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

3

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

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25

## 26 Summary

- 27 • The explosive 2,4,6-trinitrotoluene (TNT) is a significant, global environmental  
28 pollutant that is both toxic and recalcitrant to degradation. Given the sheer scale, and  
29 inaccessible nature of contaminated areas, phytoremediation may be a viable clean-up  
30 approach. Here, we have characterised a *Drosophila melanogaster* (Meigen, 1830)  
31 glutathione transferase (*DmGSTE6*) which has activity towards TNT.
- 32 • Recombinantly-expressed, purified *DmGSTE6* produces predominantly 2-  
33 glutathionyl-4,6-dinitrotoluene, and has a 2.5-fold higher  $V_{\max}$ , and 5-fold lower  $K_m$   
34 than previously characterised TNT-active *Arabidopsis thaliana* (L.) Heynh  
35 (*Arabidopsis*) GSTs. Expression of *DmGSTE6* in *Arabidopsis* conferred enhanced  
36 resistance to TNT, and increased ability to remove TNT from contaminated soil  
37 relative to wild-type plants.
- 38 • *Arabidopsis* lines overexpressing TNT-active GSTs *AtGST-U24* and *AtGST-U25*  
39 were compromised in biomass production when grown in the absence of TNT. This  
40 yield drag was not observed in the *DmGSTE6* expressing *Arabidopsis* lines. We  
41 hypothesise that increased levels of endogenous TNT-active GSTs catalyse excessive  
42 glutathionylation of endogenous substrates, depleting glutathione pools, an activity  
43 that *DmGST* may lack.
- 44 • In conclusion, *DmGSTE6* has activity towards TNT, producing a compound with  
45 potential for further biodegradation. Selecting or manipulating plants to confer  
46 *DmGSTE6*-like activity could contribute towards development of phytoremediation  
47 strategies to clean up TNT from polluted military sites.

48

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

51

## 52 **Introduction**

53 The explosive compound 2,4,6-trinitrotoluene (TNT) has been extensively used by the  
54 military worldwide for many decades. TNT is remarkably resistant to biodegradation and is  
55 now classed as a possible human carcinogen and serious environmental pollutant by the  
56 United States Environmental Protection Agency (2014). In the US alone, there are an  
57 estimated 10 million hectares of military land contaminated with munitions components  
58 (United States Defense Science Board Task Force. 1998; United States General Accounting,  
59 2004), and many contaminated sites in Europe and Asia (Kalderis *et al.*, 2011; Pichtel, 2012).  
60 For example, the Werk Tanne former ammunition site in Germany, detonated in 1944, is  
61 heavily contaminated with TNT (Eisentraeger *et al.*, 2007). Increased environmental  
62 awareness is now compelling governments to identify sites of explosives contamination and  
63 put together remediation strategies (Lima *et al.*, 2011). However, a major challenge to  
64 cleaning-up these sites is the sheer scale and hazardous nature of many contaminated sites,  
65 which rules-out many strategies such as excavation, land fill and off-site treatments, as  
66 prohibitively expensive. Phytoremediation may be a viable alternative approach.

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

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

90 In a recent study a *Drosophila melanogaster* glutathione transferase (*DmGSTE6*) was found  
91 to display outstanding activity toward TNT (Mazari & Mannervik, 2016). This research  
92 describes the characterisation of *DmGSTE6*, which has greater activity towards TNT than  
93 *AtGST-U24* and *AtGST-U25*. We have engineered *Arabidopsis* plants to express *DmGSTE6*,  
94 and assessed its potential for the *in planta* detoxification of TNT, with the aim of developing  
95 such technologies for the phytoremediation of TNT-contaminated military training ranges.

## 96 **Materials and Methods**

### 97 *Chemicals*

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

### 100 *Expression of DmGSTE6 in Escherichia coli and Arabidopsis*

101 The *DmGSTE6* gene (NCBI accession number NT\_033778) was cloned into pET-  
102 YSBLIC3C, expressed in *E. coli* and purified as described in Gunning *et al.* (2014). For  
103 expression in *Arabidopsis*, *DmGSTE6* was cloned into the intermediary pART7 vector. The  
104 subsequent DNA cassette containing *DmGSTE6*, flanked by CaMV-35S promoter and *ocs*  
105 terminator regions, was transferred into the binary vector pART27 using *NotI* restriction sites  
106 (Gleave, 1992). The pART27 vector contains a selectable marker, *nptII*, which confers  
107 resistance to kanamycin. Following transformation, using the floral dipping method (Clough  
108 & Bent, 1998), primary transformants were identified by screening on agar plates containing  
109 half-strength Murashige and Skoog medium (Murashige & Skoog, 1962) ( $\frac{1}{2}$  MS) plus 50

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

### 113 *GST assays using CDNB*

114 Conjugating activity of the purified proteins, and crude extracts from rosette leaves, was  
115 assessed using the model GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) as described  
116 previously (Colville & Smirnoff, 2008). Briefly, the reaction, carried out at 20°C, comprised  
117 100 mM potassium phosphate buffer pH 6.5, 5 mM reduced glutathione (GSH) and 500 ng of  
118 purified *DmGSTE6* and was initiated by addition of 1 mM CDNB to a total volume of 1 ml.  
119 Increase in absorbance at A<sub>340</sub> measured spectrophotometrically.

### 120 *GST assays using TNT*

121 Reactions, carried out at 30°C, contained 100 mM potassium phosphate buffer, 10 µg of  
122 purified *DmGSTE6* and 5 mM GSH and were initiated by addition of TNT to a final volume  
123 of 250 µl. Reactions were stopped by the addition of trichloroacetic acid, to a final  
124 concentration of 10% (v/v), and samples analysed by HPLC.

125 Control reactions using *AtGST-U25* contained 150 µg of enzyme. The glutathione peroxidase  
126 activity (GPOX) assays were performed according to Edwards & Dixon (2005) with  
127 modifications (Gunning *et al.* 2014). Michaelis-Menten K<sub>m</sub> and V<sub>max</sub> parameters for  
128 Lineweaver-Burke plots were calculated using Sigma Plot v. 12.0.

### 129 *Measurement of TNT and products*

130 The TNT, ADNT and conjugates were analysed by HPLC using a Waters (Milford USA)  
131 HPLC system (Waters 2695 separator and Waters Photodiode array detector) with Waters X-  
132 Bridge C18 column (300 × 4.5 mm, 5 µm). The mobile phases for the gradient conditions  
133 were as reported in Gunning *et al.* (2014), with the exception of data presented in Figure 7  
134 (see later) which used the following: mobile phase A, acetonitrile; mobile phase B, 50 mM  
135 NaH<sub>2</sub>PO<sub>4</sub>, pH 2.7, with 85% (v/v) phosphoric acid. The gradient ran: 0 min 0 % A 100 % B,  
136 6 min 0 % A 100 % B, 11 min 50 % A 50 % B, 25 min 100 % A 0 % B, 30 min 0 % A 100 %  
137 B, runtime 30 min. Peaks were identified and quantified using purified conjugates as  
138 described in Gunning *et al.* (2014). The expected retention times were: TNT, 30.9 min;

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

#### 141 *Nitrite measurement*

142 Nitrite production was measured using Griess assays according to the method of French et al.  
143 (1998) with modifications as described in Gunning *et al.* (2014).

