

Regioselectivity of glucosylation of caffeic acid by a UDP-glucose:glucosyltransferase is maintained *in planta*¹

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Caffeic acid is a phenylpropanoid playing an important role in the pathways leading to lignin synthesis and the production of a wide variety of secondary metabolites. The compound is also an antioxidant and has potential utility as a general protectant against free radicals. Three glucosylated forms of caffeic acid are known to exist: the 3-*O*- and 4-*O*-glucosides and the glucose ester. This study describes for the first time a glucosyltransferase [UDP-glucose:glucosyltransferase (UGT)] that is specific for the 3-hydroxyl, and not the 4-hydroxyl, position of caffeic acid. The UGT sequence of *Arabidopsis*, UGT71C1, has been expressed as a recombinant fusion protein in *Escherichia coli*, purified and assayed against a range of substrates *in vitro*. The assay confirmed that caffeic acid as the preferred substrate when compared with other hydroxycinnamates, although UGT71C1 also exhibited substantial activity towards flavonoid substrates,

known to have structural features that can be recognized by many different UGTs. The expression of UGT71C1 in transgenic *Arabidopsis* was driven by the constitutive cauliflower mosaic virus 35 S (CaMV35S) promoter. Nine independent transgenic lines were taken to homozygosity and characterized by Northern-blot analysis, assay of enzyme activity in leaf extracts and HPLC analysis of the glucosides. The level of expression of UGT71C1 was enhanced considerably in several lines, leading to a higher level of the corresponding enzyme activity and a higher level of caffeoyl-3-*O*-glucoside. The data are discussed in the context of the utility of UGTs for natural product biotransformations.

Key words: *Arabidopsis* family 1 glucosyltransferase, over-expression, recombinant protein, target profiling.

INTRODUCTION

Caffeic acid (3,4-dihydroxycinnamic acid) is a key secondary metabolite occurring throughout the Plant Kingdom. The metabolite exists as the monomeric free acid, a cross-linked oligomer [1,2] or a conjugate with other organic compounds to form glucosides [3–6] and esters [7–12]. The metabolite is of fundamental importance in the plant, playing a key role in phenylpropanoid metabolism and acting as a precursor for lignin biosynthesis [13]. Caffeic acid has also attracted considerable interest as an antioxidant since it has proven ability to scavenge reactive oxygen species [14–16]. In this context, studies have demonstrated that the compound can protect linoleic acid and low-density-lipoprotein from oxidation [17–20] and prevent tissue and DNA from oxidative damage [21,22]. Supplementation experiments in rats, resulting in lipoproteins with higher oxidation resistance, also suggest that the caffeic acid can be used successfully as a dietary source of antioxidant [23].

Glucosylation of caffeic acid is known to lead to the formation of three different conjugates: 1-*O*-caffeoylglucose [4], a high-energy glucose ester, acting as a transient intermediate in the formation of other metabolites [24], and two glucosides, caffeoyl-3-*O*-glucoside and caffeoyl-4-*O*-glucoside [11,12]. Although the aglycone and the two *O*-glucosides have the same level of antioxidant activity, the free acid is oxidized readily when exposed to light irradiation [25]. This observation has focused attention on the need to produce higher quantities of the glucosides for

potential commercial applications. Since glucosylation is difficult to accomplish chemically [26], the possibility of increasing yield of the glucosides *in planta* has been raised.

We have recently identified 107 sequences containing the UDP-glucose:glucosyltransferase (UGT) signature motif in the *Arabidopsis thaliana* genomic database [27,28]. This has provided a foundation to analyse the multigene family in detail and to use a genomic strategy to start to identify the substrates recognized by the individual enzymes. Through large-scale screening of recombinant UGTs we discovered catalytic activities towards plant hormones, benzoates and a range of different phenylpropanoids, including those involved in sinapate metabolism and lignin biosynthesis [29–31]. The initial screen identified only five enzymes with substantial activity towards a set of hydroxycinnamate substrates [30]. Three of the five enzymes were found to produce glucose esters, including one enzyme, UGT84A1, that formed 1-*O*-caffeoylglucose. Two of the enzymes produced 4-*O*-glucosides, but neither recognized caffeic acid. Subsequently, further work demonstrated that another of the UGTs initially screened, UGT71C1, whereas not producing the glucose ester nor the 4-*O*-glucoside of caffeic acid, did in fact produce the 3-*O*-glucoside. Given the wide significance of caffeic acid glucosides, both *in planta* and as potential dietary supplements, we have now examined the activity of UGT71C1 in greater detail. This study describes the findings obtained and investigates the potential use of this enzyme for increasing the yield of the glucoside(s) in transgenic plants.

