ORIGINAL ARTICLE

Recombinant expression and functional characterisation of regiospecific flavonoid glucosyltransferases from *Hieracium pilosella* L.

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Abstract Five glucosyltransferases were cloned by RT-PCR amplification using total RNA from *Hieracium pilosella* L. (Asteraceae) inflorescences as template. Expression was accomplished in *Escherichia coli*, and three of the HIS-tagged enzymes, UGT90A7, UGT95A1, and UGT72B11 were partially purified and functionally characterised as UDP-glucose:flavonoid *O*-glucosyltransferases. Both UGT90A7 and UGT95A1 preferred luteolin as substrate, but possessed different regiospecificity profiles. UGT95A1 established a new subgroup within the UGT family showing high regiospecificity towards the C-3′ hydroxyl group of luteolin, while UGT90A7 primarily yielded the 4′-*O*-glucoside, but concomitantly catalysed also the formation of the 7-*O*-glucoside, which could account for this flavones glucoside in *H. pilosella* flower heads. Semi quantitative expression profiles revealed that UGT95A1 was expressed at all stages of inflorescence development as well as in leaf and stem tissue, whereas UGT90A7 transcript abundance was nearly limited to flower tissue and started to develop with the pigmentation of closed buds. Other than these enzymes, UGT72B11 showed rather broad substrate acceptance, with highest activity towards flavones and flavonols which have not been reported from *H. pilosella*. As umbelliferone was also readily accepted, this enzyme could be involved in the glucosylation of coumarins and other metabolites.

Abbreviations
GT	Glycosyltransferases
UGT	UDP-Glycosyltransferases
PSPG	Plant secondary product glycosyltransferases
RACE	Rapid amplification of cDNA ends
HSCCC	High-speed counter current chromatography

Introduction

Mouse-ear hawkweed, *Hieracium pilosella* L. (Asteraceae) is a perennial herb attributed with diuretic properties (Bolle et al. 1993; Beaux et al. 1999) and traditionally utilised for the treatment of diarrhoea, enteritis, bronchitis, pharyngitis, and stomatitis (Dombrowicz et al. 1992). Furthermore, both the ethanol and water-soluble extracts showed some anti-inflammatory activity (Bolle et al. 1993; Trouillas et al. 2003). The plant accumulates cinnamic acids, such as 4-coumaric, ferulic, and caffeic acid, as well as some derivates (chlorogenic, 3,5-dicaffeoylquinic, and 4,5-dicaffeoylquinic acids), besides benzoic acids (salicylic, 4-hydroxybenzoic, 4-hydroxyphenylacetic, syringic, and protocatechuic acid) (Dombrowicz et al. 1992; Zidorn et al. 2002, 2005). Additionally the coumarin umbelliferone (Duquénois and Greib 1953; Bate-Smith et al. 1968) and various triterpenoids (taraxasterol, Ψ-taraxasterol, taraxerol, α-amyrin, β-amyrin, lupeol, fern-7-en-3β-ol) were reported from different tissues (Gawrońska-Grzywacz and

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Krzaczek 2007). The plant also accumulates a set of flavones, particularly glycosides of the rare isoetin and luteolin, as well as the latter aglycone itself (Supplementary Fig. 1S) (Haag-Berrurier and Duquénois 1962, 1963; Harborne 1978; Zidorn et al. 2002, 2005).

Besides the numerous contributions to plant protection against biotic and abiotic stresses like UV radiation, microbial invasion, or herbivore activity (Harborne and Williams 2000; Simmonds 2003; Martens and Mithöfer 2005), flavones can also act as signalling molecules, for example in the interaction of flowers (copigmentation) and bees or between legumes and nitrogen-fixing bacteria (Harborne and Williams 2000; Martens and Mithöfer 2005). Medical properties of flavones include protective effects against certain kinds of cancer and prevention of cardiovascular diseases (Arts and Hollman 2005; Martens and Mithöfer 2005), as well as effects on the central nervous system (Wang et al. 2005; Coleta et al. 2006, 2008). Furthermore, flavones are likely to contribute to the diuretic effect of *H. pilosella*, given that they bind to the adenosine A1 receptor (Ji et al. 1996; Hasrat et al. 1997; Ingkaninan et al. 2000) and exert antagonistic effects in the kidney (Welch 2002; Modlinger and Welch 2003).

Plants accumulate most flavones as their glycosides, and luteolin-7-*O*-β-D-glucoside, luteolin-7-*O*-β-D-glucuronide, luteolin-4′-*O*-β-D-glucoside, and isoetin-4′-*O*-β-D-glucuronide were reported from *H. pilosella* (Haag-Berrurier and Duquénois 1963; Zidorn et al. 2002, 2005). Glycosylation generally leads to reduced chemical reactivity and enhanced water solubility of secondary metabolites, facilitating their transport and storage (Jones and Vogt 2001). The transfer of a sugar moiety from an activated donor to an acceptor molecule is generally catalysed by members of the superfamily of glycosyltransferases (GTs). The carbohydrate-active enzymes database (www.cazy.org) currently itemises 89 sequence-based families (Campbell et al. 1997; Coutinho et al. 2003) with over 20,000 entries. GTs belonging to Family 1 (UGTs) display a conserved amino acid motif near the C-terminus, use UDP-activated sugars (mainly UDP-glucose) as donors, and various types of low molecular-weight molecules as acceptors (Mackenzie et al. 1997). Plant UGTs possess an adjusted version of the conserved amino acid region named PSPG motif (plant secondary product glycosyltransferase) (Hughes and Hughes 1994; Paquette et al. 2003), and acceptors include metabolites like plant hormones, phenylpropanoids, flavonoids, coumarins, terpenoids, cyanohydrins, thiohydroxamates, and alkaloids (Vogt and Jones 2000; Bowles et al. 2006). The broad spectrum of acceptors and the altered chemical features of glycosides suggest that UGTs play major roles in stabilization and colour determination of pigments, the adjustment of plant growth regulators, and plant defences against microbial and viral infections (Lim and Bowles

2004; Gachon et al. 2005; Bowles et al. 2005, 2006). This is also mirrored in the number of PSPG genes reported. In *Arabidopsis thaliana* 112 full-length UGTs were assigned (Paquette et al. 2003), while in *Medicago truncatula* at present 164 sequences are listed putatively encoding UGTs (Modolo et al. 2007).

This report describes the cloning, expression, and characterisation of five UGTs from *H. pilosella* flower heads, as well as their expression profiles in different tissues and the classification within the UGT family.

