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Selection of plants for roles in phytoremediation: the importance of glucosylation

Melissa Brazier-Hicks, Lesley A. Edwards and Robert Edwards*

Centre for Bioactive Chemistry, Durham University, Durham DH1 3LE, UK

Summary

Over-expression and transposon mutagenesis in root cultures of Arabidopsis thaliana demonstrated the importance of the family 1 glycosyltransferase UGT72B1 in catalysing the \( N \)-glucosylation of the persistent pollutant 3,4-dichloroaniline (DCA). In phytotoxicity studies with DCA in seedlings, over-expression of UGT72B1 enhanced sensitivity, whereas the knockouts were more resistant than the controls. In contrast, manipulating the expression of UGT72B1 had no effect on the \( O \)-glucosylation, or toxicity, of chlorophenols. When \( N \)-glucosylation was disrupted in plants, radioactivity derived from \(^{14}\text{C}\)-DCA became covalently bound into high molecular weight insoluble material, principally associated with the lignin fraction. This suggested that insolubilization into stable cell wall components represented a more effective mechanism of DCA detoxification than the formation of \( N \)-glycosidic conjugates. A screen of plants used in remediation, identified low levels of \( N \)-glucosyltransferase activity in switchgrass and high activities in reed canary grass. When incubated with \(^{14}\text{C}\)-DCA, reed canary grass plants accumulated soluble \( N \)-glycosides of DCA, whereas switchgrass formed insoluble residues. Consistent with the results obtained in studies with Arabidopsis, phytotoxicity trials with DCA demonstrated that switchgrass was more tolerant than reed canary grass. Our studies provide a new biochemical basis for selecting plants for useful remediating traits towards specific classes of pollutants.

Introduction

The use of plants to phytoremediate environments contaminated with organic pollutants has attracted considerable interest as representing a low-cost and sustainable solution to environmental improvement, as compared with costly intervention using chemical or engineering approaches (Meagher, 2000). As part of a long-term programme we have been interested in identifying useful proteins in plants which confer tolerance to synthetic organic compounds, including pollutants, which could then be harnessed in biotechnological applications such as bioremediation. One group of proteins that can be readily identified as being potentially useful biocatalysts for detoxifying xenobiotics are the family 1 glycosyltransferases (UGT). These enzymes catalyse the \( O \)-, \( S \)- and \( N \)-glyco-conjugation of a diverse range of synthetic compound acceptors using NDP-activated sugar donors (Schröder et al., 2001). As such, they are a very important group of bioconjugating, or phase 2, enzymes that are involved in the detoxification of multiple pollutants and pesticide metabolites in plants (Cole and Edwards, 2000).

In recent studies we identified a UGT in Arabidopsis thaliana, termed UGT72B1, which catalysed the conjugation of both 3,4-dichloroaniline (DCA) and 2,4,5-trichlorophenol (TCP) with glucose (Loutre et al., 2003). Both DCA and TCP are persistent pollutants which are widespread in environments exposed to effluents from chemical industries, or heavy pesticide usage. In the European Union both compounds are designated priority pollutants (Harvey et al., 2002). When UGT72B1 was selectively disrupted by transposon mutagenesis, the resulting ugt72B1 knockout Arabidopsis plants were impaired in their ability to glucosylate DCA, but were unaffected in their conjugation of TCP (Brazier-Hicks and Edwards, 2005). Unexpectedly, ugt72B1 seedlings showed an increased tolerance to DCA as compared with wild-types, prompting us to further investigate the role of UGT72B1 in...
xenobiotic detoxification \textit{in planta}, by generating and testing the respective over-expressors (UGT72B1-OEs). Using the results obtained from metabolism and toxicity studies in these \textit{Arabidopsis} lines, we have then used this biochemical information to select for plant species able to tolerate and remediate DCA.