Abbreviations used: UGT, UDP-glucose:glucosyltransferase; CaMV35S promoter, cauliflower mosaic virus 35 S promoter.

¹ This work is dedicated to the memory of Professor Jeffrey Harborne, with appreciation for his contribution in the identification of caffeoyl-3-*O*-glucoside and many other secondary metabolites.

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The nucleotide sequence for UGT71C1 reported in this paper has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number AC005496.

EXPERIMENTAL

Materials

The majority of the phenolic compounds used in this study were purchased from Sigma-Aldrich. *m*-Coumaric acid was purchased from Fluka Chemika. Eriodictyol, luteolin and cyanidin were purchased from Apin Chemicals.

Plasmids

The UGT71C1 DNA fragment was amplified from *A. thaliana* Columbia genomic DNA by PCR with oligomer 5'-GGGT-GATCAGGTACCATGGGGAAGCAAGAAGATG-3' (5' primer) and 5'-CGGAATTCGTCGACTACTTACTTATAGAAACGCCG-3' (3' primer) since previous sequence analysis has suggested that this UGT sequence does not contain any introns [28]. The DNA fragment was amplified and purified following the conditions described previously [32], and was subcloned into the *Bam*HI and *Sma*I sites on the multiple cloning site of the glutathione S-transferase (GST) gene fusion vector pGEX-2T (Amersham Biosciences) to generate the fusion plasmid.

To construct the binary vector in order to express UGT71C1 cDNA in *A. thaliana* under the control of the cauliflower mosaic virus 35 S (CaMV35S) promoter, the UGT71C1 cDNA fragment was subcloned into the *Kpn*I and *Sal*I sites on the multiple cloning site of the binary vector pJR1Ri.

Recombinant UGT71C1 purification and glucosyltransferase activity assay

Recombinant UGT71C1 was purified as a GST-UGT71C1 fusion protein from *Escherichia coli* carrying the expression plasmid and the glucosyltransferase activity was measured following the method described previously [30]. Each assay mix (200 μ l) contained 1 μ g of recombinant protein, 50 mM Tris/HCl, pH 7.0, 14 mM 2-mercaptoethanol, 5 mM UDP-glucose and 1 mM phenolic substrate. The reaction was carried out at 30 °C for 30 min, and was stopped by the addition of 20 μ l of trichloroacetic acid (240 mg/ml), quick-frozen and stored at -20 °C prior to reversed-phase HPLC analysis. The specific enzyme activity was expressed in nmol of phenolic compound glucosylated/s (nkat) by 1 mg of protein in a 30-min reaction.

Plant transformation

The transgenic plants were made in *A. thaliana* ecotype Columbia background. The binary plasmid carrying UGT71C1 cDNA was introduced into *A. thaliana* via *Agrobacterium tumefaciens*-mediated vacuum infiltration [33]. To select the transgenic plants, seeds collected from vacuum-infiltrated plants were surface-sterilized with 10% (v/v) Chlorox solution (Scientific Laboratory Supplies, Nottingham, U.K.) with a drop of Tween 80 followed by 70% (v/v) ethanol, and were germinated on ATS agar [10 g/l sucrose, 5 ml/l 1 M KNO₃, 2.5 ml/l 1 M KPO₄, pH 5.5, 2 ml/l 1 M MgSO₄, 2 ml/l 1 M Ca(NO₃)₂, 2.5 ml/l 20 mM EDTA, 1 ml/l micronutrients and 0.8% (w/v) Oxoid agar] containing kanamycin (50 mg/l) and cefotaxime (50 mg/l). All plants were homozygous for the transgene. The control plant, designated Ri, was transformed with an empty pJR1Ri vector.

The plants were grown in Levington's seed and modular compost in a controlled environment of 16 h:8 h light/dark cycle (22 °C, \approx 150 μ mol of photons \cdot m⁻² \cdot s⁻¹ in the light; 18 °C in

the dark). After 4 weeks, aerial tissues were harvested and frozen in liquid nitrogen for RNA and crude-protein extraction.