Materials and methods

Chemicals and plant material

If not stated otherwise all general chemicals were purchased from Carl Roth (Karlsruhe, Germany) and Sigma-Aldrich (Taufkirchen, Germany). ADP-[U-^{14}C]glucose (10.1 GBq/mmol) and UDP-[U-^{14}C]glucose (12.1 GBq/mmol) were obtained from GE Healthcare UK (Little Chalfont, UK). UDP-[U-^{14}C]glucuronic acid (10.1 GBq/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, USA). Flavonoid substrates and authentic glucosides were obtained from Carl Roth, Fluka (Buchs, Switzerland), and Extrasynthese (Genay, France). Isoetin was from our laboratory collection. All enzymes were purchased from Fermentas (St. Leon-Rot, Germany). Sequencing and primer synthesis was performed by MWG-Biotech (Ebersberg, Germany). Plasmids were purchased from Novagen (Merck Chemicals, Darmstadt, Germany) or Invitrogen (Karlsruhe, Germany).

H. pilosella was obtained from Plogstedt nursery (Attaching, Germany) and grown in the local experimental garden. For RNA extraction, tissues were collected from leaves (<2 cm in length), stems, and flowers. Flowers were harvest at developing stages as follows: stage 1, closed round inflorescence with first signs of pigmentation (∼4.5 mm in diameter); stage 2, pigmenting closed inflorescence (∼7 mm in length, ∼5.4 mm in diameter); stage 3, fully pigmented closed inflorescence (∼9 mm in length, ∼6 mm in diameter); stage 4, pigmented gradually widening inflorescence (∼9 mm in length, ∼7 mm in diameter); stage 5, fully opened flower head (∼14 mm in length, ∼21 mm in diameter). Flowers for the preparation of cDNA were harvested at the developing stages 1–3. All plant tissues were immediately frozen in liquid nitrogen and stored at −80°C until use.

Enzyme preparation from flower tissue

All steps were performed at 4°C. Flowers from the developmental stage 3 were ground in a mortar in the presence of

100 mM Tris–HCl pH 7.5 (supplemented with 28 mM 2-mercaptoethanol and 10 mM sodium ascorbate) and Dowex 1 × 2 (200–400 mesh). The supernatant from 10,000g centrifugation (10 min) served as protein source.

Isolation of RNA and cDNA-cloning of UGTs

Total RNA was isolated from each tissue using the RNeasy Plant Mini Kit from Qiagen (Hilden, Germany). Degenerated oligonucleotide primer 5′-AC(AGCT) CA(CT) TG(CT) GG(GC) TGG AAC-3′ was designed for 3′-RACE based on the conserved PSPG motif (Isayenkova et al. 2006) using the *H. pilosella* RNA as template. The cDNA fragments were cloned, sequenced, and full-length cDNAs were generated by 5′-RACE using gene specific primers. Full-length cDNAs were amplified using end-to-end oligonucleotide primer sets for each gene modified with appropriate restriction sites (Supplementary Table 1S). The PCR products were ligated into the pCR2.1 T/A vector and sequenced. The plasmids were digested with the corresponding restriction enzymes, and resulting DNA fragments were ligated into the restriction sites of pET-15b expression vector possessing six N-terminal histidine residues.

Semi-quantitative RT-PCR from plant tissues

Total RNA (5 μg) each of leaves, stems, and the five developmental stages of flower heads were used for reverse transcription. Equivalent amounts of cDNA were used for PCR with the gene specific end-to-end primer sets (Supplementary Table 1S), using 25 or 30 amplification cycles (one replication). As a control, a fragment of the 18S ribosomal RNA was amplified in parallel using primers 18SrRNAf/r (Supplementary Table 1S). To compare the expression intensity of the UGTs with genes belonging to the flavonoid biosynthesis pathway, semi-quantitative RT-PCR was performed with gene-specific primer sets (Supplementary Table 1S) under the same conditions as mentioned above for flavone synthase II (FNS II, accession number EU561012), flavonoid 3′-hydroxylase (F3′H, DQ319866), flavanone 3β-hydroxylase (FHT, EU561014), dihydroflavonol 4-reductase (DFR, EU561013), and anthocyanin synthase (ANS, EU561015) from *H. pilosella*. PCR products were separated by 1.5% (w/v) agarose gel electrophoresis.

Heterologous expression, purification of the proteins, and molecular mass determination

Transformed *E. coli* BL21 (DE3) cells were pre-cultured overnight in LB medium supplemented with ampicillin (100 μg/ml), and 10 ml of the culture was transferred into 1,000 ml of the same medium (both at 37°C). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM when OD_{600} reached 0.6. The culture was further incubated for 5 h at 26°C.

For protein purification, cells were collected by centrifugation for 10 min at 4,000g and re-suspended in 10 ml lysis buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole) containing 0.2% desoxycholate and 1% Triton X-100. After sonication (150 W, 24 kHz, 3 min) the slurry was cleared by centrifugation for 10 min at 10,000g, and the enzyme purification was carried out by immobilized metal affinity chromatography (Profinity IMAC Resins, Bio-Rad-Laboratories, Hercules, CA, USA) of the supernatant using the batch-mode purification of histidine proteins (gravity flow) in empty PD-10 columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. The purified protein solution was desalted through pre-packed PD-10 columns Sephadex G-25 M (GE Healthcare Bio-Sciences AB). Protein concentration was measured as described by Bradford 1976, and purity was determined by Coomassie brilliant blue stained SDS-PAGE.

The molecular mass of the native UGTs was determined by size-exclusion chromatography on a Fractogel EMD Bio Sec (S) column (Superperformance 16, Merck) calibrated with LMW Gel Filtration Calibration Kit reference proteins (GE Healthcare, UK) the flow rate was 1 ml/min. The molecular mass of the denatured UGTs was confirmed by 12.5% SDS-PAGE.

Enzyme assays

The assay for the native protein extract from plant tissue consisted of 100 mM Tris–HCl pH 7.5, 925 Bq UDP-[U-^{14}C]glucose or UDP-[U-^{14}C]glucuronic acid, 150 μM luteolin or isoetin, and 50 μl of the crude protein extract (200 μl total). Incubation length was 30 min at 30°C. Ethyl acetate extracts were analysed via thin-layer chromatography (TLC) and Bioimaging (FLA 2000 Fujifilm, Tokyo, Japan) with the AIDA software (Raytest, Straubenhardt, Germany).