Results

Effect of manipulating the expression of UGT72B1 on the metabolism of xenobiotics in \textit{Arabidopsis}

To investigate the effects of over-expression of UGT72B1 on the metabolism of xenobiotics, a UGT72B1-pCAMBIA 3300 construct was created and transformed into \textit{Arabidopsis} (Dixon \textit{et al}., 2003). Seed from homozygous T3 lines were used to create root cultures for metabolism and protein expression studies. Root cultures were used, as UGT72B1 expression could be accurately monitored by Western blotting without interference from comigrating Rubisco protein (Brazier-Hicks and Edwards, 2005). In addition, the use of root cultures facilitated the quantitative monitoring of the distribution of the radioactivity between the plant and root medium and helped reduce the large-scale loss of volatile radioactivity seen with $^{14}$C-DCA in whole plant studies (Brazier-Hicks and Edwards, 2005). Levels of immunoreactive polypeptides in the T3 UGT72B1-pCAMBIA transformant lines were determined by immunoblotting using a specific anti-UGT72B1-serum (Brazier-Hicks and Edwards, 2005). Two lines showing enhanced expression of UGT72B1 were identified, with one line selected for further characterization and designated UGT72B1-OE. Enhanced levels of the UGT72B1 polypeptide were determined in UGT72B1-OE roots as compared to the wild-type (Figure 1a). However, as determined by Western blotting, the level of immunoreactive protein in the ugt72b1 roots was below the limit of detection (2 ng) as determined with recombinant UGT72B1. The extracts were assayed for O-glucosyltransferase (OGT) conjugating activity towards chlorophenols and N-glucosyltransferase (NGT) activity towards chloroaniline substrates using UDP-$^{14}$C-glucose as the sugar donor (Table 1). As compared with control plants, knocking out the expression of UGT72B1 reduced extractable enzyme activity towards DCA (101-fold) and 2,4,5-trichloroaniline (11-fold), while with the chlorophenols, 27% and 63% of OGT activity was retained with the substrates TCP and triclosan, respectively (Table 1). Over-expression of UGT72B1 gave a four- to sevenfold enhancement in NGT activity and a two- to fourfold increase in OGT, depending on the substrate assayed.

To determine the effect of perturbing UGT72B1 expression on the conjugation of chlorinated xenobiotics \textit{in planta}, radiolabelled DCA and TCP were individually fed to wild-type, UGT72B1-OE and ugt72b1 root cultures. As described previously, knocking out the expression of UGT72B1 had no significant effect on uptake of the radioactivity following feeding with $^{14}$C-TCP (Brazier-Hicks and Edwards, 2005). Thus, over an 8-h incubation, 54.3% ± 6.9% (mean ± variation, $n = 2$) of the recovered radioactivity was present in the roots of the wild-types, as compared with a figure of 59.5% ± 8.3% in the knockouts. Similarly increasing the expression of the enzyme had no detectable effect on the quantitative partitioning of the radioactivity, with 59.2% ± 2.3% recovered in the roots. In each of these studies with $^{14}$C-TCP the overall recoveries of radioactivity were similar, being in the range...
70.2 ± 6.6% of the applied dose. Similar losses in recovered radioactivity were observed in earlier metabolism studies (Brazier-Hicks and Edwards, 2005), although it seems unlikely that these are due to direct volatilization of TCP (boiling point, 253 °C). Quantification of the radioactive metabolites by thin layer chromatography (TLC) showed that the O-glucoside of $[^{14}C]$-TCP was the major extractable metabolite in both the roots and the medium of all three lines representing just under 50% (49.1% ± 5.6%) of the total recovered radioactivity in each case after an 8-h incubation. It therefore appeared that Arabidopsis OGTs, other than UGT72B1, with activity towards TCP masked the effect of varying UGT72B1 expression on the rates of O-conjugation of this chlorophenol in the root cultures. Thus, several Arabidopsis UGTs other than UGT72B1 are known to actively glucosylate TCP (Messner et al., 2003).

