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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 (*Dm*GSTE6) which has activity towards TNT.

32 - Recombinantly-expressed, purified *Dm*GSTE6 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 *Dm*GSTE6 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 *At*GST-U24 and *At*GST-U25
39 were compromised in biomass production when grown in the absence of TNT. This
40 yield drag was not observed in the *Dm*GSTE6 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 *Dm*GST may lack.

44 - In conclusion, *Dm*GSTE6 has activity towards TNT, producing a compound with
45 potential for further biodegradation. Selecting or manipulating plants to confer
46 *Dm*GSTE6-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 *At*GST-U24 and *At*GST-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 $nmol.min^{-1}.mg^{-1}$) 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 (*Dm*GSTE6) was found
91 to display outstanding activity toward TNT (Mazari & Mannervik, 2016). This research
92 describes the characterisation of *Dm*GSTE6, which has greater activity towards TNT than
93 *At*GST-U24 and *At*GST-U25. We have engineered Arabidopsis plants to express *Dm*GSTE6,
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* Dm*GSTE6 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 *Not*I 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) (½ 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 *Dm*GSTE6 and was initiated by addition of 1 mM CDNB to a total volume of 1 ml.
119 Increase in absorbance at A_{340} 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 *Dm*GSTE6 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 *At*GST-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_m and V_{max} 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_2PO_4, 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 µl of 80% acetone (v/v) , centrifuged at 12,000 g for two min at 4
147 °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)12-18 using
151 Superscript II reverse transcriptase (Thermo Fisher Scientific, Waltham, USA) containing
152 RNAsin (Promega, Madison, USA) at 42 °C for 2 h, before inactivation at 70 °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'-GCCGCTTTCAAAATGCAGAC-
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 ΔΔCt 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 ½
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^{-2} s^{-1} 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, *Dm*GSTE6 was analysed by SDS-PAGE
182 for purity and integrity. Figure 1a shows the purified *Dm*GSTE6, with an expected size of
183 approximately 25 kDa. The enzyme kinetics for *Dm*GSTE6 using TNT as substrate, were
184 determined and the Michaelis-Menten plot is shown in Figure 1b. The V_{max} and K_m values
185 were 235 ± 3.9 $nmol.min^{-1}.mg^{-1}$ and 269.5 ± 17.5 µM respectively. While GPOX activity was
186 detected in purified *At*GST-U25, GPOX activity was not detected for *Dm*GSTE6 (results not
187 shown).

188 Previous studies have shown that, dependent on pH and temperature, *At*GST-U25 produces
189 three different TNT-conjugates as shown in Figure 2a. The TNT conjugating activity by
190 *Dm*GSTE6 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 *Dm*GSTE6 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 *Dm*GSTE6, confirming the absence of non-enzymatic conjugation and
197 the stability of TNT at the different pH values tested. TNT-conjugating activity of *Dm*GSTE6
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 *Dm*GSTE6 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, *Dm*GSTE6 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 *Dm*GSTE6
206 produced significantly higher amounts of nitrite than *At*GST-U25; nitrite was not detected
207 with *At*GST-U24 which is unable to produce conjugate 3. Nitrite release was not observed
208 from the denatured *Dm*GSTE6 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 *Dm*GSTE6 towards ADNTs and HADNTs was tested, but no conjugated products
217 were detected (data not shown).

