

A genomic approach to isoflavone biosynthesis in kudzu (*Pueraria lobata*)

XianZhi He · Jack W. Blount · Shujun Ge · Yuhong Tang · Richard A. Dixon

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Abstract Roots of kudzu (*Pueraria lobata*) are a rich source of isoflavone *O*- and *C*-glycosides. Although *O*-glycosylation of (iso)flavonoids has been well characterized at the molecular level, no plant isoflavonoid *C*-glycosyltransferase genes have yet been isolated. To address the biosynthesis of kudzu isoflavonoids, we generated 6,365 high-quality expressed sequence tags (ESTs) from a subtraction cDNA library constructed using RNA from roots that differentially accumulate puerarin. The ESTs were clustered into 722 TCs and 3,913 singletons, from which 15 family I glycosyltransferases (UGTs) were identified. Hierarchical clustering analysis of the expression patterns of these UGTs with isoflavone synthase (IFS) in a range of tissues identified UGTs with potential functions in isoflavone glycosylation. The open reading frames of these UGTs were expressed in *E. coli* for functional analysis, and one was shown to preferentially glycosylate isoflavones at the 7-*O*-position. In addition, ESTs corresponding to chalcone synthase, chalcone reductase, chalcone isomerase (CHI) and 2-hydroxyisoflavanone dehydratase were identified. Recombinant CHI proteins had high activities with both 6'-deoxy- and 6'-hydroxy chalcones, typical of Type II CHIs. Establishment of this

EST database and identification of genes associated with kudzu isoflavone biosynthesis and glycosylation provide a new resource for metabolic engineering of bioactive kudzu isoflavones.

Keywords Glycosyltransferase · Chalcone isomerase · Isoflavone biosynthesis · *Pueraria lobata*

Introduction

Pueraria lobata (Willd.) Ohwi is a member of the Leguminosae family commonly known as “kudzu”. *Puerariae Radix*, the dried root of the kudzu plant, has been used for centuries in Chinese herbal medicine for prevention of cardiovascular disease and rehabilitation of patients with stroke. The major secondary metabolites accumulating in kudzu roots are isoflavones such as daidzein, genistein, formononetin and their glucosides. Puerarin, the 8-*C*-glucoside of daidzein (Rong et al. 1998), is considered the main active principal of *Puerariae Radix* (Oshima et al. 1988). Interest in the use of puerarin for treatment of blood circulation associated disorders has increased in recent years (Liang et al. 2005; Yeung et al. 2006; Wu et al. 2007), and acute administration of kudzu root extract or puerarin leads to improved glucose tolerance in rodent models of metabolic syndrome and to improved insulin sensitivity (Meezan et al. 2005). The hydrolyzable 7-*O*-glucoside of daidzein, daidzin, may have an opposite effect on glucose tolerance (Meezan et al. 2005). *C*-glycosyl-isoflavones can also play roles in plant defense against insect predation and pathogen infection (Byrne et al. 1996; Cortes-Cruz et al. 2002; Ni et al. 2008).

Several reports have described the cloning of genes encoding flavonoid *O*-glycosyltransferases (Jones et al.

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Present Address:

S. Ge
College of Agronomy, Agricultural University of Hebei,
2596 Lekai South Street, 071001 Baoding, Hebei, China

X. He · J. W. Blount · Y. Tang · R. A. Dixon (✉)
Plant Biology Division, Samuel Roberts Noble Foundation,
2510 Sam Noble Parkway, Ardmore, OK 73401, USA
e-mail: radixon@noble.org

2003; Achnine et al. 2005; Sawada et al. 2005; Kim et al. 2006; Li et al. 2007; Modolo et al. 2007; Pang et al. 2008). Recently, plant *C*-glycosyltransferases involved in the detoxification of 2,4,6-trinitrotoluene (TNT) (Gandia-Herrero et al. 2008) or formation of *C*-glycosyl flavones in cereals (Brazier-Hicks et al. 2009) have been cloned. However, little is known of the enzymes that *C*-glycosylate isoflavones. By analogy with the formation of daidzin from daidzein catalyzed by a 7-*O*-glucosyltransferase utilizing UDP-glucose as sugar donor (Modolo et al. 2007), it would be logical to presume that daidzein is also the precursor for puerarin biosynthesis catalyzed by an isoflavone 8-*C*-glucosyltransferase. However, early in vivo labeling studies showed that the glycosylation reaction in *C*-glycosyl flavone biosynthesis likely occurs prior to flavone formation (Wallace and Grisebach 1973; Inoue and Fujita 1977). Thus, ^{14}C labeled 4',5,7-trihydroxyflavanone (naringenin) was incorporated in a parallel manner into apigenin (flavone) 7-*O*-glucoside and apigenin 8-*C*-glucoside (vitexin) and into luteolin 7-*O*-glucoside and luteolin 8-*C*-glucoside (orientin) (Wallace and Grisebach 1973). However, radioactivity from ^{14}C labeled apigenin and luteolin was only detected in *O*-glycosylated and *O*-methylated flavones, but not in *C*-glycosylflavones (Wallace et al. 1969). These findings were rationalized when it was shown that 2-hydroxyflavanones were the substrates for glycosylation leading to *C*-glycosyl flavones with enzyme preparations in vitro (Kerscher and Franz 1987), and this was confirmed for the recently cloned cereal flavonoid *C*-glycosyltransferases (Brazier-Hicks et al. 2009).

Precursor labeling studies also suggest a non-classical route to *C*-glycosyl-isoflavones. Thus, ^{14}C -labeled chalcone (isoliquiritigenin), but not isoflavone (daidzein), was efficiently incorporated into puerarin in kudzu roots, supporting the idea that the *C*-glycosylation reaction of puerarin biosynthesis occurs prior to isoflavone formation (Fig. 1). Further competitive feeding studies with isoliquiritigenin and liquiritigenin suggested that *C*-glycosylation in kudzu might take place at the chalcone rather than the flavanone level (Inoue and Fujita 1977) (Fig. 1). The key step in isoflavone biosynthesis is the migration of the aryl group (B-ring) of a flavanone precursor from C-2 to the adjacent C-3 to generate the isoflavone skeleton. This enzymatic process is a two-step reaction (Kochs and Grisebach 1986; Hashim et al. 1990; Crombie and Whiting 1992). The first step is the 2-hydroxylation of the C-ring associated with the aryl migration to generate 2-hydroxyisoflavanone, catalyzed by a cytochrome P-450-dependent monooxygenase, generally but misleadingly called isoflavone synthase (IFS). cDNAs encoding this enzyme have been cloned from several plant species (Akashi et al. 1999; Steele et al. 1999; Jung et al. 2000). The second step is the dehydration of 2-hydroxyisoflavanone to yield isoflavone,

catalyzed by a 2-hydroxyisoflavanone dehydratase (HID). cDNAs encoding HID have been cloned and characterized from licorice (*Glycyrrhiza echinata*) and soybean (*Glycine max*) (Akashi et al. 2005). 2-HID has also been purified to apparent homogeneity from kudzu cell cultures, and shown to convert 2,7,4'-trihydroxyisoflavanone to daidzein with kinetic parameters similar to those of the soybean HID (Hakamatsuka et al. 1998; Akashi et al. 2005). However, the kudzu HID has not been characterized at the DNA sequence level.

