the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2008) doi: 10.1111/j.1365-313X.2008.03653.x

Detoxification of the explosive 2,4,6-trinitrotoluene in Arabidopsis: discovery of bifunctional *O*- and *C*-glucosyltransferases

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Received 14 July 2008; accepted 1 August 2008.
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Summary

Plants, as predominantly sessile organisms, have evolved complex detoxification pathways to deal with a diverse range of toxic chemicals. The elasticity of this stress response system additionally enables them to tackle relatively recently produced, novel, synthetic pollutants. One such compound is the explosive 2,4,6-trinitrotoluene (TNT). Large areas of soil and groundwater are contaminated with TNT, which is both highly toxic and recalcitrant to degradation, and persists in the environment for decades. Although TNT is phytotoxic, plants are able to tolerate low levels of the compound. To identify the genes involved in this detoxification process, we used microarray analysis and then subsequently characterized seven uridine diphosphate (UDP) glycosyltransferases (UGTs) from *Arabidopsis thaliana* (Arabidopsis). Six of the recombinantly expressed UGTs conjugated the TNT-transformation products 2- and 4-hydroxylaminodinitrotoulene, exhibiting individual bias for either the 2- or the 4-isomer. For both 2- and 4-hydroxylaminodinitrotoulene substrates, two monoglucose conjugate products, confirmed by HPLC-MS-MS, were observed. Further analysis indicated that these were conjugated by either an *O*- or *C*-glucosidic bond. The other major compounds in TNT metabolism, aminodinitrotoluenes, were also conjugated by the UGTs, but to a lesser extent. These conjugates were also identified in extracts and media from Arabidopsis plants grown in liquid culture containing TNT. Overexpression of two of these UGTs, 743B4 and 73C1, in Arabidopsis resulted in increases in conjugate production, and enhanced root growth in 74B4 overexpression seedlings. Our results show that UGTs play an integral role in the biochemical mechanism of TNT detoxification by plants.

Keywords: glucosyltransferase, 2,4,6-trinitrotoluene, Arabidopsis, phytoremediation, detoxification, explosive.

Introduction

The nitroaromatic explosive 2,4,6-trinitrotoluene (TNT) is a highly persistent, organic pollutant, introduced into the environment by human activities. Contamination with TNT results principally from the manufacture, packaging, use and decommissioning of explosives. It is a primary target for remediation because of its toxicity (it is a designated class-C carcinogen) and the global extent of environmental contamination. Current remediation strategies are insufficient to tackle the scale of the problem, and phytoremediation is emerging as a feasible alternative. Although numerous studies have investigated the tolerance of plants to TNT using soil-based approaches, remarkably little is known about the fundamental molecular biology and biochemistry involved in the detoxification of this phytotoxin (Hannink *et al.*, 2002). Active uptake into the roots has been observed (Pavlostathis *et al.*, 1998), although little TNT is transported to the aerial parts of the plant (Sens *et al.*, 1998).

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2008		**00-00-2008 to 00-00-2008**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Detoxification of the explosive 2,4,6-trinitrotoluene in Arabidopsis: discovery of bifunctional O- and C-glucosyltransferases	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **CNAP, Department of Biology,University of York,PO Box 373,York YO10 5YW, UK,**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**12**	

The electron-withdrawing properties of the nitro groups of TNT make the aromatic ring electron deficient. This favours reductive transformation reactions in plants: most commonly the reduction of a nitro group via a nitroso intermediate to give hydroxylaminodinitrotoluene (HADNT), and then aminodinitrotoluene (ADNT) (Hannink *et al.*, 2007; Sens *et al.*, 1998). Both the 2- and the 4- isomers of HADNT and ADNT intermediates have been detected in plants (Hannink *et al.*, 2002; Wang *et al.*, 2003), including Arabidopsis (Subramanian *et al.*, 2006), and we have shown that expression of a bacterial nitroreductase, *nfsI*, in tobacco increases the rate of TNT reduction, thereby conferring enhanced resistance (Hannink *et al.*, 2001) to TNT.

The reduction of TNT is a classical 'phase-1' activation step in the detoxification of xenobiotics. 'Phase 2' in this process is the conjugation of the activated xenobiotic with polar donor molecules, such as sugars, glutathione and amino acids (Coleman *et al.*, 1997). Both 2- and 4-ADNTs conjugated to one or more six-carbon units have been identified in plant extracts (Bhadra *et al.*, 1999; Harvey *et al.*, 1990), and it is anticipated that glycosyltransferases are involved. Further studies have identified mono and diglycoside conjugates of the less stable 2- and 4-HADNT intermediates (Subramanian *et al.*, 2006; Vila *et al.*, 2005; Wayment *et al.*, 1999), and it is likely that HADNT intermediates had not previously been detected because of their low chemical stability. Following conjugation, which usually occurs in the cytosol, 'phase 3' involves either compartmentation of the conjugates into the vacuole or hydrolysis and release of the compound into the apoplast region. Alternatively, the conjugates can be incorporated into insoluble residues within the cell-wall matrix (Brazier-Hicks *et al.*, 2007). To identify the genes involved in the detoxification of TNT, we performed microarray analysis of TNT-treated Arabidopsis plants, and identified seven UDP glycosyltransferases (UGTs) that were upregulated in response to TNT exposure. We present data on their biochemical characterization, demonstrating *in vitro* activity and the production of conjugated intermediates *in vivo*.

Results

Upregulation of specific UGTs in response to TNT

A TNT concentration of 60 μM for 6 h was established to be non-lethal whilst still inhibiting seedling growth. The liquid culture growth conditions were also optimized to reduce the background expression of stress response genes. Microarray analysis was performed using pair-wise replicates, comparing 14-day-old seedlings treated with TNT for 6 h with seedlings treated with the TNT solvent dimethylformamide (DMF) only. Analysis of the microarray data revealed 633 genes with increased expression levels of between 2- and 204-fold, with a median value of 3.0 (expressed by 50% of the genes), relative to the DMF-treated control levels. Less than 10% of these 633 genes were upregulated by 12-fold or higher. Annotation of these data revealed seven UGTs that exhibited increased transcript levels of between 14- and 173-fold in response to TNT treatment (Table 1). These seven UGTs are all members of family glycosyltransferases that transfer sugar from a donor molecule, usually UDP-glucose, to a small, hydrophobic or lipophillic acceptor molecule. Expression levels of these UGTs, tested using semi-quantitative RT-PCR, confirmed that all were upregulated in response to TNT exposure (Figure 1a,b), although UGT73B4, which exhibited the highest fold increase in the microarray results (173-fold), did not exhibit the highest level of expression in the RT-PCR analysis.