218 *Expression of* Dm*GSTE6 in Arabidopsis*

219 To assess the ability of *Dm*GSTE6 to conjugate and detoxify TNT *in planta*, Arabidopsis
220 lines expressing *Dm*GSTE6 were generated. Seven, homozygous *Dm*GSTE6 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 *Dm*GSTE6 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 *At*GST-U24, which does not produce conjugate 3, and *At*GST-
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 *At*GST-U25 over-
231 expressing lines, and thus more conjugate 3, confirming that these lines had a higher
232 conjugation activity *in planta* than the *At*GST-U25 over-expression line. Protein extracts
233 from wild type and the *At*GST-U24 over-expression line generated amounts of free nitrite
234 close to those of the *At*GST-U25 over-expression line. This was probably the result of
235 endogenous *At*GST-U25 present in those samples; approximately half of the conjugates
236 produced *in vivo* by *At*GST-U25 are predicted to be conjugate 3, with concomitant release of
237 nitrite, whereas *At*GST-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 *At*GST-U24 and
239 *At*GST-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 *At*GST-U24
241 and *At*GST-U25 over-expression lines. The appearance of the wild type, dGST and *At*GST-
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 *At*GST-U24 and *At*GST-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* Dm*GSTE6-expressing Arabidopsis*

250 To assess the ability of the *Dm*GSTE6-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 *At*GST-
253 U24 and *At*GST-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^{-1} 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^{-1} TNT, a
259 concentration found to completely inhibit growth for wild type; and *At*GST-U24 and *At*GST-
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^{-1} TNT; and 2.8 and 4.8-fold higher at 200 mg kg^{-1} 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^{-1} 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 µM 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 μM 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 μM 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* Dm*GST*

295 *Dm*GSTE6 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, *Dm*GSTE6 has both an increased affinity
298 towards TNT (K_m = 269.5 ± 17.5 μM) and significantly higher V_{max} (235 ± 3.9 nmol.min^{-}
299 ^{1}.mg^{-1}**)** than values reported by Gunning *et al.* (2014) for endogenous *At*GST-U24 and
300 *At*GST-U25 (K_m = 1644 ± 113.2 and 1210 ± 85.7 μM; Vmax = 92.3 ± 2.6 and 98.39 ± 3
301 nmol.min^{-1}.mg^{-1}, for *At*GST-U24 and *At*GST-U25 respectively).

302 The pH optimum for *Dm*GSTE6 activity towards TNT of pH 9.0, is in agreement with that
303 observed for both *At*GST-U24 and *At*GST-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
307 al., 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 *Dm*GSTE6 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 *Dm*GSTE6 expressing Arabidopsis lines, the yield drag observed in *At*GST-U24 and
313 *At*GST-U25 overexpressing Arabidopsis lines grown in the absence of TNT, was absent. It is
314 possible that over-expression of *At*GST-U24 and *At*GST-U25 causes damage via excessive

315 glutathionylation of endogenous substrates, and subsequent depletion of GSH pools;
316 *Dm*GSTE6 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 *At*GST-U24 and *At*GST-U25 overexpressing lines; the increased affinity and
319 activity of *Dm*GSTE6 for TNT, observed in the studies on purified protein, compared to the
320 *At*GST-U24 and *At*GST-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 *Dm*GSTE6, 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 *Dm*GSTE6 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, 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.

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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.

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.** *At*MRP2,
476 an Arabidopsis ATP binding cassette transporter able to transport glutathione S-
477 conjugates and chlorophyll catabolites: functional comparisons with *At*mrp1. *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 14C 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 ^{14}C-TNT in different
517 biochemical compartments of the monocotyledonous *Triticum aestivum*.
518 *Environ.Pollut.* **104**: 113-119.

