Expression of a Drosophila glutathione transferase in Arabidopsis confers ability to detoxify the environmental pollutant, and explosive, 2,4,6-trinitrotoluene.

Brief heading: Drosophila glutathione transferase detoxifies 2,4,6-trinitrotoluene.

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Total word count	4,198	Discussion	1,219
Introduction	539	Number of Figures	7
Materials and Methods	924	Number of Tables	0
Results	1,516	Number of Supporting Information files	0

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Summary

- The explosive 2,4,6-trinitrotoluene (TNT) is a significant, global environmental pollutant that is both toxic and recalcitrant to degradation. Given the sheer scale, and inaccessible nature of contaminated areas, phytoremediation may be a viable clean-up approach. Here, we have characterised a *Drosophila melanogaster* (Meigen, 1830) glutathione transferase (*Dm*GSTE6) which has activity towards TNT.

- Recombinantly-expressed, purified *Dm*GSTE6 produces predominantly 2-glutathionyl-4,6-dinitrotoluene, and has a 2.5-fold higher V_{max}, and 5-fold lower K_m than previously characterised TNT-active *Arabidopsis thaliana* (L.) Heynh (Arabidopsis) GSTs. Expression of *Dm*GSTE6 in Arabidopsis conferred enhanced resistance to TNT, and increased ability to remove TNT from contaminated soil relative to wild-type plants.

- Arabidopsis lines overexpressing TNT-active GSTs *At*GST-U24 and *At*GST-U25 were compromised in biomass production when grown in the absence of TNT. This yield drag was not observed in the *Dm*GSTE6 expressing Arabidopsis lines. We hypothesise that increased levels of endogenous TNT-active GSTs catalyse excessive glutathionylation of endogenous substrates, depleting glutathione pools, an activity that *Dm*GST may lack.

- In conclusion, *Dm*GSTE6 has activity towards TNT, producing a compound with potential for further biodegradation. Selecting or manipulating plants to confer *Dm*GSTE6-like activity could contribute towards development of phytoremediation strategies to clean up TNT from polluted military sites.

Key words: Arabidopsis, conjugation, detoxification, *Drosophila melanogaster*, environmental pollutant, glutathione transferase, phytoremediation, 2,4,6-trinitrotoluene.

Introduction

The explosive compound 2,4,6-trinitrotoluene (TNT) has been extensively used by the military worldwide for many decades. TNT is remarkably resistant to biodegradation and is now classed as a possible human carcinogen and serious environmental pollutant by the United States Environmental Protection Agency (2014). In the US alone, there are an estimated 10 million hectares of military land contaminated with munitions components (United States Defense Science Board Task Force. 1998; United States General Accounting, 2004), and many contaminated sites in Europe and Asia (Kalderis *et al.*, 2011; Pichtel, 2012). For example, the Werk Tanne former ammunition site in Germany, detonated in 1944, is heavily contaminated with TNT (Eisentraeger *et al.*, 2007). Increased environmental awareness is now compelling governments to identify sites of explosives contamination and put together remediation strategies (Lima *et al.*, 2011). However, a major challenge to cleaning-up these sites is the sheer scale and hazardous nature of many contaminated sites, which rules-out many strategies such as excavation, land fill and off-site treatments, as prohibitively expensive. Phytoremediation may be a viable alternative approach.

TNT is not readily degraded in the environment due to the electron-withdrawing properties of the three nitro groups of TNT which render the aromatic ring particularly resistant to oxidative attack and ring cleavage (Qasim *et al.*, 2009); the main route of aromatic compounds by soil microbes. Instead microbial flora catalyse a series of reductive reactions, producing predominantly hydroxylamino dinitrotoluene (HADNT) and amino dinitrotoluene (ADNT) and further reduced derivatives (Rylott *et al.*, 2011b). In plants, HADNT and ADNT can be conjugated to sugars, for example, to glucose by UDP-glucosyltransferases (Gandia-Herrero *et al.*, 2008), and it has recently been shown that glutathione transferases can conjugate the TNT molecule directly (Gunning *et al.*, 2014; Rylott *et al.*, 2015). Two *Arabidopsis thaliana* (L.) Heynh (Arabidopsis) glutathione transferase (GST) genes, *AtGST-U24* and *AtGST-U25*, are specifically upregulated in response to TNT exposure, and their gene products catalyse the formation of three characterised TNT glutathionyl-products (Gunning *et al.*, 2014). The removal of a nitro group in one of the three products, 2-glutathionyl-4,6-dinitrotoluene, has the potential to be more amenable to subsequent biodegradation in the environment, a property that could be applied *in planta* for the

detoxification of TNT in the field. Expression of *At*GST-U24 and *At*GST-U25 in Arabidopsis conferred increased ability to take up and detoxify TNT; however, in the absence of TNT, overexpression of these GSTs caused a reduction in plant biomass; an effect with deleterious implications for xenobiotic detoxification (Gunning *et al.*, 2014). In a more recent study, two poplar GSTs, *PtGST-U16* and *PtGST-U24*, were found to be strongly upregulated in response to TNT. However, the encoded enzymes exhibited only low (< 0.05 $nmol.min^{-1}.mg^{-1}$) specific activity toward TNT, and are unlikely to play a major role in the detoxification of TNT in poplar (Musdal & Mannervik, 2015).

In a recent study a *Drosophila melanogaster* glutathione transferase (*Dm*GSTE6) was found to display outstanding activity toward TNT (Mazari & Mannervik, 2016). This research describes the characterisation of *Dm*GSTE6, which has greater activity towards TNT than *At*GST-U24 and *At*GST-U25. We have engineered Arabidopsis plants to express *Dm*GSTE6, and assessed its potential for the *in planta* detoxification of TNT, with the aim of developing such technologies for the phytoremediation of TNT-contaminated military training ranges.

Materials and Methods

Chemicals

TNT was provided by the Defence Science and Technology Laboratory (DSTL) (Fort Halstead, Kent, United Kingdom).