Conjugation of TNT-transformed intermediates

The UGTs were expressed as recombinant fusion proteins in *Escherichia coli*, were purified by affinity purification, and were then screened for activity towards the 2- and 4-HADNT and ADNT isomers using UDP-glucose as the donor molecule. A previous study (Lim *et al.*, 2003) demonstrated that many of the groups within UGT family 1 had activity towards the hydroxycoumarin, scopoletin, although overall activity varied between individual UGTs. This indicates that basic features of substrate recognition are retained across the family. We therefore used scopoletin as a model substrate to test for activity in the seven recombinant UGTs. In agreement with *Lim et al.* (2003), UGT74B4, UGT73C1, UGT73C6, UGT73B2 and UGT73B5 exhibited activity towards scopoletin, and additionally we detected activity for UGT74E2. No activity was detected for UGT75B1, and only weak activity (>0.1 nkat mg^{-1}) was reported previously (Lim *et al.*, 2003) (results not shown). Table 2 shows that six of the seven UGTs were found to have activity towards both HADNT and ADNT substrates; no significant activity was detected from UGT75B1, the least upregulated of the seven UGTs in the microarray results (Table 1). The specific activities of all six UGTs were approximately three orders of magnitude greater with HADNT than with ADNT substrates, and the 4-HADNT and 4-ADNT isomers gave significantly higher activities than their respective 2-HADNT and 2-ADNT isomers. There were differences between the relative activities of the UGTs with the 2- and 4- isomers: UGT73B5 exhibited the highest specific activity with 4-HADNT, yet exhibited no significant activity with 2-HADNT, whereas UGT73B4 exhibited equal levels of activity with either 2- or 4-HADNT isomers (Table 2). The pH optimum of UGT73B4 was measured over pH 6.5–8.5 using Tris-HCl and phosphate buffers, and pH 7.0 was found to be the optimum, with similar activities seen with either buffer (results not shown). Although activity of UGTs has been shown to be enhanced by the addition of the reducing agents DTT or β-mercaptoethanol (Hou *et al.*, 2004; Loutre *et al.*, 2003), this effect could not be determined, as addition of 2–20 μM of either

Table 1 Fifty of the most upregulated transcripts, from the subset of 633 genes with increased expression levels of between 2- and 204-fold, in 14-day-old Arabidopsis seedlings treated for 6 h with 60 μM 2,4,6-trinitrotoluene (TNT). Results are presented as the fold difference in transcript level compared with control plants, treated with dimethylformamide (DMF) solvent only. Glycosyltransferases are highlighted in bold. All measurements were performed as described in the Experimental procedures

Gene number	UGT classification	Fold increase	Transcript name
At3g54520		204.7	hypothetical protein
At2g15490	**UGT73B4**	**173.4**	**putative glucosyltransferase**
At4g04490		155.6	putative receptor-like protein kinase
At1g05680	**UGT74E2**	**118.7**	**putative indole-3-acetate beta-glucosyltransferase**
At1g73120		109.7	hypothetical protein
At4g24110		85.3	putative protein
At2g36750	**UGT73C1**	**83.6**	**putative glucosyl transferase**
At5g24110		72.0	WRKY family transcription factor
At1g57630		64.1	disease resistance protein (TIR class), putative
At2g36790	**UGT73C6**	**59.4**	**putative glucosyl transferase**
At2g41730		57.5	hypothetical protein
At1g01480		55.3	1-aminocyclopropane-1-carboxylate synthase
At1g78410		48.1	expressed protein
At1g17180		45.9	glutathione transferase, putative
At2g22880		39.9	hypothetical protein
At1g26380		38.8	FAD-linked oxidoreductase family
At2g20800		38.6	putative NADH-ubiquinone oxireductase
At2g20720		37.5	hypothetical protein
At1g17170		37.0	glutathione transferase, putative
At2g02010		31.3	glutamate decarboxylase
At2g26560		30.6	patatin, putative
At2g29460		30.4	glutathione transferase, putative
At4g01870		30.3	hypothetical protein
At3g60420		29.7	putative protein
At2g41380		27.4	putative embryo-abundant protein
At4g25200		26.5	mitochondrion-localized small heat shock protein
At4g34135	**UGT73B2**	**26.1**	**glucosyltransferase -like protein**
At3g28740		25.1	cytochrome p450 family
At3g50930		24.1	BCS1 protein-like protein
At3g20340		24.0	unknown protein
At3g01830		23.3	expressed protein
At4g33070		21.2	pyruvate decarboxylase-1 (Pdc1)
At2g15480	**UGT73B5**	**21.1**	**putative glucosyltransferase**
At3g25250		21.0	protein kinase, putative
At2g04050		18.8	hypothetical protein
At1g24140		18.4	putative metalloproteinase
At4g37290		18.4	expressed protein
At2g04070		18.2	hypothetical protein
At2g03760		18.0	putative steroid sulfotransferase
At2g38250		17.9	putative GT-1-like transcription factor
At2g29470		17.3	glutathione transferase, putative
At2g32030		17.1	putative alanine acetyl transferase
At2g04040		15.8	hypothetical protein
At2g37430		15.5	putative C2H2-type zinc finger protein
At2g18190		15.5	putative AAA-type ATPase
At2g47520		14.7	putative AP2 domain transcription factor
At5g57220		14.5	cytochrome p450 family
At1g05560	**UGT75B1**	**13.9**	**UDP-glucose transferase(UGT1)**
At1g76690		13.7	12-oxophytodienoate reductase (OPR2)

reagent resulted in the reduction of the HADNT substrate to ADNT (results not shown).

The HPLC analysis of the conjugated products, presumed to be monoglucosides (MGs), of the 2- and 4-HADNT substrates revealed two distinct peaks, with retention times of 11.3 and 12.0 min (Figure 2a, 12.0-min peak only) for 2-HADNT, and 11.8 and 12.7 min for 4-HADNT (Figure 2b). These product peaks had spectra similar to the substrates (Figure 2c,d). The six UGTs differed in their bias towards the formation of the four detected conjugates. Using the

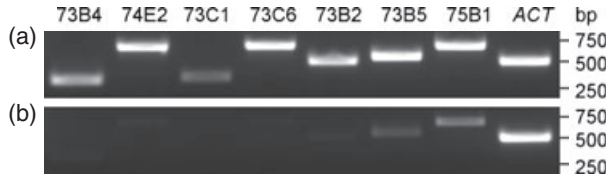

Figure 1. Semi-quantitative RT-PCR and SDS-PAGE analysis of UDP glycosyltransferases (UGTs) upregulated by treatment with 2,4,6-trinitrotoluene (TNT). RT-PCR was performed on cDNA from 14-day-old seedlings treated with (a) TNT or (b) dimethylformamide (DMF) solvent-only control. Levels of the *ACTIN2* gene: *ACT*. Molecular weight markers are shown in nucleotide base pairs (bp).

2-HADNT substrate, UGT73C1 produced almost exclusively HADNT-MG 11.3, whereas the five remaining UGTs formed predominantly HADNT-MG 12.0 (Figure 2e). Using the 4-HADNT substrate, UGT73B4 produced equal quantities of both HADNT-MG 11.8 and HADNT-MG 12.7 isomers, whereas UGT74E2 principally produced HADNT-MG 11.8, and the remaining UGTs produced the HADNT-MG 12.7 conjugate (Figure 2f).

The primary products of the glucosyltransferase activity were analysed by mass spectrometry, and their masses corresponded to the expected glucosylated compounds: *m/z* 374 $[M\text{-}H]^-$ for the HADNTs, and *m/z* 358 $[M\text{-}H]^-$ for the ADNTs. Both peaks obtained from each of the HADNTs are monoglucosides with the same masses. For 2-HADNT, the isomer eluted at 12.0 min had a main fragment of *m/z* 212 $[M\text{-}H]^-$-162 that corresponded to the cleavage of a unit of glucose, which is indicative of the presence of an *O*-glucosidic bond. The loss of oxygen in the cleavage also generated a main fragment with a mass of *m/z* 196, which corresponded to the ADNT form (Figure 3a), as reported previously (Vila *et al.*, 2005). For the peak eluted at 11.3 min, the main fragment corresponded to the loss of water (*m/z* 356). A fragment with a mass *m/z* 254, $[M\text{-}H]^-$-120 corresponded to a fragmentation $^{0,2}X$, in agreement with the fragmentation of the *C*-glucosides of flavonoids (Figure 3b) (Cuyckens and Claeys, 2004). During this study, attempts to obtain milligram quantities of this compound for NMR analysis failed, and the position of the glucose moiety could not be assigned to a particular carbon atom of TNT. For the product of 4-HADNT, eluted at 11.8 min, the same $^{0,2}X$ fragmentation was found, with a further loss of water (*m/z* 236), and one or two NO· (*m/z* 206 and 176, respectively). Thus, the 2-HADNT-glucoside that eluted at 11.3 min and the 4-HADNT-glucoside that eluted at 11.8 min were assigned as *C*-glucosides, whereas their isomers corresponded to *O*-glucosides. This was further supported by the hydrolysis of the *O*-glucosides to the original HADNTs by the activity of β-glucosidase, and by the resistance to the same treatment of the *C*-glucosides.

