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Cloning and functional characterisation of two regioselective flavonoid glucosyltransferases from *Beta vulgaris*

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Dedicated to Rodney Croteau on the occasion of his 60th birthday.

Abstract

Two full-length cDNAs encoding flavonoid-specific glucosyltransferases, UGT73A4 and UGT71F1, were isolated from a cDNA library of *Beta vulgaris* (Amaranthaceae) cell suspension cultures. They displayed high identity to position-specific betanidin and flavonoid glucosyltransferases from *Dorotheanthus bellidiformis* (Aizoaceae) and to enzymes with similar substrate specificities from various plant families. The open reading frame of the sequences encode proteins of 476 (UGT73A4) and 492 (UGT71F1) amino acids with calculated molecular masses of 54.07 kDa and 54.39 kDa, and isoelectric points of 5.8 and 5.6, respectively. Both enzymes were functionally expressed in *Escherichia coli* as His- and GST-tagged proteins, respectively. They exhibited a broad substrate specificity, but a distinct regioselectivity, glucosylating a variety of flavonols, flavones, flavanones, and coumarins. UGT73A4 showed a preference for the 4′- and 7-OH position in the flavonoids, whereas UGT71F1 preferentially glucosylated the 3- or the 7-OH position. Glucosylation of betanidin, the aglycone of the major betacyanin, betanin, in *B. vulgaris* was also observed to a low extent by both enzymes. Several *O*-glycosylated vitexin derivatives isolated from leaves of young *B. vulgaris* plants and rutin obtained from *B. vulgaris* tissue culture are discussed as potential endogenous products of UGT73A4 and UGT71F1. The results are analyzed with regard to evolution and specificity of plant natural product glucosyltransferases.

Keywords: Glucosyltransferase; *Beta vulgaris*; Chenopodiaceae; Flavonoids; Betanin; Substrate specificity

1. Introduction

In higher plants secondary metabolites are often converted to their glycoconjugates by UDP-glucose-dependent glycosyltransferases (UGTs), which transfer nucleotide diphosphate activated sugars to low molecular-weight substrates. Sugar acceptors include all major classes of secondary metabolites, including phenylpropanoids, terpenoids, cyanohydrins, alkaloids, and betacyanins (Vogt and Jones, 2000; Bowles et al., 2005; Gachon et al., 2005). Glycosylation of low molecular-weight compounds increases water solubility, may detoxify xenobiotics, enhance pathogen resistance, or regulate cellular homeostasis (Kaminaga et al., 2003; Brazier-Hicks and Edwards, 2005; Langlois-Meurinne et al., 2005; Lim and Bowles, 2004). As biotechnological targets, UGTs might be used to improve food or crop quality (Frydman et al., 2004) or be applied as regioselective biocatalysts (Arend et al., 2001; Lim et al., 2002). The respective enzymes constitute a highly divergent polyphyletic multigene family classified into 75 members (Mackenzie et al., 1997; Coutinho et al., 2003; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Family 1 contains the UGTs involved in plant natural product modification and can be identified based on a 44 amino acid

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C-terminal consensus motif (Mackenzie et al., 1997; Li et al., 2001; Ross et al., 2001; Hughes and Hughes, 1994). Those enzymes which have been functionally expressed exhibit either distinct or broad substrate specificities (e.g. Martin et al., 1999; Miller et al., 1999; Yamazaki et al., 1999; Vogt et al., 1999; Taguchi et al., 2001). Functional genomics combined with metabolite profiling support the challenging tasks to predict *in vivo* and *in vitro* the substrate preference of recombinant UGTs (Richman et al., 2005; Gachon et al., 2005; Lim et al., 2003), already effected by a single amino acid change (Kubo et al., 2004).

Betanin (betanidin 5-*O*-glucoside) is the major pigment in red beet (*Beta vulgaris*) classified as a member of the Amaranthaceae (Cuénod et al., 2002). Functionally equivalent betanin and other betacyanins have replaced the chromogenic anthocyanins in the plant order Caryophyllales (Clement and Mabry, 1996; Strack et al., 2003). Glucosylation in betacyanin containing plants has been shown to proceed either at the level of betanidin (Heuer and Strack, 1992; Heuer et al., 1996; Vogt et al., 1997) or at the level of *cyclo*-DOPA (Sasaki et al., 2004, 2005a,b). To elucidate the biosynthetic steps in *B. vulgaris* and identify glucosyltransferases potentially involved in the biosynthesis of betanin, its major red pigment, we have cloned and functionally characterized two new regioselective flavonoid UGTs by homology screening. In addition, we have purified and characterized several endogenous vitexin derivatives, with the aim of identifying potential endogenous products of these UGTs. Results are discussed in terms of molecular evolution of plant natural product glucosyltransferases.

2. Results and discussion

2.1. Cloning, expression and purification of recombinant UGT73A4 and UGT71F1 from B. vulgaris

A cDNA library from *B. vulgaris* cell suspension culture was used as a template in PCR reactions with degenerate primers derived from the ''PSPG''-box (Hughes and Hughes, 1994). Positive clones were picked, analyzed for correct size, partially sequenced and compared to the databases. The deduced protein sequences of two isolated full-length clones, termed UGT73A4 and UGT71F1, based on the nomenclature by Mackenzie et al. (1997), exhibited the highest homology with 64% and 59% identity at the amino acid level to UGT73A5 and UGT71F2 from *D. bellidiformis*, respectively, whereas both sequences shared only 24% identity to each other. Only one member of each clone was found in the screening approach.

To verify the identity of the recombinant UGTs from *B. vulgaris*, both full-length clones were functionally expressed in *E. coli*. The overall amount of both recombinant enzymes in crude bacterial extracts was very low, complicating the purification and determination of enzyme properties. Attempts to achieve higher specific activities in yeast or insect cell cultures were unsuccessful due to very low enzyme yields (data not shown). Purification of the recombinant N-terminal His-tag fusion protein of UGT73A4 to near homogeneity was achieved via a three-step protocol, including ammonium sulfate precipitation, hydrophobic interaction and metal affinity chromatography, with an overall yield of less than 0.2 mg/l culture. It was essential to purify UGT73A4 from the crude bacterial extract by HIC prior to affinity chromatography. Otherwise, the enzyme did not bind to the subsequent affinity matrix. SDS–PAGE of the final concentrated preparation showed one major band at 58 kDa (Fig. 1A), that correlated with the activity-elution profiles on Phenylsepharose and immobilized-metal affinity chromatography. Attempts to eliminate minor impurities failed, since enzyme activity was lost after subsequent gel filtration or ion exchange chromatography. Western-blot detection with a monoclonal mouse anti-His-tag antibody was also performed and the recombinant His-tag fusion protein of UGT73A4 was verified with an anti-mouse alkaline phosphatase coupled antibody conjugate and formation of the colored nitroblue tetrazolium/bromo-chloro-indolylphosphate (NBT/BCIP) (Fig. 1B).

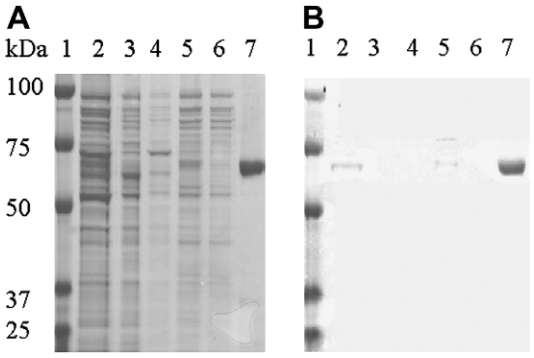

Fig. 1. (A) SDS–PAGE of selected fractions from His-tagged UGT73A4 purification steps. Proteins were stained with Coomassie brilliant blue. lane 1, molecular weight marker; lane 2, crude protein extract (20 μg); lane 3 crude protein extract from cells (20 μg), transformed with empty vector pQE30 (20 μg); lane 4, crude extract from untransformed M15 p[Rep4] cells (10 μg); lane 5, pooled UGT73A4 fractions after Phenylsepharose 4L-CB (10 μg); lane 6 flow-through Talon™ metal affinity chromatography (10 μg); lane 7, UGT73A4 metal affinity chromatography purified UGT73A4 (10 μg); (B) detection of (partly) purified UGT73A4 fractions by Western Blot. The membrane was incubated with a monoclonal mouse Anti-His-IgG-AP antibody (Invitrogen, Karlsruhe, Germany) that binds the recombinant 6x-His-tag fusion protein. Protein was detected with an anti-mouse, alkaline phosphatase coupled antibody conjugate (Sigma) and formation of the colored nitroblue tetrazolium/bromo-chloro-indolylphosphate (NBT/BCIP) complex.

