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1 Expression in grasses of multiple transgenes for degradation of munitions compounds on live fire
2 training ranges

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8

9 **Running title:** Transgenic grasses degrade the explosive RDX.

10

11 **Keywords:** phytoremediation; RDX; TNT; switchgrass; monocot promoters; stacked genes.

12

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14

15 **Summary**

16 The deposition of toxic munitions compounds, such as hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine
17 (RDX), on soils around targets in live-fire-training ranges is an important source of groundwater
18 contamination. Plants take up RDX but do not significantly degrade it. Reported here is the
19 transformation of two perennial grass species, switchgrass (*Panicum virgatum*) and creeping
20 bentgrass (*Agrostis stolonifera*), with the genes for degradation of RDX. These species possess a
21 number of agronomic traits making them well-equipped for the uptake and removal of RDX from root
22 zone leachates. Transformation vectors were constructed with *xplA* and *xplB*, which confer the ability
23 to degrade RDX, and *nfsI*, which encodes a nitroreductase for the detoxification of the co-
24 contaminating explosive 2, 4, 6-trinitrotoluene (TNT). The vectors were transformed into the grass
25 species using *Agrobacterium tumefaciens* infection. All transformed grass lines showing high
26 transgene expression levels removed significantly more RDX from hydroponic solutions and retained
27 significantly less RDX in their leaf tissues than wild type plants. Soil columns planted with the best-
28 performing switchgrass line were able to prevent leaching of RDX through a 0.5 m root zone. These
29 plants represent a promising plant biotechnology to sustainably remove RDX from training range soil,
30 thus preventing contamination of groundwater.

31 **Introduction**

32 Continual military activity over nearly a century has resulted in the contamination of land and
33 groundwater by high explosives, in particular, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and 2,
34 4, 6-trinitrotoluene (TNT). These compounds enter the environment through manufacturing, military
35 use, and the decommissioning of outdated explosives. Human toxicity associated with TNT includes
36 aplastic anemia, and hepatitis, while RDX affects the central nervous system (Deng et al., 2014). Both
37 RDX and TNT are listed by the EPA as possible human carcinogens. More than 100 military bases
38 and explosives-manufacturing facilities in the USA are now contaminated with these chemicals,
39 which are highly recalcitrant to degradation in the environment. Live fire training at military bases has
40 resulted in the contamination of soils around targets with particulates of RDX and TNT, which leach

41 into the soil environment. The groundwater at these sites is at risk of contamination by the relatively
42 mobile RDX, increasing the likelihood that the health risk will spread to drinking water sources
43 beyond the military bases (Rivera et al., 1998). In contrast to RDX, TNT binds tightly to soil surfaces
44 and is a lesser threat to groundwater. However, TNT is highly phytotoxic and its presence as a co-
45 contaminant can hinder clean-up operations for RDX (Rylott and Bruce, 2009).

46

47 In the US, the clean-up of active ranges contaminated with explosives has been estimated by the US
48 Department of Defense to cost between US\$16 billion and US\$165 billion (United States General
49 Accounting Office 2004). A sustainable and potentially low-cost alternative for remediating
50 munitions contaminated soils is phytoremediation: the use of plants to degrade the pollutants.
51 Although plants are able to take up RDX, translocating it to the aerial tissues, degradation of RDX by
52 plants is low (Just and Schnoor, 2004; Winfield, 2004), resulting in the persistence of RDX in soil
53 environments. Studies in poplar tissues (*Populus deltoides* x *nigra* DN-34) show that RDX in leaf
54 tissue is partially reduced to hexahydro-1-nitroso-3, 5-dinitro-1, 3, 5-triazine (MNX) and hexahydro-1,
55 3-dinitroso-5-nitro-1, 3, 5-triazine (DNX). Further transformation of RDX, MNX, and DNX results in
56 the formation of formaldehyde, methanol, and carbon dioxide through a photolytic mechanism (Van
57 Aken et al., 2004).

58 The process of TNT uptake by plants, observed in a number of species, is hampered by its acute
59 phytotoxicity (Johnston et al., 2015). Although plants have only a limited ability to detoxify TNT, the
60 biochemical pathways involved have been well studied in *Arabidopsis*. Following uptake, TNT is
61 transformed by oxophytodienoate reductases (OPRs; Beynon et al., 2009), then conjugated by uridine
62 diphosphate glycosyltransferases (UGTs; Gandia-Herrero et al., 2008). Conjugation directly to the
63 TNT molecule by glutathione transferases (GSTs) has also been demonstrated (Gunning et al., 2014).
64 In order to increase the effectiveness of phytoremediation, it is possible to express transgenes
65 involved in metabolism, uptake, or transport of specific pollutants in genetically modified plants. This

66 strategy combines the advantages of plants including their high biomass and energy levels, ease of
67 cultivation, and water uptake of plants with the diverse catabolic capabilities of bacteria.

68 Bacterial genes encoding enzymes for the degradation and transformation of RDX and TNT,
69 respectively, have been identified. *Rhodococcus rhodochrous* strain 11Y was isolated from
70 explosives-contaminated soils and found to grow on RDX as the sole source of nitrogen. The RDX
71 degradation system was subsequently characterized and found to comprise a novel fused flavodoxin-
72 cytochrome P450 XplA and partnering flavodoxin reductase XplB (Seth-Smith et al., 2002, Rylott *et*
73 *al.*, 2006, Rylott *et al.*, 2011b). The *xplA* gene and its reductase partner *xplB* were expressed together
74 in *Arabidopsis* (*Arabidopsis thaliana*) and the transformants found to remove RDX from liquid
75 culture and soil leachate at rates significantly faster than those of untransformed plants (Jackson et al.,
76 2007). The *nfsI* gene from *Enterobacter cloacae* encodes a nitroreductase (NR) that transforms TNT
77 (Bryant et al., 1991). *Arabidopsis* plants engineered with *xplA*, *xplB*, and *nfsI* were able to degrade
78 RDX and detoxify TNT, suggesting that plants adapted to training range conditions could reduce
79 RDX contamination *in situ* if the plants were similarly transformed (Rylott et al., 2011a).

80 Several perennial grass species are well-adapted to the environmental conditions found on training
81 range conditions in temperate regions (Palazzo et al., 2005). This study focuses on the expression of
82 *xplA*, *xplB*, and *nfsI* in switchgrass (*Panicum virgatum*) and creeping bentgrass (*Agrostis stolonifera*)
83 for the phytoremediation of RDX and TNT in soils such as those found at training ranges in the USA.
84 High throughput *Agrobacterium*-mediated transformation techniques are well established for
85 switchgrass (Li and Qu, 2011; Ramamoorthy and Kumar, 2012; Xi et al., 2009) and for creeping
86 bentgrass (Zhou et al., 2013). Transformation vectors were constructed using a multiple gene
87 transformation vector system, pNSAT, which was based on the pSAT versatile vector system (Chung
88 et al., 2005). The pNSAT vectors all contained *xplA*, *xplB*, and *nfsI* along with the selection marker
89 gene, *hygromycin B phosphotransferase (hpt)*, which were driven by either monocotyledon-specific
90 promoters or the 35s promoter. This is the first report of the genetic transformation of grasses for
91 phytoremediation.

92 **Results**

93 **Vector construction**

94 To produce grass lines expressing multiple transgenes, the pSAT vector series were selected (Chung
95 et al., 2005) and further modified. In order to identify putative transformants, the *hyg* gene, which
96 encodes resistance to hygromycin, was employed. Selection efficiency of transformants was further
97 enhanced by replacing the promoter and terminator regions in the expression cassette *ocs-hpt-ocs* in
98 pSAT1a with 35S promoter and terminator regions to produce the cassette pSAT1a-35S-*hpt*-35T. The
99 *xplA*, *xplB*, and *nfsI* genes were inserted into pSAT6a, pSAT4a, and pSAT7a to produce pSAT6a-*xplA*,
100 pSAT4a-*xplB*, and pSAT7a-*nfsI*, respectively. Then the expression cassettes of 35S-*hpt*-35ST, *rbc*-
101 *xplA*-*rbcT*, 35S-*xplB*-35ST, *act-nfsI*-*agsT* were excised from the pSAT vectors with the appropriate
102 homing endonucleases and sequentially inserted into the corresponding restriction sites of the binary
103 vector pPZP-RCS2 to produce pRCS2-ABNR-HR, as shown in Figure 1a.