Steady-state kinetics of UGT73B4

Following the analysis by mass spectrometry presented in Figure 3, a more detailed biochemical characterization on UGT73B4 was performed. UGT73B4 was chosen for further characterization because it was the most highly upregulated UGT in the group of 633 genes from the microarray data. When the activity of UGT73B4 was assayed using the 2-HADNT substrate, a peak of 2-HADNT-MG conjugate was observed prior to the production of a second compound of higher polarity, designated 2-HADNT-unknown (Figure 4a). This compound was derived from the activity of the enzyme with UDP-glucose on the purified *O*-MG (results not shown), and was not hydrolysed by treatment with β-glucosidase. The *C*-MG of 2-HADNT, also incubated with the enzyme, was not found to be a substrate. This final product was further HPLC purified and analysed by mass spectrometry (Figure 3c). A diglucoside conjugate of 2-HADNT has a nominal molecular mass of 537 and a predicted $[M\text{-}H]^-$ ion of *m/z* 536. However, the largest ion that could be found for this peak had an *m/z* value of 501. When subjected to mass spectrometry product ion scan (MSn) analyses, this ion produced two major fragments (*m/z* 272 and 228) that further fragmented into *m/z* 254, 210, 179 and 143, for the *m/z*-272 fragment, and *m/z* 180

Table 2 Specific activities of UDP glycosyltransferases (UGTs) towards the hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT) isomers. The specific activity was defined in nmol of substrate converted into glucose conjugate (nanokatal, nkat) by 1 mg of protein. Not detected (n/d): no significant activity detected (<0.001 nkat mg^{-1} protein)

	Specific activity (nkat mg^{-1} protein)			
UGT	2-HADNT	4-HADNT	2-ADNT	4-ADNT
73B4	1.10 ± 0.18	1.15 ± 0.09	0.0082 ± 0.0006	0.0126 ± 0.0014
74E2	0.43 ± 0.01	1.79 ± 0.19	0.0023 ± 0.0003	0.0179 ± 0.0017
73C1	0.82 ± 0.01	2.13 ± 0.16	0.0052 ± 0.0013	0.0033 ± 0.0004
73C6	0.78 ± 0.03	1.92 ± 0.13	0.0022 ± 0.0010	0.0073 ± 0.0010
73B2	n/d	4.21 ± 0.05	0.0064 ± 0.0022	0.0279 ± 0.0056
73B5	0.53 ± 0.04	0.97 ± 0.01	0.0021 ± 0.0008	0.0058 ± 0.0003
75B1	n/d	n/d	n/d	n/d

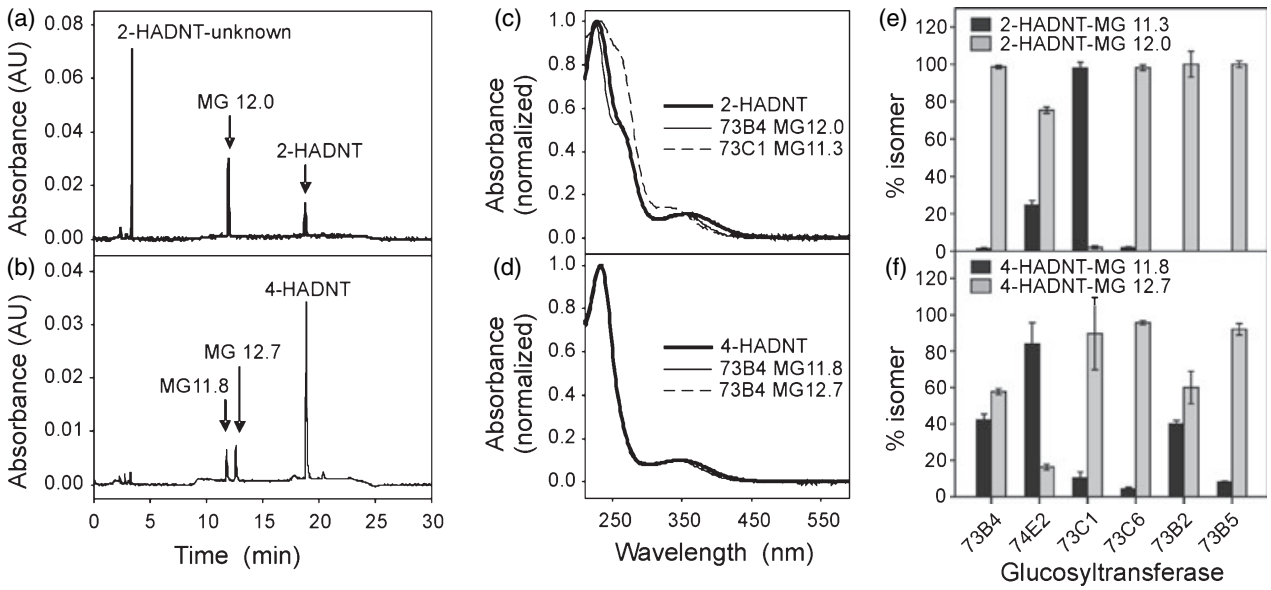

Figure 2. HPLC and spectral analysis of 2,4,6-trinitrotoluene (TNT) transformed hydroxylaminodinitrotoluene (HADNT) intermediates and glucoconjugates produced by UDP glycosyltransferase 73B4 (UGT73B4), and substrate isomer bias of UGT73B4. (a) 2-HADNT substrate; (b) 4-HADNT substrate. Absorbance spectra for (c) 2-HADNT, UGT73B4 produced 2-HADNT-MG 12.0 and UGT73C1 produced 2-HADNT-MG 11.3. (d) 4-HADNT, UGT73B4 produced 4-HADNT-MG 11.8, and UGT73B4 produced 4-HADNT-MG 12.7. (e) The percentages of 2-HADNT-MG isomers with retention times of 11.3 and 12.0 produced. (f) The percentages of 4-HADNT-MG isomers with retention times of 11.8 and 12.7 produced. Results were the means of three replicates ± standard deviations.

and 150, for the *m/z*-228 fragment. None of these fragments were consistent with neutral losses of sugar moieties. Therefore, although the compound was derived from the activity of the UGT73B4-purified enzyme using, as a substrate, HPLC-purified 2-HADNT-*O*-monoglucoside, and in a medium only containing UDP-glucose, it could not be confirmed as a diglucoside of HADNT.

When recombinant UGT73B4 was assayed using 4-HADNT as the substrate (Figure 4c), the HPLC analysis yielded MGs with retention times of 11.8 and 12.7 min. Conjugates more polar than these MGs were not detected. The kinetic results are presented in Table 3. As the solubility limits of the TNT derivatives were less than 500 μM, kinetic parameters were estimated using nonlinear regression fitting or, in the case of 4-HADNT, by linear fitting to pseudo first-order kinetics (details are given in the Experimental procedures). Whereas the K_m values and specific activities for ADNTs were relatively low, with 4-HADNT clearly having the highest specific activity, 2-HADNT-MG exhibited the highest k_{cat}/K_m value. Taken together with the K_m values, which are lower than for 4-HADNT, it is clear that for UGT73B4, 2-HADNT-MG is the preferred substrate.

