FEBS Letters 580 (2006) 6915–6920

Phenylpropanoid glycosyltransferases from osage orange (*Maclura pomifera*) fruit☆

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Received 16 November 2006; accepted 23 November 2006

Available online 4 December 2006

Edited by Ulf-Ingo Flügge

Abstract **Flavonoids and isoflavonoids are well known for their beneficial effects on human health and their anti-insect and anti-microbial activities in plants. Osage orange fruit is rich in prenylated isoflavones and dihydrokaempferol and its glucoside. Four glycosyltransferases were identified from a collection of osage orange fruit expressed sequence tags. Biochemical characterization suggested that the glycosyltransferase UGT75L4 might be responsible for glucosylation of dihydrokaempferol in vivo, although this enzyme exhibited broad substrate recognition toward isoflavonoids and flavonoids in vitro. UGT88A4 was active on coumarin substrates. Identification of highly active phenylpropanoid glycosyltransferases will facilitate the metabolic engineering of glycosylated natural products in plants.**

Keywords: Glycosyltransferase; Osage orange fruit; Flavonoid; Isoflavonoid

1. Introduction

Osage orange (*Maclura pomifera*) is a tree in the Moraceae family [1]. The common name is derived from its fruit, which resembles the shape of an orange, and from the fact that its hardwood was used by the Osage Indian tribe to make bows. Osage orange is native to Southern Oklahoma and Northern Texas, and is planted throughout the United States. Although the fruit is not edible for humans, its extract exhibits anti-microbial and anti-insect activities, and the Native Americans have used osage orange for cancer treatment [2].

Osage orange fruit accumulates a group of flavonoid, isoflavonoid and triterpene compounds [3–7]. Two predominant isoflavones, pomiferin and osajin, are derived from the simple isoflavone genistein by prenyl substitutions ([7]; Fig. 1A). In addition to its anti-microbial activity, pomiferin is also a strong antioxidant, with similar activity to vitamins C and E [8]. Kaempferol-7-*O*-glucoside and dihydrokaempferol-7-*O*-glucoside were identified from ethyl acetate extracts of osage orange fruit, and were the only flavonoids extracted from stems and leaves [4,6]. The triterpenes lupeol, butyrospermol, lupane-3β, 20-diol and 19α-H-lupeol were also reported to be present in the fruit [3,5].

Flavonoids and isoflavonoids share common biosynthetic routes from L-phenylalanine or tyrosine [9]. Studies have identified their roles in plant disease resistance, and they hold great potential as nutraceuticals [10,11]. Flavonoids and isoflavonoids exist in plants mostly in their glycosylated forms. Glycosylation alters their stability, solubility and activity, and facilitates their transport to the vacuole for long-term storage. The attachment of sugar groups to flavonoid/isoflavonoid compounds is catalyzed by members of a large family of enzymes, the uridine diphosphate glycosyltransferases (UGTs) [12]. The three dimensional structures of two plant family 1 UGTs, both active on a range of flavonoid and isoflavonoid substrates in vitro, have recently been solved. The acceptor molecule and the sugar donor bind to the N-terminal and C-terminal halves of the protein, respectively [13,14].

We here report the isolation and functional characterization of UGTs from osage orange fruit. UGT75L4 likely accounts for in vivo glucosylation of dihydrokaempferol (Fig. 1A). The broad spectrum substrate recognition of UGT75L4 toward flavonoids/isoflavonoids, and functionality of UGT88A4 toward coumarins, are also described.

2. Materials and methods

2.1. Plant material and chemicals

Osage orange fruit was collected locally (Ardmore, Oklahoma) in August 2004. Fruit was cut into small pieces, frozen in liquid nitrogen and stored at −80 °C. Flavonoid and isoflavonoid aglycone and glycoside standards were purchased from Indofine (Hillsborough, NJ) and Apin (Oxon, UK), respectively.