The recombinant UGT71F1 was expressed as a C-terminal GST-tag fusion protein and affinity purified on glutathione-agarose (Table 2). SDS–PAGE analysis revealed one major band at 85 kDa of the fusion protein in fractions which exhibited the highest activity (Fig. 2A). The enrichment of the GST fusion protein during the purification step was verified by Western blot analysis (Fig. 2B). All attempts to further purify the recombinant UGT71F1 by

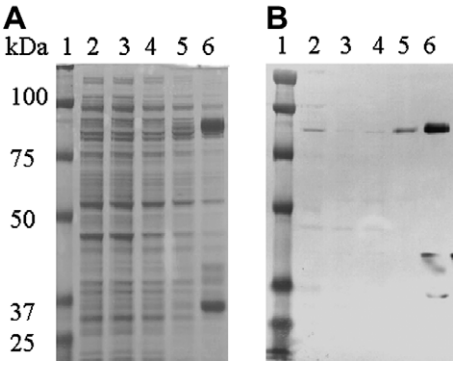

Fig. 2. (A) SDS–PAGE of protein from the partially purification of GST-tagged UGT71F1. Proteins were stained with Coomassie brilliant blue. lane 1, molecular weight marker; lane 2 crude protein extract (20 μg); lane 3, flow-through of affinity column (20 μg); lane 4 wash fraction of affinity column without detectable enzyme activity (10 μg); lane 5 fraction with 20% activity (10 μg); lane 6, fraction with 90% enzyme activity (10 μg). (B) Detection of partially purified UGT71F1 fractions by Western Blot. The membrane was incubated with a monoclonal mouse antibody with high affinity to the 26 kDa GST domain from *S. japonicum* (Novagen, Madison, USA) that binds the recombinant GST-tag fusion protein. Protein was detected with a goat-anti-mouse, alkaline phosphatase coupled antibody conjugate and formation of the colored nitroblue tetrazolium/bromo-chloro-indolylphosphate (NBT/BCIP) complex.

conventional column chromatography failed and led to complete loss of enzyme activity. Purification by dye-ligand chromatography successfully used for the homologous enzyme UGT71F2 from *D. bellidiformis* cell suspension cultures (Vogt et al., 1997) was also not possible.

2.2. Functional characterization

2.2.1. General properties

The general properties of both enzymes are summarized in Table 1. There was no requirement for the bivalent cations Mg^{2+}, Mn^{2+} or Ca^{2+} for enzyme activities and no effect was observed upon the addition of EDTA in the range from 0.2 to 1 mM. However, Cu^{2+} and Co^{2+} were inhibitory (100% inhibition near 40 μM) on both enzyme activities, which is not surprising, as the sensitivity of other UGTs towards copper has been reported (Latchinian-Sadek and Ibrahim, 1991; Ishikura and Mato, 1993). UGT73A4 was insensitive to dithioerythritol or 2-mercaptoethanol, thus disulfide bond formation is not essential for enzyme stabilization. UGT73A4 can be stored at 4 °C or frozen at −20 °C or −80 °C for several months without requiring addition of stabilizing agents such as bovine serum albumin (BSA) or glycerol. In contrast, UGT71F1 could not be frozen nor stored at 4 °C as 50% activity was lost within 24 h.

Table 1
Biochemical properties of recombinant His-tagged UGT73A4 and GST-tagged UGT71F1

Properties	UGT73A4	UGT71F1
Molecular mass (kDa) of tag-free proteins[a]	54.07	54.39
Structure	Monomer	Monomer
pH optimum[b]	6.0	7.0–9.0
Temperature maximum	30 °C	37 °C
Energy of activation[c]	37.6 kJ mol^{-1}	38.8 kJ mol^{-1}
Isoelectric point[d]	5.8	5.6
Requirement for bivalent cations	None	None

[a] Determined by MALDI-TOF-MS analysis with molecular masses of tags subtracted.
[b] Determined with a three-component buffer mixture (Ellis and Morrison, 1982).
[c] Determined by Arrhenius-plots.
[d] Calculated by use of the software package DNASTAR (Lasergene, Madison, Wisconsin).

Chemical modification of UGT73A4 by amino acid-modifying reagents showed inhibition by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and *p*-chloro-mercuribenzene sulfonic acid (PCMBS). DIDS has been reported to react with the ε-amino group of lysine (Goffner et al., 1994) and inhibited UGT73A4 and UGT71F1 activity at 10 μM. With PCMBS, used to oxidize free SH groups, the UGT73A4 activity was completely inhibited at 10 μM, whereas the activity of UGT71F1 was reduced to 22%. Treatment with diethyl pyrocarbonate (DEPC), indicative for the involvement of a histidine in the active center (Hans et al., 2004; Shao et al., 2005) led to a complete loss only of UGT73A4 activity at 1 mM. However, the partially purified recombinant UGT71F1 fusion protein was not affected by DEPC in the range from 10 μM to 1 mM, in contrast to the previously characterized native UGT71F2 from *D. bellidiformis* (Vogt et al., 1997). Phenylglyoxal, used to modify arginine residues, did not show any effect on the enzyme activities.

2.2.2. Substrate specificity and kinetic data

In vitro studies of the recombinant UGTs revealed that both enzymes catalyze the regioselective transfer of glucose to hydroxyl groups of various flavonoids, e.g. flavonols, flavones, flavanones, as well as coumarins (Table 2). Initial studies indicated that incubation of crude protein extracts from *B. vulgaris* cell suspension cultures and from young plants with 20 μM quercetin resulted in the formation of three major quercetin glucosides, the 7-*O*-, 3-*O*-, and preferentially the 4′-*O*-glucoside, identified from their UV spectral data and comparison with authentic standards (data not shown). Experiments with crude recombinant UGT73A4 showed glucosylation of the 4′-OH, the 7-OH, and the 3′-OH with a preference for the 4′-OH position (Fig. 3). Therefore, observed 3-*O*-glucosylation in crude extracts (data not shown) presumably is the result of another UGT (see below). Further analyses with a broad spectrum of flavonoids (Table 2) indicates the preferred direction of glucosylation is governed by the presence or absence of a hydroxyl group at C-3′. When this group is present (quercetin, myricetin, and quercetagetin), 4′-OH glycosylation is preferred while in its absence 7-OH glucosylation is observed. In enzyme

Table 2
Substrate specificity of recombinant His-tagged UGT73A4 (3.4 μg protein, 30 °C, 10 min) and GST-tagged UGT71F1 (13.4 μg protein, 30 °C, 20 min) and comparison of relative enzyme activities

Substrate	UGT73A4 (relative activity)	OH-group glucosylated	UGT71F1 (relative activity)	OH-group glucosylated
Quercetin	100	4′-OH, 7-OH	100	3-OH, 7-OH
Betanidin	(10)[a]	5-OH	(28)[a]	5-OH, 6-OH
cyclo-DOPA	n.d.[b]	–	n.d.	–
Myricetin	80	4′-OH (?)	n.d.	–
Kaempferol	81	7-OH	17	3-OH
Kaempferol-4′-OMe	42	7-OH	n.m.[b]	3-OH (?)
Kaempferol-7-OMe	n.d.	–	n.m.	3-OH (?)
Quercetagetin	28	4′-OH (?)	84	3-OH, 7-OH
Lluteolin	100	7-OH	17	7-OH
Aapigenin	90	7-OH	40	7-OH
Eriodictyol	48	7-OH	n.d.	–
Vitexin	n.d.	–	n.d.	–
Naringenin	10	7-OH	n.d.	–
Pinocembrin	25	7-OH	57	7-OH
Chrysin	70	7-OH	n.d.	–
Esculetin	77	6-OH	n.d.	–
Scopoletin	n.d.	–	n.d.	–
Caffeic acid	75	3-OH (?)	n.d.	–
p-Coumaric acid	n.d.	–	n.d.	–
Salicylic acid	n.d.	–	n.d.	–
Naphthol	n.d.	–	n.d.	–

Substrates were used with a final concentration of 20 μM. 100% correspond to 490 pkat/mg protein (UGT73A4) or 62 pkat/mg protein (UGT71F1). Data represent the average of triplicates. Standard deviations in this experiment for all substrates was less than 10% of relative activities.
(?), Identification tentative.
[a] Relative activities for betanidin as the substrate varied strongly with the individual batch of purified enzyme (see also Table 3).
[b] n.d. = no product detected; n.m. not measured.