104 The pSAT vector sets are tailored to function in dicot species, but to achieve optimal expression in
105 monocot species it is necessary to use native monocot promoters (Mann et al., 2012). The rice actin
106 promoter (*Osact*) and maize ubiquitin promoter (*Zmubi*) are widely used in monocot crops due to their
107 ability to direct high levels of near constitutive gene expression (Cornejo et al., 1993; McElroy et al.,
108 1990). The switchgrass ubiquitin promoter (*Pvubi*) has strong constitutive expression in switchgrass
109 and rice (Mann et al., 2011). These three promoters were used to replace the 35S promoter in
110 pSAT1a-35S, the *mas* promoter in pSAT3a and the *rbc* promoter in pSAT6a, producing pNSAT1a,
111 pNSAT3a, and pNSAT6a, respectively (Figure 1b). The selectable marker gene *hpt*, and the target
112 genes *xplA*, *xplB*, and *nfsI* were inserted into pNSAT1a, pNSAT6a, pNSAT3a, and pNSAT4a to
113 produce *Osact-hpt*-35ST, *Pvubi-xplA*-*rbcT*, *Zmubi-xplB*-*masT*, and 35S-*nfsI*-35ST cassettes. These
114 expression cassettes were then integrated into the corresponding homing restriction sites of pPZP-
115 RCS2 to produce pRCS2-NABNR (Figure 1c).

116 **Functional evaluation of the pNSAT vectors**

117 To validate the functionality of the expression cassettes of pNSAT, the green-fluorescent protein-
118 encoding *gfp* gene was inserted into pNSAT1a to produce pNSAT1a-*gfp* and a second visual reporter
119 gene (*gus*) encoding β -glucuronidase was inserted into pNSAT3a and pNSAT6a to produce
120 pNSAT3a-*gus* and pNSAT6a-*gus*. The GFP and GUS were transformed, separately in epidermal cells
121 of onion using the biolistic method and transiently expression of GFP and GUS visualized using
122 fluorescent and light microscopy, respectively. Detailed methods are provided in the Supplement. As
123 shown in Figure 2, transient expression of GFP and GUS were observed in the cytosol of epidermal
124 cells.

125 **Production of transgenic grasses and molecular analysis**

126 Embryogenic calli of creeping bentgrass were infected with *Agrobacterium* strain EHA105 harboring
127 pRCS2-ABNR-HR or pRCS2-NABNR and then placed on callus induction medium (CIM) containing
128 the selection agent hygromycin (100 mg/L). After three weeks of selection, hygromycin resistant calli
129 (Figure 3a) were transferred to regeneration medium containing hygromycin to develop plantlets,
130 which were later transferred to soil (Figure 3b and c). To confirm the expression of the transgenes in
131 the hygromycin-resistant plantlets, qRT-PCR analysis was conducted.

132 The results presented in Figure S1a show that all four pRCS2-NABNR transformed creeping
133 bentgrass lines expressed *xplA* to similar levels ($p = 0.15$), while the expression level of *xplB* in line
134 N19 was more than twice that of the other lines ($p = 0.044$). The expression level of *nfsI* was much
135 lower than for *xplA* and *xplB*. Relative to *xplA* expression, the levels of *nfsI* transcript were $0.56 \pm$
136 0.02 , 0.06 ± 0.03 , 0.15 ± 0.01 and 0.04 ± 0.04 for the lines N5, N14, N18 and N19, respectively.
137 Western blot analysis of the transformed creeping bentgrass lines, shown in Figure S1b, revealed the
138 presence of a single 60 kDa band following immunoblot analysis using the XplA antibody, and
139 corresponded in size to the XplA protein. A single 45 kDa band was detected by immunoblot analysis
140 using the XplB antibody, and corresponded in size to the XplB protein. Bands were not seen on blots
141 probed using an antibody to the nitroreductase protein (NR), the product of *nfsI*.

142 To produce transformed switchgrass, friable type II embryogenic callus (Burris et al., 2009) was used
143 for infection with *Agrobacterium* strain EHA105 harboring the pRCS2-NABNR vector. The calli
144 were screened on CIM with 100 mg/L hygromycin for two weeks in the first round of selection. The
145 surviving calli from the first selection round were transferred for further selection on CIM with 200
146 mg/L hygromycin (Figure 3d). Vigorously growing calli during the third round selection were
147 transferred to regeneration medium containing 50 mg/L hygromycin for plant development (Figure
148 3e). After two months, healthy plants were transferred to soil (Figure 3f). To monitor transgene
149 transcript levels, qRT-PCR was conducted on the transformed switchgrass (Figure 4a). The relative
150 levels of *xplA* and *xplB* were broadly similar across all transformed lines, with plant line N4
151 exhibiting the highest levels of expression for these transgenes.

152 The *xplA-xplB-nfsI* expressing *Arabidopsis* line 7D, published by Rylott et al., (2011a) was used as a
153 guide; transgene expression in this line conferred significant ability to remove RDX and TNT from
154 contaminated media (Rylott et al., 2011a); however, while the expression levels of *xplA* and *xplB*
155 were all significantly higher than 7D in the transformed switchgrass lines, direct comparisons cannot
156 be made. The levels of *nfsI* transcripts were significantly lower than for *xplA* and *xplB* in all the
157 switchgrass lines, with the exception of line N7. In agreement with the relatively low transcript levels
158 observed for *nfsI*, the western blot analyses presented in Figure 4b and c show that levels of NR were
159 low when compared to the expression for XplA and XplB, and too low for band intensities to be
160 accurately determined. As seen with the transcript levels for *xplA* and *xplB*, the protein levels of XplA
161 and XplB were broadly similar across all the lines tested, with only a three-fold difference in
162 transcript and protein expression levels, with line N1 producing the highest levels of XplA and XplB
163 protein.

164 **RDX uptake and degradation by transformed grasses**

165 To determine the uptake rate of RDX by the transformed grasses the plants were grown in liquid
166 culture. The experiment used open test tubes to allow for maximum transpiration; physical losses of
167 RDX were minimal due to the low volatility of RDX (Xiong et al., 2009).

168 For creeping bentgrass, the RDX uptake from the medium is shown in Figure 5a. All three creeping
169 bentgrass lines removed RDX from the medium faster than the wild type control line. After three days,
170 the medium of creeping bentgrass line N19 contained significantly ($p=0.010$) less RDX than medium
171 from wild type plants and this difference increased during further culture. The RDX removal by line
172 N19 was not only significantly greater than that of wild type, but also greater than lines N5 and N18
173 ($p < 0.05$). After nine days of culture, lines N5 and 18 also removed significantly more RDX from the
174 media than wild type ($p = 0.03$ for N5 and 0.05 for N18).

175 To determine accumulation of RDX in leaf tissue, RDX was extracted and analyzed by HPLC at day
176 16 of the uptake experiments. RDX levels were highest in wild type creeping bentgrass tissues (Figure
177 5b) while lower levels of RDX were detected in plant lines N5 and N18; RDX was not detected in line
178 N19.

179 The accumulation of RDX in the leaf tissue of wild type creeping bentgrass was correlated with a
180 three-fold depression of the growth of the plants compared to wild type plants grown in medium
181 without RDX (Table 1). This decrease in biomass occurred despite the presence of sufficient nitrogen
182 in the MS medium (20.6 mM NH_4^+ and 39.4 mM NO_3^-). In contrast, the transformed creeping
183 bentgrass gained 1.5 to 2.5-fold more biomass than wild type bentgrass when cultured in the presence
184 of RDX.

185 For switchgrass, the course of RDX uptake from the medium are shown in Figure 6a. All three
186 transgenic switchgrass lines removed RDX from the medium at significantly faster rates than the wild
187 type plants ($p = 0.051$, 0.0014 and 0.0016 for lines N1, 2 and 3 respectively at day 3. $p=0.0043$ for
188 line N1 at day 7). Figure 6b shows that RDX was not detectable in the transgenic switchgrass plants,
189 whereas wild type plants contained $0.058 \text{ mg RDX per g}$ of leaf tissue. To confirm that the lack of
190 accumulation of RDX in the transgenic switchgrass tissues was caused by degradation, rather than by
191 dilution in growing plant tissue, switchgrass plants were exposed to 20 mg/L RDX in MS medium for
192 36 hours . After this time, leaf tissue from wild type plants contained $0.207 \pm 0.002 \text{ mg/g RDX}$ ($n = 3$
193 $\pm \text{SD}$) whereas RDX was not detected in leaf tissue of transformed switchgrass.

