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

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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 characterized a *Drosophila melanogaster* 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 Maximal Velocity (V_{max}), and five-fold lower Michaelis Constant (K_m) than previously characterized TNT-active *Arabidopsis thaliana* (Arabidopsis) GSTs. Expression of *Dm*GSTE6 in Arabidopsis conferred enhanced resistance to TNT, and increased the 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 hypothesize 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.

Introduction

The explosive compound 2,4,6-trinitrotoluene (TNT) has been extensively used by the military world-wide 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). There are an estimated 10 million ha of military land contaminated with munitions components in the USA alone (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 as a consequence of 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 degradation 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 (GSTs) can conjugate the TNT molecule directly (Gunning *et al.*, 2014; Rylott *et al.*, 2015). Two *Arabidopsis thaliana* (Arabidopsis) *GST* genes, *AtGST-U24* and *AtGST-U25*, are specifically up-regulated in response to TNT exposure, and their

gene products catalyse the formation of three characterized 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 had a nitro group removed, and thus 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 *GST* genes 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 (*Populus trichocarpa*) *GST* genes, *PtGST-U16* and *PtGST-U24*, were found to be strongly up-regulated in response to TNT. However, the encoded enzymes exhibited only low (< 0.05 nmol min^{-1} mg^{-1}) specific activity towards 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* (Meigen, 1830) glutathione transferase (*Dm*GSTE6) was found to display outstanding activity towards TNT (Mazari & Mannervik, 2016). This research describes the characterization 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, UK).

Expression of DmGSTE6 in *Escherichia coli* and Arabidopsis

The *DmGSTE6* gene (National Center for Biotechnology Information (NCBI) accession number NT_033778) was cloned into pET-YSBLIC3C, expressed in *Escherichia coli* and purified as described in Gunning *et al.* (2014). For expression in *Arabidopsis thaliana* (L.) Heynh., *DmGSTE6* was cloned into the intermediary pART7 vector. The subsequent DNA cassette containing *DmGSTE6*, flanked by the cauliflower mosaic virus (CaMV) *35S* promoter and octopine synthase (*ocs*) terminator regions, was transferred into the binary vector pART27 using *Not*I restriction sites (Gleave, 1992). The pART27 vector contains a selectable marker, neomycin phosphotransferase II (*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^{-1} kanamycin. T_2 lines with kanamycin-resistance segregation ratios indicative of single insertional events were selected, and independent T_3 and T_4 generation plants, homozygous for kanamycin resistance, were used in subsequent experiments.

GST assays using 1-chloro-2,4-dinitrobenzene (CDNB)

The conjugating activity of the purified proteins, and the 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 the addition of 1 mM CDNB to a total volume of 1 ml. The increase in absorbance at wavelength 340 nm was 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 were analysed by high-performance liquid chromatography (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 Michaelis Constant (K_m) and Maximal Velocity (V_{max}) parameters for Lineweaver–Burke plots were calculated using SIGMA PLOT v. 12.0 (Systat Software Inc. (SSI), San Jose, CA, USA).

Measurement of TNT and products

TNT, ADNT and conjugates were analysed by HPLC using a Waters (Milford, MA, USA) HPLC system (Waters 2695 separator and Waters Photodiode array detector) with a 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 Fig. 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, and 30 min 0% A 100% B, with run time 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 (Waters).

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) and centrifuged at 12 000 ***g*** for 2 min at 4°C, and the supernatant was assayed spectrophotometrically at 645 and 663 nm.

Gene expression

Plant RNA was extracted from 3-wk-old rosette leaves using the Isolate II RNA plant kit (Bioline, London, UK) and cDNA was synthesized using oligo(dT)12-18 with Superscript II reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) containing RNAsin (Promega, Madison, WI, USA) at 42°C for 2 h, before inactivation at 70°C for 15 min. Synthesized cDNA was purified using the Wizard DNA Clean Up System (Promega) and quantified. Quantitative reverse transcription PCR (qPCR), using the primers dqPCR1_F 5′-GGACGACGGTCACTA CATCT-3′ and dqPCR1_R 5′-GCCGCTTTCAAAATGCAG AC-3′, was performed using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR green reporter dye. Data were normalized to the expression levels of the internal control gene *ACTIN2* (*ACT2*; At3g18780) using primers qActinF 5′-TACAGTGTCTGGATCGGTGGTT-3′ and qActinR 5′-CGGCCTTGGAGATCCACAT-3′, and the comparative ΔΔCt method (Livak & Schmittgen, 2001) was used to calculate the mean fold change in expression of *DmGSTE6*.