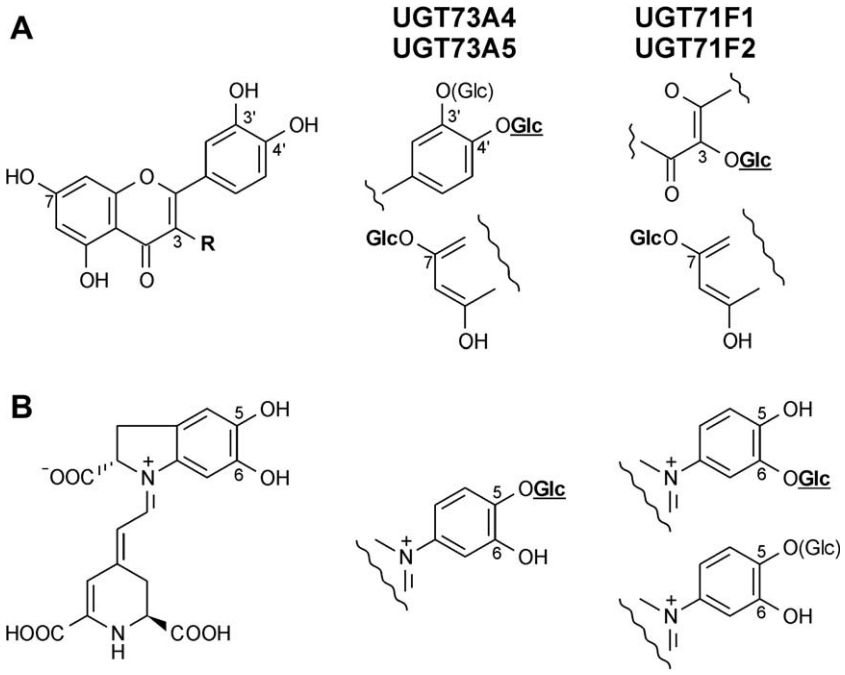

Fig. 3. Position of glucose attachment by UGTs from *B. vulgaris* (UGT73A4 and UGT71F1), and from livingstone daisy, *D. bellidiformis* (UGT73A5 and UGT71F2). (A) Flavonoids like quercetin (R = OH) or flavones like luteolin (R = H); (B) betanidin as substrates. Preferred specificities are marked in bold letters and underlined. (Glc) additional glucose attachment sites catalysed by the *B. vulgaris* enzymes.

assays with purified, enriched recombinant protein, the formation of quercetin 7,4′-*O*-diglucoside was detected, and also traces of the 3′-*O*-glucoside (Fig. 4A). Therefore, only the flavonoid B-ring is attacked, but multiple products formed, if a dihydroxylated B-ring system is present. This is exemplified with kaempferol and quercetin, where only the B-ring of the latter is glucosylated (Table 2). This preference has already been verified by docking experiments and energy minimization described for the related UGT73A5 from *D. bellidiformis* (Hans et al.,

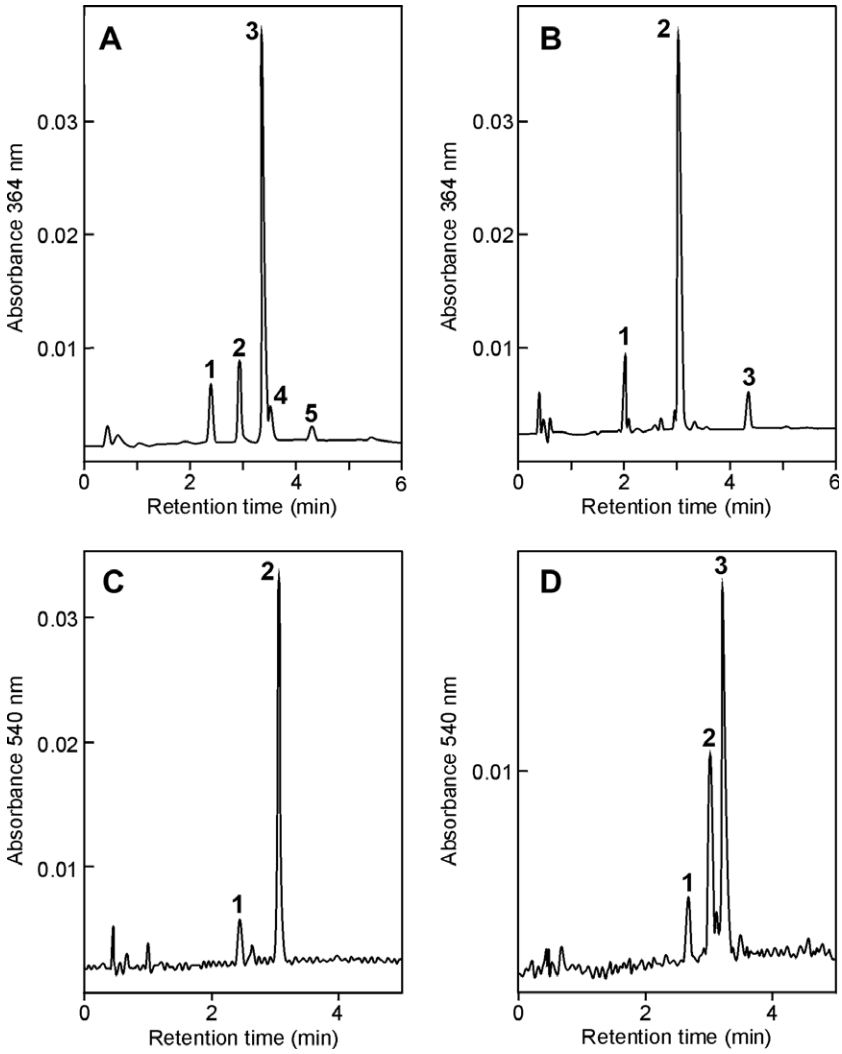

Fig. 4. (A) HPLC analyses of glucosyltransferase assays with 20 μM quercetin (detection wavelength at 364 nm) and purified UGT73A4. Peak identification: (1) quercetin 7,4′-di-*O*-glucoside; (2) quercetin 7-*O*-glucoside; (3) quercetin 4′-*O*-glucoside; (4) quercetin 3′-*O*-glucoside; (5) substrate quercetin. (B) HPLC analyses of glucosyltransferase assays with 20 μM quercetin (detection wavelength at 364 nm) with partially purified UGT71F1. Peak identification: (1) quercetin 3,7 di-*O*-glucoside; (2) quercetin 3-*O*-glucoside; (3) substrate quercetin. (C) Purified UGT73A4 with 10 μM betanidin (detection wavelength at 540 nm). Peak identification: (1) betanin; (2) substrate, betanidin. (D) Partially purified UGT71F1with 10 μM betanidin (detection wavelength at 540 nm). Peak identification: (1) betanin; (2) gomphrenin I; (3) substrate betanidin. Differences in detection sensitivity are due to the different concentrations of betanidin and quercetion used in the assays.

2004). Based on their acceptor specificities, UGT73A4 and UGT73A5 could be involved in the biosynthesis of flavone glycosides in *B. vulgaris* or flavonol glycosides in *D. bellidiformis*, respectively.

In contrast, the same set of substrates indicated that UGT71F1 transfers glucose to the 3-OH when this is present and less efficiently to the 7-OH group in its absence (Fig. 4B and Table 2). Hydroxyl groups on the B-ring are not glucosylated, although they affect relative activities (see Table 2, 3-OH glycosylation is in the order quercetin > kaempferol > myricetin). UGT71F1 is a potential candidate for the observed 3-*O*-glucosylation in crude *B. vulgaris* extracts. The low transcript levels, and the observed promiscuity towards either the 5- or the 6-OH of betanidin does not suggest any essential role of this enzyme for betanin biosynthesis in red beet cell cultures, from which it was isolated. However, *in vivo* cellular and subcellular compartmentalisation, availability of substrates, and multi enzyme-complex association play an essential role besides specificity (Hansen et al., 2003) and do not rule out this possibility. Lim et al. (2003) published enzymatic data for recombinant UGT71C1, a putative caffeic acid 3-UGT whose catalytic efficiency towards quercetin was 13 times higher than towards caffeic acid. Nevertheless, transformation of *Arabidopsis thaliana* with the UGT71C1 cDNA, showed that the recombinant enzyme glucosylated caffeic acid *in planta* at the 3-OH position, as expected.

With regard to donor preference, only UDP-glucose, but not UDP-galactose or ADP-glucose were accepted by both enzymes, consistent with the data from UGT73A5 and UGT71F2 from *D. bellidiformis*.

Table 3
Apparent K_m and V_{max} values of His-tagged UGT73A4 and GST-tagged UGT71F1, estimated from Hanes plots (not shown)

Substrate	K_m (μM)	V_{max} (pkat μg^{-1})	V_{max}/K_m (pkat μg^{-1} μM^{-1})	Efficiency (%)
UGT73A4				
Quercetin	11.0	16.4	1.5	100
Apigenin	19.2	12.8	0.7	47
Betanidin	333	0.2	6.0×10^{-4}	0.04
UGT71F1				
Quercetin	2.4	0.7	0.3	100
Apigenin	26.8	0.1	3.4×10^{-3}	1.1
Betanidin	29.0	0.03	1.0×10^{-3}	0.3

See Table 2.