Expression of Dm*GSTE6 in* Escherichia coli *and Arabidopsis*

The *DmGSTE6* gene (NCBI accession number NT_033778) was cloned into pET-YSBLIC3C, expressed in *E. coli* and purified as described in Gunning *et al.* (2014). For expression in Arabidopsis, *DmGSTE6* was cloned into the intermediary pART7 vector. The subsequent DNA cassette containing *DmGSTE6*, flanked by CaMV-*35S* promoter and *ocs* terminator regions, was transferred into the binary vector pART27 using *Not*I restriction sites (Gleave, 1992). The pART27 vector contains a selectable marker, *nptII*, which confers resistance to kanamycin. Following transformation, using the floral dipping method (Clough & Bent, 1998), primary transformants were identified by screening on agar plates containing half-strength Murashige and Skoog medium (Murashige & Skoog, 1962) (½ MS) plus 50

mg/L kanamycin. T2 lines with kanamycin-resistance segregation ratios indicative of single insertional events were selected, and independent, T3 and T4 generation plants, homozygous for kanamycin resistance, were used in subsequent experiments.

GST assays using CDNB

Conjugating activity of the purified proteins, and crude extracts from rosette leaves, was assessed using the model GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) as described previously (Colville & Smirnoff, 2008). Briefly, the reaction, carried out at 20°C, comprised 100 mM potassium phosphate buffer pH 6.5, 5 mM reduced glutathione (GSH) and 500 ng of purified *Dm*GSTE6 and was initiated by addition of 1 mM CDNB to a total volume of 1 ml. Increase in absorbance at A_{340} measured spectrophotometrically.

GST assays using TNT

Reactions, carried out at 30°C, contained 100 mM potassium phosphate buffer, 10 µg of purified *Dm*GSTE6 and 5 mM GSH and were initiated by addition of TNT to a final volume of 250 µl. Reactions were stopped by the addition of trichloroacetic acid, to a final concentration of 10% (v/v), and samples analysed by HPLC.

Control reactions using *At*GST-U25 contained 150 µg of enzyme. The glutathione peroxidase activity (GPOX) assays were performed according to Edwards & Dixon (2005) with modifications (Gunning *et al.* 2014). Michaelis-Menten K_m and V_{max} parameters for Lineweaver-Burke plots were calculated using Sigma Plot v. 12.0.

Measurement of TNT and products

The TNT, ADNT and conjugates were analysed by HPLC using a Waters (Milford USA) HPLC system (Waters 2695 separator and Waters Photodiode array detector) with Waters X-Bridge C18 column (300 × 4.5 mm, 5 µm). The mobile phases for the gradient conditions were as reported in Gunning *et al.* (2014), with the exception of data presented in Figure 7 (see later) which used the following: mobile phase A, acetonitrile; mobile phase B, 50 mM NaH_2PO_4, pH 2.7, with 85% (v/v) phosphoric acid. The gradient ran: 0 min 0 % A 100 % B, 6 min 0 % A 100 % B, 11 min 50 % A 50 % B, 25 min 100 % A 0 % B, 30 min 0 % A 100 % B, runtime 30 min. Peaks were identified and quantified using purified conjugates as described in Gunning *et al.* (2014). The expected retention times were: TNT, 30.9 min;

conjugate 1, 16.7 min; conjugate 2, 20.2 min; conjugate 3, 21.0 min. Integration was performed at 250 nm with Empower Pro Software.

Nitrite measurement

Nitrite production was measured using Griess assays according to the method of French et al. (1998) with modifications as described in Gunning *et al*. (2014).

Chlorophyll measurement

Chlorophyll was extracted based on the method of Arnon (1949). Briefly, 100 mg of fresh tissue was ground in 500 µl of 80% acetone (v/v) , centrifuged at 12,000 g for two min at 4 °C and the supernatant assayed spectrophotometrically at 645 and 663 nm.

Gene expression

Plant RNA was extracted from three-week-old rosette leaves using the Isolate II RNA plant kit (Bioline, London, UK) and cDNA was synthesised using oligo(dT)12-18 using Superscript II reverse transcriptase (Thermo Fisher Scientific, Waltham, USA) containing RNAsin (Promega, Madison, USA) at 42 °C for 2 h, before inactivation at 70 °C for 15 min. Synthesised cDNA was purified using Wizard DNA Clean Up System (Promega) and quantified. Quantitative reverse transcription PCR (qPCR), using the primers dqPCR1_F 5'-GGACGACGGTCACTACATCT-3' and dqPCR1_R 5'-GCCGCTTTCAAAATGCAGAC-3',was performed using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) with SYBR green reporter dye. Data were normalised to expression levels of the internal control gene (*ACT2*, At3g18780) using primers qActinF 5'-TACAGTGTCTGGATCGGTGGTT-3' and qActinR 5'-CGGCCTTGGAGATCCACAT-3', and the comparative ΔΔCt method (Livak & Schmittgen, 2001) used to calculate the mean fold change in expression of *DmGSTE6*.

Agar plate experiments

Seeds were stratified for three days then germinated and grown on agar plates containing ½ MS and a range of TNT concentrations (dissolved in DMSO; final DMSO concentration 0.05% (v/v)). To determine the surface area of roots, Adobe Camera Raw ver. 6.0 software was used to remove non-root background from each image. The surface area, in pixels, was then determined using Adobe Photoshop software.

Liquid culture experiments.

Eight seven-day-old seedlings were transferred to each 100 ml conical flask containing 20 mL of ½ MS medium plus 20 mM sucrose. Plants were grown for two weeks under 20 µmol m^{-2} s^{-1} light on a rotary shaker at 130 rpm. After this time, the medium was replaced with 20 ml of 20 mM sucrose amended with 250 µM TNT and a range of GSH concentrations.

Soil studies

The TNT-contaminated soil studies, and subsequent isolation of TNT and ADNTs, were conducted as previously described in Rylott *et al*., (2011a).

Statistical Analysis

Data were analysed for statistical significant using ANOVA, with post hoc Tukey's HSD, using SPSS version 22 software.

Results

Activity of DmGSTE6

Following recombinant expression and purification, *Dm*GSTE6 was analysed by SDS-PAGE for purity and integrity. Figure 1a shows the purified *Dm*GSTE6, with an expected size of approximately 25 kDa. The enzyme kinetics for *Dm*GSTE6 using TNT as substrate, were determined and the Michaelis-Menten plot is shown in Figure 1b. The V_{max} and K_m values were 235 ± 3.9 $nmol.min^{-1}.mg^{-1}$ and 269.5 ± 17.5 µM respectively. While GPOX activity was detected in purified *At*GST-U25, GPOX activity was not detected for *Dm*GSTE6 (results not shown).