Agar plate experiments

Seeds were stratified for 3 d then germinated and grown on agar plates containing ½ MS and a range of TNT concentrations (dissolved in dimethyl sulfoxide (DMSO); final DMSO concentration 0.05% (v/v)). To determine the surface area of roots, Adobe Camera Raw v.6.0 software was used to remove nonroot background from each image. The surface area, in pixels, was then determined using Adobe Photoshop software.

Liquid culture experiments

Eight 7-d-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 2 wk 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 honest significant difference (HSD), using Spss v.22 software (IBM Analytics, Armonk, NY, USA).

Results

Activity of DmGSTE6

Following recombinant expression and purification, *Dm*GSTE6 was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) for purity and integrity. Fig. 1(a) shows the purified *Dm*GSTE6, with an expected size of

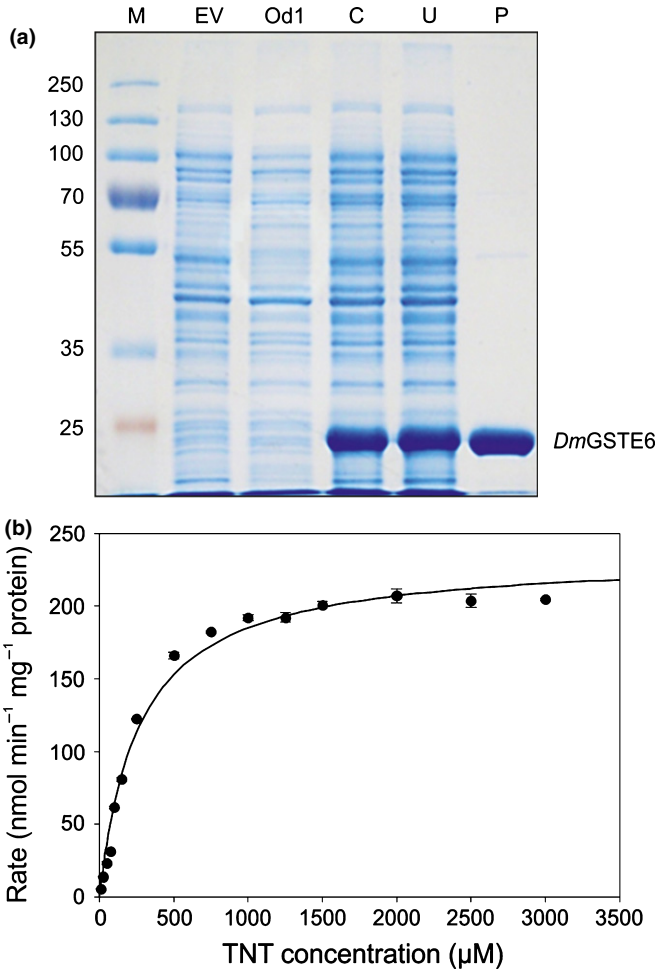

Fig. 1 Analysis of purified *Drosophila melanogaster* glutathione transferase E6 (*Dm*GSTE6). (a) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel showing recombinantly expressed and purified *Dm*GSTE6. M, molecular weight marker (kDa); EV, protein extract from cells transformed 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 2,4,6-trinitrotoluene (TNT) performed at pH 9.0 and 30°C. Values represent the mean of three reactions ± SE.

c. 25 kDa. The kinetic parameters for *Dm*GSTE6 using TNT as substrate were determined and the Michaelis–Menten plot is shown in Fig. 1(b). 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 Fig. 2(a). The TNT-conjugating activity of *Dm*GSTE6 was maximal at pH 9.0, where almost 50% of the initial TNT was conjugated within 1 h (Fig. 2b); at pH 5.5 the enzyme exhibited < 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 nonenzymatic 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 (Fig. 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 Fig. 2(d) show that, across the three pH values tested, *Dm*GSTE6 produced conjugate 3 to nitrite ratios of 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). *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 concentrations of nitrite were detected in the absence of GSH. As 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, polymerization reactions can also occur between the TNT molecules, reducing the number of exposed nitro groups. The presence of enzyme could reduce polymerization by binding TNT molecules into the active site or in noncatalytic 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).

