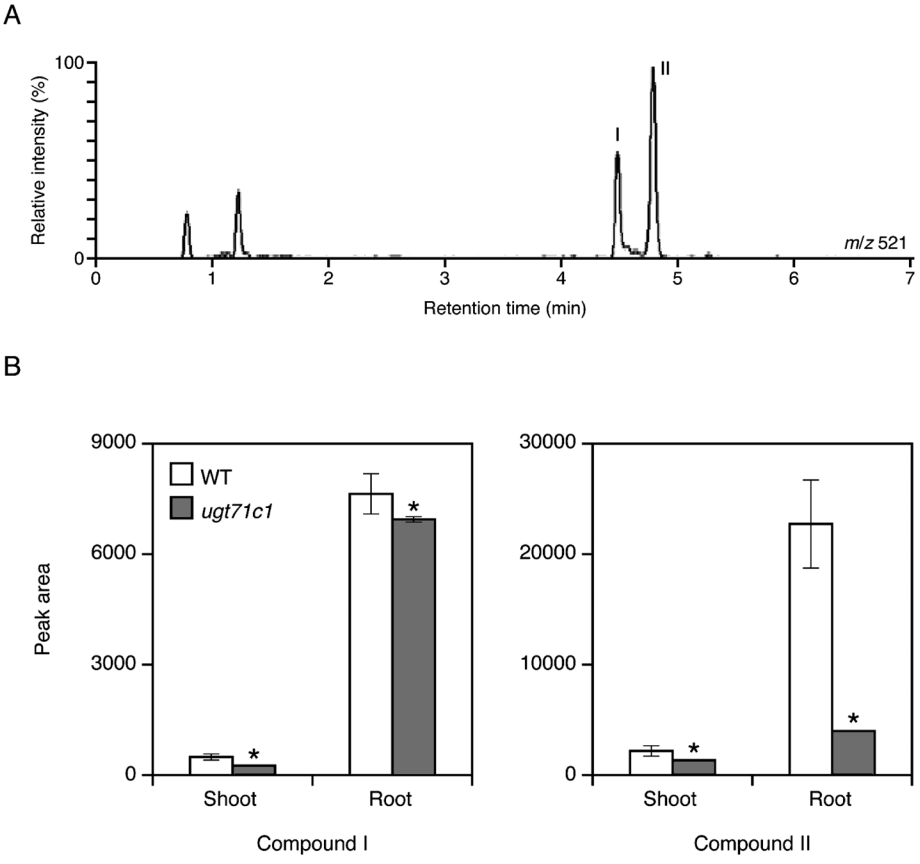

Figure 3. Measurement of lariciresionol glucosides by UPLC-MS/MS. (A) Extracted ion chromatogram (EIC) (*m*/*z* 521) of the root extract from the WT. Two major peaks named compound I and II were detected. (B) Peak areas of compound I and II in the shoots and the roots of the WT and *ugt71c1* plants. All measurements were performed in triplicate. The values represent the mean±SD. Significant differences between WT and *ugt71c1* samples were determined at $p<0.05$ by Student's *t*-test and are indicated with asterisks.

Lignans were quantified by UPLC-MS/MS (ACQUITY TQD; Waters, Milford, MA, USA) with an ACQUITY UPLC HSS C18 1.8 μm (2.1×100 mm) column (Waters) at 40°C with water/acetonitrile/acetic acid mixture as a solvent system in the negative mode. The parameters for MS in the full-scan mode and multiple reaction monitoring (MRM) were described previously (Okazawa et al. 2011; Tamura et al. 2014). Significant changes in the lignan profile were observed in *ugt71c1* compared with that of the wild type (WT), Col-0 (Figure 2). An increase in pinoresinol content in *ugt71c1* was accompanied by a decrease in pinoresinol glucoside content. The pinoresinol glucoside content was higher than that of pinoresinol in *ugt71c1*; however, the decreased content

