Arabidopsis glucosyltransferase UGT74B1 functions in glucosinolate biosynthesis and auxin homeostasis

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Summary

Glucosinolates are a class of secondary metabolites with important roles in plant defense and human nutrition. Here, we characterize a putative UDP-glucose:thiohydroximate S-glucosyltransferase, UGT74B1, to determine its role in the Arabidopsis glucosinolate pathway. Biochemical analyses demonstrate that recombinant UGT74B1 specifically glucosylates the thiohydroximate functional group. Low \( K_m \) values for phenyl-acetothiohydroximic acid (approximately 6 \( \mu \)M) and UDP-glucose (approximately 50 \( \mu \)M) strongly suggest that thiohydroximates are in vivo substrates of UGT74B1. Insertional loss-of-function \( ugt74b1 \) mutants exhibit significantly decreased, but not abolished, glucosinolate accumulation. In addition, \( ugt74b1 \) mutants display phenotypes reminiscent of auxin overproduction, such as epinastic cotyledons, elongated hypocotyls in light-grown plants, excess adventitious rooting and incomplete leaf vascularization. Indeed, during early plant development, mutant \( ugt74b1 \) seedlings accumulate nearly threefold more indole-3-acetic acid than the wild type. Other phenotypes, however, such as chlorosis along the leaf veins, are likely caused by thiohydroximate toxicity. Analysis of UGT74B1 promoter activity during plant development reveals expression patterns consistent with glucosinolate metabolism and induction by auxin treatment. The results are discussed in the context of known mutations in glucosinolate pathway genes and their effects on auxin homeostasis. Taken together, our work provides complementary in vitro and in vivo evidence for a primary role of UGT74B1 in glucosinolate biosynthesis.

Keywords: glucosinolate pathway, glucosyltransferase, thiohydroximates, thioglycosides, auxin, secondary metabolism.

Introduction

Secondary metabolism is central to the ability of plants to cope with biotic and abiotic challenge. Among the large number of described natural products, the best studied have proved to be primary mediators of plant interactions with other organisms, including competitors, commensals, and natural enemies. This mediation frequently involves physiological manipulation of the interacting organism, a fact long exploited by humans in their use of these compounds as medicines. A broader view of the utility of natural products includes their potential for disease prevention, particularly for serious and intransigent illnesses such as cancer. An example receiving considerable attention at present is a class of amino acid derivatives known as glucosinolates.

The glucosinolates, found almost exclusively in the order Brassicales (Rodman et al., 1998), are a diverse class of natural products defined by a common glucose comprising a sulfonated oxime and a \( \beta \)-thioglucose residue. A variable side chain is responsible for the chemical diversity of glucosinolates (Fahey et al., 2001; Rodman et al., 1998; Wittstock and Halkier, 2002). Plant tissue damage leads to glucosinolate hydrolysis and formation of unstable intermediates that spontaneously rearrange to isothiocyanates, thiocyanates, nitriles, oxazolidine-2-thiones, or epithioalkanes (Fahey et al., 2001; Wittstock and Halkier, 2002). Many of these products are biologically active and have been implicated in plant defense against pathogens and herbivores (Kliebenstein et al., 2002; Lambrix et al., 2001; Tierens et al.,...
2001), as attractants of specialist pests (Giamoustaris and Mithen, 1995), and as dietary inducers of detoxification enzymes that favorably modify carcinogen metabolism in mammals (Mithen et al., 2000; Talalay and Zhang, 1996). The wide range of biological activities of glucosinolate breakdown products and the intricate interactions of the glucosinolate pathway with auxin homeostasis in cruciferous plants (Bartel et al., 2001; Ljung et al., 2002) has sparked interest in glucosinolate metabolism.

Bioisostasis of glucosinolates proceeds in three phases: (i) side chain elongation of amino acids by incorporation of methylene groups, (ii) formation of the glucone moiety to produce primary glucosinolates, and (iii) secondary modifications of the side chain to generate the known spectrum of glucosinolate compounds. Identification of glucosinolate pathway genes in Arabidopsis followed by biochemical and genetic studies confirmed the tripartite biosynthetic concept (Mikkelsen et al., 2002; Wittstock and Halkier, 2002). Most enzymes of the second phase, which is common to all glucosinolates, have been cloned and characterized. These include amino acid-specific cytochromes P450 of the CYP79 family catalyzing aldoxime formation (Hansen et al., 2001a; Hull et al., 2000; Mikkelsen et al., 2000; Naur et al., 2003a; Reintanz et al., 2001, 2004; Wittstock and Halkier, 2000), members of the aldoxime-metabolizing CYP83 family (Bak and Feyereisen, 2001; Bak et al., 2001; Hemm et al., 2003; Naur et al., 2003b), and a C-S lyase that generates thiohydroximates (Mikkelsen et al., 2004). Glucosylation of the thiohydroximate yields a desulfoglucosinolate, which is then sulfated to complete the glucone structure. Although a cDNA encoding a thiohydroximate S-glucosyltransferase (S-GT) has been cloned from *Brassica napus* and partially characterized (Marillia et al., 2001), the in vivo function of any S-GT in glucosinolate biosynthesis remains to be established. Likewise, the final step of the core pathway, catalyzed by a sulfotransferase, awaits gene identification.

Genetic screens for developmental mutations in Arabidopsis identified several biosynthetic genes of the glucosinolate pathway that not only impair glucosinolate accumulation, but also disturb auxin homeostasis. Loss-of-function mutations in genes encoding a C-S lyase (*sur1*/*alf1*/his3/*rty*), CYP83B1 (*sur2/*rmt1*) and CYP79F1 (*bus/*sps*) increase indole-3-acetic acid (IAA) accumulation (Barlier et al., 2000; Boerjan et al., 1995; Delarue et al., 1998; Reintanz et al., 2001; Tantikanjana et al., 2001). As the *sur2* and *sur1* mutations block post-aldoxime reactions, the elevated IAA levels can be explained by the accumulation of indole-3-acetaldoxime (IAOx), which can be converted to IAA (Ljung et al., 2002). Recent studies in Arabidopsis established IAOx as a metabolic branchpoint in auxin and indole glucosinolate biosynthesis (Bak and Feyereisen, 2001; Bak et al., 2001; Zhao et al., 2002). An explanation for the high IAA phenotype is less obvious for the cyp79f1 mutants, which are defective in the formation of aldoximes from short-chain methionine derivatives (Hansen et al., 2001a; Reintanz et al., 2001; Tantikanjana et al., 2001).

The *B. napus* S-GT has a single likely ortholog in Arabidopsis, UGT74B1 (Petersen et al., 2001). In this study, we provide evidence for a biological role of UGT74B1 in glucosinolate biosynthesis. The substrate specificity and kinetic parameters of recombinant UGT74B1 demonstrate that it is a UDP-glucose:thiohydroximate S-glucosyltransferase. Characterization of plants harboring null alleles of *UGT74B1* reveals reduced glucosinolate concentrations, elevated levels of free and total IAA, as well as phenotypes consistent with IAA overproduction and thiohydroximate toxicity. Thus, our work demonstrates that UGT74B1 acts in glucosinolate biosynthesis and suggests that a block in thiohydroximate glucosylation perturbs auxin homeostasis.