Apparent K_m and V_{max} values for a selected set of substrates of UGT73A4 and UGT71F1 are listed in Table 3 for the partially purified enzymes. The values were estimated graphically from Hanes plots. Apparent K_m values suggest that both recombinant enzymes accept quercetin (among other flavonoids) as the preferred substrate *in vitro*. The catalytic efficiency for betanidin is strikingly low for both UGT71F1 and UGT73A4. Determination of kinetic data was complicated due to inhibitory effects of the two flavonoid substrates above 20 μM concentration. Two important features distinguish the new enzymes from their homologues in *D. bellidiformis*. UGT71F1 does not discriminate between the 5- or the 6-OH group of its potential substrate betanidin, leading to the simultaneous formation of betanin and gomphrenin I, respectively (Fig. 4D). Catalytic efficiencies of both enzymes with betanidin however, were far below the activities with the flavonoids tested (Table 2) The low efficiencies of both UGTs towards betanidin as compared to the flavonoids make their *in vivo* role in betanin biosynthesis questionable. In case of UGT73A4 it also varied strongly with betanidin as a substrate, ranging from below 1% up to 20% as compared to quercetin (see also Table 2). This discrepancy was never observed with any other substrate. Speculations of artificial protein aggregation appear unlikely, since ultracentrifugation experiments showed purified UGT73A4 is monomeric in solution (Lilie, H., personal communication).

Another candidate gene would be the inducible and, from comparison of available amino acid sequence (57% sequence identities), very similar glucosyltransferase reported from *B. vulgaris* (Sepulveda-Jimenez et al., 2005; Acc. No. AAO88911). An antisense approach performed by these authors with its partial sequence resulted in a reduced amount of betanin in *B. vulgaris* plantlets. Since no full length sequence or any substrate specificity data of this gene are available, this putative betanidin-UGT cannot be annotated (Fig. 7). If any the above described enzymes are involved in betanin biosynthesis in *B. vulgaris*, this remains to be proven. Glucosylation of betanidin in *B. vulgaris* might otherwise be performed by a *cyclo*-DOPA-UGT (Wyler et al., 1984) although the actual amounts of *cyclo*-DOPA glucoside present in *B. vulgaris* tissues were shown to be 100-fold lower than those previously reported (Schliemann, W., personal communication). Reports of *cyclo*-DOPA-UGTs correlating with the accumulation of betacyanins in some Caryophyllales have been reported (Sasaki et al., 2004). The results were corroborated by cloning and functional expression of two novel UGT cDNAs from *Mirabilis jalapa* and *Celosia cristata*. The corresponding proteins are able to glucosylate *cyclo*-DOPA in betacyanin biosynthesis (Sasaki et al., 2005a). The same authors also reported that glucuronidation to amaranthin in *Amaranthus caudatus* takes place at the *cyclo*-DOPA glucoside rather than at the betanin level (Sasaki et al., 2005b). Until now, there is no experimental evidence of these enzymatic functions in *B. vulgaris* yet.

2.3. Analysis of transcript accumulation levels

Detection of UGT73A4 transcripts by Northern blots indicated gene expression throughout seedling development of *B. vulgaris*, strongest at 4–5 weeks after sowing, (Fig. 5A) consistent with light induction of betacyanin biosynthesis (French et al., 1973; Giudici de Nicola et al., 1974; Leathers et al., 1992). A reduced amount of transcript is observed in older plants (Fig. 5A). Transcript

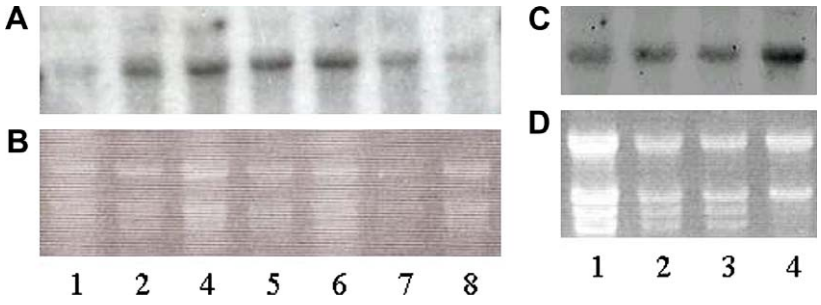

Fig. 5. RNA gel blot analysis of UGT73A4 expression. (A) Time course of transcript levels during seedling development of *B. vulgaris* cultivar. (B) Corresponding RNA panels. The numbers represent the weeks after sowing. (C) Transcript levels of three ecotypes of *B.* maritima four weeks after sowing and of *B. vulgaris* cell suspension cultures. Lane 1, ecotype Thessaloniki 296; 2, ecotype Wales 307; 3, ecotype Camargue 310; 4, *B. vulgaris* cell suspension culture. (D) Corresponding RNA-panels. Ten micrograms of total RNA were loaded onto each lane.

analyses in different tissues (leaves, hypocotyls and stems) indicated no tissue-specific expression of the UGT73A4 (data not shown). Similar to the cultivar analyzed, the transcript can also be detected 4 weeks after sowing in three different ecotypes of *B. maritima* representing original wild-type *Beta* varieties from various ecosystems (Fig. 5C). A stronger signal is observed with mRNA from *B. vulgaris* cell cultures (Fig. 5C), from where the library was constructed. Consistent with its unclear role in *B. vulgaris* plantlets, the presence of UGT71F1 transcript could not be confirmed by Northern blot analysis or RT-PCR. A weak signal by RT-PCR was detected in *B. vulgaris* cell suspension cultures (data not shown).

2.4. Flavonoid glycosides from B. vulgaris

In order to identify potential endogenous flavonoid substrates of UGT73A4 and UGT71F1, eight-week-old plants and cell suspension cultures were extracted and the predominant flavonoid secondary metabolites were isolated and rigorously characterized (Fig. 6a and b). A combination of electrospray mass spectra and high resolution MS data afforded the molecular formulae of HPLC-separated compounds. Methylation analysis allowed identification of the sugar moieties present in each compound (Table 4). Nuclear magnetic resonance spectroscopic data allowed unambiguous structural elucidation of five major glycosylated vitexin (C-glucosylated flavone) derivatives. 2″-glucosylvitexin (**1**), 2″-*O*-glucosyl-6″-malonylvitexin (**2**), 2″-*O*-xylosylvitexin (**3**), 7-*O*-glucosylvitexin (**4**) and 7-*O*-glucosyl-4′-methoxyvitexin (**5**) (Fig. 6a).

The five compounds fall into two structural categories (Fig. 6b), namely three derivatives with disaccharide substituents at C-8 (**1**–**3**) and two derivatives with monosaccharide substituents at both, carbons 7 and 8 of the flavone structure (**4** and **5**). In each case, the structure of one compound from each category (**1** and **4**) was established unambiguously by the use of 2D homonuclear and

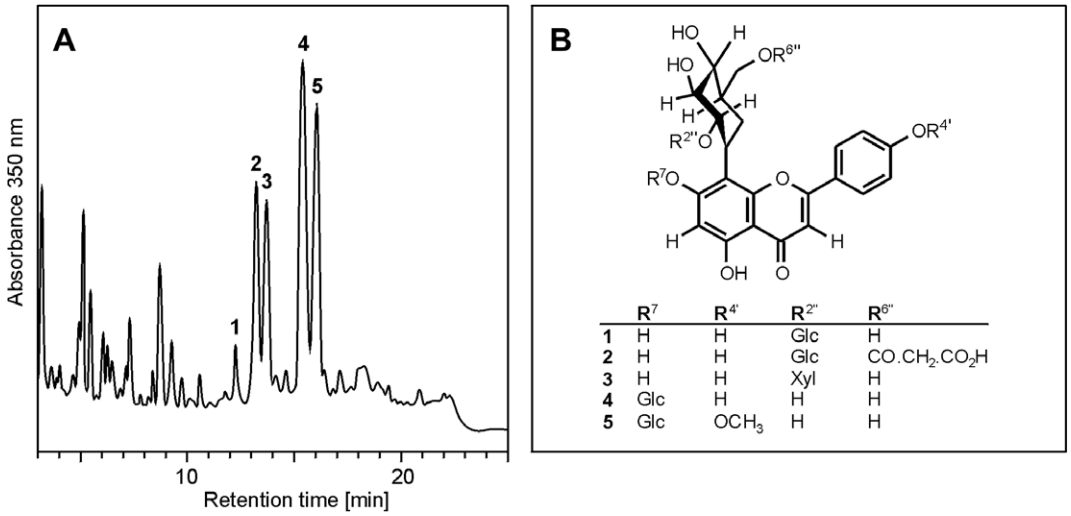

Fig. 6. (A) HPLC analysis of methanolic extracts from *B. vulgaris* plants (eight week old) with detection at 350 nm. Peak identification: **1**, 2″-glucosylvitexin; **2**, 2″-glucosyl-6″-malonylvitexin; **3**, 2″-xylosylvitexin; **4**, 7-glucosylvitexin; **5**, 7-glucosyl-4′-methoxyvitexin. (B) Structure and substitution pattern of vitexin conjugates isolated from *B. vulgaris* plants.

