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Field trial demonstrating phytoremediation of the military explosive RDX by *XplAB*-expressing switchgrass

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Abstract

The explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a major component of munitions, is used extensively on military training ranges. As a result, widespread RDX pollution in groundwater and aquifers in the United States is now well documented. RDX is toxic, but its removal from training ranges is logistically challenging, lacking cost-effective and sustainable solutions. Previously, we have shown that thale cress (*Arabidopsis thaliana*)

engineered to express two genes, *xplA* and *xplB,* encoding RDX-degrading enzymes from the soil bacterium *Rhodococcus rhodochrous* 11Y can break down this xenobiotic in laboratory studies. Here, we report the results of a 3-year field trial of XplAB-expressing switchgrass (*Panicum virgatum*) conducted on three locations in a military site. Our data suggest that XplAB switchgrass has *in situ* efficacy, with potential utility for detoxifying RDX on live-fire training ranges, munitions dumps and minefields.

Main text

During World War II, RDX production peaked at 15,000 tons a month in the United States, and 7000 tons a month in Germany[1]. A major component in many munitions[1], the demand for RDX is still high with a market size of $10.43 billion in 2018[2]. But RDX is toxic to soil organisms and humans, recalcitrant to degradation in the environment, and extremely mobile, readily moving into the groundwater[3-7]. These properties have resulted in the buildup of RDX in soil and groundwater to levels that have then threatened, or affected, human health, such as contamination of a sole source aquifer at Cape Cod[8], and exposure of individuals to RDX in the Bickford Case in Utah[9].

Global RDX-contaminated areas are hard to map: historical explosives contamination is poorly recorded, and for reasons of security and political sensitivity, locations of current military sites are not openly documented. Extended Data Figure 1 maps global locations of explosives contamination, with examples of specifically RDX contamination additionally listed, and described in Supplementary Table 1. Supplementary Table 2 demonstrates the scale of RDX contamination in the United States. Contamination is not just limited to active military ranges, but includes decommissioned ranges, munitions dump sites and minefields; extensive RDX plumes emanate from manufacturing sites[8,9].

The removal of this pollutant is an intractable challenge: the scale is enormous, in the US alone, 10 million hectares of military land is contaminated with munitions components[10,11]. Contamination is heterogeneously spread[8,10], often unmapped, with unexploded ordnance restricting ground-access. Furthermore, armed forces need live-fire training, and there is an urgent need to contain and perpetually remediate continuing contamination on active ranges. Traditional remediation methods such as land fill, incineration or advanced oxidation, are more suited to small-scale, highly polluted areas, where clean-up time is short (< 3 years), and overall costs are relatively low[12]. The US Department of Defense (DoD) estimated that the remediation of its active ranges alone using currently-available methodologies would cost between $16 billion and $165 billion[11]. To address this remediation challenge, the testing of novel cost-effective technologies is necessary.

Previously, we isolated a soil bacterium, *Rhodococcus rhodochrous* 11Y, with the ability to degrade RDX. The genes responsible, *xplA* and *xplB,* encoded a unique class VI cytochrome P450 XplA, and accompanying flavodoxin reductase, XplB[13-15]. The XplAB system catalyzes the reductive denitration of RDX to form, under aerobic conditions, 4-nitro-2,4-diazbutanal (NDAB), and anaerobically methylenedinitramine (MEDINA; Fig. 1a)[13]. Although MEDINA is converted to formaldehyde and nitrous oxide, products that are mineralizable in soils, the aerobic product NDAB has been detected in groundwater from RDX-contaminated soils[16].

Using plants to remediate environmental pollutants has many potential benefits: they are minimally disruptive, promote restoration ecology, and are aesthetically pleasing, with high levels of public acceptance. Furthermore, for longer-term (>3 years) projects, phytoremediation has the potential to be low maintenance and cost effective. In plants,

following uptake, RDX is located almost exclusively in the aerial tissues[17,18]. Unlike bacteria, plants have little inherent ability to degrade RDX[19], with stored RDX becoming biologically available through the food chain via herbivory, or returned as RDX back to the soil when the plant dies[20]. We have previously demonstrated, in soil-based laboratory studies, that *Arabidopsis thaliana* (Arabidopsis) plants transformed with *xplAB* are able to remove saturating levels of RDX from soil leachate[13,14]. In these XplAB-expressing plants, RDX is broken down into inert, naturally occurring plant metabolites that are then incorporated into the plant, with no plant harvesting necessary to remove the contaminated material from the site.

Transgenic phytotechnologies, using model plant species and laboratory and pot-based studies have shown great promise for remediating persistent organic pollutants from the environment, but testing efficacy in-the-field is a significant bottleneck to their implementation[20]. Firstly, transformation methodologies exist for only a few phytoremediation-suitable species. Secondly, obtaining the necessary licensing to grow transgenic plants requires substantial paperwork and oversight. Thirdly, field-relevant species are relatively large, such as trees and perennial grasses, require geographically large-scale (> 0.5 hectare) trials, and can take several years to establish. Finally, field trials, by their very nature, are open to uncontrollable, adverse weather events. Thus, large-scale trials can be prohibitively expensive and time-consuming (>3 years) and without the guarantee of clear-cut results.

Towards translation of our RDX-degrading technology into the field, we have transformed switchgrass (cultivar 'Alamo'), with a vector containing *xplA* and *xplB* (Fig. 1b)[21]. These genes were expressed using ubiquitin promoters from grass species giving near constitutive

100 expression throughout the plant [21]. Laboratory studies on soil columns showed that the
101 XplAB-expressing switchgrass lines were able to remove RDX from soil leachate
102 significantly faster than untransformed plants. Wild-type plants were initially able to stop
103 RDX leaching, but as RDX accumulated in the leaves, uptake declined indicating the plants
104 were saturated with RDX. Furthermore, whereas wild-type plants accumulated up to 0.058
105 mg RDX per g tissue, RDX was not detected in the XplAB-expressing lines, with
106 calculations indicating that RDX degradation was limited by uptake in the transpiration
107 stream, rather than the enzymatic capacity of XplAB[21].
108
109 Here we report the results from the first field trial of this technology, at Fort Drum military
110 range, NY, USA using XplAB-expressing switchgrass lines[21]. A permit from the US
111 Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS)
112 was required to enable controlled release of the transgenic plants into the environment. For
113 the permit, the following data were collected, in collaboration with the US Army Garrison
114 Fort Drum, Environmental and Integrated Training Area Management, and Land
115 Rehabilitation and Maintenance groups: descriptions of the proposed site (rainfall amounts,
116 GPS coordinates, and orientation), demonstration of controlled site access, soil, flora and
117 fauna surveys. Data comparing the wild-type lines with the transgenic lines in greenhouse
118 studies were also provided. A specific condition of the permit was the removal of all
119 developing flower heads from the plots.
120
121 The demonstration site, located on a Plainfield sandy soil, contained a mean of 92 % sand,
122 with small amounts of silt and clay (analyzed across 0 to 1 m depths). A plot cross-section is
123 shown in Figure 2a and an aerial view of the plots is shown in Extended Data Figure 2a. The
124 site layout was based on a completely randomized design with three soil treatments (no RDX,

1 mg/kg and 100 mg/kg RDX), three vegetation treatments (no plant control; NPC, wild type, untransformed switchgrass; WT, and XplAB-expressing switchgrass; TG) replicated three times for a total of 27 plots (Fig. S2b). On the 100 mg/kg plots, RDX was applied at 76 g per m^2 (0.68 kg/plot), a concentration that is within the distribution patterns of RDX reported around a low order detonation of, on average, 3.3 g m^{-2}, with peaks at 96 g m^{-2} [22]. Results are presented here for the NPC, WT and TG 100 mg/kg plots.