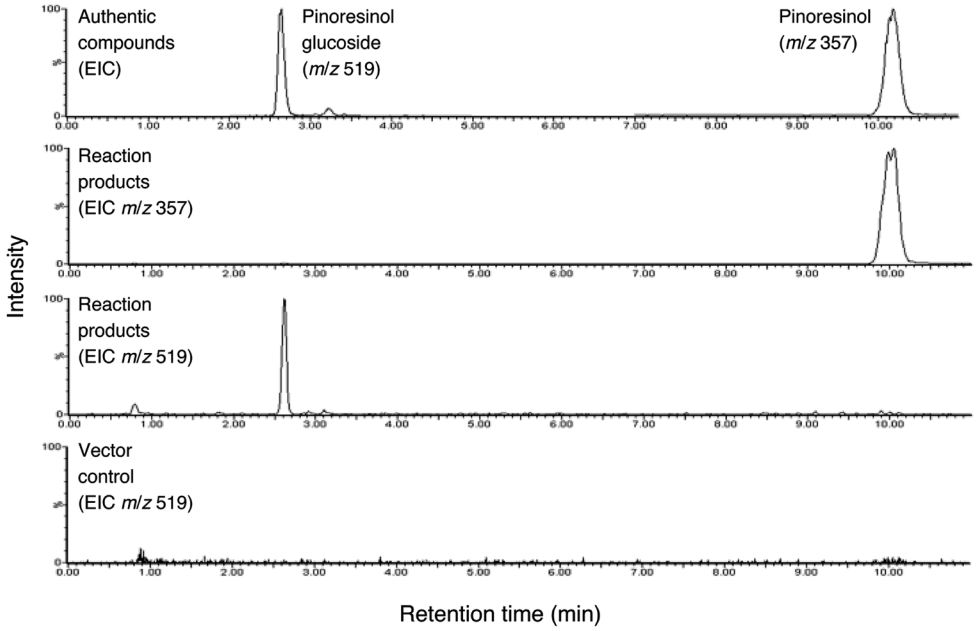

Figure 4. The glucosyltransferase activity of UGT71C1 expressed in *E. coli* towards pinoresinol was confirmed by UPLC-MS/MS. Pinoresinol and pinoresinol glucoside were detected in the EICs (*m*/*z* 357 and 519, respectively) at the same retention times as the authentic compounds.