Previous studies have shown that, dependent on pH and temperature, *At*GST-U25 produces three different TNT-conjugates as shown in Figure 2a. The TNT conjugating activity by *Dm*GSTE6 was maximal at pH 9.0, where almost 50 % of the initial TNT was conjugated within an hour (Figure 2b); at pH 5.5 the enzyme exhibited less than 1 % of the activity at pH 9.0. Of the three TNT-GSH conjugates identified previously (Gunning *et al.*, 2014), *Dm*GSTE6 produced almost exclusively conjugate 3 across the pH range tested. Small amounts of conjugate 2 were produced at pH 8.0 and higher, while conjugate 1 was not detected. No significant changes were observed in the TNT concentration of control reactions

containing denatured *Dm*GSTE6, confirming the absence of non-enzymatic conjugation and the stability of TNT at the different pH values tested. TNT-conjugating activity of *Dm*GSTE6 was detected across the full range of temperatures tested (from 4 °C to 50 °C), with maximal activity at 30 °C (Figure 2c). At all these temperatures *Dm*GSTE6 produced almost entirely conjugate 3, with low but progressively increasing levels of conjugate 2 produced from 20 to 42 °C.

Conjugate 3 production should result in the concomitant stoichiometric release of nitrite 1:1. To measure nitrite production, Griess assays were used. The results presented in Figure 2d show that across the three pH values tested, *Dm*GSTE6 produced conjugate 3 to nitrite ratios at close to 1:1 (1: 1.18 at pH 6.5; 1:0.92 at pH 8.0 and 1:1.14 at pH 9.5). The *Dm*GSTE6 produced significantly higher amounts of nitrite than *At*GST-U25; nitrite was not detected with *At*GST-U24 which is unable to produce conjugate 3. Nitrite release was not observed from the denatured *Dm*GSTE6 control, but low levels of nitrite were detected in the absence of GSH. Since the amount of nitrite increased with increasing pH, this release is probably the result of alkaline hydrolysis. Qasim et al. (2009) have reported significant alkaline hydrolysis of TNT in aqueous solutions at high pH. Under such alkaline conditions, polymerisation reactions can also occur between the TNT molecules, reducing the number of exposed nitro groups. The presence of enzyme could reduce polymerisation by binding TNT molecules into the active site or in non-catalytic ligand binding sites that have been previously identified in plant GSTs (Dixon et al., 2011), allowing further alkaline hydrolysis to occur. Conjugating activity of *Dm*GSTE6 towards ADNTs and HADNTs was tested, but no conjugated products were detected (data not shown).

Expression of Dm*GSTE6 in Arabidopsis*

To assess the ability of *Dm*GSTE6 to conjugate and detoxify TNT *in planta*, Arabidopsis lines expressing *Dm*GSTE6 were generated. Seven, homozygous *Dm*GSTE6 expressing lines, were assayed for CDNB-conjugating activity. As shown in Figure 3a, the seven lines exhibited a range of activities. Lines dGST-1, 2 and 3 which had 2.4, 1.6 and 2.1-fold respectively more CDNB activity in roots than wild type plants were selected for further analysis. To confirm that *Dm*GSTE6 was expressed in the lines, qPCR was used to measure transcript levels. Figure 3b confirmed that all three lines were expressing the transgene.

To establish whether the dGST lines had increased ability to produce conjugate 3, root protein extracts were assayed for TNT-derived nitrite release using the Griess assay. As controls, lines over-expressing *At*GST-U24, which does not produce conjugate 3, and *At*GST-U25, which produces conjugate 3, were included. The results in Figure 3c demonstrated that all three dGST lines produced higher amounts of free nitrite than the *At*GST-U25 over-expressing lines, and thus more conjugate 3, confirming that these lines had a higher conjugation activity *in planta* than the *At*GST-U25 over-expression line. Protein extracts from wild type and the *At*GST-U24 over-expression line generated amounts of free nitrite close to those of the *At*GST-U25 over-expression line. This was probably the result of endogenous *At*GST-U25 present in those samples; approximately half of the conjugates produced *in vivo* by *At*GST-U25 are predicted to be conjugate 3, with concomitant release of nitrite, whereas *At*GST-U24 produces almost exclusively conjugate 2 (Gunning *et al.*, 2014).

To compare the resistance of the dGST plant lines to TNT with that of the *At*GST-U24 and *At*GST-U25 over-expression lines, the plants were grown for 20 days on ½ MS agar plates containing a range of TNT concentrations, alongside wild type and the selected *At*GST-U24 and *At*GST-U25 over-expression lines. The appearance of the wild type, dGST and *At*GST-U24 plants at the end of the experiment is given in Figure 4a. Concentrations of TNT up to 7 µM were probably not toxic enough to induce symptoms, since no significant differences in root surface area were recorded among the different plant lines (Figure 4b). However, at higher TNT concentrations, all of the dGST lines displayed higher root surface areas than either wild type or the *At*GST-U24 and *At*GST-U25 over-expression lines. In more detail, when grown on ½ MS agar plates containing 30 µM TNT, line dGST-3 displayed a 4.4-fold higher root surface area than wild type.

Contaminated soil studies on Dm*GSTE6-expressing Arabidopsis*

To assess the ability of the *Dm*GSTE6-expressing lines to remediate TNT from soil, the lines were grown for six weeks in soil contaminated with TNT. The appearance of the plants after six weeks is shown in Figure 5a. Earlier studies reported that the over-expression of *At*GST-U24 and *At*GST-U25 resulted in reduced plant biomasses in the absence of TNT; however, the shoot and root biomasses of the dGST lines were indistinguishable from the wild type lines when grown in the absence of TNT. As predicted from earlier studies (Rylott *et al.*,

2011a; Gunning *et al.*, 2014), at TNT concentrations above 50 mg.kg^{-1} TNT, wild type plants appeared chlorotic and severely stunted. On the contrary, the dGST lines appeared green, with less stunting. All three dGST lines were able to continue growing at 200 mg.kg^{-1} TNT, a concentration found to completely inhibit growth for wild type; and *At*GST-U24 and *At*GST-U25 over-expressing lines. The shoot and root biomasses were recorded after six weeks, and are presented in Figure 5b and c. In TNT-contaminated soil, both dGST-1 and dGST-3 produced significantly more root and shoot biomass than wild type; although line dGST-2 was not significantly different from wild type. Line dGST-1 exhibited the greatest resistance to TNT toxicity, attaining shoot and root biomasses 2.4 and 3.2-fold higher than wild type at 100 mg kg^{-1} TNT; and 2.8 and 4.8-fold higher at 200 mg kg^{-1} TNT, respectively. To gauge the ability of the dGST lines to remove TNT from the contaminated soil, the levels of TNT, and ADNT, resulting from the transformation of TNT by soil-based microbial communities, were determined. To do this, soil from the pots containing 50 mg kg^{-1} TNT that the plants had been growing in for six weeks was used. At this concentration aerial biomass was not significantly different between the lines. As shown in Figure 6, levels of TNT and ADNT from soil in which the dGST lines had been grown were significantly lower than in the soil from wild type plants.