Northern blot analysis

Total RNA was extracted from the transgenic plants following the method of Verwoerd et al. [34]. An aliquot of total RNA (10 μ g) was separated on 0.6 M formaldehyde/1.4% (w/v) agarose containing 0.5 μ g/ml ethidium bromide, blotted on to Hybond-N membrane, UV-cross-linked (120 mJ) and probed with radiolabelled DNA fragment prepared with random primer.

Crude-protein extraction from transgenic plants and the glucosyltransferase activity assay

To obtain the crude protein extract from the transgenic plants, 1 g of frozen tissue was ground to fine powder in liquid nitrogen using a pestle and mortar. Extraction buffer [1 ml; 25 mM Tris/Mes, pH 6.5, 10% (v/v) glycerol, 20 mM 2-mercaptoethanol, 1 mM PMSF and 1% polyvinylpyrrolidone (w/w of aerial tissue)] was added to the powder and the slurry was thawed on ice. Subsequently the slurry was mixed vigorously and centrifuged at 10 500 g and 4 °C for 20 min. Then 850 μ l of the supernatant was collected and transferred to a 1.5-ml microfuge tube and centrifuged further at 15 000 g and 4 °C for 5 min. The resulting supernatant was collected for glucosyltransferase activity assay. The protein concentration assay was carried out with Bio-Rad Protein Assay Dye using BSA as a reference protein.

To investigate the glucosyltransferase activity of the crude protein extracts prepared from plant tissues, 50 μ l of crude protein extracts (containing 0.1–0.3 mg of total protein) were mixed with 1 mM phenolic compound, 5 mM UDP-glucose and 50 mM Tris/HCl, pH 7.0, in a 100- μ l reaction. The reactions were incubated at 30 °C for 30 min and were stopped by the addition of 10 μ l of trichloroacetic acid (240 mg/ml). The reaction mixtures were analysed subsequently using reversed-phase HPLC.

HPLC analysis of *in vitro* reaction mixture

Reversed-phase HPLC (SpectraSYSTEM HPLC systems and UV6000LP photodiode array detector; ThermoQuest) analysis was carried out using a Columbus 5 μ C₁₈ column (250 mm \times 4.60 mm; Phenomenex). A linear gradient of acetonitrile in water (all solutions contained 0.1% trifluoroacetic acid) at 1 ml/min over 20 min was used to separate the glucose conjugates from their aglycone. The HPLC methods were as follows: *o*-coumaric acid and *m*-coumaric acid, $\lambda_{260 \text{ nm}}$, 10–50% acetonitrile; *p*-coumaric acid, $\lambda_{260 \text{ nm}}$, 10–25% acetonitrile; caffeic acid, $\lambda_{296 \text{ nm}}$, 10–16% acetonitrile; ferulic acid and sinapic acid, $\lambda_{296 \text{ nm}}$, 10–40% acetonitrile; eriodictyol, $\lambda_{264 \text{ nm}}$, 10–75% acetonitrile; luteolin, $\lambda_{324 \text{ nm}}$, 10–70% acetonitrile; quercetin, $\lambda_{334 \text{ nm}}$, 10–75% acetonitrile; catechin, $\lambda_{278 \text{ nm}}$, 10–15% acetonitrile; cyanidin, $\lambda_{524 \text{ nm}}$, 10–35% acetonitrile.

HPLC analysis of the glucosides in transgenic plants

To analyse the amounts of the glucosides of interest in the transgenic plants, 1 g of plant tissue was freeze-dried and homogenized in 5 ml of methanol. *m*-Coumaric acid was added as an internal control to monitor sample loss during the extraction. The slurry was left at room temperature for 30 min followed by centrifugation at 5000 g. The supernatant was collected and dried *in vacuo*. The powder was dissolved in 1 ml of