**Results**

**Recombinant UGT74B1 catalyzes thiohydroximate-specific glucosylation**

To test the hypothesis that UGT74B1 functions in glucosinolate biosynthesis, we first studied its enzymatic properties *in vitro*. We used phenylacetothiohydroximate (PATH) as a test substrate because it is readily synthesized (Ettlinger and Lundein, 1957), occurs naturally as a glucosinolate precursor (Schultz and Wagner, 1956), and has been used for previous biochemical studies of thiohydroximate glucosyltransferase activity (Matsuo and Underhill, 1969). The reaction to be assayed is shown in Figure 1(a). We expressed in *Escherichia coli* full-length UGT74B1 and a truncated protein, UGT74B1C, that lacks the highly conserved UGT signature motif (Mackenzie et al., 1997), purified both (His<sub>6</sub>)<sup>+</sup>-tagged polypeptides by affinity chromatography (Figure S1), and analyzed *in vitro* glucosylation reactions by HPLC with bacterial extracts (Figures 1b and 2) or with purified protein (Table 1). We synthesized the PATH substrate and verified its identity by mass spectrometry (Figure S2a). The expected reaction product of PATH glucosylation, desulfobenzylglucosinolate (dsBGS), was identified by its retention time and A<sub>226nm</sub>/A<sub>390nm</sub> ratio, both of which were identical to those of the authentic standard obtained by enzymatic desulfation of commercially available benzylglucosinolate. In addition, we confirmed the identity of the glucosylation product, dsBGS, by mass spectrometry (Figure S2b). When the *in vitro* glucosylation reaction included UGT74B1 and PATH, the dsBGS product was formed. However, if PATH was omitted or UGT74B1C replaced the full-length protein, formation of dsBGS was not detectable (Figure 1b). Thus, recombinant UGT74B1 catalyzes glucosylation of a thiohydroximate substrate.

Next, we tested UGT74B1 activity against three alternate substrates: phenylacetic acid (PAA), IAA, and salicylic acid.
PAA is the carboxylate analog of PATH and was chosen as the most direct test of UGT74B1 specificity for thiohydroximates. IAA and SA are aromatic carboxylic acids that we tested for two reasons. First, plants lacking a functional UGT74B1 gene have morphological phenotypes consistent with defects in IAA conjugation (see below), suggesting that IAA may be an in vivo substrate of UGT74B1. Secondly, two enzymes of the UGT74 family, UGT74F1 and UGT74F2, glucosylate SA (Lim et al., 2002). As a high degree of amino acid sequence similarity can sometimes predict substrate specificity (Lim et al., 2003a), we tested SA as a potential substrate of UGT74B1. We expressed recombinant UGT84B1 and UGT74F2 (data not shown), which we used in control reactions with IAA, PAA and SA as substrates (Jackson et al., 2001). UGT74B1 glucosylates PATH (Figure 2a) but is inactive toward IAA, PAA, and SA (Figure 2b,c). Both IAA and PAA are substrates of UGT84B1 (Figure 2b,c), which only shows a trace activity with PATH (Figure 2a). Despite its close phylogenetic relationship with UGT74B1 (Lim et al., 2003a), UGT74F2 does not glucosylate PATH (data not shown), but has the expected activity toward SA (Figure 2d). Thus, our results demonstrate that recombinant UGT74B1 is specific for thiohydroximate(s) and that IAA is not a substrate in vitro.

Purified recombinant UGT74B1 is active over a broad pH range with a maximum at pH 6, and the enzyme is stable between pH 5 and pH 9 for at least 30 min, which is three
Isolation and genetic analysis of UGT74B1 T-DNA insertion mutants

Having confirmed the predicted biochemical activity of UGT74B1, we next explored its in vivo function by reverse genetics in Arabidopsis. The University of Wisconsin T-DNA collection (Krysan et al., 1999) contained two independent insertion alleles in the Wassilewskija background. The T-DNA-UGT74B1 junctions were amplified by polymerase chain reaction (PCR) and sequenced to determine the exact structure of the insertion site. One allele, ugt74b1-1, harbors the T-DNA insert in the first exon, whereas a second allele, ugt74b1-3, carries the insertion in the only intron of UGT74B1 (Figure 3a). A 28-base pair sequence of unknown origin separates the T-DNA left border and flanking intron sequence in ugt74b1-3. DNA sequencing across the T-DNA right border indicated that both alleles are insertions, rather than translocations. Southern analysis was consistent with a single T-DNA insertion for both lines, which was corroborated by the fact that in populations derived from parents heterozygous for either insert, the kanamycin resistance phenotype segregated as a single dominant gene (data not shown). We recovered and verified a third allele, ugt74b1-2, from the Syngenta Arabidopsis Insertion Line collection (Sessions et al., 2002) in the Columbia background, which carries the T-DNA insert in the second exon (Figure 3a). Genetic analysis of ugt74b1-2 is consistent with a single gene conferring resistance to the Basta herbicide (data not shown).

We noted a dwarfed phenotype for one quarter of the progeny of each original ugt74b1/+ isolate. Segregation analysis of the F1 and F2 progeny of backcrosses to the appropriate wild type confirmed that the morphological aberration of each ugt74b1 allele is caused by a single recessive gene (data not shown). PCR genotyping revealed a strict cosegregation of the dwarf phenotype with the homozygous mutant genotype, while plants heterozygous for either insertion allele were indistinguishable from the wild type (data not shown). Because the three ugt74b1 alleles display very similar morphological phenotypes and glucosinolate chemotypes (see below), only ugt74b1-1 was characterized in detail.

Analysis of steady-state UGT74B1 mRNA levels by semi-quantitative RT-PCR demonstrated expression in the wild type but failed to detect UGT74B1 transcripts in the homozygous ugt74b1-1 mutant (Figure 3b). As the T-DNA insertion site in ugt74b1-1 is located in the first exon and upstream of the UGT signature motif necessary for glucosyltransferase activity (Mackenzie et al., 1997), it is unlikely that ugt74b1-1 plants express a functional UGT74B1 protein. Thus, we consider ugt74b1-1 to be a bona fide null allele.

ugt74b1 Mutants are severely dwarfed and partially sterile

When grown on agar and compared with wild type, homozygous ugt74b1-1 seedlings produce curled leaves and short petioles, and develop chlorosis along major vascular bundles of the leaves (Figure 4a, Table 2). On soil, the slow-growth phenotype of ugt74b1-1 is exaggerated, which causes late flowering, both chronologically and developmentally (Figure 4e,f; Table 2). In addition, ugt74b1-1 plants are partially sterile, which is likely due to defective flower development (Figure 4f). A number of morphological characteristics of ugt74b1-1 are consistent with auxin hypersensitivity or overaccumulation. Relative to wild type, light-grown mutant seedlings developed elongated hypocotyls and epinastic cotyledons, while the hypocotyls

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### Table 1 Kinetic parameters for purified recombinant UGT74B1

<table>
<thead>
<tr>
<th>PATH</th>
<th>$K_\text{m}$ (μM, n = 3)</th>
<th>$k_{\text{cat}}$ (sec$^{-1}$)</th>
<th>$k_{\text{cat}}/K_\text{m}$ (mm$^{-1}$ sec$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>PATH</td>
<td>5.8 ± 3.1</td>
<td>1.0</td>
<td>172</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>48 ± 23</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

times the assay length (data not shown). For the analysis of kinetic parameters, all assays were performed at pH 6.0. The kinetic parameters of UGT74B1 are reported in Table 1. The low $K_\text{m}$ for PATH is consistent with the proposed role of UGT74B1 in glucosinolate synthesis.
of dark-grown mutant seedlings were slightly shorter (Figure 4b, Table 2). Rosette leaves of ugt74b1-1 are incompletely vascularized (Figure 4c), a phenotype also reported for null mutations in the CYP79F1 gene (Hansen et al., 2001a; Reintanz et al., 2001; Tantikanjana et al., 2001). Quantification of the vascular phenotype showed that ugt74b1-1 plants produced about 50% less vein length per unit leaf area than the wild type (Table 2). Finally, when ugt74b1-1 and wild-type seedlings were grown under conditions to promote adventitious root formation (Tian and Reed, 1999), the mutant developed over 20 times the adventitious roots of the wild type (Figure 4d, Table 2).