Table 4

Compound[a]	$[M+H]^+$	$[M+Na]^+$	M	Structure	Sugar analysis
(a) Positive ion ESI-MS data and sugar analyses of vitexin derivatives of B. vulgaris					
1	595.2	617.2	594	Hexose/hexose	Glucose
2	681.2	703.1	680	Hexose/hexose/malonoyl	Glucose
3	565.1	587.1	564	Hexose/pentose	Glucose/xylose
4		617.2	594	Hexose/hexose	Glucose
5	609.2	631.2	608	Hexose/hexose/OMe	Glucose

Compound	$[M+H]^+$ found	$[M+H]^+$ calculated	Molecular formula
(b) High-resolution positive ion ESI-MS data of vitexin derivatives of B. vulgaris			
1	595.166	595.1663	$C_{27}H_{30}O_{15}$
2	681.167	680.1667	$C_{30}H_{32}O_{18}$
3	565.155	564.1557	$C_{26}H_{28}O_{14}$
4	595.168	595.1663	$C_{27}H_{30}O_{15}$
5	609.184	609.1819	$C_{28}H_{32}O_{15}$

[a] Compounds **1**–**3** with a disaccharide substitution at C-8; compounds 4 and 5 with monosaccharide substituent both at C-7 and C-8.

heteronuclear ^{1}H and ^{13}C NMR spectroscopic techniques. Subsequently, the structure of other members were determined by comparison of the ^{1}H NMR data. The ^{1}H and ^{13}C NMR spectroscopic data are given in Tables 5 and 6, respectively.

In all compounds (**1**–**5**), two distinct sets of signals of unequal intensities were observed in the ^{1}H spectra with the largest chemical shift differences being observed for H-2′/6′ and certain sugar protons (Table 5), and were indicative of slow conformational exchange (see below). For the full analyses of **1** and **4**, the flavonoid and β-glucopyranosyl fragments in the molecules were identified from correlations in the 2D ^{1}H COSY spectra. The presence of a C-bound β-glucopyranosyl unit was evident from the 10 Hz coupling between H-1″ and H-2″. Heteronuclear 2D correlations allowed assignments of all the ^{1}H and ^{13}C signals, and further afforded the linkages between the various moieties. The observation of three ^{13}C–^{1}H correlations from H-1″ to carbons (C-7,-8 and -9) of the A ring in the HMBC spectra confirmed the linkage of the C-bound sugar to C-8. The three-bond correlation of H-1‴ to C-2″ in **1** and to C-7 in **4** determined the position of the second β-glucopyranosyl units. An alternative arrangement in which the C-bound sugar was at C-6 in ring A could be

Table 5
^{1}H NMR spectroscopic data of vitexin derivatives **1**–**5** from *B. vulgaris* with disaccharide substituents at C-8, **1**–**3**, and with monosaccharide substituents at both *O*-7 and C-8,**4** and **5**, in CD_3OD

	Compound									
	1		**2**[c]		**3**[b]		**4**		**5**	
	Major	Minor	Major	Minor	Major	Minor	Major	Minor	Major	Minor
Percentages[d]	65	35	65	35	72	28	77	23	79	21
Shifts (ppm)										
H-3	6.64	6.64	6.67	6.65	6.65	6.63	6.71	6.70	6.73	6.73
H-6	6.29	6.30	6.30	6.30	6.27	6.29	6.72	6.72	6.76	6.75
H-2′/6	8.03	7.90	7.98	7.90	8.02	7.90	8.05	7.92	8.15	8.01
H-3′/5′	6.99	6.99	7.03	6.99	6.99	6.99	6.98	6.98	7.14	7.14
4′-OCH_3									3.94	3.94
H-1″	5.10	5.18	5.10	5.18	5.07	5.20	5.11	5.08	5.12	5.09
H-2″	4.37	4.37	4.43	4.37	4.35	4.16	4.05	4.31	4.05	4.31
H-3″	3.77	3.80	3.79	3.81	3.93-3.71	3.93-3.71	3.58	3.57	3.59	
H-4″	3.72	3.60	3.76	3.62	3.93-3.71	3.93-3.71	3.70		3.74	
H-5″	3.50	3.55	3.70	3.79	3.51	3.57	3.51		3.57-3.47	
H-6″A	4.00	3.93	4.54	4.57	3.99	3.93-3.71	4.00		3.99	
H-6″B	3.84	3.81	4.40	4.39	3.84	3.93-3.71	3.84		3.87	
H-1‴	4.24	4.35	4.25	4.36	4.18	4.18	5.08	5.10	5.09	5.11
H-2‴	3.04	2.99	3.04	2.98	3.01		3.62		3.62	
H-3‴	3.17	3.25	3.17	3.24	3.11		3.57-3.47		3.57-3.47	
H-4‴	3.20	3.11	3.20	3.10	3.27-3.11		3.57-3.47		3.57-3.47	
H-5‴	2.76	2.93	2.76	2.93	A3.27-3.11, B 2.76-2.59		3.54		3.57-3.47	
H-6‴A	3.41	3.43	3.41	3.45-3.39			3.95		3.95	
H-6‴B	3.32	3.43	3.31	3.45-3.39			3.78		3.78	
Couplings[a]										
2′–3′ + 2′–5′	8.7	8.7	8.7	8.7	8.7	8.7	8.8	8.8	8.9	8.9
1″–2″	10.0	9.8	10.1	9.8	10.0	9.8	10.0	9.7	10.0	
2″–3″	8.8		8.9	9.0	8.4		8.9	8.8		
3″–4″	9.0	9.1	9.0	9.1			9.4		9.4	
4″–5″	9.2	9.1	9.0	9.3			9.4		9.4	
5″–6″A	1.8		1.7		1.8		2.1		2.2	
5″–6″B	5.5		5.2		5.3		5.8		5.7	
6″A–6″B	12.1		12.1		12.0		12.1		12.2	
1‴–2‴	7.8	7.8	7.8	8.1	7.2		7.6	7.3	7.6	7.6
2‴–3‴	8.9	9.2	9.0	9.1	8.5		9.1		9.1	
3‴–4‴	9.0	9.2	9.0	9.1	8.3					
4‴–5‴	9.0	9.3	9.0	9.1						
5‴–6‴A	3.7		3.5				2.1		2.1	
5‴–6‴B	3.0		3.0				5.4		5.4	
6‴A–6‴B	11.2		11.4				12.2		12.2	

[a] Overlap of signals allowed only those couplings shown to be evaluated.
[b] The identity of the second sugar moiety could not be identified from the NMR data at 300 MHz.
[c] The signals from the malonoyl moiety were not observed due to deuterium exchange.
[d] From peak height of H-2′/6′ signals.

Table 6
^{13}C NMR spectroscopic data of vitexin derivatives **1** and **4** from *B. vulgaris* in CD_3OD

	Compounds			
	1		**4**[e]	
	Major	Minor	Major	Minor
Shifts (ppm)				
C-2	166.5	166.3	167.0	166.6
C-3	103.8	104.0	E	
C-4	184.3[a]	184.2[a]	184.2	
C-5	[c]	[c]	162.9	
C-6	99.5	100.8	99.7	
C-7	164.6	164.9	163.0	
C-8	[d]	[d]	107.9	
C-9	158.3	157.7	157.0	
C-10	[d]	[d]	107.7	107.1
C-1′	123.7	123.7	123.2	
C-2′/6′	130.1	129.7	130.2	129.7
C-3′/5′	117.0	117.1	117.0	
C-4′	[c]	[c]	163.0	162.9
C-1″	73.6	74.9	75.2	75.8
C-2″	81.7	82.4	73.3	72.6
C-3″	80.2	79.8	80.2	80.5
C-4″	72.1	71.3	72.1	
C-5″	82.9	82.9	82.9	82.6
C-6″	62.9	62.6[b]	62.9	
C-1‴	105.8	106.1	102.8	
C-2‴	75.8	76.1	74.7	
C-3‴	77.7	77.9	77.6	
C-4‴	71.1	71.3	70.9	
C-5‴	77.1	77.5	78.3	
C-6‴	62.3	62.5[b]	62.2	

[a,b] Assignments interchangeable.
[c] 163.0, 162.8 × 2, 162.7.
[d] 105.6, 105.5, 105.1, 104.5.
[e] The ^{13}C shifts were evaluated from correlations in the HMBC spectrum, hence the shift of C-3 is not determinable.

excluded from careful assignment of the carbons of ring A. This was confirmed from through space ^1H–^1H correlations observed in the 2D ROESY spectra. In these spectra H-2′/6′ showed through-space interactions with the protons of the C-bound β-glucopyranosyl system. For **1** there was a strong correlation between H-1″ and H-1‴ and in **4** of H-1‴ with H-6 that confirmed the respective positions of the second sugar moiety. Finally the observation of exchange peaks in the latter spectrum also rationalized the observation of two conformations in the molecule that must arise from slow rotation about the C8–C1 sugar bond.

To the best of our knowledge compounds **2**, **4** and **5** are new vitexin conjugates. Compound **1** has been first reported from *Desmodium trifolium* and has recently been identified as a characteristic marker of the genus *Cryptocoryne* (Araceae) (Adinarayana and Syamasundar, 1982; Franke et al., 2006). Vitexin 2″-*O*-xyloside (compound **3**) and a 6″-*O*-malonyl derivative (the 2″ xylosyl equivalent of compound **2**) have been previously reported from plants of *B. vulgaris* subspecies *cycla* (Gil et al., 1998). Vitexin 7-*O*-glucosides have not been reported from *B. vulgaris*.