194 These data demonstrate that all the RDX taken up by transformed switchgrass was degraded within
195 the timescale of the experiment and suggests that RDX degradation is limited by uptake in the
196 transpiration stream. As reported in other studies (Jackson et al., 2007; Rylott et al., 2011a; Rylott et
197 al., 2006; Sabbadin et al., 2009) the RDX transformation products MNX and DNX were not detected
198 in either the creeping bentgrass or switchgrass liquid culture studies.

199 Switchgrass transformants were propagated by inducing cluster shoots from their nodes and the
200 propagated lines tested for RDX uptake from liquid culture. Consistent with the activity determined
201 from the parent transformed plants, the propagated plants had similar rates of RDX removal and
202 degradation (Figure S2), confirming that expression of *xplA* and *xplB* in switchgrass can be
203 transferred during vegetative propagation.

204

205 **TNT resistance of transformed grasses**

206 When cultured in ½ MS medium containing 4.5 mg/L TNT both pRCS2-ABNR-HR transformed line
207 N1 and wild type creeping bentgrass plants survived and grew. The ABNR-HR line removed TNT
208 from the medium more rapidly than the wild type (Figure S3a). While there was no difference in the
209 morphology of the aerial parts of wild type or transformed bentgrass, the root morphology was
210 affected by exposure to TNT. The density of mature root hairs was greater for transformed plants
211 compared to that of wild type bentgrass after 15 days (Figure S3b and S3c). This result demonstrated
212 that TNT in liquid medium depressed the development of the root system of creeping bentgrass, and
213 that the expression of *nfsI* enhanced resistance of plants to TNT. This observation is similar to the
214 finding of TNT toxicity resistance in *Arabidopsis* transformed with *nfsI* (Hannink et al., 2001).

215 In contrast, we found no difference in TNT resistance between pRCS2-NABNR transformed and wild
216 type switchgrass and creeping bentgrass. The root systems of both transformants and wild type plants
217 were repressed in MS medium containing 4.5 mg/L TNT. This may be explained by a lack of NR
218 protein production, since the western blot for NR in switchgrass showed only weak bands, although
219 *nfsI* transcript was observed by qRT-PCR.

220 **Column studies with switchgrass**

221 To gain an understanding of the ability of the transgenic grass lines to remove RDX from soils on
222 military ranges, column studies containing a sand and gravel mix were conducted. The RDX was
223 applied to the columns containing wild type or line N1 switchgrass plants and flushed out three times
224 over the course of two months. Following the first two applications, RDX was undetectable in the
225 leachates of columns containing wild type and transgenic switchgrass, but wild type leaf tissue
226 contained significantly more RDX than transgenic leaf tissue (data not shown). The results of the third
227 application of RDX are shown in Figure 7a. About one fourth of the applied RDX was recovered in
228 the leachate from the wild type columns, whereas RDX was not detected in the leachate from the
229 transgenic columns. In the leaf tissues, RDX level was significantly less ($p = 0.0044$) in the transgenic
230 leaf tissue compared to the wild type tissue (Figure 7b).

231 **Discussion**

232 The use of transgenic plants has been proposed for the phytoremediation of pollutants including
233 metals, explosives, petroleum, solvents, and polycyclic aromatic hydrocarbons (Bizily et al., 2000;
234 Chen et al., 2010; Doty et al., 2007; Doty et al., 2000; Karavangeli et al., 2005, Rylott et al. 2015),
235 and demonstrated in tobacco, Arabidopsis, and Populus. For application to soil remediation, species
236 such as grasses are desirable, but transformation of grasses with phytoremediation genes has not been
237 demonstrated. This work focuses on the development of creeping bentgrass and switchgrass lines
238 transformed with *xplA*, *xplB*, and *nfsI* for the degradation of RDX and detoxification of TNT on live-
239 fire training ranges. These perennial grasses provide year-round cover, are adaptable to different
240 environmental conditions on training ranges and have wide geographic range. They also have highly
241 dense and deep rooting systems which provide accessibility to and uptake of RDX and TNT. In
242 addition, these species provide good erosion control, nesting and invertebrate habitats. Together, the
243 studies here show that these transformed species can take up and degrade RDX more efficiently than
244 wild type plants.

245 These results demonstrate that, compared to transformed creeping bentgrass, transformed switchgrass
246 degraded RDX more efficiently. The RDX remained at a detectable concentration in most
247 transformed lines of creeping bentgrass; while RDX was not detected in any of the four transformed
248 lines of switchgrass examined. This high activity may have been due to the promoter used to drive the
249 expression of *xplA*, the ubiquitin promoter, which was cloned from switchgrass (Mann et al., 2011).

250 Once RDX enters the plant roots it is transported to leaf tissue in the transpiration stream. Studies in
251 poplar tissues (*Populus deltoides* x *nigra* DN-34) show that RDX in leaf tissue is partially reduced to
252 MNX and DNX. (Van Aken et al., 2004). In agreement with other studies (Jackson et al., 2007; Rylott
253 et al., 2011; Rylott et al., 2006; Sabbadin et al., 2009) these metabolites were not detected in the liquid
254 culture studies reported here and it is likely that that these compounds are either produced at very low
255 levels or are rapidly conjugated by the plant.

256 Despite high uptake and translocation rates, the ability of wild type plants to degrade RDX is low
257 (Just and Schnoor, 2004; Winfield, 2004). Thus when plants are grown in RDX-contaminated media,
258 RDX accumulates in the leaf tissue, limiting the capacity of the plant to remove further RDX from the
259 soil. As shown here, the accumulation of RDX in the leaf tissue of wild type creeping bentgrass also
260 suppresses plant growth (Table 1). Not only is the RDX-degrading ability of plants inherently low,
261 RDX accumulated in plant tissue is likely to re-enter the soil and potentially leach to groundwater
262 following plant senescence. These factors limit the usefulness of wild type plants for the
263 phytoremediation of RDX in training ranges and provide an explanation for the persistent pollution of
264 groundwater under vegetated training ranges. The results of the column studies with wild type
265 switchgrass are consistent with these findings, showing that wild type plants were initially able to stop
266 RDX leaching, but that RDX accumulated in the wild type leaves, and uptake by wild type plants
267 subsequently declined.

268 Although transformation of creeping bentgrass with the *nfsI*-containing vector pRCS-ABNR-HR
269 conferred increased TNT resistance, the level of resistance was poor when compared to the
270 performance of *nfs*-transformed tobacco and Arabidopsis in other studies (Hannink et al., 2001; Rylott

271 et al., 2011a,). Furthermore, switchgrass transformed with the *nfsI*-containing vector pRCS2-NABNR
272 did not have increased resistance to TNT compared to the wild type. The results presented here
273 indicate that the lack of TNT resistance in the *nfsI*-transformed grass species is due to low
274 transcription *nfsI*, and possibly due to poor performance of the 35S promoter in these monocot species
275 (McElroy et al., 1990). Another explanation for the lack of increased resistance is that the *xplB* and
276 *nfsI* expression cassettes were transcribed in opposite directions in the pRCS2-NABNR vector,
277 possibly yielding antisense RNA by read-through transcription, which triggered silencing (Kooter et
278 al., 1999). In future work, the 35S promoter in pRCS2-NABNR will be replaced with a monocot
279 specific promoter to enhance the expression level of *nfsI* and to optimize the transcription direction of
280 different cassettes. These studies underline the importance of promoter choice in monocot
281 transformation studies. In recent years, several constitutive promoters cloned from monocot plant
282 species have been shown to drive high expression of reporter genes in monocot hosts (Kamo, 2003;
283 Park et al., 2010). Alternatively, there have been reports of virus promoters successfully used for
284 foreign gene expression in monocot plant species (Schenk et al., 2001; Schenk et al., 1999).

285 Rather than using a bacterial nitroreductase transgene to confer enhanced resistance to TNT
286 phytotoxicity, there are alternative approaches. As with RDX, plants have only a limited ability to
287 detoxify TNT, the elucidation of these pathways in *Arabidopsis* has shown that OPRs, UGTs, and
288 GSTs are all involved and that overexpression of these TNT-detoxification encoding genes can
289 significantly enhance tolerance of *Arabidopsis* to TNT (Beynon et al., 2009; Gandia-Herrero et al.,
290 2008; Gunning et al., 2014). Furthermore, a recent study has shown that the mutation of
291 monodehydroascorbate reductase 6 in *Arabidopsis* greatly enhanced TNT tolerance (Johnston et al.,
292 2015), a finding that could perhaps be applied in to other species using non-transgenic gene editing
293 techniques.