Overexpression of UGT73B4 and UGT73C1

Following evidence that these UGTs are able to conjugate the transformation products of TNT, UGT73B4 and UGT73C1 were overexpressed in Arabidopsis. Five, independent, homozygous lines were analysed for each UGT. The RT-PCR data in Figure 5(a) shows that the UGT73B4 overexpressing transgenic lines exhibited a range of increased levels of UGT73B4 transcripts compared with wild type. A similar range of increased levels of UGT73C1 transcript was also seen in the UGT73B4 overexpressing lines. To determine if these increases conferred an increased ability to detoxify TNT, and a resulting increase in tolerance to TNT, seeds were germinated and grown on media containing 7 μM TNT. This was the lowest concentration at which significant root-length inhibition is seen in wild-type seedlings. After 2 weeks, the root lengths of seedlings overexpressing UGT73B4 were significantly longer than in wild-type seedlings, whereas the root lengths of lines overexpressing UGT73C1 were unaltered from wild-type levels (Figure 5b,c). In liquid-culture experiments, no enhanced removal of TNT was observed in the transgenic lines with respect to wild type (Figure 6a). All lines produced a transient accumulation of HADNTs during the first hour of the experiment, but subsequently mainly ADNTs were detected, reaching the highest concentration after 3 days (Figure 6b). The glucosylated compound 4-HADNT-*C*-glucoside was also detected during the first 2 days, after which, the same glucosylated compound of 4-ADNT seen with *in vitro* activity of the UGTs (Table 2) accumulated in the medium, and remained until the end of the experiment (Figure 6c). The level of this glucosylated metabolite was 28–41% higher in both the transgenic lines than in the wild type at day four (WT, 4.2 ± 0.6 μM; UGT73B4, 5.9 ± 0.2 μM; UGT73C1, 5.4 ± 0.4 μM). There were no significant differences in the biomass of the plant material of the

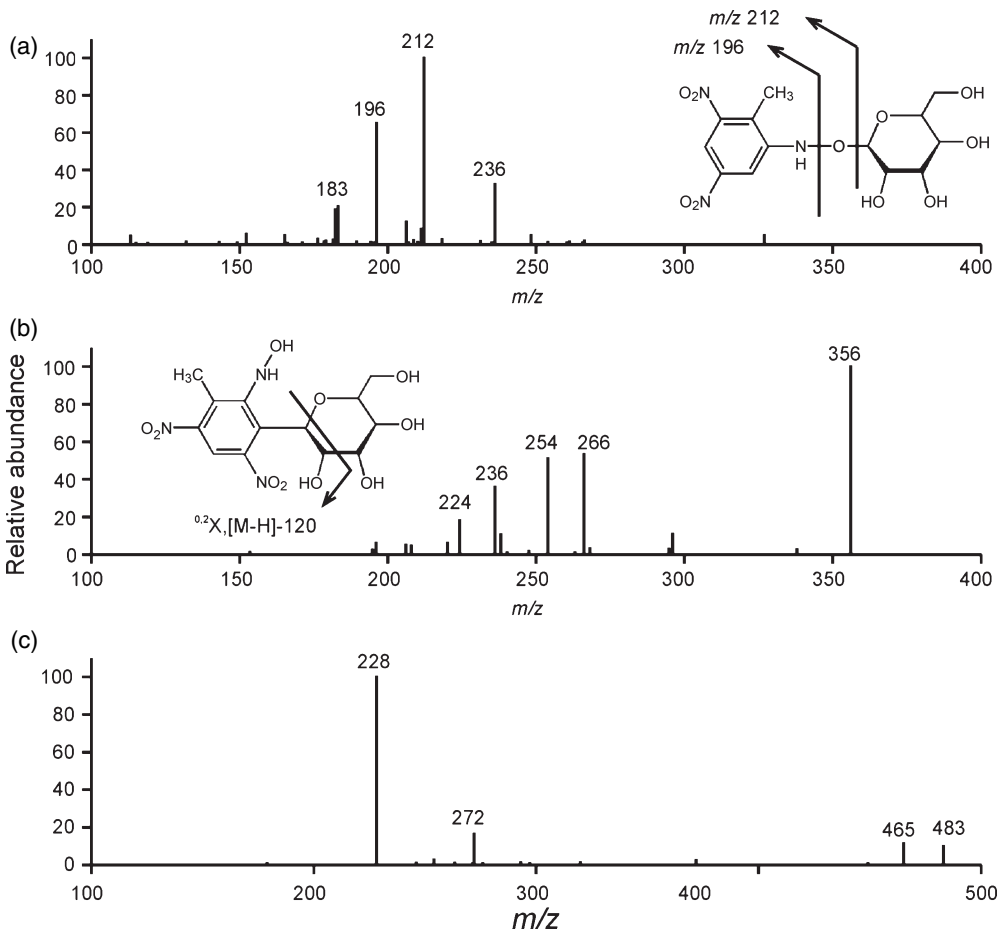

Figure 3. ESI-MS2 fragmentation patterns for the glucosylated 2-hydroxylaminodinitrotoluene (2-HADNT) derived compounds. (a) 2-HADNT-*O*-glucoside *m/z* 374 $[M\text{-}H]^-$; (b) 2-HADNT-*C*-glucoside *m/z* 374 $[M\text{-}H]^-$; and (c) 2-HADNT unknown compound.

transgenic lines, with respect to the wild-type plants, after TNT feeding (data not shown). The TNT metabolites in the plant material were extracted and analysed by HPLC-ESI-MS, and glucosylated compounds were identified by comparison of retention times and MSn fragmentation with standards, confirming *in planta* the activities previously characterized *in vitro*: 4-HADNT-*C*-glucoside, 2-HADNT-*O*-glucoside, 4-HADNT-*O*-glucoside, 2-ADNT-glucoside and 4-ADNT-glucoside (Figure 6d).

Further analysis of the main metabolites found in the media revealed the presence of around 50% of each of the 2- and 4-ADNT isomers at day four (results not shown). Thus, an equimolar mixture of ADNT isomers was used to explore the toxicity of ADNTS in wild-type and UGT73B4-transformed plants. On agar plates containing 7, 50 and 200 μM TNT, no differences between wild-type and UGT73B4 line A4 seedling growth was observed. In liquid culture experiments containing equimolar mixtures of 200 μM ADNTs, as seen in the TNT feeding experiments, overexpression of the glucosyltransferase did not promote the removal of the ADNTs from the medium (Figure 7a). However, the 4-ADNT-glucoside accumulated more quickly in the media: twice as much ADNT glucoside was detected for both isomers in the plant material of UGT73B4-transformed plants, relative to wild type (Figure 7b).