2.2. RNA isolation, cDNA library construction, EST sequencing and analysis

Total RNA from osage orange fruit was extracted as previously described [15]. One microgram total RNA was used as template for reverse transcription reaction and a directional cDNA library was constructed using the Creator SMART cDNA library construction kit following the manufacturer's instructions (Clontech, Mountain View, CA). Nine hundred and sixty clones were picked randomly and plasmids were purified and sequenced with the M13 forward primer. Vector and poor quality sequences were removed. The cleansed EST sequences were assembled into 590 unigenes. The unigene sequences were searched against the NCBI non-redundant database

☆The GenBank accession numbers for the nucleotide sequences reported in this paper are: UGT72B9 (DG985177); UGT88A4 (DG985176); UGT71A13 (DG985178); UGT75L4 (DG985179).

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Abbreviations: HPLC, high performance liquid chromatography; GT, glycosyltransferase; UGT, UDP-glycosyltransferase; RACE, rapid amplification of cDNA ends; LC–MS, liquid chromatography–mass spectrometry

0014-5793/$32.00
doi:10.1016/j.febslet.2006.11.056

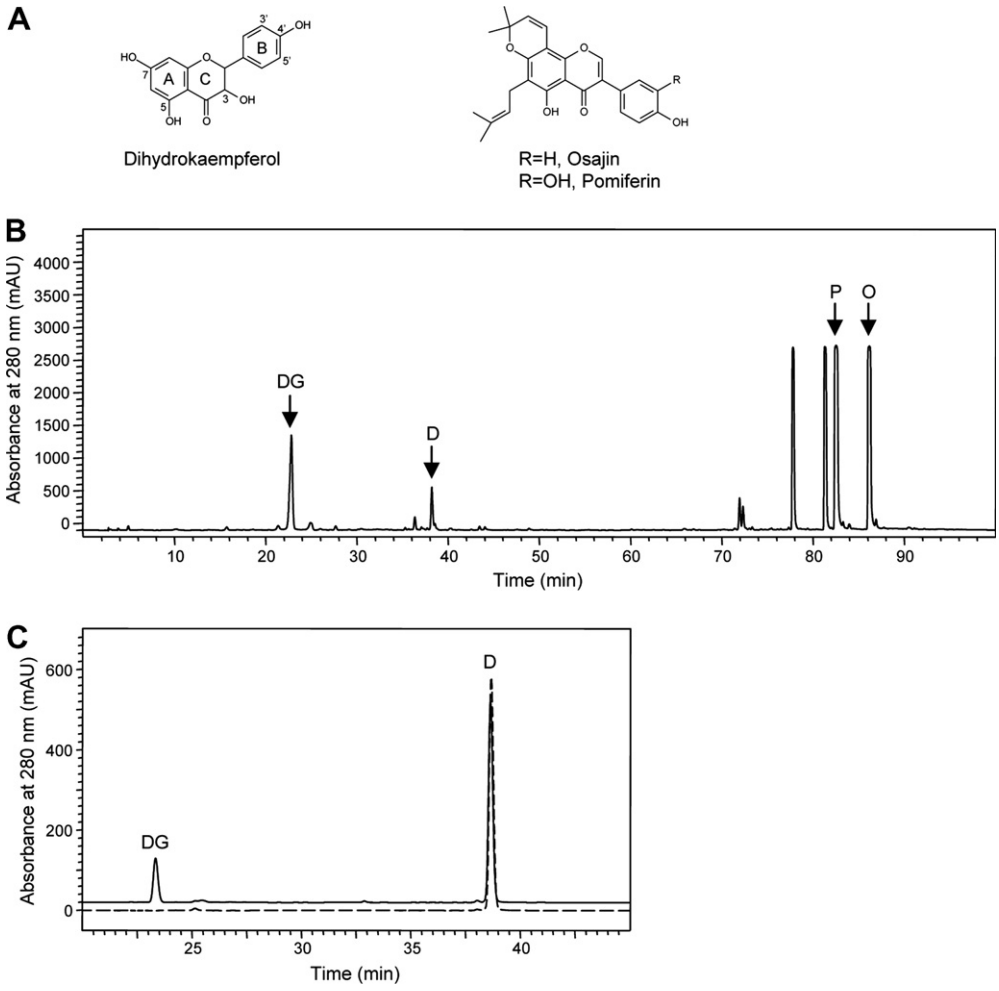

Fig. 1. (A) Chemical structures of flavonoid and isoflavonoid aglycones identified from osage orange fruit. (B) HPLC elution profile of ethyl acetate extract of osage orange fruit. Fractions of major peaks were collected and subjected to LC–MS analysis (Supplementary Fig. 1). (C) HPLC elution profiles of UGT assay mixtures with UDP-glucose and dihydrokaempferol, incubated in the presence (solid trace) or absence (dashed trace) of purified UGT75L4. D, dihydrokaempferol; DG, dihydrokaempferol glucoside; P, pomiferin; O, osajin.

using the BLASTX algorithm [16]. 5′-RACE PCR reactions were performed for ESTs that lacked the 5′ sequence. Primers used for rapid amplification of cDNA ends (RACE) PCR, sequencing and TOPO cloning of the full length cDNAs are listed in Supplementary Table 1.

Deduced amino acid sequences of UGTs from osage orange, Arabidopsis, *Medicago truncatula* (UGT71G1) and *Vitis vinifera* (VvGT1) were aligned using the ClustalW algorithm [17]. This alignment was used to construct a neighbor-joining tree using the PAUP program. Positions with gaps were excluded from the distance calculation. Bootstrap analysis was performed to test the reliability of the branches (1000 replicates). GenBank accession numbers for UGT71G1 and VvGT1 are AAW56092 and AF000372, respectively. The Arabidopsis UGT sequences were obtained from http://www.p450.kvl.dk/UGT.shtml.