With $[^{14}C]$-DCA, the effect of knocking-out UGT72B1 was to reduce the initial rate of uptake of the xenobiotic from the media into the roots, suppress the levels of soluble radioactivity accumulating in the plant tissues and greatly enhance the formation of non-extractable insoluble radioactive residues (Figure 2). Surprisingly, over-expression of UGT72B1 had a negligible effect on the overall distribution of soluble radioactivity accumulating in the root tissues and in the media, although as compared with wild-type cultures, a reduction in insoluble residue formation was determined at 24 h (Figure 2). Total recoveries of radioactivity over the period of the experiment were 67.4% ± 3.7% (mean ± variation, n = 2) of the applied dose in the wild-type cultures, 64.3% ± 2.1% for the over-expressors, and 54.2% ± 2.7% for the knockouts. Figures for recoveries were similar to those determined in earlier studies and were due to the volatilization of $[^{14}C]$-DCA from the media, with this being greater in the ugt72b1 cultures (Brazier-Hicks and Edwards, 2005). To monitor the effect of varying UGT72B1 expression on the formation of specific DCA metabolites, the methanol-extractable radioactive residues in the roots and medium from knockout, wild-type and over-expressing lines were analysed by TLC and autoradiography (Figure 1b). The major radioactive metabolites in both the medium and the roots were parent DCA and DCA-N-glucoside, respectively, with the identity of these metabolites confirmed by HPLC-MS (Loutre et al., 2003). The more minor polar metabolites were additional uncharacterized N-glycosidic conjugates (Loutre et al., 2003). The results demonstrated that while the ugt72B1 plants were impaired in their ability to conjugate DCA, over-expression of UGT72B1 had a negligible effect on N-glucosylation of the xenobiotic.

The metabolism studies demonstrated that UGT72B1 played an important role in determining whether or not radioactivity

| Substrates | GT activity toward xenobiotics (pmol/min/mg protein) |
|------------|-----------------------------------------------|
|            | Wild-type | UGT72B1-OE | ugt72B1          |
| NGT        |           |           |                  |
| 3,4-Dichloroaniline (DCA) | 101.1 ± 14.9 | 459.8 ± 27.7 | 1.0 ± 0.2 |
| 3,4,5-Trichloroaniline | 3.3 ± 0.0 | 22.4 ± 2.8 | 0.3 ± 0.0 |
| OGT        |           |           |                  |
| 2,4,5-Trichlorophenol (TCP) | 32.8 ± 6.2 | 129.7 ± 0.8 | 9.1 ± 1.3 |
| Triclosan   | 14.7 ± 0.0 | 34.1 ± 5.9 | 9.4 ± 1.0 |

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derived from $^{14}$C-DCA became incorporated into the insoluble, or ‘bound’ residues. To investigate the nature of the bound residues, wild-type and knockout cultures were treated with $^{14}$C-DCA for 24 h to promote incorporation. Following solvent extraction, the insoluble residue was then sequentially extracted with chemical and enzymic treatments to fractionate the insoluble radioactive residues as being associated with definable bio-macromolecules (Langbartels and Harms, 1985). As compared with the wild-types, the ugt72b1 plants contained higher levels of solubilized radioactivity in all of the fractions derived from the methanol-insoluble residue (Table 2). In both sets of samples, the single greatest recovery of radioactivity was obtained following extraction with dioxane-based solvents, corresponding to the selective solubilization of the lignin fraction (Langbartels and Harms, 1985). In the knockout plants, approximately 25% of the total radioactivity extracted from the plant material was present in the lignin fraction, as compared with a figure of around 10% in the controls.