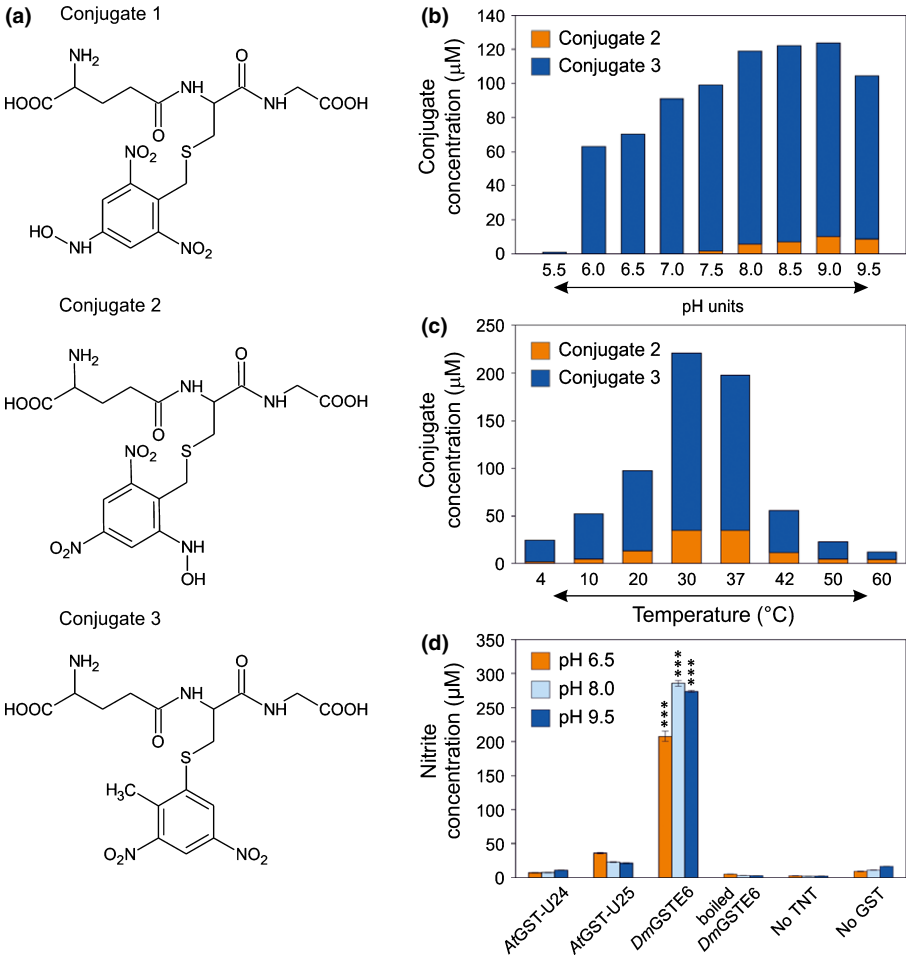

Fig. 2 2,4,6-trinitrotoluene (TNT) conjugate production by *Drosophila melanogaster* glutathione transferase E6 (*Dm*GSTE6). (a) Chemical structures of the three TNT conjugates. TNT-conjugate production profiles for *Dm*GSTE6 over (b) variable pH at 20°C and (c) variable temperature at pH 9.0; $n = 3 \pm$ SE. Reactions were performed over 1 h using 10 μg of enzyme, 200 μM TNT and 5 mM glutathione (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 h, using 500 μM TNT, at 20°C; $n = 5 \pm$ SE; ***, $P < 0.001$; statistically significantly different from *At*GST-U25 at that pH.