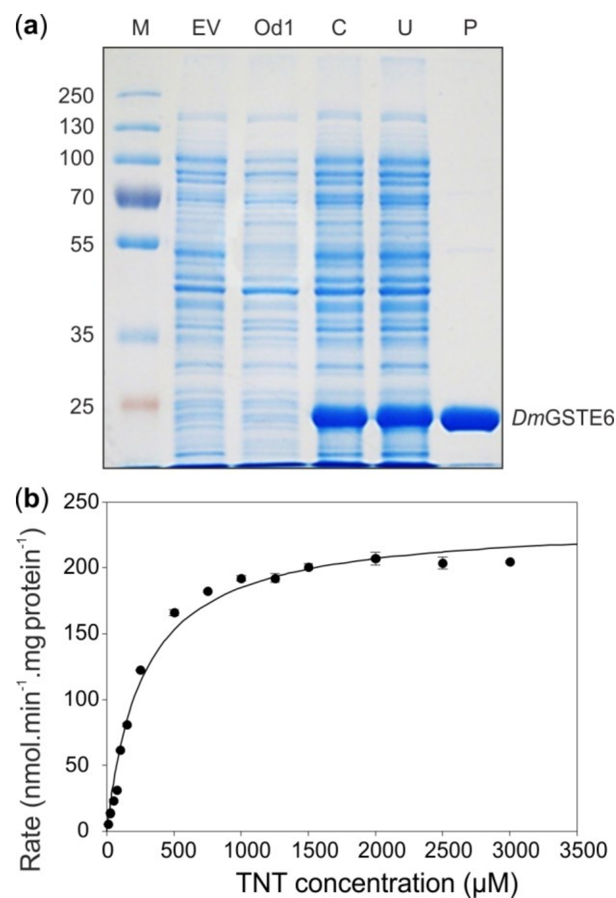
519 **Talmage SS, Opresko DM, Maxwell CJ, Welsh CJ, Cretella FM, Reno PH, Daniel FB.**
520 **1999.** Nitroaromatic munition compounds: environmental effects and screening
521 values. *Rev. Environ. Contam Toxicol.* **161**: 1-156.
522 **Tommasini R, Vogt E, Fromenteau M, Hortensteiner S, Matile P, Amrhein 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 Aquisition and Technology,*
532 *Washington, D.C.* Website: 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-
537 03/documents/ffrrofactsheet_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.
558
559

560 **Figure Legends**

561 **Figure 1**

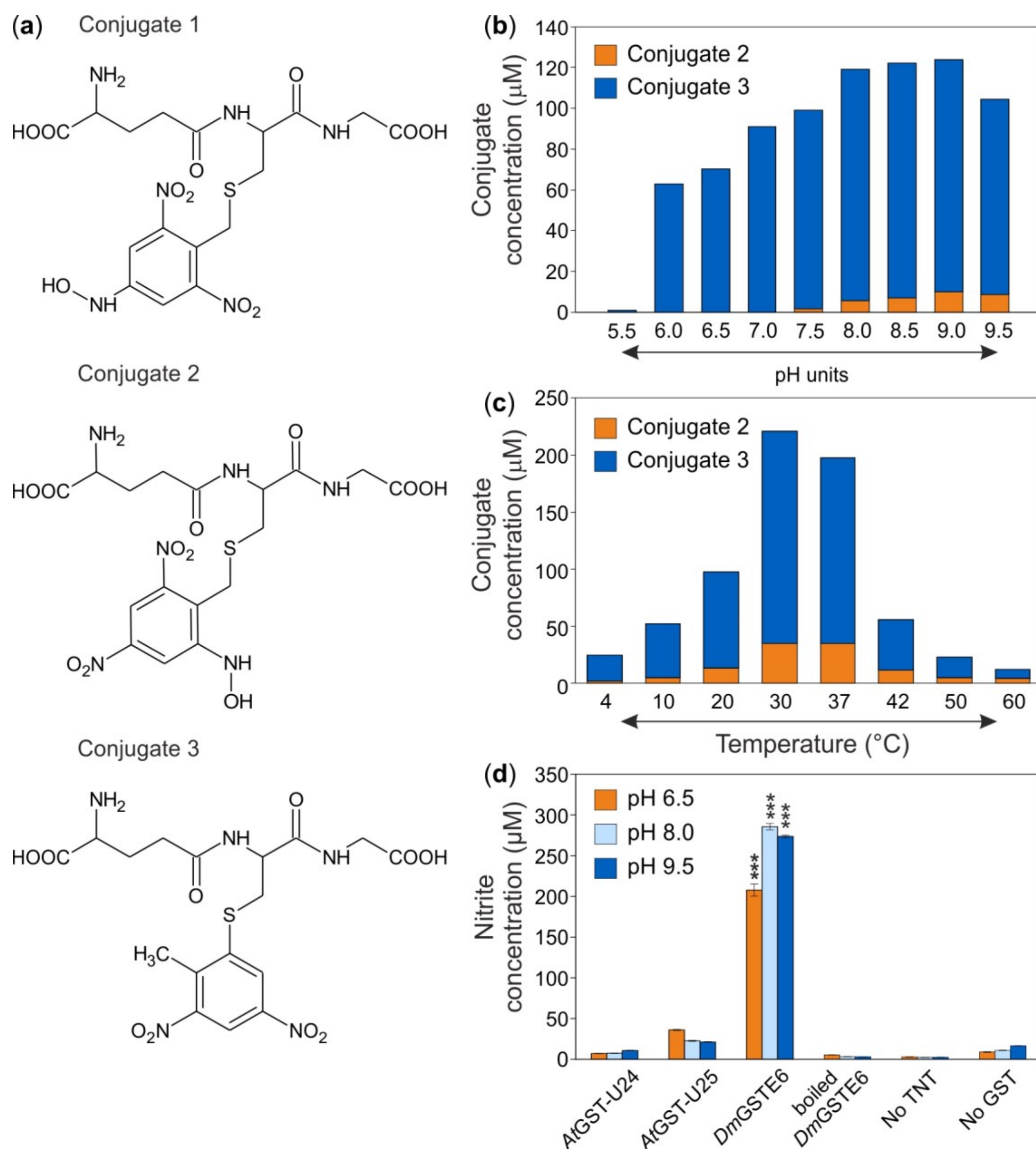
562 Analysis on purified *Drosophila melanogaster* *Dm*GSTE6. (**a**) SDS-PAGE gel showing
563 recombinantly expressed and purified *Dm*GSTE6. M, molecular weight marker (kDa); EV,
564 protein extract from cells transformed with the empty vector; OD1, protein extract from
565 cultures with optical density 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 *Dm*GSTE6 with
568 TNT performed at pH 9.0 and 30 °C. Values represent the mean of three reactions ± se.