**Role of UGT72B1 in determining the toxicity of chlorinated anilines and phenols to Arabidopsis plants**

The metabolism and enzyme assays all pointed to an important role for UGT72B1 in regulating the metabolism of chloroanilines. To determine the effect of manipulating UGT72B1 expression on the phytoxicity of DCA, seedlings from the wild-type, ugt72b1 and UGT72B1-OE lines of Arabidopsis were germinated on agar containing increasing concentrations of the pollutant (Figure 3). As reported previously (Loutre et al., 2003), the ugt72b1 seedlings were found to tolerate concentrations of DCA greater than 25 μM much better than the wild-type. In contrast, the over-expressors showing an enhanced sensitivity to DCA at concentrations above 10 μM. In subsequent studies, the toxicity trials were extended to include 2,4-dichloroaniline, 3,4,5-trichloroaniline, 2,4,5-trichlorophenol, 2,3,4-trichlorophenol, 2,3,6-trichlorophenol and triclosan (Figure S1). With the two chloroanilines, the results mirrored those observed with DCA, with reduced expression...
Role of glucosylation in phytoremediation

of UGT72B1 reducing toxicity and increased expression enhancing sensitivity. With the chlorophenols, no significant effect on tolerance was determined after varying UGT72B1 expression through either knockout or over-expression (Figure S1).

Screening plants for useful traits in DCA phytoremediation based on relative NGT activity

Our results with Arabidopsis demonstrated that the titres of NGT conjugating activity towards DCA determined tolerance towards the chloroaniline. Since NGTs active in DCA conjugation are not unique to Arabidopsis (Pflugmacher and Sandermann, 1998), plants with potential utility in phytoremediation were screened for NGT activity. The first plants selected were Brassica napus, Brassica juncea and Brassica nigra, which have proven useful in remediation studies with inorganic and organic pollutants in both laboratory and field studies (Marchiol et al., 2004). Conveniently, these Brassica species are also closely related to Arabidopsis. In addition, switchgrass (SWG) and reed canary grass (RCG) were selected as two fast-growing grasses with similar growth habits, which are finding increasing applications both in remediation and as biofuel crops. For example, RCG and SWG have been used in field trials to remediate explosives and polyaromatic hydrocarbons, respectively (reviewed by Singh and Jain, 2003). The three Brassica species all contained very low NGT activity towards DCA, with conjugating activity towards TCP was determined in the range 5.6–10.3 pmol/min/mg protein (Table 3), which was similar to that determined for Arabidopsis plants (8.5 pmol/min/mg). Since the Brassica species had such similar GT activities to one another, they were unsuitable for comparing the role of metabolism in determining the toxicity of DCA, or indeed TCP. Also because the size of the plants was so different from Arabidopsis, comparative metabolism and phytotoxicity studies between the Brassica and the model species would have been of little quantitative value. Instead, while the two cultivars of RCG showed high NGT activity, both SWG lines showed very low activities towards DCA (Table 3). All of the grasses had similar OGT activities towards TCP. Thus, these two grass species offered a better system to compare the roles of NGTs in determining the routes and rates of metabolism and subsequent sensitivity to chloroanilines.

RCG and SWG were incubated with [14C]-DCA for 24 h and the methanolic extracts then analysed by TLC (Figure S2). Both the Palaton and Bamse cultivars of RCG formed DCA-N-glucoside as a major metabolite. Two more polar metabolites were also observed, which based

Table 3 Glucosyltransferase (GT) activities towards xenobiotics in crude protein extracts from the foliage of a range of plant species. Values are the means ± SD (n = 3).

| Plant species (cultivar) | 3,4-Dichloroaniline | 2,4,5-Trichlorophenol |
|-------------------------|---------------------|-----------------------|
| Arabidopsis             | 14.9 ± 0.8          | 8.5 ± 0.7             |
| Brassica napus          | 0.2 ± 0.0           | 5.6 ± 0.8             |
| Brassica juncea         | 0.5 ± 0.0           | 8.3 ± 0.4             |
| Brassica nigra          | 1.0 ± 0.4           | 10.3 ± 0.5            |
| Reed canary grass ‘Bamse’ | 6.1 ± 0.7       | 2.1 ± 0.2             |
| Reed canary grass ‘Palaton’ | 6.3 ± 0.2         | 1.8 ± 0.0             |
| Switchgrass ‘Shelter’   | 0.2 ± 0.0           | 1.3 ± 0.0             |
| Switchgrass ‘Trailblazer’ | 0.5 ± 0.4         | 2.3 ± 0.4             |