Expression of *DmGSTE6* 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 Fig. 3(a), 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. Fig. 3(b) shows that all three lines were expressing the transgene.

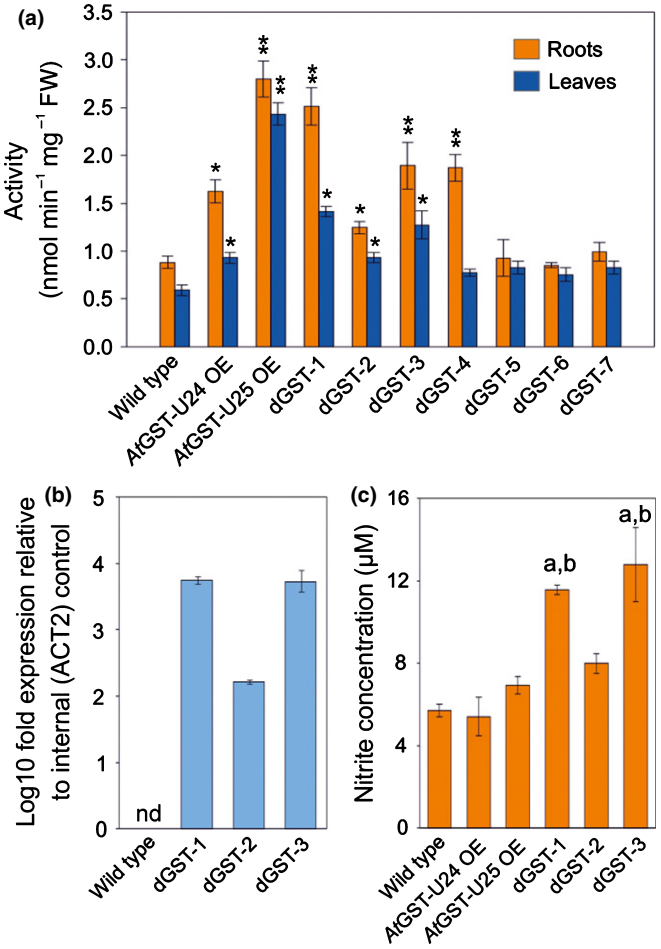

Fig. 3 (a) Conjugation activity in leaf and root protein extracts from Arabidopsis wild type, *Drosophila melanogaster* glutathione transferase E6 (*Dm*GSTE6)-expressing lines, and *At*GST-U24- and *At*GST-U25-overexpressing (OE) lines assayed using 1-chloro-2,4-dinitrobenzene (CDNB) substrate. Rosette leaves were from 6-wk-old plants grown in uncontaminated soil. Roots were from 2-wk-old plants grown vertically on agar plates containing ½ Murashige and Skoog (MS) medium. Statistically significant difference from wild type: *, $P < 0.05$; **, $P < 0.01$. (b) Expression of *Dm*GSTE6 transcript using quantitative polymerase chain reaction (qPCR) in 14-d-old Arabidopsis plants grown on uncontaminated soil; nd, not detected. (c) Nitrite released during conjugation of 2,4,6-trinitrotoluene (TNT) by *Dm*GSTE6, measured using the Griess assay. Reactions were performed over 3 h, using 500 μM TNT, at 20°C; $n = 5 \pm$ SE. 'a' denotes statistically significantly different from the wild type ($P < 0.01$) and 'b' from the GST-U24/GST-U25 OE lines ($P < 0.05$).

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 overexpressing *At*GST-U24, which does not produce conjugate 3, and *At*GST-U25, which produces conjugate 3, were included. The results shown in Fig. 3(c) demonstrated that all three dGST lines produced higher amounts of free nitrite than the *At*GST-U25-overexpressing lines, and thus more conjugate 3, confirming that these lines had a higher conjugation activity *in planta* than the *At*GST-U25 overexpression line. Protein extracts from wild type and the *At*GST-U24 overexpression line generated amounts of free nitrite close to those of the *At*GST-U25 overexpression 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 overexpression lines, the plants were grown for 20 d on ½ MS agar plates containing a range of TNT concentrations, alongside wild type and the selected *At*GST-U24 and *At*GST-U25 overexpression lines. The appearance of the wild-type, dGST and *At*GST-U24 plants at the end of the experiment is shown in Fig. 4(a). Concentrations of TNT up to 7 μM were probably not toxic enough to induce symptoms, as no significant differences in root surface area were recorded among the different plant lines (Fig. 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 overexpression 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 6 wk in soil contaminated with TNT. The appearance of the plants after 6 wk is shown in Fig. 5(a). Earlier studies reported that the overexpression 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 those of 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. In contrast, 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-overexpressing lines. The shoot and root biomasses were recorded after 6 wk, and are presented in Fig. 5(b,c). In TNT-contaminated soil, both dGST-1 and dGST-3 produced significantly more root and shoot