It is noteworthy that the 4'-*O*-methylation of isoflavones occurs at the level of the 2-hydroxyisoflavanone prior to the action of HID (Akashi et al. 2000; Akashi et al. 2003). It is therefore possible that the 8-*C*-glycosylation in the formation of puerarin may also occur at the level of 2-hydroxyisoflavanone rather than chalcone as previously suggested, since neither CHI nor IFS have been reported to use glycosylated conjugates as substrates, and *C*-glycosylated chalcones have not been described as kudzu metabolites.

Analysis of expressed sequence tags (EST) has greatly accelerated gene discovery and facilitated global gene expression profiling, especially in non-model plant species. Examples include the discovery of many genes involved in secondary metabolism, including the biosynthesis of monoterpenes and sesquiterpenes (Lange et al. 2000; Shimada et al. 2004), monoterpene indole alkaloids (Murata et al. 2006), diterpenes (Brandle et al. 2002), carotenoids (Jako et al. 2002) and prenylflavonoids (Nagel et al. 2008). This approach has been particularly successful for the discovery of natural product glycosyltransferases (Achnine et al. 2005; Tian et al. 2006; Richman et al. 2005; Modolo et al. 2007). However, there are currently no EST or genomic resources available from *Pueraria lobata*.

We here apply an EST-based functional genomics approach to identify genes involved in the biosynthesis of isoflavones in kudzu, with special emphasis on glycosyl transfer reactions.

Materials and methods

Plant materials

Field-collected kudzu plants were obtained from approximately 3 miles southwest of the Samuel Roberts Noble Foundation, Ardmore, Oklahoma. Seeds of commercial kudzu plants were purchased from Kudzu Kingdom Division of Suntop Inc., PO Box 98, Kodak, TN 37764, USA.

Plants were maintained in the greenhouse and propagated by cuttings. Morphological features of the two kudzu lines are shown in Supplemental Fig. 1. Seed and plant

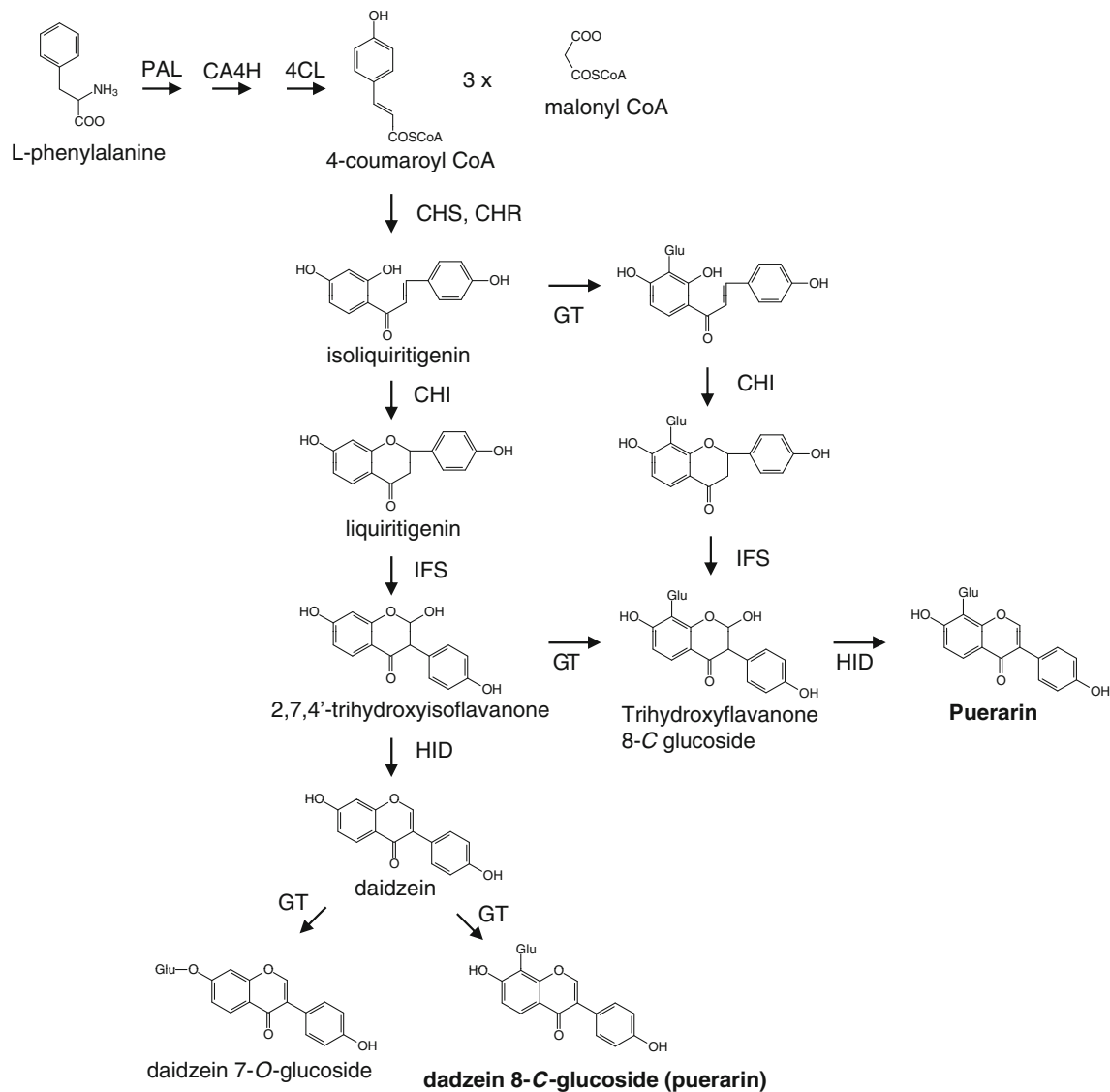


Fig. 1 Potential biosynthetic pathways to puerarin, the daidzein 8-C-glycoside in kudzu. Isoliquiritigenin could be glycosylated at the 3'-C position by a chalcone glycosyltransferase (GT) to form a chalcone C-glycoside, which is then isomerized to liquiritigenin C-glycoside by chalcone isomerase (CHI). The latter could then be converted to puerarin by the sequential actions of isoflavone synthase

(IFS) and trihydroxyflavanone C-glycoside dehydratase (HID). Alternatively, the trihydroxyisoflavanone C-glycoside, which is then converted to puerarin by HID. In the simplest model, daidzein is the direct substrate for puerarin formation catalyzed by a C-GT

materials are available to researchers on request from the authors.