Figure 3 Phytotoxicity of DCA in Arabidopsis seedlings showing modified expression of UGT72B1. Seeds of wild-type, knock-out (ugt72b1) and over-expressing (UGT72B1-OE) lines were germinated on agar containing 0–100 µM DCA. Scoring data and additional phytotoxicity trials with other xenobiotics are given in the supplementary data.

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on their co-chromatography with DCA metabolites isolated from Arabidopsis, were uncharacterized N-glycosidic conjugates (Loutre et al., 2003). In the two SWG lines, DCA-N-glucoside and one of the polar N-glycosides was also observed at lower abundance, with the major extractable metabolite residing near the origin. The radioactivity in the extractable fraction from the RCG plants but not in SWG, which was surprising based on the lower NGT activities in the early stages of polymerization, with reactions with phenoxy radicals leading to the DCA substituting at the benzylic α-position of the lignin side chains (Lange et al., 1998), as shown in Figure 4. In Arabidopsis, where N-glucosylation is the major route of DCA metabolism (Lao et al., 2003), we have identified competing pathways of sugar conjugation and bound residue formation. Thus, when NGT activity is suppressed in planta, DCA is directed into the lignin fraction.

Discussion

Chloroanilines such as DCA are common environmental pollutants and pesticide metabolites and are known to undergo three major alternative routes of metabolism in higher plants, namely, N-glucosylation, N-malonylation or bound residue formation (Schmidt et al., 1994). A schematic diagram showing these three reactions is shown in Figure 4. In the case of the insoluble plant residues, DCA is known to be incorporated into several plant macromolecules, predominantly lignin (reviewed by Sandermann, 2004). As observed in our fractionation studies, incorporation of chloroanilines occurs in the early stages of polymerization, with reactions with phenoxy radicals leading to the DCA substituting at the benzylic α-position of the lignin side chains (Lange et al., 1998), as shown in Figure 4. In Arabidopsis, where N-glucosylation is the major route of DCA metabolism (Lao et al., 2003), we have identified competing pathways of sugar conjugation and bound residue formation. Thus, when NGT activity is suppressed in planta, DCA is directed into the lignin fraction. This competition model for DCA also seems to operate in the grasses RCG and SWG. In the RCG plants, which contained high levels of NGT activity, DCA was metabolized to the N-glucoside, whereas in SWG, which had low levels of this conjugating activity, bound residues predominated. Insoluble residue formation has also been reported in plants that

### Table 4

| Radioactivity derived from [14C]-DCA (nmol) | Reed canary grass | Switchgrass |
|------------------------------------------|------------------|--------------|
|                                          | Palaton          | Bamse        | Trailblazer | Shelter |
| Medium                                   | 24.7 ± 1.8       | 24.6 ± 0.9   | 24.7 ± 1.0 | 23.6 ± 1.5 |
| Plant extract                            | 4.6 ± 1.1        | 3.9 ± 0.0    | 1.7 ± 0.2  | 2.4 ± 0.9  |
| Plant non-extract                        | 0.3 ± 0.1        | 0.2 ± 0.0    | 3.1 ± 0.2  | 3.9 ± 0.4  |
| Total                                    | 29.6             | 28.7         | 29.5       | 29.9       |