2.3. Total flavonoid extraction, UGT enzyme assay and HPLC analysis

Osage orange fruit was ground in liquid nitrogen. The fine powder was extracted with ethyl acetate and dried under a stream of nitrogen. Dried residue was resuspended in methanol and injected on a reverse phase high performance liquid chromatography (HPLC) column as previously described, with a slight modification of the HPLC gradient [18]. Elution from 50% B (acetonitrile) to 100% B was for 24 min, instead of 4 min as in the original program.

Osage orange UGTs were subcloned into the pENTR-D vector and then recombined into pDEST17 expression vector (Invitrogen, Carlsbad, CA). Insert-containing plasmids were transformed into *E. coli* BL21 (DE3) competent cells by electroporation. Cells were grown at 37 °C until the OD_{600} reached 0.8, and protein expression was induced by adding isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM. Induction was at 16 °C overnight, after which the cells were harvested at $3000 \times g$ for 20 min. The His-tagged proteins were purified using MagneHis kits following the manufacturer's instructions (Promega, Madison, WI). Protein concentration was determined by the Bradford assay [19]. The yields of recombinant proteins were: 58 ± 8 μg per 10 ml *E. coli* culture ($n = 3$) for UGT71A13, 49 ± 5 μg ($n = 3$) for UGT72B9, 62 ± 11 μg (n = 5) for UGT75L4 and 55 ± 7 μg ($n = 3$) for UGT88A4.

The UGT reaction mix (total volume of 50 μl) contained 2 μg purified recombinant protein, 200 μM flavonoid/isoflavonoid/coumarin substrate, 5 mM UDP-glucose or UDP-glucuronic acid, and 50 mM Tris–HCl, pH 7.0. The reaction mix was incubated at 30 °C for 3 h and stopped by adding 2 μl of trichloroacetic acid (TCA; 240 mg/ml). Ten microliter of methanol was added to each reaction mix, and 50 μl of the reaction was injected on a reverse phase HPLC column and separation performed as described previously [18]. Identification of glycosylated product was based on both retention time and UV spectrum, by comparison either to authentic standards or to aglycones, where the conjugates showed similar UV spectra but altered retention time. Liquid chromatography-mass spectrometry (LC–MS) analysis was carried out as previously described [20].