Chemicals

Flavonoid and isoflavonoid acceptor substrates were purchased from Indofine (Hillsborough, NJ). Naringenin chalcone was purchased from Apin Chemicals (Oxon, UK). 2, 4',7-Trihydroxyisoflavanone was produced by incubation of liquiritigenin (7, 4'-dihydroxyflavanone) with yeast microsomes containing recombinant *Medicago truncatula*

IFS, as described previously (Liu et al. 2006). All other chemicals were from Sigma-Aldrich (St. Louis, MO).

HPLC analysis of secondary metabolites from young kudzu roots

One gram of young root tissues was ground to powder in liquid nitrogen and extracted overnight with 5 ml of acetone at 4°C. The extract was centrifuged at 3,500 rpm for 30 min. The supernatant was dried under a stream of nitrogen, the residues were resuspended in methanol, and

an aliquot was analyzed by reverse-phase HPLC (Hewlett Packard 1100 system) on a 5- μ m C18 column (250 \times 4.6 mm, Waters spherisorb 5- μ m ODS2) using the solvent gradient described previously (He et al. 2008).

cDNA library construction

Total RNA was isolated from roots of the field-collected and commercial kudzu plants separately using an RNeasy plant mini kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). cDNA was synthesized from the two RNA preparations using the Super Smart PCR cDNA synthesis kit (Clontech, Mountain View, CA). cDNA subtraction was performed with the commercial kudzu root cDNA as the driver using the PCR-select cDNA subtraction kit according to the manufacturer's protocol (Clontech). The cDNA pools were cloned into pGEM T-easy vectors (Promega, Madison, WI).

DNA template preparation and sequencing

The cDNA library was plated onto LB-ampicillin plates containing IPTG and X-gal, and white colonies were picked into 384-well blocks containing 120 μ l TB-ampicillin medium. The cultures were allowed to grow overnight in a HiGro incubator shaker. Plasmid DNA was prepared using a Biomek FX workstation using standard protocols. Sequencing was performed on a 3730 sequencer using BigDye3.1 (Applied Biosystems, Foster City, CA).

Cloning and expression of kudzu glycosyltransferases

Glycosyltransferase ESTs, which were not full length, were extended by 5'-or 3'-rapid amplification of the cDNA ends (RACE) using the Smart RACE cDNA amplification kit (Clontech). Primers for the cDNA end amplification are listed in Supplemental Table 1. Full-length clones were obtained by RT-PCR based on the RACE sequence information. Full-length cDNAs of GT03H14, GT03H24, GT04F14 and GT07O02 were cloned into the protein expression vector pET28a with restriction enzyme adaptors, and all other UGTs were introduced into pENTR/D-TOPO vector to create entry clones. The insert in entry clones was then transferred into the destination vector pDEST17 by LR recombination reaction (Invitrogen, Carlsbad, CA) for protein expression in *E. coli*. Primers for the restriction enzyme- and Gateway-based cloning of the glycosyltransferases are listed in Supplemental Table 2. Protein was expressed in *E. coli* BL21(DE3) pLysS (Novagen, Madison, WI) with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) induction overnight at 16°C and purified using a MagneHis protein purification kit (Promega).

Assay of *O*-glycosyltransferase activity

Enzyme reactions were performed with 1–3 μ g of enzyme in 50- μ l reaction volumes containing 50 mM Tris-HCl pH 7.5, 5 mM UDP-glucose and 250 μ M acceptor substrate at 30°C for 3 h. The reactions were stopped with 10- μ l (240 mg/ml) trichloroacetic acid (TCA) and the products were analyzed by HPLC as described previously (He et al. 2008). For kinetic analysis of UGT04F14, a 50- μ l reaction mix containing 50 mM Tris-HCl, pH 7.5, 5 mM UDP-glucose, 0–500 μ M genistein and 1.5 μ g of enzyme was incubated at 30°C for 1 h. The reaction was stopped with TCA prior to HPLC analysis. Kinetic parameters were determined by hyperbolic regression analysis (<http://homepage.ntlworld.com/john.easterby/hyper32.html>).

Cloning and expression of chalcone isomerase

Kudzu chalcone isomerase 2 (CHI2) was identified from the EST collection of the kudzu cDNA library described above. Kudzu chalcone isomerase 1 (CHI1) was cloned based on the cDNA sequence previously reported (D63577) (Terai et al. 1996). Full-length CHI 1 and CHI 2 were PCR amplified with the primer pairs: (CHI 1) 5'-CA CCATGGCGGCAGCAGCAGC-3' and 5'-TCAGACTAT AATGCCGTGGCT-3'; and (CHI 2) 5'-CACCATGGC CACTCCAGCATCC-3' and 5'-CTAAGGATTGTTGGC CTCTTTGAG-3'. The full length clones were then introduced into pENTR/D-TOPO vector to create entry clones, which were introduced into destination vector pDEST17 by LR recombination reaction for protein expression in *E. coli* (Invitrogen, Carlsbad, CA). The recombinant protein was expressed in *E. coli* BL21 (DE3) pLysS (Novagen) after induction with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) overnight at 16°C. Protein was purified using a MagneHis protein purification kit (Promega).

Assay of CHI activities

CHI assays were performed at 25°C in a 50- μ l reaction containing 50 mM Tris-HCl, pH 7.5, 1% ethanol, 250–500 μ M substrate and 1–5 μ g protein. The reactions were stopped by the addition of 50 μ l methanol, vortexed and centrifuged for 15 min at 13,000 rpm. The supernatant was used for HPLC assay as described above.

Real-time quantitative RT-PCR

Total RNA was isolated from different developmental stages of roots, nodes and internodes and treated with Turbo DNA-free DNase I (Ambion, Austin, TX). RNA integrity was evaluated with an Agilent 2100 Bioanalyzer.

cDNA synthesis was performed using the DNaseI-treated RNAs and Superscript III reverse transcriptase (Invitrogen).

The PCR primers for real-time qRT-PCR were designed using Primer Express Software 3.0 for Real-Time PCR from Applied Biosystems (Supplemental Table 3) using the default parameters. PCR reactions were performed in an optical 384-well plate with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Reactions contained 5 μ l 2 \times Power SYBR Green Master Mix reagent (Applied Biosystems), 2 μ l of a 1:20 dilution of cDNA, and 2 μ l of gene-specific primer pair (1 μ M) in a final volume of 10 μ l. PCR reactions were performed as described (Czechowski et al. 2005). Data were collected and analyzed using SDS 2.2.1 Software (Applied Biosystems). PCR efficiency was estimated using LinRegPCR software (Ramakers et al. 2003) and transcript levels were determined by relative quantification (Pfaffl 2001) using the actin gene from the kudzu EST collection as a reference. Transcript cluster analysis was performed using Spotfire Software (TIBCO Spotfire).