### Table 5

| Phytoxicity study (plant height in cm) | Reed canary grass | Switchgrass |
|----------------------------------------|------------------|--------------|
|                                       | Palaton          | Bamse        | Trailblazer | Shelter |
| Control                                | 26.5 ± 2.0       | 30.8 ± 2.6   | 24.5 ± 0.2 | 24.5 ± 1.3 |
| 10 µM DCA                              | 29.3 ± 2.1*      | 30.0 ± 5.5*  | 26.2 ± 2.8*| 25.0 ± 1.7*|
| 50 µM DCA                              | 20.5 ± 2.1†      | 18.7 ± 2.1†  | 21.1 ± 1.7†| 22.0 ± 1.9†|
| 100 µM DCA                             | 16.3 ± 3.5†      | 13.2 ± 1.4†  | 20.7 ± 2.0†| 19.0 ± 0.6†|

*no significant difference (P > 0.1).
†P < 0.01; ‡P < 0.001 in comparison with control values in each case.
use a combination of $N$-glucosylation and $N$-malonylation to metabolize DCA, such as soybean, wheat and carrot (Bockers et al., 1994; Schmidt et al., 1994). As shown in Figure 4, in such plants $N$-malonylation will also compete for DCA with the $N$-glucosylation and bound residue pathways (Winkler and Sandermann, 1989; Gareis et al., 1992). Conjugation by $N$-glucosylation and $N$-malonylation both direct DCA for extracellular deposition (Gareis et al., 1992; Lao et al., 2003). Once exported, these conjugates are only poorly reabsorbed (Lao et al., 2003). However, although the DCA-$N$-malonate is described as being stable to chemical and microbial hydrolysis (Winkler and Sandermann, 1989), this is not the case with DCA-$N$-glucoside (Winkler and Sandermann, 1992). Thus, following its export into the rhizosphere, the glucoside will undergo chemical and microbial hydrolysis to release DCA, which will then be re-imported into the plant via the roots (Figure 4). In plants that predominantly metabolize DCA by $N$-glucosylation, this cyclical re-absorption of the pollutant means that its incorporation into the cell wall is the most effective mechanism of sequestration. Once bound, the residues have the potential to be completely removed from the environment if the plant is harvested, or will return to the soil as partly degraded lignin derivatives once the dead tissue is acted on by microbes (Harvey et al., 2002; Sandermann, 2004). In the current report, based on the assumption that all plants have the capacity to peroxidatively cross-link DCA into cell wall components, we have only considered low glucosylating activities as a metabolic trait likely to predispose plants to hyperaccumulate bound residues. This is likely to be a reasonable model based on the widespread occurrence of NGT activity towards DCA in higher plants (Pflugmacher and Sandermann, 1998). However, a further useful refinement for a predictive screen for bound residue formation in different species would be the determination of the less widely reported DCA-$N$-malonyltransferase activity (Lao et al., 2003).

By disrupting the expression of UGT72B1 in Arabidopsis, our studies have demonstrated the practical importance of the competition between soluble and insoluble pathways of DCA detoxification in determining tolerance to this pollutant. We have then shown that the inverse relationship between the rates of $N$-glucosylation and phytotoxicity of DCA also appear to operate in two grass species. While additional factors may also regulate the toxicity of DCA in the grasses, the feeding studies demonstrated that the chloroaniline was taken up with similar efficiency in all the cultivars of RCG and SWG tested, demonstrating that differences in tolerance were not due to variations in the bioavailability of the pollutant. Although we cannot demonstrate that the grasses fundamentally differ in their sensitivity to DCA, our results are consistent with SWG being more tolerant than RCG to DCA because of its greater capacity to incorporate the pollutant into a stable insoluble residue.