Role of glutathione in TNT detoxification

It has previously been shown that plants with GST-enhanced ability to detoxify TNT by conjugation have depleted GSH levels when grown in the presence of TNT (Gunning *et al.*, 2014). With the hypothesis that GSH is limiting GST-catalysed detoxification of TNT, the application of exogenous GSH to liquid culture systems was tested to see if GSH could enhance a potentially limiting supply of endogenous GSH. Plants were grown in liquid cultures containing TNT, and a range of GSH concentrations, and TNT uptake monitored for one week (Figure 7).

In the absence of GSH, the dGST/1 line, as expected, removed TNT more quickly than wild type plants, with significantly more removed after 24 hours (67 and 49% respectively of the TNT, $P < 0.05$, Figure 7a). When 100 μM of GSH was present in the medium, the rate of TNT uptake increased for both wild type and dGST/1 plants, again with significantly more TNT removed after 24 hours (83 and 64 % respectively of the TNT, $P < 0.01$, Figure 7b).

Increasing the GSH concentration to 250 µM enhanced TNT uptake only slightly in dGST/1 plants and did not enhance the uptake in wild-type plants, which displayed a lower TNT uptake rate than that observed in the absence of GSH (Figure 7c). When 1000 µM GSH was present, a strong toxic effect was observed on the plants which became chlorotic (Figure 7d). To quantify the toxic effect of GSH on the plants, chlorophyll content was measured at the end of the experiment. Total chlorophyll content decreased in both dGST-1 and wild type plant lines in a dose-dependent manner with increasing concentrations of GSH (Figure 7e).

Discussion

Activity of Dm*GST*

*Dm*GSTE6 was found to catalyse the conjugation of GSH to TNT producing almost exclusively conjugate 3, 2-glutathionyl-4,6-dinitrotoluene, and concurrently a 1:1 stoichiometric release of nitrite. Furthermore, *Dm*GSTE6 has both an increased affinity towards TNT (K_m = 269.5 ± 17.5 µM) and significantly higher V_{max} (235 ± 3.9 nmol.min^{-1}.mg^{-1}**)** than values reported by Gunning *et al.* (2014) for endogenous *At*GST-U24 and *At*GST-U25 (K_m = 1644 ± 113.2 and 1210 ± 85.7 µM; Vmax = 92.3 ± 2.6 and 98.39 ± 3 nmol.min^{-1}.mg^{-1}, for *At*GST-U24 and *At*GST-U25 respectively).

The pH optimum for *Dm*GSTE6 activity towards TNT of pH 9.0, is in agreement with that observed for both *At*GST-U24 and *At*GST-U25 (Gunning et al., 2014) and can at least partly be attributed to ionisation of the sulfhydryl group of GSH, which has a pKa of 9.4, forming the reactive thiolate anion (Dixon & Edwards, 2010). However, within the roots, the site of TNT detoxification in dicot and grass species (Sens et al., 1998; Sens et al., 1999; Brentner et al., 2010), the pH of the cytosol is estimated to be within the range of 6.5 to 7.9 (Scott & Allen, 1999; Moseyko & Feldman, 2001; Tournaire-Roux et al., 2003). Although the activity of *Dm*GSTE6 is lower at pH7.5 than at pH9.0, our studies indicate that only conjugate 3 would be produced within the roots.

Is TNT detoxification GSH limited?

In the *Dm*GSTE6 expressing Arabidopsis lines, the yield drag observed in *At*GST-U24 and *At*GST-U25 overexpressing Arabidopsis lines grown in the absence of TNT, was absent. It is possible that over-expression of *At*GST-U24 and *At*GST-U25 causes damage via excessive

glutathionylation of endogenous substrates, and subsequent depletion of GSH pools; *Dm*GSTE6 could lack activity towards these plant-endogenous substrates. However, the enhanced resistance and ability to take up TNT observed in the dGST lines was similar to that reported for the *At*GST-U24 and *At*GST-U25 overexpressing lines; the increased affinity and activity of *Dm*GSTE6 for TNT, observed in the studies on purified protein, compared to the *At*GST-U24 and *At*GST-U25 enzymes, did not translate into the predicted further increases in resistance and ability to take up TNT when expressed *in planta*.

We hypothesise that the constraint on TNT uptake and detoxification is due to limiting GSH levels in the root cytosol. The GSH abundance in the Arabidopsis cytosol is predicted to be in the range of 1 to 3 mM (Meyer et al., 2001; Meyer & Fricker, 2002), a concentration that is likely to be high enough to efficiently detoxify TNT in the presence of sufficient GST activity. However, it is possible that GSH levels become limited because they are utilised by other biochemical processes, compartmentalised to secure the GSH levels of specific organelles or the actual GSH levels are lower than those reported. In support of this hypothesis is the observation that the addition of exogenous GSH increased the ability of both wild type and dGST plants to remove TNT from liquid media. This is in agreement with the findings of Zechman *et al*. (2011) who reported that low pollen germination rates induced by treatment with the GSH synthesis inhibitor buthionine sulfoximine could be restored by the addition of 1 mM GSH to the growth media without any toxic effects. In addition to the requirement for GSH by *Dm*GSTE6, TNT phytotoxicity is caused by its redox cycling activity (Johnston *et al.,* 2015). As GSH is important for redox homeostasis, depletion of GSH via TNT conjugation could compound the phytotoxicity of remaining TNT.