For measurement of the steady-state kinetics of UGT75L4, the UGT assay mix (total volume of 50 μl) contained 2 μg purified recombinant protein, 50 mM Tris–HCl, pH 7.0, 10 μM UDP-[U-^{14}C]

glucose, 490 μM non-labeled UDP-glucose, and 0–500 μM dihydrokaempferol, dihydroquercetin, kaempferol, genistein or isoliquiritigenin. The reaction was carried out at 30 °C for 30 min, terminated by adding 5 μl TCA (240 mg/ml), and extracted with 250 μl ethyl acetate. Two hundred μl of the extract was used for radioactivity counting (Beckman LS6500). Kinetic parameters were calculated using the Hyper32 program (http://homepage.ntlworld.com/john.easterby/hyper32.html).

3. Results and discussion

3.1. Flavonoids and isoflavonoids in fruit of osage orange

To identify the major flavonoid and isoflavonoid constituents of the osage orange fruit sample, total flavonoids were extracted from fruit with ethyl acetate and separated on reverse phase HPLC (Fig. 1B). The peak eluting at 38.5 min was identified as the dihydroflavonol dihydrokaempferol (Fig. 1A) based on comparison of its retention time and UV spectrum with those of an authentic standard. The peak eluting at 23 min showed a UV spectrum almost identical to that of dihydrokaempferol, and LC–MS analysis revealed a hexose conjugate of dihydrokaempferol (Supplementary Fig. 1). Two major peaks eluting at the end of the gradient, and therefore hydrophobic in nature, were confirmed to be the prenylated isoflavones pomiferin and osajin (Fig. 1A) based on their UV and MS spectra (Supplementary Fig. 1). The compounds in the two major UV-absorbing peaks eluting just prior to osajin and pomiferin were not identified, but are unlikely to be glycosides based on their high retention times.

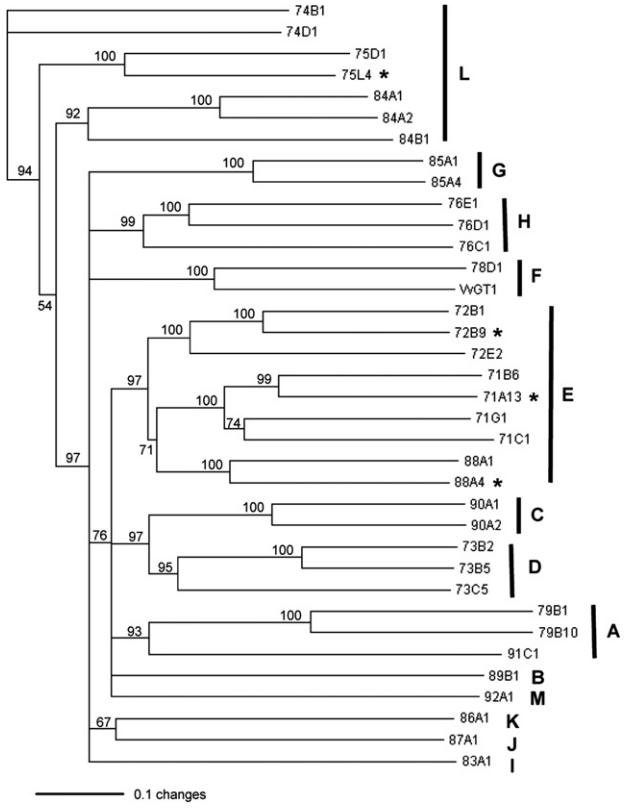

Fig. 2. Neighbor-joining tree of osage orange UGTs, representative Arabidopsis UGTs, *Medicago* UGT71G1 and *Vitis* VvGT1. Branch lengths are drawn to scale and boot strap values are indicated by the branches (1000 replicates). Thirteen major groups of Arabidopsis UGTs are delineated. Osage orange UGTs are indicated by asterisks.