The results of our study are important for plant biotechnology for two reasons. First, they provide a biochemical basis for deploying plants in specific applications for the removal of specific chloroaniline pollutants from the environment. While DCA is only classified as a priority pollutant in the European Union, related chloroanilines are commonly determined pollutants worldwide, being degradation products of pesticides and intermediates in the synthesis of azo dyes. As long-lived contaminants, they are particularly toxic to aquatic organisms and the utility of plants expressing low levels of NGTs to scavenge these compounds from the environment to form stable bound residues has a number of attractions (Harvey et al., 2002). Since many pollutants have the potential to undergo metabolism to both soluble and bound residues (Sandermann, 2004), a greater knowledge of how these pathways compete for detoxification in planta could be extremely useful in selecting plants for the remediation of specific pollutants, especially if these traits can be harnessed in emerging bioenergy crops. In addition to providing new tools for phytoremediation, our studies also give new insight into the factors regulating bound residue formation in plants. Thus, understanding the competition between routes of soluble and insoluble residue formation is an important biotechnological tool in predicting the metabolism of crop protection agents and the form in which xenobiotics enter the food chain (Sandermann, 2004).
Experimental procedures

Plants

Plants and root cultures of wild-type and transgenic Arabidopsis thaliana (Columbia) were maintained as described previously (Brazier-Hicks and Edwards, 2005). Seeds of B. napus, B. juncea and B. nigra were obtained from Herbsied, Wokingham, UK, and of RCG (Phalaris arundinacea var. Palaton and Bamse) and SWG (Panicum virgatum var. Shelter and Trailblazer) from Semundo Ltd. (Great Abington, Cambridge). For the metabolism studies, Brassica plants and the grasses were placed on moist tissue paper for 3 days in the light and then grown in Arthur Bower’s multipurpose compost for 21 days. Arabidopsis plants over-expressing UGT72B1 were generated by subcloning UGT72B1 from pET11d into the expression vector cassette PRT108 using Ncol and BamHI sites. This was then digested with HindIII and ligated into the binary vector pCAMBIA 3300 (CAMBIA, Canberra, Australia). The vector was used to transform Arabidopsis plants (Col-0) and the resulting seed subjected to selection with glucosinate ammonium using a previously described procedure (Dixon et al., 2003). Selection was repeated through a further two generations to produce the homozygous T3 UGT72B1-OE transformed plants. The T-DNA knockout line ugt72B1 was available from previous studies (Brazier-Hicks and Edwards, 2005).

Feeding studies with radiolabelled xenobiotics

For metabolism studies Arabidopsis root cultures (14 days old) were dosed for 24 h with 4 µM [U-14C]-TCP (74 MBq/mmol, Sigma, Poole, Dorset, UK) or [U-14C]-DCA (769.6 MBq/mmol, Sigma), the latter diluted with cold DCA to a final specific activity of 74 MBq/mmol. Cultures were harvested at 4 h, 8 h, 16 h and 24 h and the roots and media separated and extracted as described previously (Brazier-Hicks and Edwards, 2005). Extracted radioactive residues were quantified by liquid scintillation counting (LSC) prior to TLC using the solvent system chloroform : methanol : water (60 : 35 : 8 v/v). Radioactive metabolites were located by autoradiography and quantified by LSC. Where possible, identification was based on co-chromatography with reference metabolites that had previously been identified by mass spectrometry (Loutre et al., 2003). The total bound residue remaining after extraction with methanol was combusted using a sample oxidizer and the ¹⁴CO₂ released captured and quantified by LSC (Brazier-Hicks and Edwards, 2005).

For metabolism studies with RCG and SWG, the stems of 14-day-old plants were placed in 4 µM [¹⁴C]-DCA prepared at the same specific activity as described for the studies with Arabidopsis root cultures. After 24 h, the plant tissue and feeding solution were extracted and analysed by LSC and TLC.