The standard assays for the recombinant proteins consisted of 100 mM buffer (KH_2PO_4/K_2HPO_4, pH 8.0, Tris–HCl pH 7.0 or pH 7.6), aglycone (50 or 250 μM), 2.5 μM UDP-[U-^{14}C]glucose (5.5 kBq), supplemented with unlabelled UDP-glucose to 500 μM, and the enzymes (200 μl total). The reaction mixture, in absence of the enzyme, was pre-incubated at 25, 30, or 45°C for 5 min. The reaction was started by adding 3 or 5 μg of the purified protein. After incubation for 3 or 5 min, the reaction was stopped by adding 5 μl trichloroacetic acid (240 mg/ml) and extracted with 500 μl ethyl acetate. As a control, BL21 cells containing an empty pET-15b vector were assayed for activity

under the same conditions. Alternatively, purified enzyme solution was heated to 95°C for 10 min prior to the enzyme assay. UDP-glucose, ADP-glucose, and UDP-glucuronic acid were tested for donor activity. As acceptors various phenylpropanoids at a final concentration of 50 μM were examined.

The radioactivity of the product was determined in a 400 μl aliquot of the ethyl acetate phase by liquid scintillation counting (1214 RackBeta liquid scintillation counter, LKB Wallac, Turku, Finland) after addition of 5 ml of Rotiszint® eco plus scintillation cocktail. Alternatively, the reaction products were applied to cellulose TLC plates (Merck) for separation in solvents 30% aqueous acetic acid or BAW (butanol–acetic acid–water 6:1:2, by vol.), and the radioactivity pattern was monitored by Bioimaging.

For determination of pH optima, glucosyl formation was measured at a fixed buffer concentration of 200 mM using glycine-HCl, sodium acetate, potassium phosphate, Tris–HCl, and sodium glycinate buffers between pH 2.0 and 10.0. The effect of temperature was examined between 5 and 60°C. To assess the metal ion requirements, 100 mM aqueous solutions of various salts, including NaCl, $CaCl_2$, $CoSO_4$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $NiSO_4$, and $ZnSO_4$ were prepared. Standard assays were supplemented to a final concentration of 0.05 mM or 0.5 mM. For checking the effect of various amino acid modifying- and reducing-agents, diethyl pyrocarbonate (DEPC, 1 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), N-ethylmaleimide (NEM, 1 mM), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS, 1 mM), ethylenediaminetetraacetic acid (EDTA, 1 mM and 5 mM), dithiothreitol (DTT, 5 mM), and 2-mercaptoethanol (50 mM) were added to the standard enzyme assays.

Kinetic data of the recombinant UGTs were determined from Lineweaver–Burk plots for the donor substrate UDP-glucose (10 μM–2 mM) and the acceptors luteolin, eriodictyol, and kaempferol (2.5 μM–1 mM) in the present of a saturating concentration of their counterpart. All results are the means of three replications.

Biotransformation and high-speed countercurrent chromatography (HSCCC)

E. coli BL21 (DE3) cells containing the different expression vectors were grown in LB medium until OD_{600} reached 0.6 (at 37°C), completed with IPTG to a final concentration of 1 mM, and incubated for 5 h (at 26°C). Up to 20 mg of luteolin, eriodictyol, kaempferol, or quercetin dissolved in DMSO were then added. After an additional 48 h the cell cultures and media was acidified with HCl and extracted twice with ethyl acetate. The ethyl acetate layer was evaporated to dryness in a rotary evaporator, residues were re-suspended in a biphasic mixture of chloroform–metha-

nol–butanol–water (10:10:1:6, by vol.), and subjected to the HSCCC system (Zinsser Analytic, Frankfurt, Germany), equipped with a Merck-Hitachi L-6000 pump (Tokyo, Japan) and a manual sample injection valve with a 10 ml loop.

The 420 ml coil was first filled with the lower phase as the stationary phase, then the upper mobile phase was pumped into the head end of the column at a flow rate of 1 ml/min, while the coil rotated in a forward direction at 600*g*. After the hydrodynamic equilibrium was reached, the sample was injected through the valve. The effluent from the tail end of the column was collected in 10 ml fractions, and each sample was analysed by TLC. The fractions containing the pure glucoside were combined, and identification was carried out by NMR spectroscopy.

Product identification

For product identification the standard assay was supplemented with unlabelled UDP-glucose. The extracted ethyl acetate was evaporated, residues were dissolved in methanol–water (2:1, v/v), applied to a HPLC column (EC 250/4.6 Nucleosil 100-10 C18, Macherey-Nagel, Düren, Germany), and separated in 1.5% phosphoric acid (solvent A) and acetonitrile (solvent B) using a step gradient of 0–13 min from 15 to 55% B in A, 13–15 min from 55 to 15% B in A, and 15–20 min, 15% B. The elution of the aglycone and its glucosides was monitored at 260 nm for flavonols, at 265 nm for flavones, at 285 nm for flavanones and caffeic acid, and at 290 nm for DHQ.

The ^{1}H-NMR spectrum was recorded on the dried purified HSCCC fraction solubilised in 0.6 ml CD_3OD at 600.1328156 MHz on a Bruker Avance III spectrometer using a 5 mm inverse cryoprobe. Data acquisition was made under TopSpin version 2.1 (Bruker BioSpin, Karlsruhe, Germany). Eight scans of 64 K data points were acquired with a spectral width of 12 ppm, acquisition time of 4.48 s, and recycle delays of 5 s at 300 K. The obtained free-induction-decay was Fourier transformed, manually phased, and baseline corrected. The resulting spectra were shifted so that the methanol signal was aligned to 3.31 ppm. The obtained spectrum was compared to NMR database of flavonoids (Moco et al. 2006).

Results

Previous assays employing native enzyme extracts from flower tissue and UDP-[U-^{14}C]glucose or UDP-[U-^{14}C]glucuronic acid in combination with the aglycones luteolin or isoetin yielded several radioactively labelled glycosides on TLC (data not shown). These results led us to attempt the cDNA cloning of UGTs from *H. pilosella*.

A degenerated forward PCR primer was designed based on the conserved PSPG motif and used with the oligo-dT-Primer (reverse primer). Amplicons from 300 to 550 bp length were sub-cloned, sequenced, and examined for UGT similarity. Individual full-length clones were generated by nested 5′-RACE. Through sequence alignments, five different cDNAs encoding UGTs were assigned and named according to the UGT naming committee (http://som.flinders.edu.au/FUSA/ClinPharm/UGT/index.html).