#### 144 *Chlorophyll measurement*

145 Chlorophyll was extracted based on the method of Arnon (1949). Briefly, 100 mg of fresh  
146 tissue was ground in 500  $\mu$ l of 80% acetone (v/v), centrifuged at 12,000 g for two min at 4  
147  $^{\circ}$ C and the supernatant assayed spectrophotometrically at 645 and 663 nm.

#### 148 *Gene expression*

149 Plant RNA was extracted from three-week-old rosette leaves using the Isolate II RNA plant  
150 kit (Bioline, London, UK) and cDNA was synthesised using oligo(dT)<sub>12-18</sub> using  
151 Superscript II reverse transcriptase (Thermo Fisher Scientific, Waltham, USA) containing  
152 RNasin (Promega, Madison, USA) at 42  $^{\circ}$ C for 2 h, before inactivation at 70  $^{\circ}$ C for 15 min.  
153 Synthesised cDNA was purified using Wizard DNA Clean Up System (Promega) and  
154 quantified. Quantitative reverse transcription PCR (qPCR), using the primers dqPCR1\_F 5'-  
155 GGACGACGGTCACTACATCT-3' and dqPCR1\_R 5'-GCCGCTTTCAAATGCAGAC-  
156 3', was performed using an ABI 7000 Sequence Detection System (Applied Biosystems,  
157 Foster City, USA) with SYBR green reporter dye. Data were normalised to expression levels  
158 of the internal control gene (*ACT2*, At3g18780) using primers qActinF 5'-  
159 TACAGTGTCTGGATCGGTGGTT-3' and qActinR 5'-CGGCCTTGGAGATCCACAT-3',  
160 and the comparative  $\Delta\Delta$ Ct method (Livak & Schmittgen, 2001) used to calculate the mean  
161 fold change in expression of *DmGSTE6*.

#### 162 *Agar plate experiments*

163 Seeds were stratified for three days then germinated and grown on agar plates containing  $\frac{1}{2}$   
164 MS and a range of TNT concentrations (dissolved in DMSO; final DMSO concentration  
165 0.05% (v/v)). To determine the surface area of roots, Adobe Camera Raw ver. 6.0 software  
166 was used to remove non-root background from each image. The surface area, in pixels, was  
167 then determined using Adobe Photoshop software.



168 *Liquid culture experiments.*

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

173 *Soil studies*

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

176 *Statistical Analysis*

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

## 179 **Results**

180 *Activity of DmGSTE6*

181 Following recombinant expression and purification, *DmGSTE6* was analysed by SDS-PAGE  
182 for purity and integrity. Figure 1a shows the purified *DmGSTE6*, with an expected size of  
183 approximately 25 kDa. The enzyme kinetics for *DmGSTE6* using TNT as substrate, were  
184 determined and the Michaelis-Menten plot is shown in Figure 1b. The V<sub>max</sub> and K<sub>m</sub> values  
185 were 235 ± 3.9 nmol.min<sup>-1</sup>.mg<sup>-1</sup> and 269.5 ± 17.5 µM respectively. While GPOX activity was  
186 detected in purified *AtGST-U25*, GPOX activity was not detected for *DmGSTE6* (results not  
187 shown).

188 Previous studies have shown that, dependent on pH and temperature, *AtGST-U25* produces  
189 three different TNT-conjugates as shown in Figure 2a. The TNT conjugating activity by  
190 *DmGSTE6* was maximal at pH 9.0, where almost 50 % of the initial TNT was conjugated  
191 within an hour (Figure 2b); at pH 5.5 the enzyme exhibited less than 1 % of the activity at pH  
192 9.0. Of the three TNT-GSH conjugates identified previously (Gunning *et al.*, 2014),  
193 *DmGSTE6* produced almost exclusively conjugate 3 across the pH range tested. Small  
194 amounts of conjugate 2 were produced at pH 8.0 and higher, while conjugate 1 was not  
195 detected. No significant changes were observed in the TNT concentration of control reactions

196 containing denatured *DmGSTE6*, confirming the absence of non-enzymatic conjugation and  
197 the stability of TNT at the different pH values tested. TNT-conjugating activity of *DmGSTE6*  
198 was detected across the full range of temperatures tested (from 4 °C to 50 °C), with maximal  
199 activity at 30 °C (Figure 2c). At all these temperatures *DmGSTE6* produced almost entirely  
200 conjugate 3, with low but progressively increasing levels of conjugate 2 produced from 20 to  
201 42 °C.

202 Conjugate 3 production should result in the concomitant stoichiometric release of nitrite 1:1.  
203 To measure nitrite production, Griess assays were used. The results presented in Figure 2d  
204 show that across the three pH values tested, *DmGSTE6* produced conjugate 3 to nitrite ratios  
205 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 *DmGSTE6*  
206 produced significantly higher amounts of nitrite than *AtGST-U25*; nitrite was not detected  
207 with *AtGST-U24* which is unable to produce conjugate 3. Nitrite release was not observed  
208 from the denatured *DmGSTE6* control, but low levels of nitrite were detected in the absence  
209 of GSH. Since the amount of nitrite increased with increasing pH, this release is probably the  
210 result of alkaline hydrolysis. Qasim et al. (2009) have reported significant alkaline hydrolysis  
211 of TNT in aqueous solutions at high pH. Under such alkaline conditions, polymerisation  
212 reactions can also occur between the TNT molecules, reducing the number of exposed nitro  
213 groups. The presence of enzyme could reduce polymerisation by binding TNT molecules into  
214 the active site or in non-catalytic ligand binding sites that have been previously identified in  
215 plant GSTs (Dixon et al., 2011), allowing further alkaline hydrolysis to occur. Conjugating  
216 activity of *DmGSTE6* towards ADNTs and HADNTs was tested, but no conjugated products  
217 were detected (data not shown).

#### 218 *Expression of DmGSTE6 in Arabidopsis*

219 To assess the ability of *DmGSTE6* to conjugate and detoxify TNT *in planta*, *Arabidopsis*  
220 lines expressing *DmGSTE6* were generated. Seven, homozygous *DmGSTE6* expressing lines,  
221 were assayed for CDNB-conjugating activity. As shown in Figure 3a, the seven lines  
222 exhibited a range of activities. Lines dGST-1, 2 and 3 which had 2.4, 1.6 and 2.1-fold  
223 respectively more CDNB activity in roots than wild type plants were selected for further  
224 analysis. To confirm that *DmGSTE6* was expressed in the lines, qPCR was used to measure  
225 transcript levels. Figure 3b confirmed that all three lines were expressing the transgene.

