Catalytic Key Amino Acids and UDP-Sugar Donor Specificity of a Plant Glucuronosyltransferase, UGT94B1: Molecular Modeling Substantiated by Site-Specific Mutagenesis and Biochemical Analyses1[C][OA]*

Sarah A. Osmani, Søren Bak, Anne Imberty, Carl Erik Olsen, and Birger Lindberg Møller*

VVKR Research Centre Pro-Active Plants, DK–1871 Frederiksberg C, Copenhagen, Denmark (S.A.O., S.B., C.E.O., B.L.M.); Plant Biochemistry Laboratory, Department of Plant Biology and Biotechnology (S.A.O., S.B., B.L.M.), and Department of Natural Sciences (C.E.O.), University of Copenhagen, DK–1871 Frederiksberg C, Copenhagen, Denmark; and Centre de Recherches sur les Macromolécules Végétales, CNRS, 38041 Grenoble cedex 09, France (A.I.)

The plant UDP-dependent glucosyltransferase (UGT) BpUGT94B1 catalyzes the synthesis of a glucuronosylated cyanidin-derivated flavonoid in red daisy (Bellis perennis). The functional properties of BpUGT94B1 were investigated using protein modeling, site-directed mutagenesis, and analysis of the substrate specificity of isolated wild-type and mutated forms of BpUGT94B1. A single unique arginine residue (R25) positioned outside the conserved plant secondary product glycosyltransferase region was identified as crucial for the activity with UDP-glucuronic acid. The mutants R22S, R25G, and R25K all exhibited only 0.5% to 2.5% of wild-type activity with UDP-glucuronic acid, but showed a 3-fold increase in activity with UDP-glucose. The model of BpUGT94B1 also enabled identification of key residues in the acceptor pocket. The mutations N123A and D152A decreased the activity with cyanidin 3-O-glucoside to less than 15% of wild type. The wild-type enzyme activity toward delphinidin-3-O-glucoside was only 5% to 10% of the activity with cyanidin 3-O-glucoside. Independent point mutations of three residues positioned near the acceptor B ring were introduced to increase the activity toward delphinidin-3-O-glucoside. In all three mutant enzymes, the enzymatic activity toward both acceptors was reduced to less than 15% of wild type. The model of BpUGT94B1 allowed for correct identification of catalytically important residues, within as well as outside the plant secondary product glycosyltransferase motif, determining sugar donor and acceptor specificity.

Glycosylation of bioactive plant natural products (secondary metabolites), xenobiotics, and hormones provides a means to regulate their activity, to secure safe storage in specific cellular compartments, and to modulate their chemical properties, such as solubility and stability important for their in planta function (Jones and Vogt, 2001; Kristensen et al., 2005). The glycosylation process is catalyzed by glycosyltransferases (GTs), enzymes found in organisms across all phyla. In mammals, glycosylation plays an important role in detoxification, e.g. by glucuronosylation of xenobiotics, while in plants, glycosylation constitutes the last step in the biosynthesis of numerous plant natural products. These include major classes like terpenes, phenylpropanoids, cyanogenic glucosides, and glucosinolates. Vitus vinifera contains more than 200 different aglycones conjugated to Glc (Sefton et al., 1993, 1994), and for the flavonol quercetin alone, 300 different glycosides have been identified (Jones and Vogt, 2001). It remains an open question how the specificity of GTs is controlled. High specificity would indicate the requirement for many more enzymes than would be predicted from the genome sequencing programs, whereas a low specificity would pose problems with respect to undesired side reactions like inactivation of plant hormones (Ford, 1998; Jones et al., 1999). Further knowledge of the in vivo substrate specificity of GTs is important to understand the basis for plant plasticity with respect to natural product synthesis and adaptation to abiotic and biotic environmental stress factors. Despite low primary sequence similarity, the secondary and tertiary structures of GTs are highly conserved. So far, the crystal structures available show that the GTs adopt of two distinct folds, designated GT-A fold and GT-B fold, with an emerging...
third fold being identified as GT-A-like (http://www.cermav.cnrs.fr/glyco3d/). The GTs are currently divided into 91 families (http://www.cazy.org/fam/acc_GT.html) based on sequence identity scores (Campbell et al., 1997; Coutinho et al., 2003). Family 1 GTs contain a large number of GTs that use a UDP-activated sugar as donor in the enzymatic reaction, and these are often referred to as the UDP-dependent glucosyltransferases (UGTs; Mackenzie et al., 1997; Lim and Bowles, 2004). The family 1 UGTs constitute a large multiprotein family. They share a conserved sequence motif at the C-terminal end, the UGT-defining sequence (Mackenzie et al., 1997). In Arabidopsis (Arabidopsis thaliana), 120 UGT-encoding genes have been identified (Paquette et al., 2003), and phylogenetic analyses have shown these to group into tree clades. The major clade is plant specific and contains the Arabidopsis UGTs known to be involved in plant natural product synthesis, control of plant hormone homeostasis, and detoxification of xenobiotics (Vogt and Jones, 2000; Jones and Vogt, 2001; Paquette et al., 2003; Lim and Bowles, 2004). These plant UGTs have an expanded UGT-defining sequence, denoted the plant secondary product GT (PSPG) motif (Hughes and Hughes, 1994; Paquette et al., 2003).

New UGT sequences are obtained too fast to be accompanied by functional characterization (Coutinho et al., 2003). Bioinformatic analyses for accurate prediction of sequence features important for defining the substrate specificity of a particular UGT have not yet been developed. State of the art may be exemplified by the recent development of a program to predict acceptors for bacterial GTs glycosylating antibiotics (Kamra et al., 2005). This program uses a knowledge-based approach and relies on available biochemical and crystal structure data. This means that high homology of the query sequence with the templates is essential to achieve reliable predictions. It also underscores the need for substantial additional amounts of biochemical and structural data to enable development of bioinformatic programs with reliable predictive power. This is especially important for the GTs, as annotations based on primary structure analysis have proven difficult, and substrate specificity can be highly diverse for UGTs within groups defined by phylogenetic analyses (Richman et al., 2005; Modolo et al., 2007). The biochemical data available on UGTs enable some conclusions on UGT acceptor specificity to be drawn. Several UGTs have broad acceptor specificity as demonstrated by their ability to glycosylate a large number of structurally quite different aglycones in vitro (Vogt et al., 1997; Vogt and Jones, 2000; Hansen et al., 2003; Kramer et al., 2003; Modolo et al., 2007). Other UGTs are highly specific for a single acceptor (Fukuchi-Mizutani et al., 2003; Kramer et al., 2003). An emerging picture is that UGTs show regiospecificity toward the part of the substrate molecule harboring the site of glycosylation (Vogt et al., 1997; Kramer et al., 2003; Lim et al., 2003b, 2004; Richman et al., 2005; He et al., 2006). This would be in accordance with the broad acceptor specificity of some UGTs, as the presence of a specific structural domain to be glycosylated can be presented on otherwise differing molecules. Biochemical studies seem to support regiospecificity as a key parameter for UGT substrate specificity. At the same time it is also evident that the presence of a specific structural domain will not by itself assure activity, but should be considered in conjunction with the overall structure of the aglycone (Richman et al., 2005; He et al., 2006; Kogawa et al., 2007; Modolo et al., 2007). Several studies of biochemically described UGTs show some conservation of regiospecificity within phylogenetic groups (Vogt and Jones, 2000; Lim et al., 2003a; Morita et al., 2005; Yonekura-Sakakibara et al., 2007; Caputi et al., 2008).