Amino acid sequence alignment

Deduced amino acid sequences of glycosyltransferases from kudzu and *Arabidopsis thaliana* were aligned using the ClustalW algorithm (Thompson et al. 1994).

Accession numbers

The cDNA sequence data for this article can be found in the GenBank/EMBL data libraries under the following accession numbers: GT01K01 (HQ219038), GT02J01 (HQ219039), GT03H14 (HQ219040), GT03H24 (HQ219041), GT04F14 (HQ219042), GT07O02 (HQ219043), GT10J15 (HQ219044), GT12D15 (HQ219045), GT12P06 (HQ219046), GT14A05 (HQ219047), GT14K13 (HQ219048), GT14M03 (HQ219049), GT18P15 (HQ219050), GT19J14 (HQ219051), GT21C20 (HQ219052), IFS (HQ219053), HID (HQ219054) and CHI2 (HQ219055). The kudzu EST sequences have been deposited in dbEST (<http://www.ncbi.nlm.nih.gov/dbEST/>) with dbEST ID #: 71178728-71185092 (GenBank accession ID #: HO703606-HO709970).

Results and discussion

Isoflavone accumulation in kudzu root and stem tissues

Kudzu roots produce many secondary metabolites, with isoflavone C- and O- glucosides predominating. We obtained seeds of kudzu from a commercial source, and also collected root and stem cuttings from a plant growing

wild in a field hedge SW of Ardmore, Oklahoma. HPLC analysis indicated that young roots derived from the field-collected kudzu isolate produced significant amounts of puerarin in addition to other isoflavone O-glucosides (e.g., the 7-O-glucosides of daidzein, genistein and biochanin A), whereas no puerarin was detected in roots of the commercially obtained kudzu material (Fig. 2a, b). The commercial kudzu line was morphologically very similar to the field-collected material, but could possibly be a different *Pueraria* species. It did, however, accumulate the major isoflavone 7-O-glucosides characteristic of kudzu roots (Fig. 2a, b).

Kudzu grows as a vine, and cuttings of field-collected kudzu established in the greenhouse produced long runners with distinct nodes and internodes (Fig. 3a, b). Analysis of these tissues indicated that puerarin levels increased with developmental age, with lowest levels in the first (youngest) internode to highest in the most mature (6th) node (Fig. 3). The higher levels in nodes than in internodes are likely due to the presence of adventitious root initials in the nodal regions (Fig. 3c), as puerarin levels are high in roots. We also detected a number of isoflavone aglycones (daidzein, genistein, formononetin) and their O-glucosides in the runners; their concentrations followed a similar developmental pattern to that of puerarin (Fig. 3c, d).

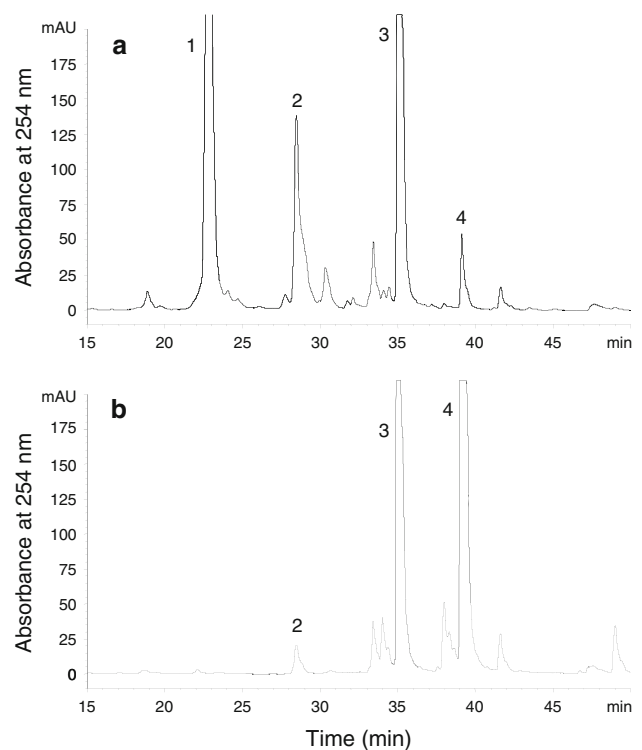


Fig. 2 HPLC profiles of extracts from young roots obtained from field-collected and commercial kudzu plants. **a** Field collected. **b** Commercial. Peak 1 puerarin, peak 2 daidzein 7-O-glucoside, peak 3 formononetin 7-O-glucoside, peak 4 genistein 7-O-glucoside

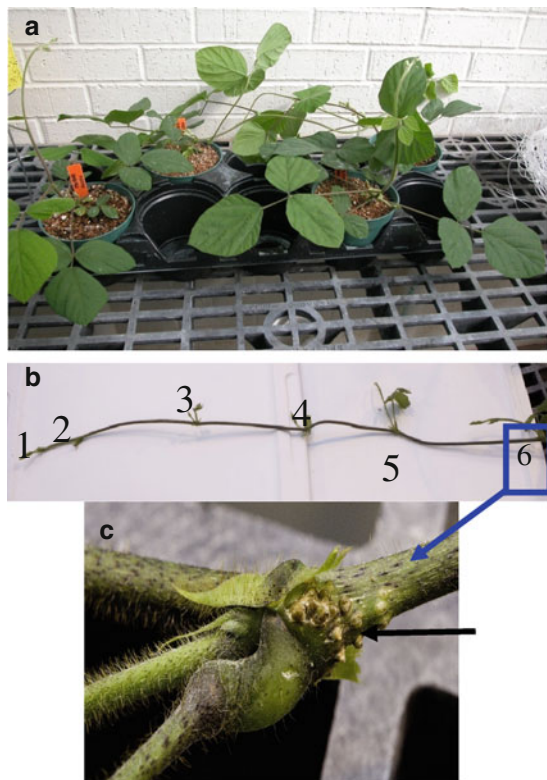
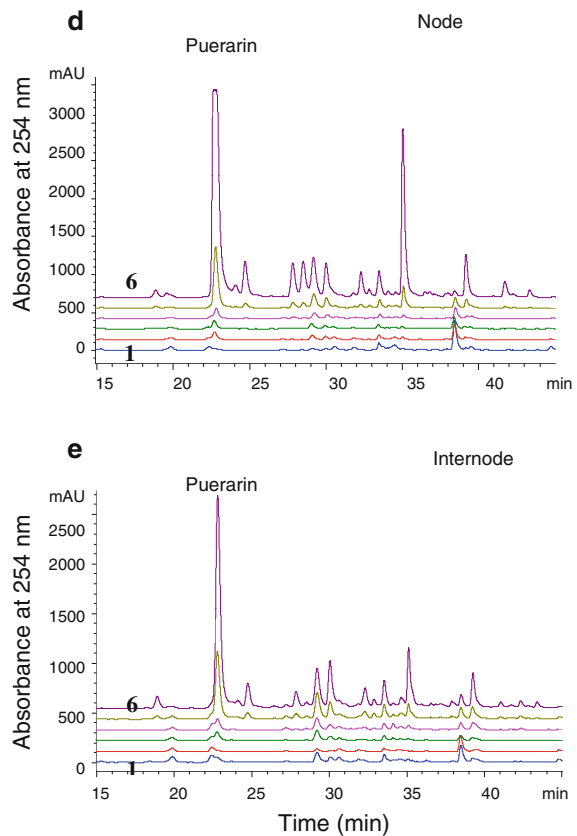