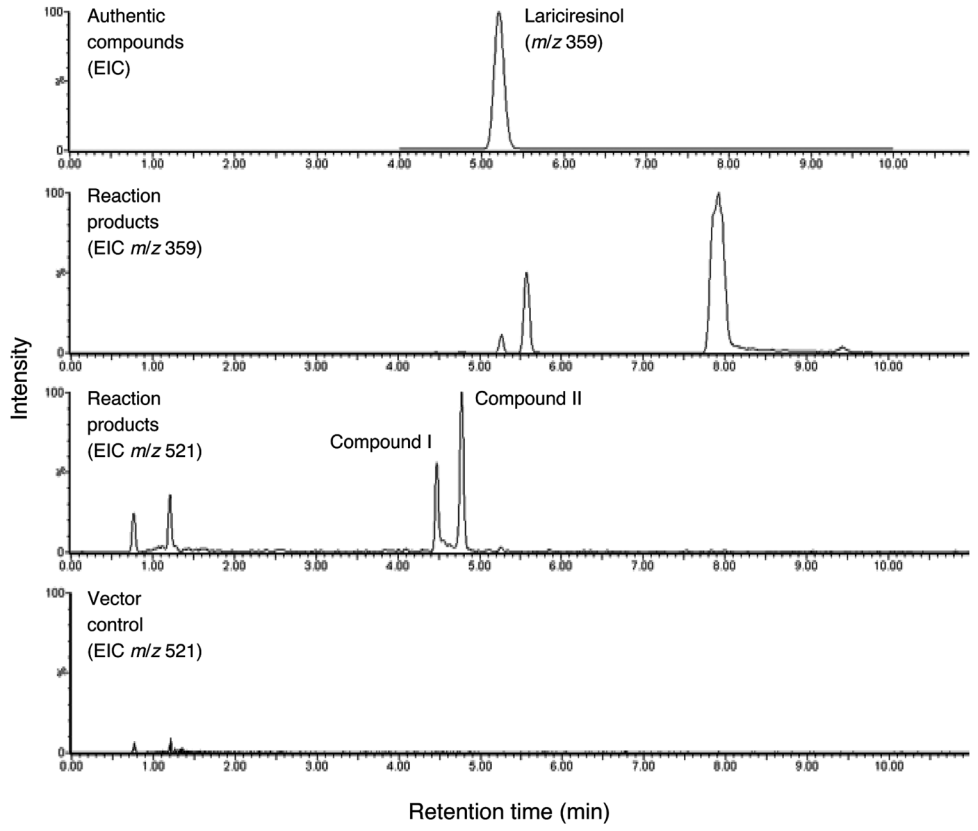

Figure 5. The glucosyltransferase activity of UGT71C1 expressed in *E. coli* towards lariciresinol was confirmed by UPLC-MS/MS. Lariciresinol was detected in the EIC (*m*/*z* 359) at the same retention time as the authentic compounds. Lariciresinol glucosides were detected in the EIC (*m*/*z* 521) at the same retention times as compounds I and II in the root extract from the WT (Figure 3).

of glucoside seemed to correspond to the increased content of pinoresinol (ca. 5 ng/mg dry weight). On the other hand, lariciresinol content in *ugt71c1* was lower that that in the WT. Decrease of lariciresinol content in the roots of *ugt71c1* suggests that lariciresinol biosynthesis by PrRs might be influenced by the metabolic alternation caused by the loss-of-function of UGT71C1. Because the authentic lariciresinol glucosides were not available, their levels were evaluated as peak areas in the extracted ion chromatogram (EIC) at *m*/*z* 521. Two peaks corresponding to lariciresinol glucosides, which were monitored with a MRM transition, *m*/*z* 521.1 (molecular ion)>359.2 (aglycon fragment), were detected in the chromatogram of the root extract from the WT and named compound I and compound II (Figure 3A). Compound II was a major component of the WT sample and dramatically decreased in the *ugt71c1* mutant (Figure 3B). Compounds I and II were expected to be lariciresinol glucosides and its isomer, since they have similar mass fragmentation patterns. Previously, two forms of lariciresinol glucosides, lariciresinol-4-*O*-*β*-D-glucoside and lariciresinol-4′-*O*-*β*-D-glucoside, were isolated from *A. thaliana* (Figure 1) (Kim et al. 2012). Moreover, lariciresinol can be converted to the isomers such as isolariciresinol under acidic conditions (Sicilia et al. 2003).

Next, UGT71C1 was heterologously expressed in *Escherichia coli* and its activity towards lignans was investigated. The open reading frame of the *UGT71C1* gene (At2g29750) was amplified by PCR from the cDNAs prepared from the roots of WT with a forward primer, 5′-CAC CAT GGG GAA GCA AGA AGA TGC AGA GCT-3′, and a reverse primer, 5′-CTA CTT ACT TAT AGA AAC GCC GTC GAT CAA-3′, and subcloned into a pENTR/D-TOPO vector (Life Technologies, Carlsbad, CA, USA). The subcloned DNA was transferred by recombination using LR Clonase II (Life Technologies) into pGEX-5X-1-GW, which was constructed by inserting a GATEWAY conversion cassette (frame B) into the SmaI site of pGEX-5X-1 (GE Healthcare, Little Chalfont, UK). The constructed plasmid, pGEX-5X-1-GW-*UGT71C1*, was transferred into *E. coli* BL21 (DE3). Protein expression was induced by 0.5 mM IPTG at 20°C for 24 h. Cells were harvested by centrifugation (6,000×*g*, 10 min). The pellet was sonicated in 10 mM sodium phosphate buffer (pH 7.4) and centrifuged (15,000×*g*, 15 min) and the supernatant was used as crude enzyme solution. The reaction mixture containing 350 *μ*l enzyme solution, 1 mM UDP-glucose and 1 mM pinoresinol or lariciresinol in a total volume of 500 *μ*l of 10 mM sodium phosphate buffer (pH 7.4) was incubated at 37°C for 30 min. The reactant was analyzed by UPLC-MS/MS.