The fate of TNT

We have shown that *Dm*GSTE6 catalyses a denitration step producing 2-glutathionyl-4,6-dinitrotoluene. Based on studies of herbicides and other xenobiotics (Edwards *et al.*, 2011), TNT-conjugates are thought to be imported into the vacuole; two characterised glutathione-conjugate ABC transporters, MRP1 and MRP2 (Lu *et al.*, 1998; Tommasini *et al.*, 1998), are up-regulated in Arabidopsis, in response to TNT (Gandia-Herrero *et al.*, 2008). Once in the vacuole, further processing of GST-conjugated xenobiotics can occur to salvage the cysteinylglycine, γ-glutamylcysteine and cysteine derivatives (Grzam *et al.*, 2006; Ohkama-

346 Ohtsu *et al.*, 2007); but, the downstream processing of 2-glutathionyl-4,6-dinitrotoluene is
347 not known. It is possible that it, or a cleaved dinitro-derivative, could be released upon
348 evacuolation as tissues are converted into woody biomass, but dinitrotoluene-degrading
349 activities have not been reported in plants, so mineralisation *in planta* appears unlikely.
350 However, fungi and bacteria with the ability to mineralise dinitrotoluene have been reported
351 (Valli *et al.*, 1992; Johnson & Spain, 2003) and biodegradation of 2-glutathionyl-4,6-
352 dinitrotoluene by soil microbes during decomposition at the end of the plant life cycle is
353 plausible.

354 *Potential of transgenic plants for TNT detoxification*
355 The TNT pollution on military training ranges is heterogenic with levels of 100 mg/kg soil
356 not uncommon and hotspots in excess of 10,000 mg/kg (Jenkins et al., 2006, Talmage et al.,
357 1999). Arabidopsis is not a field-applicable species, but the studies presented here were
358 conducted within the lower bounds of TNT contamination found on military ranges, and
359 demonstrate that plants expressing increased levels of TNT-active GSTs are more tolerant to
360 TNT. When compared to earlier studies expressing bacterial enzymes such as nitroreductases
361 (Hannink *et al*., 2001, Rylott *et al*., 2011a) and pentaerythritol tetranitrate reductase, (French
362 *et al*., 1999), the GST-linked increase is only moderate. Such expression of bacterial
363 reductases in plants increases the conversion of TNT to HADNT and ADNT, which can
364 condense to form diarylamines (van Dillewijn *et al*., 2008b; Wittich *et al*., 2008) or be
365 subsequently converted to sugar conjugates (Gandia-Herrero *et al.*, 2008) and it is likely that
366 in the longer term, these compounds become incorporated into plant macromolecular
367 structures such as lignin.

368 However, HADNT and ADNT are not substrates for *Dm*GSTE6 and the results presented
369 here indicate that increasing the flux towards production of conjugate 3, requires an increase
370 in glutathione levels. Arabidopsis plants with increased levels of Ɣ-glutamyl cysteine
371 synthase have increased levels of GSH (Dhankher *et al*., 2002), and could perhaps be
372 combined with *Dm*GST activity.

373 In tandem with the development of transgenic plants for phytoremediation, it is important to
374 understand the impact such modified plants could have on training range ecosystems. While

there are many studies on the ecotoxicity of TNT, such analyses on transgenic plants are still needed.

Developing field-applicable plant species

While Arabidopsis is an excellent model system for elucidating, and manipulating, the mechanisms of TNT detoxification, species with different attributes are required for effective remediation of TNT from the environment. Such species would need to be fast-growing, and able to flourish in the harsh environments found on military training ranges. Species could include monocots such as switchgrass (*Panicum virgatum* L.), trees such as willow (*Salix* spp. L.) and poplar (*Populus* spp. L.) and species native to the contaminated region, like the shrub *Baccharis halimifolia* L. which is found on TNT-contaminated training ranges in North America (Ali et al., 2014). A combination of traditional breeding could be combined with genetic modification techniques to enhance TNT detoxification in these species. Indeed studies by van Dillewijn et al., (2008a) have shown that transgenic approaches, using bacterial nitroreductases, can be successfully extrapolated to a tree species for the remediation of TNT. Towards this aim, advancements in genomics and gene editing could be used to screen or manipulate *Dm*GSTE6-like activity in field-applicable species; a trait that would contribute to the development and use of plants able to remediate TNT and re-vegetate explosives-polluted sites.

Acknowledgements

This work was funded by the Strategic Environmental Research and Development Program of the US Department of Defense as well as by the Swedish Research Council. KT acknowledges funding from a Burgess studentship.

KT, BM, ELR and NCB planned and designed the research.

TK, MMR, IG, AMAM and ELR performed experiments and analysed data.

TK, BM, ELR and NCB wrote the manuscript.

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Figure Legends

Figure 1

Analysis on purified *Drosophila melanogaster* *Dm*GSTE6. (**a**) SDS-PAGE gel showing recombinantly expressed and purified *Dm*GSTE6. M, molecular weight marker (kDa); EV, protein extract from cells transformed with the empty vector; OD1, protein extract from cultures with optical density 0.8-1 at 600 nm before the induction of the protein expression; C, crude protein extract from cells after the 60 h period of expression; U, unbound fraction of the purification process; P, purified protein. (**b**) Michaelis-Menten plots of *Dm*GSTE6 with TNT performed at pH 9.0 and 30 °C. Values represent the mean of three reactions ± se.