Discussion

In Arabidopsis, 107 putative family-1 UGTs have been identified: defined by the presence of a carboxy terminal consensus sequence involved in the binding of the protein to the UDP moiety of the sugar nucleotide (Bowles, 2002; Lim and Bowles, 2004; Lim *et al.*, 2001). It has been demonstrated that many of the family members have distinct substrate specificities for endogenous plant compounds and xenobiotics (reviewed in Bowles *et al.*, 2006). The Arabidopsis GT1 family has been subcategorized into 14 groups (Ross *et al.*, 2001). The seven TNT-induced UGTs fall into group D (UGT74B4, UGT73C1, UGT73C6, UGT73B2 and UGT73B5) or group L (UGT74E2 and UGT75B1), and members of group D have been shown to be commonly expressed in response to environmental

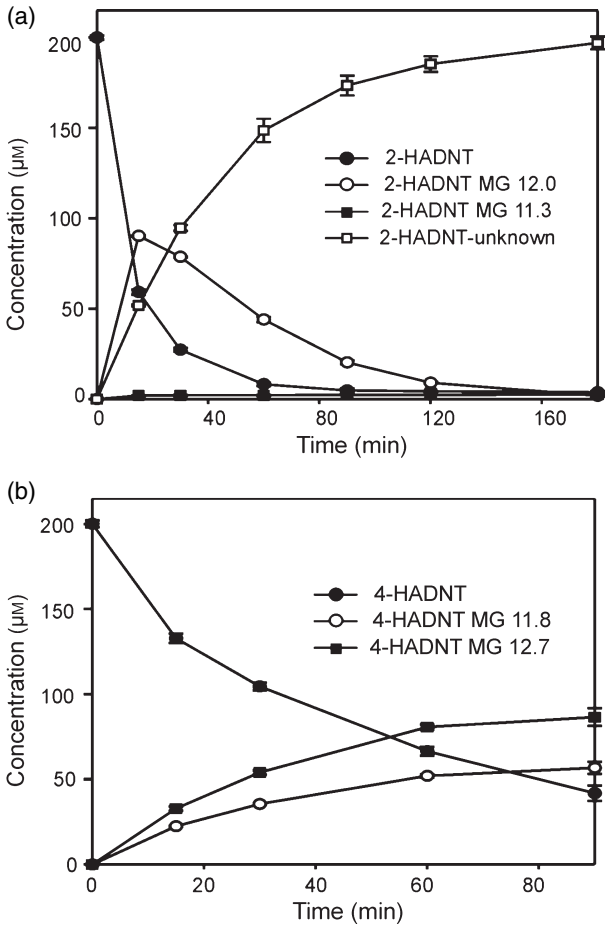

Figure 4. Time course of UDP glycosyltransferase 73B4 (UGT73B4) activity with (a) 2-hydroxylaminodinitrotoluene (2-HADNT) substrate showing the production of 2-HADNT monoglucosides (2-HADNT-MGs) and 2-HADNT-unknown. (b) 4-HADNT showing production of 4-HADNT-MG 11.8 and 4-HADNT-MG 12.7. The results are the means of three replicate measurements ± standard deviations.

Table 3 Steady-state kinetics of UDP glycosyltransferase 73B4 (UGT73B4) analysed over a range of 2,4,6-trinitrotoluene (TNT) derivatives. The kinetic parameters K_m and V_{max} were estimated using nonlinear regression analysis. The k_{cat} values were calculated from the V_{max} values. For 4-HADNT, a pseudo first-order kinetic model ([S] $\ll$ K_m) was used to calculate the ratio V_{max}/K_m, and then to calculate k_{cat}/K_m. Results represent means from three replicates ± standard deviations

Substrate	K_m (mM)	V_{max} (nkat mg^{-1})	k_{cat}/K_m (mM^{-1} sec^{-1})
2-HADNT	0.95 ± 0.29	4.36 ± 0.96	0.354 ± 0.029
2-HADNT-MG	0.44 ± 0.03	3.32 ± 0.15	0.585 ± 0.019
4-HADNT	–	–	0.223 ± 0.039
2-ADNT	0.15 ± 0.04	0.008 ± 0.001	0.0041 ± 0.0006
4-ADNT	0.29 ± 0.09	0.021 ± 0.003	0.0055 ± 0.0009

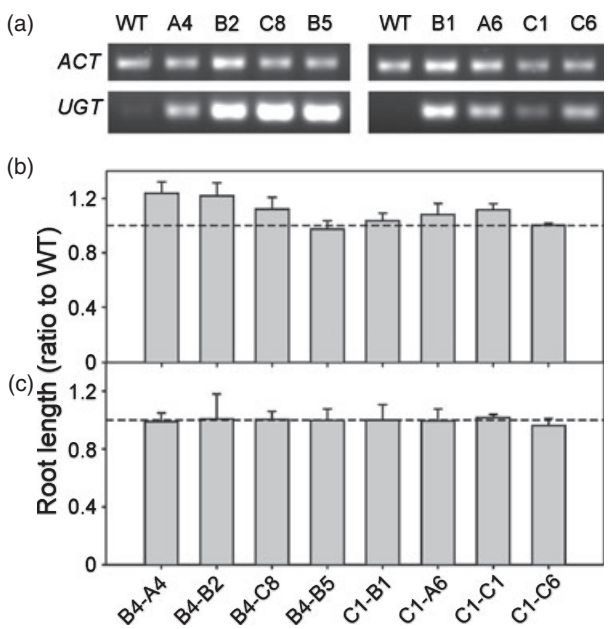

Figure 5. Overexpression of UDP glycosyltransferase 73B4 (UGT73B4) and UGT73C1, and root length comparisons on media treated with and without 2,4,6-trinitrotoluene (TNT). (a) Semiquantitative RT-PCR data showing expression levels in rosette leaves of five, independent, homozygous lines overexpressing either UGT73B4 or UGT73C1. *ACT*, levels of the *ACTIN2* gene; WT, wild type; RNA was pooled from five plants per line. (b) Root lengths of transgenic lines, relative to wild type (set to one), of 10-day-old seedlings grown on solid media containing 7 μM TNT, or (c) without TNT. Results are from 20 seedlings from three replica plates.

and pathogen-induced stress (Langlois-Meurinne *et al.*, 2005).

Several reports have demonstrated that Arabidopsis UGTs rapidly metabolize xenobiotic pollutants by *O*- or *N*-glucosylation (Brazier-Hicks and Edwards, 2005; Lao *et al.*, 2003; Loutre *et al.*, 2003; Messner *et al.*, 2003). Recently, a glucosyltransferase with bifunctional *N*- and *O*- activity involved in xenobiotic metabolism has been reported (Brazier-Hicks *et al.*, 2007), and a *Streptomyces* (S.) *fradiae* urdamycin *C*-glycosyltransferase has been shown to catalyse the production of both *O*- and *C*-glycosides (Dürr *et al.*, 2004). The six UGTs characterized here produced, with differing preference, two monoglucose conjugates from each HADNT and ADNT isomer substrate: an *O*-glucoside and a *C*-glucoside in each case. This was confirmed by mass spectrometry masses, fragmentation patterns, comparisons with published data (Cuyckens and Claeys, 2004) and β-glucosidase cleavage analysis. Subsequent analysis of liquid culture grown, TNT-treated, Arabidopsis demonstrated the production of isomers of both HADNT and ADNT glucosides *in vivo*.

Following the data presented here, we propose the detoxification pathway for TNT as presented in Figure 8, where, following reduction, both HADNT and ADNT intermediates are glucosylated. *In vivo*, this pathway is likely to be more complex, with the reduction of more than