As shown in Figure 2b and Extended Data Fig. 3, the plants did not establish vigorous growth until the third growing season (2018). At the end of the trial, the average aerial tissue biomass was 0.53 kg/ m^2. Typical biomass yields for switchgrass are 1.08 kg/m^2 [23], thus, at the end of the three-year trial, the switchgrass had reached 50 % of its potential biomass. 'Alamo', is primarily adapted to southern regions of the USA (below 38-39°N latitude); the field site was located at 44°, 4'47.03" N. Growing 'Alamo' at a suboptimal geographical range is likely to have contributed to reduced vigor in the trial. This choice of mismatched cultivar, was a deliberate component for the APHIS permit, designed to reduce the likelihood of cross-pollination with closely-related, native species, and was in addition to the removal of flower heads, during the field trial. Heavy rainfall, coupled with the lined-plot design meant that excess water needed to be pumped from the plots, and it is likely that initial plant growth was limited by water-logging, and subsequent loss of nutrients in the excess water removed from the plots. Throughout the final season of growth, an all-purpose fertilizer was applied. Although previous studies have shown that XplA-[14] and XplAB-[21] expressing plants have increased plant biomass when compared to unmodified plants, on the plots, the presence of RDX did not meaningfully affect plant biomass levels in either XplAB-expressing or unmodified plants (Extended Data Fig. 4). This finding demonstrates that RDX toxicity is not

149 a major issue to unmodified switchgrass at 100 mg/kg soil, a concentration commonly found
150 on RDX-contaminated military sites[22].
151
152 During the 2018 growing season, levels of RDX in the aerial tissues of unmodified plants
153 grown on the 100 mg/kg RDX plots varied within plots, between plots, and between monthly
154 samplings. RDX was not detected in any of the aerial tissues sampled from the transgenic
155 lines over the entire 2018 growing season, and across all three 100 mg/kg RDX plots, (Fig.
156 2c), confirming XplA activity in all the transgenic lines throughout the growing season.
157 Analysis of transcript and protein levels in tissue harvested from the plots in 2018 (Extended
158 Data Fig 5) showed that the transgenes in the three independently transformed plant lines
159 were expressed at levels comparable to those reported earlier[21]. At the end of the trial, the
160 mean RDX concentration in the wild-type switchgrass from each of the three plots (Extended
161 Data Fig. 6) was 0.025 ± 0.02 mg/g dry weight. Previous measurements of tissue RDX levels
162 using RDX-saturated soil column studies measured a maximum of 0.23 mg/g for wild-type
163 switchgrass. Under these column conditions, RDX was detected in the XplAB-expressing
164 switchgrass line N1, but at just 4 % of that in the unmodified switchgrass, suggesting that
165 0.23 mg/g is indicative of the likely upper limit for the RDX-degrading capacity of the
166 transgenic lines in-the-field[21], and that the XplAB-expressing plants in the 2018 season were
167 at approximately 11 % of their RDX uptake capacity.
168
169 It is likely that, the aerobic route of RDX degradation (Fig. 1a) is followed *in planta*, with the
170 production of NDAB, nitrite and formaldehyde. Nitrite and formaldehyde are readily
171 metabolized by plants. Structurally, NDAB shares similarity with allantoin, an intermediate
172 in the purine catabolic pathway which requires an amidohydrolase for its decomposition.
173 *Methylobacterium* sp. strain JS178, which is able to degrade NDAB[26], also grew on allantoin,

174 the presence of which induced the synthesis of enzyme(s) required for NDAB degradation.
175 Allantoin is also a key intermediate in the assimilation, metabolism, transport, and storage of
176 nitrogen in plants. As we have not observed accumulation of NDAB in our plant-based
177 studies in Arabidopsis or switchgrass[12, 13, 21, 25], it is possibly further degraded in plants by an
178 allantoin amidohydrolase.
179
180 Analysis of soil core samples from across the plots (Extended Data Fig. 7a) showed that the
181 RDX was distributed heterogeneously across the plot. Unexpectedly, given its documented
182 mobility in soils, RDX had not significantly moved below the 0-5 cm depth in the plots.
183 However, higher concentrations tended to be clustered furthest away from the culvert areas,
184 suggesting localized gradients of RDX occurred, as indicated by arrows on Figure S7b.
185
186 Levels of RDX in soil lysimeters (Extended Data Fig. 7c) indicated that there was no
187 significant difference in the levels of soil water RDX in the NPC, WT, and TG plots across
188 the 2018 growing season. However, the mean RDX level in the TG plot tanks was
189 significantly less ($P = 0.021$) than in the tanks on the NPC plots, and at each month sampled,
190 the RDX level was always lower than that in the NPC and WT plot tanks (Extended Data Fig.
191 7c).
192
193 Figure 2d shows that across all three years, the concentration of RDX in the excess water
194 pumped from the TG plots was always less than from either the NPC or WT plots. In the
195 2018 growing season, water pumped from the plots planted with the XplAB-expressing
196 switchgrass contained significantly less ($P = 0.015$) RDX than water pumped from the NPC
197 plots.

198 As shown in Extended Data Figure 8, less water was pumped from the plots containing the
199 XplAB-expressing plants than from the WT plots. This difference in water pumped from the
200 plots, was irrespective of the presence of RDX. Leaf surface areas (Extended Data Fig. 9) of
201 the wild type and transgenic lines were not significantly different from each other.
202 Furthermore, transpiration rates measured for line N1, were not significantly different from
203 wild-type plants (Extended Data Fig. 10). Differences in water uptake were not observed in
204 laboratory experiments.
205
206 The levels of transpiration from each plot were estimated from the excess water pumped from
207 the plots minus the rainfall measurements for the 2018 season. While it was not possible to
208 determine the level of evaporation from the plots, given the near canopy closure shown in
209 Figure 2b, and S3, this factor was considered to be relatively low compared to that lost
210 through transpiration. Multiplying the mean RDX concentrations in the soil water (Extended
211 Data Fig. 7c) by the calculated volume of water transpired during the 2018 growing season,
212 we calculated that the TG plants took up, and metabolized, significantly (P = 0.015) more
213 RDX from the plots than the WT plants (Fig. 2e), and equivalent to an RDX removal rate by
214 the XplAB-expressing plants of 27 kg RDX per hectare. Based on our data in Arabidopsis[14,
215 24], and grass species[21, 25], it is likely that the wild-type plants did not significantly degrade any
216 RDX.
217
218 In summary, the results presented here demonstrate that XplAB-expressing switchgrass is an
219 effective tool for the remediation of RDX from military ranges. We have also developed a
220 method for transforming RDX-degrading ability into western wheatgrass (*Pascopyrum*
221 *smithii*)[25], a species native to many US ranges. Expected benefits are a cost effective and

sustainable *in situ* method for preventing contamination of groundwater beneath military ranges.

Methods

Plot dosing and planting.

A plot cross-section is shown in Figure 2a. Each plot had a surface area of 3.0 m x 3.0 m with a 0.5 m depth. Lysimeters, placed between the plot upper liners and secondary liners, were used to monitor for RDX contamination in the event of a breach in the integrity of the upper liner. To simulate particulate munitions contamination around targets on training ranges, the 100 mg/kg plots were surface dosed with fine particulate RDX mixed with sand at 76 g RDX per m^2 (0.68 kg/plot), and irrigated with water.

To produce the estimated 3000 plants needed for the demonstration site, switchgrass vegetative micropropagation techniques were used[27]. Prior to planting, randomly-selected XplAB-expressing plants were tested using PCR, as described[21] to confirm the presence of the transgenes, and used in soil-column studies to verify RDX-degrading activity. Each vegetated plot was planted with 150 clones, each 15 - 20 cm high, of control WT or TG plants, spaced on a 20 x 20 cm grid as detailed in Figures S2c and S2d. For the TG plots, three, independently transformed lines, N1, N2 and N5[21], were planted, as described in Figure S2d. The plants were established for one month before dosing with RDX.