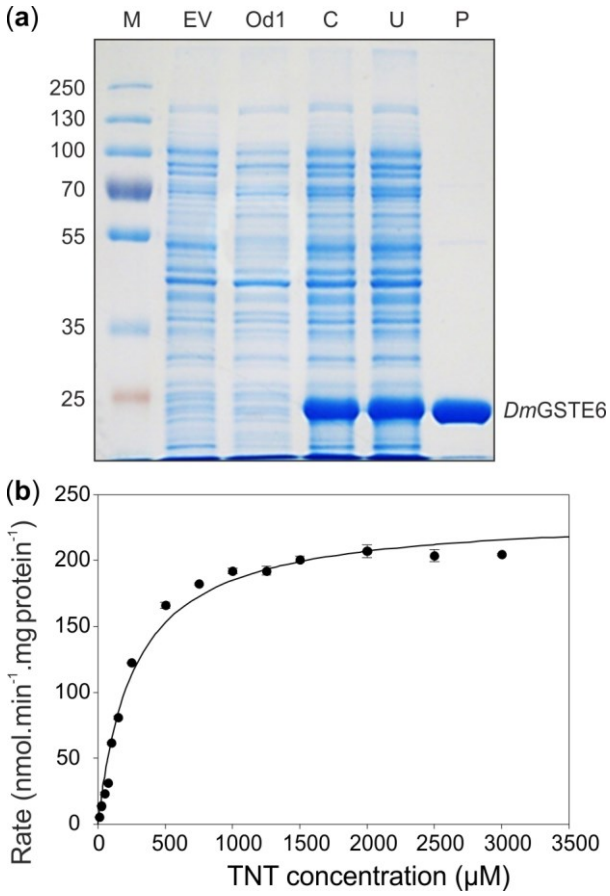


Figure 2

TNT-conjugate production by *Drosophila melanogaster* *Dm*GSTE6. (a) Chemical structures of the three TNT-conjugates. TNT-conjugate production profiles for *Dm*GSTE6 over variable (**b**) pH, at 20 °C and (**c**) variable temperature at pH 9.0, n = 3 ± se. Reactions were performed over 1 hour using 10 µg of enzyme, 200 µM TNT and 5 mM GSH. (**d**) Nitrite released during conjugation of TNT by 10 µg *Dm*GSTE6, 100 µg *At*GST-U24 and 100 µg GST-U25; measured using the Griess assay. Reactions were performed over 3 hours, using 500 µM TNT, at 20 °C, n = 5 ± se; *** P <0.001 statistically significant from *At*GST-U25 at that pH.

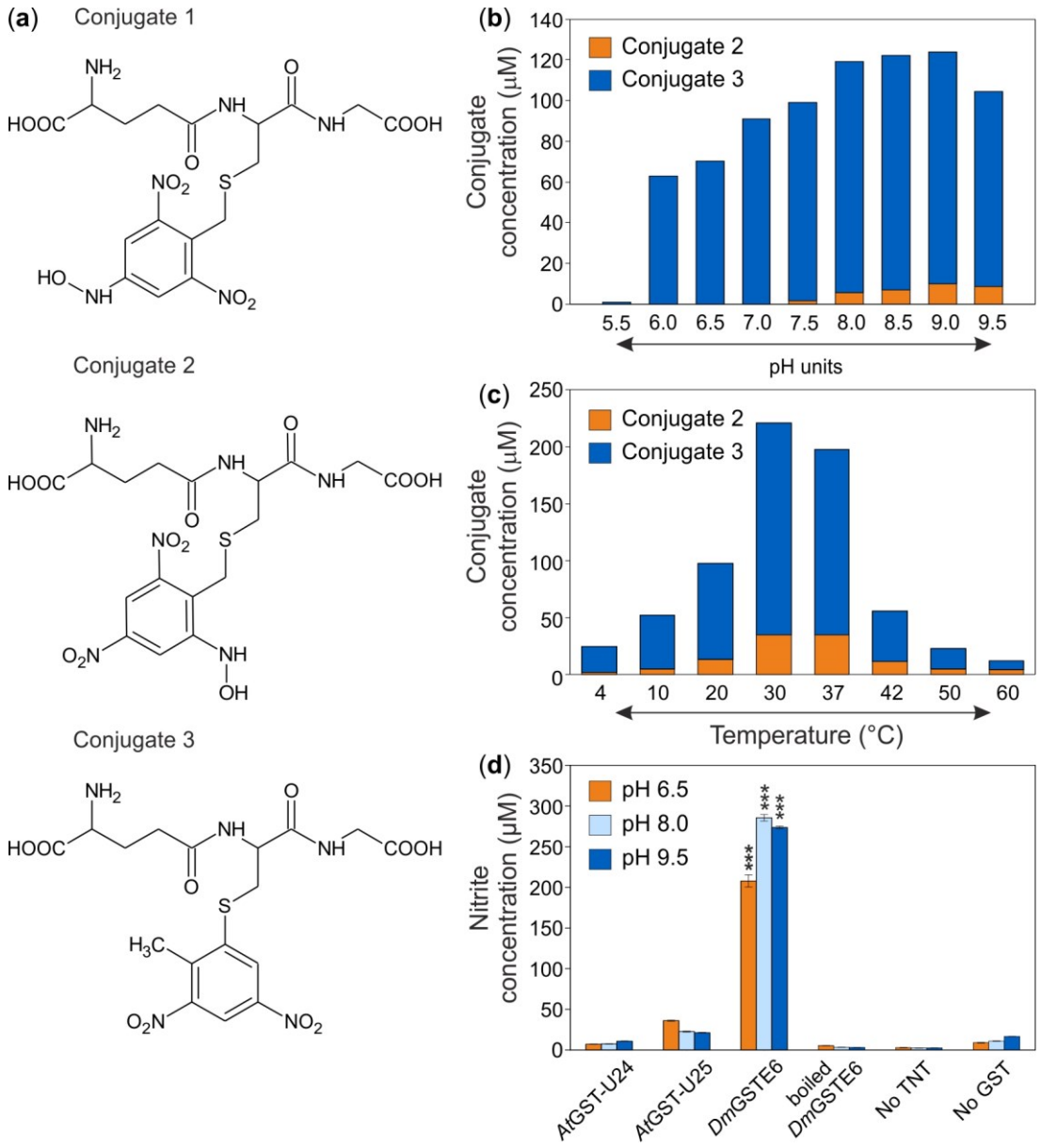


Figure 3

(**a**) Conjugation activity in leaf and root protein extracts from Arabidopsis wild type, *Drosophila melanogaster* *Dm*GSTE6 expressing lines, and *At*GST-U24 and *At*GST-U25 overexpressing (OE) lines assayed using CDNB substrate. Rosette leaves were from six-week-old plants grown in uncontaminated soil. Roots were from two-week-old plants grown vertically on agar plates containing ½ MS medium. * P <0.05, ** P <0.01, statistically significant from wild type. (**b**) Expression of *Dm*GSTE6 transcript using qPCR on 14 day old Arabidopsis grown on uncontaminated soil; N/D, not detected. (**c**) Nitrite released during conjugation of TNT by *Dm*GSTE6, measured using the Griess assay. Reactions were performed over 3 hours, using 500 µM TNT, at 20 °C, n = 5 ± se; 'a' denotes statistically significant from the wild type (P <0.01) and 'b' from the GST-U24/GST-U25 OE lines (P <0.05).