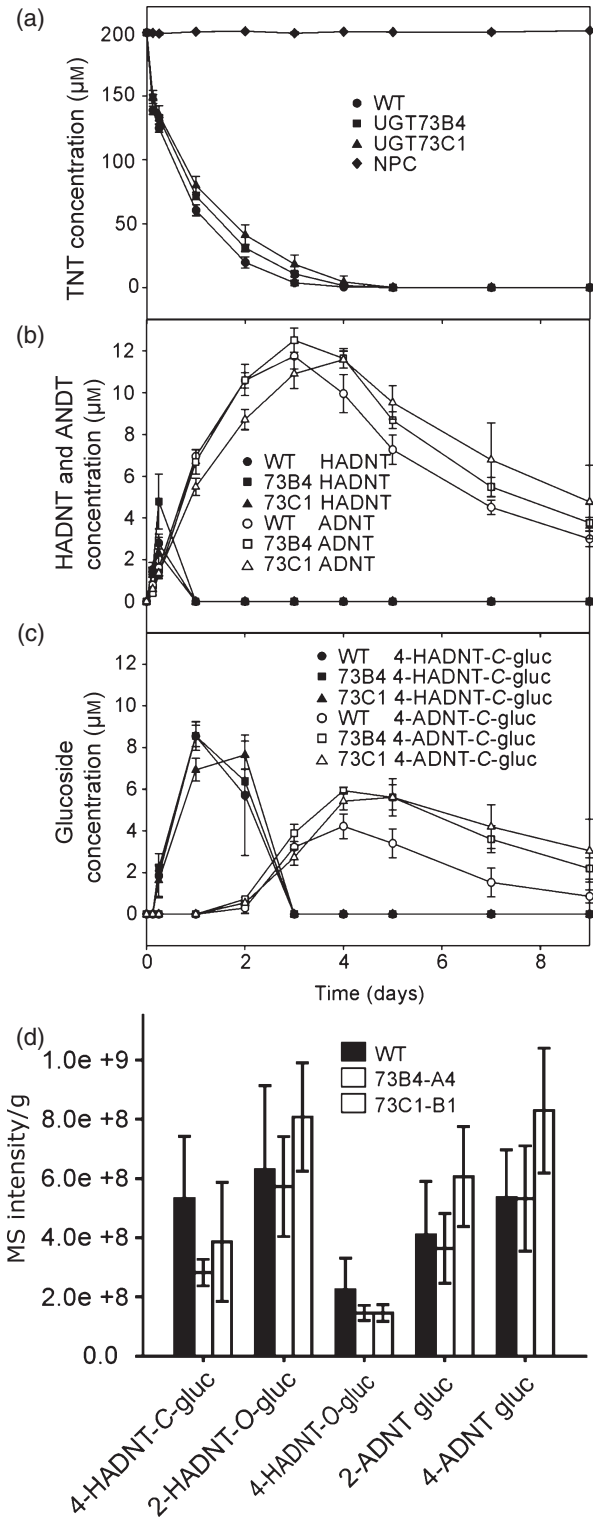

Figure 6. 2,4,6-Trinitrotoluene (TNT) feeding to wild type (WT), UDP glycosyltransferase 73B4 (UGT73B4) and UGT73C1 expressing lines. (a) TNT uptake from the media, (b) levels of hydroxylaminodinitrotoluene (HADNT) and aminodinitrotoluene (ADNT), and (c) levels of 4-HADNT-*C*-glucosides and 4-ADNT-*C*-glucosides in the media. (d) TNT-derived glucosides extracted from the plant material after 9 days. NPC, no plant control.

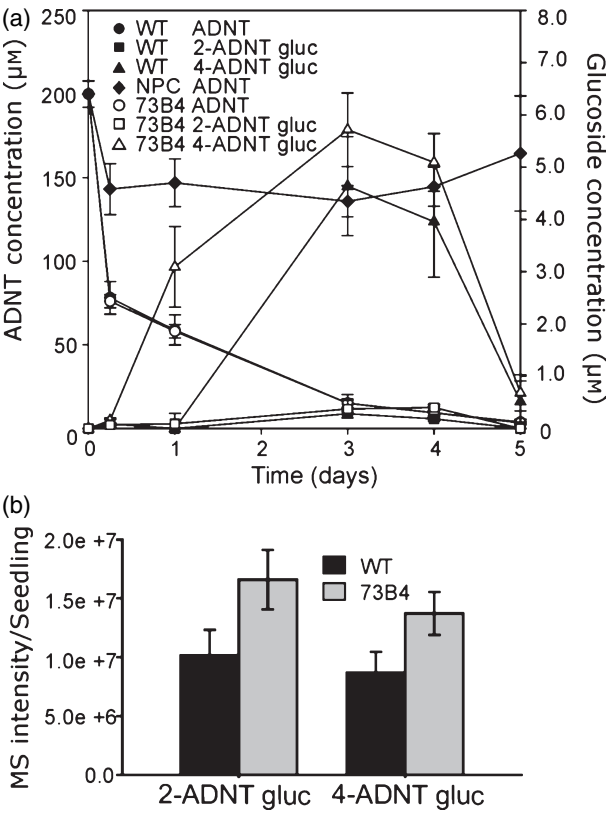

Figure 7. Uptake of aminodinitrotoluenes (ADNTs) from media, and accumulation of glucosides in plant tissue by wild type (WT) and UDP glycosyltransferase 73B4 (UGT73B4) lines. (a) Uptake of 200 μM equimolar mixture of 2- and 4-ADNTs, and levels of ADNT glucosides detected in the media with time. (b) ADNT glucosides extracted from the plant material after 5 days.

one nitro group and subsequent conjugation, by *O*- or *C*-glycosidic bond types, to one or more sites on the reduced TNT molecule, by a sugar molecule.

Compartmentation of glycosylated intermediates

No targeting signals or membrane-spanning signals have been identified in plant UGTs (Li *et al.*, 2001), and the reaction is presumed to occur in the cytosol. Evidence suggests that TNT-detoxified products may be subsequently stored in the vacuole or cell wall. In *Phaseolus vulgaris* fed ^{14}C labelled TNT, over half (57%) of the recovered radioactivity was found in the non-polar, lignin fraction. Isolated TNT-derived intermediates and conjugates were found to be distributed evenly between cytosolic and cell wall fractions, with the lignin fraction totalling 27% of the cell wall fraction (Sens *et al.*, 1998, 1999). These findings suggest that TNT conjugates are incorporated into the cell wall matrix, and sequestration to the vacuole may also occur, as has been shown for