294 In conclusion, this is the first report of genetically transformed grasses for the phytoremediation of the
295 explosive, and environmental pollutant, RDX. Creeping bentgrass and switchgrass were successfully
296 transformed with the bacterial genes to confer RDX degradation, *xplA* and *xplB*. Both transformed
297 grasses were able to degrade RDX at substantially higher rates than untransformed plants; in the best

298 performing lines preventing the accumulation of RDX in the plant tissues. The use of these plants is a
299 promising biotechnology to prevent contamination of groundwater under live fire training ranges by
300 degrading RDX taken up from the root zone.

301 **Experimental procedures**

302 **Plant materials, explant sterilization, and callus induction**

303 A commercial lowland switchgrass cultivar, Alamo, was used for this study. Mature seeds of Alamo
304 were surface-sterilized in 20 % bleach for 30 min, rinsed three times with sterile water, and left
305 overnight in the dark at 24°C. On the second day, the sterilization procedure was repeated as
306 described above. Embryogenic callus induction, infection and selection of transformed switchgrass
307 plantlets were carried out as described in Li and Qu, (2011). The method for callus induction and
308 transformation of creeping bentgrass followed the previous protocol of Lee et al., (2011).

309 **Plasmid construction and transformation protocol**

310 The 35S cassette was released from pSAT4a (Chung et al, 2005) as an *AgeI-NotI* fragment and used
311 to replace the *ocs* expression cassette in pSAT1a to produce pSAT1a-35S. The hygromycin resistance
312 gene, *hygromycin B phosphotransferase (hpt)*, was cloned by PCR from pcambia1301 and inserted
313 into pSAT1a-35S to produce pSAT1a-35S-*hpt*. All the primers used in this paper are shown in Table
314 S1. The 35S-*hpt*-35ST expression cassette was released from pSAT1a-35S-*hpt* as an *AscI* fragment
315 and inserted into the binary vector pPZP-RCS2 to produce *prcs2-35S-hpt*. The *xplA*, *xplB*, and *nfsI*
316 genes were cloned by PCR from the vectors pMLBart-*xplA*, pART27-*xplB*, and pART27-*nfsI* (Rylott
317 et al, 2011a), using AccuPrime taq DNA polymerase high fidelity (Invitrogen) for amplification. The
318 *xplA* gene was inserted into the pSAT6a vector to produce pSAT6a-*xplA*. The expression cassette *rbc-*
319 *xplA-rbc* T was released as a *PI-PspI* fragment from pSAT6a-*xplA* and inserted into the binary vector
320 pRCS2-35S-*hpt* to produce pRCS2-6*xplA*. The *xplB* gene was inserted into pSAT4a to produce
321 pSAT4a-*xplB*, and the expression cassette 35S-*xplB*-35ST was released from pSAT4a-*xplB* as an *I-*
322 *SceI* fragment and inserted into pRCS2-*xplA* to produce *prcs2-6xplA-4xplB*. The *nfsI* gene was cloned
323 and inserted into pSAT7a to produce pSAT7a-*nfsI*, and the expression cassette *act-nfsI-agsT* was

324 released from pSAT7a-*nfsI* as a PI-*TliI* fragment and inserted into *prcs2-6xplA-4xplB* to produce
325 pRCS2-ABNR-HR.

326 To enhance the expression level of transgenes in switchgrass, three monocot specific promoters were
327 cloned from the pANIC vector system (Mann et al, 2012) by PCR and used to replace the promoters
328 in the pSAT vectors to produce a new set of vectors, which were designated the pNSAT vectors. The
329 actin promoter from rice (*Oryza sativa*) and the ubiquitin promoters from corn (*Zea mays*) and
330 switchgrass (*Panicum virgatum*) were cloned by PCR using the pANIC vector as a template, replacing
331 the promoters in pSAT1a-35S, pSAT3a, and pSAT6a, respectively, to produce the pNSAT1a,
332 pNSAT3a, pNSAT6a cloning vectors. The *hpt*, *xplA*, *xplB*, and *nfsI* genes were inserted into
333 pNSAT1a, pNSAT6a, pNSAT3a, and pSAT4a, respectively, and the expression cassettes of these
334 genes were inserted into the pRCS2 binary vector to produce pRCS2-NABNR .

335 The binary vectors pRCS2-ABNR-HR and pRCS2-NABNR were transferred into *Agrobacterium*
336 strain EHA105 by the freeze-thaw method (Chen et al., 1994) and the resulting strain, EHA105
337 (pRCS2-ABNRHR/pRCS2-NABNR) was grown in LB medium with 50 mg/L rifampicin, 100 mg/L
338 spectinomycin, and 300 mg/L streptomycin for infection of the embryogenic callus of switchgrass and
339 creeping bentgrass.

340 **Molecular analysis of transgenic plants**

341 For PCR analysis, the DNasy plant mini kit (Qiagen, Valencia, CA, USA) was used to purify DNA
342 from hygromycin-resistant plants. The PCR reactions were carried out by amplifying the expression
343 cassette region of the *xplB* gene, including parts of the promoter and terminator sequences (Table S1).

344 For transcript analysis, mRNA was extracted from mature creeping bentgrass, switchgrass leaf blades
345 and Arabidopsis six-week-old rosette leaves using the Isolate II RNA Plant Kit (Biolone). Five
346 micrograms of total RNA was used to synthesize cDNA using oligo (dT) 12-18 primers (Invitrogen)
347 and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was
348 performed using a StepOne Plus real-time PCR detection system with SYBR green (Applied
349 Biosystems). Bentgrass values were normalized to the 5.8S gene. Switchgrass values were normalized

350 to the switchgrass reference gene eIF-4a (Gimeno et al., 2014; Genbank accession number
351 GR877213). Primers sequences for *ACT2*, *xplA*, *xplB* and *nfsI* were as reported previously (Rylott et
352 al., 2011a). Transcript abundance was expressed relative to the levels of the *xplA-xplB-nfsI* expressing
353 Arabidopsis line 7D (Rylott et al., 2011a).

354 **Protein extraction and immunoblot analyses**

355 For protein expression analysis, eight micrograms of crude protein extract from leaf tissues was
356 loaded per lane. Antibodies were used as reported previously, XplA, (Rylott et al., 2006); XplB,
357 (Jackson et al., 2007) and NR (Rylott et al., 2011a). Three replicate blots were made for each protein
358 and band intensities quantified from pixel measurements of western blot images using ImageJ
359 software.

360 **RDX uptake by transformed switchgrass and creeping bentgrass**

361 Wild type and transformed grass plants with similar biomass and at the same development status were
362 selected and cultured in 5 mL ½ MS media without sugar and supplied with RDX at 40 mg/L for
363 creeping bentgrass and 20 mg/L for switchgrass under 16 h light, 8 h dark photoperiod at 25 °C for
364 15 days. The concentration of RDX in the medium was assayed at regular time intervals. The volume
365 of medium was refilled back to 5 mL with water every time before sampling. After 15 days culture,
366 the RDX concentration in plant tissue was also analyzed. Plant tissues (100 mg) were collected and
367 freeze dried using a Labconco Freezone 4.5 Liter Freeze Dry System (Labconco, Kansas, USA) and
368 ground to powder using a Fast Prep 24 (MP Biomedicals, LLC., Solon, USA). The plant tissue
369 powders were immersed in 1 mL methanol and incubated for 12 hours at room temperature with
370 shaking. The tubes were then centrifuged twice at 13000 rpm for 10 min. The supernatant (800 uL)
371 was collected for HPLC analysis.

372 **HPLC quantification of aqueous RDX**

373 RDX concentrations in culture media were analyzed with a modular Waters HPLC system consisting
374 of a Waters 717 autosampler, two Waters 515 HPLC pumps, and a Waters 2996 photodiode array

375 detector. A 4.6- by 250-mm Waters C18 column was used for separation under conditions similar to
376 those outlined previously (Andeer et al., 2013), with concentration determined based on absorbance at
377 240 nm. Peak integrations and analyses were conducted using Millennium32 software (Waters,
378 Milford, MA). The limit of detection of RDX by this method is 0.01 mg/L.