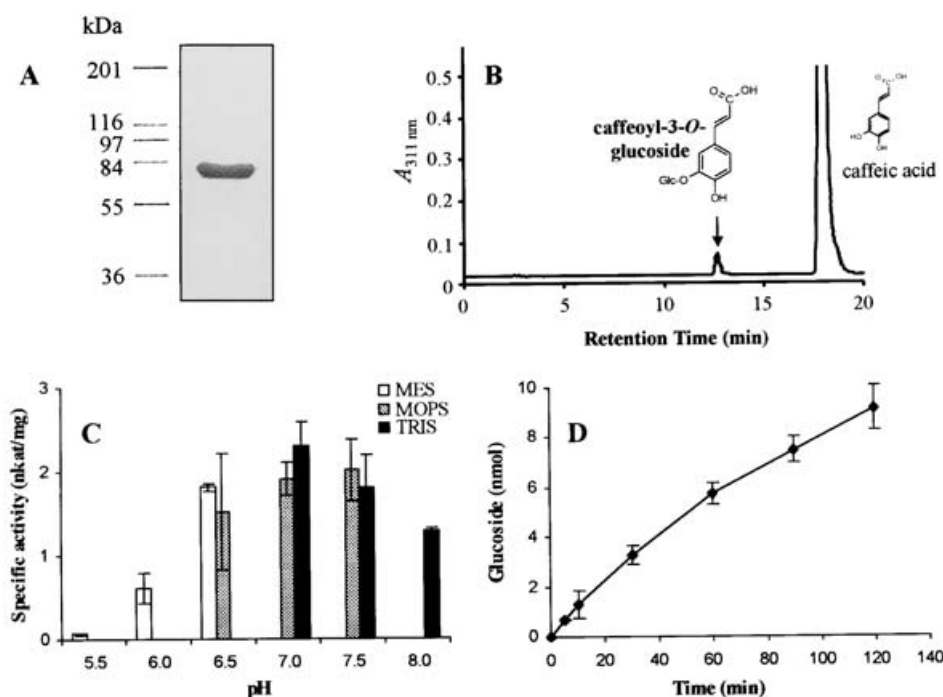


Figure 1 UGT activity towards the 3-OH of caffeic acid

(A) The fusion protein UGT71C1 (1 μ g) was purified from *E. coli* using glutathione-coupled Sepharose, analysed using SDS/PAGE [10% (w/v) gel], and visualized with Coomassie Brilliant Blue staining. (B) HPLC chromatograph of a standard reaction mix containing recombinant UGT71C1, UDP-glucose and the substrate caffeic acid. (C) pH optimum of UGT71C1 activity was measured over the range pH 5.5–8.0 in the reactions (200 μ l) containing 50 mM buffer, 1 μ g of recombinant enzyme, 1 mM caffeic acid, 5 mM UDP-glucose and 14 mM 2-mercaptoethanol. The specific enzyme activity was expressed as nmol of caffeic acid glucosylated/s (nkat) by 1 mg of protein in 30 min at 30 °C. (D) The time course of UGT71C1 activity was studied by measuring the amount of caffeic acid glucosylated by 1 μ g of recombinant enzyme in 50 mM Tris/HCl, pH 7.0. The results represent means \pm S.D. from three replicates.

80% methanol, passed through octadecyl cartridge (JT Baker Bakerbond, prewashed with 80% methanol) and collected for HPLC analysis. The HPLC analysis was carried out as described in the previous section with some modifications. The methanol extract was separated with a linear gradient of acetonitrile in water (10% at 0 min, 16% at 80 min, 45% at 100 min and 100% at 105 min). The flow rate was 1 ml/min. The UV spectra between 200 and 400 nm were recorded. To determine the retention time of the glucosides of interest, the plant extracts were spiked with glucosides synthesized from the *in vitro* reaction.

¹H- and ¹³C-NMR analyses

The caffeoyl-3-*O*-glucoside for NMR analysis was purified using HPLC and a Gilson FC204 fraction collector. The purified glucoside was freeze-dried and resuspended in deuterated methanol. The NMR spectra were acquired on a Bruker AMX 500-MHz NMR spectrometer at 22 °C. The data were processed and analysed using Bruker XWIN-NMR software version 2.6.

RESULTS

Characterization of the activity of UGT71C1 towards caffeic acid and other substrates *in vitro*

A recombinant fusion protein of UGT71C1 was purified from *E. coli* (Figure 1A) and assayed *in vitro* against caffeic acid. As shown in Figure 1(B), the enzyme produced one product, which was confirmed by ¹H- and ¹³C-NMR as the 3-*O*-glucoside of caffeic acid (Table 1). The pH optimum and the linearity of the

Table 1 ¹H- and ¹³C-NMR spectral data of caffeic acid and caffeoyl-3-*O*-glucoside

¹H- and ¹³C-NMR spectra were recorded in deuterated methanol at 500 and 125 MHz respectively. Chemical shifts are given on a p.p.m. scale with tetramethylsilane as internal standard. The position on the aromatic ring begins with the carbon joining the propanoic acid.