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

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

#### 249 *Contaminated soil studies on DmGSTE6-expressing Arabidopsis*

250 To assess the ability of the *DmGSTE6*-expressing lines to remediate TNT from soil, the lines  
251 were grown for six weeks in soil contaminated with TNT. The appearance of the plants after  
252 six weeks is shown in Figure 5a. Earlier studies reported that the over-expression of *AtGST-*  
253 *U24* and *AtGST-U25* resulted in reduced plant biomasses in the absence of TNT; however,  
254 the shoot and root biomasses of the dGST lines were indistinguishable from the wild type  
255 lines when grown in the absence of TNT. As predicted from earlier studies (Rylott *et al.*,

256 2011a; Gunning *et al.*, 2014), at TNT concentrations above 50 mg.kg<sup>-1</sup> TNT, wild type plants  
257 appeared chlorotic and severely stunted. On the contrary, the dGST lines appeared green,  
258 with less stunting. All three dGST lines were able to continue growing at 200 mg.kg<sup>-1</sup> TNT, a  
259 concentration found to completely inhibit growth for wild type; and *AtGST-U24* and *AtGST-*  
260 *U25* over-expressing lines. The shoot and root biomasses were recorded after six weeks, and  
261 are presented in Figure 5b and c. In TNT-contaminated soil, both dGST-1 and dGST-3  
262 produced significantly more root and shoot biomass than wild type; although line dGST-2  
263 was not significantly different from wild type. Line dGST-1 exhibited the greatest resistance  
264 to TNT toxicity, attaining shoot and root biomasses 2.4 and 3.2-fold higher than wild type at  
265 100 mg kg<sup>-1</sup> TNT; and 2.8 and 4.8-fold higher at 200 mg kg<sup>-1</sup> TNT, respectively. To gauge  
266 the ability of the dGST lines to remove TNT from the contaminated soil, the levels of TNT,  
267 and ADNT, resulting from the transformation of TNT by soil-based microbial communities,  
268 were determined. To do this, soil from the pots containing 50 mg kg<sup>-1</sup> TNT that the plants had  
269 been growing in for six weeks was used. At this concentration aerial biomass was not  
270 significantly different between the lines. As shown in Figure 6, levels of TNT and ADNT  
271 from soil in which the dGST lines had been grown were significantly lower than in the soil  
272 from wild type plants.

### 273 *Role of glutathione in TNT detoxification*

274 It has previously been shown that plants with GST-enhanced ability to detoxify TNT by  
275 conjugation have depleted GSH levels when grown in the presence of TNT (Gunning *et al.*,  
276 2014). With the hypothesis that GSH is limiting GST-catalysed detoxification of TNT, the  
277 application of exogenous GSH to liquid culture systems was tested to see if GSH could  
278 enhance a potentially limiting supply of endogenous GSH. Plants were grown in liquid  
279 cultures containing TNT, and a range of GSH concentrations, and TNT uptake monitored for  
280 one week (Figure 7).

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

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

## 293 Discussion

### 294 *Activity of DmGST*

295 *DmGSTE6* was found to catalyse the conjugation of GSH to TNT producing almost  
296 exclusively conjugate 3, 2-glutathionyl-4,6-dinitrotoluene, and concurrently a 1:1  
297 stoichiometric release of nitrite. Furthermore, *DmGSTE6* has both an increased affinity  
298 towards TNT ( $K_m = 269.5 \pm 17.5 \mu\text{M}$ ) and significantly higher  $V_{\text{max}}$  ( $235 \pm 3.9 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ )  
299 than values reported by Gunning *et al.* (2014) for endogenous *AtGST-U24* and  
300 *AtGST-U25* ( $K_m = 1644 \pm 113.2$  and  $1210 \pm 85.7 \mu\text{M}$ ;  $V_{\text{max}} = 92.3 \pm 2.6$  and  $98.39 \pm 3$   
301  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ , for *AtGST-U24* and *AtGST-U25* respectively).

302 The pH optimum for *DmGSTE6* activity towards TNT of pH 9.0, is in agreement with that  
303 observed for both *AtGST-U24* and *AtGST-U25* (Gunning *et al.*, 2014) and can at least partly  
304 be attributed to ionisation of the sulfhydryl group of GSH, which has a pKa of 9.4, forming  
305 the reactive thiolate anion (Dixon & Edwards, 2010). However, within the roots, the site of  
306 TNT detoxification in dicot and grass species (Sens *et al.*, 1998; Sens *et al.*, 1999; Brentner *et al.*,  
307 2010), the pH of the cytosol is estimated to be within the range of 6.5 to 7.9 (Scott &  
308 Allen, 1999; Moseyko & Feldman, 2001; Tournaire-Roux *et al.*, 2003). Although the activity  
309 of *DmGSTE6* is lower at pH7.5 than at pH9.0, our studies indicate that only conjugate 3  
310 would be produced within the roots.

### 311 *Is TNT detoxification GSH limited?*

312 In the *DmGSTE6* expressing Arabidopsis lines, the yield drag observed in *AtGST-U24* and  
313 *AtGST-U25* overexpressing Arabidopsis lines grown in the absence of TNT, was absent. It is  
314 possible that over-expression of *AtGST-U24* and *AtGST-U25* causes damage via excessive

315 glutathionylation of endogenous substrates, and subsequent depletion of GSH pools;  
316 *DmGSTE6* could lack activity towards these plant-endogenous substrates. However, the  
317 enhanced resistance and ability to take up TNT observed in the dGST lines was similar to that  
318 reported for the *AtGST-U24* and *AtGST-U25* overexpressing lines; the increased affinity and  
319 activity of *DmGSTE6* for TNT, observed in the studies on purified protein, compared to the  
320 *AtGST-U24* and *AtGST-U25* enzymes, did not translate into the predicted further increases in  
321 resistance and ability to take up TNT when expressed *in planta*.

322

323 We hypothesise that the constraint on TNT uptake and detoxification is due to limiting GSH  
324 levels in the root cytosol. The GSH abundance in the Arabidopsis cytosol is predicted to be in  
325 the range of 1 to 3 mM (Meyer et al., 2001; Meyer & Fricker, 2002), a concentration that is  
326 likely to be high enough to efficiently detoxify TNT in the presence of sufficient GST  
327 activity. However, it is possible that GSH levels become limited because they are utilised by  
328 other biochemical processes, compartmentalised to secure the GSH levels of specific  
329 organelles or the actual GSH levels are lower than those reported. In support of this  
330 hypothesis is the observation that the addition of exogenous GSH increased the ability of both  
331 wild type and dGST plants to remove TNT from liquid media. This is in agreement with the  
332 findings of Zechman *et al.* (2011) who reported that low pollen germination rates induced by  
333 treatment with the GSH synthesis inhibitor buthionine sulfoximine could be restored by the  
334 addition of 1 mM GSH to the growth media without any toxic effects. In addition to the  
335 requirement for GSH by *DmGSTE6*, TNT phytotoxicity is caused by its redox cycling  
336 activity (Johnston *et al.*, 2015). As GSH is important for redox homeostasis, depletion of  
337 GSH via TNT conjugation could compound the phytotoxicity of remaining TNT.