379 **TNT uptake by transformed creeping bentgrass**

380 Wild type and transformed creeping bentgrass plantlets were cultured in 30 mL liquid ½ MS medium
381 amended with TNT at 4.5 mg/L in flasks at 20°C with shaking. The light intensity is at 13.875
382 $\mu\text{mol}/\text{m}^2\cdot\text{s}$. Each flask contained three independent creeping bentgrass plantlets with biomass of about
383 100 mg and each treatment was repeated four times. The growth of the roots was observed and the
384 root hairs were photographed after 15 days culture.

385 **Column studies**

386 Twelve polyvinyl chloride (PVC) columns were constructed with PVC tubing (90mm diameter, 0.5m
387 long). Media for the columns was a mix of 75% gravel and 25% sand.

388 Eight matching columns were planted, four each, with wild type and transgenic grasses and the
389 grasses were grown to over 0.5 m and pruned back to about 0.5 m uniform height. Then the columns
390 were dosed with equal amounts of RDX on the first, third and fifth day of the first week, and, as
391 needed, again on the following week on the same schedule. The RDX was dosed using with aliquots
392 of 125 mL of RDX solution containing approximately 7.5 mg RDX. The void volume of the planted
393 columns was approximately 1.5 L. Following each dosing, the planted columns were incubated for
394 one week with 125 mL 1X Hoagland's medium. Two days after the final RDX dosing, the planted
395 columns were flushed with 5 L DI water and the effluent was collected in 500 mL aliquots, which
396 were sampled for analysis of RDX. A total of 5 L DI water was used to flush the columns clean of
397 RDX, until RDX was undetectable by HPLC, usually 3.5 to 4.5 L.

398 **Data analysis**

399 Data were analyzed for statistical significance using ANOVA in Microsoft Excel software (Microsoft
400 Excel 2016 MSO). When ANOVA analysis gave a significant difference, Fishers Least Significant
401 Difference (LSD) method was performed to compare the means to each other and the statistical
402 significance labeled by lower case letters in the figures.

403 **Acknowledgements**

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405 ESTCP-201436.

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545

546

| Plant line | Initial biomass (g) | Final biomass (g) | Biomass gain (g) |
|-----------------|---------------------|-------------------|---------------------------|
| Control, no RDX | 0.132±0.012 | 0.552±0.028 | 0.42±0.038 ^a |
| Wild type | 0.136±0.009 | 0.266±0.021 | 0.131±0.029 ^d |
| N5 | 0.138±0.004 | 0.337±0.034 | 0.199±0.033 ^{cd} |
| N18 | 0.132±0.004 | 0.342±0.039 | 0.21±0.037 ^c |
| N19 | 0.131±0.005 | 0.455±0.033 | 0.324±0.035 ^b |

547

548 **Table 1.** The effect of RDX on the growth of wild type and *xplA-xplB-nfsI* transformed creeping
549 bentgrass.

550 Creeping bentgrass plants were cultured in 5 mL liquid ½ MS medium dosed with RDX at 40 mg/L.

551 The masses of wild type and transformants (N5, N18, N19) were measured after 16 days culture and

552 the biomass gains calculated. The transformed lines of N18 and N19 accumulated more biomass than

553 wild type during the time course. Controls consisted of wild type plantlets cultured in MS medium

554 without RDX. Letters indicate biomass gains were significantly different ($p < 0.05$) from other lines (n

555 = 3 ± SE).

556

557

558 **Figure Legends**

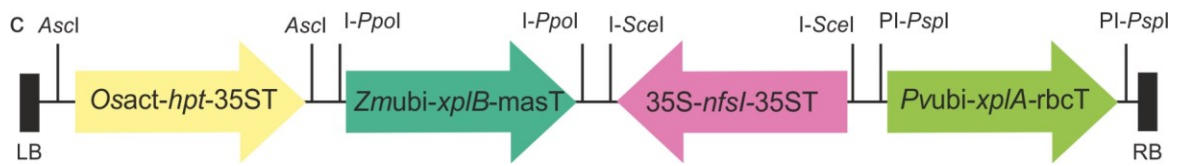
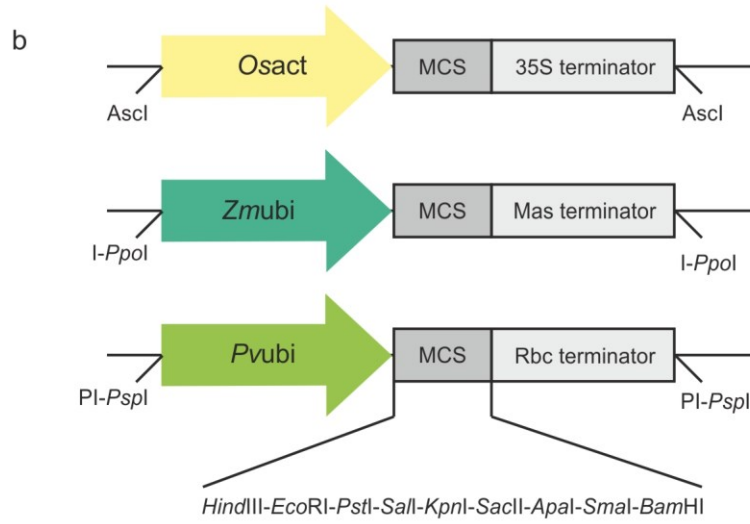
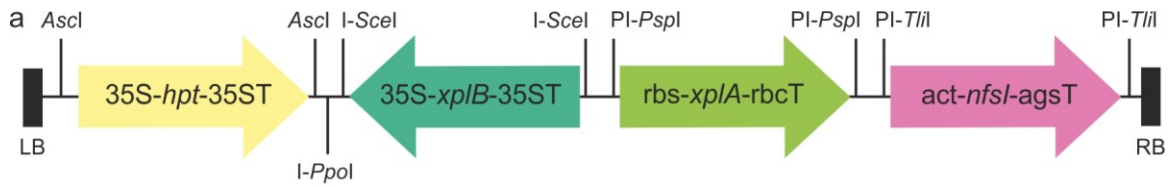
559 **Figure 1.** Construction of vectors for transformation of the grasses.

560 a) T-DNA region of the binary vector plasmid pRCS2-ABNR-HR. The RDX degradation gene *xplA*,
561 flavodoxin reductase gene *xplB*, and TNT detoxifying nitroreductase gene *nfsI* were constructed into
562 versatile cloning vector pSATs (Chung et al 2005). Arrows show the direction of transcription.

563 b) The *Osact*, *Zmubi*, and *Pvubi* promoters were used to replace the promoters in the pSAT vectors
564 resulting in pNSAT1a, pNSAT3a, and pNSAT6a respectively.

565 c) T-DNA region of the binary vector plasmid pRCS2-NABNR. The *hpt*, *xplA*, *xplB*, and *nfsI* genes
566 were constructed into pNSAT1a, pNSAT6a, pNSAT3a, and pSAT4a respectively. The expression
567 cassettes of these genes were integrated into the binary vector pPZP-RCS2 to produce pRCS2-
568 NABNR.

569 Abbreviations: 35s, CaMV 35s; *rbc*, rubisco small subunit; *act*, actin; *ags*, agropine synthase; *Osact*,
570 *Oryza sativa* actin promoter; *Zmubi*, *Zea mays* ubiquitin promoter; *Pvubi*, *Panicum virgatum*
571 (switchgrass) ubiquitin promoter; RB left border; RB right border.



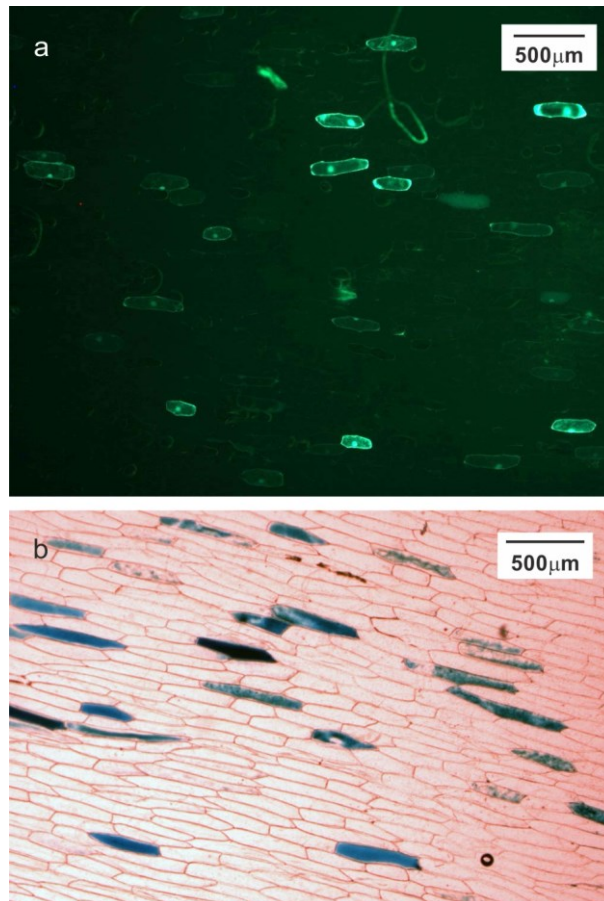
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575 **Figure 2.** Functional evaluation of the pNSATs vectors using transient expression reporter genes in
576 the cytosol of epidermal onion cells.