UGT88A8 contained an open reading frame of 1,389 bp encoding 463 amino acids. UGT88A9 and UGT72B11 were both 1,398 bp in length and encoded proteins consisting of 466 amino acids. UGT90A7 was 1,401 bp long representing 467 amino acids, and UGT95A1 (1,635 bp) contained an open reading frame encoding 545 amino acids (Supplementary Fig. 2S). Among the annotated PSPGs and *A. thaliana* UGTs, *H. pilosella* UGT72B11 (accession number EU561016) was closest to arbutin synthase from *Rauvolfia serpentina* (CAC35167) with 68% identity, followed by *A. thaliana* UGT72B1 (CAB80916) with 63% identity, and *Maclura pomifera* UGT72B9 (ABL85472) with 60% identity. The amino acid sequence of *H. pilosella* UGT88A8 (EU561017) revealed 53% identity with UGT88A4 from *M. pomifera* (ABL85471), 51% identities with UGT88B1 from *Stevia rebaudiana* (AAR06919) and *Rosa hybrida* anthocyanidin 5,3-*O*-glucosyltransferase (BAD99560), and 49% identity with *A. thaliana* UGT88A1 (BAB01151). As *H. pilosella* UGT88A8 and UGT88A9 share an identity of 79%, UGT88A9 (EU561018) was also closest to these GTs with 54% identity towards *M. pomifera* UGT88A4 and 49% identity towards *R. hybrida* anthocyanidin 5,3-*O*-glucosyltransferase. *H. pilosella* UGT90A7 (EU561019) was closest to UGT90A1 (56%), UGT90A3 (50%), and UGT90A2 (50%) from *A. thaliana* (AAC64220, ABE66156, NP_172511). The highest sequence similarity of *H. pilosella* UGT95A1 (EU561020) was found with *A. thaliana* UGT73C3 (37%), UGT73C2 (35%), and UGT92A1 (35%) (AAL85061, AAD20152, CAB88253). Consequently, this UGT classifies to a novel subfamily (UGT95A).

A phylogenetic tree was constructed based on 59 PSPG sequences (Fig. 1). *H. pilosella* UGT88A8 and UGT88A9 cluster with other GTs from this subfamily, which contains enzymes with regiospecificity for the 7-OH group of flavonoids (analogue 4′-OH of chalcones) (Nagashima et al. 2000; Ono et al. 2006; Noguchi et al. 2007) as well as a glucosyltransferase which catalyses two glucosylation steps at the 5-OH and 3-OH position of anthocyanidin (Ogata et al. 2005) and UGT88A1 from *A. thaliana* glucosylating hydroxyls at four sites of quercetin (Lim et al. 2004). It should be noted that UGT88A4 from *M. pomifera* did not exhibit any activity towards flavonoids and isoflavonoids (Tian et al. 2006), but produced glucosides from coumarin

substrates. *H. pilosella* UGT72B11 also belongs to group E of the classification scheme (Fig. 1). Besides the arbutin synthase from *R. serpentina* (Hefner et al. 2002) mentioned above, subfamily 72 contains *A. thaliana* glucosyltransferases, including UGT72B1, which possesses both *N*- and *O*-glucosyltransferase activity. This enzyme was assigned to the detoxification of xenobiotics (Loutre et al. 2003; Brazier-Hicks and Edwards 2005; Brazier-Hicks et al. 2007), whereas the UGTs 72E1-E3 are involved in the glucosylation of monolignols, hydroxycinnamic acids, and hydroxycinnamic aldehydes (Lim et al. 2001, 2005; Lanot et al. 2006). *H. pilosella* UGT90A7 belongs to group C and, to the best of our knowledge, an enzyme of this subfamily has never been characterised. *H. pilosella* UGT95A1 does not fit any of the groups classified, in agreement with the fact that it is the first enzyme of a new subfamily.

Expression profiles of the UGTs and genes belonging to the flavonoid biosynthesis pathway were analysed via semi-quantitative RT-PCR (Fig. 2). During the five developmental flower head stages as well as in leaves and stems UGT95A1 was expressed with equal intensity, while the abundance of the other four UGT transcripts was less in the stems of *H. pilosella*. FNS II and F3′H which represent 'early' genes of the flavonoid biosynthesis pathway were expressed concomitantly with the glucosyltransferases, while transcripts of the 'later' genes FHT and DFR were detectable only in some flower head stages after 30 cycles of amplification. The ANS, which was cloned from UV treated suspension cultures, was not detectable under these conditions at any stage of the tissues. To identify the substrate specificity of the five UGTs, the cDNAs were cloned into the expression vector pET-15b in *E. coli* BL21 (DE3) cells. UGT90A7, UGT95A1, and UGT72B11 could easily be purified by immobilized metal affinity chromatography (IMAC) yielding from 5 to 9 mg/l of culture broth after 5 h of incubation with IPTG. By contrast UGT88A8 and UGT88A9 yielded negligible enzyme quantities (Fig. 3a). However, eluates of UGT88A8 after IMAC purification showed activity with flavones, flavonols, caffeic acid, esculetin, catechol, resorcinol, and hydroquinone, and UGT88A9 glucosylated flavanones and flavonols as well as apigenin, luteolin, and DHQ. Variations in incubation time and/or temperature did not improve the yields, and UGT88A8 and UGT88A9 were therefore not characterised any further.

The molecular mass of the enzymes was determined using a calibrated Fractogel EMD Bio Sec column. Each enzyme pre-purified through IMAC chromatography eluted in a single peak of enzyme activity, indicating an apparent Mr of 53,300, 55,000, and 61,700 Da (UGT72B11, UGT90A7, and UGT95A1, respectively) for the native UGTs. The separation by SDS-PAGE (Fig. 3b) suggested monomeric enzymes, since the Mr of the denatured