Plot maintenance, sampling and decommissioning.

245 All plots were maintained at a constant soil moisture level (40 %) using a Campbell Scientific
246 CR1000 data logger system coupled to a CS650 soil moisture and temperature sensor to
247 control both the sump pump and irrigation systems.
248 During the growing seasons, all plots were checked weekly and seedling weeds removed. In
249 compliance with the APHIS-released permit, the grass plants were checked for developing
250 flower heads which were detached and destroyed. From April to Oct 2018, a complete 15-15-
251 15 all-purpose fertilizer (Miracle Grow) was applied every week at a rate of 16 g/m^2.
252
253 Over the winter season, plots were covered with polythene-covered cloches, to reduce plant
254 losses to cold weather and to reduce excess water/snow accumulation in the plots.
255
256 At the start and end of the trial, 5cm diameter soil cores were taken from five equidistant
257 locations, and at three depths, across the plots. At monthly intervals across the 2018 growing
258 season, plants were sampled from rows 2, 3, 4, 7,8, 9, 12, 13, and 14 and from plants 2
259 through 9 to remove bias from edge effects. One plant per row was sampled every two weeks
260 from June through September. The selected plant was cut 15cm above the soil surface and
261 allowed to regrow.
262
263 At the end of the trial, all plant material was incinerated. The RDX in plot tanks was
264 degraded by addition of 10 g/L $Ca(OH)_2$ per L storage water, RDX in the soil was treated
265 with 5 kg/m^2 $Ca(OH)_2$. Water and soil samples were extracted and analyzed using HPLC to
266 verify all RDX had been destroyed. The soil pH on the plots was restored by addition of 4
267 kg/m^2 $Al_2(SO_4)$ and re-seeded with a mix of 30 % fine fescue (*Festuca arundinacea*), 10 %
268 sheep fescue (*Festuca ovina*), 30 % Kentucky bluegrass (*Poa pratensis*) and 30 % perennial
269 ryegrass (*Lolium perenne*). While this approach was suitable for small-scale removal of

RDX, the application of $Ca(OH)_2$ is not cost-effective at the field-scale required, and would negatively affect soil organisms, with cascade effects on food webs.

Extraction and quantification of RDX.

All samples were analyzed using a method adapted from EPA method 8330A[28]. Briefly, soil samples were air dried at room temperature in the dark, ground, then a 5 g subsample, extracted with 10 mL acetonitrile, and incubated overnight on a rotary shaker. The supernatant was filtered through 0.45 µm PTFE filters, then analyzed using HPLC. RDX was extracted from 100 mg ground plant tissue in 1.4 mL acetonitrile on a rotary shaker overnight, centrifuged 10 min at 12,000 g. The supernatant was applied to a 1 g florisil, 1 g alumina sample preparation column[29], and the concentrated, filtered samples analyzed using HPLC.

HPLC analysis was conducted using a modular Waters system consisting of a Waters 717+ autosampler, two Water 515 HPLC pumps and a Waters 9926 photodiode array detector. A 250 x 4.6 mm Hypersil Gold reverse phase column (ThermoFisher) was used for separation with a 60: 40 mobile phase (HPLC-grade water: 83 % methanol/ 17 % acetonitrile) at 1 mL/min and monitored at 254 nm with frequent confirmation of RDX identification by spectral analysis using the photo diode array. Peak integration and data analysis were conducted using the Millennium software (Waters).

Determination of leaf surface area and transpiration rates.

Polyvinyl chloride (PVC) columns were constructed with PVC tubing (90 mm diameter, 0.5 m long), and filled with a mix of 75 % gravel and 25 % sand. Plant were grown in the columns at 25 °C, 16-hour light and 8-hour dark photoperiod, 110 µmol m^{-2} s^{-1} light intensity to a height of 50 cm. Transpiration efficiency was measured on second youngest, fully-

295 expanded leaves using a SC-1 leaf porometer (Decagon Devices, Inc.). Ten leaves were
296 sampled for each line. Leaf surface areas were determined using WinRhizo (2011 Regent
297 Instruments).
298
299 **Statistical analyses.**
300 Statistical analyses were performed using IBM SPSS Statistics software version 25. All data
301 were tested for homogeneity of variance using Levene's test, for data with $P > 0.05$,
302 statistical significance was performed using one-way analysis of variance (ANOVA)
303 followed by Tukey's *post hoc* test. For data with $P < 0.05$, statistical significance was
304 performed using the Independent-Samples Kruskal-Wallis test, *post hoc* pairwise
305 comparisons were then performed using Dunn's[30] procedure with a Bonferroni correction for
306 multiple comparisons, and adjusted *P* values presented.
307
308 **Data and biological materials availability**
309 The data that support the findings of this study, and biological materials, are available from
310 the corresponding authors on reasonable request.
311

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Author contributions

T.J.C., E.L.R., A.J.P., S.E.S., and N.C.B. conceived the study and designed the experiments. T.J.C. was responsible for plot design, construction and sample collection. T.J.C., L.Z. and R.M.R. performed biomass and RDX quantification. E.L.R. T.J.C., S.E.S., L.Z. and N.C.B. analyzed data. E.L.R performed statistical analysis, and L.Z. performed all other experiments. E.L.R. and N.C.B. wrote the manuscript. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Data availability

The data that support the findings of this study are available from the corresponding authors on request.