### 338 *The fate of TNT*

339 We have shown that *DmGSTE6* catalyses a denitration step producing 2-glutathionyl-4,6-  
340 dinitrotoluene. Based on studies of herbicides and other xenobiotics (Edwards *et al.*, 2011),  
341 TNT-conjugates are thought to be imported into the vacuole; two characterised glutathione-  
342 conjugate ABC transporters, MRP1 and MRP2 (Lu *et al.*, 1998; Tommasini *et al.*, 1998), are  
343 up-regulated in Arabidopsis, in response to TNT (Gandia-Herrero *et al.*, 2008). Once in the  
344 vacuole, further processing of GST-conjugated xenobiotics can occur to salvage the  
345 cysteinylglycine,  $\gamma$ -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 evacuation 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 *DmGSTE6* 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  $\gamma$ -glutamyl cysteine  
371 synthase have increased levels of GSH (Dhankher *et al.*, 2002), and could perhaps be  
372 combined with *DmGST* 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

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

### 377 *Developing field-applicable plant species*

378 While *Arabidopsis* is an excellent model system for elucidating, and manipulating, the  
379 mechanisms of TNT detoxification, species with different attributes are required for effective  
380 remediation of TNT from the environment. Such species would need to be fast-growing, and  
381 able to flourish in the harsh environments found on military training ranges. Species could  
382 include monocots such as switchgrass (*Panicum virgatum* L.), trees such as willow (*Salix*  
383 spp. L.) and poplar (*Populus* spp. L.) and species native to the contaminated region, like the  
384 shrub *Baccharis halimifolia* L. which is found on TNT-contaminated training ranges in North  
385 America (Ali et al., 2014). A combination of traditional breeding could be combined with  
386 genetic modification techniques to enhance TNT detoxification in these species. Indeed  
387 studies by van Dillewijn et al., (2008a) have shown that transgenic approaches, using  
388 bacterial nitroreductases, can be successfully extrapolated to a tree species for the  
389 remediation of TNT. Towards this aim, advancements in genomics and gene editing could be  
390 used to screen or manipulate *DmGSTE6*-like activity in field-applicable species; a trait that  
391 would contribute to the development and use of plants able to remediate TNT and re-vegetate  
392 explosives-polluted sites.

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397

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

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

400 TK, BM, ELR and NCB wrote the manuscript.

401



402 **References**

- 403 **Ali A, Zinnert JC, Muthukumar B, Peng Y, Chung SM, Stewart CN, Jr. 2014.**  
404 Physiological and transcriptional responses of *Baccharis halimifolia* to the explosive  
405 "composition B" (RDX/TNT) in amended soil. *Environ Sci Pollut Res Int* **21**: 8261-  
406 8270.
- 407 **Arnon DI. 1949.** Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta*  
408 *vulgaris*. *Plant Physiol* **24**: 1-15.
- 409 **Brentner L, Mukherji S, Walsh S, Schnoor J. 2010.** Localization of hexahydro-1,3,5-  
410 trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT) in poplar and  
411 switchgrass plants using phosphor imager autoradiography. *Environmental Pollution*  
412 **158**: 470-475.
- 413 **Clough SJ, Bent AF. 1998.** Floral dip: a simplified method for *Agrobacterium*-mediated  
414 transformation of *Arabidopsis thaliana*. *Plant J.* **16**: 735-743.
- 415 **Colville L, Smirnoff N. 2008.** Antioxidant status, peroxidase activity, and PR protein  
416 transcript levels in ascorbate-deficient *Arabidopsis thaliana* vtc mutants. *J Exp Bot*  
417 **59**: 3857-3868.
- 418 **Dhankher OP, Li Y, Rosen BP, Shi J, Salt D, Senecoff JF, Sashti NA, Meagher RB.**  
419 **2002.** Engineering tolerance and hyperaccumulation of arsenic in plants by combining  
420 arsenate reductase and gamma-glutamylcysteine synthetase expression. *Nat*  
421 *Biotechnol* **20**: 1140-1145.
- 422 **Dixon D, Sellars J, Edwards R. 2011.** The Arabidopsis phi class glutathione transferase  
423 AtGSTF2: binding and regulation by biologically active heterocyclic ligands.  
424 *Biochemical Journal* **438**: 63-70.
- 425 **Dixon DP, Edwards R. 2010.** Glutathione transferases. *Arabidopsis Book* **8**: e0131.
- 426 **Edwards R, Dixon D, Cummins I, Brazier-Hicks M, Skipsey M, Schroder P, Collins C.**  
427 **2011.** New Perspectives on the Metabolism and Detoxification of Synthetic  
428 Compounds in Plants. *Organic Xenobiotics and Plants: From Mode of Action To*  
429 *Ecophysiology* **8**: 125-148.
- 430 **Edwards R, Dixon DP. 2005.** Plant glutathione transferases. *Methods Enzymol* **401**: 169-  
431 186.

432 **Eisentraeger A, Reifferscheid G, Dardenne F, Blust R, Schofer A. 2007.** Hazard  
433 characterization and identification of a former ammunition site using microarrays,  
434 bioassays, and chemical analysis. *Environ Toxicol Chem* **26**: 634-646.

435 **French CE, Nicklin S, Bruce NC. 1998.** Aerobic degradation of 2,4,6-trinitrotoluene by  
436 *Enterobacter cloacae* PB2 and by pentaerythritol tetranitrate reductase.  
437 *Appl. Environ. Microbiol.* **64**: 2864-2868.

438 **French CE, Rosser SJ, Davies GJ, Nicklin S, Bruce NC. 1999.** Biodegradation of  
439 explosives by transgenic plants expressing pentaerythritol tetranitrate reductase.  
440 *Nat. Biotechnol.* **17**: 491-494.

441 **Gandia-Herrero F, Lorenz A, Larson T, Graham IA, Bowles DJ, Rylott EL, Bruce NC.**  
442 **2008.** Detoxification of the explosive 2,4,6-trinitrotoluene in *Arabidopsis*: discovery  
443 of bifunctional O- and C-glucosyltransferases. *Plant J* **56**: 963-974.

444 **Gleave AP. 1992.** A versatile binary vector system with a T-DNA organisational structure  
445 conducive to efficient integration of cloned DNA into the plant genome. *Plant*  
446 *Mol. Biol.* **20**: 1203-1207.

447 **Grzam A, Tennstedt P, Clemens S, Hell R, Meyer AJ. 2006.** Vacuolar sequestration of  
448 glutathione S-conjugates outcompetes a possible degradation of the glutathione  
449 moiety by phytochelatin synthase. *FEBS Lett* **580**: 6384-6390.

450 **Gunning V, Tzafestas K, Sparrow H, Johnston EJ, Brentnall AS, Potts JR, Rylott EL,**  
451 **Bruce NC. 2014.** *Arabidopsis* glutathione transferases U24 and U25 exhibit a range  
452 of detoxification activities with the environmental pollutant and explosive, 2,4,6-  
453 trinitrotoluene. *Plant Physiol* **165**: 854-865.