577 a) Fluorescence microscopy showing GFP expression following particle bombardment with
578 pNSAT1a-GFP (*OsAct-GPF-35S*). b) Histochemical staining of GUS expression following particle
579 bombardment with pNSAT3a/6a-GUS (*ZmUbi-GUS-Mas*; *PvUbi-GUS-rbc*).



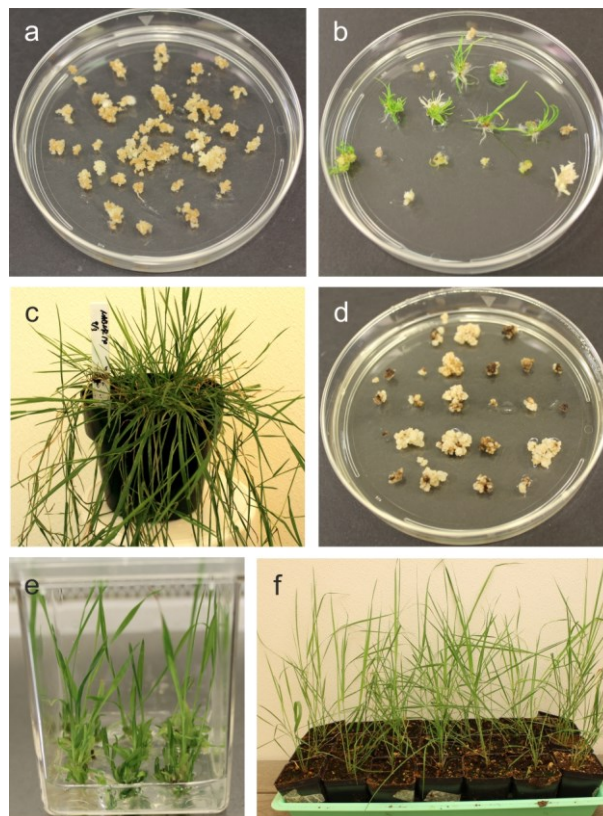
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581

582 **Figure 3.** Production of transgenic creeping bentgrass and switchgrass.

583 a) Appearance of embryogenic calli of creeping bentgrass infected with *Agrobacterium* harboring
584 pRCS2-NABNR after 3 weeks of culture on callus induction medium with hygromycin. b)
585 Hygromycin resistant calli on regeneration medium with hygromycin and c) transgenic plants in soil.
586 d) Appearance of embryogenic calli of switchgrass infected with *Agrobacterium* harboring pRCS2-
587 NABNR after 4 weeks of culture on callus induction medium with hygromycin. e) Hygromycin
588 resistant plantlets on regeneration medium with hygromycin and f) genetically transformed plants in
589 soil.

590



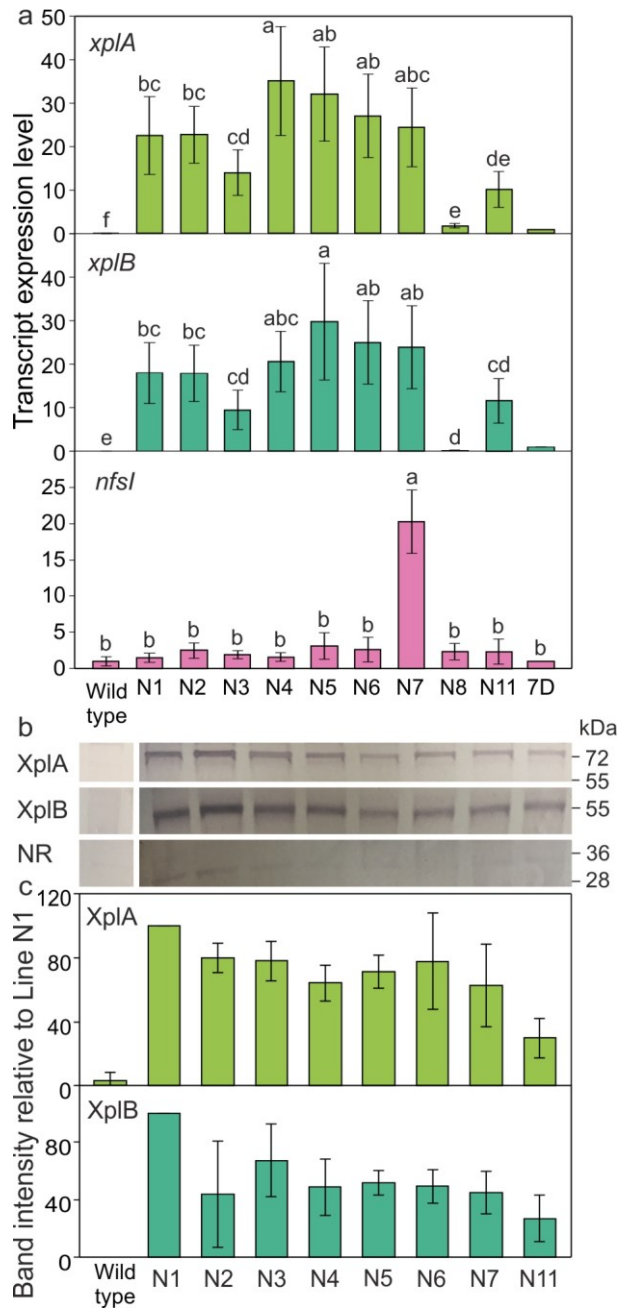
591

592 **Figure 4.** Molecular characterization of *xplA-xplB-nfsI* transformed switchgrass.

593 a) Transcript abundance measured using quantitative RT- PCR on plant lines transformed with *xplA*,
594 *xplB* and *nfsI*. Values were normalized to the switchgrass reference gene *eIF-4a* (Gimeno et al., 2014).
595 *Arabidopsis* values were normalized to the reference gene *ACT2*. All values are relative to the
596 expression levels of the *xplA-xplB-nfsI* expressing *Arabidopsis* line 7D (Rylott et al., 2011; n = 4 ±
597 SE).

598 b) Western blot analysis on leaf blades of switchgrass lines expressing XplA, XplB and nitroreductase
599 (NR) protein. c) Band intensities were quantified for XplA and XplB expression. Levels were
600 normalized to the Coomassie-stained RUBSICO large subunit, results are from three replicate blots ±
601 SE.

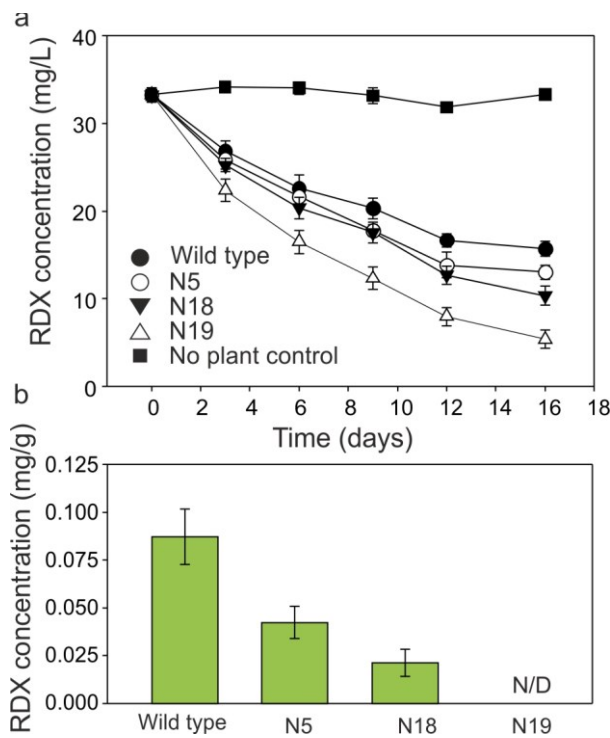
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604 **Figure 5.** Uptake of RDX by *xplA-xplB-nfsI* transformed creeping bentgrass grown in liquid culture.