**Table 2** Morphological phenotypes of ugt74b1-1 and wild-type seedlings grown on agar

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>ugt74b1-1</th>
<th>Ws-0</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypocotyl length&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>3.9 ± 0.4</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>light-grown (mm)</td>
<td>6</td>
<td>8.5 ± 1.1</td>
<td>10.8 ± 2.0</td>
</tr>
<tr>
<td>dark-grown (mm)</td>
<td>14</td>
<td>6.8 ± 0.4</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>Rosette leaves (no.)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14</td>
<td>2.3 ± 0.5</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>Petiole length (mm)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14</td>
<td>15.6 ± 6.9</td>
<td>2.5 ± 6.9</td>
</tr>
<tr>
<td>Adventitious roots (no.)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14</td>
<td>2.2 ± 0.4</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>Vein length/unit leaf area (mm&lt;sup&gt;e&lt;/sup&gt;)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5</td>
<td>14</td>
<td>2.2 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

<sup>a</sup>n = 9–14 seedlings.
<sup>b</sup>n = 6 plants.
<sup>c</sup>n = 34–38 leaves.
<sup>d</sup>n = 9–13 seedlings.
<sup>e</sup>n = 5 leaves.

**Loss of UGT74B1 decreases glucosinolate levels**

To evaluate an expected in vivo role of UGT74B1 in glucosinolate biosynthesis, we compared glucosinolate profiles of 3-week-old wild type and homozygous ugt74b1-1 mutant plants (Figure 5a). Three major glucosinolates are found in leaves of the Wassilewskija accession: the methionine-derived 3-methylsulfinylpropyl glucosinolate (S3) and the tryptophan derivatives, 3-indolylmethyl glucosinolate (IM) and 3-indolylmethyl glucosinolate (IM).

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**Figure 4**. Morphological phenotypes of ugt74b1 mutants.
(a) Independent ugt74b1 T-DNA insertion alleles cause similar morphological phenotypes that are restored to wild type by molecular complementation. From left to right: a wild type (Ws-0), ugt74b1-1, ugt74b1-1, and UGT74B1-complemented ugt74b1-1 seedling. Plants were grown under axenic conditions for 2 weeks.
(b) Six-day-old ugt74b1-1 seedlings (right) have elongated hypocotyls and epinastic cotyledons when compared with wild type (left).
(c) Leaves of the ugt74b1-1 mutant have less vasculature than comparable wild-type leaves. The upper tracing shows the veins of the wild type, the lower those of the ugt74b1-1 mutant. The leaves are from 3-week-old plants.
(d) The ugt74b1-1 mutant (right) develops substantially more adventitious roots than the wild type (left) when grown under conditions that promote adventitious rooting (Tian and Reed, 1999).
(e) Growth of wild type (left) and ugt74b1-1 (right) plants on soil for 4 weeks.
(f) The left panel shows a mature wild type (left) and ugt74b1-1 (right) plant (8-week old). The right panel contains enlarged pictures of the ugt74b1-1 plant. Defective flowers of ugt74b1-1 result in highly reduced seed set.
and 4-methoxy-3-indolylmethyl glucosinolate (4IM). Relative to wild type, the ugt74b1-1 line accumulates less than 50% of both S3 and IM, but contains wild-type levels of 4IM. The ugt74b1-3 mutant shows the same glucosinolate chemotype, and line ugt74b1-2, which is in the Columbia background and has a different wild-type glucosinolate composition than the Wassilewskija accession (Haughn et al., 1991; Kliebenstein et al., 2001a), displays a similar reduction (40%) in all glucosinolates, including 4IM (data not shown). As with the morphological phenotypes, the low glucosinolate chemotype of the three ugt74b1 alleles is recessive and cosegregates with the homozygous mutant genotype, whereas the glucosinolate profiles of ugt74b1/þ heterozygotes are indistinguishable from wild type (data not shown).

Molecular complementation rescues the ugt74b1-1 phenotype

To exclude the possibility that the phenotypes observed in the ugt74b1-1 mutant are caused by a secondary mutation closely linked to the T-DNA insert, we generated ugt74b1-1 complementation lines that are transgenic for a 3-kb genomic fragment of the Wassilewskija UGT74B1 locus. The morphological appearance (Figure 4a) and glucosinolate profiles (Figure 5a) of the complementation line are indistinguishable from those of wild-type plants, indicating that loss of UGT74B1 function is responsible for the observed morphological phenotypes and the low glucosinolate chemotype.

Loss of UGT74B1 increases IAA levels

As at least some of the morphological phenotypes of the ugt74b1-1 line appear to be auxin-related, we measured free and total IAA content at two stages of plant development. We analyzed plants grown on agar in order to minimize the growth-stage differences between the mutant and wild type. Six-day-old mutant seedlings accumulated about threefold higher concentrations of free IAA and about 50% more IAA conjugates relative to the wild type, whereas levels of both free and total IAA were similar for 3-week-old wild type and ugt74b1-1 plants (Figure 5b). It is therefore likely that at least some of the aberrant morphological phenotypes observed during early development of ugt74b1-1 seedlings are caused by increased IAA concentrations.

Phenylacetothiohydroximic acid is toxic

We examined two possible biological activities of thiohydroximates, which presumably accumulate in ugt74b1 plants. A number of structurally diverse compounds can act as auxins, including various IAA derivatives, as well as the naturally occurring PAA (Ludwig-Müller and Cohen, 2002). We reasoned that the thiohydroximic acid analogs of these compounds, such as indole-3-acetothiohydroximic acid (IATH), have potential auxin activity that may contribute to the high auxin phenotypes of ugt74b1-1 mutants. As IATH is not commercially available, we tested PATH, the thiohydroximate analog of PAA, for its ability to induce expression of the DR5::GUS reporter (Ulmasov et al., 1997). As expected, IAA and PAA (each at 10−5 M) induced GUS expression in 5-day-old seedlings; however, none of the PATH concentrations tested (10−7–10−3 M) had this effect (data not shown). It is therefore unlikely that aromatic and indolyl thiohydroximates have auxin activity.

A previous report on the herbicidal properties of thiohydroximates (Franz, 1971) and the observation that PATH suppresses DR5::GUS expression at 1 mM (data not shown) led us to examine PATH toxicity by double-staining of Arabidopsis roots with fluorescein diacetate (FDA) and...
propidium iodide (PI). When compared with mock-treated seedlings, incubation of roots with PATH as low as 15 μM abolished FDA staining, indicating PATH-induced root cell death (Figure 6). Control seedlings resumed rapid growth upon transfer to agar, whereas most PATH-treated seedlings failed to recover (data not shown). Thus, phenotypes of ugt74b1 mutants that are not easily rationalized by IAA overaccumulation, such as leaf chlorosis (Figure 4a), are likely to be caused by thiohydroximate toxicity.