569

570 **Figure 2**

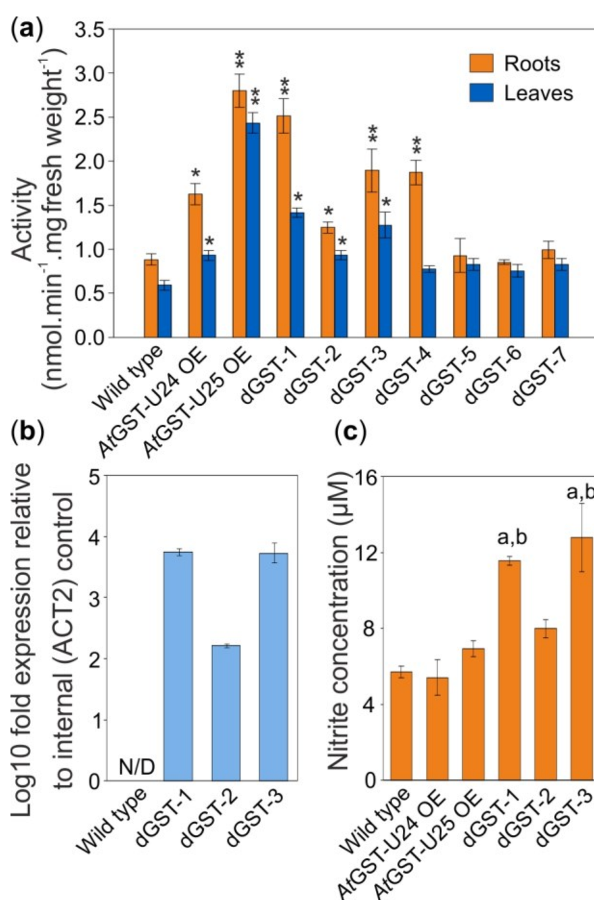
571 TNT-conjugate production by *Drosophila melanogaster Dm*GSTE6. (a) Chemical structures
572 of the three TNT-conjugates. TNT-conjugate production profiles for *Dm*GSTE6 over variable
573 (**b**) pH, at 20 °C and (**c**) variable temperature at pH 9.0, n = 3 ± se. Reactions were performed
574 over 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 *Dm*GSTE6, 100 µg *At*GST-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 *At*GST-U25 at that pH.