Fig. 1 Phylogenetic tree to group the UGT polypeptide sequences. Multiple sequence alignments were performed with ClustalX and the tree was calculated with the neighbour-joining method and constructed using the programme NJPlot. The human UGT1A1 was defined as out-group. *H. pilosella* UGTs are marked with *asterisks* and bootstrap values over 950 are included by the branches (1,000 replicates). Clusters I–IV are defined for plant GTs based on substrate specificity and/or regioselectivity (Vogt and Jones 2000; Sawada et al. 2005; Yonekura-Sakakibara et al. 2007; Noguchi et al. 2007). Also included is the phylogenetic analysis of the UGTs from *Arabidopsis thaliana*, which are arranged in 14 subgroups (A–N) (Ross et al. 2001; Li et al. 2001). The abbreviations and GenBank accession numbers are shown in Supplementary Fig. 3S
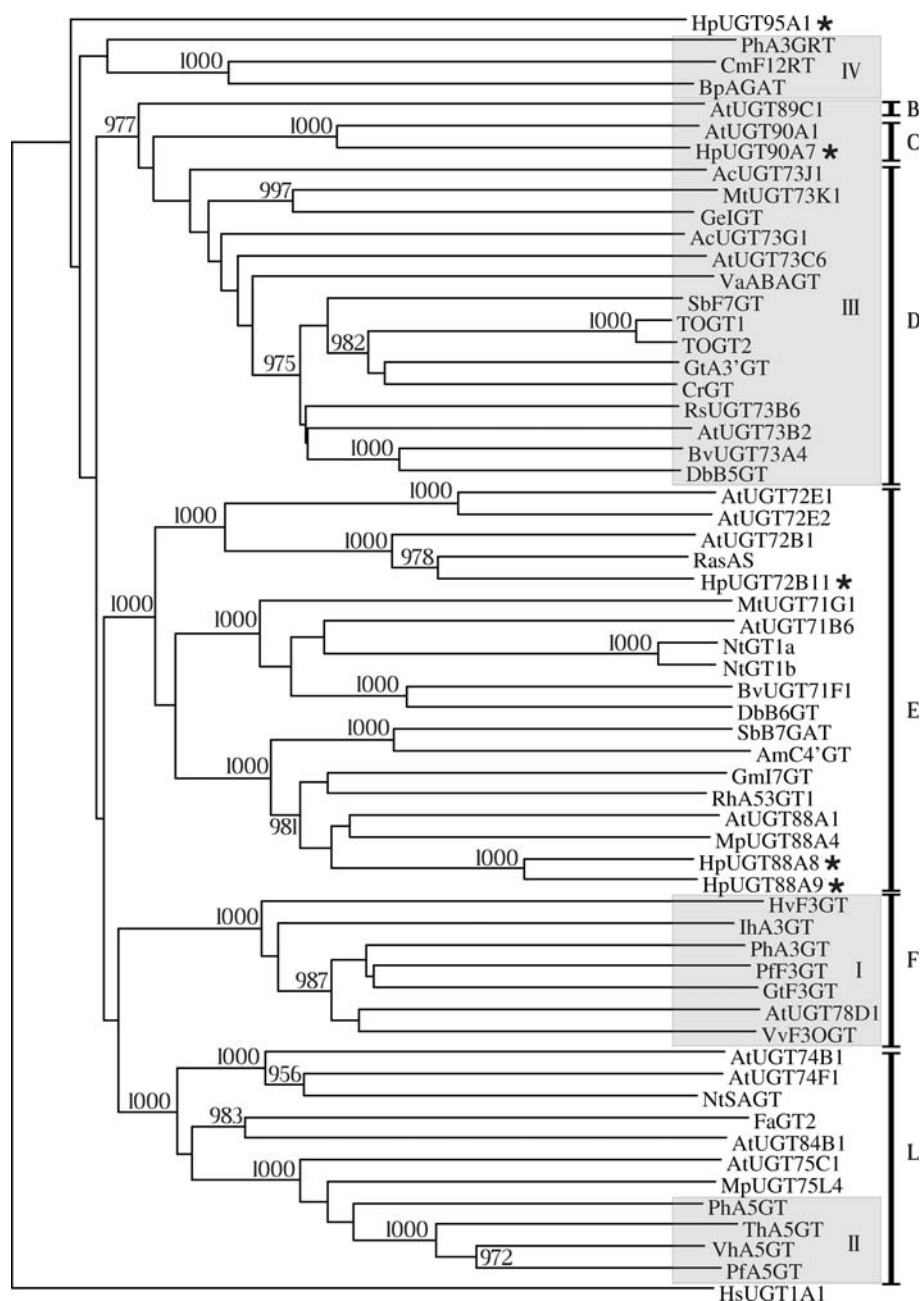
proteins (UGT72B11 53,600 Da, UGT90A7 56,000 Da, UGT95A1 65,700 Da) corresponded to the Mr determined by SEC chromatography.

The pH optimum of UGT72B11 activity was observed in potassium phosphate buffer of pH 8.0 at an optimum temperature of 45°C. Both UGT90A7 and UGT95A1 showed highest activity in Tris–HCl buffer with optimum reaction rates at pH of 7.0 for UGT90A7 and 7.6 for UGT95A1. The assay temperatures were best at 30 and 25°C, respectively.

Bivalent cations were not required for UGT activities, but the addition of $MnSO_4$ up to 0.5 mM resulted in a small stimulation (+17%) of UGT72B11 activity (data not shown). NaCl, CaCl, or $MgSO_4$ had no or only marginal influence on the glucosylation kinetics (Supplementary Table 2S). The addition of $CuSO_4$, $FeSO_4$, or $ZnSO_4$ to 0.5 mM concentration led to the complete loss of UGT90A7 and UGT95A1 activities, while UGT72B11 activity was only reduced to 12, 13, and 89%, respectively. Many flavonoid glucosyltransferases are inhibited by Zn^{2+}, while some anthocyanidin-*O*-glucosyltransferases are known to keep up moderate activity in the presence of even 1 mM Zn^{2+} (Kamsteeg et al. 1978; Yabuya et al. 2002; Fukuchi-Mizutani et al. 2003; Kogawa et al. 2007). $CoSO_4$ at 0.5 mM almost completely inhibited UGT95A1, whereas UGT72B11 and UGT90A7 exhibited commensurable activities (38 and 45%) at the same concentration. The glucosylation
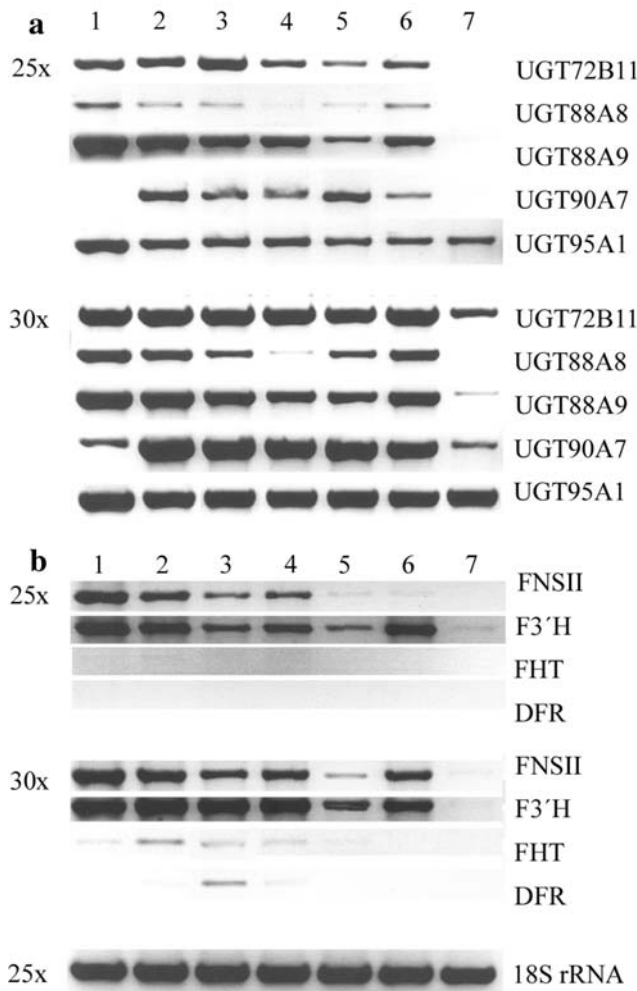
Fig. 2 Semi-quantitative RT-PCR transcript analysis of *H. pilosella* UGTs (**a**) and of genes assigned to flavonoid biosynthesis (**b**) in different organs and during flower head development. *Lanes 1–5* correspond to the developmental stages of flower heads defined in the text. *Lane 6*, leaves; *Lane 7*, stems. The ANS transcript was not detectable