UGTs recognizing several different sugar donors have been identified. These include UDP-Glc, UDP-Gal, UDP-Xyl, UDP-Rha, and UDP-GlcUA. In plant UGTs, the most common sugar donor is UDP-Glc. Several conserved residues, most of which are found in the PSPG motif of plant UGTs, interact with the sugar donor (Shao et al., 2005; Thorsoe et al., 2005; Offen et al., 2006; Li et al., 2007). The sugar donor preference of a specific UGT is often very narrow, showing little or no activity with alternative sugars. Replacement of a single amino acid residue has been shown to change the sugar specificity of Aralia cordata UGT78A2 (GaT; Kubo et al., 2004) and Medicago truncatula UGT71G1 (He et al., 2006). Similar results have been obtained from other GTs (Qasba et al., 2005). Nevertheless, no conserved amino acid residues have been identified as general determinants of sugar specificity (Ouzzine et al., 2002; Kubo et al., 2004; Modolo et al., 2007; Yonekura-Sakakibara et al., 2007).

Functional characterization of a UGT requires time-demanding biochemical studies as well as the study of site-specific mutants. Knowledge derived from crystal structures is an important tool for the rational design of mutants for such biochemical studies. So far the crystal structures of 11 family 1 GTs have been solved. Ten are listed at http://www.cermav.cnrs.fr/glyco3d/ and the structure coordinates of all 11 are available at http://www.rcsb.org/pdb/home/home.do. These GTs all adopt the GT-B fold. Obtaining GT crystals has proven difficult (Breton et al., 2006) and structure solution progresses slowly in comparison to the rate of appearance of new UGT sequences. To circumvent this bottleneck in the study of UGTs, homology modeling has proved to be a strong tool for the identification of potentially important residues (Hans et al., 2004; Thorsoe et al., 2005). The recently solved crystal structures of the first plant UGTs, MtUGT71G1 from M. truncatula (Shao et al., 2005) and VvGT1 from V. vinifera (Offen et al., 2006), essentially add confidence to the structures derived from homology modeling.

A plant UGT with affinity for the alternative sugar UDP-GlcUA (Fig. 1) UGT94B1 has been identified in red daisy (Bellis perennis; Sawada et al., 2005). Together with the UGT UBGAT from Scutellaria baicalensis (Nagashima et al., 2000), these are the only character-
ized plant UGTs using UDP-GlcUA as the sugar donor. BpUGT94B1 shows specificity for the acceptor cyanidin 3-O-glucoside, whereas delphinidin 3-O-glucoside is a poor acceptor (Fig. 1). UGT94B1 is unique both with respect to sugar donor and acceptor specificity by using the alternative sugar donor UDP-GlcUA and by forming a diglycoside (Fig. 1). To gain an understanding of the molecular structure governing acceptor as well as donor specificity of this enzyme, a model of BpUGT94B1 was constructed using coordinates of the two plant crystal structures VvGT1 (Offen et al., 2006) and MtUGT71G1 (Shao et al., 2005) as scaffolds. Residues constituting the substrate pocket in the model were identified. The significance of these residues for activity and substrate specificity was investigated by site-specific mutagenesis and by biochemical characterization of the catalytic properties of the isolated mutant proteins. A unique Arg residue (R25) situated outside the conserved PSPG motif was identified as essential for BpUGT94B1 activity with the sugar donor UDP-GlcUA. In addition, several residues forming the acceptor pocket were identified as important for the catalytic properties. Based on these findings, the conservation of primary and secondary structure in relation to substrate specificity of UGTs is discussed.

RESULTS

Homology Modeling of UGT94B1

Prediction of red daisy UGT94B1 sary structure identified 16 potential α-helices and 13 potential β-strands. The secondary structure predictions were used for an alignment of BpUGT94B1 with sequences of the plant enzymes MtUGT71G1 (Shao et al., 2005) and VvGT1 (Offen et al., 2006; Fig. 2), for which crystal structure coordinates have been published. The BpUGT94B1 amino acid sequence has approximately 26% and approximately 20% overall sequence identity to MtUGT71G1 and VvGT1, respectively, as determined by ClustalX alignment.

The structural alignment (Fig. 2) was entered into the Composer function of the Sybyl protein modeling software and divided into 17 structurally conserved regions (SCRs) and 18 loops (Fig. 2). All 18 SCRs and 13 of the 18 loops were constructed using the coordinates of the MtUGT71G1 and VvGT1 crystal structures as templates. The missing loops were loops 1, 7, 9, 11, and 18. Loop 1 corresponds to the N-terminal end and loop 18 to the C terminus. Construction of loop 1 (residues 1–9), loop 7 (residues 156–162), and loop 11 (residues 232–242) was performed by search in the GT crystal structure database. Two loops were left out of the model. Loop 9 (residues 204–208) was left out because it could not be modeled without major energy constraints. The N-terminal loop 18 was also left out, as no acceptable template for this region was found.