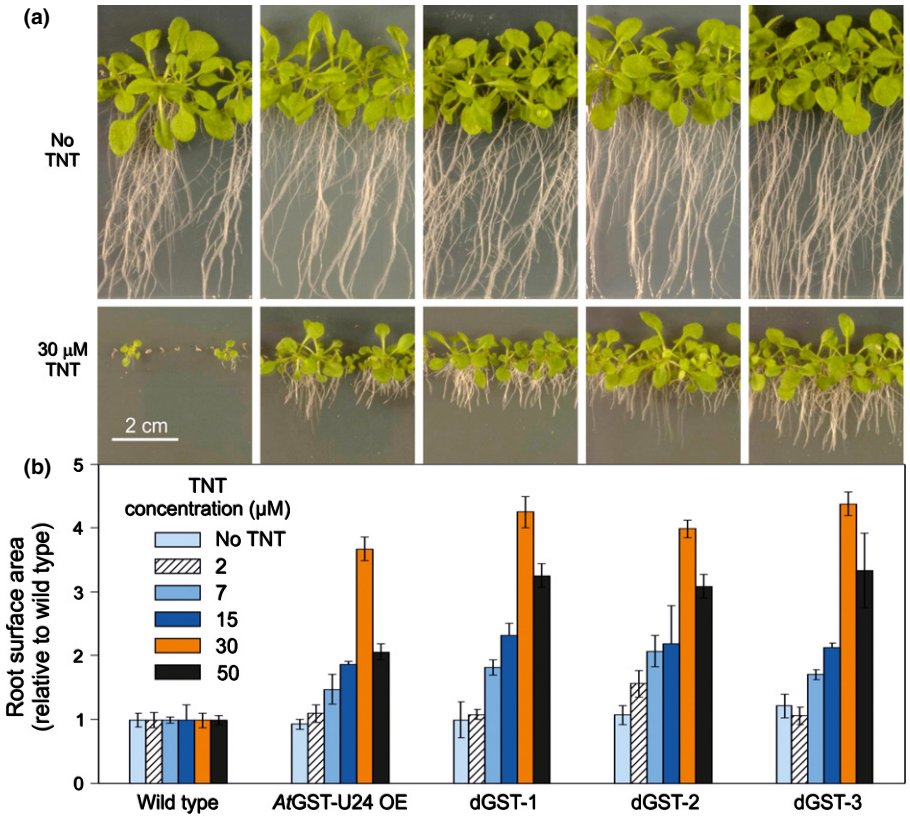

Fig. 4 (a) Appearance of Arabidopsis wild type, *Drosophila melanogaster* glutathione transferase E6 (*Dm*GSTE6)-expressing lines, and GST-U24-overexpressing (OE) seedlings grown for 20 d on ½ Murashige and Skoog (MS) agar plates in the absence of 2,4,6-trinitrotoluene (TNT), or in the presence of 30 µM TNT. (b) Root surface area of 20-d-old plants grown on ½ MS agar plates containing a range of TNT concentrations; $n = 60 \pm$ SE.