578

579 **Figure 3**

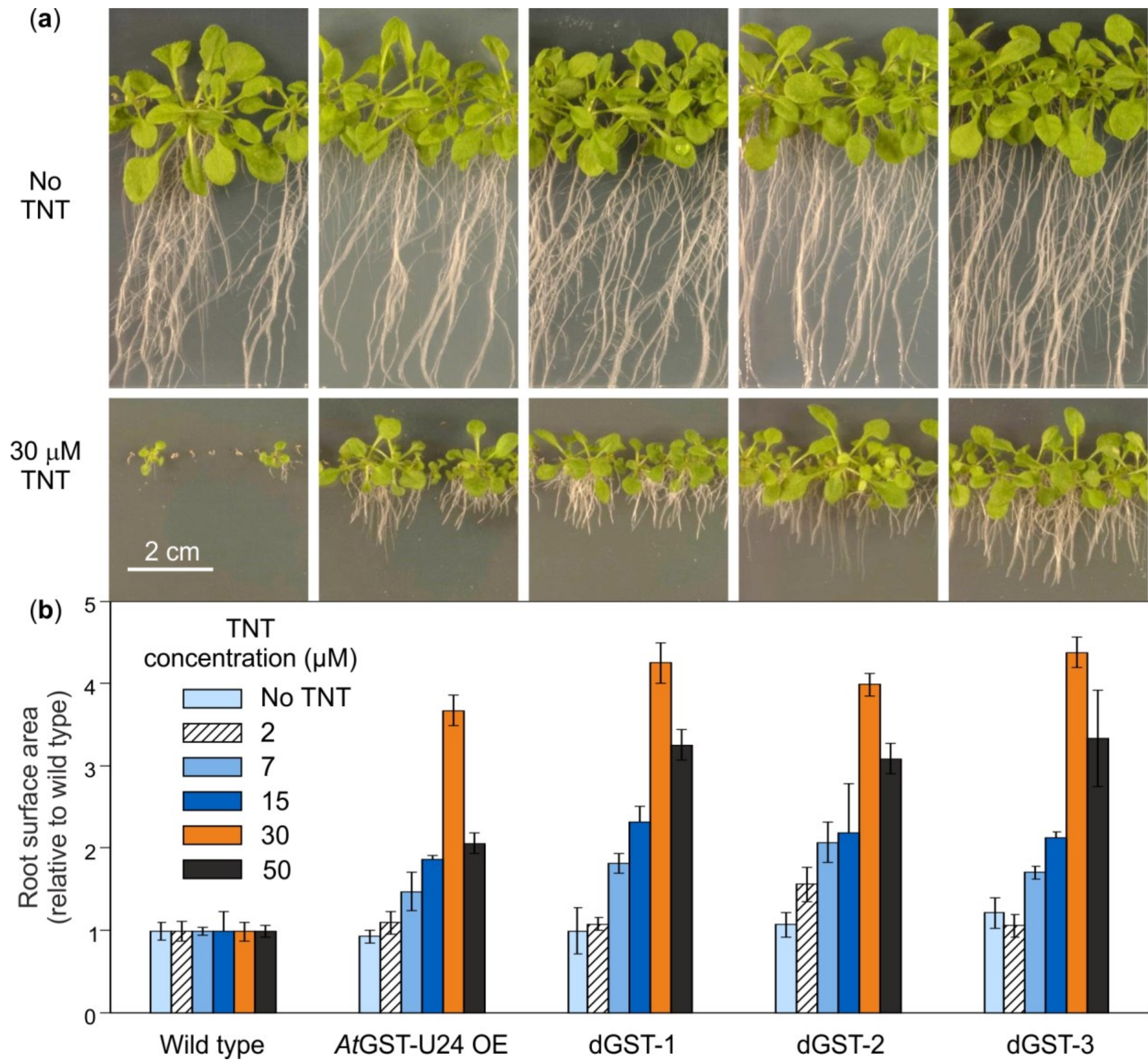
580 (**a**) Conjugation activity in leaf and root protein extracts from Arabidopsis wild type,
581 *Drosophila melanogaster Dm*GSTE6 expressing lines, and *At*GST-U24 and *At*GST-U25
582 overexpressing (OE) lines assayed using CDNB substrate. Rosette leaves were from six-
583 week-old plants grown in 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 *Dm*GSTE6 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 *Dm*GSTE6, measured using the Griess assay. Reactions were
588 performed over 3 hours, using 500 μM TNT, at 20 °C, n = 5 ± 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).