The model was optimized by several steps of energy minimization of side chains and loop regions (Imberty et al., 2006). Chirality and charges of all amino acids were verified. Stereochemistry of the backbone structure was verified by consultation of Ramachandran maps followed by optimization of torsion angles and energy. In the final model, no residues were in disallowed regions, and only 15 of the 423 amino acid residues in the model were in generously allowed regions. The PROCHECK (Laskowski et al., 1996) overall g factor evaluating all torsion angles and bond lengths was –0.46, indicating a high-quality model (Bhattacharya et al., 2008). The g factors for the two template structures VvGT1 and MtUGT71G1 were 0.13 and 0.24, respectively. The overall model quality was also validated by calculation of the ProSA Z score (Wiederstein and Sippl, 2007). The Z score for the BpUGT94B1 model was calculated to be –8.29. Z scores for experimentally determined x-ray structures of proteins of similar size (around 450 amino acids) lie in the range of –6 to –13. The Z score of the model was thus in the range of scores typical for proteins of similar size. For comparison the calculated Z scores for the template structures MtUGT71G1 and VvGT1 were –12.75 and –10.39, respectively. The ProSA residue score calculates local model quality. In general, positive values indicate problematic zones. Looking at the residue score for the BpUGT94B1 model, approximately 90% of the residues in the model lie between 0 and –2, as also observed for the template structures. Two short regions of the N terminus (approximately 10 amino acids) show a score of 0.5 while the linker region has a score of 1.

Docking of Sugar Donor and Acceptor into the Model

In vitro, BpUGT94B1 catalyzes the regiospecific transfer of GlcUA from UDP-GlcUA to the 2'-OH of cyanidin 3-O-β-glucoside and cyanidin 3-O-β-6’-O-malonylg glucoside (Fig. 1; Sawada et al., 2005). The
sugar donor and acceptor were inserted into the model by homology docking (Imberty et al., 2006). Docking of the BpUGT94B1 sugar donor UDP-GlcUA was performed guided by the coordinates of the crystal structure of MtUGT71G1 crystallized with UDP-Glc. The acceptors cyanidin 3-O-β-glucoside or cyanidin 3-O-β-6-O-malonylglucoside were inserted guided by the position of acceptors in the crystal structures of the V. vinifera UGT VvGT1 containing the sugar acceptor kaempferol (Offen et al., 2006) and the Amycolatopsis orientalis GT GtfD crystallized with the acceptor L-vancosamine (Mulichak et al., 2004). The VvGT1 acceptor is a flavonoid, as is the acceptor in BpUGT94B1, whereas the glycosylated position of the GftD acceptor is the 2′-OH group of a Glc residue, as is also the case in BpUGT94B1. Docking was followed by energy optimization of amino acid side chains surrounding the substrates.

Analyzing the Model

The tertiary structure of the model takes a GT-B-fold conformation with two distinct domains formed by the N- and C-terminal parts of the primary structure (Fig. 3). In the final model, the N-terminal domain folds into seven α-helices and a β-sheet composed of seven parallel β-strands. The C-terminal domain folds into eight α-helices, whereas the ninth predicted α-helix is positioned in the C terminus that was left out of the model. In the C-terminal domain, five of the predicted six β-strands are formed in the predicted regions and fold into a parallel β-sheet.

The N- and C-terminal domains form a deep cleft accommodating a tight binding site for the sugar acceptor and sugar donor deeply buried inside the protein (Fig. 4, A–E). The sugar donor is positioned so that it mainly interacts with residues in the C-terminal domain, whereas the acceptor mainly interacts with residues in the N-terminal domain (Fig. 4, B–E).

Sugar Donor Interactions

In the BpUGT94B1 model, several residues are positioned so that they may interact with the sugar donor using the optimized docking software Sybyl and used to construct the model of BpUGT94B1. SCR positions are highlighted in gray above the alignment. Loops are numbered and named above the alignment. The highly conserved PSPG motif is underlined. Amino acid residues within this motif that are predicted to interact with the sugar donor in the model of BpUGT94B1 are marked with stars. Amino acid residues in BpUGT94B1 mutated in this study are marked with triangles.

**Figure 2.** Amino acid sequence alignment of BpUGT94B1 against MtUGT71G1 and VvGT1. The alignment is performed guided by conservation of secondary structure, either as predicted (BpUGT94B1 see “Materials and Methods”) or observed from the solved crystal structures (UGT71G1 and VvGT1). α-Helices are highlighted in turquoise and β-strands in pink. The alignment was entered into the modeling software Sybyl and used to construct the model of BpUGT94B1. SCR positions are highlighted in gray above the alignment. Loops are numbered and named above the alignment. The highly conserved PSPG motif is underlined. Amino acid residues within this motif that are predicted to interact with the sugar donor in the model of BpUGT94B1 are marked with stars. Amino acid residues in BpUGT94B1 mutated in this study are marked with triangles.
these residues are situated within the PSPG motif that is highly conserved between different UGTs (BpUGT94B1 residues 315–358; Fig. 2). Nine PSPG residues are predicted to interact with the sugar donor in the BpUGT94B1 model (Fig. 5A). These nine residues possess similar positions in the crystal structure of MtUGT71G1, with five being identical and four being similar residues (Fig. 2).

In the BpUGT94B1 model, the residues Q355, D357, and Q358 are all positioned near the sugar moiety of the sugar donor (Fig. 5A). The residue V316 lies within H-bond distance to the uridine ring of the donor. The position of the uridine ring is further stabilized by aromatic ring stacking with the indole ring of W315. E341 can interact with the Rib ring of the donor. H333, S337, and S338 can form H bonds with the α-P, while S264 and maybe Q355 can interact with the β-P. Two highly conserved residues outside the PSPG motif are the residues corresponding to BpUGT94B1 H22 and D121. These are directly involved in the catalytic process and have been shown to be crucial for activity in many plant UGTs (Hans et al., 2004; Shao et al., 2005; Offen et al., 2006). In the BpUGT94B1 model these residues are positioned in a similar manner as in the MtUGT71G1 and VvGT1 crystals, favoring a similar catalytic mechanism. The conserved position of UDP-sugar donor interacting residues (PSPG motif residues) and catalytically important residues (H22 and D121) between the model and the template structures confirm a high degree of confidence of the model for conserved regions.

Less conserved residues outside the PSPG motif also exhibit interaction with the sugar donor. These residues are S143 that can H bond with the sugar moiety and is paralleled by a Thr residue in MtUGT71G1, S21 that interacts with the β-P, and the positively charged R25 that is positioned near the negatively charged carboxylate group of the GlcUA (Fig. 5B).

Sugar Acceptor Interactions

The residues in the BpUGT94B1 model that form the acceptor pocket are mainly positioned in the N-terminal domain. A hydrophobic environment is created by the residues W17, P88, P89, F122, L148, L151, P174, I187, Y186, and F356. Additional residues forming part of the acceptor pocket are T92, D121, N123, D152,
K175, N176, Q355, and D357. An analysis of the model following docking of the acceptor cyanidin 3-O-glucoside shows that the acceptor is held in place by a tight fit of the surrounding amino acid residues. Stabilizing interactions are also envisioned between specific amino acid residues and the acceptor. The residues N123 and T92 are positioned close to the sugar part of the acceptor (Fig. 5A), and the acceptor B ring can be stabilized by H-bond formation to D152 (Fig. 5C). The A and C rings of the cyanidin moiety can be stabilized by aromatic ring stacking with the side chain of F356.