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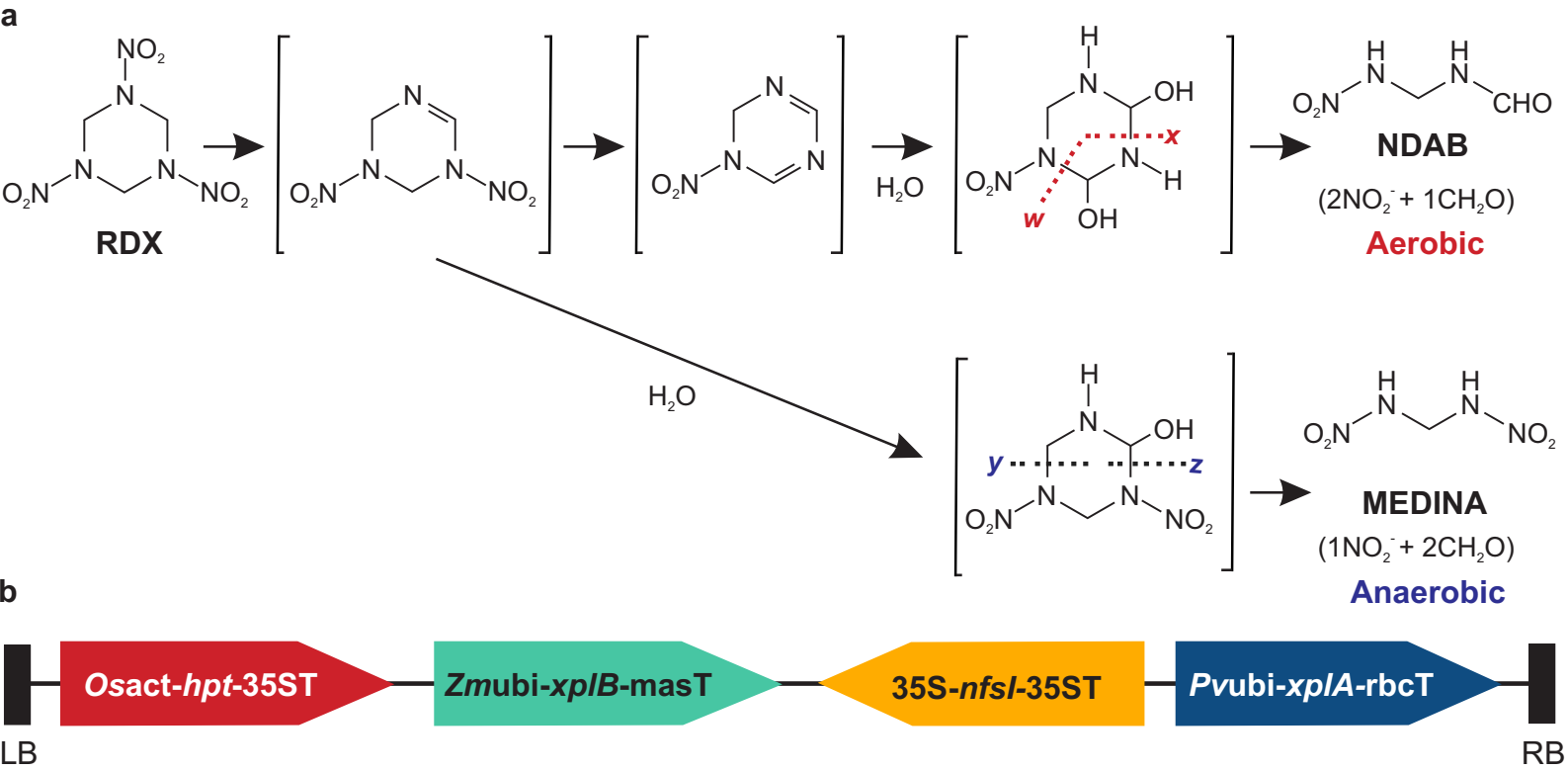
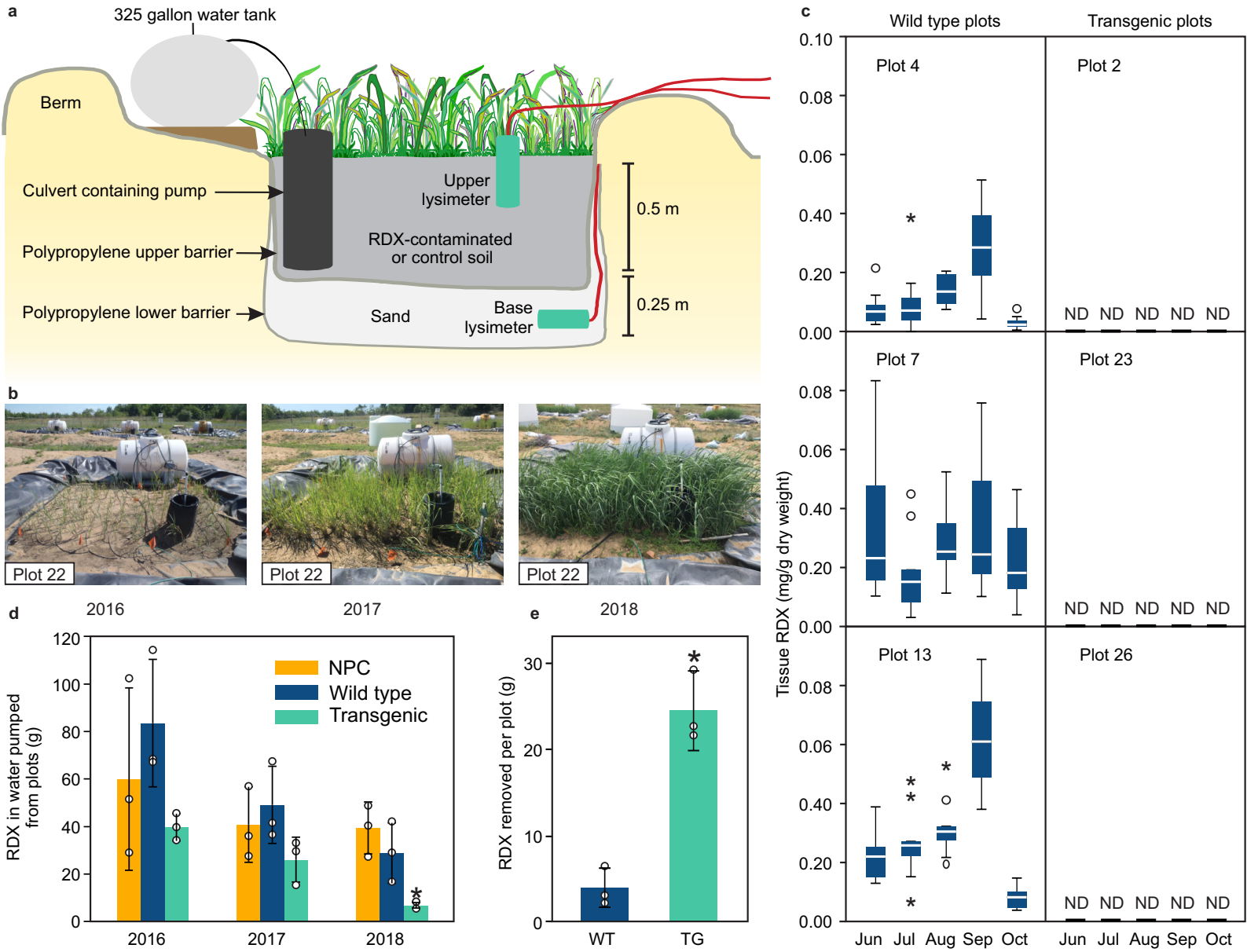
Figure Legends

Figure 1: Pathway of RDX catabolism by XplAB, and transgene cassette. (a) Ring cleavage occurs at wx and yz. Compounds in brackets are hypothetical, adapted from[12]. (**b)** The cassette transformed into the switchgrass lines is described in Zhang et al (2017)[21]. The transgenes *xplA*, *xplB*, *nfsI*, and the selectable marker gene *hyg*, which encodes resistance to hygromycin, were controlled by the rice actin (*Os*act), maize ubiquitin (*Zm*ubi), cauliflower mosaic virus 35S, and switchgrass ubiquitin (*Pv*ubi) promoters, respectively.

Figure 2: Plot design and performance. (a) Schematic showing a cross-section through a vegetated plot. **(b)** Appearance of the switchgrass on representative plots at the end of each growing season. **(c)** Levels of RDX in aerial tissues harvested across the 2018 growing season (ND = Not Detected, n = tissue from 9 plants). The top and bottom box lines show the first and third quartiles, the line in the middle of each box is the median. The whiskers show the maximum and minimum values, with the exceptions of outliers (circles) and extremes (asterisks). Outliers are at least 1.5 box lengths from the median, and extremes are at least 3 box lengths from the median. (d) Levels of RDX in the excess water pumped from the plots over the three-year duration of the trial (NPC; No Plant Control, n = 3 plots ± SD, circles show data points, * represents significantly different, $P = 0.015$, to NPC 2018). **(e)** Calculated level of RDX degraded in the transpiration stream from plants in the 100 mg/kg RDX plots over the 2018 growing season (WT; wild type, TG; transgenic, n = 3 plots ± SD). The statistical significance was performed using one-way analysis of variance followed by Tukey's *post hoc* test, * represents significantly, $P = 0.015$.