**UGT74B1 is expressed in a highly organized pattern comprising three domains**

We generated transgenic UGT74B1::GUS plants for histochemical analysis of tissue-specific UGT74B1 promoter activity during plant development. Multiple Arabidopsis lines transgenic for the GUS gene under control of the UGT74B1 promoter and entire intergenic region between At1g24100 and At1g24110 (total of 751 bp) failed to express the reporter for unknown reasons, possibly due to the presence of insulator sequences in the intergenic region. However, six independently lines transformed with the GUS gene under control of a 435-bp UGT74B1 promoter fragment showed qualitatively similar patterns of GUS expression, although strength of expression varied slightly from line to line (data not shown). GUS expression was evident in every organ and was organized in three overlapping domains: the vasculature, the apical meristems, and the junctions of organs or branches (Figure 7). Control plants transgenic for the promoterless GUS gene did not reveal any detectable reporter expression (data not shown).

Reporter activity is strongly associated with the plant vasculature in every organ examined, including the placenta and the funiculus (Figure 7a). However, GUS staining is not detectable in immature vascular tissue (Figure 7d, inset), and is quite weak in the vascular bundles of petals (Figure 7i). GUS staining in a cross section of the inflorescence stem appears to include some or all of the phloem (Figure 7c). A second domain exhibiting strong GUS expression includes the apical meristems of root, shoot, and inflorescence (Figure 7d,g), but excludes the emerging lateral roots; GUS expression does not begin in these meristems until the lateral roots are approximately 5 mm long (Figure 7e, inset). Finally, UGT74B1::GUS is highly expressed in all tissue junctions, including the transitions between different organs such as the shoot-root junction, the transition between the peduncle and floral organs, and the connective (Figure 7d,k,m), as well as branch points within organs (Figure 7b,e inset). At least some of the staining of the immature siliques (Figure 7o) may be interpreted as being associated with the junction of the two gynoecia. The apparently complex staining pattern associated with flower development (Figure 7g–o) may be interpreted in terms of the three expression domains described above, with two additional observations: (i) expression of UGT74B1::GUS in the elongating carpel is diffuse and not closely associated with the vasculature (Figure 7h), and (ii) staining intensity decreases as organs age and approach senescence (Figure 7, compare m and n). The remainder of the observed staining is vasculature-, meristem-, or junction-associated.

We independently confirmed expression of UGT74B1 in multiple organs by quantitative RT-PCR with total RNA prepared from different wild-type tissues. Relative to flowers, UGT74B1 transcripts were up to 19-fold more abundant in roots, stems, leaves, and siliques (Figure 7f).

**High IAA concentration activates UGT74B1 expression**

We used transgenic UGT74B1::GUS seedlings to test whether UGT74B1 is induced by exogenous application of PATH, IAA or methyl jasmonate (MeJA). A modest increase in UGT74B1 expression was previously reported for treatment of Arabidopsis plants with MeJA (Mikkelsen et al., 2003), which we confirmed for roots of 6-day-old seedlings (Figure 7p). Interestingly, treatment of seedlings with high concentrations of IAA (100 μM) caused strong UGT74B1::GUS expression in cotyledons but not in roots (Figure 7p). IAA at 10 μM and PATH at all concentrations tested did not further activate the UGT74B1 promoter relative to control seedlings (data not shown).

**Discussion**

**UGT74B1 functions in glucosinolate biosynthesis**

Several genes of the glucosinolate pathway remain to be identified or warrant functional analysis to establish their...
roles in planta (Mikkelsen et al., 2002; Wittstock and Halkier, 2002). Here, we have characterized Arabidopsis UGT74B1 and demonstrated its function in glucosinolate biosynthesis by complementary in vitro and in vivo studies. Partially purified S-GT enzyme preparations from several cruciferous species have been characterized (GrootWassink et al., 1994; Guo and Poulton, 1994; Jain et al., 1989; Matsuo and Underhill, 1971; Reed et al., 1993), and a cDNA has recently been cloned from B. napus (Marillia et al., 2001). However, only qualitative assays with PATH as substrate have been performed to authenticate the recombinant S-GT enzyme (Marillia et al., 2001).

Our biochemical and genetic characterization of Arabidopsis UGT74B1 is consistent with its function as an UDP-glucosethiohydroximate S-glucosyltransferase (EC 2.4.1.195) in glucosinolate biosynthesis, indicating that Arabidopsis UGT74B1 is orthologous to Brassica S-GT. Recombinant UGT74B1 converts a thiohydroximate substrate, PATH, into its corresponding desulfoglucosinolate. Our data demonstrate that UGT74B1 is specific for the thiohydroximate functional group and does not glucosylate the related carboxylate group, or a hydroxyl group, as tested for PAA, IAA, and SA (Figure 2). This result is in agreement with previous observations that post-aldoxime enzymes in glucosinolate biosynthesis are highly specific for the functional group but less selective for the aglycone side chain (Wittstock and Halkier, 2002). The low $K_m$ of purified recombinant UGT74B1 for PATH (6 μM) suggests that toxic thiohydroximates are indeed in vivo substrates of UGT74B1. A similarly low $K_m$ was previously reported for recombinant CYP83B1 (3 μM), which converts the reactive IAOx to S-alkylindole thiohydroximates in vivo (Bak et al., 2001). The $K_m$ for PATH of recombinant Arabidopsis UGT74B1 is about 10-fold lower than that reported for S-GT purified from B. napus. However, the characterized B. napus enzyme preparation contained several isoforms, and the lowest PATH concentration tested (50 μM) equaled the reported $K_m$ value (Reed et al., 1993). Unfortunately, the kinetic parameters and substrate specificity of recombinant S-GT from B. napus were not determined (Marillia et al., 2001).

The ugt74b1-1 mutant accumulates about 50% less 3-methylsulfinylpropyl and 3-methylindolyl glucosinolates, whereas the content of 4-methoxy-3-methylindolyl glucosinolate is not affected (Figure 5a). This suggests low specificity of UGT74B1 for the side chain of thiohydroximates and the possibility that additional glucosyltransferases play roles in glucosinolate biosynthesis. Low side chain specificity of UGT74B1, which remains to be tested with additional thiohydroximate substrates, would be in agreement with properties of the Brassica enzyme (Reed et al., 1993) and with the observation that Arabidopsis plants convert non-endogenous aldoximes into glucosinolates (Mikkelsen et al., 2002). Functional redundancy of enzymes in glucosinolate biosynthesis is not without precedence. Previous work has identified two pairs of closely related enzymes with overlapping substrate specificities: CYP79F1 and CYP79F2, which oxidize chain-elongated methionine derivatives (Chen et al., 2003), and CYP83A1 and CYP83B1, which oxidize alkyl and indolyl aldoximes, respectively (Bak and Feyereisen, 2001; Bak et al., 2001; Hansen et al., 2001b; Hemm et al., 2003; Naur et al., 2003b). The partially overlapping substrate specificities of CYP83A1 and CYP83B1 explain the incomplete reduction in indole glucosinolate accumulation in sur2 null mutants, which are blocked in the conversion of IAOx by CYP83B1 (Bak et al., 2001). On the contrary, the sur1 mutant does not accumulate any glucosinolate to detectable levels, indicating that the SUR1-encoded C-S lyase functions as a non-redundant enzyme in glucosinolate biosynthesis (Mikkelsen et al., 2004). Thus, the partial reduction in glucosinolate concentrations in ugt74b1 mutants may reflect functional redundancy of thiohydroximate glucosylation in glucosinolate synthesis or, alternatively, non-specific detoxification of reactive thiohydroximate intermediates by glucosylation. Conjugation of toxic thiol compounds by glucosylation is a common detoxification reaction and occurs in insects, plants, and mammals (Gessner and Acara, 1967; Kaslander, 1963; Kaslander et al., 1961). It is therefore conceivable that additional glucosyltransferases with broader substrate specificity exist in Arabidopsis that are capable of detoxifying thiohydroximate intermediates. In fact, UGT84B1 may be one such enzyme, considering its trace activity toward PATH (Figure 2a).