Fig. 3 Production of puerarin in the stems of kudzu vines (field-collected isolate). **a** Vines growing in the greenhouse. **b** Runner showing the different internodes. **c** Close-up of a nodal region (node 6)

Generation of a kudzu root cDNA library and EST collection

The above-described field-collected and commercial kudzu roots provided material for a subtractive library approach toward the identification of genes involved in puerarin biosynthesis. A subtraction library with the commercial kudzu root cDNA as the driver was therefore constructed, to enrich for genes involved in *C*-glycosyl isoflavone biosynthesis (see “Materials and methods” section). To generate the ESTs, the inserts of 7,466 randomly picked cDNA clones were partially sequenced with M13 forward primer. The 6,365 high-quality sequences (average 538 bp in length) were clustered into 722 tentative consensus sequences (TCs) and 3,913 singletons to yield 4,635 apparent unigenes. These unisequences were annotated by BLAST search against the NCBI NR database, and 2,023 of the ESTs (43.6%) had significant sequence similarity to genes with assigned functions, 1,485 ESTs (32%) showed sequence similarity to genes with unknown function and 1,127 ESTs (24.3%) had no database match (Supplemental Fig. 2a). Gene ontology analysis showed that the largest



showing adventitious roots. **d** HPLC profiles showing isoflavonoids in successive nodes **e** As above, for successive internodes

group of ESTs was involved in catalytic activity (30%), and 2% were associated with transcription regulation activity (Supplemental Fig. 2b), which will provide a resource for future regulatory gene discovery. Only 32 ESTs were predicted to be involved in (iso)flavonoid biosynthesis based on our current understanding of the pathway (Tian et al. 2008); each gene was represented by only a single EST except for chalcone synthase (5 ESTs), chalcone reductase (10 ESTs), isoflavone synthase (3 ESTs) and isoflavone reductase (9 ESTs).

Genes of isoflavone biosynthesis in kudzu roots

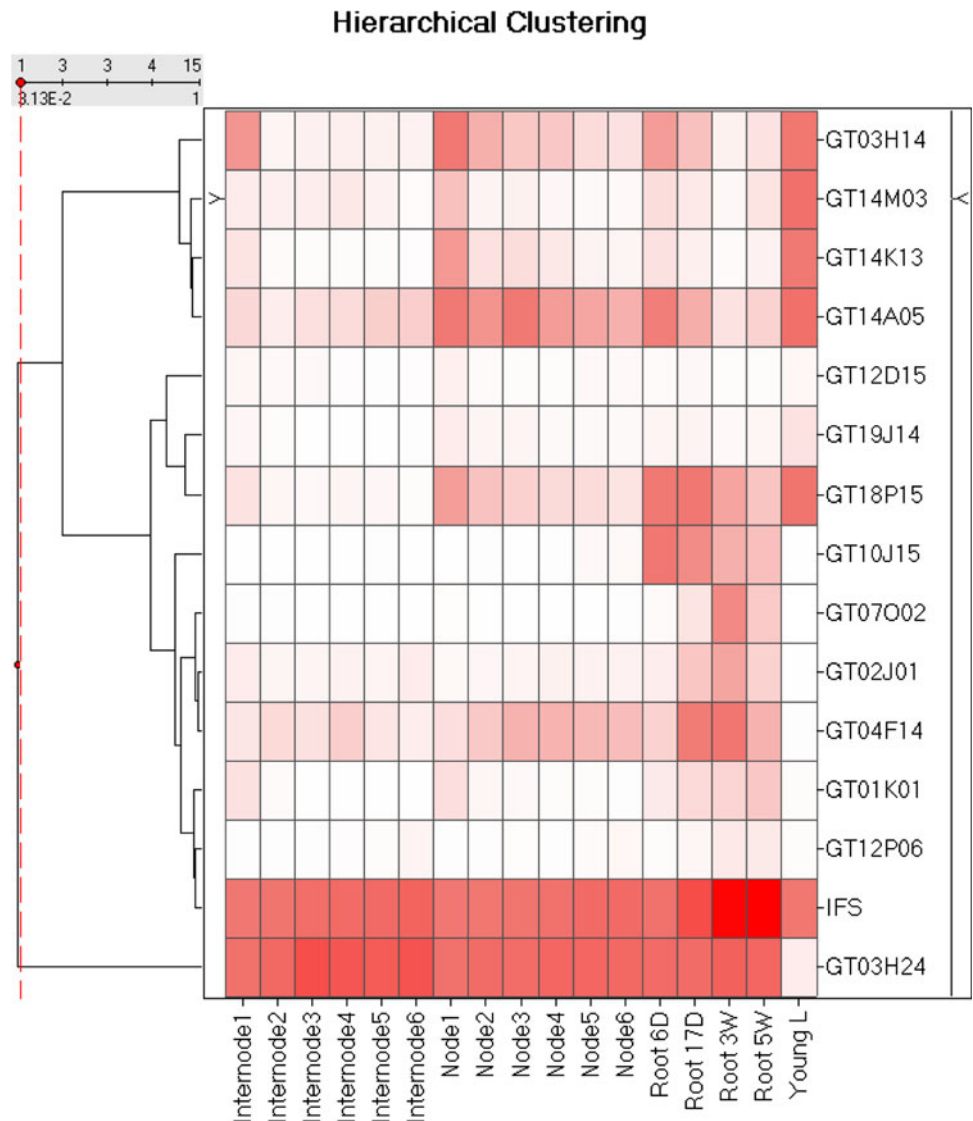
Analysis of the kudzu root EST collection allowed us to identify gene sequences corresponding to most of the enzymes of isoflavone biosynthesis, including CHI, IFS and HID (Table 1). The kudzu IFS is most closely related to soybean IFS (AF195798) with 92.7% amino acid sequence identity. qRT-PCR confirmed that *IFS* transcripts were highly expressed in kudzu root and runner tissues that actively produce puerarin (Fig. 4). IFS is the entry point enzyme in the biosynthesis of isoflavonoids, and its