Extended Data Figure 1: Map of explosives contamination across the globe. Stars indicate documented RDX contamination, with details listed in Supplementary Table 1.


a
RDX
H2O
NDAB
(2NO2- + 1CH2O)
Aerobic
H2O
MEDINA
(1NO2- + 2CH2O)
Anaerobic
b
Osact-hpt-35ST
Zmubi-xplB-masT
35S-nfsI-35ST
Pvubi-xplA-rbcT
LB
RB


a
325 gallon water tank
Berm
Culvert containing pump
Polypropylene upper barrier
Polypropylene lower barrier
Upper lysimeter
RDX-contaminated or control soil
Sand
Base lysimeter
0.5 m
0.25 m
b
Plot 22
Plot 22
Plot 22
c
Wild type plots
Transgenic plots
Plot 4
Plot 2
Plot 7
Plot 23
Plot 13
Plot 26
Tissue RDX (mg/g dry weight)
ND
Jun Jul Aug Sep Oct
d
2016
2017
2018
NPC
Wild type
Transgenic
RDX in water pumped from plots (g)
e
RDX removed per plot (g)
WT
TG

Supplementary Tables and Figures

Table S1: Documented RDX contamination located in Figure S1.

Number	Country	Location
1	**Afghanistan**	Significant concentrations of RDX recorded in soil between warheads stored on site Astana, a village in the Panjshir Valley of the Parwan Province of Afghanistan[20]
2	**Australia**	Soil for enrichments obtained from a disused site of munitions manufacture and storage site contaminated with RDX [21], RDX-contaminated groundwater in Victoria[22].
3	**Belgium**	Soil samples from training ranges[13]
4	**Canada**	Contaminated soil at military sites in Alberta, New Brunswick, Quebec, Ontario, British Columbia[1].
5	**Guam**	A US island territory in Micronesia. RDX detected in surface water runoff at the discharge point of the Almagosa River into the Fena Reservoir[23].
6	**Germany**	The Kolberger Heide munition dumping ground in the southwest Baltic Sea near Kiel, Germany. The site contains approx. 30 000 tons of munitions from World War II comprising mainly TNT, RDX, and DNB (1,3- dinitrobenzene) [24].
		RDX in soil and groundwater samples from a former plant for the production of explosives during World War II at Elsnig (Saxony) [25].
7	**India**	Soil for enrichment studies collected from an explosives-contaminated site in Panchkula, Haryana, India[26].

8	**Italy**	RDX found in soil samples collected in Sardinia[17].
9	**Israel**	Process wastes from an RDX manufacturing plant used for microbial enrichment studies[27]. RDX contaminated groundwater from a coastal aquifer[28].
10	**Puerto Rico**	Vieques island used for military training 1941 to 2001, contaminated with explosives including RDX at levels threatening human health[8].
11	**South Korea**	RDX from a military gunnery range found in groundwater towards the Imjin River, near Seoul[29].
		Artillery ranges operated by the Korean Army of South Korea[7].
12	**Switzerland**	Between 1920 and 1967 roughly 4600 tonnes (t) of ammunition waste dumped at Lake Thun, including RDX[15].
13	**Sweden**	RDX found at Alvdalen shooting range, Sweden[30]
14	**Taiwan**	RDX found in surface soil at military shooting ranges in Kinmen County[31].
15	**UK**	Soil samples from a site heavily contaminated with RDX and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) [32], and **s**oil samples used for enrichment cultures[13].
16	**Ukraine**	Military training range soil[13.]
17	**US**	See Table S2 for details

3

4

Table S2: Examples of RDX contamination in American States.

This list is not exhaustive; many sites are under continuous monitoring and clean-up, and identification of new sites continues.

State		Location
Alabama	AL	Redstone Arsenal[33]
Alaska	AK	Donnelly Training Area[34], Fort Richardson[35], Fort Wainwright[36]
Arizona	AZ	Camp Navajo, Bellemont[37]
California	CA	Fort Ord[34], 29 Palms[38]
Colorado	CO	Fort Carson[34]
Florida	FL	Elgin Air Force Base[39]
Hawaii	HI	Pohakuloa Schofield Barracks[34]
Illinois	IL	Joliet Army Ammunition Plant[1], Savanna Army Depot[40], Crab Orchard National Wildlife Refuge[41]
Iowa	IA	Iowa Army Ammunition Plant[40]
Kentucky	KY	Blue Grass Army Depot[42]
Louisiana	LA	Louisiana Army Ammunition Plant[40], Fort Polk[34]
Maryland	MD	Aberdeen Proving Ground[40]
Massachusetts	MA	Camp Edwards, Massachusetts Military Reserve[43], Cape Cod and Otis Air National Guard Base[44]
Mississippi	MS	Camp Shelby[34]
Missouri	MO	Fort Leonard Wood[34]
Nebraska	NE	Cornhusker Army Ammunition Plant[40]
Nevada	NV	Hawthorne Army Depot[45]

New Jersey	NJ	Picatinny Arsenal[46]
New Mexico	NM	Fort Bliss[34], Holloman Air Force Base, Los Alamos National Laboratory[47]
Oregon	OR	Umatilla Army Depot[40], a munitions storage and handling depot in Hermiston, Oregon[48, 49]
South Carolina	SC	Fort Jackson[50]
Tennessee	TN	Holston Army Ammunition Plant in Kingsport[1], Milan Army Ammunition Plant[40]
Texas	TX	Fort Hood[34], Holston Army Ammunition Plant[1], Pantex Plant[51]
Utah	UT	Tooele Army Depot (North Area) [52, 53]
Virginia	VA	Radford Army Ammunition Plant, Radford[54]
Washington	WA	Bangor Naval Submarine Base & Ordnance Disposal[34], Camp Bonneville, Yakima Training Center[40]
Wisconsin	WI	Barksdale Explosives Plant[1]
Wyoming	WY	Camp Guernsey[34]

Figure S1: Map of explosives contamination across the globe. Country names link to source information. Stars indicate documented RDX contamination, with details listed in Table S1.

(This figure is provided as a pdf with interactive hyperlinks)

Figure S2: Design of the field plots and layout.

(**a**) Randomized block lay-out of the plots. (**b**) Location of the plant lines on each plot. (**c**) Location of each of the three, independently-transformed XplAB-expressing lines N1, N2 and N5. (**d**) Aerial photograph of the plot taken September 2015 before planting.

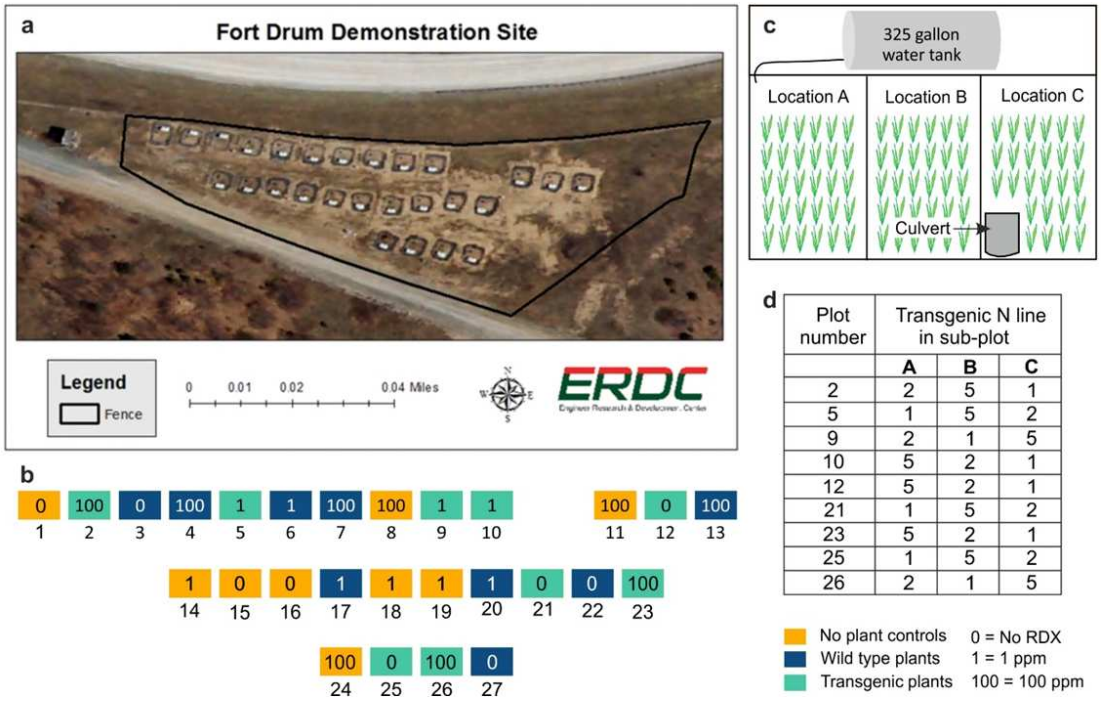


Figure S3: Appearance of the plants at the end of each growing season over the three-year trial.