¹ H- and ¹³ C-NMR spectral data (p.p.m.)				
Position	Caffeic acid		Caffeoyl-3- <i>O</i> -glucoside	
	δ_H	δ_C	δ_H	δ_C
C1	–	128.1	–	127.6
C2	7.02 (1H, <i>d</i> , <i>J</i> = 2.0 Hz)	115.2	7.47 (1H, <i>d</i> , <i>J</i> = 2.0 Hz)	117.0
C3	–	146.7	–	146.0
C4	–	149.4	–	150.6
C5	6.77 (1H, <i>d</i> , <i>J</i> = 8.0 Hz)	116.6	6.84 (1H, <i>d</i> , <i>J</i> = 8.5 Hz)	117.8
C6	6.92 (1H, <i>dd</i> , <i>J</i> = 8.0, 2.0 Hz)	122.8	7.13 (1H, <i>dd</i> , <i>J</i> = 8.5, 2.0 Hz)	125.6
C7	7.53 (1H, <i>d</i> , <i>J</i> = 16.0 Hz)	146.9	7.45 (1H, <i>d</i> , <i>J</i> = 14.5 Hz)	146.6
C8	6.21 (1H, <i>d</i> , <i>J</i> = 15.5 Hz)	116.3	6.33 (1H, <i>d</i> , <i>J</i> = 14.5 Hz)	116.1
C9	–	171.5	–	170.4
Glc-1			≈ 4.86 (signal interrupted)	103.9
Glc-2			} 3.40–3.50(4H, <i>m</i>)	74.5
Glc-3				78.0
Glc-4				71.0
Glc-5				77.2
Glc-6			3.93 (1H, <i>dd</i> , <i>J</i> = 12.0, 2.0 Hz)	62.4
			3.71 (1H, <i>dd</i> , <i>J</i> = 12.0, 5.5 Hz)	

enzyme activity under the assay conditions used are shown in Figures 1(C) and 1(D). Whereas UGT71C1 recognized only the 3-*O*H of caffeic acid, it was unclear whether this regioselectivity

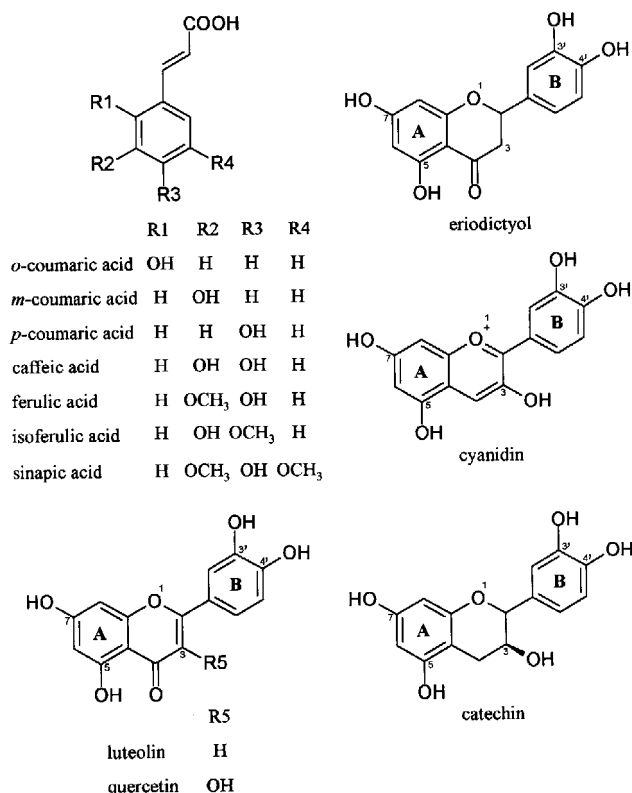


Figure 2 Chemical structures of the compounds described in this study

Table 2 Glucosyltransferase activity of UGT71C1 towards different substrates

The specific enzyme activity was expressed in nmol of substrate converted into its glucose conjugate/s (nkat) by 1 mg of protein in the presence of 1 mM substrates in 30 min. The Michaelis–Menten kinetics of UGT71C1 glucosyltransferase activity were measured over a range of 0–5 mM substrate. The K_m (mM) and k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$) values were derived using data from the Lineweaver–Burk plot. The results represent the means \pm S.D. from three replicates. –, not applicable; N.D., not determined.