The possibility to dock cyanidin 3-O-6′-O-malonylglucoside (Fig. 1) as an alternative acceptor was also investigated. Despite the tight fit of the enzyme around the smaller acceptor cyanidin 3-O-glucoside, it was possible to accommodate the acceptor cyanidin 3-O-6′-O-malonylglucoside within the acceptor pocket with favorable energy. This is accomplished when the malonic acid is bent toward the aglycone, which is also the energetically favored configuration of the molecule (Fig. 4, D and E).

**Test of the Model by Site-Directed Mutagenesis Activity of UGT94B1**

To study the substrate specificity and enzyme kinetic properties of BpUGT94B1, a full-length cDNA clone encoding the enzyme was obtained from petals of red daisy. An N-terminal His-tag was added for heterologous expression in *Escherichia coli* and subsequent affinity purification (Fig. 6). The three-dimensional structure of UGTs suggests that N-terminal His-tagging would position the tag on the surface of the protein far from the active site, i.e. at a position that would not be expected to interfere with protein folding and activity (Fig. 3).

The isolated His-tagged enzyme was highly specific with respect to the sugar donor UDP-GlcUA. Activity with other activated sugars such as UDP-Glc, UDP-Gal, and UDP-Xyl was very low. The activity with UDP-Glc was <0.5% of the activity with UDP-GlcUA. The \( K_m \) value for UDP-GlcUA was determined to be 1.1 ± 0.1 mM (Fig. 7; Table I), whereas the \( K_m \) value for UDP-Glc was 7.3 ± 0.9 mM (Fig. 8; Table I). The enzyme exhibited high activity using cyanidin 3-O-
glucoside as acceptor. The \( K_m \) value for cyanidin 3-O-glucoside was estimated to be 0.8 ± 0.2 mM. Substrate inhibition of BpUGT94B1 at acceptor concentrations above 1 mM prevented a more accurate assessment of this \( K_m \) value (Fig. 9). Enzyme activity with the acceptor delphinidin-3-O-glucoside (Fig. 1) was of only 5% to 10% of the activity observed with cyanidin 3-O-glucoside.

Modulation of Sugar Donor Specificity

Residues in the PSPG motif of plant UGTs have previously been shown to be involved in sugar donor binding of UGTs (Kubo et al., 2004; Shao et al., 2005; Thorsøe et al., 2005; Offen et al., 2006). These residues are highly conserved and might not explain the various sugar specificities observed for plant UGTs. The model of BpUGT94B1 was analyzed to identify residues responsible for the high specificity of this enzyme for UDP-GlcUA. This identified an Arg residue (R25) far from the PSPG motif that interacted with the sugar part of the donor. The positively charged side chain of R25 is positioned pointing toward the negatively charged carboxylate group of the UDP-GlcUA of the sugar donor (Fig. 5B). This could serve to stabilize binding of the negatively charged sugar residue in the active site, explaining why BpUGT94B1 exhibits high specificity toward UDP-GlcUA. In other plant UGTs, the position corresponding to BpUGT94B1 R25 is often a Ser or Pro. To investigate the role of the R25 residue experimentally, the mutations R25S, R25P, R25G, and R25K were introduced into BpUGT94B1. The mutant R25S mirrors S25 in MtUGT71G1 whereas R25P mirrors P23 in VvGT1. The R25G mutation would be expected to completely abolish a specific interaction with the charged carboxylate group of UDP-GlcUA, whereas the positively charged Lys side chain in the R25K mutation retains the positive charge but introduces a shorter side chain compared to Arg.

All mutated enzymes were tested for activity with the sugar donors UDP-GlcUA, UDP-GalUA, UDP-Glc, UDP-Xyl, and UDP-Gal, using cyanidin 3-O-glucoside as the acceptor. All the mutants exhibited significantly decreased activity (relative activity <2.5% of wild type) with UDP-GlcUA (Table I; Fig. 10). Despite the severe decrease in activity with UDP-GlcUA for the R25S mutant, the \( K_m \) value for UDP-GlcUA only increased from 1.1 ± 0.1 mM to 1.4 ± 0.3 mM. In contrast, the \( K_m \) value for the mutant R25K increased to 7.0 ± 0.5 mM (Fig. 7; Table I).

BpUGT94B1 and mutants R25S, R25G, R25P, and R25K were tested for their ability to use UDP-Glc as the sugar donor. Three of the four mutants (R25S, R25G, and R25K) showed 3-fold activity increases using UDP-Glc as donor compared to the wild type (Fig. 10). \( K_m \) for UDP-Glc was determined for the mutant R25S and was 2.9 ± 0.5 mM (Fig. 8; Table I) as compared to 7.3 ± 0.9 mM for the wild-type enzyme (Fig. 8; Table I), and the relative \( K_{cat} \) value was more than twice as high as for the wild-type enzyme. No activity was detected with UDP-GalUA and UDP-Xyl. In contrast to the wild-type enzyme, the mutants R25S, R25G, and R25K showed marginal activity with UDP-Gal.

Modulation of Sugar Acceptor Specificity

In vivo, BpUGT94B1 glucuronosylates cyanidin 3-O-glucoside at the 2''-OH (Fig. 1; Toki et al., 1991; Sawada et al., 2005). When delphinidin-3-O-glucoside was used as substrate, the conversion efficiency dropped to 5% of that obtained with cyanidin 3-O-glucoside. Delphinidin 3-O-glucoside has a 3'',4'',5''-hydroxylation pattern at the B ring compared to the 3',4'-hydroxylation pattern of cyanidin (Fig. 1). To identify amino acid residues in BpUGT94B1 involved in acceptor interac-
tions and enabling the enzyme to discriminate so effectively between the di- and trihydroxylated acceptors, the model of BpUGT94B1 was analyzed. Residues I187, P174, L148, and D152 were found to form part of the acceptor pocket and accommodate the acceptor B ring (Fig. 5C). The amino acids I187, P174, and L148 provide a hydrophobic environment for the acceptor and the residue D152 is positioned to form a stabilizing H bond with the 3'-OH of the acceptor. Accommodation of a delphinidin acceptor with an additional OH group on the B ring would require more space around the B ring, but also propose an additional OH group to interact in H-bond formation compared to cyanidin. To test if more space or amino acids proposing H-bond interactions could increase activity with delphinidin 3-O-glucoside, the point mutations I187A, I187S, P174G, D152A, and L148A were independently introduced in the BpUGT94B1 sequence. The residues I187, P174, and L148 were changed into the amino acid Ala or Gly with very small or no side chains to provide more space in this area of the acceptor pocket. The mutant I187S was made as the Ser residue could offer a stabilizing interaction in the form of H-bond formation with the acceptor. The mutant D152A was made to test whether the predicted H-bond formation between this residue and the acceptor was of importance for substrate interaction and activity.