605 a) Concentration of RDX in culture medium over the course of the experiment. After three days, the
606 medium from line N19 contained significantly ($p=0.010$) less RDX than medium from wild type
607 plants and after nine days, lines N5 and N18 had also removed significantly more RDX from the
608 media than wild type ($p = 0.03$ for N5 and 0.05 for N18). b) Concentration of RDX in creeping
609 bentgrass tissue after 16 days. Letters indicate RDX concentrations in tissue were significantly
610 different ($p<0.05$) from other lines ($n = 3 \pm SE$, N/D = none detected).

611

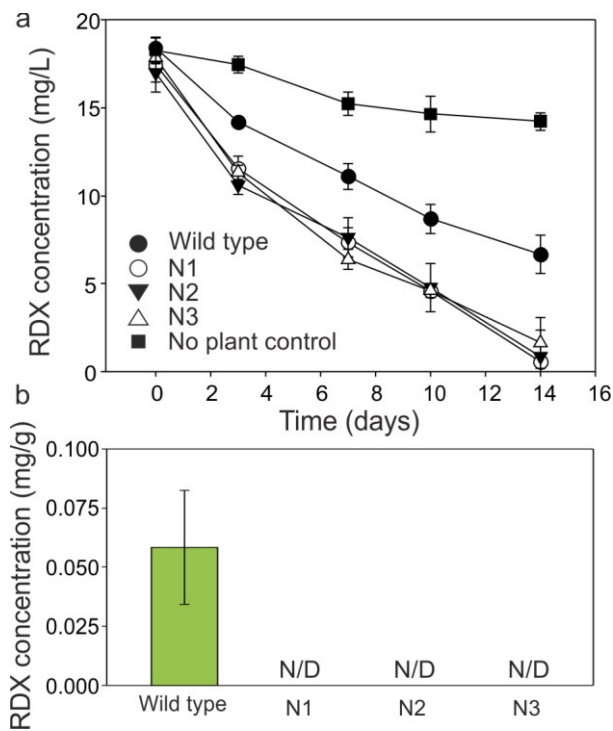


612

613 **Figure 6.** Uptake of RDX by *xplA-xplB-nfsI* transformed switchgrass grown in liquid culture.

614 a) Concentration of RDX in culture medium over the course of the experiment. All three transgenic
615 lines removed RDX from the medium at significantly faster rates than the wild type plants ($p = 0.051$,
616 0.0014 and 0.0016 for lines N1, 2 and 3 respectively at day 3; $p=0.0043$ for line N1 at day 7). b)
617 concentration of RDX in switchgrass tissue after 14 days ($n= 3 \pm SE$, N/D = none detected).

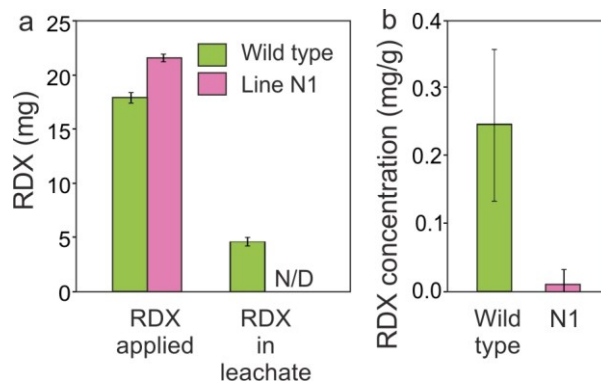
618



619

620 **Figure 7.** Recovery of RDX applied to wild-type and *xplA-xplB-nfsI* transformed switchgrass in
621 column experiments.

622 a) Mass of RDX applied as solutions containing 30 mg/L, and mass recovered in the leachate by
623 flushing each column with 5 L water. b) RDX level was significantly less ($p = 0.0044$) in the
624 transgenic leaf tissue compared to the wild type leaf tissue in the column experiments after 14 days (n
625 $= 4 \pm SE$, N/D = none detected).



626

627

628 **Supplementary Materials**

629

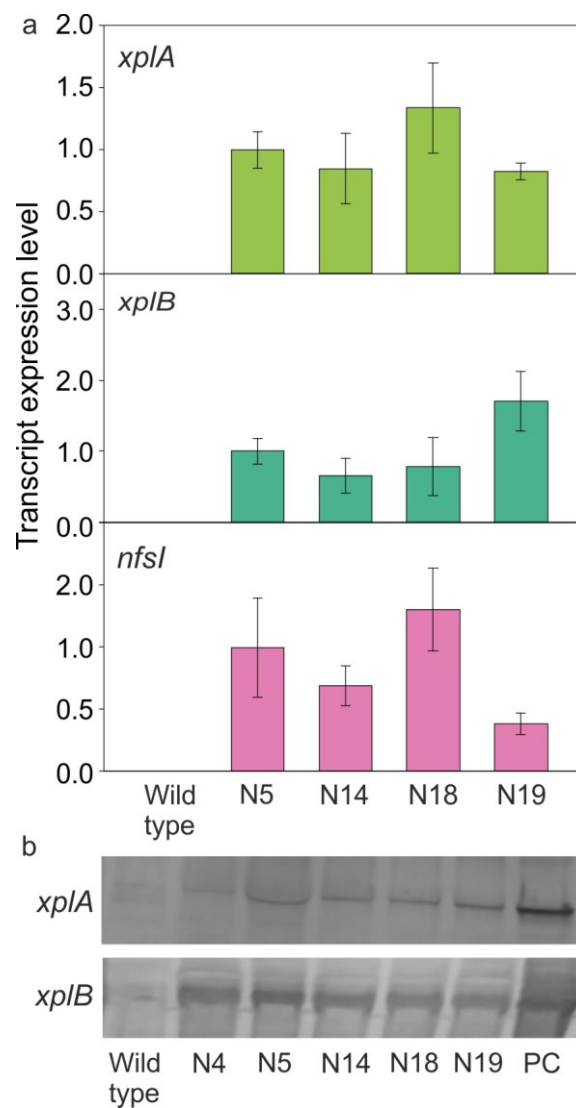
630

631 **Supplemental Figure 1.** Molecular characterization of transgene creeping bentgrass.

632 a) Transcript abundance measured using quantitative RT- PCR on plant lines transformed with *xplA*,
633 *xplB* and *nfsI*. Values were normalized to the creeping bentgrass reference gene 5.8SrRNA and are
634 relative to the expression levels of line N5 ($n = 3 \pm SE$).

635 b) Western blot analysis on leaf blades of creeping bentgrass lines expressing XplA, XplB and
636 nitroreductase (NR) protein (PC, positive control; total soluble protein isolated from *xplA-xplB-nfsI*
637 expressing *Arabidopsis* line 7D (Rylott et al., 2011).

638

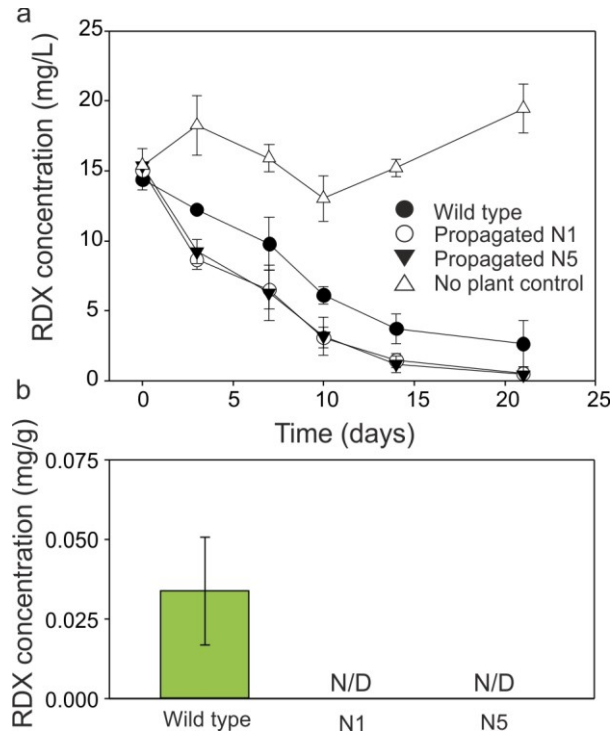


639

640

641 **Supplemental Figure 2. Uptake of RDX by propagated plants from *xplA-xplB-nfsI* transformed**
 642 **switchgrass grown in liquid culture**
 643 a) Concentration of RDX in culture medium over the course of the experiment and b) concentration of
 644 RDX in switchgrass tissue after 16 days (n = 3 ± SE, N/D = none detected).