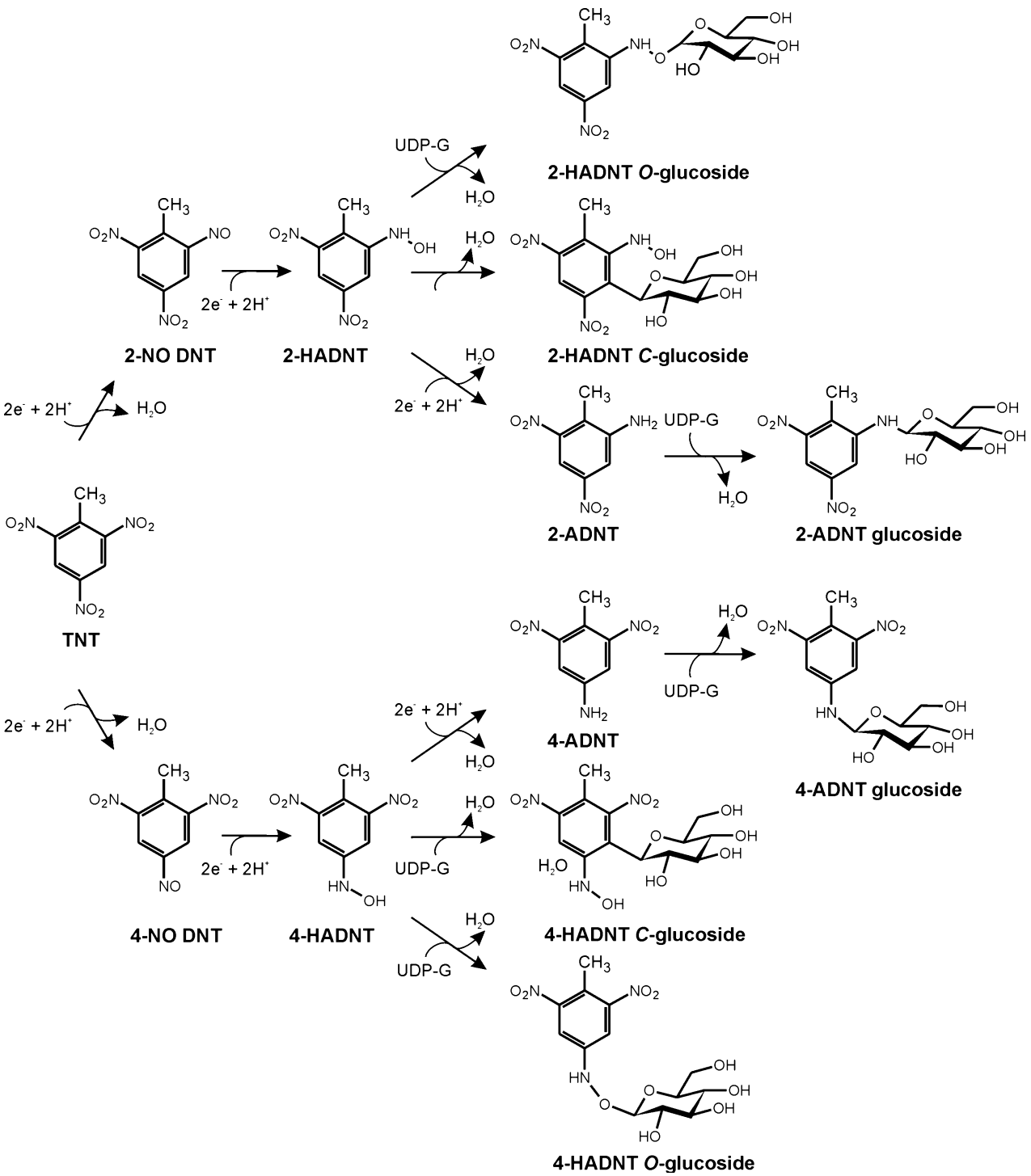

Figure 8. Proposed glucosylation pathway of 2,4,6-trinitrotoluene (TNT) in Arabidopsis.

chlorinated phenols and aniline conjugates (Pascal-Lorber *et al.*, 2003). Studies in tobacco cell suspension cultures, which lack appreciable levels of lignin, identified significantly higher levels of mono and diglycoside conjugates of 2- and 4-HADNT than are found in whole-plant cultures (Vila *et al.*, 2005), possibly because of the lack of an appreciable lignin sink for incorporation of these conjugates. Whereas genes associated with lignin synthesis were not appreciably upregulated after 6 h in our microarray data, in SAGE data from Arabidopsis roots

treated with TNT for 24 h, a number of genes associated with lignin biosynthesis were upregulated (including phenyl ammonium lyase, cinnamate 4-hydroxylase, 4-coumarate:CoA ligase, hydroxycinnamoyltransferase, cinnamoyl-CoA reductase, caffeic acid *O*-methyltransferase and cinnamyl alcohol dehydrogenase) (Ekman *et al.*, 2003). It is possible that the TNT-conjugated products of the UGTs identified here are also incorporated into insoluble, bound residues within the cell wall matrix.

Our results show that seedlings overexpressing UGT73B4 were significantly longer than wild-type seedlings when grown on 7 μM TNT, and that in liquid culture studies, levels of glucosylated 4-ADNTs were significantly higher in the transgenic lines than in the wild type, indicating that UGTs can be altered to modify the ability of plants to tolerate and detoxify TNT. Although little is currently known regarding the biochemical and molecular genetic mechanisms of detoxification of TNT in plants, an understanding of these systems may allow the identification and breeding of robust native plant species with highly active detoxification pathways for phytoremediation of contaminated sites. This study establishes the role of UGTs in the detoxification of TNT, and provides insight into the elucidation of the complete detoxification pathway of this toxic and widespread pollutant.

Experimental procedures

Biochemicals

The TNT was kindly provided by the Defence Science and Technology Laboratory (http://www.dstl.gov.uk). The HADNT and ADNT isomers were obtained from Accustandard (http://www.accustandard.com). HADNT- and ADNT-MGs were produced by UGTs and purified using HPLC.

Treatment of Arabidopsis plants

Arabidopsis, ecotype Col0, seeds were stratified for 3 days at 4°C in the dark on agar plates containing half-strength MS (1/2 MS) medium (Murashige and Skoog, 1962) with 20 mM sucrose. Plates were transferred to 125 μM m^{-1} sec^{-2} light, with a 16-h photoperiod, at a 20–18°C day and night temperature for 24 h, and then 10 seedlings were transferred to 500-ml conical flasks containing 100 ml 1/2 MS medium, 20 mM sucrose and 1x Gamborg's vitamin solution (Sigma-Aldrich, http://www.sigmaaldrich.com), and were grown at 130 rpm, with 20 μM m^{-1} sec^{-2} light, for 13 days. Flasks were dosed with 60 μM TNT dissolved in 60 μl DMF, or with 60 μl of DMF only, and after 6 h RNA was harvested.

Extractions of TNT transformation products from plants

Five 14-day-old seedlings, grown in 50 ml of 1/2 MS + sucrose media in 100-ml flasks were treated with 100 μM TNT for 6 h. The freeze-dried, ground, plant tissue was extracted twice with MeOH and was then analysed by LC-MS.

RNA isolation, microarray hybridization and data analysis

The RNA was extracted using the Qiagen RNAeasy kit (Qiagen, http://www.qiagen.com), and RNA integrity was determined using Bioanalyser Agilent 2100 chip technology (http://www.agilent.com). The cDNA synthesis and Arabidopsis ATH1 genome array chip labelling and hybridization were performed according to the manufacturer's instructions (Affymetrix; http://www.affymetrix.com/support/technical/manual/expression_manual.affx), and three independent, pair-wise replicates were performed. Chips were analysed further using GENESPRING software (version 6.0; Silicon Genetics, http://www.chem.agilent.com). Following Lowess normalization, the fold change was calculated by dividing the TNT-treatment value by the control value. A one-way ANOVA Welch's *t*-test ($P = 0.05$) was performed, and then genes with a twofold and higher increase in gene expression were selected for further analysis. These genes were annotated using gene ontology software (GENESPRING) based on information from the International Arabidopsis Genome Initiative sequencing project, in collaboration with The Institute for Genome Research. Hybridization quality was measured by comparing the expression ratios of control genes on the arrays that were within the range recommended by Affymetrix.

Semiquantitative RT-PCR

The cDNA was synthesized from 10 μg of DNase-treated RNA using 2 μl of Superscript II according to the manufacturer's protocol (Invitrogen, http://www.invitrogen.com). PCR was performed using serial dilutions of cDNA and the following conditions: 95°C for 2 min, then 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 1 min and then 72°C for 10 min. The *actin2* gene (GenBank Accession No. NM_180280) was used as a constitutively expressed control. Primer sequences were as follows: UGT73B4, 5′-CAACAGGGGATTTGAGGAGA-3′ and 5′-AACCTCCCTCACCGCTTTAT-3′; UGT74E2, 5′-TGATTTGCCGTCTTTCCTC-3′ and 5′-ACAAACCCATCACCTTCTGC-3′; UGT73C1, 5′-CGCACATAATGCACCAAAAC-3′ and 5′-ATCAATGTCCGCCTTGTTTC-3′; UGT73C6, 5′-CTTCAAGGAGGCAAGGTCTG-3′ and 5′-GCCTTGTGAGCTGATTCTCC-3′; UGT73B2, 5′-CAACAGGGGATTTGAGGAGA-3′ and 5′-AACCTCCCTCACCGCTTTAT-3′; UGT73B5, 5′-GAATGCCTAAAATGGCTGGA-3′ and 5′-GAGTTCCATCCGCAATGAGT-3′; GT75B1, 5′-CCACCGCATTTTCTACTGGT-3′ and 5′-CGTGGGAAGTAAAGGACCAA-3′; and actin, 5′-CTTACAATTTCCCGCTCTGC-3′ and 5′-GTTGGGATGAACCAGAAGGA-3′.