The mutated proteins were assayed for their activity toward the acceptors cyanidin 3-O-glucoside and delphinidin 3-O-glucoside. For both acceptors, the mutated proteins all showed less than 15% of the activity obtained with the wild-type protein. The most severe effect was observed with the D152A mutant, in which the activity toward cyanidine 3-O-glucoside dropped to less than 5% of wild type (Fig. 10). Km values of the mutant enzymes with respect to the two sugar acceptors were not determined because of the observed substrate inhibition (Fig. 9). To observe if the affinity for the sugar donor could account for the reduced relative activity of the mutants, the Km values for the sugar donor UDP-GlcUA were determined for the mutants L148A D152A and I187A (Fig. 7; Table I). The mutated proteins had Km values for the sugar donor UDP-GlcUA 1 to 3 times higher than wild type.

Analysis of the acceptor pocket in the model identified an additional residue predicted to be important for acceptor stabilization in the active site. This was the residue N123 that proposes H bonding to the sugar part of the acceptor. To verify a role of this residue for activity, the mutation N123A was introduced in the mutant. The mutated proteins had Km values for the sugar donor UDP-GlcUA 1 to 3 times higher than wild type.

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Table 1. Kinetic parameters for wild-type BpUGT94B1 and point mutants

<table>
<thead>
<tr>
<th>Enzyme/substrate</th>
<th>Km ± sD (mM)</th>
<th>Relative kcat ± sD (min⁻¹)</th>
<th>Relative kcat/Km (min⁻¹ mM⁻¹)</th>
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<tr>
<td>Wild type UDP-GlcUA</td>
<td>1.1 ± 0.1</td>
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<td>R25S UDP-GlcUA</td>
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<tr>
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<tr>
<td>Wild type UDP-Glc</td>
<td>7.3 ± 0.9</td>
<td>100 ± 5</td>
<td>13.7</td>
</tr>
<tr>
<td>R25S UDP-Glc</td>
<td>2.9 ± 0.5</td>
<td>218 ± 14</td>
<td>75.2</td>
</tr>
<tr>
<td>Wild type cyanidin 3-O-glucoside</td>
<td>0.8 ± 0.2</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Figure 8. Km curves for the sugar donor UDP-Glc with wild-type and R25S mutant BpUGT94B1 enzymes using 0.65 mM cyanidin 3-O-glucoside as acceptor. Product formation is expressed as percentage of wild-type product formation per molecule of enzyme that is set to 100% at 25 μM UDP-Glc. The experimental points used for calculation of kinetic parameters by fitting to the Michaelis-Menten equation are shown by points and the fitted curve by a line. [See online article for color version of this figure.]
BpUGT94B1 sequence. Introduction of an Ala residue would abolish any interaction between this residue and the acceptor. The N123A mutation caused relative enzyme activity to decrease to less than 15% of wild type for both acceptors (Fig. 10).

DISCUSSION

Recent crystallization of four plant UGTs, MtUGT71G1 (Shao et al., 2005), VvGT1 (Offen et al., 2006), MtUGT85H2 (Li et al., 2007), and AtUGT72B1 (Brazier-Hicks et al., 2007), provides structural templates for homology modeling of plant UGTs. This increases the reliability of plant UGT modeling, formerly performed using crystal structure coordinates of more distant bacterial family 1 GTs as scaffolds (Hans et al., 2004; Thorsoe et al., 2005). UGT94B1 from red daisy exhibits high substrate specificity both with respect to the sugar donor and the sugar acceptor (Sawada et al., 2005). In this study a model of BpUGT94B1 was constructed to reveal structural features responsible for the tight specificity of this enzyme and to identify strategies to change the substrate specificity.

In the molecular model of BpUGT94B1, the substrates are tightly fitted in the cleft between the N- and C-terminal domains deeply buried within the enzyme (Fig. 4). This suggests cleft opening as a prerequisite for substrate binding. This is in accordance with studies of several GT crystal structures, suggesting accommodation of substrates within GT-B-fold GTs proceeds by slight movements of the linker region between the N- and C-terminal domains (Hu et al., 2003; Mulichak et al., 2003; Shao et al., 2005).

Sugar Donor Specificity of UGT94B1

In the model of BpUGT94B1, the positively charged side chain of R25 is positioned so that it points toward the carboxylate group of the UDP-GlcUA sugar donor. The crucial importance of the residue R25 was substantiated by biochemical analyses of BpUGT94B1 R25 mutants. Mutant proteins R25S, R25G, R25K, and R25P all possessed only 0.5% to 2.5% of wild-type activity with the sugar donor UDP-GlcUA. For the R25K mutant, this was paralleled by a 7-fold increase in $K_m$. These results demonstrated the importance of R25 for enzyme activity with UDP-GlcUA as sugar donor.

In contrast to the observed loss of activity with UDP-GlcUA, the three point mutants R25S, R25G, and R25K showed a 3- to 4-fold increase in activity with UDP-Glc as sugar donor paralleled by a decrease of $K_m$ for UDP-Glc from 7.3 mM for the wild-type BpUGT94B1 to 2.9 mM for the R25S mutant. Even the R25K mutation that retains a positively charged side chain shows loss of activity with UDP-GlcUA and increased activity with UDP-Glc. Apparently even a minor displacement of the positive charge away from the GlcUA, as mediated by the shorter side chain of the Lys residue as compared to Arg, is enough to abolish activity with UDP-GlcUA as the sugar donor. The mutant R25P showed decreased activity with both sugar donors. The severity of this mutation probably reflects that the Pro residue causes a changed torsion of the backbone.

The crystal structures of UGTs show the majority of interactions with the UDP-sugar donor to be provided by amino acid residues within the highly conserved PSPG motif (Shao et al., 2005; Offen et al., 2006; Brazier-Hicks et al., 2007). This is in accordance with the fact that the major part of the donor is the invariant nucleotide unit. In the BpUGT94B1 model, nine of the 14 amino acid residues positioned close to the donor are from the PSPG motif. Of these nine residues, five are identical and four are conserved when compared to the crystal structure of BpUGT94B1.

