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# The in vitro substrate regiospecificity of recombinant UGT85B1, the cyanohydrin glucosyltransferase from *Sorghum bicolor*

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Dedicated to the memory of Professor Jeffrey B. Harborne

**Abstract**

The in vitro substrate specificity of UDP-glucose:*p*-hydroxymandelonitrile-*O*-glucosyltransferase from *Sorghum bicolor* (UGT85B1) was examined using a range of potential acceptor molecules, including cyanohydrins, terpenoids, phenolics, hexanol derivatives and plant hormones. Qualitative enzyme activity assays employing 20 different putative substrates were performed and 15 proved to be glucosylated using recombinant UGT85B1 isolated from *Escherichia coli*. $K_m$ and $k_{cat}$ values were determined for nine of these substrates including mandelonitrile, geraniol, nerol and β-citronellol, 2-hydroxy-3-methoxybenzyl alcohol, 1-hexanol, *cis*-3-hexen-1-ol, 3-methyl-3-buten-1-ol and 3-methyl-2-buten-1-ol. UGT85B1 has a rather broad substrate specificity in vitro but shows regiospecificity, demanding the presence of a sterically unhindered hydroxyl group e.g. as part of a cyanohydrin function, as a primary alcohol or as a phenolic hydroxyl group and being influenced by the stereochemistry and/or interactive chemistry of the substituents on the hydroxyl-bearing carbon atom.


*Keywords:* *Sorghum bicolor*; Gramineae; UDPG-glucosyltransferase; UGT85B1; In vitro regiospecificity; Natural products; Cyanohydrins; Terpenoids; Geraniol

## 1. Introduction

Glycosylation reactions are catalyzed by glycosyltransferases, and these enzymes are found in all phylae. In plants, glycosylation typically occurs as one of the last steps in natural product synthesis (e.g. Cheng et al., 1994; Vogt and Taylor, 1995; Jones et al., 2000) and glycosides are found among all major classes of natural products, e.g. phenolics (Landtag et al., 2002), terpenoids (Kita et al., 2000), flavonoids (Vogt and Taylor, 1995; Miller et al., 1999), glucosinolates (Reed et al., 1993) and cyanogenic glucosides (Jones et al., 1999).

Glycose is a highly polar molecule and glucosylation of hydrophobic aglycones is important to stabilize labile aglycones, to reduce their toxic properties and to facilitate their compartmentalization, transport and storage (Coleman et al., 1997; Jones et al., 2000). Such examples include the aglucone precursors of cyanogenic glucosides, which must be glycosylated to prevent dissociation and release of toxic hydrogen cyanide.

Glycosyltransferases comprise a large and divergent polyphyletic multigene super-family (Mackenzie et al., 1997). Glycosyltransferases are currently divided into 65 different families on basis of biochemical and sequence similarities (Campbell et al., 1997; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Glycosyltransferases involved in plant natural product synthesis belong to family 1. Glycosyltransferases involved in natural product synthesis are characterized by a consensus sequence near the C-terminus ascribed to UDPG binding (Hughes and Hughes, 1994) and are designated UDPG-glycosyltransferases (UGTs) (Mackenzie et al., 1997).

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The fully sequenced *Arabidopsis thaliana* genome has revealed 112 proposed full-length family 1 UGTs and eight apparent pseudo genes (Paquette et al., 2003). Heterologous expression of predicted UGT sequences and subsequent substrate assays has lead to functional characterization of a few *A. thaliana* UGTs (e.g. Jackson et al., 2001; Lim et al., 2001, 2002). In addition, a family 1 UGT designated *p*-hydroxymandelonitrile-*O*-glucosyltransferase catalyzing the final step in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* was isolated, cloned, and heterologously expressed in *Escherichia coli* (Jones et al., 1999). This UGT has subsequently been assigned UGT85B1 by the UGT Nomenclature Committee (Mackenzie et al., 1997). The labile cyanohydrin substrate of UGT85B1 is provided from the amino acid L-tyrosine by action of two membrane located cytochrome P450 enzymes, CYP79A1 and CYP71E1 (Sibbesen et al., 1995; Koch et al., 1995; Kahn et al., 1997; Bak et al., 1998).