Substrate	Specific activity (nkat/mg)	K_m (mM)	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{s}^{-1}$)
Hydroxycinnamates			
Caffeic acid	2.9 ± 0.8	0.32	0.91
<i>o</i> -Coumaric acid	1.5 ± 0.2	0.80	0.24
<i>m</i> -Coumaric acid	1.2 ± 0.2	0.69	0.27
<i>p</i> -Coumaric acid	0	–	–
Ferulic acid	0	–	–
Isoferulic acid	2.8 ± 0.2	N.D.	N.D.
Sinapic acid	0	–	–
Flavonoids			
Quercetin	1.4 ± 0.4	0.09	12.00
Luteolin	0.7 ± 0.1	0.12	4.73
Eriodictyol	0	–	–
Catechin	0	–	–
Cyanidin	0	–	–

would always be maintained. Thus a range of compounds was assayed in which free hydroxyl groups were available at different positions on the benzene ring (Figure 2). As shown in Table 2, predictably no activity was shown towards the 4-OH position on other phenylpropanoids, but the enzyme did recognize the 3-OH of *m*-coumaric acid and the 2-OH of *o*-coumaric acid.

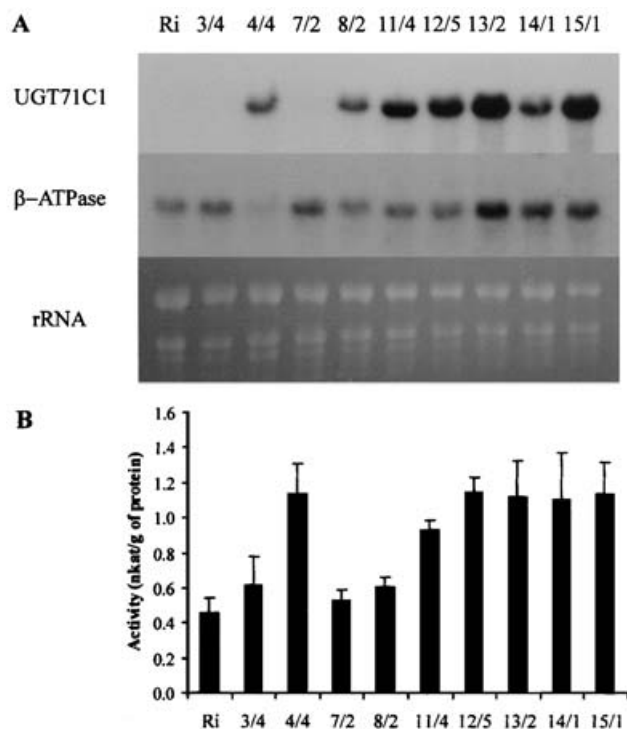


Figure 3 Analysis of the transgenic plants over-expressing UGT71C1 cDNA under the control of the CaMV35S promoter

(A) Aerial tissues from 4-week-old transgenic plants and the control plant Ri were used to analyse the steady-state levels of mRNA. Total RNA (10 μg) was analysed by 1.4% (w/v) agarose gel (bottom panel). After electrophoresis, the RNA was transferred on to nylon membrane and subsequently probed with radiolabelled UGT71C1 (top panel) and β -ATPase (middle panel) DNA fragments. (B) Crude protein extracts were prepared from the aerial tissues of 4-week-old transgenic plants and the control plant Ri. The UGT activity was measured in the reactions (100 μl) containing 50 mM Tris/HCl, pH 7.0, 0.1–0.3 mg of total protein, 1 mM caffeic acid and 5 mM UDP-glucose. The specific enzyme activity was expressed as nmol of caffeic acid glucosylated/s (nkat) by 1 g of protein in 30 min of reaction time at 30 $^{\circ}\text{C}$. The results represent means \pm S.D. from three replicates.