Figure 9. $K_m$ curve for the sugar acceptor cyanidin 3-O-glucoside for wild-type BpUGT94B1 enzyme using 2.5 mM UDP-GlcUA as donor in the experiments. Product formation is expressed as percentage of product formation at 1 mM cyanidin 3-O-glucoside. $K_m$ was estimated from the experimental points obtained at acceptor concentrations below 1 mM, by fitting to the Michaelis-Menten equation. The theoretical fitted curve is shown by a dot and dash line. [See online article for color version of this figure.]

Figure 10. Relative activity of wild-type and mutant BpUGT94B1 enzymes using three different sugar acceptor and sugar donor combinations. Sugar acceptor and donor were present at fixed concentrations of 0.650 mM and 2.5 mM, respectively. Substrates and incubation times were adjusted to obtain less than 10% conversion. The data presented are the mean values of four independent determinations. SDs for each data set are shown. [See online article for color version of this figure.]
to the PSPG motif in MtUGT71G1 (Fig. 2). Despite the high conservation of sugar donor interacting PSPG motif residues, most UGTs are highly specific with respect to their sugar donor preference. Several studies have searched for residues within the PSPG motif responsible for specificity toward specific sugars but with very limited success (Kubo et al., 2004; Shao et al., 2005; Offen et al., 2006). The R25 residue of BpUGT94B1 lies outside the PSPG motif and is neither conserved among plant UGTs nor in mammalian UGTs known to use UDP-GlcUA as sugar donor. This was demonstrated by Clustal alignments of a large number of UGT amino acid sequences (results not shown), including the plant UGT UBGAT from *S. baicalensis* that also uses UDP-GlcUA as the sugar donor (Nagashima et al., 2000). Alignment of the *S. baicalensis* UBGAT with BpUGT94B1 suggests an Ala to take the R25 position, and there are no charged residues nearby this position. The mechanism responsible for BpUGT94B1 activity with UDP-GlcUA thus seems to be specific for this particular UGT and is not a conserved feature of UDP-glucuronosyltransferases. In the *Sorghum bicolor* UGT85B1 sharing 24% amino acid identity with BpUGT94B1, a Pro residue is found at the position that corresponds to R25 in BpUGT94B1. Replacement of this Pro residue with an Arg residue did not confer activity with UDP-GlcUA (data not shown). Presence of an Arg residue at this position is therefore not sufficient for activity with UDP-GlcUA. The involvement of N-terminal residues as decisive for sugar donor specificity has also been observed for UGT93A2 (ZOG1) from *Phaseolus lunatus* and UGT93A1 (ZOX1) from *Phaseolus vulgaris*, where domain swap identified a region of the N-terminal domain to be determinant for ZOG1 activity with UDP-Glc (Martin, 2000).

The observation that a single amino acid residue in BpUGT94B1 may be decisive with respect to UDP-sugar specificity is in accordance with other studies of the sugar specificity of UGTs. In the *A. cordata* UGT78A2 (GaT), a single residue (H374) was observed to dictate specificity for UDP-Gal (Kubo et al., 2004). This residue lies within the PSPG motif and is conserved in galactosyltransferases. Sugar specificity was shifted to UDP-Glc by the point mutation H374Q. However, the inverse mutation in *S. baicalensis* UGT UBGAT did not confer galactosyltransferase activity, illustrating that several other structural features have to be in place to mediate such a shift in specificity (Hans et al., 2004; Kubo et al., 2004). Other studies add to the conclusion that UDP-sugar recognition is highly complex (Thorsoe et al., 2005; Kohara et al., 2007; Modolo et al., 2007; Yonekura-Sakakibara et al., 2007). The sugar residue Rha has the same C-4 configuration as Gal. In the Arabidopsis rhamnosyltransferase UGT89C1, a His residue is present at the position corresponding to H374 in the AcUGT78A2 galactosyltransferase. However, this is not the case for the rhamnosyltransferase AtUGT78D1 (Yonekura-Sakakibara et al., 2007), and in MtUGT78G1 that uses both UDP-Glc and UDP-Gal as sugar donor, this position is occupied by a Gln residue (Modolo et al., 2007). These observations on the specificity of UGTs with respect to the sugar donor lead to the conclusion that multiple amino acid residues contribute to the determination of sugar specificity. Thus, while single amino acid residues might be decisive for sugar recognition of a specific UGT, these are not general determinants of sugar specificity.

Studies of GTs from animals support the involvement of single residues as determinants of sugar specificity in specific GTs (Qasba et al., 2005). In the human GT-A-fold enzyme GlcAT-I involved in proteoglycan biosynthesis, the mutation H308R switches the sugar donor specificity of the enzyme from UDP-GlcUA to UDP-Glc. In addition, an Arg residue R277 was found to be of crucial importance for the glucuronosylation to proceed (Ouzzine et al., 2002). This Arg residue was suggested to stabilize the acidic group of the sugar donor in a similar fashion to that proposed for R25 of BpUGT94B1 in this study. Most of the plant UGTs so far studied and all of the four crystallized plant UGTs are glucosyltransferases. As more biochemical data, crystal structures, and modeling work on UGTs with other sugar preferences become available, a more comprehensive knowledge of key residues in sugar donor recognition is likely to emerge.

**Sugar Acceptor Specificity of BpUGT94B1**

BpUGT94B1 glucuronosylates cyanidine 3-O-glucoside at the 2'-OH position (Fig. 1). A very tight acceptor specificity of BpUGT94B1 is evidenced by the low activity with the acceptor delphinidin 3-O-glucoside, relative activity being only 5% of the activity with cyanidin 3-O-glucoside. Cyanidin 3-O-glucoside and delphinidin 3-O-glucoside offer the same structural epitope for glycosylation at the 2'-OH position, and only differ by the presence of an additional hydroxyl group on the B ring of the aglycone (Fig. 1). This shows that the entire structure of the substrates and not just the site of the accepting atom is being recognized by BpUGT94B1.