Levels of 4M glucosinolates are unaffected in lines ugt74b1-1 and ugt74b1-3 (Ws-0), but are reduced in

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**Figure 7.** UGT74B1::GUS expression during Arabidopsis development.

For one representative transgenic line are shown.

(a) Nearly mature silique stained for 1 h (inset: close-up of the same).
(b) Branch of inflorescence stem stained overnight (the subtending leaf was removed).
(c) Cross section of the stem shown in (b) from an area 1 cm below the branch point.
(d) Seedling (8-day old) stained overnight (inset: root tip of the same plant).
(e) Mature rosette leaf stained for 1 h (inset: emerging lateral root from mature plant).
(f) UGT74B1 mRNA expression levels in different tissues relative to flowers as determined by quantitative RT-PCR (see Experimental procedures). Mean ± SD are given ($n = 3$).

(g-o) Flowers from a single inflorescence stained overnight. The scale bars in (g) and (k) both represent 1 mm and apply to (g-j) and (k-o), respectively.

(p) UGT74B1::GUS expression in response to external methyl jasmonate (MeJA) (75 μM) and IAA (100 μM). Six-day-old seedlings were exposed to MeJA or IAA for 6 h and stained overnight.

(n)-Homomethionine  Tryptophan

\[ \begin{align*}
(\text{bus/sps}) & \xrightarrow{\text{CYP79F1}} \text{CYP79B1} \\
& \xrightarrow{\text{CYP79B2}} \text{CYP79B3} \\
\text{Alkyl aldoximes} & \xrightarrow{\text{IAOx}} \text{IAA} \\
\text{ref1} & \xrightarrow{\text{CYP83A1}} \text{S-Alkylthiohydroximates} \\
& \xrightarrow{\text{NIT1}, \text{NIT2}, \text{NIT3}} \text{IAN} \\
\text{sur1} & \xrightarrow{\text{C-S Lyase}} \text{Co, Cu, Fe Chelates} \\
& \xrightarrow{\text{UGT74B1}} \text{IAA} \\
& \xrightarrow{\text{S-Glucose}} \text{IAOx} \\
& \xrightarrow{\text{S-Glucose}} \text{IAOx} \\
& \xrightarrow{\text{S-Glucose}} \text{IAOx} \\
& \xrightarrow{\text{S-Glucose}} \text{IAOx}
\end{align*} \]

Figure 8. The glucosinolate pathway and its possible interactions with auxin metabolism.

Metabolic consequences of UGT74B1 gene inactivation include increased auxin production, possibly by accumulation of indole-3-acetaldoxime (IAOx), as shown for the sur2 mutant, or by spontaneous decomposition of indole-3-acetoxyhydroximate to indole-3-acetonitrile (IAN) and elemental sulfur. An ‘X’ indicates a point where gene inactivation leads to IAA accumulation. In addition, thiohydroximates are known to chelate essential metal ions. Auxin treatment induces SUR2 (Barlier et al., 2000) and UGT74B1 (this study).

ugt74b1-2 plants (Col). As 4IM is derived directly from IM (Elliott and Stowe, 1971), our results indicate that 4IM concentrations are actively regulated in the Ws-0 accession, perhaps by induction of side chain modification enzymes (Mikkelsen et al., 2003). Natural variation in genes controlling side chain modification may explain the observed difference between the ugt74b1 alleles in different genetic backgrounds (Kliebenstein et al., 2001b).

Transgenic Arabidopsis lines that constitutively overexpress UGT74B1 under control of the CaMV 35S promoter contain wild-type levels of all glucosinolates and are wild type in appearance (C.D. Grubb and S. Abel, unpublished data). These data are consistent with the observation that overexpression of SUR1, which generates thiohydroximates in glucosinolate biosynthesis, does not affect glucosinolate accumulation and overall plant morphology (Mikkelsen et al., 2004). SUR1 and UGT74B1 function downstream in the core pathway (Figure 8). Therefore, both enzymes are likely not rate-limiting under physiological conditions and, when overexpressed, may not enhance carbon flow toward glucosinolate production.

Finally, a biosynthetic role for UGT74B1 in glucosinolate metabolism is further supported by its expression pattern during plant development (Figure 7), which conspicuously overlaps with those reported for the glucosinolate biosynthetic genes CYP79F1 and CYP79F2 (Chen et al., 2003; Reintanz et al., 2001; Tantikanjana et al., 2001). Moreover, the observed UGT74B1 expression in vascular tissues likely includes the sulfur-rich S cells that accumulate high concentrations of glucosinolates in regions just abaxial to vascular bundles (Koroleva et al., 2000).

Taken together, our results provide strong evidence for a primary role of UGT74B1 in glucosinolate biosynthesis.

UGT74B1 participates in auxin homeostasis

Our data further indicate that UGT74B1 is one of several glucosinolate-related genes whose inactivation causes auxin-related phenotypes (Figure 8). Other such genes include BUS/SPS/CYP79F1 (Reintanz et al., 2001; Tantikanjana et al., 2001), SUR2/CYP83B1 (Bak et al., 2001), and SUR1 (Boerjan et al., 1995; Celenza et al., 1995; Mikkelsen et al., 2004). Interestingly, loss-of-function mutations in genes coding for post-aldoxime enzymes that act sequentially in the synthesis of indole glucosinolates, sur2, sur1, and ugt74b1, share similar high-auxin growth phenotypes, including elongated hypocotyls, epinastic cotyledons, or increased adventitious root formation, and are characterized by a reduction in indole glucosinolate synthesis and concomitant overaccumulation of IAA (Barlier et al., 2000; Boerjan et al., 1995; Celenza et al., 1995; Delarue et al., 1998; Mikkelsen et al., 2004). A diminished ability of ugt74b1 plants to metabolize thiohydroximates may lead to accumulation of endogenous C-S lyase substrates, as detected in sur1 plants (Mikkelsen et al., 2004), and accumulation of IAOx, as shown for sur2 lines (Barlier et al., 2000). IAOx is likely converted to IAA via indole-3-acetaldehyde (Bak et al., 2001; Helmlinger et al., 1987; Rajagopal and Larsen, 1972) or via indole-3-acetonitrile (Ludwig-Müller and Hilgenberg, 1990). While loss of the single SUR1 gene is lethal, the semi-sterile sur2 and ugt74b1 mutants are able to overcome the adverse effects of auxin deregulation and survive, suggesting that redundant cytochromes P450 and glucosyltransferases alleviate overaccumulation of IAOx in sur2 and ugt74b1 plants, respectively.

Activation of the UGT74B1 promoter by exogenous IAA (Figure 7p) is consistent with previous observations that indole glucosinolate biosynthesis plays an active role in auxin homeostasis (Bak et al., 2001; Barlier et al., 2000). In addition to overaccumulation of IAA in the sur2 mutant, Barlier et al. (2000) reported that exogenous auxin induces
SUR2/CYP83B1. This suggests that expression of SUR2/CYP83B1, UGT74B1 and possibly SUR1 is coordinately induced by IAA in a negative feedback loop regulating IAA biosynthesis.