Whereas UGT71C1 did not glucosylate ferulic acid, the enzyme did recognize isoferulic acid, which has a hydroxyl group on the 3 position, to form the corresponding glucoside (Table 2). More complex compounds were analysed, such as the flavonoids, with a 3'-OH group on the benzene ring similar to that of caffeic acid. The data show that UGT71C1 glucosylated quercetin. Interestingly, the products formed with quercetin were found to be the 3'-*O*-glucoside, equivalent to that observed for caffeic acid, and the 7-*O*-glucoside together with the 7,3'-di-*O*-glucoside. The identities of these products have been confirmed by NMR within a wider study of regioselective glucosylation of quercetin by many members of the UGT family (E.-K. Lim and D.J. Bowles, unpublished work). Similarly, when luteolin was the substrate, identical hydroxyl groups to those on the closely related quercetin were again glucosylated (results not shown).

Expression of UGT71C1 in transgenic *Arabidopsis* plants

The expression of UGT71C1 in transgenic *Arabidopsis* plants was driven by the constitutive CaMV35S promoter. Independent transgenic lines were taken to homozygosity and were characterized together with a control line transformed with an empty vector (Ri). Figure 3(A) shows the steady-state levels of mRNA corresponding to UGT71C1 in aerial tissues of 4-week-old plants

of each of the lines. Of the nine independent lines analysed, seven expressed the transgene at high level (4/4, 8/2, 11/4, 12/5, 13/2, 14/1 and 15/1) and two showed negligible expression (3/4 and 7/2). Figure 3(B) shows results of assays *in vitro* in which leaf extracts from each of the transgenic lines were incubated with caffeic acid. The changes in the level of UGT activity in the extracts corresponded to the changes in steady-state level of the mRNAs shown in Figure 3(A). Thus constitutive over-expression of UGT71C1 in *Arabidopsis* leads to an elevated level of UGT activity in the green tissue measured.

Identification of the caffeoyl-3-*O*-glucoside in transgenic lines over-expressing UGT71C1

Two approaches were used to analyse the extracts for the glucoside. Measurement of the retention time following application of the sample to the HPLC system was complemented by use of a photo-diode array detector. Thus the metabolite peak was characterized by both retention time and photo-diode array profile. Figure 4(A) shows two typical HPLC chromatographs. These compare the target profiles of leaf extracts from the control line (Ri) and a high-expressor line (4/4). There is a peak at 15.56 min present only in the transgenic lines. This peak co-elutes with a standard caffeoyl-3-*O*-glucoside and its identity was further confirmed by its photo-diode array profile (Figure 4B).

The same procedure was applied to all of the transgenic lines constructed. Levels of caffeoyl-3-*O*-glucoside were found generally to correspond again to levels of recombinant enzyme activity (comparing Figures 4C and 3B) and ranged from less than 0.5 to 2.0 nmol/g of fresh weight. The HPLC analysis was extended to investigate the existence of additional glucosides of the substrates recognized *in vitro* by UGT71C1. No other increased glycosides were detected, neither quercetin glucosides nor rhamnosides (results not shown).

DISCUSSION

Earlier work from our laboratory identified a number of UGTs from *Arabidopsis* that recognized hydroxycinnamic acids [30]. Those studies focused on the recombinant enzymes capable of producing glucose esters and 4-*O*-glucosides, including the UGTs involved in sinapate metabolism and monolignol glucosylation. This study extends the analysis of regioselective glucosylation through the identification and characterization of a UGT capable of producing the 3-*O*-glucoside of caffeic acid. We show that UGT71C1 recognizes only the 3-OH position of caffeic acid and that this regioselectivity is retained when the gene encoding the UGT is expressed *in planta*.

Whereas the recombinant enzyme glucosylates caffeic acid *in planta*, the yield of product in transgenic *Arabidopsis* was surprisingly low. There are several possible explanations for this observation. First, caffeic acid is at a central position leading to different biosynthetic pathways of secondary metabolism. For example, the metabolite is at the branch point leading to caffeoyl-CoA or ferulic acid [35]. The precursor of caffeic acid is also the entry point into flavonoid biosynthesis and benzoate metabolism [36]. Thus it is likely that at any one time levels of the free acid will be low, and this could constrain glucoside accumulation even under circumstances in which UGT activity is high. In a recent study, the expression of caffeoyl-CoA 3-*O*-methyltransferase in alfalfa was knocked out and the consequences on metabolite levels were analysed [37]. Blocking the methylation in this way led to the accumulation of a glucosylated species of caffeic acid, which in further analyses was shown to be the glucose ester (R. Dixon,