Recombinant UGT purification

Recombinant UGTs containing an N-terminal, 26-kDa glutathione S-transferase (GST) were constructed as described previously (Lim *et al.*, 2003). The UGT-GST fusion proteins were expressed in *Escherichia coli* strain BL21, grown at 37°C in Luria Broth containing 50 μg ml^{-1} ampicillin to an A_{600} of 0.8–1.0, prior to induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 24 h at 20°C. Cells were harvested by centrifugation at 5000 ***g*** for 5 min, resuspended in ice-cold phosphate-buffered saline, 0.2 mM phenylmethylsulphonyl fluoride (PMSF), and were then lysed using a French Press. Proteins in the supernatant were collected using glutathione-coupled Sepharose 4B beads (Amersham Biosciences, http://www.amersham.com) according to the manufacturer's instructions. The protein level was quantified using the Bio-Rad Protein Assay Dye (http://www.bio-rad.com/), with bovine serum albumin as a reference, and was then analysed by 10% SDS-PAGE.

Glucosyltransferase activity assay

Unless otherwise stated, the assay was based on that of Lim *et al.* (2001), with modifications: the assay mix (65 μl) contained 6.5 μg of purified recombinant protein, 10 mM $MgCl_2$ and 5 mM UDP-G, with 200 μM of either HADNT or ADNT. Reactions were initiated with enzyme at 30°C, and were terminated by the addition of a tenth of the assay volume of trichloroacetic acid (TCA) (240 mg ml^{-1}). Samples were stored at –80°C prior to analysis using HPLC. Because of the low solubility limits of TNT derivatives, the highest concentration assayed was 500 μM. Kinetic parameters were estimated from experiments varying the substrate concentration within the range of TNT solubility. The MGs used as substrates were HPLC purified from 2-HADNT glucosylation media. Data analysis was carried out by using nonlinear regression fitting (Marquardt, 1963), using SigmaPlot v10.0 (2006; Systat Software Inc., http://www.systat.com). The lack of activity saturation using the 4-HADNT substrate prevented an accurate estimation of K_m using nonlinear regression fitting, and thus the ratio V_m/K_m was determined by linear fitting to pseudo first-order kinetics ($[S] \ll K_m$).

HPLC analysis

Reverse-phase HPLC was performed using a Waters HPLC system (Waters Separator 2695 and Waters Absorbance Detector 2996; Waters, http://www.waters.com) and a Techsphere 80 5-μM C_{18} column (250 × 4.6 mm; Chromacol, http://www.chromacol.com). Mobile phase A (used for ADNTs conjugation analyses): 0–9 min, 70% distilled H_2O (solvent A), 30% acetonitrile (solvent B); 9–10 min gradient to 50% A, 50% B; then 10–15 min 50% A, 50% B. Mobile phase B (used for HADNT conjugation analyses): 0–5 min, 90% A, 10% B; 5–7 min gradient to 70% A, 30% B; then 7–14 min 70% A, 30% B; 14–15 min gradient to 50% A, 50% B; and then 15–20 min at 50% A, 50% B. Mobile phase C (used for glucosides identification in plant incubation complex mixtures): 0–5 min, 90% A, 10% B; 5–14 min gradient to 70% A, 30% B; then 14–21 min gradient to 50% A, 50% B; and then 21–29 min at 50% A, 50% B.

Liquid chromatography–mass spectrometry of the ADNT and HADNT glucose conjugates

Reverse-phase HPLC-MSn was performed using a TSP HPLC system coupled to an LCQ ion-trap mass spectrometer (Thermo Electron Corporation, http://www.thermo.com). Separation was achieved using a 5-μM LUNA phenyl-hexyl column (250 × 3 mm; Phenomenex, http://www.phenomenex.com) at 30°C. Mobile phases were as follows: A, water; B, acetonitrile; C, methanol. The gradient program was as follows: 0–2 min, isocratic 90% A, 2% B, 8% C; 2–20 min, linear gradient to 20% B, 80% C; 20–25 min, isocratic 20% B, 80% C; 25–25.1 min return to isocratic start conditions; 25.1–30 min, re-equilibration time. The flow rate was 0.5 ml min^{-1}. For analyses of TNT-reduced glucose conjugates in plant material, the chromatographic system described in the HPLC analysis section was used. The injection volume was 25 μl. In all cases, the column eluate was monitored at 230 and 340 nm with a PDA detector (Thermo Electron Corporation) before electrospray ionization (ESI) at the LCQ interface. Ions were generated in negative ESI mode with the following source conditions: source voltage, 4.5 kV; capillary temperature, 200°C; capillary voltage, –20 V; sheath gas flow, 65 units; auxiliary gas flow, 30 units. A source-induced dissociation (SID) voltage of 10 V was applied to minimize dimerization. Full-scan mass spectra were obtained from three averaged microscans in the range 150–600 *m/z*, with the automatic gain control set to 1×10^7. Ions were further subjected to data-dependent collisionally induced fragmentation in the ion trap. MS2 ions were generated from the most intense ions in the full-scan mass spectra, and MS3 ions were generated from the most intense MS2 ions. An isolation width window of 3 *m/z* and normalized collisional energy of 35% was used for MS2 and MS3 scans. Dynamic exclusion was set up so that six MSn scans were collected before the parent ion was put on the exclusion list for 0.5 min. This generally allowed up to three, closely or co-eluting peaks in the full-scan ms trace to be automatically selected for MSn analysis. Extracted ion chromatograms from the full-scan mass spectra were integrated using Xcalibur 1.2 (Thermo Electron Corporation) to give the peak area data for specific compounds, which were subsequently identified by reference to standards and MSn spectra. Where appropriate, mass-accurate high-resolution MS and MS2 spectra were collected from HPLC-purified infused samples on an LTQ-Orbitrap mass spectrometer (ThermoFisher, http://www.thermofisher.com) operated in negative ESI mode at a resolution setting of 100 000 FWHM at *m/z* 400.

Overexpression of UGT73B4 and UGT73C1 in Arabidopsis

The UGT cDNAs were cloned into the binary vector system, pMLBart (Gleave, 1992), under the control of the CaMV 35S promoter and ocs terminator. The primers designed were as follows: UGT73B4, 5′-ACCTCGAGAACAATGAACAGAGAGC-3′ and 5′-ATTCTAGACTACTTTCTACCATTCA-3′; and UGT73C1, 5′-ACAAGCTTAACAATGGCATCGGAAT-3′ and 5′-ATTCTAGATCATTTCTTGGGTTGTTC-3′. The binary constructs were introduced into Arabidopsis plants, ecotype Col0, by *Agrobacterium*-mediated transformation using floral dipping. Transformants were selected on medium supplemented with glufosinate-ammonium (50 μg ml^{-1}). Homozygous T_3 generation plants were used in this study.

Transgenic plant experiments

Arabidopsis seeds were germinated on agar plates containing 1/2 MS media + 20 mM sucrose with TNT dissolved in DMF (final concentration 0.0035%). ADNTs were dissolved in acetonitrile (final concentration 0.135%). Liquid culture experiments were performed as described in Rylott *et al.* (2006). For incubation with ADNTs, 5 ml of media was added to flasks containing eight, 16-day-old seedlings.

Acknowledgements

This work was funded by the Strategic Environmental Research and Development Program (SERDP) of the US Department of Defense and the Garfield Weston Foundation. FGH acknowledges EMBO for a fellowship award.

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Accession numbers: The microarray data are publicly available via the Nottingham Arabidopsis Stock Centre microarray database (http://affymetrix.arabidopsis.info/) under experiment ID NASCARRAYS-472.