Analytical HPLC indicated the amounts present in the plant extract were **4** > **5** > **2** ~ **3** > **1** (Fig. 6a). In agreement with substrate and position specificities described above, the major endogenous flavonoid glycosides are those glycosylated at 7-OH. It is interesting to note that the C-glucosylated apigenin derivative, vitexin is not further *O*-glucosylated by UGT73A4 (data not shown). If this enzyme is associated with the formation of vitexin conjugates, *C*-glucosylation in *B. vulgaris* may occur after *O*-glucosylation. *C*-glycosylation is an uncharacterized phenomenon in plants since the first reports from Kerscher and Franz (1987).

Besides betanin, only rutin (quercetin 3-*O*-rutinoside) could be verified as one flavonoid glycoside among a large array of hydroxycinnamic acid conjugates in the cell cultures (Bokern et al., 1991). Whether UGTF1, which is capable of glucosylating quercetin at position 3-OH, is involved in rutin biosynthesis remains to be seen. It is not unusual that cell cultures do not produce the same set of secondary metabolites as those present in the intact plants, although the respective transcripts or proteins are present (Cacace et al., 2003; Heuer and Strack, 1992; Vogt et al., 1997).

2.5. Sequence analysis and evolutionary aspects

The heterogeneity of the overall UGT superfamily is striking with an average sequence identity of less than 20%. Only a few amino acids outside of the PSPG-box, such as a histidine close to the N-terminal part and an aspartate/glutamate residue 100 amino acids further downstream are conserved and both participate in the conserved reaction mechanism (Hans et al., 2004; Thorsøe et al., 2005; Shao et al., 2005). Based on the classification of plant family 1 UGTs into 13 groups, A–N (Ross et al., 2001; Bowles et al., 2005), UGT73A4 and UGT71F1 cluster within different flavonoid glycosylating clades of selected plant natural product glycosyltransferases (Fig. 7). UGT73A4, together with UGT73A5 is placed within group D (Ross et al., 2001), together with a similar 2′-chalconaringenin UGT from carnation (*Dianthus caryophyllus*) (Ogata et al., 2004). A common evolutionary origin is supported by their close to 60% sequence identity, a preference for polyhydroxylated phenylpropanoids, and shared regioselectivity for flavonoids, respectively. Interestingly, the 2′-chalconaringenin UGT, does not glucosylate betanidin, consistent with the observed lack of betacyanins in the Caryophyllaceae. A partial sequence from *B. vulgaris* (AA088911.1), potentially involved in betanidin glucosylation (Sepulveda-Jimenez et al., 2005), is also associated with this cluster, but since its N-terminal 100 amino acids have not been identified, functional annotation was not yet performed. Among several stress-inducible enzymes from various plant species (Horvath and Chua, 1996; Truesdale et al., 1996; Frassinet-Tachet et al., 1998; Langlois-Meurinne et al., 2005), this cluster harbors flavonoid A- and B-ring modifiers from *Scutellaria*

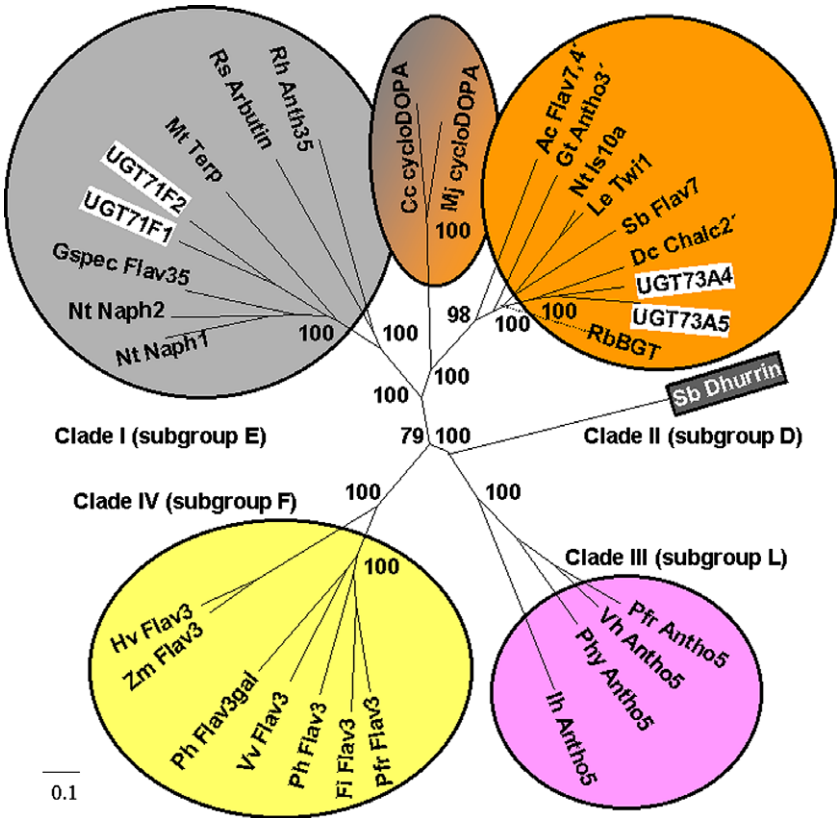

Fig. 7. Bootstrapped tree of flavonoid glycosylating UGT73A4 and UGT71F1 homologues in plants. Multiple alignments from UGT73A4 and UGT71F1 full-length sequences were evaluated with Clustal W and the tree was created with Treecon (Van de Peer and De Wachter, 1994). Clades I–IV correlate to the larger family 1 groups E, D, F, and L, respectively (Ross et al., 2001; Bowles et al., 2005; Gachon et al., 2005). The *cyclo*-DOPA-UGTs are more closely associated to clade II than clade I. For clarity, bootstrap values are displayed only for selected branches.Accession numbers and abbreviations: Clade I UGTs from *Rosa hybrida*, anthocyanin 3,5-glucosyltransferase, BAD 99560; *Rauwolfia serpentina*, Rs, arbutin synthase, CAC35167; *Medicago truncatula*, Mt, terpene glucosyltransferase AAW56092; *Dorotheanthus bellidiformis* UGT71F2, AF374004; *B. vulgaris* UGT71F1, AY526081; *Gentiana* sp. flavonoid-UGT, Gspec Flav35; *Nicotiana tabacum*, Naphthol-UGTs, Nt Naph2 and Nt Naph1 (BAB60721 and BAB60720). *Celosia cristata*, Cc BAD91804, *cyclo*DOPA-UGT; *Mirabilis jalapa* Mj, *cyclo*DOPA-UGT, BAD91803. Clade II UGTs from *Allium cepa*, Ac Flav74′, AAP88406; *Gentiana triflora*, Gt Antho3′, BAC54092; *Nicotiana tabacum*, Nt Is10a, U32643; *Lycopersicum esculentum* Twi1-UGT, X85138; *Scutellaria baicalensis* flavone 7-UGT, Sb Flav7, BAA83484; *Dianthus caryophyllus* chalcone UGT, Dc Chalc2′, BAD52007; *B. vulgaris* UGT73A4, AY526080; *Dorotheanthus bellidiformis* UGT73A5, Y18871; *B. vulgaris* putative betanidin UGT, RbBGT AA088911; Clade III, anthocyanin 5-UGTs (Antho5) from *Perilla frutescens*, Pfr, AB013596; *Verbena hybrida*, Vh, BAA36423; *Petunia hybrida*, Ph, AB02745; *Iris hollandica*, Ih (putative), BAD06874. Clade IV, flavonol-/anthocyanidin-3UGTs (Flav3) from *Perilla frutescens*, Pfr, AB002818; *Forsythia intermedia*, Fi, AAD21086; *Petunia hybrida*, Ph, AB027454; *Vitis vinifera*, Vv, AF000371; *Zea mays*, X13501; *Hordeum vulgare*, Hv, CAA33729. The dhurrin UGT from *Sorghum bicolor*, Sb, AF199453 was used as an outgroup.

baicalensis (Hirotani et al., 2000), *Allium cepa* (Willits et al., 2003), and *Gentiana triflora* with exclusive specificity for the 3′-OH of anthocyanins only (Fukuchi-Mizutani et al., 2003). The cloned *cyclo*-DOPA UGTs from *M. jalapa* and *C. cristata* (Sasaki et al., 2005a) are placed close to this heterogenous subgroup D within the UGT superfamily.

UGT71F1 from *B. vulgaris* and betanidin 6-UGT (UGT71F2) from *D. bellidiformis* belong to group E of family 1 UGTs (Ross et al., 2001). Its members share a preference for hydroxylated phenolic ring systems and accept various substrates, like terpenoids or xenobiotics, like naphthol (Arend et al., 2001; Shao et al., 2005; Taguchi et al., 2003). The promiscuous terpenoid/flavonoid-UGT71G1 from *Medicago truncatula* was the first UGT whose crystal structure has been resolved (Shao et al., 2005). The unique bifunctional anthocyanin 3,5-UGT identified in *Rosa hybrida* completes this cluster of diverse enzymes (Ogata et al., 2005).