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 concentrations 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 6 wk was used. At this concentration, aerial biomass was not significantly different between the lines. As shown in Fig. 6, concentrations 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 concentrations when grown in the presence of TNT (Gunning *et al.*, 2014). With the hypothesis that GSH is limiting GST-catalysed detoxification of TNT, exogenous GSH was added to the liquid culture systems to see if supplemented GSH could complement a potentially limiting supply of endogenous GSH. Plants were grown in liquid cultures containing TNT, and a range of GSH concentrations, and TNT uptake was monitored for 1 wk (Fig. 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 h (67 and 49%, respectively, of the TNT; $P < 0.05$; Fig. 7a). When 100 µM 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 h (83 and 64%, respectively, of the TNT; $P < 0.01$; Fig. 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 (Fig. 7c). When 1000 µM GSH was present, a strong toxic effect was observed on the plants, which became chlorotic (Fig. 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 (Fig. 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 had both an increased affinity towards TNT ($K_m = 269.5 \pm 17.5$ µM) and significantly higher V_{max} (235 ± 3.9 nmol min^{-1} mg^{-1}) than

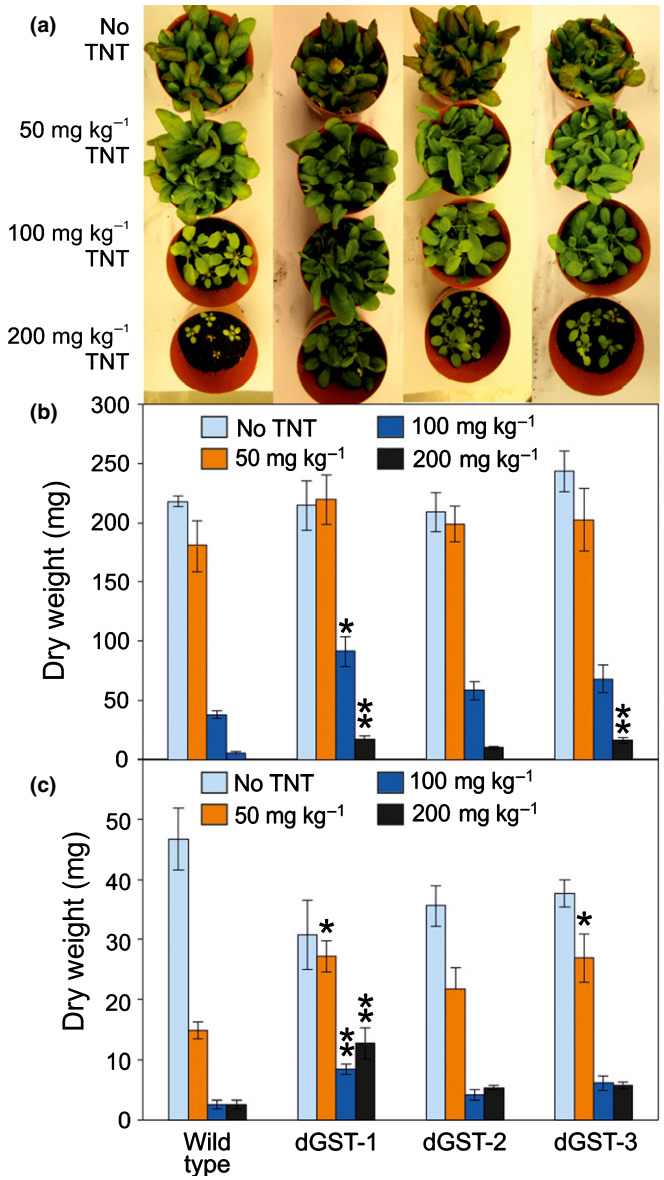

Fig. 5 (a) Appearance of Arabidopsis plants grown in soil contaminated with a range of 2,4,6-trinitrotoluene (TNT) concentrations for 6 wk. (b) Shoot and (c) root biomasses of Arabidopsis plants grown for 6 wk in soil contaminated with a range of TNT concentrations. WT, wild type, untransformed; dGST/1-3, independent homozygous lines expressing *Drosophila melanogaster* *Dm*GSTE6; $n = 8 \pm$ SE; statistically significant difference from wild type: *, $P < 0.05$; **, $P < 0.01$.