Fig. 3 a Western blotting of crude extracts from *E. coli* expressing single UGTs of *H. pilosella*. The expression time in *E. coli* was five (UGT72B11, UGT90A7, and UGT95A1) or 15 h (UGT88A8 and UGT88A9), and the blots were developed with monoclonal anti-poly-histidine antibody produced in mouse followed by goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma, Taufkirchen, Germany). *Lane 1*, molecular mass marker; *lanes 2–6*, extracts expressing UGT72B11 (5 µg protein), UGT88A8 (20 µg), UGT88A9 (20 µg), UGT90A7 (5 µg), or UGT95A1 (5 µg). Proteins which are displayed in Fig. 3b are marked with an *asterisk*. **b** SDS-PAGE separation of IMAC purified UGTs from *H. pilosella*. *Lane 1*, molecular mass marker, *Lane 2–4*, UGT72B11, UGT90A7, UGT95A1 (2 µg protein/Lane). Proteins were stained with Coomassie brilliant blue

reaction of UGT90A7 was moderately reduced to 65% by 0.5 mM $NiSO_4$, while the activities of UGT72B11 and UGT95A1 were considerably suppressed to 14 and 1% under these conditions.

All three glucosyltransferases were strongly inhibited by DEPC and DIDS (Supplementary Table 3S). PMSF reduced the activity of UGT90A7 by half. The same level of inhibition was caused by NEM on the glucosylation activity of UGT72B11 and UGT90A7, while UGT95A1 activity was not affected. The thiols DTT and 2-mercaptoethanol repressed only the activity of UGT72B11, and EDTA had a negligible influence on the activities of all three UGTs.

The five UDP-glucosyltransferases of *H. pilosella* utilised UDP-glucose as glycosyl donor, whereas UDP-glucuronic acid or ADP-glucose was not accepted. The acceptor specificity of the UGTs was determined with a variety of
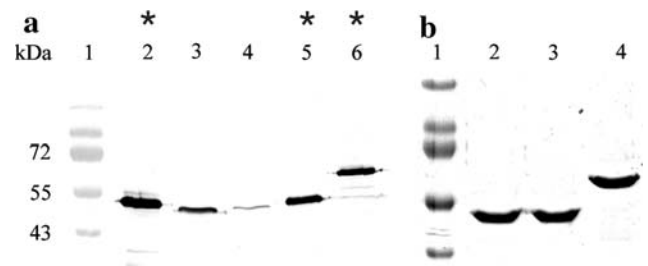
aglycones, including flavonoids, coumarins, and cinnamic acids. The three closer examined UGTs not only accepted multiple aglycones, but also yielded mostly more than one product from a single aglycone (Table 1). Some of these products could not be fully identified due to unavailability of authentic reference glucosides.

UGT90A7 showed highest activity with eriodictyol and luteolin (Table 1), the latter flavone occurring in the flower heads of *H. pilosella*. Also the flavonol quercetin was conjugated to an equivalent extent, leading to the assumption that a hydroxyl at C-3′ is important for the activity. In the presence of C-3′ hydroxyl group (luteolin and quercetin), the main product formed was the 4′-*O*-glucoside with only traces of another glucoside detectable (Fig. 4). In its absence two glucosides were also generated with the main product being the 7-*O*-glucoside. The importance of the 3′-hydroxyl for the activity and regiospecificity had been reported before for the UGT73A4 from *Beta vulgaris* (Isa-yenkova et al. 2006) and the UGT73A5 from *Dorothean-thus bellidiformis* (Vogt et al. 1999). The minor product in the assay with luteolin besides the 4′-*O*-glucoside was identified as the 7-*O*-glucoside. Both glucosides (Fig. 5) also accumulate in the flower heads of *H. pilosella* (Zidorn et al. 2002, 2005). Kinetic data for UGT90A7 were recorded with eriodictyol and luteolin at concentrations between 2.5 µM and 1 mM, but concentrations above 50 µM inhibited the enzyme reaction. Catalytic parameters were therefore calculated from data up to this concentration (Table 2). The V_{max}/K_m ratios suggested a slight bias in specificity of UGT90A7 towards luteolin.

H. pilosella UGT95A1 also showed the highest activity with *ortho*-hydroxylated flavonoids, i.e. luteolin and

Table 1 Substrate specificities of recombinant UGTs from *H. pilosella*

Substrate	UGT90A7			UGT95A1			UGT72B11		
	a	b	c	a	b	c	a	b	c
Naringenin	27	2	7-OH	ND			4	1	7-OH
Eriodictyol	100	1		21	1	3′-OH*	3	NM	
Apigenin	16	2	7-OH	2	NM		16	2	7-OH
Luteolin	100	2	4′-OH*, 7-OH	100	1	3′-OH*	9	2	4′-OH, 7-OH
Scutellarein	30	2		9	2		44	2	
Baicalein	37	2	7-OH	12	2	7-OH	100	2	7-OH
Chrysin	3	NM		ND			17	1	
Kaempferol	14	2	7-OH	26	1	7-OH	75	3	3-OH*, 7-OH
Quercetin	74	2	3-OH, 4′-OH	62	2	3′-OH*	78	3	3-OH, 4′-OH
Myricetin	58	2		15	4	3-OH	2	NM	
Morin	5	1		1	NM		5	1	
DHQ	13	2		11	2		ND		
Cinnamic acid	ND			ND			ND		
Caffeic acid	1	NM		8	1		ND		
2-Coumaric acid	ND			1	NM		ND		
3-Coumaric acid	ND			ND			ND		
4-Coumaric acid	7	ND		ND			ND		
Ferulic acid	ND			ND			ND		
Chlorogenic acid	ND			ND			ND		
Umbelliferone	ND			ND			15	1	
Esculetin	ND			ND			7	1	
Catechol	ND			ND			25	1	
Resorcinol	ND			ND			17	1	
Hydroquinone	ND			ND			3	NM	