454 **Hannink NK, Rosser SJ, French CE, Basran A, Murray JA, Nicklin S, Bruce NC. 2001.**  
455 Phytodetoxification of TNT by transgenic plants expressing a bacterial nitroreductase.  
456 *Nat. Biotechnol.* **19**: 1168-1172.

457 **Jenkins TF, Hewitt AD, Grant CL, Thiboutot S, Ampleman G, Walsh ME, Ranney TA,**  
458 **Ramsey CA, Palazzo AJ, Pennington JC. 2006.** Identity and distribution of residues  
459 of energetic compounds at army live-fire training ranges. *Chemosphere* **63**, 1280-  
460 1290.

461 **Johnston EJ, Rylott EL, Beynon E, Lorenz A, Chechik V, Bruce NC. 2015.**  
462 Monodehydroascorbate reductase mediates TNT toxicity in plants. *Science* **349**: 1072-  
463 1075.

464

465 **Johnson GR, Spain JC. 2003.** Evolution of catabolic pathways for synthetic compounds:  
466 bacterial pathways for degradation of 2,4-dinitrotoluene and nitrobenzene. *Appl*  
467 *Microbiol Biotechnol* **62**: 110-123.

468 **Kalderis D, Juhasz Albert L, Boopathy R, Comfort S 2011.** Soils contaminated with  
469 explosives: Environmental fate and evaluation of state-of-the-art remediation  
470 processes (IUPAC Technical Report). *Pure and Applied Chemistry*. 1407-1484.

471 **Lima DR, Bezerra ML, Neves EB, Moreira FR. 2011.** Impact of ammunition and military  
472 explosives on human health and the environment. *Rev Environ Health* **26**: 101-110.

473 **Livak KJ, Schmittgen TD. 2001.** Analysis of relative gene expression data using real-time  
474 quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**: 402-408.

475 **Lu YP, Li ZS, Drozdowicz YM, Hortensteiner S, Martinoia E, Rea PA. 1998.** *AtMRP2*,  
476 an Arabidopsis ATP binding cassette transporter able to transport glutathione S-  
477 conjugates and chlorophyll catabolites: functional comparisons with *Atmrp1*. *Plant*  
478 *Cell* **10**(2): 267-282.

479 **Mazari AMA, Mannervik B. 2016.** Drosophila GSTs display outstanding catalytic  
480 efficiencies with the environmental pollutants 2,4,6-trinitrotoluene and 2,4-  
481 dinitrotoluene. *Biochemistry and Biophysics Reports* **5**: 141-145.

482 **Meyer AJ, Fricker MD. 2002.** Control of demand-driven biosynthesis of glutathione in  
483 green Arabidopsis suspension culture cells. *Plant Physiol* **130**: 1927-1937.

484 **Meyer AJ, May MJ, Fricker M. 2001.** Quantitative *in vivo* measurement of glutathione in  
485 Arabidopsis cells. *Plant J* **27**(1): 67-78.

486 **Moseyko N, Feldman L. 2001.** Expression of pH-sensitive green fluorescent protein in  
487 *Arabidopsis thaliana*. *Plant Cell and Environment* **24**(5): 557-563.

488 **Murashige T, Skoog F. 1962.** A revised medium for rapid growth and bioassay with tobacco  
489 tissue cultures. *Physiol.Plant.* **15**: 473-496.

490 **Musdal Y, Mannervik B. 2015.** Substrate specificities of two tau class glutathione  
491 transferases inducible by 2,4,6-trinitrotoluene in poplar. *Biochim Biophys Acta* **1850**:  
492 1877-1883.

493 **Ohkama-Ohtsu N, Zhao P, Xiang C, Oliver D. 2007.** Glutathione conjugates in the vacuole  
494 are degraded by gamma-glutamyl transpeptidase GGT3 in Arabidopsis. *Plant Journal*  
495 **49(5)**: 878-888.

496 **Pichtel J. 2012.** Distribution and fate of military explosives and propellants in soil: A review.  
497 *Applied and Environmental Soil Science* **2012**: 1-33.

498 **Qasim M, Gorb L, Magers D, Honea P, Leszczynski J, Moore B, Taylor L, Middleton**  
499 **M. 2009.** Structure and reactivity of TNT and related species: application of  
500 spectroscopic approaches and quantum-chemical approximations toward  
501 understanding transformation mechanisms. *J Hazard Mater* **167**: 154-163.

502 **Rylott EL, Budarina MV, Barker A, Lorenz A, Strand SE, Bruce NC. 2011a.**  
503 Engineering plants for the phytoremediation of RDX in the presence of the co-  
504 contaminating explosive TNT. *New Phytol* **192**: 405-413.

505 **Rylott EL, Gunning V, Tzafestas K, Sparrow H, Johnston EJ, Brentnall AS, Potts JR,**  
506 **Bruce NC. 2015.** Phytodetoxification of the environmental pollutant and explosive  
507 2,4,6-trinitrotoluene. *Plant Signal Behav* **10**: e977714.

508 **Rylott EL, Lorenz A, Bruce NC. 2011b.** Biodegradation and biotransformation of  
509 explosives. *Curr Opin Biotechnol* **22**: 434-440.

510 **Scott AC, Allen NS. 1999.** Changes in cytosolic pH within Arabidopsis root columella cells  
511 play a key role in the early signaling pathway for root gravitropism. *Plant Physiol*  
512 **121**: 1291-1298.

513 **Sens C, Scheidemann P, Klunk A, Werner D. 1998.** Distribution of <sup>14</sup>C TNT and  
514 derivatives in different biochemical compartments of *Phaseolus vulgaris*.  
515 *Environ.Sci.Pollution Res.* **5**: 202-208.

516 **Sens C, Scheidemann P, Werner D. 1999.** The distribution of <sup>14</sup>C-TNT in different  
517 biochemical compartments of the monocotyledonous *Triticum aestivum*.  
518 *Environ.Pollut.* **104**: 113-119.

519 **Talmage SS, Opresko DM, Maxwell CJ, Welsh CJ, Cretella FM, Reno PH, Daniel FB.**  
520 **1999.** Nitroaromatic munition compounds: environmental effects and screening  
521 values. *Rev. Environ. Contam Toxicol.* **161:** 1-156.

522 **Tommasini R, Vogt E, Fromenteau M, Hortensteiner S, Matile P, Amrhein N,**  
523 **Martinoia E. 1998.** An ABC-transporter of *Arabidopsis thaliana* has both  
524 glutathione-conjugate and chlorophyll catabolite transport activity. *Plant J* **13:** 773-  
525 780.

526 **Tournaire-Roux C, Sutka M, Javot H, Gout E, Gerbeau P, Luu D, Bligny R, Maurel C.**  
527 **2003.** Cytosolic pH regulates root water transport during anoxic stress through gating  
528 of aquaporins. *Nature* **425(6956):** 393-397.