Defective IAA conjugation by glucosylation may be the simplest explanation of the high auxin phenotype of ugt74b1 plants. However, UGT74B1 does not glucosylate IAA in vitro (Figure 2b), and the ugt74b1-1 line accumulates higher concentrations of both free and conjugated IAA than the wild type (Figure 5b). Therefore, it is unlikely that UGT74B1 functions directly in auxin metabolism. Likewise, we did not obtain evidence that aromatic and indolyl thiohydroximates directly contribute to the observed high-auxin phenotype by mimicking natural auxins such as PAA and IAA. This possibility is suggested by the functional similarity of the thiohydroximate and carboxylate group, and by structural requirements for auxin binding to Auxin-binding Protein 1, which include a planar aromatic ring-binding platform, a carboxylate binding site, and a hydrophobic transition region separating the two binding sites (Edgerton et al., 1994). However, it is possible that an untested alternate route to IAA contributes to auxin accumulation in ugt74b1 mutants (Figure 8). Thiohydroximates spontaneously decompose in proteic solutions to elemental sulfur and nitriles (Ettlinger and Lundeen, 1957). IATH accumulating in ugt74b1 plants would decompose to indole-3-acetonitrile, which can be converted to IAA by the action of nitrilases (Normanly et al., 1997).

Thiohydroximate toxicity

Among known glucosinolate-related mutations, chlorosis associated with leaf vascular strands appears to be unique for ugt74b1. Chlorosis has not been reported for sur1, cyp83b1, or cyp79f1 mutants, all of which display severe phenotypic defects (Bojer et al., 1995; Delarue et al., 1998; Reintanz et al., 2001; Tantikanjana et al., 2001). This phenotype is likely caused by thiohydroximate toxicity, for which several mechanistic explanations are possible. Thiohydroximates form stable tris- and bis-chelates with copper, cobalt, and iron (Mitchell et al., 1977; Walter and Schaumann, 1971). It is therefore conceivable that accumulation of thiohydroximates in ugt74b1 mutants interferes with the metabolism and biochemical functions of these essential metals.

Conclusion

Our biochemical and genetic studies provide compelling evidence that UGT74B1 is a glucosyltransferase in glucosinolate biosynthesis. However, loss of UGT74B1 gene function causes only partial reduction in glucosinolate accumulation, suggesting the presence of additional enzymes that glucosylate thiohydroximates. Such glucosyltransferases may (i) be specific for the thiohydroximate functional group but have overlapping specificities for the side chain, (ii) play more general roles in detoxification of reactive thiol compounds, or (iii) use a broader range of substrates with minor activities toward thiohydroximates. The Arabidopsis genome contains more than 100 genes predicted to encode Family 1 glucosyltransferases (Lim et al., 2003b). We are currently testing recombinant enzymes encoded by this large gene family to determine which members are active toward thiohydroximates. Reverse genetic studies will reveal if any of these genes code for bona fide glucosinololate biosynthetic enzymes.

Experimental procedures

Synthesis of phenylacetoxythiohydroxamic acid

PATH was synthesized according to Ettlinger and Lundeen (1957). The Grignard reagent and all other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Successful synthesis was confirmed by NMR and mass spectrometry.

Expression and purification of recombinant UGT polypeptides

An Arabidopsis cDNA corresponding to UGT74B1 (At1g24100) was identified and a CDNA clone (APD53c08 F 3' ) obtained from the Kazusa DNA Research Institute (Chiba, Japan), which was authenticated by DNA sequencing. Primers GTsense (5'-TGAGCTGCA-TGATGCGGAAACAACCCCTAAAGG-3') and GTanti (5'-GAATC-AAAAGCTTATCTTCTCTTATAAAC-3') were used to amplify the UGT74B1 ORF and to introduce Spel and HindIII restriction sites. The PCR product was subcloned into the corresponding restriction sites of expression vector pQE30 (Qiagen, Valencia, CA, USA). The resulting plasmid, pQGT18, supports expression of a fusion protein, comprising the complete sequence of UGT74B1 and an additional N-terminal, (His)6-tag containing sequence (MRGSHHHHHHSGAC). The insert of pQGT18 was verified by DNA sequencing. Digestion of pQGT18 with EcoRV and HindIII followed by fill-in and re-ligation resulted in pQGT18Ac which encodes a truncated protein in which the C-terminal 199 amino acids of UGT74B1 are replaced with a single alanine (UGT74B1Ac). Both plasmids were transformed into E. coli strain M15[pREP4] (Qiagen) for protein expression. Expression of UGT84B1 and UGT74F2 used the same strategy as for UGT74B1, with the modifications noted below. For UGT84B1, the primers were 84B1F (5'-GCATGCATGGGCAGATGGGCTA-3') and 84B1R (5'-CGCA-GTGGACGGATTGTGATAC-3'), which incorporate restriction sites Spel and PstI, respectively. The cDNA was amplified using a floral cDNA library as a template. For UGT74F2, the primers were 74F2F (5'-TGAGCTGCA-TGATGCGGAAACAACCCCTAAAGG-3') and 74F2R (5'-TGCTGCACTATTTGCTCTGAAACCTTG-3'), which incorporate restriction sites BamHI and PstI, respectively. The cDNA was amplified from a plasmid (clone no. 144H18) obtained from the Arabidopsis Biological Resource Center. The amplified fragment was cloned into vector pQE31 (Qiagen). Recombinant proteins were purified under denaturing conditions essentially according to Morgan et al. (1999). Purification of recombinant polypeptides under native conditions was based on protocols from the QAexpressionist kit (Qiagen) and from published reports (Jackson et al., 2001; Lim et al., 2002). Escherichia coli M15 cultures
were grown at 20°C in 80 ml 2x YT media containing 100 µg ml⁻¹ ampicillin and 25 µg ml⁻¹ kanamycin. Recombinant proteins were expressed at 20°C for 24 h by induction with the pellet and the resin was resuspended in 3 ml sonication buffer (50 mM Na₂HPO₄-NaH₂PO₄, 300 mM NaCl, 1 mg ml⁻¹ lysozyme), pH 7.8. After incubation on ice for 30 min, the suspension was sonicated (5 x 1 min) and cleared by centrifugation (20 min, 20,000 g), and the crude bacterial extract was incubated at 4°C with 100 µl N2-NTA agarose (Qiagen). The agarose beads were washed three times with cold sonication buffer, pH 6.3, and subsequently eluted with cold sonication buffer, pH 4.0.

Assay of glucosyltransferase transferase activity

For qualitative assays of glucosyltransferase activity, the reaction mix (200 µl) contained 32.5 mM MES-KOH, pH 6.0, 5 mM MgSO₄, 5 mM UDP-glucose, 0.065% (v/v) β-mercaptoethanol, 5 mM substrate (PATH, IAA, PAA, or SA), 5% (v/v) ethanol (the substrate solvent) and 40 µl of crude E. coli extract (2 µg µl⁻¹). After incubation for 2 h at 30°C, the reaction mix was heated for 10 min at 95°C and extracted three times with 300 µl of ethyl acetate. The combined ethyl acetate extracts were evaporated, and the residue dissolved in 150 µl of water for HPLC analysis.