3.2. Osage orange ESTs representing phenylpropanoid metabolic enzymes

To identify genes encoding enzymes involved in glycosylated flavonoid and prenylated isoflavone biosyntheses in osage orange fruit, a cDNA library was constructed using total RNA isolated from fruit tissue. Around 1000 ESTs were sequenced and assembled into 590 unigenes. ESTs encoding biosynthetic enzymes in the phenylpropanoid pathway, including caffeic acid 3-*O*-methyltransferase, cinnamyl alcohol dehydrogenase, 4-coumarate-CoA ligase, chalcone synthase and vestitone reductase, were present in the cDNA library (Supplementary Table 2).

Notably, four ESTs showed high similarity to UGTs (Supplementary Table 2). UGT72B9 was present as a full length EST, while UGT71A13, UGT75L4 and UGT88A4 lacked 5′ regions and their full length sequences were therefore obtained by 5′-RACE PCR (Supplementary Table 1). To investigate the evolutionary relationships between the osage orange UGTs and other plant UGTs, a neighbor-joining tree was constructed using a sequence alignment of the osage orange enzymes, representative Arabidopsis UGTs, UGT71G1 (*Medicago truncatula*) and VvGT1 (*Vitis vinifera*) (Fig. 2). The Arabidopsis UGTs were previously classified into 13 distinct lineage groups [21,22]. The osage orange UGT71A13, UGT72B9 and UGT88A4 clustered within group E, while UGT75L4 belonged to group L, suggesting that UGT75L4 evolved from a different ancestor than the other three UGTs (Fig. 2).

3.3. UGT75L4 is potentially involved in dihydrokaempferol glucosylation in vivo

Open reading frames of UGT72B9, UGT88A4, UGT71A13 and UGT75L4 were subcloned in the expression vector pDEST17. The plasmid constructs were functionally expressed in *E. coli* BL21 (DE3) cells, and the recombinant, N-terminal His-tagged fusion proteins were purified using MagneHis beads.

Total flavonoid extracts from osage orange fruit contained only dihydrokaempferol as the major glucosylated product (Fig. 1B). To search for UGT(s) that can glycosylate dihydrokaempferol, purified recombinant proteins of the four osage orange UGTs were incubated with dihydrokaempferol and UDP-glucose. Only UGT75L4 was able to convert dihydrokaempferol to its glucosylated form (Fig. 1C), as confirmed by HPLC retention time, UV spectrum, and LC–MS analysis (Supplementary Fig. 1). The other three UGTs showed no activity toward dihydrokaempferol (data not shown) (see Fig. 3).

Steady-state kinetics for UGT75L4 were determined with dihydrokaempferol and dihydroquercetin (3′-hydroxy dihydrokaempferol) (Table 1). K_m values for dihydroquercetin (23.05 μM) and dihydrokaempferol (20.51 μM) were similar, indicating that the binding affinities of the two dihydroflavonols for UGT75L4 were comparable. However, the enzyme showed much higher substrate specificity (k_{cat}/K_m) for dihydroquercetin than for dihydrokaempferol (Table 1), even though only glucosylated dihydrokaempferol was detected in the fruit extract (Fig. 1B).

3.4. UGT75L4 has broad substrate recognition for flavonoids and isoflavonoids

To investigate the overall acceptor spectrum of UGT75L4, a group of flavonoid and isoflavonoid substrates (structures