529 **United States Defense Science Board Task Force. 1998.** Unexploded ordnance (UXO)  
530 clearance, active range UXO clearance, and explosive ordnance disposal (EOD)  
531 programs. *Office of the Undersecretary of Defense for Acquisition and Technology,*  
532 *Washington, D.C.* Website: [www.dtic.mil/cgi-bin/GetTRDoc?AD=ADA345904](http://www.dtic.mil/cgi-bin/GetTRDoc?AD=ADA345904), last  
533 accessed 7-Oct-16.

534 **United States Environmental Protection Agency. Jan 2014** Technical Fact Sheet –  
535 2,4,6-Trinitrotoluene (TNT). Website:  
536 [https://www.epa.gov/sites/production/files/2014-](https://www.epa.gov/sites/production/files/2014-03/documents/ffirofactsheet_contaminant_tnt_january2014_final.pdf)  
537 [03/documents/ffirofactsheet\\_contaminant\\_tnt\\_january2014\\_final.pdf](https://www.epa.gov/sites/production/files/2014-03/documents/ffirofactsheet_contaminant_tnt_january2014_final.pdf), last accessed 7-  
538 Oct-16.

539 **United States General Accounting Office. 2004.** Department of Defense operational ranges,  
540 more reliable cleanup cost estimates and a proactive approach to identifying  
541 contamination are needed. *Report to Congressional Requesters.* Website:  
542 <http://www.gao.gov/new.items/d04601.pdf>, last accessed 7-Oct-16.

543

544 **Valli K, Brock BJ, Joshi DK, Gold MH. 1992.** Degradation of 2,4-dinitrotoluene by the  
545 lignin-degrading fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **58:**  
546 221-228.

547 **van Dillewijn P, Couselo J, Corredoira E, Delgado A, Wittich R, Ballester A, Ramos J.**  
548 **2008a.** Bioremediation of 2,4,6-trinitrotoluene by bacterial nitroreductase expressing  
549 transgenic aspen. *Environ. Sci. Technol.* **42:** 7405-7410.

550 **van Dillewijn P, Wittich RM, Caballero A, Ramos JL. 2008b.** Type II hydride transferases  
551 from different microorganisms yield nitrite and diarylamines from polynitroaromatic  
552 compounds. *Appl Environ Microbiol* **74**: 6820-6823.

553 **Wittich RM, Haidour A, Van Dillewijn P, Ramos JL. 2008.** OYE flavoprotein reductases  
554 initiate the condensation of TNT-derived intermediates to secondary diarylamines and  
555 nitrite. *Environ.Sci.Technol.* **42**: 734-739.

556 **Zechmann B, Koffler BE, Russell SD. 2011.** Glutathione synthesis is essential for pollen  
557 germination *in vitro*. *BMC Plant Biol* **11**: 54.

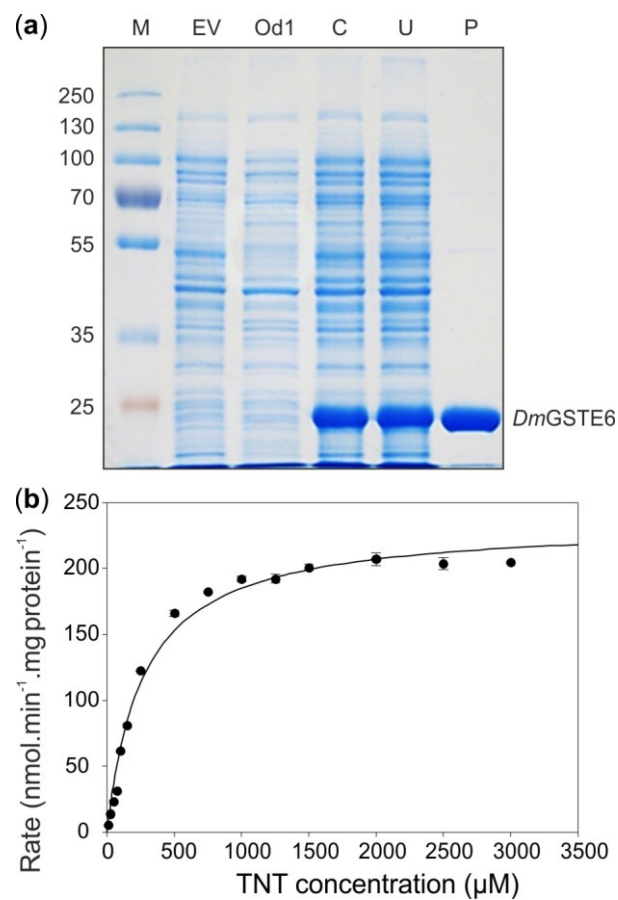
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560 **Figure Legends**

561 **Figure 1**

562 Analysis on purified *Drosophila melanogaster* *DmGSTE6*. (a) SDS-PAGE gel showing  
563 recombinantly expressed and purified *DmGSTE6*. M, molecular weight marker (kDa); EV,  
564 protein extract from cells transformed with the empty vector; OD1, protein extract from  
565 cultures with optical density 0.8-1 at 600 nm before the induction of the protein expression;  
566 C, crude protein extract from cells after the 60 h period of expression; U, unbound fraction of  
567 the purification process; P, purified protein. (b) Michaelis-Menten plots of *DmGSTE6* with  
568 TNT performed at pH 9.0 and 30 °C. Values represent the mean of three reactions  $\pm$  se.