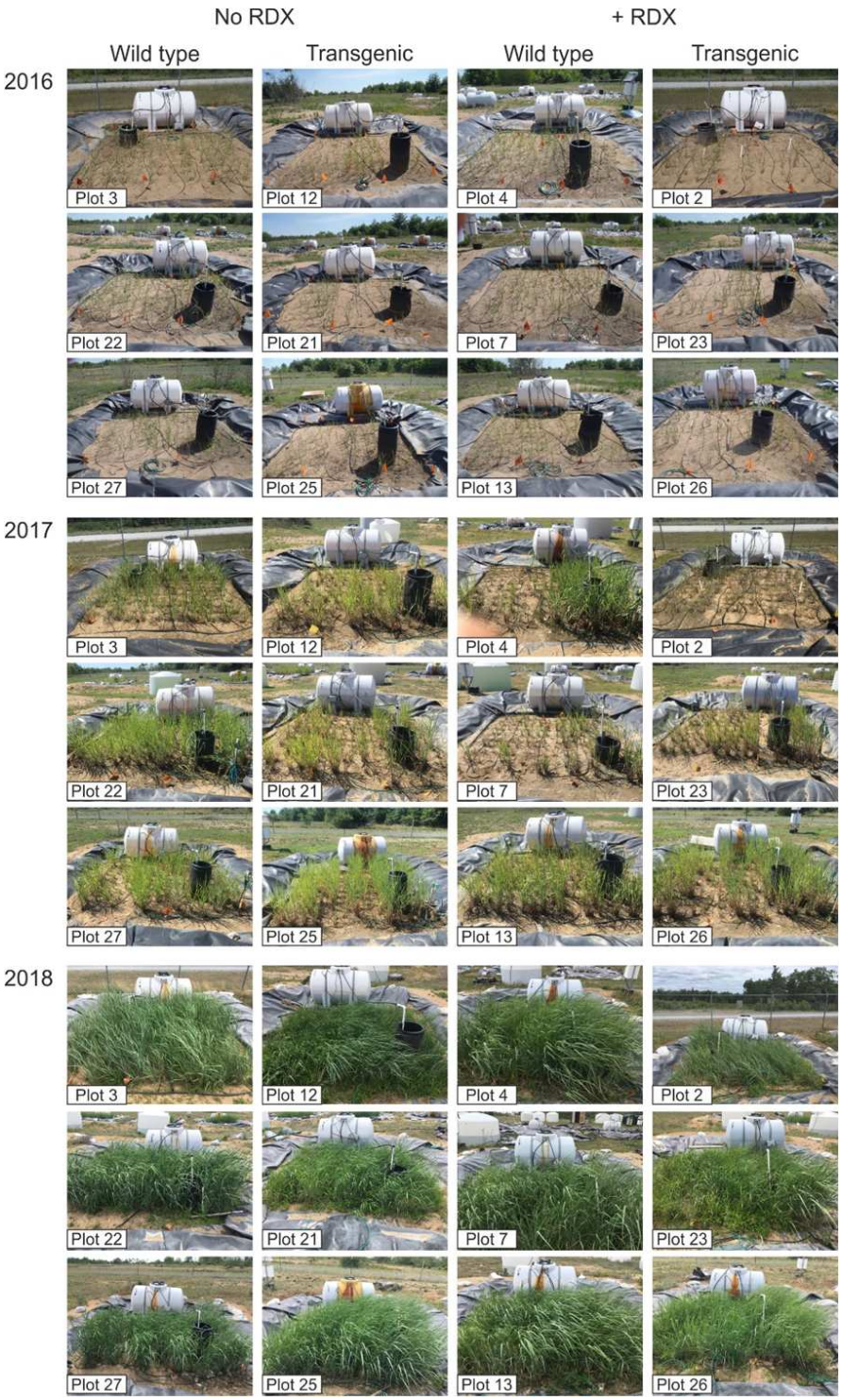


Figure S4: Aerial tissue weights following harvest at the end of the field trial.

(**a**) Biomass of the WT (wild type) and individual (TG) transgenic N lines on uncontaminated and RDX-treated plots (n = 45(WT), n = 15(TG) plants). Statistical significance was performed using one-way analysis of variance followed by Tukey's *post hoc* test. $P<0.05$ was considered as significant. On No RDX plots, line N5 mean biomass was significantly ($P < 0.004$) lower than N2. (**b**) Comparison of biomass from individual plots (n = 15 plants). A Kruskal-Wallis H test showed the distributions of biomass values for the No RDX plots were statistically significantly different ($P = 0.002$). Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons, and presenting adjusted *P* values. This *post hoc* analysis revealed that the plant biomass from TG plot 12 was statistically significantly different from TG plots 21 and 26 ($P = 0.013$). (**c**) Total biomass for each of the treatment types WT and transgenic (TG) plants (n = 3 plots). (**d**) Appearance of representative tissue samples during harvest.

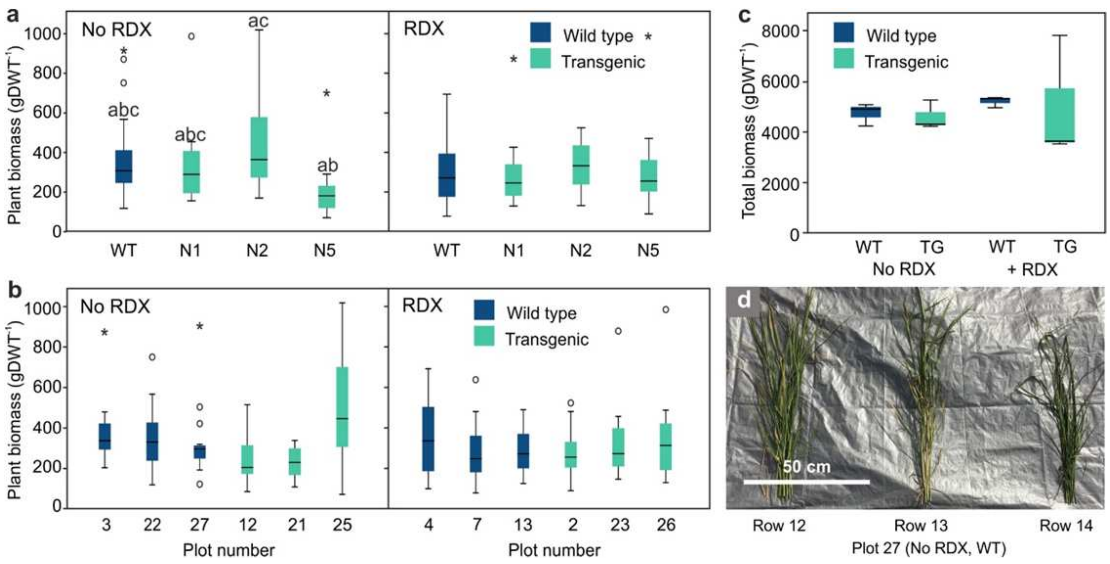


Figure S5: Total tissue RDX from plots over the 2018 growing season.