591

592 **Figure 4**

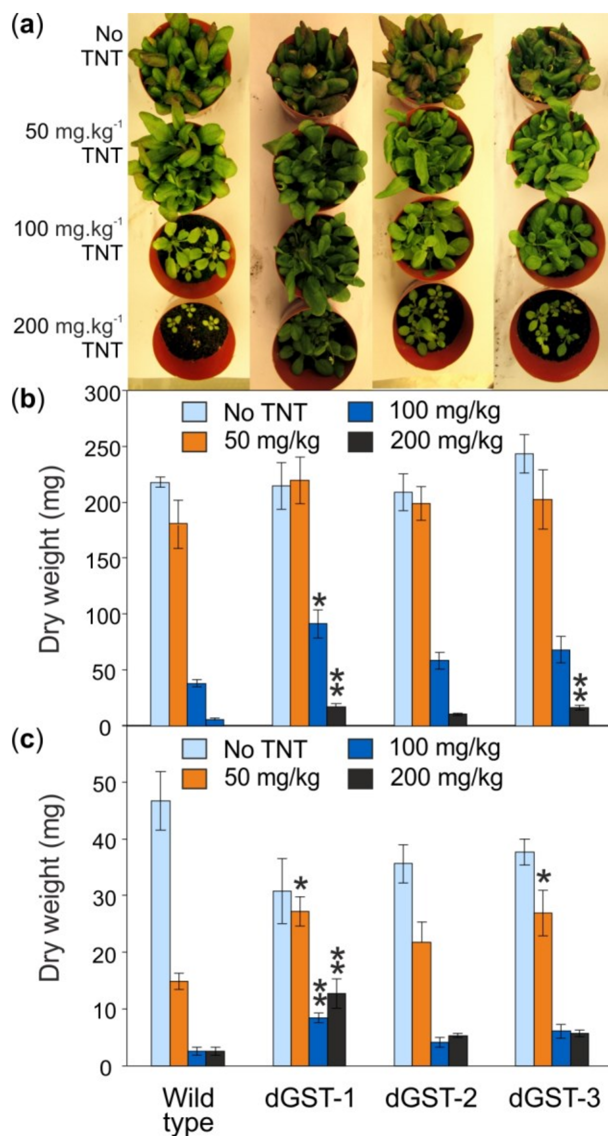
593 (a) Appearance of Arabidopsis wild type, *Drosophila melanogaster Dm*GSTE6 expressing
594 lines, and GST-U24 overexpressing (OE) seedlings grown for 20 days on ½ MS agar plates
595 in the absence of TNT, or in the presence of 30 μM TNT. (b), Root surface area of 20 day-old
596 plants grown on ½ MS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