Lane a, relative activity (%), and 100% corresponds to 3.4 pkat/μg for UGT72B11, 2.0 pkat/μg for UGT90A7, and 5.4 pkat/μg for UGT95A1; Lane b, number of products from assays conducted with 50 μM aglycone and 500 mM UDP-glucose under standard conditions examined by TLC (UDP-[U-14C]-glucose) and HPLC (unlabelled UDP-glucose); Lane c, site of conjugation. Identification was carried out by comparison of HPLC retention times and UV spectra with those of authentic reference samples. Products which were additionally identified by biotransformation and NMR spectroscopy are marked with an *asterisk*

NM not measured, *ND* no product detected

quercetin (Table 1). NMR spectroscopy documented that luteolin, quercetin, or eriodictyol were conjugated to their 3′-*O*-glucosides. However, the flavones baicalein and scutellarein, as well as the flavonol kaempferol, which do not possess a hydroxyl group at C-3′ also served as acceptors leading to the formation of one product in case of kaempferol (Fig. 4) and two products from the flavones. Since UGT95A1 clearly preferred luteolin as substrate, kinetic data were only determined for this flavone (Table 2).

UGT72B11 glucosylated most aglycones used in the assays showing highest activities with baicalein, quercetin, and kaempferol (Table 1). Hydroquinone, the main substrate of arbutin synthase from *R. serpentina* (Hefner et al. 2002), which shares 68% identity with UGT72B11, was a poor substrate. UGT72B11 also conjugated predominantly the 4′-hydroxyl of 3′,4′-hydroxylated flavonoids (Fig. 4). However, the substitution pattern was not important for the turnover rate, because similar rates were observed with 4′-hydroxylated or 3′,4′-dihydroxylated flavonoids (Table 1). At least in case of the flavones, the presence of a 6-hydroxyl group seemed to be more relevant for activity (baicalein and scutellarein). A number of flavones and flavonols, including kaempferol, which have not been

described from *H. pilosella*, served as substrate in UGT72B11 assays. Initial assays were conducted with kaempferol and reproducibly revealed considerable glucosylation activity. Kinetic parameters were therefore evaluated with this acceptor (Table 2).

Discussion

To obtain information on catalytical function and substrate specificity of plant derived UGTs is a big challenge in the post genomic area but still limited to a few plant species. Therefore, all five full-length cDNAs cloned from *H. pilosella* flower buds coding for putative UGTs were heterologously over-expressed in *E. coli*. Proteins from each clone were estimated to be in the range of 53–62 kDa, a molecular weight typical for UGTs involved in secondary plant metabolism (Vogt and Jones 2000). The developmental expression profiles of the analysed genes do not allow to assign a distinct glucosyltransferase activity to the flavonoid biosynthesis pathway. For all five UGTs and the FNS II/F3′H a slightly higher transcription activity was observed in the earlier flower head stages (beginning of

Fig. 4 HPLC separation from assays of UGT90A7, UGT72B11, and UGT95A1 with apigenin, luteolin, kaempferol, or quercetin. The aglycone concentration was 50 μM (UGT90A7) or 250 μM (UGT72B11, UGT95A1), and products were identified by comparison with reference samples: *1* apigenin 7-*O*-glucoside *2* apigenin *3* luteolin 7-*O*-glucoside *4* luteolin 4′-*O*-glucoside *5* luteolin 3′-*O*-glucoside *6* luteolin *7* kaempferol 3-*O*-glucoside *8* kaempferol 7-*O*-glucoside *9* kaempferol *10* quercetin 3-*O*-glucoside *11* quercetin 4′-*O*-glucoside *12* quercetin. Unidentified products are marked with an *asterisk*
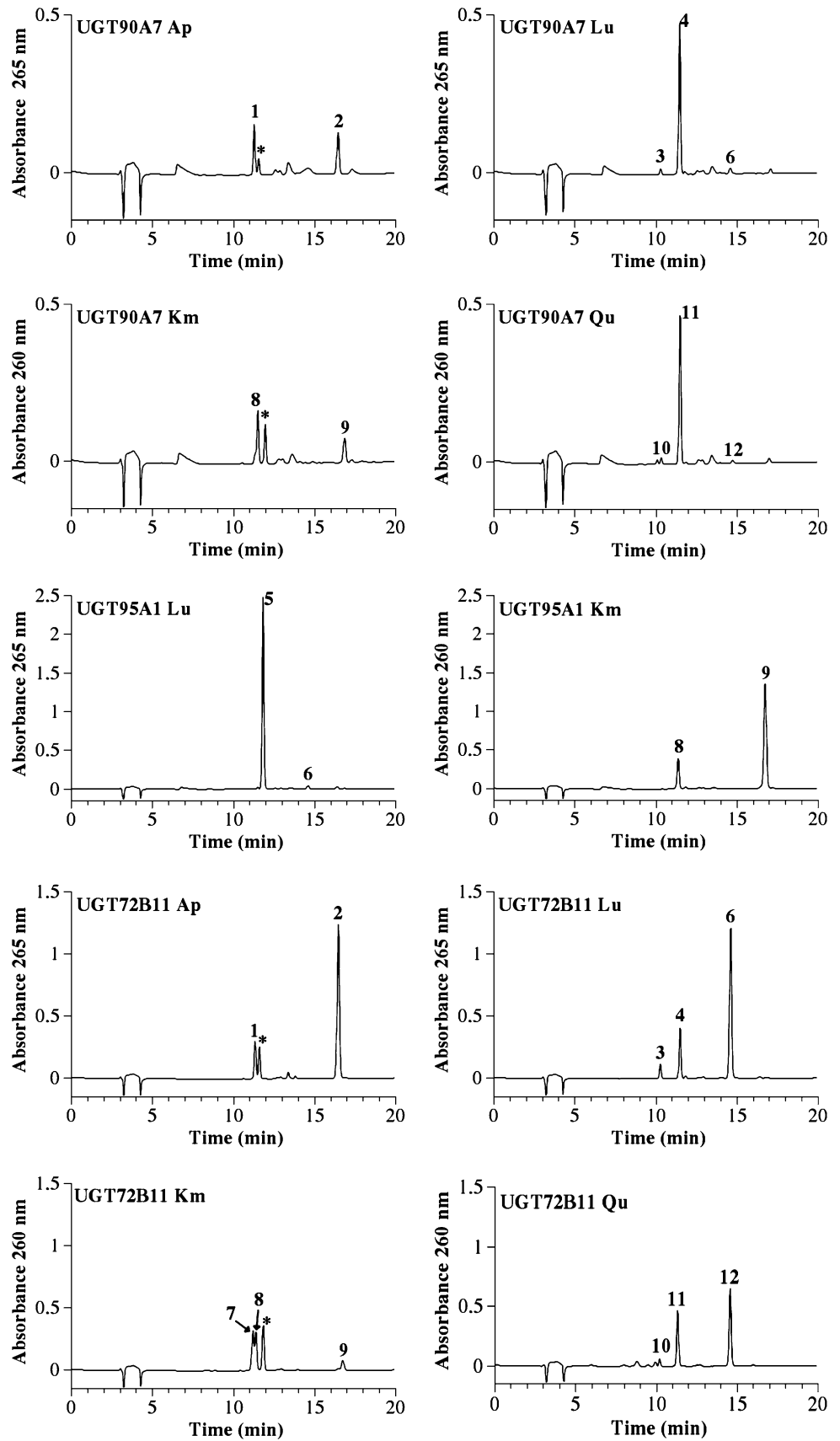
Fig. 5 Reaction scheme illustrating the transfer of a glucosyl group to luteolin catalysed by UGT90A7
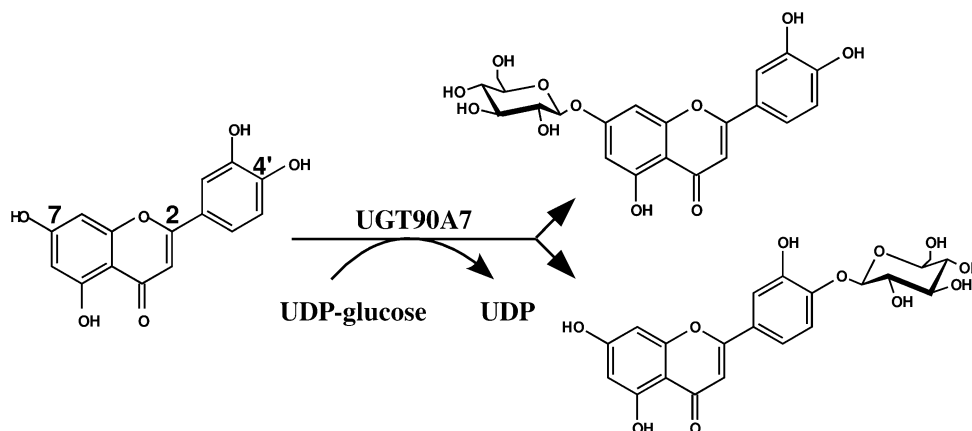