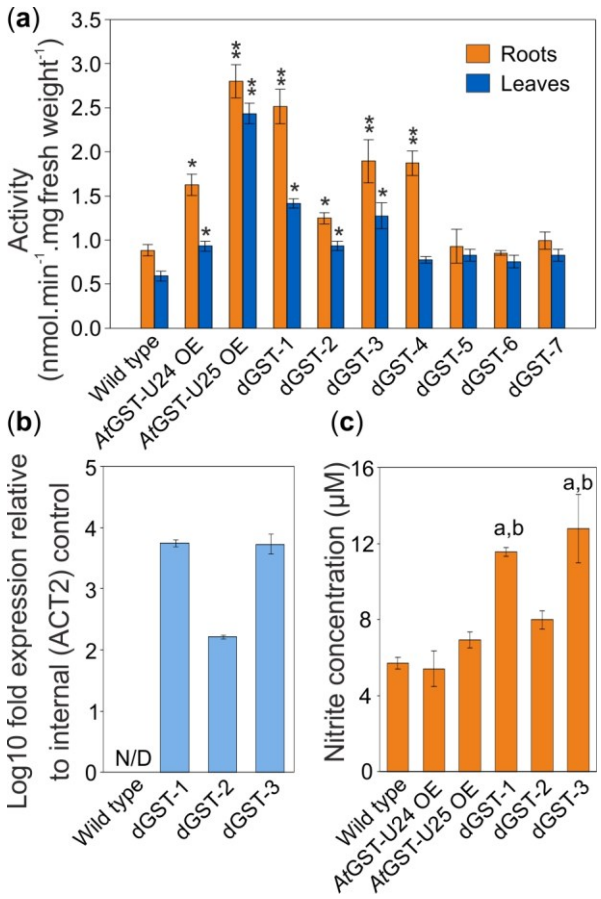
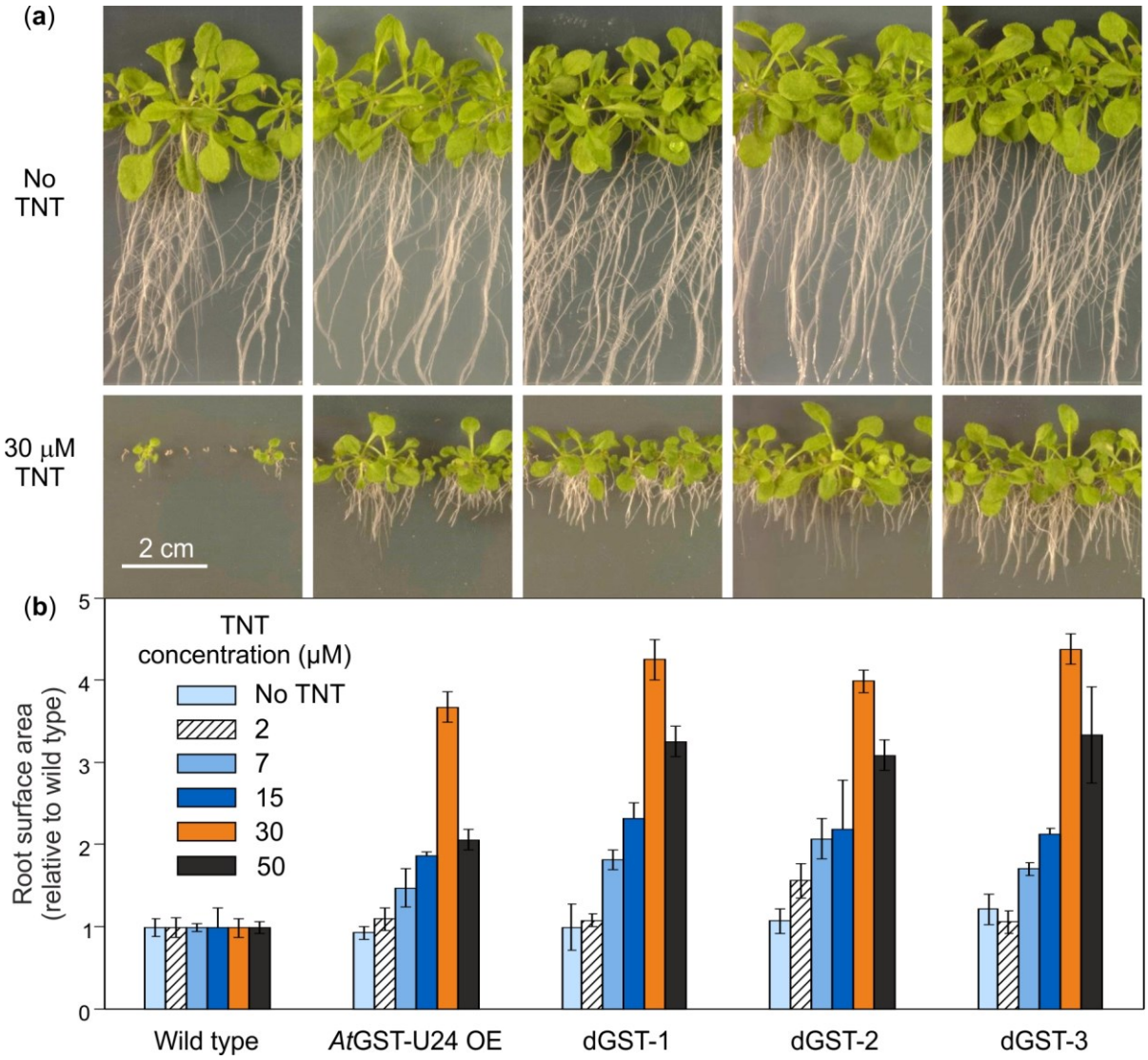