2.6. Concluding remarks

In contrast to the flavonoid and anthocyanin 3-OH and 5-OH regiospecific groups F and L, clusters D and E have developed independently (Vogt, 2002) and may be derived from unknown ancestors modifying polyhydroxylated metabolites, considered a primitive feature in terms of evolution (Swain, 1975). While conserving their ancestral properties, during evolution, they acquired new functions dependent on the subset of substrates synthesized by individual species. A combination of knockout-mutations combined with functional proteomics and metabolomics may further decipher this apparent redundancy of UGTs in plant secondary metabolism.

3. Experimental

3.1. Plant material

Cell-suspension cultures from *B. vulgaris* (Bokern et al., 1991) cultivated at the IPB were grown at 24 °C under a 16-h day regimen on a shaker operating at 120 rpm. Subcultivation was carried out every 14 days by transferring 0.5–1 g cell inoculum into a MS medium (Murashige and Skoog, 1962) containing 30 g L^{-1} sucrose, 440 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 100 mg L^{-1} *myo*-inositol, 21.71 mg L^{-1} Na_2EDTA, 15.7 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 2 mg L^{-1} glycine, 0.1 mg $\cdot L^{-1}$ vitamin B1, 0.5 mg L^{-1} nicotinic acid and 0.5 mg L^{-1} vitamin B6 in 250 ml Erlenmeyer flasks. *B. vulgaris* seedlings (*B. vulgaris* L. subsp. *vulgaris* "Cylindra" and subsp. *maritima*, Ecotype 296, 307, and 310 obtained from H. Knueffer, IPK, Gatersleben, Germany) were grown from seeds in a greenhouse and cultivated in soil under natural-daylight conditions.

3.2. High performance liquid chromatography and purification of compounds for structural elucidation

The analytical reversed-phase chromatography was performed as follows: Solvent A (1.5% phosphoric acid in H_2O) and solvent B 100% CH_3CN). Identification and quantification of enzymatically formed betacyanins was performed with the following gradient system: within 3.8 min from 5% B to 25% B in A at a flow rate of 1 ml min^{-1} (detection at 540 nm). Standard conditions for detection of enzymatically formed quercetin glucosides was a gradient from 10% B to 48% B in A within 4.5 min at a flow rate of 1 ml min^{-1}. Other flavonoid derivatives were separated with the following gradient system: within 6.5 min from 15% B to 60% B in A; detection wavelengths: 540 nm for betacyanins, 520 nm for anthocyanins, 350 nm for flavonol-3-*O*-derivatives and luteolin, 365 nm for flavonols and flavonol 4′- and 7-*O*-derivatives or 285 nm for L-Dopa, caffeic acid and their presumed glucosides. Quantification of flavonoids and caffeic acid was achieved by standard curves with the respective reference compounds. Data were obtained by a liquid chromatograph, equipped with a photodiode array detector, an autosampler and data processor (Millenium software) from Waters Millipore (Eschborn, Germany). Betacyanins and quercetin glucosides were separated with an EC 50/4 Nucleosil 120-5 C_{18} column (5 cm length) (Macherey and Nagel, Düren, Germany). Other flavonoid derivatives were separated on an EC 125/4.6 Nucleosil 120-5 C_{18} column (12 cm length).

For isolation and identification of vitexins from *B. vulgaris* plantlets, 4–5 week old plants (500 g) were macerated in a Waring blender and extracted with H_2O (2L, 4:1) MeOH at room temperature for 30 min, concentrated, fractionated on polyamide SC-6 with a H_2O–MeOH gradient of increasing MeOH concentrations (Winter and Herrmann, 1984). Individual fractions were subjected to preparative HPLC. Solvent A: H_2O and solvent B, MeOH, with a A:B gradient of increasing B. For preparative HPLC, a Waters Delta 600 (Millipore) was equipped with a 10 μm-Nucleosil 100-10 C_{18} column (VarioPrep; 250 × 40 mm i. d., Macherey-Nagel, Düren, Germany). The purification and isolation of the vitexin derivatives was performed with a flow rate of 20 mL min^{-1} and detection at 350 nm with the following solvent and gradient system. Solvent A: 1.5% (v/v) AcOH in H_2O and B, 100% CH_3CN; isocratic run for 15 min with 100% A, followed by a linear gradient from 0% B to 80% B in A within 75 min. Isolated compounds (amounts from 0.5 to 1.5 mg) were concentrated to dryness and the structures determined by mass spectrometry and NMR spectroscopy. All compounds, including the new compounds **2**, **4** and **5**, were fully characterized by (+) ESI-MS, HR-(+)ESI-MS, sugar analysis (Table 4), ^1H NMR spectroscopy (Table 5) and ^{13}C NMR spectroscopy (**1** and **4** only, Table 6).

3.3. NMR as mass spectroscopy

^1H (1D and 2D COSY and ROESY) and ^{13}C (1D and 2D HMQC and HMBC) NMR spectra were recorded at 300 K on a Brüker AVANCE DMX 600, AMX 400 or DPX 300 NMR spectrometers locked to the major signal of the solvent, CD_3OD. Chemical shifts are reported in ppm relative to TMS, but were determined relative to the signals of the solvent (^1H: 3.35 ppm; ^{13}C: 49.00 ppm) and coupling in Hz. Positive ion electrospray mass spectra (ESI-MS) were recorded on a Micromass $QTOF^2$ mass spectrometer. High resolution ESI-MS were recorded on the same instrument. Sugars were identified using a standard micro-methylation technique on a Finnigan GCQ GC-MS spectrometer as described previously (Nimtz et al., 1996).

3.4. cDNA library construction and isolation of specific UGT-fragments

Total RNA from *B. vulgaris* cell suspension culture (8.25 g) was prepared using a Total-RNA Midi-Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. From 0.5 mg total RNA, 5.8 μg poly(A)+ RNA was isolated using oligo-dT-Oligotex beads (Qiagen). A λ-Zap cDNA library (Stratagene, Heidelberg, Germany) was constructed according to the manufacturer's instructions, except that the original MMLV-reverse transcriptase supplied with the kit was replaced by $Superscript^{II}$ reverse transcriptase (Invitrogen). Glucosyltransferase-specific sequences were amplified from the cDNA library by polymerase chain reaction (PCR) using the degenerate forward primer 5′ACNCAYTGYGGSTGGAAC3′ derived from the consensus 'PSPG-box' of plant glucosyltransferases (Hughes and Hughes, 1994) and the T7 reverse primer 5′GATATACGACTCACTATAGGGC3′. The resulting 1200 bp and 600 bp fragments were cloned into the pGemT-easy (Promega, Madison, USA) vector and sequenced. A similarity search was performed using 'Blast' algorithms

(Altschul et al., 1997; Zhang and Madden, 1997). The 1200 bp fragment exhibited a 64% identity on the amino acid level to the betanidin glucosylating UGT73A5, whereas the 600 bp fragment exhibited 59% identity on the amino acid level to the UGT71F2 also from *D. bellidiformis*, respectively.

3.5. Characterization of new compounds

3.5.1. 2″-O-glucosyl-6″-malonylvitexin (compound **2**)

Yield 1 mg, UV (HPLC solvent A λ_{max} nm) 338, 269. ESI-MS *m/z* 681.2 $[M+H]^+$, 703.1 $[M+Na]^+$, HR-ESI-MS *m/z* 681.167 $[M+H]^+$ (calcd. for $C_{30}H_{32}O_{18}$ 681.1667). For ^{1}H NMR spectroscopic data, see Table 5.

3.5.2. 7-O-glucosylvitexin (compound **4**)

Yield: 1.5 mg, UV (HPLC solvent A λ_{max} nm) 331, 300 (sh), 269. ESI-MS *m/z* 681.2 $[M+H]^+$, 703.1 $[M+Na]^+$, HR-ESI-MS *m/z* 595.168 $[M+H]^+$ (calcd. for $C_{27}H_{30}O_{15}$ 595.1663). For ^{1}H and ^{13}C NMR spectroscopic data, see Tables 5 and 6.

3.5.3. 7-O-glucosyl-4′-methoxyvitexin (compound **5**)

Yield: 1 mg, UV (HPLC solvent A λ_{max} nm) 337, 269. ESI-MS *m/z* 681.2 $[M+H]^+$, 703.1 $[M+Na]^+$, HR-ESI-MS *m/z* 609.184 $[M+H]^+$ (calcd. for $C_{28}H_{32}O_{15}$ 609.1819). For ^{1}H NMR spectroscopic data, see Table 5.