597

598 **Figure 5**

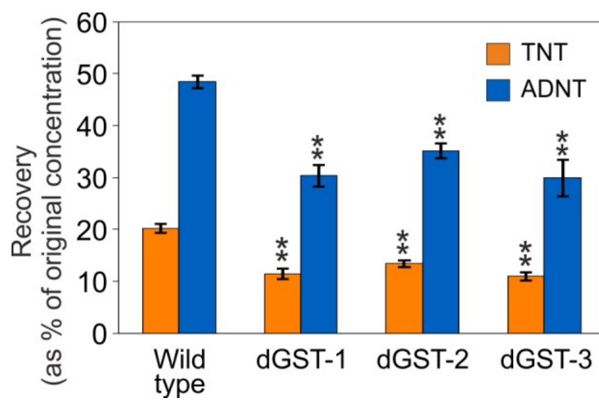
599 (**a**) Appearance of Arabidopsis plants grown in soil contaminated with a range of TNT
600 concentrations for six weeks. (**b**) Shoot and (**c**) root biomasses of Arabidopsis plants grown
601 for six weeks in soil contaminated with a range of TNT concentrations. WT, untransformed;
602 dGST/1-3,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



604

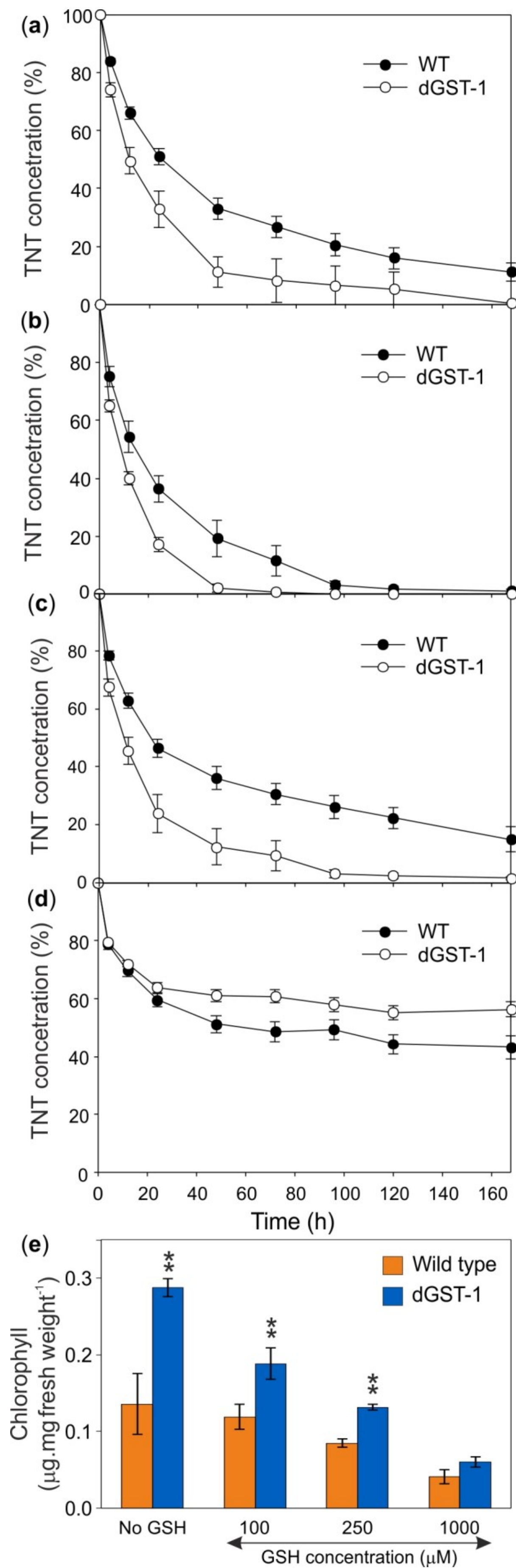
605 **Figure 6**

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.



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