For kinetic analyses, purified recombinant UGT74B1 was assayed in volumes of 100 µl of UDP-glucose concentration was varied (0.01-5 mM) in the presence of 100 µM PATH, or in volumes of 1 ml when PATH concentration was varied (2-14 µM) in the presence of 500 µM UDP-glucose. The reaction mix contained 50 mM MES-KOH, pH 6.0, 2.5 mM MgSO₄, 0.01% β-mercaptoethanol, 5% (v/v) ethanol, both substrates as indicated, and 190 ng of purified recombinant protein. Assays were performed under conditions of demonstrated linearity with respect to time and protein amount. After incubation at 25°C for up to 8 min, reactions were stopped by the addition of 10 µl of 24% (w/v) trifluoroacetic acid. Reactions were assayed by HPLC directly (for varied UDP-glucose) or were first evaporated and redissolved in 120 µl water prior to HPLC analysis (for varied PATH).

Two HPLC methods were used to separate glucosyltransferase reaction mixes. Method A (see Figure 1) is identical to the one employed for glucosinate analysis (Grubb et al., 2002). Desulfoenzymylglucosinolate was enzymatically prepared by desulphation of benzyglucosinolate (EMD Biosciences Inc., San Diego, CA, USA) as described by Grubb et al. (2002) and used as a standard for retention time to identify the product of UGT74B1-catalyzed glucosylation of PATH. Method B (see Figure 2) was developed by Lim et al. (2002) for glucosides of organic acids. Reactions were separated at 30°C on a Waters Spherisorb C₁₈ column (150 × 4.6 mm i.d., 3-µm particle size; Waters, Milford, MA, USA), using acetonitrile and water at a flow rate of 1.0 ml min⁻¹. Both eluants contained 0.1% trifluoroacetic acid. Compounds were eluted with a linear gradient from 10 to 65% acetonitrile over 20 min and detected spectrophotometrically at 226 and 296 nm. The identity of the reaction products, the glucosides of PATH, IAA, PAA, and SA, were verified by electrospray ionization-mass spectrometry analysis in negative ion mode at the UC Davis Molecular Structure Facility using an LCQ Classic mass spectrometer (Thermo Finnigan, San Jose, CA, USA).

Protein and DNA analyses

Protein concentration was determined with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s protocol, using bovine serum albumin as a standard. SDS-PAGE analysis was performed using 13.3% (w/v) acrylamide gels and standard protocols for electrophoresis and protein staining with Coomassie R-250 (Bollag et al., 1996). DNA transfer analysis was performed using standard procedures (Sambrook et al., 1989). The 712 bp KpnI-EcoRI fragment derived from plasmid pSK105 (Weigel et al., 2000), containing the promoter and 5’ end of mannopine synthase, was used to probe HindIII restricted genomic DNA (5 µg) prepared from wild type and ugt74b1 mutant plants. DNA sequencing was performed by the UC Davis Plant Genetics Facility.

Isolation of T-DNA insertion alleles of UGT74B1

The T-plant population of the University of Wisconsin T-DNA lines in the Ws-0 background (Krysan et al., 1999) was screened for T-DNA insertions in or near the UGT74B1 locus using primers GTKO5 (5’-GCAAAGCTTGAAACCGCGCATACATAGA-3’) and GTK3 (5’-GCTTATACATTGCGCCCTTTTGGATT-3’). Two insertion events were detected in superpools X20 and X23. Further rounds of screening identified the H and J pools, and finally individual plants carrying the T-DNA insertions. The two insertion alleles, named ugt74b1-1 and ugt74b1-3, were characterized at the molecular level by PCR, Southern analysis, and DNA sequencing in both directions using primers GTK5, GTK3 and JL202 (http://www.biotech.wisc.edu/Arabidopsis/index2.asp).

The Syngenta Arabidopsis Insertions Lines in the Columbia background were screened in silico by BLAST search of T-DNA flanking sequences (Sessions et al., 2002). One line (SAIL_12_F04, b1.Lb3Fa) was identified to carry a T-DNA insertion at the UGT74B1 locus. Seeds of this line were obtained, and the position of the T-DNA insert was confirmed by PCR and DNA sequencing using primers LB3 (5’-TAGCATCTGAATTTTACAAACATCTCGATAGACAC-3’) and B1F (5’-CGTTTGTATCCTGGCTTTA-3’). This insert line is referred to as ugt74b1-2.

Reverse transcriptase-mediated PCR

For semi-quantitative RT-PCR, total RNA was extracted from the indicated genotypes with the Qiagen RNeasy kit and converted to cDNA with the SuperScript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Oligomers B1F and B1R (5’-5AGACCTACCATTTTCAACTCATACATCT-3’) and TubF (5’-TTCCTGATTTGTCTGGAAGACG-3’) and TubR (5’-AGCTTCTGAGATGCTGAGTATTG-3’) were used to amplify UGT74B1 and TUBULIN cDNA templates, respectively. After 45 min at 48°C to allow reverse transcription, the reaction was heated to 94°C for 2 min, and then cycled 40 times through the program: 92°C for 30 sec, 60°C for 1 min, 88°C for 1 min.

For quantitative RT-PCR, mRNA was extracted with the TRizol reagent (Invitrogen) from the indicated tissues of wild-type plants during independent experiments. RNA was purified and treated with RNase-free DNase using the RNeasy kit (Qiagen). First-strand cDNA was prepared using the Taqman RT-PCR Reagents kit (Applied Biosystems, Foster City, CA, USA). SYBR Green PCR Master Mix was used for UGT74B1 and TUBULIN cDNA detection using the following primers of comparable amplification efficiency designed with Primer Express software (Applied Biosystems): q74B1F (5’-TTTTGTCTTCTGTTGTGGATATTTCC-3’), q74B1R (5’-GGCCACCTAACAACCAATGG-3’) and qTub (5’-GACCTTTGAGTCTGGAAGATGCTGGA-3’). Duplicate SYBR green reactions were run for each cDNA preparation using the ABI 7900 and the following cycling parameters: 95°C (1 min), 60°C (1 min), 72°C (30 s), 40 cycles. Melting curve analysis (95–60°C at 0.5°C sec⁻¹) was used at the end of each run to verify product specificity. DNA contamination and primer-dimer formation were controlled for by replicate reactions without reverse transcription.
transcriptase and without template, respectively. The results were analyzed with SDS software (version 2.1) and default settings were used for baseline and threshold values (Applied Biosystems). Cycle threshold (Ct) values correspond to the cycle number at which the fluorescence resulting from enrichment of the PCR product reaches significant levels above the background fluorescence (threshold). Transcript absence is indicated by Ct > 40 (Livak and Schmittgen, 2001). In this analysis, transcript abundance for UGT74B1 was normalized by subtracting the Ct value of the housekeeping gene (TUBULIN2) from the Ct value of the target gene (UGT74B1), which generates a ΔCt value (CtUGT74B1–CtTUBULIN2) for each tissue. As flowers had the lowest UGT74B1 mRNA abundance (ΔCt = 6.63 ± 0.08), UGT74B1 mRNA expression levels in different plant organs were normalized to those in flowers by subtracting the ΔCt value of flowers from the ΔCt value of each tissue, and then expressed as fold difference, calculated as 2^{-ΔΔCt (Organ–ΔΔCt (Flower))} (Applied Biosystems User Bulletin No. 2, ABI Prism 7700 Sequence Detection System).