Table 2 Apparent K_m and V_{max} values of recombinant UGT72B11, UGT90A7, and UGT95A1

Substrate	K_m (μM)	V_{max} (pkat/μg)	V_{max}/K_m [(pkat/(μg μM)]
UGT90A7			
UDP-glucose	436	5.2	0.01
Luteolin	9.5	4.7	0.49
Eriodictyol	11.8	4.0	0.34
UGT95A1			
UDP-glucose	400	25	0.06
Luteolin	46.8	27.2	0.58
UGT72B11			
UDP-glucose	418	7.7	0.02
Kaempferol	20.2	7.6	0.38

pigmentation), and it remains thus possible that more than one UGT is involved in the biosynthesis of flavonoid glucosides. Furthermore, a separate analysis of the individual floral organs, such as petals, stamen, and pistils might reveal an organ specific expression and a better correlation. The low and stage specific expression of the anthocyanin genes is not surprising, because *H. pilosella* flowers are yellow with only the outer ray flowers accumulating anthocyanidins on the underside of the petals in a small stripe.

All five enzymes described here could be identified as flavonoid related UGTs in vitro and exhibited a wide substrate- and regiospecificity towards not only flavones but different flavonoids and phenolic acids as well. Most of these substrates and products, e.g. flavonols, have not been described in *H. pilosella* yet. Since no FLS like sequence was obtained during our cloning studies and the expression of related flavonol/anthocyanin pathway genes such as FHT, DFR and ANS is rather low and specific compared to the transcripts of flavone biosynthesis, the absence of flavonols is likely. However, due to the described site activity of ANS (Stracke et al. 2009) the synthesis of flavonols cannot be excluded and they might escape detection due to low,

tissue specific, or spatial occurrence. UGT90A7 showed highest activity with eriodictyol and luteolin. Based on the kinetic data and the regiospecificity of *H. pilosella* UGT90A7 it is tempting to speculate that luteolin is its true in vivo substrate. The presence of luteolin, luteolin-4′-*O*-glucoside, and luteolin-7-*O*-glucoside in the flower heads support this assumption, although the formation of both glucosides by UGT90A7 needs further proof. UGT95A1 is less specific but favoured in vitro luteolin as substrate and showed regiospecificity towards the 3′-hydroxyl group of flavonoids. However, the glucosides formed in these assays have not been reported from *H. pilosella*, and the ubiquitous expression of the respective transcripts in the plant prohibits a functional assignment of UGT95A1. Sequence alignments classified UGT95A1 to a new subgroup within the Family 1 of GTs. UGT72B11 was shown to possess broad substrate specificity towards flavonoids and coumarins with preferences in vitro for flavones and flavonols, e.g. baicalein and quercetin, respectively, which have yet to be detected in *H. pilosella*. Since umbelliferone served as substrate UGT72B11 might be involved in the glycosylation of coumarins. The affinity to umbelliferone, however, is rather common among UGTs, because out of 90 *A. thaliana* UGTs 48 were capable to glycosylate hydroxycoumarins (Lim et al. 2003). Recent studies indicate that most plant UGTs are not absolutely substrate specific at least in vitro (Vogt and Jones 2000; Messner et al. 2003; Kramer et al. 2003; Lunkenbein et al. 2006; Tian et al. 2006; Suzuki et al. 2007). The here described in vitro glucosylation pattern of *H. pilosella* UGTs could therefore not be attributed directly to in vivo functions. Whereas this fact makes it difficult to assign in vivo functions to the respective enzymes, substrate specific and promiscuous UGTs, both become interesting targets for biotechnological application.

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