In planta, none of the 112 predicted family 1 UGTs of *A. thaliana* involved in glycosylation of natural products was capable of converting *p*-hydroxymandelonitrile **1** into dhurrin (Tattersall et al., 2001). Soluble extracts of *S. bicolor* were found to catalyze a strictly stereospecific glucosylation of *p*-hydroxymandelonitrile **1** to produce the (*S*)-epimer dhurrin (Rosen et al., 1975) likewise suggesting that UGT85B1 is a highly specific enzyme. In contrast, in vitro studies suggested that the enzyme has a somewhat broader specificity (Jones et al., 1999). The present study was undertaken to elucidate the substrate specificity of UGT85B1 in more detail and to investigate whether constitutive expression of UGT85B1 in transgenic *A. thaliana* would result in accumulation of new glucosides. It is concluded that the enzyme is able to glucosylate aglycones from different classes of natural products, and that its activity is largely determined by the substituents on the hydroxyl-bearing carbon atom.

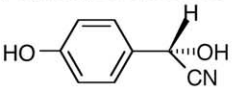
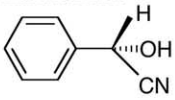
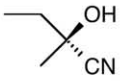
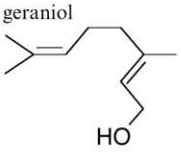
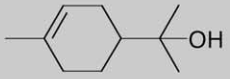
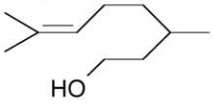
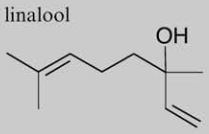
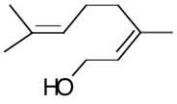
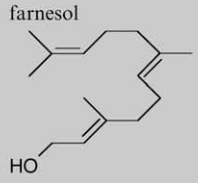
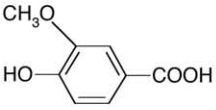
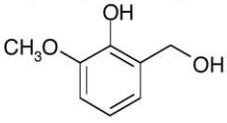
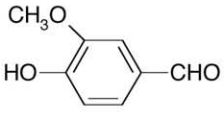
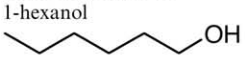
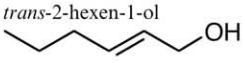
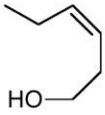
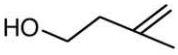
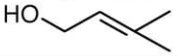
## 2. Results and discussion

UGT85B1 was heterologously expressed in *E. coli* and isolated as described by Jones et al. (1999). The ability of UGT85B1 to glucosylate cyanohydrins, terpenoids, phenolics, hexanol derivatives and plant hormones was examined (see Table 1, compounds **1**–**18**). To verify that UGT85B1 was the enzyme catalyzing glucoside formation from the different aglycones, enzyme assays were also performed using crude *E. coli* extracts obtained from cells transformed with either an empty vector (negative control) or a vector containing the UGT85B1 cDNA. In all cases, glucoside formation was caused by UGT85B1 since no products were detected in assays using extracts prepared from *E. coli* transformed with the empty vector (Fig. 1). The identity of the different glucosides was confirmed by LC–MS again using extracts from *E. coli* expressing either UGT85B1 or transformed with the empty vector as illustrated with geraniol-glucoside from geraniol **3** (Fig. 2, panels A–E). In this example, the selected ion $m/z$ 339, corresponding to the $Na^+$-adduct of geraniol-glucoside is observed at $t = 32.9$ min in extracts of *E. coli* expressing UGT85B1 (Fig. 2, panel C) but not in the negative control (Fig. 2, panel D). Fragmentation of the $m/z$ 339 ion resulted in the generation of an $m/z$ 203 ion, corresponding to fragmentation of the glucosidic linkage (Fig. 2, panel E).