To investigate residues in BpUGT94B1 responsible for this very tight substrate specificity distinguishing between cyanidin and delphinidin, independent point mutations of residues forming the acceptor pocket were made. The mutations I187S, I187A, and L148A all severely compromised activity toward cyanidin as well as delphinidin 3-O-glucoside. This showed that even minor structural changes of the residues forming the substrate-binding pocket can have a large impact on enzyme activity. To further investigate residues important for substrate interaction, two additional residues were mutated. The mutation D152A was expected to result in reduced activity because of lost ability to form a stabilizing H bond with the 4'-OH on the B ring of the two acceptors. The mutant N123A was made as this residue is positioned favorably for interaction with the sugar part of the acceptor. These mutants showed loss of activity with the D152A mu-
In Vivo Activity

In vivo the activity of BpUGT94B1 is a step in the synthesis pathway of the main anthocyanin in red daisy cyanidin 3-O-2"-O-glucuronosyl-6"-O-malonylglucoside (Fig. 1; Sawada et al., 2005). In vitro regiospecific activity at the 2"-OH position is reported for the two acceptors cyanidin 3-O-6"-O-malonylglucoside and cyanidin 3-O-glucoside (Fig. 1; Sawada et al., 2005), both intermediates in the synthesis pathway of cyanidin 3-O-2"-O-glucuronosyl-6"-O-malonylglucoside. This regiospecificity fits nicely with the model of BpUGT94B1, because suitable positioning of each of the other OH groups of the acceptor for glucuronosylation would require protein backbone rearrangement of the model. Additionally, the manual docking of either the acceptor sugar moiety 6"-OH or the B-ring 4'-OH showed that this demands the acceptor AC ring to be positioned pointing inward into the enzyme instead of outwards in the cleft region between the domains. This would require a larger movement of the domains for substrate binding and release.

The in vitro activity does not reveal whether the glucuronosylating activity of BpUGT94B1 in vivo takes place before or after introduction of malonic acid at the C 6"-OH of the cyanidin 3-O-glucoside. This cannot be deduced from the model either, because both substrates can be fitted into the acceptor pocket (Fig. 4). The site surrounding the acceptor hydroxyl group to be glucuronosylated is the same on both acceptors, but the malonic acid ester residue would take up additional space in the narrow acceptor pocket. Successful docking of the malonylated anthocyanin requires a twist of the malonic acid ester residue toward the aglycone, favored by intramolecular H-bond formation between the malonic acid and the cyanidine A ring (Dangles, 1997). In the absence of malonic acid, the residue N123 is positioned so that it can stabilize the sugar part of the donor. In the presence of the malonic acid ester, a more restricted and tight fit is envisioned to hold the acceptor in place. These observations may explain the activity with both acceptors of the otherwise very substrate-specific BpUGT94B1.

Cyanidin 3-O-2"-O-glucuronosyl-6"-O-malonylglucoside constitutes 85% of total anthocyanins in the petals of red daisy and very low amounts of the putative intermediates in the biosynthetic pathway are present (Sawada et al., 2005). Accordingly, the malonylation and glucuronosylation steps must proceed with high affinity. It has been suggested that the malonyltransferase and BpUGT94B1 are positioned in a metabolic grid where either activity may occur first (Sawada et al., 2005). This is in accordance with the observed favorable docking of both acceptor substrates into the model.

The very narrow in vitro substrate specificity of BpUGT94B1 is a general feature of several other UGTs (Fukuchi-Mizutani et al., 2003; Kramer et al., 2003; Yonekura-Sakakibara et al., 2007). The narrow substrate specificity stands in contrast to the fact that many UGTs show a very broad specificity in vitro (Hansen et al., 2003; Hefner and Stockigt, 2003; Caputi et al., 2008). This highlights the question of how narrow versus broad substrate specificity of UGTs is regulated in vivo. In vivo specificity of UGTs have been suggested to depend on their incorporation into metabolons where binding of the UGT per se could narrow its substrate specificity or serve to restrict access to its active site (Jörgensen et al., 2005; Kristensen et al., 2005; Nielsen et al., 2008).

The conclusion of this study is that a few specific amino acid residues as well as the overall size and shape of the acceptor pocket define substrate specificity. The residues in the BpUGT94B1 model forming the acceptor pocket are mostly situated in the N-terminal domain, the least well conserved domain between UGTs. The presence of several highly variable loops in the N-terminal domain adds to the possible diversity in overall shape and size of the substrate pocket of UGTs. This explains why substrate specificity cannot be assigned based on primary structure alone. It also
implies that novel combinations of donor and acceptor specificity might be obtained by combining entire N- and C-terminal domains of different UGTs. In recent studies (Cartwright et al., 2008; Weis et al., 2008) it has been possible to construct such functionally active chimeric UGTs that harbor the N- and C-terminal domains from different plant UGTs.

The importance of glucuronosylation for in planta stability, transport, and storage of bioactive natural products including anthocyanins is not yet understood. Nevertheless, engineering of specific UDP-glucuronosyltransferase activities into plants offers a wide range of interesting applications. Glucuronosylation of anthocyanins may be used to alter the coloration of flowers, fruits, and vegetables. Increased stability during exposure to high or low pH, light, elevated temperatures, oxygen, and ascorbic acid are desired properties, which may be gained by glucuronosylation and open up new uses for anthocyanins as food colorants. A chimeric UGT able to use delphinidin 3-O-glucoside as sugar acceptor and UDP-GlcUA as sugar donor would offer the possibility to produce a blue plant pigment with improved stability for use in fermented foods like yogurt. Likewise, introduction of glucuronosylated defense compounds into plants by genetic engineering may improve resistance to biotic attacks.

**MATERIALS AND METHODS**

**Homology Modeling of UGT94B1**

Homology modeling was performed according to guidelines for molecular modeling of Gfs (Imberty et al., 2006). The secondary structure of red daisy (Bellis perennis) UGT94B1 was predicted using the softwares Phyre (http://www.sbg.bio.ic.ac.uk/phyre/html/index.html) and Jpred (http://www.compbio.dundee.ac.uk/ approximately www-jpred/submit.html), and the predictions were compared to the distribution of α-helices and β-strands in the crystal structures of Medicago truncatula UGT73G1 (Shao et al., 2005) and Vitis vinifera (Shao et al., 2005). A structural alignment of UGT94B1 against these two UGT sequences was constructed manually, and SCRs were defined (Fig. 2). A model of BpUGT94B1 including all SCRs was built from the structural alignment using the Composer function of the modeling software Sybyl from Tripos. Regions between two SCRs were defined as loops. Loops that could not be modeled from the templates were modeled by screening against nonredundant protein-fold database in Composer (Sybyl modeling software from Tripos). This was the case for the loops 1, 7, 9, 11, and 18 (Fig. 2). Loop 9 residues (204–208) and the N-terminal 14 amino acids were left out as no satisfactory modeling of these loops could be made. The model was optimized by several cycles of energy minimization using Sybyl force field, and a satisfied conformational analysis of amino acids. Hydrogen atoms were added and atomic charges derived using the Pullman procedure. Model quality was verified by calculation of PROCHECK (Laskowski et al., 1992) and ProSA Z score (Wiederstein and Sippel, 2007). The Figures 3, 4, and 5 showing the model of BpUGT94B1 were made using the software Pymol (DeLano, 2002).