Plant growth

For plant growth under sterile conditions, seeds were surface sterilized and placed on solid medium containing 8 g l^{-1} agar, 5 g l^{-1} sucrose and 2.15 g l^{-1} (0.5%) Murashige-Skoog salts, pH 5.6 (Murashige and Skoog, 1962). After 1 day at 4°C, plants were subjected to 30 min of bright fluorescent light at room temperature and then returned to 4°C for an additional 24 h. Plants were then moved to a controlled growth room and grown at 22°C under illumination (60 μm m^{-2} sec^{-1}) with fluorescent light for 16 h daily. Soil grown plants were planted in Pro-Mix ‘BX’ (Premier Horticulture, Red Hill, PA, USA) at 25°C and 16 h of light, with two to three waterings per week. Water was supplemented with macronutrients (7.5 mM N, 1.7 mM P, 3 mM K, 3 mM Ca, and 1.2 mM Mg).

HPLC analysis of desulfoglucosinolates

Analysis of desulfoglucosinolates by HPLC, both for plant-derived samples and for in vitro assays of glucosyltransferase activity (dsBGS formation), was performed as previously described (Grubb et al., 2002).

GC-MS analysis of free and total IAA

Freeze-dried Arabidopsis seedlings (at least 0.1 g FW per analysis) were extracted with isopropanol/acetic acid (95:5, v/v), and three independent extractions were performed for each line. For the analysis of free and total IAA, each sample received 100 and 500 ng 13C6-IAA (Cambridge Isotope Laboratories, Andover, MS, USA), respectively, and was incubated with continuous shaking (500 rpm) for 2 h at 4°C. After centrifugation for 10 min at 10,000 × g, the supernatant was removed and evaporated to dryness under a stream of N\textsubscript{2}. For free IAA determination the residue was dissolved in methanol. After centrifugation (10 min, 10,000 × g), the supernatant was transferred to a glass vial, evaporated under a stream of N\textsubscript{2} and the residue was dissolved in 50 μl ethyl acetate. Methylation was carried out according to Cohen (1984) with diazomethane, and the sample dissolved in ethyl acetate for GC-MS analysis. Hydrolysis of conjugated IAA was performed with 5 M NaOH at 100°C under N\textsubscript{2} for 3 h. The hydrolysate was filtered and the pH adjusted to 2.5. Auxins were extracted twice with equal volumes of ethyl acetate. The organic phase was evaporated and the extract methylated as described above.

GC-MS analysis was carried out on a Varian Saturn 2100 ion-trap mass spectrometer using electron impact ionization at 70 eV, which was connected to a Varian CP-3900 gas chromatograph equipped with a CP-8400 autosampler (Varian, Walnut Creek, CA, USA). For sample analysis, 2.5 μl of the methylated sample dissolved in 20 μl ethyl acetate was injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex ZB-5 column, 30 m × 0.25 mm × 0.25 μm using He carrier gas at 1 ml min^{-1}. Injection temperature was 250°C, and the temperature program was 70°C for 1 min, followed by an increase of 20°C min^{-1} to 280°C and 5 min at 280°C. The methyl ester of IAA eluted at 11.5 min under these conditions. The settings of the mass spectrometer were as described (Campanella et al., 2003). For the determination of free and total IAA, the molecular and quinolinium ions of the methylated substance at m/z 189/195 and 130/136 (ions deriving from endogenous 13C6-IAA), respectively, were monitored in the mSIS mode (Varian Manual). Calculations were made on the basis of the quinolinium ions using the principles of isotope dilution (Ilic et al., 1996).

Histochemical analyses

Histochemical analysis of the GUS reporter enzyme activity was adapted from published methods (Jefferson et al., 1987). Sample tissues were fixed in ice-cold 80% acetone (2 × 5 min), vacuum-infiltrated with an aqueous solution consisting of 50 mM NaH\textsubscript{2}PO\textsubscript{4}, 100 mM K\textsubscript{2}Fe(CN)\textsubscript{6}, 0.5 mM K\textsubscript{3}Fe(CN)\textsubscript{6}, and 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid and incubated overnight at 37°C. Tissues were cleared overnight in 70% ethanol and imaged with a stereomicroscope (Zeiss Stemi SV11; Zeiss, Jena, Germany) equipped with a Cool Snap high-performance CCD camera.

For cell viability analysis, seedlings were first incubated for 20 min in a solution of FDA (50 ng ml^{-1}), briefly rinsed in water, and then incubated for 5 min in PI (66 ng ml^{-1}). A Leica (Wetzlar, Germany) TCS 4D confocal laser microscope was used for imaging at wavelengths specific for each stain.

Construction of GUS reporter and UGT74B1 complementation lines

The binary vector pBI121 was used for generation of GUS reporter constructs using the promoter region of UGT74B1 (position -444 to position -9, relative to the translational start codon) to drive uidA gene expression in transgenic Arabidopsis. Primers GTGUS3 (5’-GGATCCCTCAACGAAGCAGAGCTTTG-3’) and GTGUSshort (5’-AAGCTTGGAGGTTGACTTTCCAATCTTCTAT-3’) were used for amplification of the UGT74B1 promoter region. The UGT74B1 promoter fragment was cloned into vector pBI121 restricted with BamHI and HindIII, replacing the cauliflower mosaic virus 35S promoter. The authenticity of the cloned UGT74B1 promoter fragment was verified by DNA sequencing. The resulting pBI121 derivative was mobilized into Agrobacterium tumefaciens strain GV3101. Transformation of Arabidopsis (ecotype Columbia) was accomplished by spraying flowering plants with an Agrobacterium suspension in 5% sucrose and 0.05% Silwet L-77 (Lehle Seed, Round Rock, TX, USA).

For the generation of complementation lines, we amplified a 2999-bp sequence from Wo-0 genomic DNA using primers CompF (5’-TATTAAGCTTCTCTTATGTTGCGCTG-3’) and CompR (5’-CAAGGCTACGCTTTAGTGGTTTCTGAA-3’) and cloned this fragment into vector pCB302 (Xiang et al., 1999) via

restriction sites HindIII and KpnI. The resulting plasmid should generate a T-DNA (T-UGT74B1) including wild-type UGT74B1 with 5'- and 3'-regulatory sequences, as well as the BAR gene conferring Basta resistance. We used GV3101 to transform a population segregating for the ugt74b1-1 allele. T2 progeny of Basta-resistant T1 individuals were screened on kanamycin to identify homozygous ugt74b1-1 lines. One such line was isolated, which segregated Basta resistance in a 3:1 ratio. All plants with the mutant morphological phenotypes were Basta-sensitive, while the dwarfed phenotype was never observed in Basta-resistant plants (n = 35), indicating that T-UGT74B1 complements the mutation.

Statistical analysis

All claims of statistical significance are based on a one-tailed Student’s t-test, using a 0.05 level of significance as the cutoff.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2261/TPJ2261sm.htm.

Figure S1. Expression and purification of recombinant UGT74B1 and truncated UGT74B1C. (a) SDS-PAGE analysis of recombinant UGT74B1 and truncated UGT74B1C. Separations of total proteins from uninduced (U) and induced (I) Escherichia coli cells, and of proteins purified under denaturing conditions (P) are shown. (b) SDS-PAGE analysis of recombinant UGT74B1 purified under native conditions.

Figure S2. Mass spectra of the phenylacetothiohydroximic acid substrate (PATH) and the dsBGS product of the UGT74B1-catalyzed glucosylation reaction. (a) Mass spectrum of the PATH substrate, [M-H]- (m/z 166), [2M-H]- (m/z 332), [M-H]Na+ (m/z 354). (b) Mass spectrum of the dsBGS product peak of a UGT74B1-catalyzed reaction. The collected product peak gives rise to dsBGS (m/z 328), thioglucose (m/z 195), and PATH (m/z 166).

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