The in vitro substrate specificity of UGT85B1 isolated from *E. coli* extracts as previously described (Jones et al., 1999) was further examined. Fifteen of 20 tested aglycones were glucosylated by UGT85B1 as assessed by qualitative TLC analysis indicating rather broad substrate specificity (Table 1). Four cyanohydrins (**1**, **2**, **10**, **11**) were tested, two aliphatic (**2**, **11**) and two aromatic (**1**, **10**). All four were glucosylated by UGT85B1. $K_m$ and $k_{cat}$ values were only obtained for mandelonitrile **10** (Table 1) because the three other cyanohydrins did not show first-order Michaelis–Menten kinetics. The turnover number with mandelonitrile **10** as substrate ($k_{cat} = 10.6\ s^{-1}$) was 10-fold higher than observed with any other putative substrate tested. Both $k_{cat}$ and $k_{cat}/K_m$ (12 600 $s^{-1}\ M^{-1}$) values for mandelonitrile are comparable to those obtained for substrates for other UDPG glucosyltransferases (Jackson et al., 2001; von Rad et al., 2001; Lim et al., 2002). The turnover number of UGT85B1 with mandelonitrile **10** as substrate is more than 2-fold higher compared to the corresponding values for the two cytochrome P450 enzymes CYP79A1 and CYP71E1 acting in the dhurrin pathway and providing the cyanohydrin substrate. The $K_m$ and $k_{cat}$ values for CYP79A1 have been reported as 0.14 mM and approx. 4 $s^{-1}$ for CYP79A1, the rate-limiting step in the dhurrin pathway (Sibbesen et al., 1995). In aqueous solutions, cyanohydrins dissociate into a ketone or an aldehyde and hydrogen cyanide forming an equilibrium that is dependent on the initial concentration of the cyanohydrin (Reay and Conn, 1974). Furthermore, *p*-hydroxymandelonitrile **1**, the in vivo substrate of UGT85B1, and its degradation product *p*-hydroxybenzaldehyde have previously been reported to be concentration-dependent inhibitors of UGT85B1, probably due to non-selective chemical effects or due to allosteric regulation. Although this inhibitory effect was partly negated by the addition of BSA (Jones et al., 1999), the above factors may explain why no simple kinetics is obtained with acetone cyanohydrin **2**, 2-hydroxy-2-methylbutyronitrile **11** or *p*-hydroxymandelonitrile **10** as substrates.

In vitro studies using a crude UDPG-glucosyltransferase extract from *S. bicolor,* have previously shown that the sorghum UDPG-glucosyltransferase catalyzes the formation of dhurrin (*p*-hydroxy-(*S*)-

Table 1

| Substrate | | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) | Substrate | | $K_m$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| **Cyanohydrins** | | | | | | | | | |
| *p*-hydroxy-mandelonitrile  | **1** | [b] | | | mandelonitrile  | **10** | 0.84 | 10.6 | 12 600 |
| acetone cyanohydrin  | **2** | [b] | | | 2-hydroxy-2-methylbutyronitrile  | **11** | [b] | | |
| **Terpenoids** | | | | | | | | | |
| geraniol  | **3** | 0.14 | 0.10 | 690 | α-terpineol  | **12** | | | |
| β-citronellol  | **4** | 0.13 | 0.09 | 680 | linalool  | **13** | | | |
| nerol  | **5** | 1.13 | 0.06 | 50 | farnesol  | **14** | | | |
| **Phenolics** | | | | | | | | | |
| vanillic acid  | **6** | [b] | | | 2-hydroxy-3-methoxybenzyl alcohol  | **15** | 6.33 | 0.39 | 60 |
| vanillin  | **7** | [b] | | | | | | | |
| **Hexanol derivatives** | | | | | | | | | |
| 1-hexanol  | **8** | 0.66 | 0.06 | 80 | *trans*-2-hexen-1-ol  | **16** | [b] | | |
| *cis*-3-hexen-1-ol  | **9** | 0.73 | 0.03 | 40 | 3-methyl-3-buten-1-ol  | **17** | 0.69 | 0.07 | 100 |
| | | | | | 3-methyl-2-buten-1-ol  | **18** | 10.3 | 0.91 | 90 |

[a] The ability of UGT85B1 to glucosylate different substrates was examined both qualitatively and quantitatively. Farnesol **14**, linalool **13** and α-terpineol **12** (grey panel) were not glucosylated. For substrates that followed first order Michaelis–Menten kinetics, $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values are presented.

[b] Compounds that were glucosylated but did not follow first order kinetics.

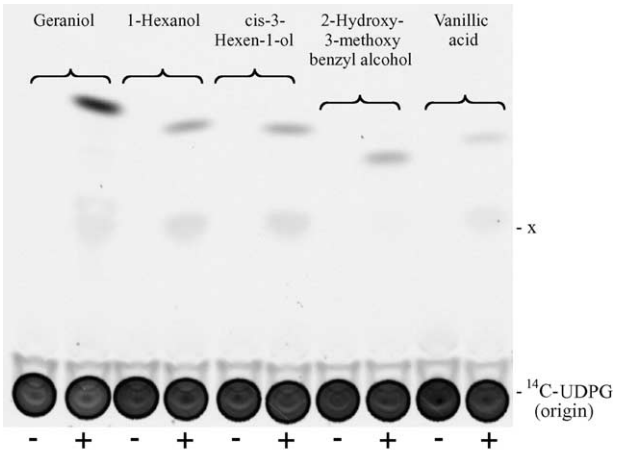



Fig. 1. Glucosylation of different aglycones using extracts prepared from *E. coli* expressing UGT85B1 (+) or transformed with the empty control vector (−) as monitored by TLC. x = unidentified impurity present in the $^{14}$C-UDPG tracer.