The results showed that UGT71C1 has glucosyltransferase activitiy towards pinoresinol and lariciresinol (Figures 4, 5). When pinoresinol was used as a substrate, pinoresinol glucoside was detected in the EIC at *m*/*z* 519, showing the same retention time as the authentic compound. No corresponding peak was detected in the experiments performed with vector control (Figure 4) or heat-denatured enzyme (data not shown). UGT71C1 did not utilized UDP-glucuronic acid as a sugar donor (data not shown). When lariciresinol was used as a substrate, two major peaks corresponding to lariciresinol glucosides were detected in the EIC at *m*/*z* 521 of the reaction products (Figure 5). Interestingly, the EIC of the reaction product was similar to that of the root extract from the WT (Figure 3A). In both cases, compound II, which was decreased in *ugt71c1*, was the major component. The results suggest that compound II is a major form of lariciresinol glucoside in *A. thaliana* and UGT71C1 is involved in its glucosylation. Involvement of UGT71C1 in the glucosylation of compound I in planta is unclear because the level of compound I in *ugt71c1* did not decreased compared with WT. The substrate specificity of UGT71C1 may differ in vitro and in vivo. Several peaks were detected in the EIC at *m*/*z* 359 after the reaction suggesting the isomerization of lariciresinol as discussed above during the incubation, since corresponding peaks were also detected in the case of vector control (data not shown). Moreover, UGT71C1 showed the glucosyltransferase activity toward other lignans such as epipinoresinol, secoisolariciresinol, mateiresinol and arctigenin (data not shown). Since glucoside of epipinoresinol was detected in *A. thaliana* previously, there is a possibility that UGT71C1 is also involved in its glucosylation (Okazawa et al. 2011). This is the first study showing that UGT71C1 has glucosyltransferase activity towards lignans in vitro. UGT71C1 is highly expressed in the roots (Lim et al. 2008), which is consistent with the fact that lignans are accumulated in the roots of *A. thaliana* (Nakatsubo et al. 2008; Okazawa et al. 2011). ATTED-II analysis indicated that *UGT71C1* is co-expressed with *PrR2* (At4g13660) (mutual rank (MR) (all): 114.8, MR (tissue): 72.0) (Obayashi et al. 2009).

Our results suggest that UGT71C1 is involved in lignan metabolism in *A. thaliana*. In a previous study, certain flavonoid glucosides were reduced by 25–30% in the seedlings of the *ugt71c1* loss-of-function mutant (Lim et al. 2008). The glucosyltransferase activity of UGT71C1 towards certain flavonoids in vitro suggests that it is involved in flavonoid glucosylation (Lim et al. 2003a; Lim et al. 2003b). The concentration of flavonoids in *A. thaliana* was reported to be approximately 200 ng/mg fresh weight at maximum (Ann Peer et al. 2001), which is comparable to the amount of lignans (approximately 30 ng/mg dry weight). Therefore, the exact substrate of UGT71C1 *in planta* remained undefined. Its possible function as a generalist enzyme with promiscuous substrate specificity that glucosylates

a number of phenylpropanoids must be considered. Detailed kinetic analyses combined with the localization analysis of UGT71C1 and its substrate may help to identify its specific substrate and the physiological role of the enzyme. There should be other UGTs that catalyze the glucosylation of lignans together with UGT71C1, because higher amounts of lignan glucosides were accumulated in *ugt71c1* than their aglycones. The involvement of the four other UGTs in the subfamily, UGT71C2–UGT71C5, should be investigated to clarify the physiological function of this UGT subfamily. Among them, UGT71C2 is the most likely candidate since it is coordinately expressed with *PrR2* in roots (MR (all): 17, MR (tissue): 16.3 by ATTED-II). Moreover, the expression of *UGT71C3* is also moderately correlated with those of *PrR2*, *UGT71C1* and *UGT71C2* (MR (all): 216, 554, and 569.7, respectively by ATTED-II). Characterization of those homologues as the candidate enzymes for lignan glucosylation will not only clarify the metabolic regulation of lignans in *A. thaliana* but also provide clues to understanding the physiological roles of lignans in planta.

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