Gene expression analysis on leaf tissue harvested from the plots in September 2018. (**a**) Transcript abundance measured using quantitative RT- PCR for *xplA* and (**b**) *xplB* expression. Conditions were as described earlier[55], with transcripts normalized to the switchgrass reference gene eIF-4a. Results are mean ± SE, represent triplicate measurements on single wild type (WT) plants from each of plots 4, 7 and13; and single plants from plots 2, 23 and 26 for each transgenic line. A two-tailed *t*-test showed the transgenic lines N1, N2 and N5 were significantly different from WT (* = $P \leq 0.022$). (**c**) Western blot analysis conducted as described earlier[55] using XplA antibody[56] and RuBisCO large subunit antibody (AS03 037, Agrisera, Sweden). Band intensities for each line, and antibody, were quantified from 8 replicate blots using ImageJ software[57], results are mean ± SE, a two-tailed *t*-test showed the transgenic lines N1, N2 and N5 were significantly different from WT (* = $P <$ 0.000).

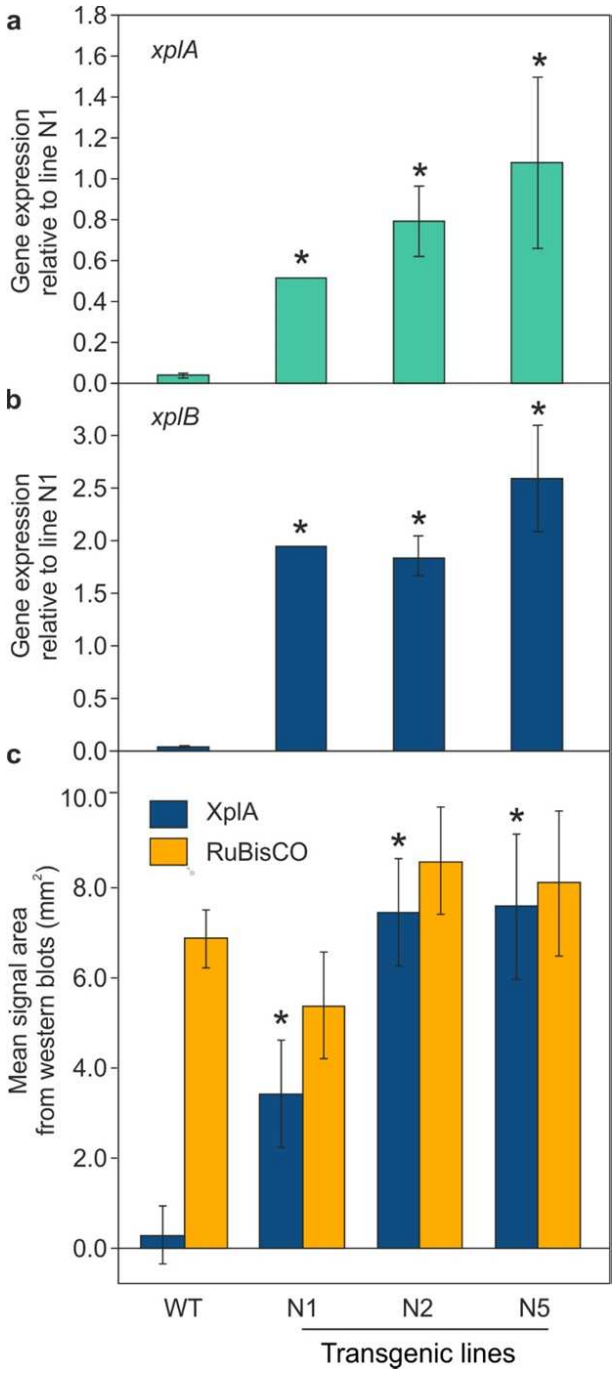

a
xplA
Gene expression relative to line N1
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
xplB
Gene expression relative to line N1
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
XplA
RuBisCO
Mean signal area from western blots (mm²)
10.0
8.0
6.0
4.0
2.0
0.0
WT
N1
N2
N5
Transgenic lines

Figure S6: Total tissue RDX from plots over the 2018 growing season.

Mean aerial tissue RDX concentration in plants from the 100 ppm RDX plots (ND, Not Detected, n = 45).

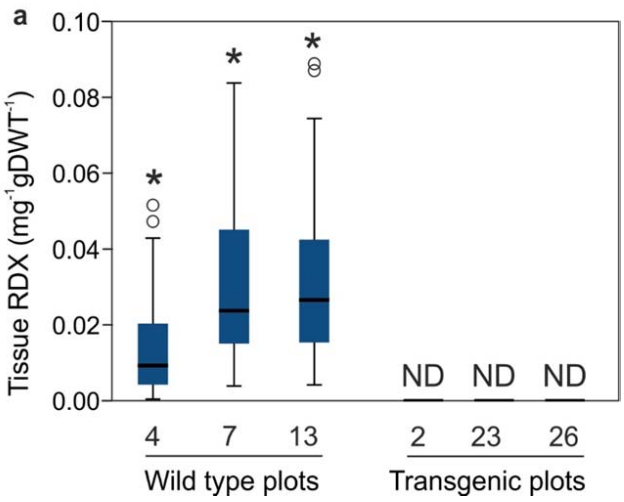
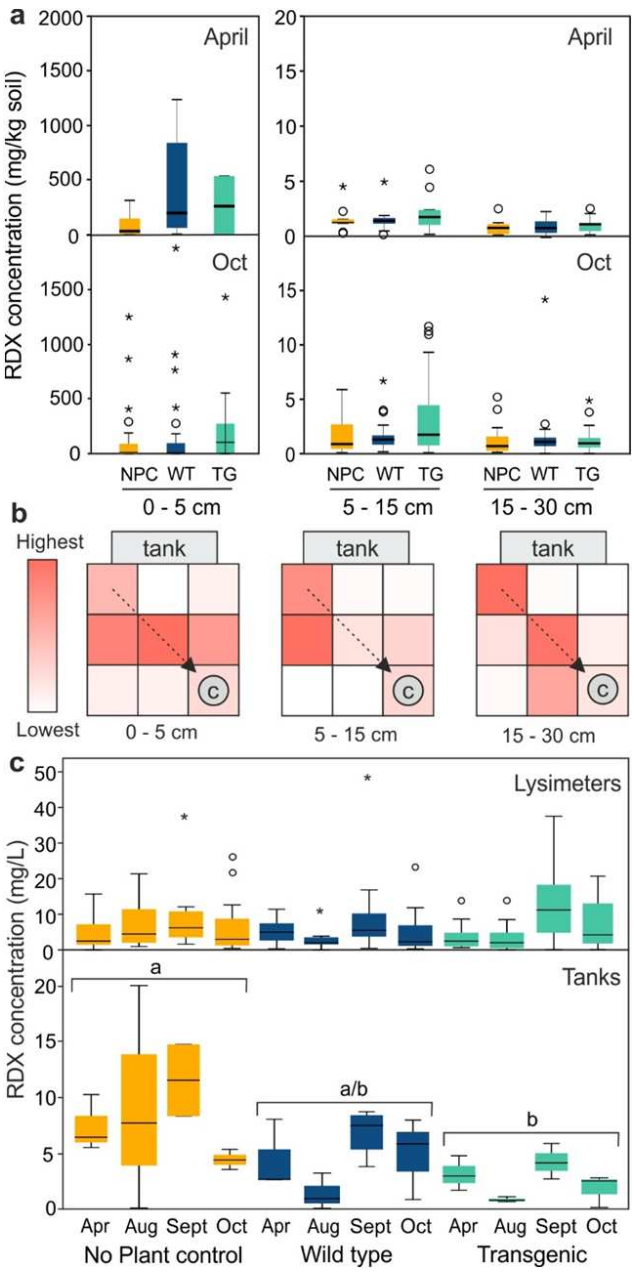


Figure S7: RDX soil, soil water and tank concentrations in 2018 growing season.