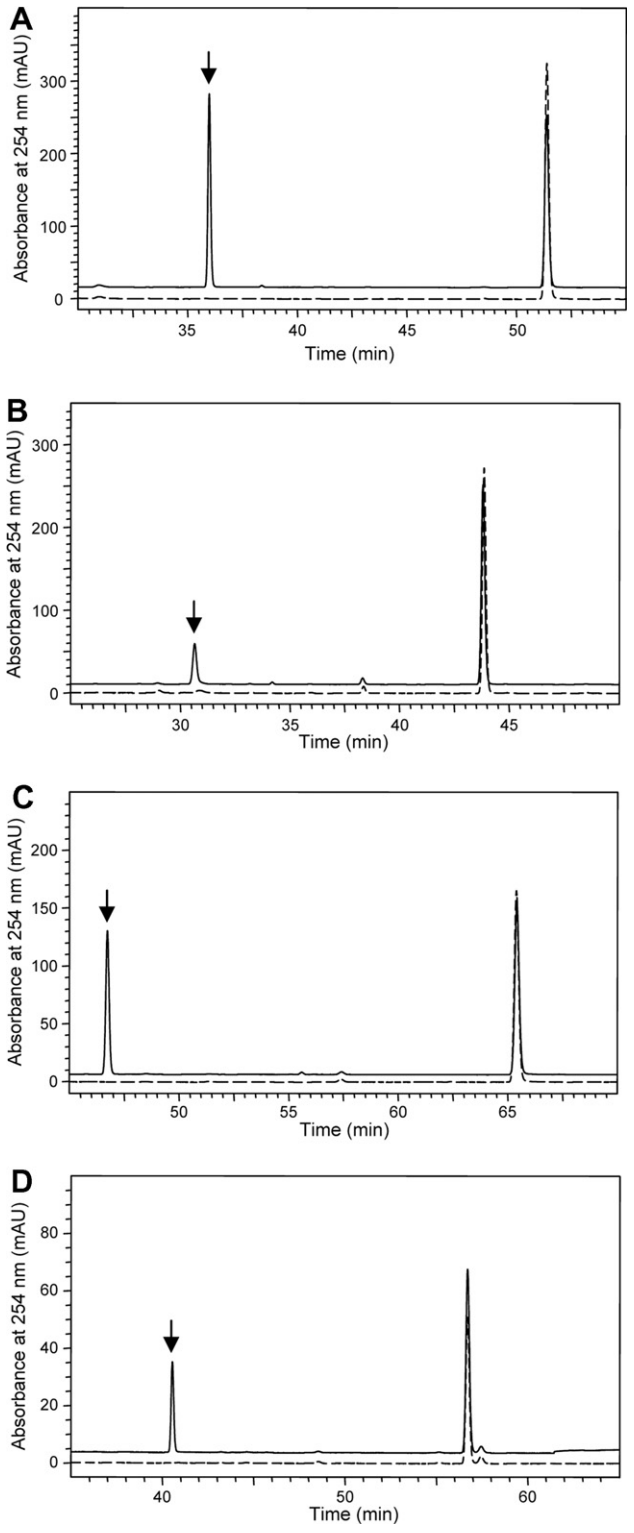

Fig. 3. HPLC elution profiles of UGT assay mixtures with UDP-glucose and the isoflavones genistein (A), daidzein (B), biochanin A (C) or formononetin (D), incubated in the presence (solid trace) or absence (dashed trace) of purified UGT75L4. Glucosylated products are indicated by arrows.

shown in Supplementary Fig. 2) were tested with the recombinant protein. The number of potential *O*-glucosylation sites for the isoflavone substrates tested were one for formononetin (7-hydroxy 4′-methoxyisoflavone), two for daidzein (7, 4′-dihydroxyisoflavone) and biochanin A (5,7-dihydroxy 4′-methoxyisoflavone), and three for genistein (5,7,4′-trihydroxyisoflavone). HPLC analysis showed that only a single product was formed with each substrate (Fig. 3), suggesting that

Table 1
Steady state kinetics of UGT75L4

	K_m (μM)	k_{cat} (10^{-4} s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Dihydrokaempferol	20.51	0.15	0.73
Dihydroquercetin	23.05	4.71	20.43
Kaempferol	2.99	25.22	843.47
Genistein	93.69	5.54	5.91
Isoliquiritigenin	59.33	8.22	13.85