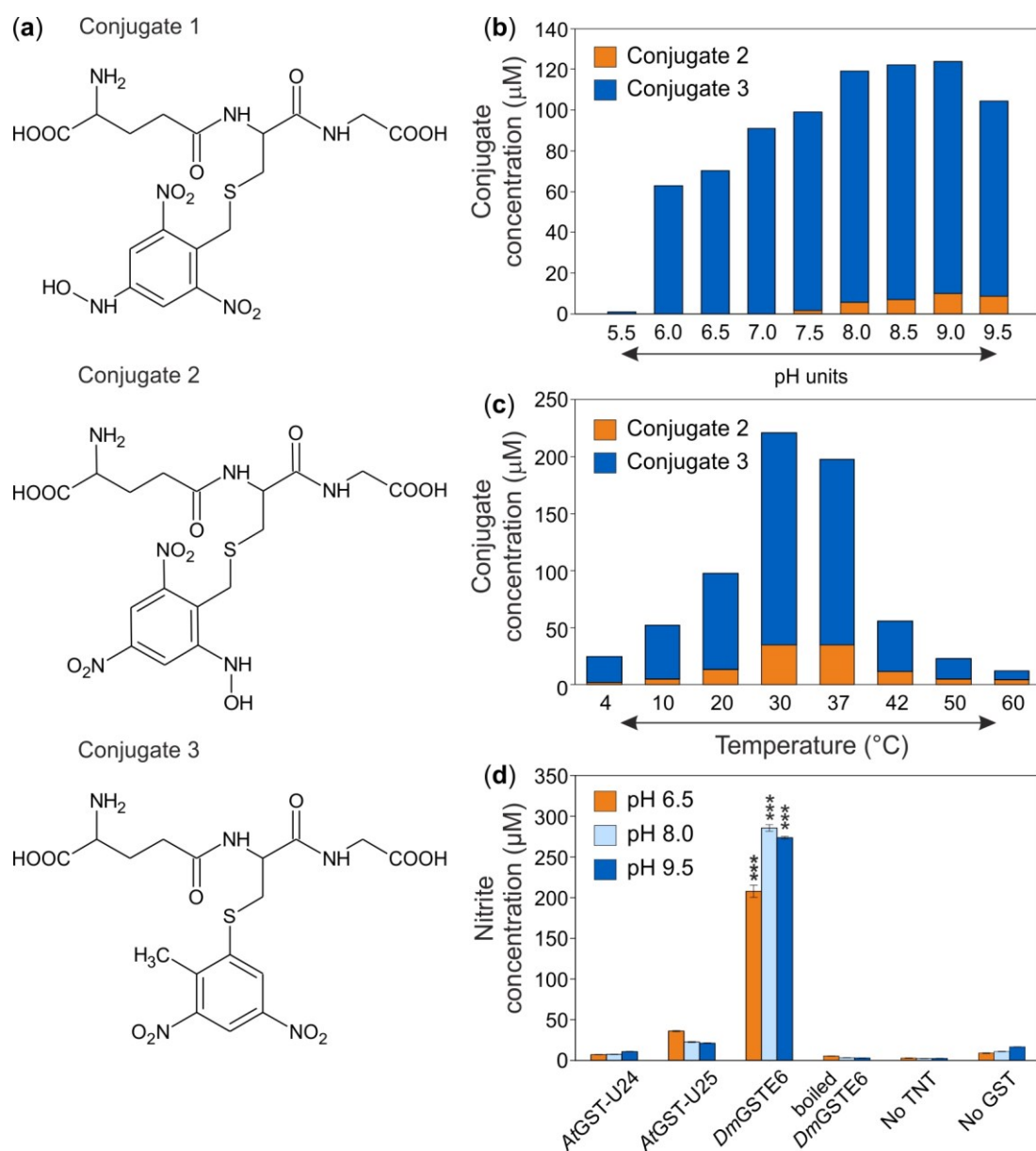


569

570

Figure 2

571 TNT-conjugate production by *Drosophila melanogaster* DmGSTE6. (a) Chemical structures  
 572 of the three TNT-conjugates. TNT-conjugate production profiles for DmGSTE6 over variable  
 573 (b) pH, at 20 °C and (c) variable temperature at pH 9.0, n = 3 ± se. Reactions were performed  
 574 over 1 hour using 10 µg of enzyme, 200 µM TNT and 5 mM GSH. (d) Nitrite released during  
 575 conjugation of TNT by 10 µg DmGSTE6, 100 µg AtGST-U24 and 100 µg GST-U25;  
 576 measured using the Griess assay. Reactions were performed over 3 hours, using 500 µM  
 577 TNT, at 20 °C, n = 5 ± se; \*\*\* P < 0.001 statistically significant from AtGST-U25 at that pH.