mandelontrile-β-D-glucopyranoside) from UDPG-glucose and (*R,S*)-*p*-hydroxymandelonitrile **1** (Reay and Conn, 1974). Accordingly, the reaction with (*R,S*)-*p*-hydroxymandelonitrile **1** is stereospecific for the (*S*)-enantiomer in agreement with the (*S*)-configuation of dhurrin. In contrast, it was demonstrated that a crude UDPG-glucosyltransferase enzyme extract from *Linum usitatissimum* (linen flax) catalyzed the formation of both lotaustralin and *epi*-lotaustralin when (*R,S*)-2-hydroxy-2-methylbutyronitrile was administered as substrate. Accordingly, the flax enzyme does not discriminate between the two enantiomeric cyanohydrins (Zilg and Conn, 1974). In the present study, racemic 2-hydroxy-2-methyl-butyronitrile **11** was tested as substrate for isolated sorghum UGT85B1 (Table 1). The glucoside obtained from these isoleucine/*threo*-isoleucine derived aglycones had the expected mass of the sodium adduct of lotaustralin/*epi*-lotaustralin ($m/z = 339$). The elution time from the reversed phase column was identical to that of an authentic lotaustralin standard isolated from *Lotus japonicus* ($t = 13.4$ min). The $m/z$ 339 component was isolated using preparative HPLC. After freeze-drying and persilylation of the collected material, GC–MS analysis again demonstrated that the product formed by UGT85B1 co-eluted with lotaustralin. Likewise, the molecular ion and fragmentation pattern corresponded to a glucoside produced from (*R,S*)-2-hydroxy-2-methyl-butyronitrile **11**. Lotaustralin has an (*R*)-configuration at the cyanohydrin carbon (Nahrstedt et al., 1987; Zilg and Conn, 1974) but the (*S*)-epimer *epi*-lotaustralin is also known to occur naturally (Pitsch et al., 1984). *epi*-Lotaustralin has been reported to elute with longer retention time compared to lotaustralin upon GC analysis (Seigler, 1975; Nahrstedt, 1981). Accordingly, our results suggest that the glucoside produced from (*R,S*)-2-hydroxy-2-methylbutyronitrile **11** by UGT85B1 has the (*R*)-configuration at the cyanohydrin function, thus being lotaustralin. This indicates that the active site of UGT85B1 is able to discriminate between the two cyanohydrin stereoisomers even when the two substituents at the cyanohydrin carbon are tiny and both aliphatic, thus exhibiting stereospecificity features different from those of the linen flax glucosyltransferase.

The ability of UGT85B1 to glucosylate an aglycone is not dependent on the presence of a cyanohydrin function. The five primary $C_5$ and $C_6$ alcohols 1-hexanol **8**, *cis*-3-hexen-1-ol **9**, *trans*-2-hexen-1-ol **16**, 3-methyl-3-buten-1-ol **17** and 3-methyl-2-buten-1-ol **18** were all glucosylated (Table 1). All tested $C_5$ and $C_6$ alcohols except *trans*-2-hexen-1-ol **16** showed normal first order Michaelis–Menten kinetics with $K_m$ values similar to that determined for mandelonitrile **10** but a 150-fold lower $k_{cat}/K_m$ value. The $K_m$ value for 3-methyl-2-buten-1-ol **17** exceeded those of the others by more than a factor of 10. Likewise, of the four different terpenoids containing primary alcohol groups, the three monoterpenoids geraniol **3**, β-citronellol **4** and nerol **5** were glucosylated whereas glucosylation was neither observed of the primary alcohol group in the sesquiterpenoid farnesol **14** nor of the tertiary alcohol groups in the monoterpenes α-terpineol **12** and linalool **13**. The glucosylation of geraniol **3**, β-citronellol **4** and nerol **5** followed first order Michaelis–Menten kinetics. The catalytic efficiency of UGT85B1 towards geraniol **3** ($K_m$ and $k_{cat}/K_m$ values of 0.14 mM and 690 $s^{-1}$ $M^{-1}$) and β-citronellol **4** ($K_m$ and $k_{cat}/K_m$ values of 0.13 mM and 680 $s^{-1}$ $M^{-1}$) indicate that these compounds could serve as substrates for UGT85B1 in vivo. The $K_m$ value for nerol **5** was a factor 10 higher compared to those of geraniol **3** and β-citronellol **4**. It is concluded that glucosylation of the above types of compounds requires the availability of a sterically unhindered primary hydroxyl group. However, proper docking of this hydroxyl group into the active site of UGT85B1 may be impeded by an improper configuration of the double bond nearest to the hydroxyl group, e.g. as observed with nerol **5** (*Z*-configuration) in comparison to geraniol **3** (*E*-configuration) or bulkiness of the side chain, e.g. as observed with farnesol **14** in comparison to geraniol **3**.