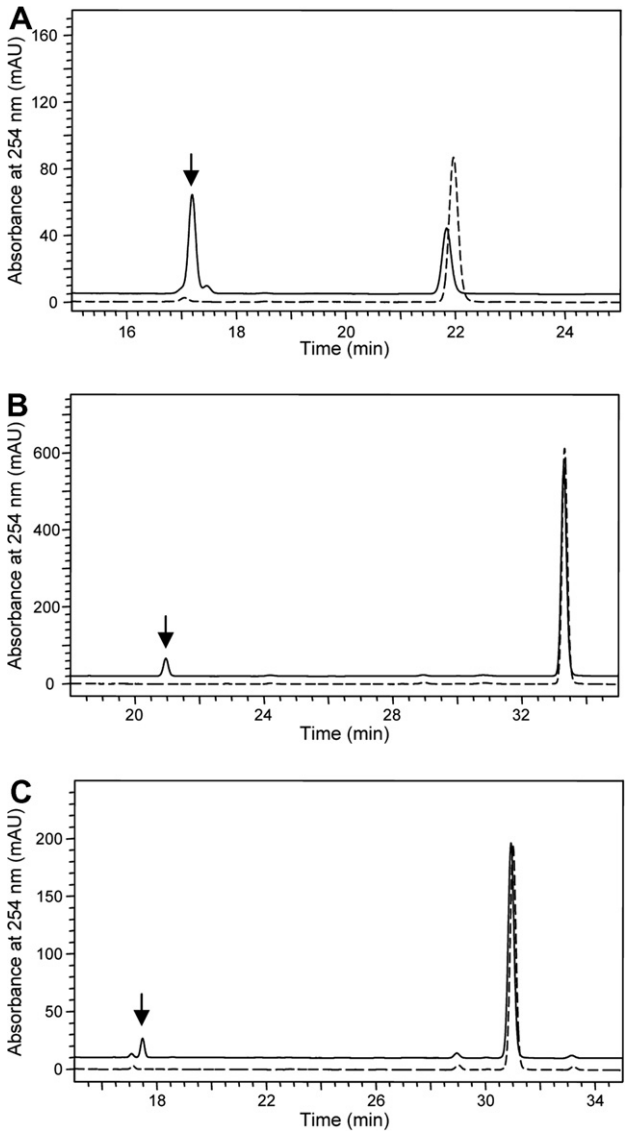

Fig. 4. HPLC elution profiles of UGT assay mixtures with UDP-glucose and the coumarins esculetin (A), scopoletin (B), or umbelliferone (C), incubated in the presence (solid trace) or absence (dashed trace) of purified UGT88A4. Glucosylated products are indicated by arrows.

glucosylation occurred on the 7-hydroxyl group of the A ring, common to all substrates. 7-*O*-glucosylation was confirmed for genistein and formononetin, for which authentic 7-*O*-glucoside standards were available.

Liquiritigenin (7,4′-dihydroxyflavanone), naringenin (5,7,4′-trihydroxyflavanone) and dihydroquercetin (3,5,7,3′4′-pentahydroxyflavanone) each gave a single glucosylated product, irrespective of the available hydroxyl groups, suggesting that UGT75L4 acted at either the 7- or 4′-hydroxyl groups of these flavanones/dihydroflavonols (Supplementary Fig. 3). In contrast, UGT75L4 was less regio-selective on flavone/flavonol substrates, which contain a C2–C3 double bond in the C-ring. Three glucosylated products were detected for apigenin (5,7,4′-trihydroxyflavone) and kaempferol (3,5,7,4′-trihydroxyflavone), suggesting that the 5-, 7-, and 4′-hydroxyl groups could all be glucosylated. However, when the 3′-position of the B ring was substituted by either hydroxylation or methoxylation, as in luteolin (5,7,3′,4′-tetrahydroxyflavone), chrysoeriol (5,7,4′-trihydroxy 3′-methoxyflavone) or quercetin (3,5,7,3′,4′-pentahydroxyflavone), only two glucosylated products were observed (Supplementary Fig. 4). This suggests that 3′-substitution of the flavonoid nucleus may affect the activity of the enzyme toward the 3- and 4′-hydroxyl groups.

The chalcone isoliquiritigenin and the coumestan coumestrol were also substrates for UGT75L4 (Supplementary Fig. 5). Isoliquiritigenin isomerizes to liquiritigenin non-enzymatically [23]. Peaks corresponding to glucosylated isoliquiritigenin (two peaks), glucosylated liquiritigenin and liquiritigenin were detected in assays with this chalcone (Supplementary Fig. 5). Coumestrol was glucosylated very efficiently (Supplementary Fig. 5). When UDP-glucuronic acid was used as sugar donor with each of the above mentioned acceptors, little or no glycosylated product was observed (data not shown).