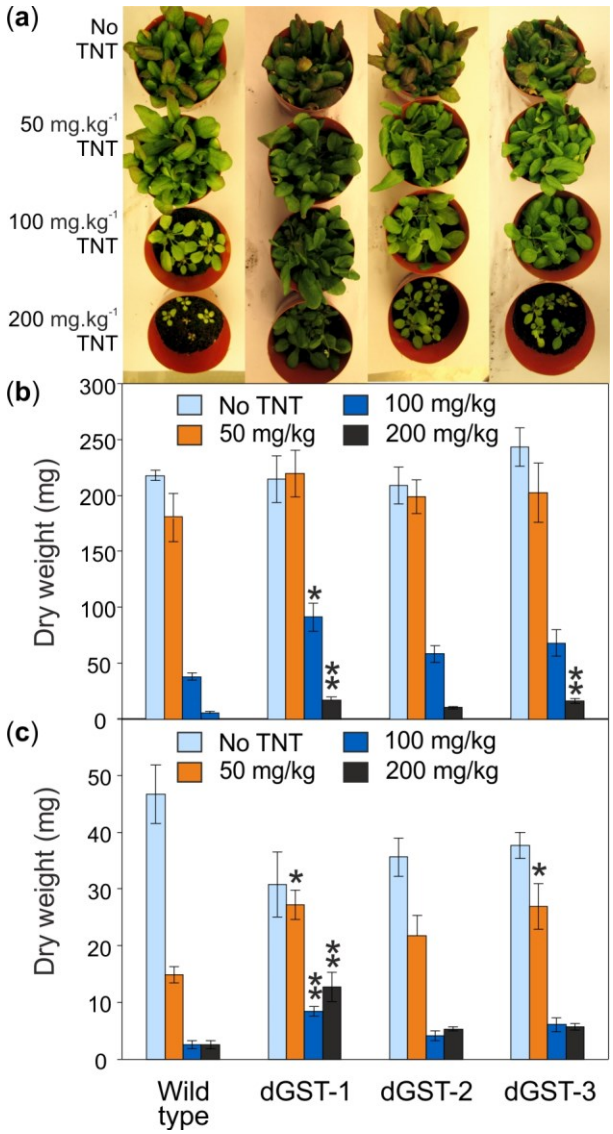
Figure 4

(a) Appearance of Arabidopsis wild type, *Drosophila melanogaster Dm*GSTE6 expressing lines, and GST-U24 overexpressing (OE) seedlings grown for 20 days on ½ MS agar plates in the absence of TNT, or in the presence of 30 µM TNT. (b), Root surface area of 20 day-old plants grown on ½ MS agar plates containing a range of TNT concentrations, n = 60 ± se.



598 **Figure 5**

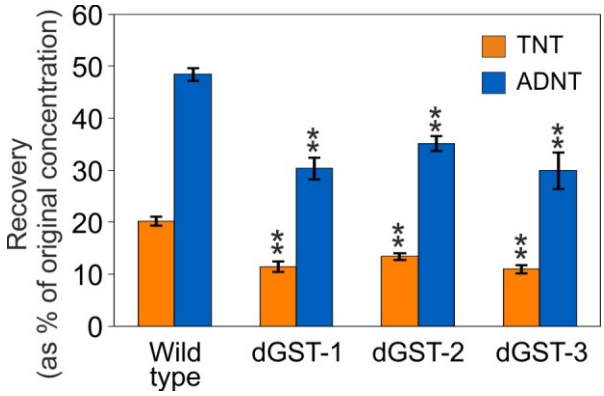
599 (**a**) Appearance of Arabidopsis plants grown in soil contaminated with a range of TNT
600 concentrations for six weeks. (**b**) Shoot and (**c**) root biomasses of Arabidopsis plants grown
601 for six weeks in soil contaminated with a range of TNT concentrations. WT, untransformed;
602 dGST/1-3, independent homozygous lines expressing *Drosophila melanogaster Dm*GSTE6, n
603 = 8 ± se; * $P < 0.05$, ** $P < 0.01$, statistically significant from wild type.



604

605 **Figure 6**

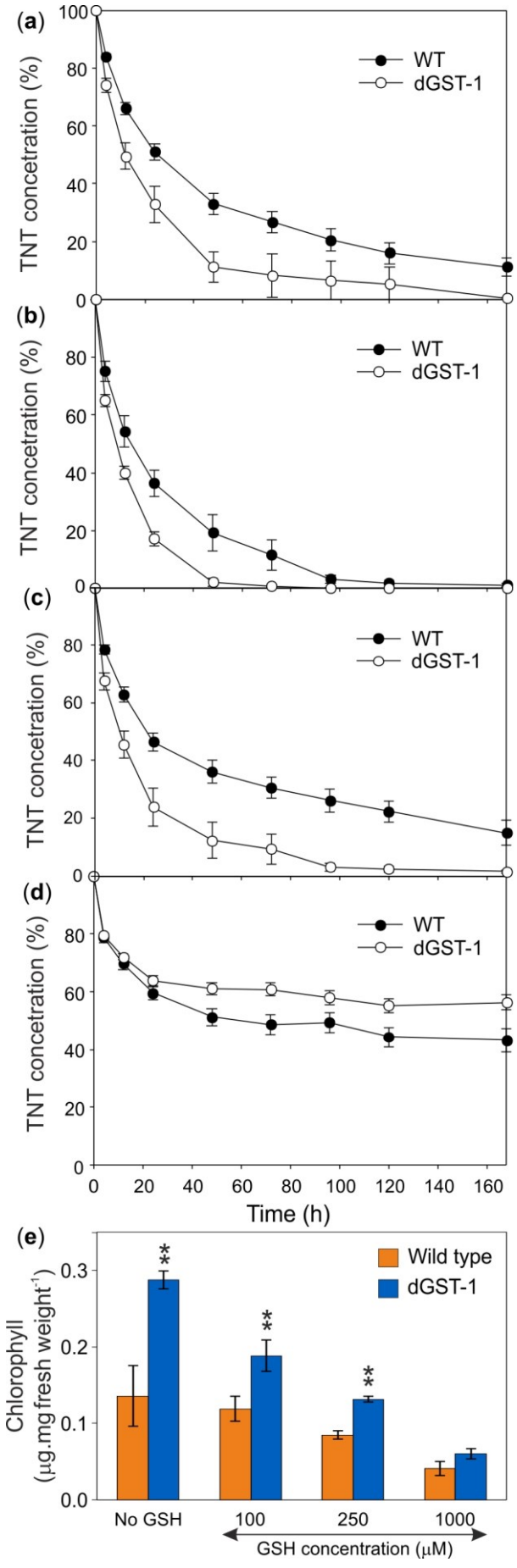
606 Levels of nitrotoluenes recovered from TNT-contaminated soil. Arabidopsis plants were
607 grown on 50 mg.kg^{-1} TNT for six weeks, n = 8 ± se; ** $P < 0.01$, statistically significant from
608 wild type.



609

610 **Figure 7**

611 Rates of TNT removal from the media by Arabidopsis plants grown in ½ MS liquid media
612 containing 250 µM TNT and (a) No GSH, (b) 100 µM, (c) 250 µM and (d) 1000 µM. (e)
613 Chlorophyll content of the plants at the end of the experiment, n = 5 ± se; ** P <0.01,
614 statistically significant from wild type.


(a)
(b)
(c)
(d)
(e)
TNT concetration (%)
WT
dGST-1
Time (h)
Chlorophyll (μg.mg fresh weight-1)
Wild type
dGST-1
No GSH
100
250
1000
GSH concentration (μM)

615