645



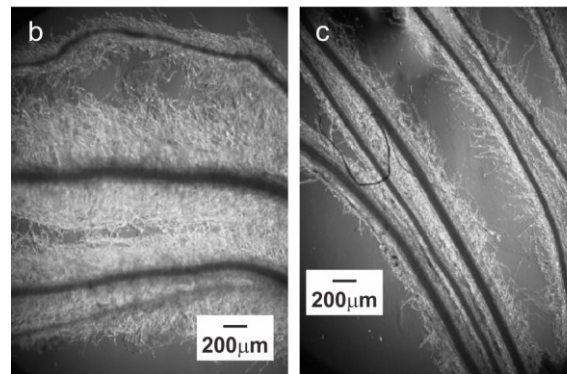
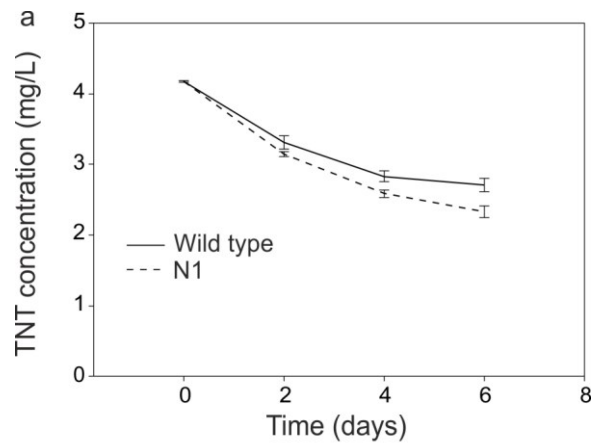
646

647

648 **Supplemental Figure 3. Studies on liquid-culture grown *xplA-xplB-nfsI* transformed creeping**
649 **bentgrass exposed to TNT.**

650 a) Concentration of TNT in culture medium over the course of the experiment ($n = 3 \pm SE$, N/D =
651 none detected). Light microscopy images of b) transformed creeping bentgrass and c) wild type roots
652 at the end of the experiment.

653



654

655 **Supplementary Table 1.** The DNA sequences of primers used in this study.

656

| Primer name | Sequence (5' - 3') | Flanking Restriction site | Details |
|-------------|----------------------------------|---------------------------|--|
| HptF3 | TTGAATTCATTATGAAAAAGCCTGAACTC | EcoRI | Clone and Insert <i>hpt</i> gene into pSAT1a-35S to produce pSAT35S- <i>hpt</i> |
| HptR3 | ATTGGATCCCTATTTCTTTGCC | BamHI | |
| XplaF5 | ttAAGCTTACCATGGccgacgtaactgtcctg | HindIII | Clone and insert <i>xplA</i> gene into pSAT6a and pnSAT6a |
| XplaR5 | TTAAGCTTTCAGGACAGGACGATCGGC | HindIII | |
| XplbF1 | ACGGTACCATGGACATCATGAGTGAAGT | KpnI | Insert <i>xplB</i> into pSAT4a and pnSAT3a |
| XplbR1 | ttGGATCCTCAGCAGACCGATTTCGGCCGGC | BamHI | |
| NrF1 | TCGAATTCAACAATGGATATCATTTCTGTCTG | EcoRI | Insert <i>nfsI</i> gene into pSAT7a and pSAT4a |
| NrR1 | TTGGATCCTCAGCACTCGGTCACAATCG | BamHI | |
| OsActinF1 | TTACCGGTCTCGAGGTCATTCATAT | AgeI | Clone and insert <i>Osactin</i> promoter into pSAT1a to produce pnSAT1a |
| OsActinR1 | TTAAGCTTTCTACCTACAAAAAAGCTCC | HindIII | |
| ZmUbiF1 | TTACCGGTTGCAGTGCAGCGTG | AgeI | Clone and insert <i>ZmUbi</i> promoter into pSAT3a to produce pnSAT3a |
| ZmUbiR1 | CCAAGCTTTGCAGAAGTAACACC | HindIII | |
| PvUbiF1 | TTACCGGTCCACTGGAGAGGG | AgeI | Clone and insert <i>PvUbi</i> promoter into pSAT6a to produce pnSAT6a |
| PvUbiR1 | TTAAGCTTGATCTGCATCTGCAGAAG | HindIII | |
| GFPF5 | TTAAGCTTATTATGGTAGATCTGACTAGT | HindIII | Clone and insert GFP gene into pnSAT1a |
| GFPR5 | ATTCTGCAGTCACACGTGGTGGTGG | PstI | |
| GUSF4 | TTGGAATTCATTATGGTAGATCTG | EcoRI | Clone and insert GUS gene into pnSAT3a and pnSAT6a |
| GUSR4 | TTAGGATCCTCACACGTGGTG | BamHI | |
| Sat4seqf1 | CGAATCTCAAGCAATCAAGC | | PCR to confirm the insertion of <i>hpt</i> cassette of pres2-abnr-hr vector in grass genome DNA. |
| Sat4seqr1 | CCTTATCTGGGAACTACTCAC | | |
| nSAT1aseqf1 | GCTGCTTCGTCAGGCTTAGAT | | Work Together with |

| | | | |
|-------------|---------------------------|--|---|
| | | | SAT4aseqr1 to do PCR to confirm the insertion of <i>hpt</i> cassette of prcs2-NABNR vector in grass genome DNA. |
| NSAT3aseqf1 | CTTGATATACTTGGATGATGGC | | PCR to confirm the insertion of <i>xplB</i> cassette of prcs2-NABNR vector in grass genome DNA. |
| Sat3aseqr1 | AGCCACGCACATTTAGGA | | |
| NSAT6aseqf1 | TGCTGTGATGCTGTTTGTG | | PCR to confirm the insertion of <i>xplA</i> cassette of prcs2-NABNR vector in grass genome DNA. |
| Sat6aseqr1 | CCGGAAACAAACAACGA | | |
| Xpla300f | CAACAACGCGATCGACATCC | | qRT-PCR of <i>xplA</i> gene |
| Xpla959r | TCGAACATCGCCTCCATCAC | | |
| XplB511F | GAATTCACCGGTTCCGATCT | | qRT-PCR of <i>xplB</i> gene |
| XplB1143R | GACTGTCCGGTCGATCACTT | | |
| Nfs1322f | TTCAACACGCCAGAAGCCA | | qRT-PCR of <i>nfsI</i> gene |
| Nfs1652r | AGCACTCGGTCACAATCGT | | |
| HptF2 | TCTTAGCCAGACGAGCGGGTTC | | qRT-PCR of <i>hpt</i> gene |
| HptR2 | TACTTCTACACAGCCATCGGTCCAG | | |
| eIF-4aF | TGATGTCATTTCAGCAAGCACAA | | eukaryotic initiation factor-4A (<i>eIFa</i>) |
| eIF-4aR | GGCATTCAACCAGGCCATAG | | |

657

658

659 **Supplemental methods**

660 **Biolistic bombardment of epidermal onion cells**

661 Biolistic bombardment was carried out using a PDS1000He biolistic gun (Bio-Rad). The onion was
662 positioned at 10 cm target distance. The micro projectiles were bombarded at a rupture pressure of
663 1100 psi. The bombarded onion was wrapped with wet paper towels and kept in a culture vessel at
664 room temperature in the dark for two days before observation.

665 **GUS assay**

666 The epidermal layer of onion cells was peeled off and assayed for GUS activity. The GUS incubation
667 buffer consisted of 2 mm α -gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid), 2 mm potassium
668 ferricyanide and potassium ferrocyanide, 50 mm sodium phosphate buffer ph 7.2, 0.2% Triton X-100.
669 The tissues were soaked in GUS incubation buffer at 37 °C overnight and then kept in 70 % ethanol
670 and observed by light microscopy.

671 **GFP observations**

672 Two days after bombardment the epidermal cells of onion were visualised using a laser scanning
673 confocal microscope (LSM5 PASCAL, Zeiss). The detection limits of the microscope were set using
674 images captured from tissue that was not bombarded with GFP. The excitation wavelength was 448
675 nm, and the images collected through TRITC filters for GFP fluorescence.

676