Table 1 Kudzu unigenes with significant sequence identity to genes of the isoflavone biosynthesis pathway

Unigene ID	GI	BLAST homology search	Bit score	E value
KudzuUG02926	P48406	Chalcone synthase [naringenin chalcone synthase]	426	8e-118
KudzuUG02533	AAM12529	Chalcone reductase [<i>Pueraria lobata</i>]	374	4e-102
KudzuUG04082	Q53B75	Chalcone isomerase [chalcone isomerase 1B-1]	404	3e-111
KudzuUG01011	AAQ20040	Isoflavone 2'-hydroxylase [<i>M.truncatula</i>]	92	9e-18
KudzuUG04588	BAD80840	2-Hydroxy isoflavanone dehydratase [<i>Glycine max</i>]	84	2e-15
KudzuUG02684	AAM12530	Isoflavone synthase [<i>Pueraria lobata</i>]	426	2e-117
KudzuUG03280	AAF17577	Isoflavone reductase [<i>Glycine max</i>]	232	9e-60

GI gene identification

Fig. 4 Hierarchical cluster analysis of transcript levels of kudzu glycosyltransferases in relation to isoflavone synthase in different kudzu tissues at different developmental stages. Tissues were harvested from internodes 1–6 and nodes 1–6 with 1 = youngest, roots at 6 days, 17 days, 3 weeks and 5 weeks, and young leaves. Transcript levels were determined by qRT-PCR, and clustering was performed using Spotfire software (TIBCO Spotfire)



over-expression has led to de novo or increased accumulation of isoflavone glycosides in various plant species (Deavours and Dixon 2005; Liu et al. 2002; Yu et al. 2000, 2003). *IFS* from kudzu provides a useful tool for metabolic engineering of puerarin.

Blast search of the kudzu full-length HID sequence (obtained by RACE from the kudzu EST collection) showed that it had 87% amino acid identity to soybean HID, 60% identity to licorice HID and possessed the residues of the putative catalytic triad (Thr, Asp and His) and

the oxyanion hole (His–Gly–Gly) as reported (Akashi et al. 2005) (Supplemental Fig. 3a). The kudzu *HID* gene is expressed in roots, but also in stems (Supplemental Fig. 3b). Recently, it was reported that overexpression of soybean *HID* in hairy roots of *Lotus japonicus* led to accumulation of daidzein and genistein at comparable levels to those found in transgenic *Arabidopsis* or alfalfa over-expressing IFS (Liu et al. 2002; Deavours and Dixon 2005), but that over-expression of licorice IFS did not lead to accumulation of isoflavones (Shimamura et al. 2007). The authors concluded that *HID* was a critical determinant for isoflavone biosynthesis in *L. japonicus* hairy root cultures. Cloning of the *HID* from kudzu may therefore provide an additional tool for metabolic engineering of isoflavonoids, including the *C*-glucosides. It is interesting to note that there was only one copy of *HID* in the genome of the field-collected kudzu material (data not shown). This implies that, if *C*-glycosylation takes place at the level of 2-hydroxyisoflavanone (see below), the kudzu *HID* would be able to use both 8-*C*-glucosyl 2,4',7-trihydroxyisoflavanone and 2,4',7-trihydroxyisoflavanone as substrates in the formation of puerarin and daidzein, respectively. Likewise, soybean *HID* can use both 4'-hydroxylated and 4'-methoxylated 2-hydroxyisoflavanones as substrates, whereas licorice *HID* is specific for 2,7-dihydroxy-4'-methoxyisoflavanone. The copy numbers of *HID* in these two latter species have not been reported (Akashi et al. 2005).

CHI catalyzes the second committed step in (iso)flavonoid biosynthesis, the isomerization of 6'-deoxychalcone (4,2',4'-trihydroxychalcone) or 6'-hydroxychalcone (4,2',4',6'-tetrahydroxychalcone) into corresponding 5-deoxyflavanone (7,4'-dihydroxyflavanone) or 5-hydroxyflavanone (5,7,4'-trihydroxyflavanone, naringenin) (Fig. 1). Type I CHIs, generally found in non-leguminous plants, catalyze conversion of only 6'-hydroxychalcones to the corresponding 5-hydroxyflavanones, whereas Type II CHIs, mostly found in leguminous plants, catalyze isomerization of both 6'-deoxy- and 6'-hydroxy chalcones. Amino acid sequence identities of around 70–80% are observed within the Type I and Type II CHI groups, whereas the between-group identities are around 50% (Shimada et al. 2003). The deduced amino acid sequences of kudzu CHIs 1 and 2 were most closely related to soybean CHI 1A and 1B1 (Ralston et al. 2005), and clustered in the Type II CHI group. Kudzu CHI 1 had 93.6% amino acid identity to soybean CHI 1A, and kudzu CHI 2 had 93.2% identity to soybean CHI 1B1 (Supplemental Fig. 4). Sequence alignment showed that both kudzu CHIs had identical active site residues to those in the *Medicago sativa* CHI crystal structure (Supplemental Fig. 5) (Jez et al. 2000).

The substrate specificities of both kudzu CHIs were determined by activity assay in vitro. Both recombinant

Table 2 Comparison of kudzu CHI and soybean CHI1A substrate specificity

Substrate	Soybean CHI	Kudzu CHI 1
2'-OH chalcone	+	+
4,2'-OH chalcone	+	+
2',4'-OH chalcone	+	+
2,2',4'-OH chalcone	–	nd
2,2',5'-OH chalcone	–	nd
4,2',4'-OH chalcone	+	+
4,2',5'-OH chalcone	+	nd
4,2',4',6'-OH chalcone	+	+
3,4,2',4'-OH chalcone	nd	+
4,2',4',6'-OH chalcone	nd	–

+ with enzyme activity, – no enzyme activity, nd not determined

CHIs were highly expressed as soluble proteins in *E. coli* and were purified using the MagneHis tag procedure. Ten chalcone substrates were used for enzyme activity determination. Both CHI1 and 2 had similar substrate preferences to soybean CHI 1A, and isomerized six out of the ten chalcones tested, including both 6'-deoxy and 6'-hydroxy chalcones (Table 2, Supplemental Fig. 6a, b). Thus, both kudzu CHI1 and 2 are Type II CHIs.

qRT-PCR analysis showed that both CHIs had very similar tissue-specific expression pattern and were highly expressed in 17-day, 24-day and 5-week-old kudzu roots, with CHI1 being expressed approximately sixfold higher than CHI 2 (Supplemental Fig. 7a, b).