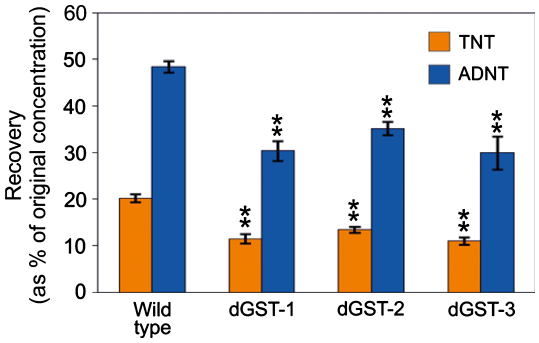

Fig. 6 Concentrations of nitrotoluenes recovered from 2,4,6-trinitrotoluene (TNT)-contaminated soil. Arabidopsis plants were grown on 50 mg kg^{-1} TNT for 6 wk; $n = 8 \pm$ SE; statistically significant difference from wild type: **, $P < 0.01$. ADNT, amino dinitrotoluene.

values reported by Gunning *et al.* (2014) for endogenous *At*GST-U24 and *At*GST-U25 ($K_m = 1644 \pm 113.2$ and 1210 ± 85.7 μM and $V_{max} = 92.3 \pm 2.6$ 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 ionization of the sulfhydryl group of GSH, which has a p*K*a 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, 1999; Brentner *et al.*, 2010), the pH of the cytosol is estimated to be within the range of 6.5–7.9 (Scott & Allen, 1999; Moseyko & Feldman, 2001; Tournaire-Roux *et al.*, 2003). Although the activity of *Dm*GSTE6 is lower at pH 7.5 than at pH 9.0, our studies indicate that only conjugate 3 would be produced within the roots.

Is TNT detoxification GSH limited?

The yield drag observed in the *At*GST-U24- and *At*GST-U25-overexpressing Arabidopsis lines grown in the absence of TNT was not observed in the *Dm*GSTE6-expressing Arabidopsis lines. It is possible that overexpression 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 were similar to those 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 with 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 the *Dm*GSTE6 was expressed *in planta*.

We hypothesize that the constraint on TNT uptake and detoxification is attributable to limiting GSH concentrations in the root cytosol. The GSH concentration in the Arabidopsis cytosol is predicted to be in the range of 1–3 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 concentrations become limited because GSH is utilized by other biochemical processes or is compartmentalized to secure the GSH concentrations of specific organelles, or that the actual GSH concentrations 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 Zechmann *et al.* (2011) who reported that low pollen germination rates induced by treatment with the GSH synthesis inhibitor