(**a**) Soil RDX concentration in the 100 ppm plots measured at three depths at the start (April n= 9 soil samples) and end (Oct n= 27 soil samples) of the growing season (NPC; No plant control, WT; wild type, TG, transgenic.

(**b**) Heat plot showing mean distribution of RDX across the 100 ppm plots at the three depths, c = position of culvert, tank = position of water tank.

(**c**) Concentration of RDX in lysimeters and tanks across the 2018 growing season. Lysimeter data: n = 12(NPC), 15(WT), 15(TG). Tank data: n = 11(NPC), 12(WT), 12(TG). For the tank data, a Kruskal-Wallis H test showed the distributions of NPC, WT and TG values were statistically significantly different (P = 0.026) from each other. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons, and presenting adjusted P values. This *post hoc* analysis revealed that the RDX concentrations in the TG tanks were statistically significantly different from those in the NPC tanks (P = 0.021).


a
April
Oct
RDX concentration (mg/kg soil)
NPC WT TG
0 - 5 cm
5 - 15 cm
15 - 30 cm
b
Highest
Lowest
tank
c
0 - 5 cm
5 - 15 cm
15 - 30 cm
c
Lysimeters
Tanks
RDX concentration (mg/L)
a
a/b
b
Apr Aug Sept Oct
No Plant control
Wild type
Transgenic

78

79

Figure S8: Excess water pumped from plots over each growing season.

Volume of excess water pumped from the plots over each growing season of the field trial (NPC; No plant control, WT; wild type, TG, transgenic, n = 3 plots). (**a**) No RDX plots. (**b**) 100ppm RDX plots. Letters denote values that were significantly between the three treatment types for that growing season. For the 2017 No RDX data, a Kruskal-Wallis H test showed the distributions of NPC, WT and TG values were statistically significantly different (P = 0.014) from each other. Pairwise comparisons were performed using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons. For all over data, one-way analysis of variance followed by Tukey's *post hoc* test was used, with P<0.05 considered as significant.

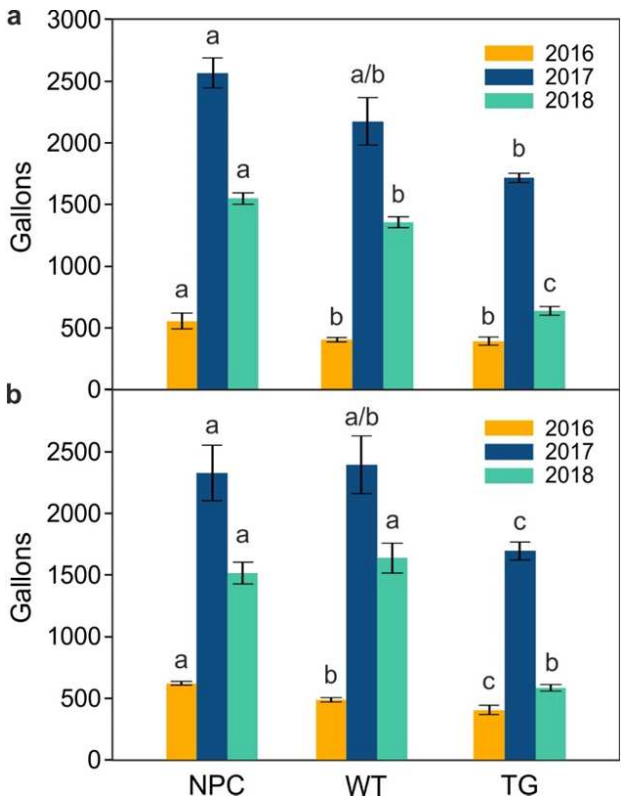


Figure S9: Leaf surface area of the plants at the end of the 2018 growing season.

(**a**) Mean leaf surface areas in the wild type (WT) and individual transgenic (TG) lines grown in the laboratory (n = 3 plants). (**b**) Comparison of mean leaf surface area of all WT and TG plants grown on the uncontaminated (No RDX) verses RDX-treated (+ RDX) plots (n = 6 plots) (**c**) Mean leaf surface areas in the WT and individual TG N lines grown in the field, independent of the presence of RDX. (**d**) Mean leaf surface areas in the WT and TG lines grown in uncontaminated verses RDX-treated.

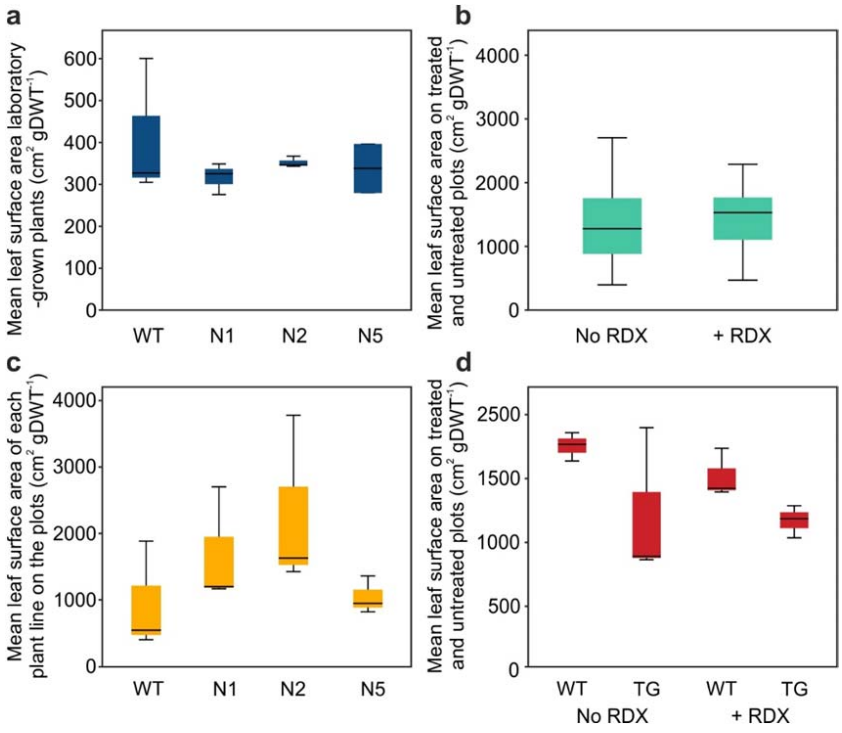
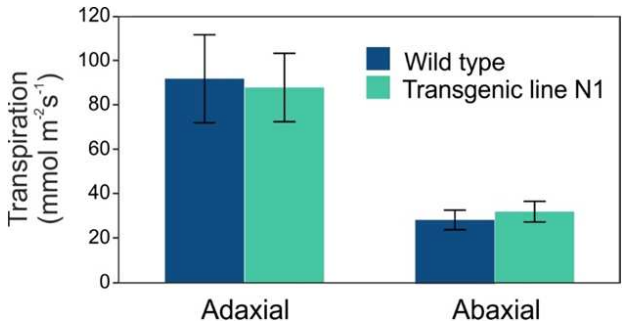


Figure S10: Leaf transpiration rates.

Leaf surface transpiration rates for wild type and transgenic line N1 from adaxial and abaxial surfaces (n = 10 leaves).



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