Identification of glycosyltransferases from the kudzu EST collection

Using keyword searching from the kudzu EST database, 15 family 1 glycosyltransferases (UGTs) were identified (Table 3). Full-length cDNAs of these UGTs were obtained by 5' and 3' RACE. Deduced amino acid sequence alignments of the 15 UGTs confirmed the presence of the conserved PSPG box (UDP-binding domain) at the C-termini (Supplemental Fig. 8a, b). The sequence identity between the kudzu UGTs was from 22 to 80%. Phylogenetic analysis of the kudzu enzymes with representative *Arabidopsis* UGTs showed that 11 out of the 15 kudzu UGTs clustered into just two groups of *Arabidopsis* UGTs (five in Group D and six in Group E) (Supplemental Fig. 9).

Group D UGTs are considered to be related to stress or defense responses. For example, expression of UGT73B3 and UGT73B5 is induced by salicylic acid in *Arabidopsis*, and T-DNA tagged mutants (*ugt73b3* and *ugt73b5*) exhibit decreased resistance to the bacterial pathogen *Pseudomonas syringae* pv *tomato-AvrRpm1* (Langlois-Meurinne et al. 2005). UGT73B3 and UGT73B4 are active in vitro toward

Table 3 Enzyme activity of kudzu glycosyltransferase GT04F14

Substrate	% of substrate converted
<i>Isoflavone</i>	
Daidzein	12.1 ^a
Genistein	13.2 ^a
Biochanin A	12.2 ^a
Formononetin	4.5 ^a
6,7,4'-OH isoflavone	12.6 ^a
7,3',4'-OH isoflavone	4.4 ^a
<i>Flavonol</i>	
Kaempferol	79.9 ^b
Quercetin	48.8 ^b
<i>Flavone</i>	
Apigenin	16.0 nd
Luteolin	13.3 ^c
Fisetin	67.7 nd
7,3',4'-OH flavone	8.4 nd
7,4'-OH flavone	5.9 nd
<i>Coumarin</i>	
Coumestrol	11.6 nd
Esculetin	64.5 nd
Scopoletin	81.6 nd

^a 7-OH glucoside, ^b3-OH glucoside, ^c4'-OH glucoside, ndposition not determined

hydroxybenzoic acids and analogs of salicylic acid (Lim et al. 2002), while UGT73C1 and UGT73C5 have been characterized as glycosylating the plant hormones *trans*-zeatin or brassinosteroid (Hou et al. 2004; Poppenberger et al. 2005). However, UGT73C6 was identified as a UDP-glucose: flavonol-3-*O*-rhamnoside-7-*O*-glucosyltransferase (Jones et al. 2003). Interestingly, UGT73B4 and UGT73C1 are capable of forming both *O*- or *C*-glucosidic bonds on 2,4,6-trinitrotoluene (TNT) (Gandia-Herrero et al. 2008). Similarly, the urdamycin glycosyltransferase (UrdGT2) of antibiotic biosynthesis from the soil bacterium *Streptomyces fradiae* displays both *C*- and *O*-glycosylation activity (Durr et al. 2004). The kudzu UGTs clustering in Group D (GT02J01, GT14A05, GT03H14, GT14K13, GT18P15) were more related to each other than to the *Arabidopsis* UGTs in this Group (Supplemental Fig. 9).

Arabidopsis Group E includes the UGT 71 and 72 families (Ross et al. 2001). Some members of the 71 family have been shown to glycosylate abscisic acid and caffeic acid (Lim et al. 2003; Priest et al. 2005). Members of the *Arabidopsis* UGT72B subfamily can detoxify pollutants such as 3,4-dichloroaniline and 2,4,5-trichlorophenol with bifunctional *O*- and *N*-glycosylation activities (Brazier-Hicks and Edwards 2005; Brazier-Hicks et al. 2007), whereas members of the UGT72E subfamily may be

involved in lignin monomer glycosylation (Lim et al. 2005; Lanot et al. 2006) and UGT72L1 from the model legume *Medicago truncatula*, preferentially *O*-glycosylates (-)-epicatechin as a key step in the biosynthesis of proanthocyanidins (Pang et al. 2008). The kudzu UGTs clustering in Group E include GT04F14, GT12D15, GT07O02, GT01K01, GT19J14 and GT03H24 (Supplemental Fig. 9). GT04F14, GT12D15 and GT07O02 are in the UGT72B subfamily and GT01K01 is closely related to the UGT72E subfamily, while GT03H24 falls in the UGT71 family.

Kudzu GT10J15 clusters in the *Arabidopsis* UGT Group A that is relatively small and includes UGT91C1, which is a member of a separate clade of sugar branch-forming glycosyltransferases (Frydman et al. 2004; Morita et al. 2005; Bowles et al. 2005). Further phylogenetic analysis of GT10J15 showed that it also clusters with sugar branch-forming glycosyltransferases (Supplemental Fig. 10). Its amino acid sequence is most closely related (74% similarity) to that of a UDP-glucose: anthocyanidin 3-*O*-glucoside-2''-*O*-glucosyltransferase from *Ipomoea nil* (Supplemental Table 4).

Supplemental Table 4 shows the mostly closely related functionally characterized plant UGTs to each of the 15 kudzu UGTs. The amino acid sequence similarities between the kudzu UGTs and the rice flavone C-GT (Brazier-Hicks et al. 2009) vary from 18% (GT10J15) to 33% (GT19J14).

Cluster analysis of expression patterns for identification of candidate isoflavone glycosyltransferases

To search for UGTs with potential functions in isoflavone glycosylation, we performed hierarchical cluster analysis of expression profiles (determined by qRT-PCR) to determine which UGTs were most tightly co-expressed with *IFS*. Tissues examined included different developmental stages of internodes, nodes, root and young leaves. Cluster analysis showed that *IFS* was expressed in all tissues examined, but with about five- to tenfold higher expression in root tissues compared to other tissues (Fig. 4). This expression pattern reflects that of isoflavone conjugate accumulation, which is highest in roots. GT12P06, GT01K01, GT04F14, GT02J01, GT07O02 and GT10J15 transcripts were highly expressed in roots compared to the other tissues, and the overall expression patterns of GT12P06 and GT01K01 were more than 97% identical to that of *IFS*, although their expression levels were much lower. GT04F14, GT02J01, GT07O02 and GT10J15 exhibited more than 85% similarity in transcript expression pattern to *IFS*, with expression levels of between 8 and 15% that of *IFS*. GT14A05, GT14K13 and GT14M03 were primarily expressed in young leaves, GT03H24 transcripts were highly expressed in all tissues except young leaves,

and GT18P15 and GT19J14 transcripts had similar expression patterns, primarily in root tissues but also high in young leaves (Fig. 4). Together, these results identify 6 out of the 15 UGTs as being candidates for isoflavone *O*- and *C*-glycosyltransferases, and these were therefore prioritized for functional analysis.