3.6. Cloning and heterologous expression of the UGT73A4 and UGT71F1 cDNAs

Screening of the cDNA library (300,000 plaque forming units each) was performed by hybridization with the [α-^{32}P]-dATP labeled, random primed 1200 bp and 600 bp probes obtained from PCR with the cDNA library. From positive clones, the corresponding recombinant pBluescript phagemids (Stratagene) were excised and used for infection of *E. coli* XL1 blue. Sequence analysis and multiple sequence alignment were performed with the software package DNASTAR (Lasergene, Madison, WI, USA). For expression, UGT73A4 was subcloned into the SphI/Sma/site of the pQE30-expression vector (Qiagen), whereas UGT71F1 was subcloned into the pDest24 vector (Gateway Technology, Invitrogen). The pQE30 expression construct was transformed into *E. coli* M15[pRep4], according to the manufacturer's instructions, whereas the pDest24 construct was transformed into BL21-CodonPlus(DE3)-RP competent cells (Stratagene), respectively. Bacteria were grown at 37 °C up to an OD_{600} of 0.6, induced by adding 200 μM IPTG and the temperature was lowered to 16 °C. After 16 h, cells were harvested by centrifuging at 5000*g* for 10 min. Pellets were resuspended in 100 mM KPi pH 7.0, treated with lysozyme (10 μg ml^{-1}) for 30 min at 4 °C, lysed by sonication at 4 °C and centrifuged for 10 min at 18000*g*. Crude extracts were assayed for UGT activity. Untransformed and pQE30 (without UGT73A4 insert) transformed M15 p[Rep4] cells (in both cases ± induction with 200 μM IPTG) were used as negative controls. For the pDest24-UGT71F1 construct, negative selection is supplied before transforming the construct into the expression strain, based on the presence of a *ccdB* gene in pDest24. Its gene product interferes with *E. coli* DNA gyrase (Bernard and Couturier, 1992), thereby inhibiting growth of most *E. coli* strains. Bacteria growing on selective media possess the positive expression clone because in these clones the *ccdB* gene is substituted by the gene of interest. Therefore, only BL21-CodonPlus(DE3)-RP cells transformed with the expression construct were grown and tested ± induction with 200 μM IPTG. In both cases, betanidin and quercetin were used as sugar acceptors to check for enzyme activity.

3.7. Purification of UGT73A4 and UGT71F1

Crude bacterial extracts were stirred for 5 min with 0.05% protamine sulfate at 4 °C to precipitate nucleic acids and centrifuged for 10 min at 20000*g*. Protein was precipitated by 85% saturated $(NH_4)_2SO_4$, centrifuged 25 min at 20000*g* and resuspended in 100 mM KPi buffer (pH 7.0) and batched overnight with Phenylsepharose 4L-CB (Amersham Biosciences, Freiburg, Germany) equilibrated with 1.3 M ammonium sulfate in 100 mM KPi (pH 7.0). Elution was performed by a stepwise gradient of decreasing $(NH_4)_2SO_4$ concentration with the UGT73A4-activity eluted from 150 mM to 10 mM $(NH_4)_2SO_4$. Fractions containing UGT73A4-activity were pooled, diluted with Talon extraction buffer (100 mM KPi (pH 7.5); 300 mM NaCl, 10% glycerin, 1 mM β-mercaptoethanol, 2.5 mM imidazole) and batched with BD Talon™ Metal Affinity Resin (Clontech, Palo Alto, CA, USA) according to the manufacturer's instruction. Active protein was eluted with a stepwise increase of the imidazole concentration (10–300 mM imidazole). The first elution fractions always exhibited the highest activity. To further purify and concentrate the UGT73A4 activity, pooled fractions from metal affinity chromatography were adjusted to 1.3 M $(NH_4)_2SO_4$ and applied to a 1 ml Phenylsepharose high performance column (Amersham Biosciences, Freiburg, Germany). Elution of active fractions was performed by a linear gradient of decreasing $(NH_4)_2SO_4$ concentration, which, after removal of unbound protein, eluted the UGT73A4 activity at 400 mM. Individual fractions containing UGT73A4 activity were checked by SDS–PAGE. The recombinant UGT71F1 was expressed as a C-terminal GST-fusion protein. Crude bacterial protein extracts were treated with 0.05% protamine sulfate and affinity purified by GST-Bind resin (Novagen, Madison, USA). Due to the small amounts of protein obtained after the first purification step, subsequent purification was not attempted. Determination of the molecular masses of UGT73A4 and UGT71F1 were performed by SDS–PAGE and subsequent tryptic digestion and MALDI-TOF MS analysis. In the case of UGT73A4, ultracentrifugation with a purified protein fraction confirmed the monomeric structure of the enzyme (Lilie, H., personal communication).

3.8. Substrates and enzyme assays

UDP-glucose and various amino acid-modifying enzyme inhibitors were purchased from Sigma (Deisenhofen). Cyclo-Dopa was prepared according to Schliemann et al. (1999). Betanidin was isolated from lyophilized *B. vulgaris* juice (Roth, Karlsruhe, Germany) upon enzymatic glucose cleavage from betanin (betanidin 5-*O*-β-glucoside) according to Heuer et al. (1996). Cyanidin chloride and kaempferol were from Roth, quercetin and myricetin from Merck (Darmstadt, Germany) and L-Dopa from Sigma. Quercetin 3-*O*-methyl ether, quercetin 4′-*O*-methyl ether, quercetin 3-*O*-glucoside and quercetin 4′-*O*-glucoside came from our own flavonoid collection. Other flavonoids, hydroxycoumarins, hydroxycinnamic acids and salicylic acid were purchased from Sigma. Eriodictiol and pinocembrin were purchased from Extrasynthèse (Genay, France). Kaempferol methylethers were obtrained from E. Wollenweber (Technische Universität Darmstadt, Germany). Glucose acceptors were dissolved in 5% aqueous DMSO to a final concentration of 20 μM. To prevent precipitation of the rather water-insoluble substrates, these were freshly prepared from 10 mM DMSO stock solutions prior to enzyme assays. Individual glucosides were identified by authentic standards, retention times and spectral properties.

The standard enzyme assay was performed in 50–80 μl. Aliquots of crude bacterial extracts (up to 50 μl) and purified fractions were incubated between 5 and 30 min at 30 °C with 30 μl assay-mixture (2 mM UDP-glucose, 20 μM quercetin or betanidin in 0.1 M KPi pH 7.0; additionally 10 mM Na-ascorbate in the case of betanidin). The reaction was terminated by adding 10 μl 15% CF_3CO_2H, centrifuged, and 20 μl of the supernatants were analyzed by HPLC.

3.9. Gel electrophoresis

Separation of proteins by SDS–PAGE was performed according to Laemmli (1970) with standard protein markers (Biorad, Munich, Germany; Invitrogen, Leek, The Netherlands). Proteins were fixed and stained by boiling for 30 s in 0.1% Serva Blue R, MeOH–H_2O (2:3) 10% AcOH in a microwave oven and destained by repeated boiling in 7% AcOH. Dilute protein (approximately 100 ng ml^{-1}) was concentrated prior to SDS–PAGE by incubating the desired amount of protein with 10 μl of hydrophobic resin StrataClean® (Stratagene, Heidelberg, Germany) according to Ziegler et al. (1997).

3.10. Kinetic properties

Apparent K_m and V_{max} values were determined using various concentrations of the donor molecule (UDP-glucose) at fixed concentrations of the acceptors and vice versa. The kinetic values were estimated from Hanes plots. All kinetic experiments were performed with partly purified protein fractions after affinity chromatography on Talon- or GST-resins, respectively. Inhibitors were dissolved either in H_2O or DMSO to give 10 mM stock solutions. Individual concentrations were diluted with aqueous DMSO to a final concentration of 2% DMSO in the enzyme assay. Amino acid-modifying inhibitors and cations were added 10 min prior to starting the assay with UDP-glucose and quercetin.

3.11. Northern blot analysis and RT-PCR

Total RNA (5 μg each) from *B. vulgaris* cell suspension culture, *B. vulgaris* seedlings (1–8 weeks old) and *B. maritima* seedlings (three different ecotypes) was prepared with the Qiagen Total-RNA Mini-Kit according to the manufacturer protocol, blotted onto Hybond N^+ membrane (Amersham, Pharmacia, Biotech) and probed with a [α-^{32}P]-dATP labelled UGT73A4 specific 750 bp N-terminal fragment. Hybridization of the blots was performed in 0.25 M sodium phosphate (pH 7.2, 250 mM NaCl, 1 mM EDTA, 7% SDS at 56 °C). Blots were washed under stringent conditions and exposed using Hyperfilm MP (Amersham, Bioscience). UGT71F1 expression analysis was performed by RT-PCR. Total RNA was isolated from BV cell suspension culture and seedlings (1–8 weeks) by the plant RNA extraction kit (Qiagen) and reverse transcribed using MMLV-H(-) point reverse transcriptase (Promega, Mannheim, Germany). PCR was performed with gene specific primers for UGT71F1: forward primer 5′ATGAGCAGCAGTACAGCAAG′ and reverse primer 5′TTACTCAAAAGTCAAAACATC3′.

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