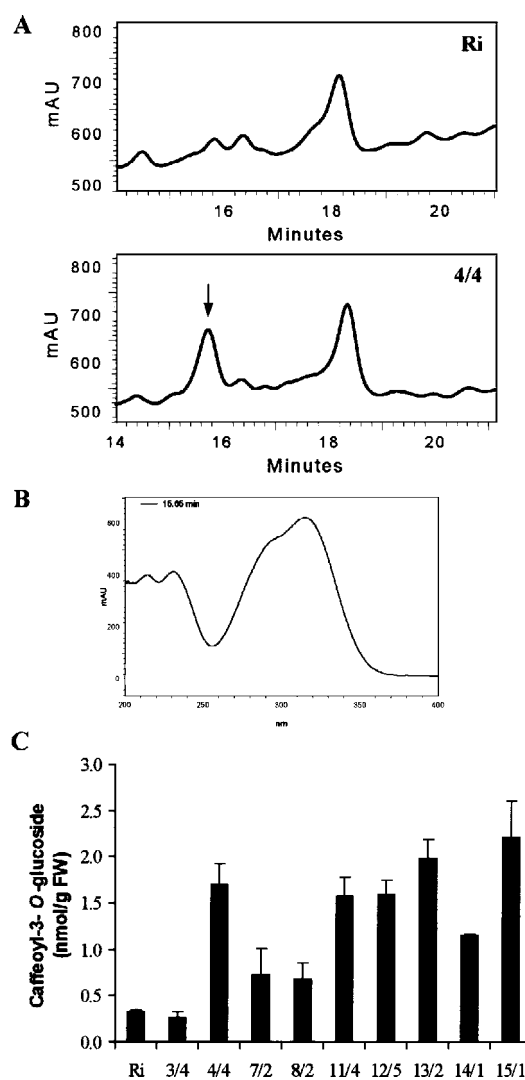


Figure 4 HPLC analysis of caffeoyl-3-*O*-glucoside in the transgenic plants over-expressing UGT71C1 cDNA under the control of the CaMV35S promoter

(A) HPLC chromatograph of the control line Ri (top panel) was compared with that of the transgenic line 4/4 (bottom panel). The peak corresponding to caffeoyl-3-*O*-glucoside is labelled with an arrow. (B) Photo-diode array profile of the peak labelled with an arrow in (A); this profile was identical with that of a standard caffeoyl-3-*O*-glucoside. (C) The amount of the caffeoyl-3-*O*-glucoside in all the transgenic lines assayed was quantified using purified caffeoyl-3-*O*-glucoside as a standard. The results represent the means \pm range from two replicates.

personal communication). These data suggest that if caffeic acid is made to accumulate abnormally, one of the detoxification mechanisms in the plant to regain cellular homeostasis is glucosylation. Thus over-expression of UGT71C1 in transgenic lines that already contain high levels of caffeic acid may increase the yield of caffeoyl-3-*O*-glucoside further.

A second reason for low yields of caffeoyl-3-*O*-glucoside could be the existence of additional metabolites *in planta* such as those compounds also recognized by the enzyme *in vitro*. However, we could not detect any additional glycosides, including those of quercetin, in the tissues and developmental stages that we analysed. It is possible that expression of UGT71C1 in other plant species may give very different results from those obtained for *Arabidopsis*, dependent on the availability and range of metabolites present. It is important to note that our study does not address the nature of the physiological substrate for

UGT71C1. Rather, our intention has been to take an enzyme of known regioselectivity and substrate recognition *in vitro* and investigate whether its gene can be used to elevate the level of the same glucoside *in vivo*.

The uses of many medicinal natural products are limited due to their lack of stability and solubility. Glucosylation can improve the solubility of many compounds, but the process is notoriously difficult to achieve by chemical synthesis [26]. Recent new methods involving the reverse action of glucosidases have been developed and, while elegant, they nevertheless remain inefficient [26]. In contrast, native plant glucosyltransferases catalyse a simple reaction with high regioselectivity, whether the enzymes are purified and function *in vitro* or whether they catalyse natural product formation *in planta*. We have now demonstrated that the regioselectivity of a recombinant UGT is maintained in a transgenic plant, and the consequences of its expression are an increase in enzyme activity and the accumulation of the appropriate glucosylation product. These data suggest that use of recombinant UGT genes may provide a useful new route for the biotransformation and selective accumulation of natural products *in planta*.

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