Fractionation of bound residues

The method for sequential fractionation of bound residues was adapted from a published procedure (Langbartels and Harms, 1985). Plant tissue (0.5 g, n = 4) was extracted with methanol (10 mL) for 4 h at 4 °C on an end-over-end mixer after homogenizing with a pestle and mortar. Solvent extraction was repeated a further four times, until no further methanol-soluble radioactivity was released. The residue was then washed with water, followed by 0.1 M K phosphate buffer pH 7.0 containing 0.15 M NaCl (4 h) to release ionically bound residues. The insoluble material was then sequentially extracted with the following treatments (each 10 mL unless specified otherwise) under the conditions given in parentheses. Amylase (2% w/v from Bacillus sp. Novozyme) in 0.1 M K phosphate buffer pH 7.0 (16 h, 37 °C) was used to hydrolyse starches; pronase (1 mg/mL from Streptomyces griseus, Fluka) in 0.1 M Tris-HCl pH 7.2 (8 h, 37 °C) was used to digest protein; 50 mM EGTA in 50 mM sodium acetate pH 4.5 (16 h, 80 °C) was used for pectin solubilization; dioxane : water (9 : 1 v/v, 16 h, 80 °C) followed by dioxane : aqueous 2 M HCl (9 : 1 v/v, 8 h, 80 °C) were used in sequence and then pooled to derive a soluble lignin extract: 4.3 M KOH was employed to digest hemicelluloses (4 h, 80 °C). The final residue was treated with concentrated H₂SO₄ to hydrolyse cellulose (1 mL, 25 °C, 4 h) and then diluted with water (9 mL) prior to LSC. Following each treatment, residual solid material were recovered by centrifugation (3900 g, 5 min) and resuspended by ultrasonication prior to digestion. Extracts (100 µL) from each extraction were radioassayed in duplicate by LSC, with the hemicellulose digest neutralized with acetic acid prior to analysis.

Phytotoxicity trials

For studies with Arabidopsis, each replicate (n = 3) consisted of a Petri dish sown with 60 seeds equally spaced out on to MS agar containing a range of concentrations (0–100 µM) of chlorinated anilines or phenols. Seedlings grown on plates containing agar alone were used as untreated controls. After 14 days, plates were scored for toxicity as follows: score of 10 = full germination and growth as observed in controls; score of 9 to 3 = 90% to 30% of growth observed in controls; score of 2 = cotyledons expanded but undergoing necrosis; score of 1 = germination halted following initial root emergence; and a score of 0 = no germination. For studies with RCG and SWG, seedlings were germinated on blotting paper and after 7 days carefully transplanted into perlite. Plants were maintained in a growth room using the conditions described above, with Phostrogen used for nutrient. After 20 days, plants were treated with DCA dissolved in water (0–100 µM) and scored for phytotoxicity after 12 days of treatment by determining plant height (Clark et al., 2004).

Enzyme analysis

Glucosyltransferase activity in crude plant extracts was determined by radioassay using UDP-[¹⁴C-glucose] as described (Loutre et al., 2003). The presence of immunodetectable UGT72B1 was determined by Western blotting using an antiserum raised against the respective recombinant protein (Brazier-Hicks and Edwards, 2005).

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Role of glucosylation in phytoremediation 635

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Effect of modified UGT72B1 expression on the tolerance of Arabidopsis seedlings to chlorinated phenols and anilines. Plants used were derived from wild-type, over-expressor (7281-0E) and knockout (ugt72b1) lines. (A) Images of plants showing a score of 10 = full germination and maximum growth (i.e. untreated plants), score of 4 = 40% of growth observed in untreated controls, score of 2 = cotyledons expanded but undergoing necrosis, score of 1 = germination halted at root elongation and score of 0 = no germination. (B) Growth/toxicity scores of 14 day old seedlings grown on different concentrations of the chloroanilines and chlorophenols.

Figure S2 Uptake and metabolism of [14C]-DCA in reed canary grass and switchgrass over a 24-h exposure. Autoradiograph of radioactive metabolites extracted from the grasses and resolved by thin layer chromatography with parent DCA and DCA-N-glucose identified.

Figure S3 Phytotoxicity of DCA in reed canary grass and switchgrass. Effect of exposing reed canary grass (Palaton) and switchgrass (Traillblazer) to 100 µM DCA for 12 days. This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1467-7652.2007.00266.x

(THis link will take you to the article abstract).

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