UGT85B1 was isolated based on its ability to glucosylate *p*-hydroxymandelonitrile **1** and had previously been shown also to glucosylate benzyl alcohol (Jones et al., 1999). When 2-hydroxy-3-methoxybenzylalcohol **8** was tested it was also glucosylated. More surprisingly, the enzyme was also able to glucosylate phenolic hydroxyl groups like in vanillic acid **6** and vanillin **7** (Table 1). Thus the increased acidity of the phenolic hydroxyl group does not prevent glucosylation although none of the reactions followed first order kinetics (data not shown). In agreement with these observations, a study

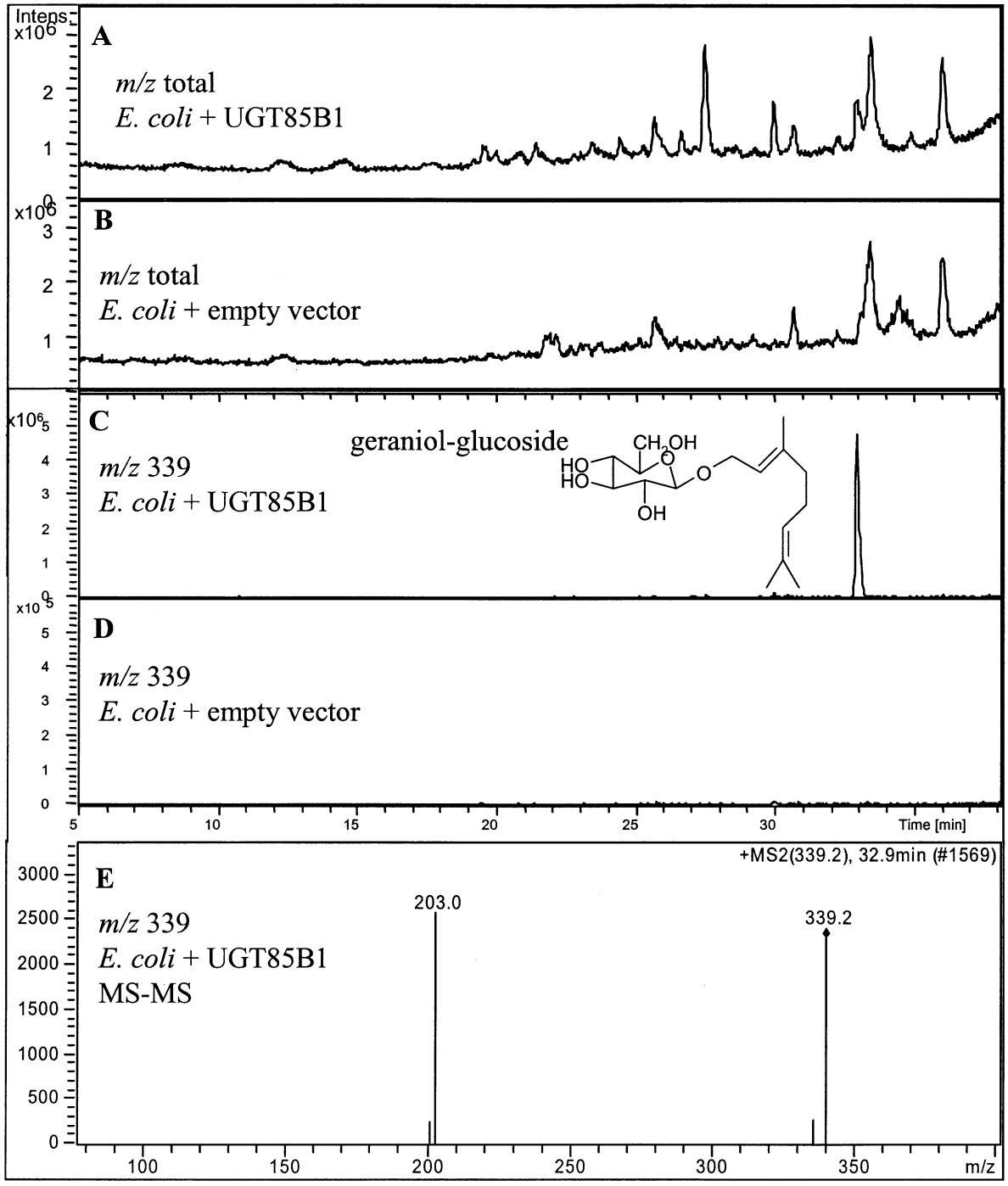



Fig. 2. Geraniol glucoside formation in extracts prepared from *E. coli* expressing UGT85B1 as verified by LC–MS and LC–MS–MS. Total (panel A + B) and selected *m*/*z* ion traces (panel C + D) are presented. A and C: *E. coli* expressing UGT85B1. B and D: *E. coli* transformed with empty control vector. Panel E: Fragmentation of the *m*/*z* 339 ion corresponding to the $Na^+$-adduct of geraniol-glucoside. The component eluting at $t = 28$ min in panel A has an *m*/*z* of 193 and is not a glucoside as verified by fragmentation analysis.

of the specificity of *A. thaliana* UGTs has previously shown that presence of additional hydroxyl groups on a benzene ring can influence glucosylation of specific sites both positively and negatively (Lim et al., 2002).

The two plant hormones, gibberellic ($GA_3$) acid and *trans*-zeatin, were not glucosylated by UGT85B1 (data not shown). Glucosylation of phytohormones in planta is thought to constitute an expedient way to regulate the level of active hormone in comparison to de novo synthesis (Jones and Vogt, 2001). Accordingly, the glucosyltransferases involved in phytohormone glucosylation reactions must either be tightly compartmentalized

or have high substrate specificity. It is therefore not surprising that UGT85B1 shows no activity towards the plant hormones tested.

The results presented on the in vitro substrate specificity of UGT85B1 and previous findings by Jones et al. (1999) let us to conclude that UGT85B1 exhibits regioselectivity rather than absolute specificity. However, this may not reflect the in vivo situation. In *Nicotiana tabacum*, a UGT73 homolog (GenBank accession number AF346431) was shown to glucosylate salicylic acid in vitro but not in vivo (Chong et al., 