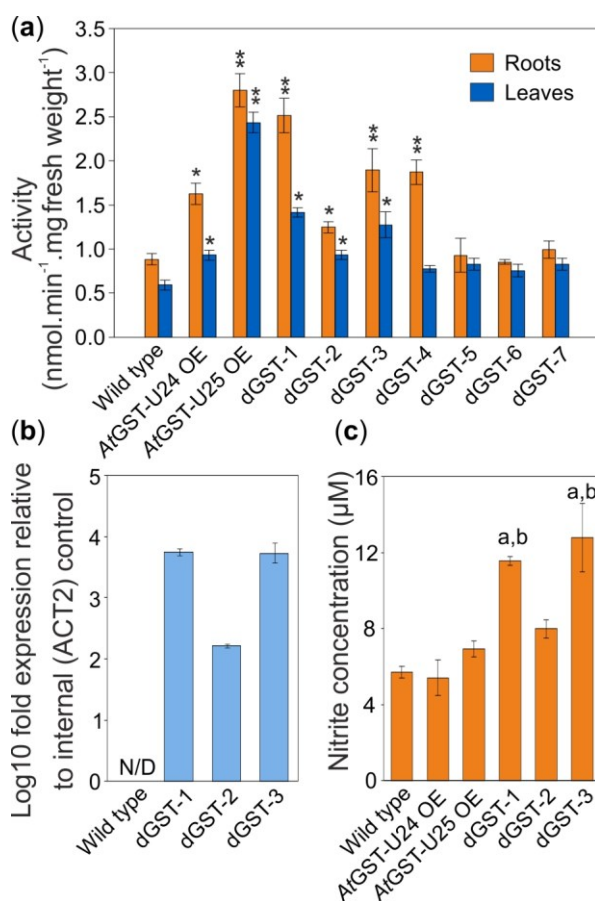


578



Figure 3

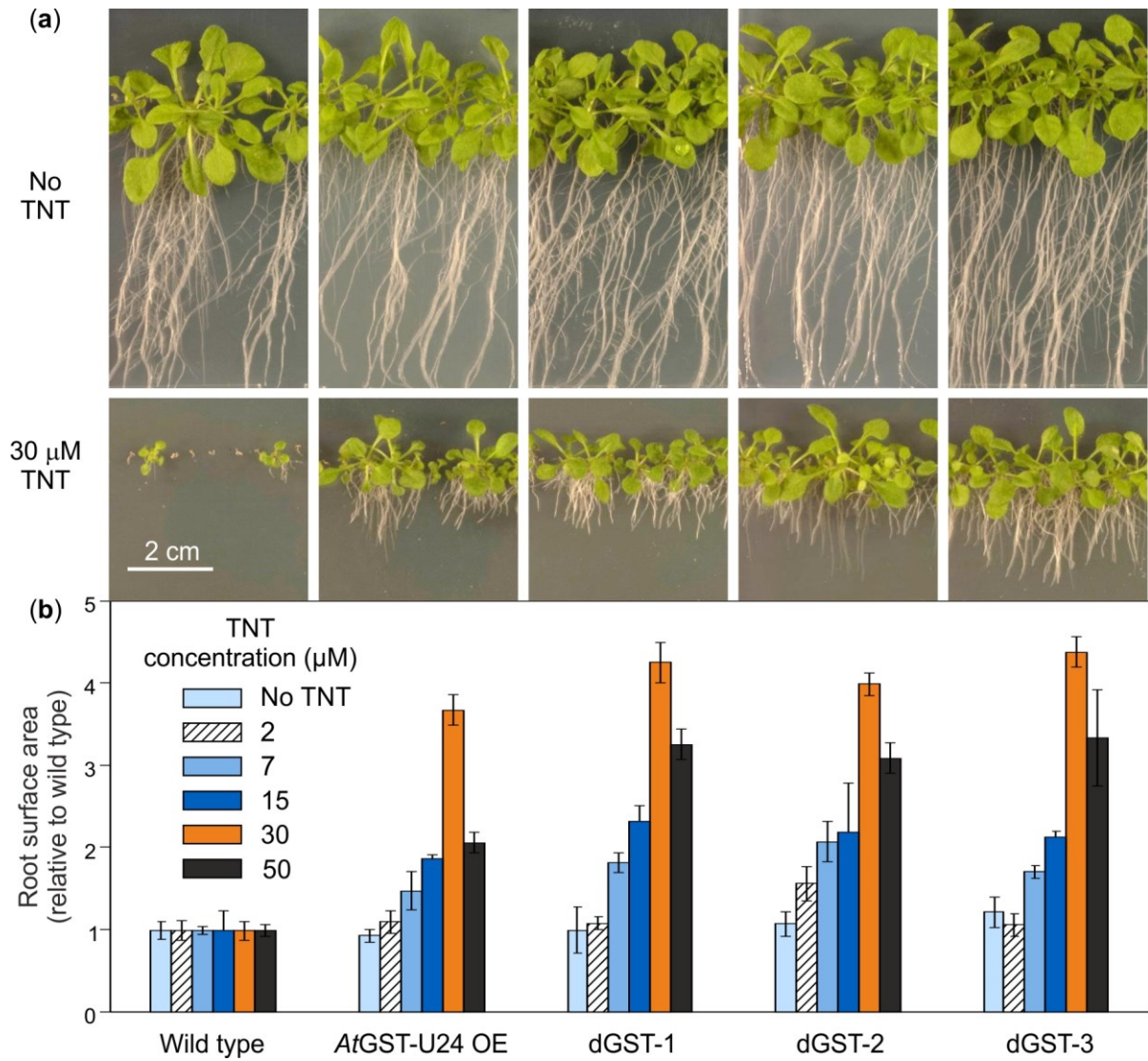
580 (a) Conjugation activity in leaf and root protein extracts from Arabidopsis wild type,  
 581 *Drosophila melanogaster* DmGSTE6 expressing lines, and AtGST-U24 and AtGST-U25  
 582 overexpressing (OE) lines assayed using CDNB substrate. Rosette leaves were from six-  
 583 week-old plants grown in uncontaminated soil. Roots were from two-week-old plants grown  
 584 vertically on agar plates containing ½ MS medium. \*  $P < 0.05$ , \*\*  $P < 0.01$ , statistically  
 585 significant from wild type. (b) Expression of *DmGSTE6* transcript using qPCR on 14 day old  
 586 Arabidopsis grown on uncontaminated soil; N/D, not detected. (c) Nitrite released during  
 587 conjugation of TNT by *DmGSTE6*, measured using the Griess assay. Reactions were  
 588 performed over 3 hours, using 500  $\mu\text{M}$  TNT, at 20 °C,  $n = 5 \pm \text{se}$ ; 'a' denotes statistically  
 589 significant from the wild type ( $P < 0.01$ ) and 'b' from the GST-U24/GST-U25 OE lines ( $P$   
 590  $< 0.05$ ).



592

**Figure 4**

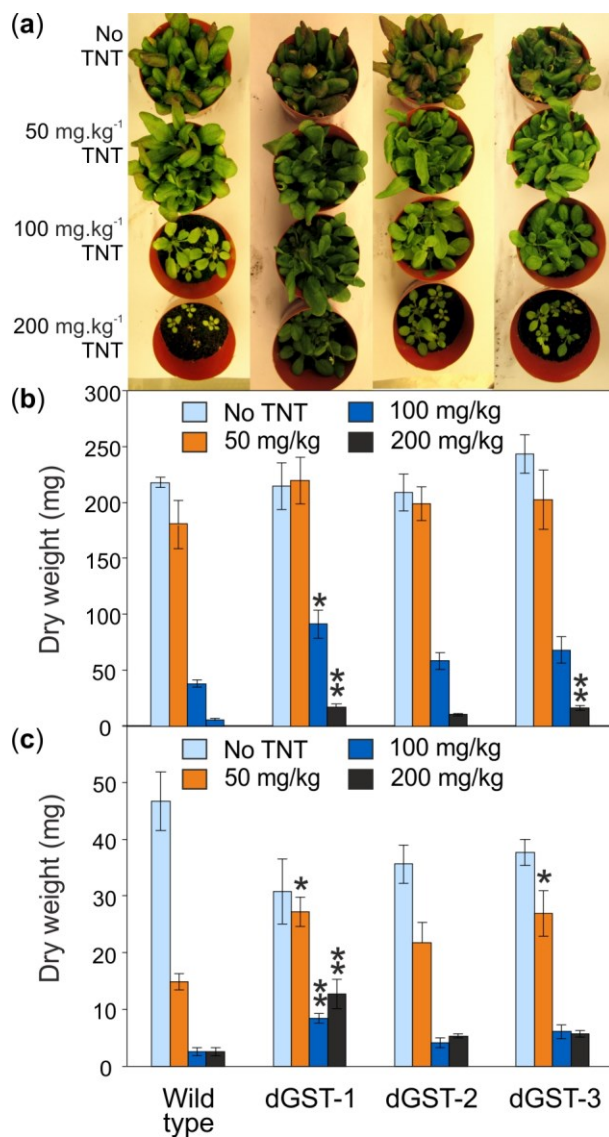
593 (a) Appearance of Arabidopsis wild type, *Drosophila melanogaster DmGSTE6* expressing  
 594 lines, and GST-U24 overexpressing (OE) seedlings grown for 20 days on ½ MS agar plates  
 595 in the absence of TNT, or in the presence of 30 µM TNT. (b), Root surface area of 20 day-old  
 596 plants grown on ½ MS agar plates containing a range of TNT concentrations, n = 60 ± se.



597

Figure 5

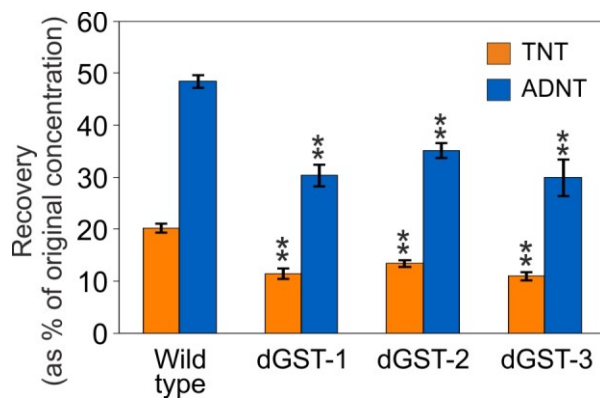
599 (a) Appearance of Arabidopsis plants grown in soil contaminated with a range of TNT  
 600 concentrations for six weeks. (b) Shoot and (c) root biomasses of Arabidopsis plants grown  
 601 for six weeks in soil contaminated with a range of TNT concentrations. WT, untransformed;  
 602 dGST/1-3, independent homozygous lines expressing *Drosophila melanogaster* DmGSTE6, n  
 603 = 8 ± se; \*  $P < 0.05$ , \*\*  $P < 0.01$ , statistically significant from wild type.



605

**Figure 6**

606 Levels of nitrotoluenes recovered from TNT-contaminated soil. Arabidopsis plants were  
607 grown on 50 mg.kg<sup>-1</sup> 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.