Functional identification of kudzu UGTs

The open reading frames of the kudzu UGTs were cloned into the protein expression vector pDEST17 with an N-terminal His tag, and the proteins were expressed in *E. coli* BL21(DE3) pLysS cells and purified using MagneHis beads. Three of the UGTs, GT03H24, GT12D15 and GT21C20, did not produce protein when expressed in *E. coli* and were not analyzed further. Enzyme activities of the remaining UGTs were determined with a range of (iso)flavonoid substrates (Supplemental Fig. 10) and UDP-glucose as sugar donor. Neither GT12P06 nor GT01K01, the two UGTs with closest expression pattern to that of IFS, showed activity with any of the substrates listed in Supplemental Fig. 11.

GT04F14 showed broad substrate specificity and was active with isoflavone, flavone, flavonol and coumarin substrates (Table 3). All isoflavones tested were glycosylated on the 7-hydroxyl group, suggesting high regioselectivity with this class of flavonoid. GT04F14 also showed very high activities with the coumarins esculetin and scopoletin (>60% conversion in the standard assay) as reported for many plant UGTs (Lim et al. 2003). A regioselectivity switching event for glycosylation of esculetin on the 6- or 7-hydroxyl group occurred between glycosyltransferase families 71 and 72 in Group E, with family 71 conjugating on the 7-OH and family 72 preferring the 6-OH (Lim et al. 2003). Since GT04F14 clusters in the family 72 clade, it is likely that it also glycosylates the 6-OH of esculetin. Kinetic parameters for GT04F14 were determined with the isoflavone genistein. The K_m value was 39.11 μM and the efficiency (K_{cat}/K_m) was 70.23 $\text{M}^{-1} \text{S}^{-1}$, values which are quite comparable to those for other plant UGTs (He et al. 2006; Tian et al. 2006). However, GT04F14 showed no activity with potential intermediates of puerarin biosynthesis, namely the chalcone isoliquiritigenin, the flavanone liquiritigenin (or naringenin), or 2,7,4'-trihydroxyisoflavanone. Since GT04F14 has quite diverse substrate specificity, it may have potential as a biocatalyst in biological fermentation systems for regioselective synthesis of diverse natural product glycosides (Lim et al. 2004; He et al. 2008).

Most of the other recombinant UGTs showed no activity with the substrates in Supplemental Fig. 11, GT03H14 exhibited weak activity toward liquiritigenin (1.4% conversion in the standard assay), GT07O02 was active toward

6,7,4'-trihydroxyisoflavone (4.1% conversion) and GT14A05 catalyzed glycosylation of luteolin on the 7-hydroxyl and of quercetin on the 3- and 4'-hydroxyls (about 1% conversion). Since GT10J15 is closely related to sugar-branching glycosyltransferases, isoflavone and flavonol glucosides such as daidzein 7-*O*-glucoside, genistein 7-*O*-glucoside and kaempferol 3-*O*-glucoside were examined as potential substrates, but no activity was detected.

Conclusions and perspectives

This study reports an EST analysis of transcripts expressed in roots of *Pueraria lobata*. The 4,635 unit ESTs provide a significant resource for comparative genomic studies between plant species for gene discovery. Genes encoding several key enzymes of isoflavone biosynthesis, including CHS, CHR, CHI, IFS and HID, were identified in the EST library and will provide tools for subsequent metabolic engineering of kudzu isoflavone glycosides.

The CHIs identified here showed broad substrate specificity similar to Type II CHIs from leguminous plants (Shimada et al. 2003). It is likely that kudzu CHI 1 and 2 will function as typical Type II CHIs in converting chalcone rather than chalcone *C*-glycoside to flavanone in vivo. Furthermore, none of the UGTs we have characterized are active with chalcone as substrate. We therefore feel that it is unlikely that *C*-glycosylation occurs at the chalcone stage during puerarin biosynthesis, although this was suggested by early labeling studies (Inoue and Fujita 1977). Subsequent to this previous hypothesis, it was shown that 4'-*O*-methylation during isoflavone biosynthesis occurs at the level of the trihydroxyisoflavanone product of IFS, not the isoflavone itself (Akashi et al. 2003), and this explains why labeled trihydroxychalcone but not daidzein is incorporated into the pterocarpan medicarpin (Dewick and Martin 1979). Similarly, 6- or 8-*C*-glycosylation of flavones occurs at the corresponding trihydroxyflavanone stage (Brazier-Hicks et al. 2009). We therefore propose that 8-*C*-glycosylation during puerarin biosynthesis likely takes place at the trihydroxyisoflavanone stage. This is supported by the observation that crude extracts from kudzu roots can convert 2,7,4'-trihydroxyisoflavanone to an unidentified intermediate of lower retention time (greater hydrophilicity), and that this compound disappears on exposure of extracts to weak acid, with a subsequent increase in the level of puerarin (Supplemental Fig. 12). The unstable intermediate is probably the 8-*C*-glucoside of the trihydroxyisoflavanone, which then dehydrates to puerarin at lower pH. However, this must remain a hypothesis until we can demonstrate the exact chemical nature of the unstable intermediate and show that it can also be formed by a recombinant kudzu UGT. To date, we have been unable to

show glycosylation of trihydroxyisoflavanone by any of the UGTs described in the present paper.

Although the *in vitro* enzyme activities of many of the kudzu UGTs have not been determined, their *in vivo* biological functions are likely associated with secondary metabolism. GT04F14 showed 7-*O*-glycosylation regio-specificity toward several isoflavones *in vitro*. Since isoflavone 7-*O*- glycosides are major metabolites accumulated in kudzu roots, it is possible that GT04F14 is involved in isoflavone glycosylation *in vivo*. It is interesting that GT04F14 shows high activity toward fisetin (>60% conversion rate), since fisetin has been reported to enhance long-term memory and protect the nervous system (Maher et al. 2006; Maher 2008). This UGT could potentially be used as a biocatalyst for production of fisetin glycosides by fermentation.

Plant natural products often have more than one sugar attached at different positions, or even have branched-sugar modifications. The latter have significant biological functions in enhancing bitterness (Frydman et al. 2004), sweetness of *Stevia rebaudiana* (Brandle and Telmer 2007) or flower color (Kondo et al. 1987). The glycosyltransferases catalyzing the secondary metabolite branch-sugar modifications have evolved into a group distinct from other glycosyltransferases, and only a few of their cDNA have been cloned and functionally characterized (Frydman et al. 2004; Richman et al. 2005, Morita et al. 2005; Sawada et al. 2005; Noguchi et al. 2008). Kudzu GT10J15 is likely an additional member to this underexploited group of glycosyltransferases, since it has high amino acid similarity to a functionally characterized sugar-branching glycosyltransferase (Morita et al. 2005) and is phylogenetically clustered in this group.

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