2002). Transgenic *A. thaliana* plants in which the sorghum UGT85B1 was expressed under control of the cauliflower mosaic virus (CaMV) 35*S* promoter had previously been prepared (Tattersall et al., 2001). The expression of a functional UGT85B1 enzyme in the transgenic plants was verified by the ability of the transgenic plants to effectively glucosylate *p*-hydroxymandelonitrile **1** as analyzed by TLC (Fig. 3). This demonstrates that UGT85B1 is active also when expressed in the absence of the cytochrome P450 enzymes that catalyze the initial steps in the biosynthesis of dhurrin. The presence of new glucosides in the transgenic *A. thaliana* plants accumulating functionally active UGT85B1 in comparison to wild-type plants was monitored by LC–MS analyses. Selective ion monitoring was used to specifically search for the presence of the glucosides shown to be produced in vitro (Table 1). No new glucosides were detected in the UGT85B1 plants in comparison to wild-type plants (data not shown). This may reflect absence of the corresponding aglycones, e.g. due to compartmentation or be taken as an indication of a different substrate specificity in vivo compared to in vitro. *p*-Hydroxymandelonitrile, **1**, the predicted in vivo substrate of UGT85B1 would not be present in wild-type *A. thaliana* because this plant does not produce cyanogenic glucosides (Halkier et al., 2002). In *S. bicolor*, dhurrin synthesis is catalyzed by three enzymes—CYP79A1, CYP71E1 and UGT85B1 (Kahn et al., 1997; Tatttersall et al., 2001). In experiments with double-labeled precursors, this pathway was shown to be highly channeled (Møller and Conn, 1980). Likewise, when the three *S. bicolor* genes were introduced into *A. thaliana* driven by the 35*S* promoter, intermediates were not detected (Tattersall et al., 2001). This would suggest that the three enzymes form a highly organized complex where the product produced by one component is readily accessible at the active site of the following enzymes. In this way, metabolic reactions that would be predicted from in vitro experiments to proceed at low speed may nevertheless proceed swiftly in vivo, e.g. if UGT85B1 is able to participate as an integrated component of a number of different highly organized multi-enzyme complexes. Such multi-enzyme complex formation may also serve to limit the access of other putative substrates to the active site of UGT85B1.