**Insertion of Sugar Donor and Acceptor into the Model**

Docking of the sugar donor was performed by homology docking (Imberty et al., 2006), where the substrate is manually inserted guided by substrate position in the crystal structure of a homologous protein. For the sugar donor UDP-GlcUA, this was guided by Permuting coordinates of the VvGT1 crystal structure with the sugar acceptor kaempferol (Oftên et al., 2006) and the bacterial family 1 GT GhD, crystallized with the acceptor 1-vancomycin (Mulchak et al., 2004). The docking was adjusted manually to optimize the position of the reactive 2'-OH group of the Glc moiety of the acceptor. Energy optimization of the amino acid side chains surrounding donor and acceptor was performed.

**Liquid Chromatography-Mass Spectrometry Analyses**

Analytical liquid chromatography-mass spectrometry was carried out using an Agilent 1100 Series LC (Agilent Technologies) hyphenated to a HCTplus ion trap mass spectrometer (Bruker Daltonics) and fitted with a Zorbax SB-C18 RRHT column (2.1 × 50 mm, 1.8 μm [Agilent], protected by a Phenomenex Gemini C18 security guard cartridge, flow rate: 0.2 mL min⁻¹). The four phases were: A, 2% formic acid in acetonitrile. The gradient program was: 0 to 8 min, linear gradient 5% to 25% (v/v) B; 8 to 9 min, linear gradient 25% to 100% B; 9 to 10.5 min 100% B, followed by equilibration at 5% B for 3.5 min.

**Cloning of UGT94B1 and Generation of Mutated Proteins by Site-Directed Mutagenesis**

For the cloning of UGT94B1, mRNA was prepared from petals of red daisy using MicrocryoA Purist kit (Ambion). CDNA was prepared from the isolated mRNA and used as template for PCR with the UGT94B1-specific primers 5’-gaagctacctaatattcactcc-3’ and 5’-gtacagctgattgtgaaaaactc-3’. Introducing KpnI and Xhol sites in the 5’ and 3’ ends, respectively. The construct was cloned first into pCR-blunt II-TOPO vector (Invitrogen) and then transferred into pET30a+ vector (Novagen).

Single point mutations were introduced by PCR using UGT94B1 inserted into the pET30a+ vector and Xhol sites as template. PCR was performed using Phusion polymerase and thermocycling parameters were 95°C 1 min, 15 cycles of 95°C 30 s, 55°C 1 min, 68°C 12 min. PCR products were digested with DpnI (1–2 h, 37°C) and transferred into competent Escherichia coli cells. Plasmids were extracted from overnight cultures of transformed colonies and sequenced to verify the correctness of mutated sequences harboring the following changes: R25P, R25S, R25K, R25G, I187A, I187S, N123A, D152A, and P174G. The primers used to introduce these point mutations were: R25P fw: 5’-gcatatagcattcatacttcctctctctgtgcttttgc3’; R25P rev: 5’-gagcaaaactaggggagatagtgactatatagc3’; R5 5fw: 5’-gcataagtcagcagcttatcttatcagcttttttttgc3’; R5 5rev: 5’-gagcaaaactaggggagatagtgactatatagc3’; R25S 5fw: 5’-gcataagtcagcagcttatcttatcagcttttttttgc3’; R25S rev: 5’-gagcaaaactaggggagatagtgactatatagc3’. The construct was cloned first into pCR-blunt II-TOPO vector (Invitrogen) and then transferred into pET30a+ vector (Novagen).

**Expression and Purification of UGT94B1 and Derivatives**

PET30a+ plasmids (Novagen) harboring BpUGT94B1 or mutated derivatives were transformed into the E. coli strain C41 cells harboring the ARS (araBAD) CIP (carrD) E. coli plasmid (DS466, own research). This strain has a chromosomally inserted λ lysosome gene (encoding λ lysosome/endolysin) inducible by λ-Ara. Cultures were inoculated and grown in Luria-Bertani media with kanamycin (overnight, 28°C). Expression of recombinant enzymes was induced by adding isopropylthio-β-D-galactoside (0.1 mM) and cell lysis prepared by addition of λ-Ara (3 mM) to express λ lysosome. Cells were then grown overnight at 20°C, harvested, and resuspended in buffer (10 mM Tris, 5 mM CaCl₂, 1 mM diithiothreitol, protease inhibitor [Roche complete tablets]). Samples were frozen at –80°C for cell membrane disruption to expose the peptidoglycan layer of the cell walls to degradation by λ lysosome. Samples were thawed, supplemented with DNase (350 μg/mL), and incubated 5 min in a 37°C water bath. Samples were added

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Enzymatic Activity and Determination of Kinetic Parameters

The activity of UGT94B1 and mutants was determined in assay mixtures (total volume: 20 μL) containing UDP-sugar donor and acceptor as specified for individual experiments, enzyme (2 μg protein), and 100 mM Tris-HCl pH 7.5. After incubation (30°C, 450 rpm, 2–60 min), the reactions were stopped by addition of 1 volume MeOH/6% formic acid. After centrifugation (10 min, 4°C, 10,000g), product formation was analyzed by liquid chromatography-mass spectrometry analysis of supernatant. Sugar acceptor concentration (cytidine 3'-O-glucoside) was 650 μM for all experiments (except determination of cytidine 3'-O-glucoside kinetic parameters). Sugar donor concentrations were as stated in Figures 7, 8, and 9 for determining kinetic parameters and were 2.5 mM for determining relative activity. For all enzymatic assays both donor and acceptor were added to obtain a 5 to 10 times substrate excess during the entire time course of the enzymatic reactions. Calculation of kinetic parameters \( k_{\text{cat}} \) (accounts as relative product formation \( V_{\text{max}} \)/mol) and relative \( V_{\text{max}} \)/mol were performed using the software R (version 2.6.2), fitting initial velocity data (exponential points) to the Michaelis Menten relation (\( V_{\text{cat}} = V_{\text{max}}[S]/k_{\text{cat}} + [S] \)) equation by means of nonlinear regression analysis.

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