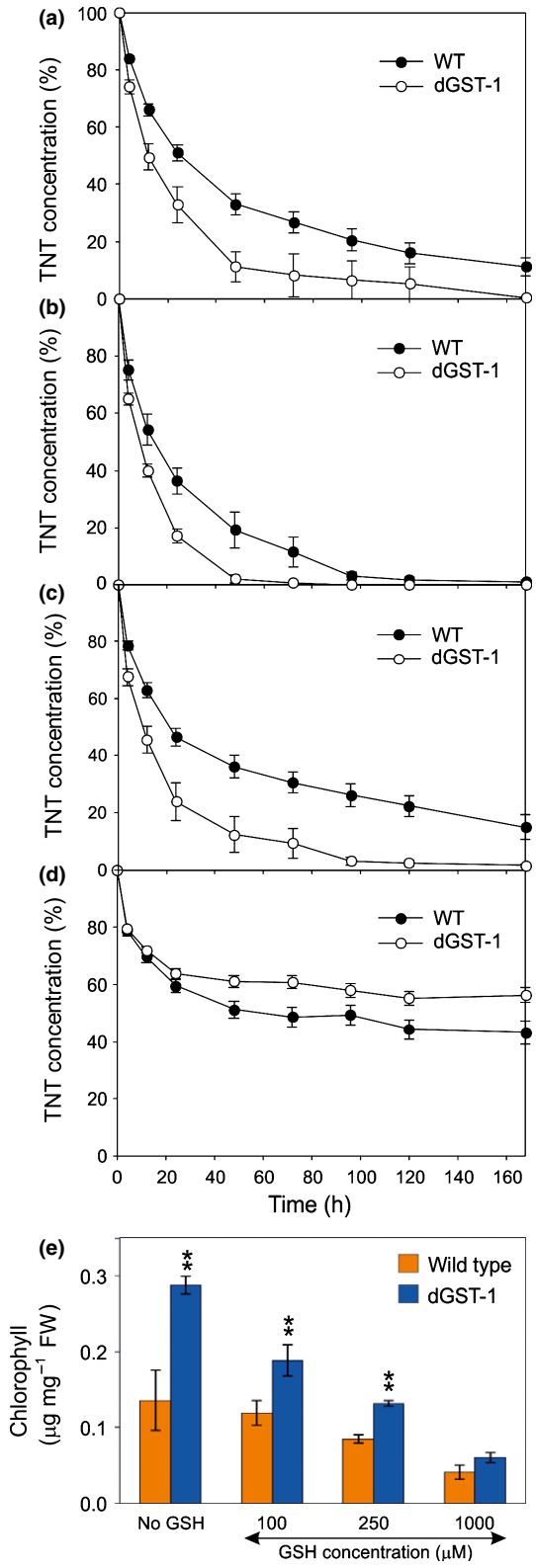

Fig. 7 Rates of 2,4,6-trinitrotoluene (TNT) removal from the media by Arabidopsis plants grown in ½ Murashige and Skoog (MS) liquid media containing 250 μM TNT and (a) no glutathione (GSH), (b) 100 μM, (c) 250 μM and (d) 1000 μM. (e) Chlorophyll content of the plants at the end of the experiment; $n = 5 \pm$ SE; statistically significant difference from wild type: **, $P < 0.01$.

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 characterized GSH-conjugate ATP-Binding Cassette (ABC) transporters, Multidrug Resistance-associated Protein 1 (MRP1) and MRP2 (Lu *et al.*, 1998; Tommasini *et al.*, 1998), are upregulated 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-Ohtsu *et al.*, 2007), but the downstream processing of 2-glutathionyl-4,6-dinitrotoluene is not known. It is possible that it, or a cleaved dinitro-derivative, could be released upon evacuolation as tissues are converted into woody biomass, but dinitrotoluene-degrading activities have not been reported in plants, so mineralization *in planta* appears unlikely. However, fungi and bacteria with the ability to mineralize dinitrotoluene have been reported (Valli *et al.*, 1992; Johnson & Spain, 2003) and biodegradation of 2-glutathionyl-4,6-dinitrotoluene by soil microbes during decomposition at the end of the plant life cycle is plausible.

Potential of transgenic plants for TNT detoxification

The TNT pollution on military training ranges is heterogenic, with concentrations of 100 mg kg^{-1} soil not uncommon and hotspots in excess of 10 000 mg kg^{-1} (Talmage *et al.*, 1999; Jenkins *et al.*, 2006). Arabidopsis is not a field-applicable species, but the studies presented here were conducted within the lower bounds of TNT contamination found on military ranges, and demonstrate that plants expressing increased levels of TNT-active GSTs are more tolerant to TNT. When compared with earlier studies expressing bacterial enzymes such as nitroreductases (Hannink *et al.*, 2001; Rylott *et al.*, 2011a) and pentaerythritol tetranitrate reductase (French *et al.*, 1999) in plants, the GST-linked increase is only moderate. Such expression of bacterial reductases in plants increases the conversion of TNT to HADNT and ADNT, which can condense to form diarylamines (van Dillewijn *et al.*, 2008b; Wittich *et al.*, 2008) or be subsequently converted to sugar conjugates (Gandia-Herrero *et al.*, 2008), and it is likely that, in the longer term, these compounds become incorporated into plant macromolecular structures such as lignin.

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

In tandem with the development of transgenic plants for phytoremediation, it is important to 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*), trees such as willow (*Salix* spp.) and poplar (*Populus* spp.) and species native to the contaminated region, like the shrub *Baccharis halimifolia* which is found on TNT-contaminated training ranges in North America (Ali *et al.*, 2014). 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.

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Author contributions

K.T., B.M., E.L.R. and N.C.B. planned and designed the research. T.K., M.M.R., I.G., A.M.A.M. and E.L.R. performed experiments and analysed data. T.K., B.M., E.L.R. and N.C.B. wrote the manuscript.

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