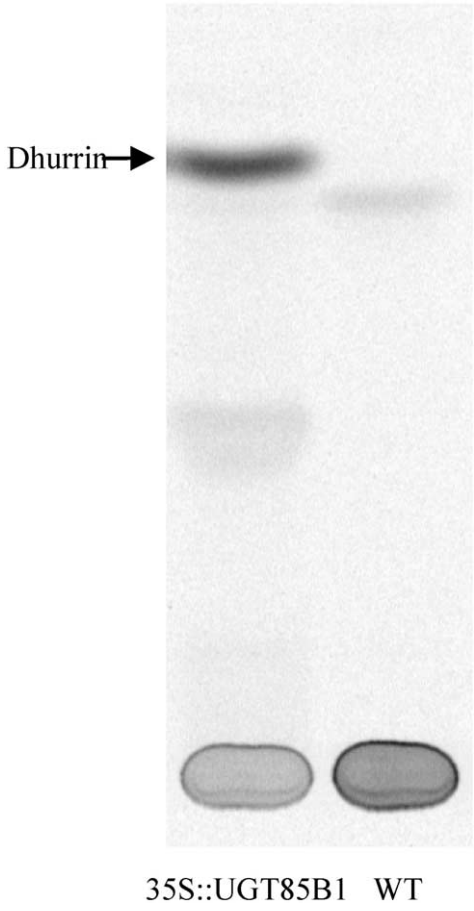



Fig. 3. Soluble protein extracts from *A. thaliana* plants expressing UGT85B1 are able to produce [$^{14}$C]-dhurrin when *p*-hydroxymendelonitrile and [$^{14}$C]-UDPG are administered as substrates. Dhurrin formation was monitored by TLC. No dhurrin production is observed in wild-type (WT).

A key issue in determining the regiospecificity for cyanohydrins is the presence of a non-bulky substituent at the position corresponding to the hydrogen atom in the chiral center of (*S*)-*p*-hydroxymandelonitrile **1**. Primary alcohols and phenolic hydroxyl groups can also be glucosylated (Table 1). In the course of evolution, regiospecificity of plant UGTs may have developed to enable plants to respond quickly to environmental challenges. The question remains whether glucosides of natural products are products of a multitude of highly specific UGTs or arise as products from a few broad-specificity UGTs. A characterization of substrate specificity of the 112 *A. thaliana* UGTs will reveal more of their role in natural product synthesis and give indications of orthologs in other species. However, genes with similar functions do not necessarily share high sequence identity (Vogt, 2002) and phylogenetic analysis should therefore always be supported by additional biochemical analysis when identifying new UGTs in other plant species.

## 3. Experimental

### 3.1. Recombinant protein and enzyme assays

Heterologous expression and subsequent isolation of UGT85B1 and enzyme assays were performed essentially as described by Jones et al. (1999). Glycerol was added to the isolated pure enzyme [final concentration: 10% (v/v)] prior to storage at −80 °C, as this resulted in the highest retainment of enzyme activity. All reaction mixtures had a total volume of 20 μl. Due to their lability, acceptor and enzyme were added last to the reaction mixture.

Qualitative analyses by thin layer chromatography (TLC) of glucosylation of potential substrates were performed in assay mixtures (30 min, 30 °C) containing 100 mM Tris HCl (pH 7.5), 3.7 μM [$^{14}$C]-UDPG (10.6 GBq/mmol, NEN™), 5 mM substrate, approx. 42 ng recombinant UGT85B1 and 10 μg BSA.

Quantitative analyses were performed in assay mixtures (4 min, 30 °C) with substrate concentrations ranging from 0.01 to 15 mM and approx. 42 ng recombinant UGT85B1. All other components were present in excess amounts: 0.2 mM UDPG, 3.7 μM [$^{14}$C]-UDPG (10.6 GBq/mmol, NEN™), 20 μg BSA, 100 mM Tris HCl (pH 7.5). Michaelis–Menten and Lineweaver–Burk plots were made to allow calculation of $K_m$ and $V_{max}$ values.

Enzyme extracts from leaves of *A. thaliana* transformed with either an empty vector or a vector containing *UGT85B1* had previously been prepared (Tattersall et al., 2001).