Kinetic analysis indicated that UGT75L4 showed higher affinity ($K_m = 2.99\ \mu M$) and catalytic efficiency ($k_{cat}/K_m = 843.47\ M^{-1}\ s^{-1}$) for kaempferol than for dihydrokaempferol (Table 1); kaempferol only differs from dihydrokaempferol by possessing a double bond between positions C2 and C3 on the C-ring. The enzyme had lower affinity for genistein and isoliquiritigenin than for kaempferol (Table 1), but catalyzed their glucosylation more efficiently than that of dihydrokaempferol, as indicated by the k_{cat}/K_m values for the isoflavone and chalcone substrates ($5.91\ M^{-1}\ s^{-1}$ and $13.85\ M^{-1}\ s^{-1}$, respectively).

UGT72B9, UGT88A4 and UGT71A13 did not exhibit activity toward any of the flavonoid or isoflavonoid substrates with either UDP-glucose or UDP-glucuronic acid as sugar donor (data not shown).

3.5. UGT88A4 is active toward coumarin substrates

It has been reported that genes encoding coumarin glycosyltransferases are induced by defense related compounds, such as salicylic acid, and biotic challenge, such as tobacco mosaic virus [24,25]. Although coumarins or coumarin glycosides were not detected in the osage orange fruit extract, coumarins are exuded from the roots of *M. pomifera* [26]. All four osage orange UGTs were tested with the coumarins umbelliferone, esculetin and scopoletin (hydroxy- and methoxy- substituted umbelliferone, respectively) (structures shown in Supplementary Fig. 2). Only UGT88A4 exhibited activity toward these compounds, being most active with esculetin (Fig. 4).

4. Conclusion and perspectives

UGT88A4 from osage orange fruit is active with coumarin substrates in vitro. Since these compounds have yet to be reported in the fruit, the true in vivo substrate for this enzyme is not yet clear. It should be noted that esculetin acts as a substrate for 48 different UGTs from Arabidopsis, representing six different phylogenetic groups (including group E) [12].

UGT75L4 has broad recognition toward flavonoid and isoflavonoid substrates, although only dihydrokaempferol glucoside was detected in the fruit extract, suggesting that dihydrokaempferol is the true in vivo substrate. The fact that the enzyme exhibits much higher catalytic efficiency with kaempferol and other flavonoids than with dihydrokaempferol highlights the difficulties of functional annotation of secondary metabolism genes based on sequence identity and in vitro expression alone. The possession of low affinity and turnover in vitro does not preclude an enzyme of secondary metabolism from functioning with a "poor" substrate in vivo. Examples include the potential involvement of a catalytically inefficient *O*-methyltransferase in flavor production in strawberry [27] and of a glycosyltransferase with preference for quercetin and genistein in the glycosylation of triterpenes in *Medicago truncatula* [18]. Interpretation of in vivo substrate specificity from in vitro measurements is dangerous in the absence of knowledge of the actual metabolites existing in vivo.

To our knowledge, UGT75L4 is the first UGT identified to act on dihydroflavonol substrates. It has been reported that dihydrokaempferol 7-*O*-glucoside can promote flowering in plants [28]. The presence of dihydrokaempferol 7-*O*-glucoside in osage orange fruit may suggest a role in fruit development. The GTs described here may have broad application in metabolic engineering of glycosylated natural products in plants.

Acknowledgements: We thank Ann Harris for high throughput sequencing, David Huhman for LC–MS data acquisition, Dr. Ji He for EST BLAST analysis, Dr. Peter McKenzie for classifying the osage orange fruit glycosyltransferases, and Drs. Luzia Modolo and Lenong Li for critical reading of the manuscript. This research was supported by the Samuel Roberts Noble Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.11.056.

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