Leaves were ground using mortar and pestle in an extraction buffer (1:5, w/v) composed of 250 mM sucrose, 100 mM Tris HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 5% PVP (w/v), 5 mM DTT and 200 μM PMSF. The supernatant obtained after centrifugation (30 min, 20,000 *g*) was used to determine the activity of UGT85B1 using 5 μl plant extract, 5 mM *p*-hydroxymandelonitrile **1** and 3.7 μM [$^{14}$C]-UDPG as outlined in the procedure for qualitative assays.

#### 3.1.1. TLC

Reaction mixtures from qualitative and quantitative assays were applied to Silica Gel 60 F254 plates (Merck), dried (1 h, rt) and developed in a solvent system consisting of ethyl acetate: acetone: dichlormethane: methanol: $H_2O$ (20:15:6:5:4, v/v/v/v/v). Plates were dried and exposed to phosphoimaging screens (Molecular Dynamics).

### 3.2. Substrates

Different compounds were tested as putative acceptors. Acceptor concentrations used for determining $K_m$ and $k_{cat}$ values are shown in parenthesis.

- *Cyanohydrins*: *p*-hydroxymandelonitrile **1**, mandelonitrile **10** (0.01–1.25 mM), acetone cyanohydrin **2**, 2-hydroxy-2-methylbutyronitrile **11**.
- *Terpenoids*: geraniol **3** (0.01–5 mM), nerol **5** (0.01–5 mM), β-citronellol **4** (0.01–5 mM), linalool **13**, α-terpineol **12**, farnesol **14**.
- *Phenolics*: vanillic acid **6**, vanillin **7**, 2-hydroxy-3-methoxybenzylalcohol **15** (0.001–0.055 mM).
- *Hexanol derivatives*: 1-hexanol **8** (0.25–7.5 mM), *trans*-2-hexen-1-ol **16**, *cis*-3-hexen-1-ol **9**, 3-methyl-3-buten-1-ol **17**, 3-methyl-2-buten-1-ol **18**.
- *Plant hormones*: gibberellic acid ($GA_3$), *trans*-zeatin.

Cyanohydrins, terpenoids, phenolics and hexanol derivatives were all dissolved in 100% EtOH. *trans*-Zeatin was dissolved in 1 M NaOH.

### 3.3. LC–MS

Assays (20 μl) were performed essentially as outlined for qualitative assays, except that crude *E. coli* extracts expressing either UGT85B1 or transformed with the empty control vector were employed instead of isolated pure protein. Likewise, 1.25 mM unlabelled UDP-glucose was included in each assay. Finally, the enzymatic reaction was stopped by addition of 2 volumes methanol (85%) and the supernatant obtained after centrifugation was subjected to LC–MS.

Analytical LC–MS was carried out using an Agilent 1100 Series LC (Agilent Technologies, Germany) coupled to a Bruker Esquire 3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). An XTerra MS C18 column (3.5 μM, 2.1 × 100 mm), flow rate of 0.2 ml min$^{-1}$, (Waters, Milford, MA) was used. The mobile phases were: A, 0.1% (v/v) HCOOH and 50 μM NaCl; B, 0.1% (v/v) HCOOH and 80% (v/v) MeCN. The gradient program was: 0–4 min, isocratic 2% (v/v) B; 4–10 min, linear gradient 2–8% B; 10–30 min, linear gradient 8–50% (v/v) B; 30–35 min, linear gradient 50–100% (v/v) B; 35–40 min, isocratic 100% B. The mass spectrometer was run in positive ion mode. Total ion current and ion traces for specific $[M+Na]^+$ adduct ions were used for locating compounds.

In order to compare the chirality of the product produced from (*R*,*S*)-2-hydroxy-2-methyl-butyronitrile **11** by UGT85B1 with that of lotaustralin, the product was isolated by preparative-LC (column: Zorbax SB-C18 5 μm 9.4×50 mm) by MS guided collection of the LC eluate containing the component affording the $[M+Na]^+$ at *m*/*z* 284. After evaporation and silylation with a mixture of BSTFA and pyridine, the product was subjected to GC–MS. GC–MS was performed in a system consisting of an HP5890 Series II gas chromatograph fitted with an SGE column (BPX5, 25 mm × 0.25 mm, 0.25 mm film thickness) and coupled to a Jeol

JMS-AX505W mass spectrometer (head pressure 100 kPa, splitless injection). The oven temperature program was as follows: 80 °C for 2 min, 80–200 °C at 20 °C/min, 200–300 °C at 5 °C/min, 300 °C for 30 min. The ion source was run in EI mode (70 eV) at 200 °C.

The retention time for the silyl derivative was 14.9 min, the same as for lotaustralin isolated from *L. japonicus* after TMS-derivatization.

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