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

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Summary

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

Introduction

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 These compounds enter the environment through manufacturing, military use, and the decommissioning of outdated explosives.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

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

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

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

Results

Vector construction

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

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

Functional evaluation of the pNSAT vectors

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

Production of transgenic grasses and molecular analysis

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

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

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

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

RDX uptake and degradation by transformed grasses

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

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

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

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

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

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

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

TNT resistance of transformed grasses

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

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

Column studies with switchgrass

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

Discussion

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

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

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

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

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

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

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

In conclusion, this is the first report of genetically transformed grasses for the phytoremediation of the explosive, and environmental pollutant, RDX. Creeping bentgrass and switchgrass were successfully transformed with the bacterial genes to confer RDX degradation, *xplA* and *xplB*. Both transformed 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 *Age*I-*Not*I 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 *Asc*I 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-*Psp*I 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 *Sce*I fragment and inserted into pRCS2-*xplA* to produce prcs2-6*xplA*-4*xplB*. The *nfsI* gene was cloned
323 and inserted into pSAT7a to produce pSAT7a-*nfsI*, and the expression cassette act-*nfsI*-agsT was

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

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

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

Molecular analysis of transgenic plants

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

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

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

Protein extraction and immunoblot analyses

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

RDX uptake by transformed switchgrass and creeping bentgrass

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

HPLC quantification of aqueous RDX

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

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

TNT uptake by transformed creeping bentgrass

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

Column studies

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

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

Data analysis

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

Acknowledgements

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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]

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

Creeping bentgrass plants were cultured in 5 mL liquid ½ MS medium dosed with RDX at 40 mg/L. The masses of wild type and transformants (N5, N18, N19) were measured after 16 days culture and the biomass gains calculated. The transformed lines of N18 and N19 accumulated more biomass than wild type during the time course. Controls consisted of wild type plantlets cultured in MS medium without RDX. Letters indicate biomass gains were significantly different ($p<0.05$) from other lines ($n = 3 \pm SE$).

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 *Os*act, *Zm*ubi, and *Pv*ubi 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; *Os*act,
570 *Oryza sativa* actin promoter; *Zm*ubi, *Zea mays* ubiquitin promoter; *Pv*ubi, *Panicum virgatum*
571 (switchgrass) ubiquitin promoter; RB left border; RB right border.


a
AscI
AscI
I-SceI
I-SceI
PI-PspI
PI-PspI
PI-TliI
PI-TliI
35S-hpt-35ST
35S-xplB-35ST
rbs-xplA-rbcT
act-nfsI-agsT
LB
RB
I-PpoI
b
Osact
MCS
35S terminator
AscI
AscI
Zmubi
MCS
Mas terminator
I-PpoI
I-PpoI
Pvubi
MCS
Rbc terminator
PI-PspI
PI-PspI
HindIII-EcoRI-PstI-SalI-KpnI-SacII-ApaI-SmaI-BamHI
c
AscI
AscI
I-PpoI
I-PpoI
I-SceI
I-SceI
PI-PspI
PI-PspI
Osact-hpt-35ST
Zmubi-xplB-masT
35S-nfsI-35ST
Pvubi-xplA-rbcT
LB
RB

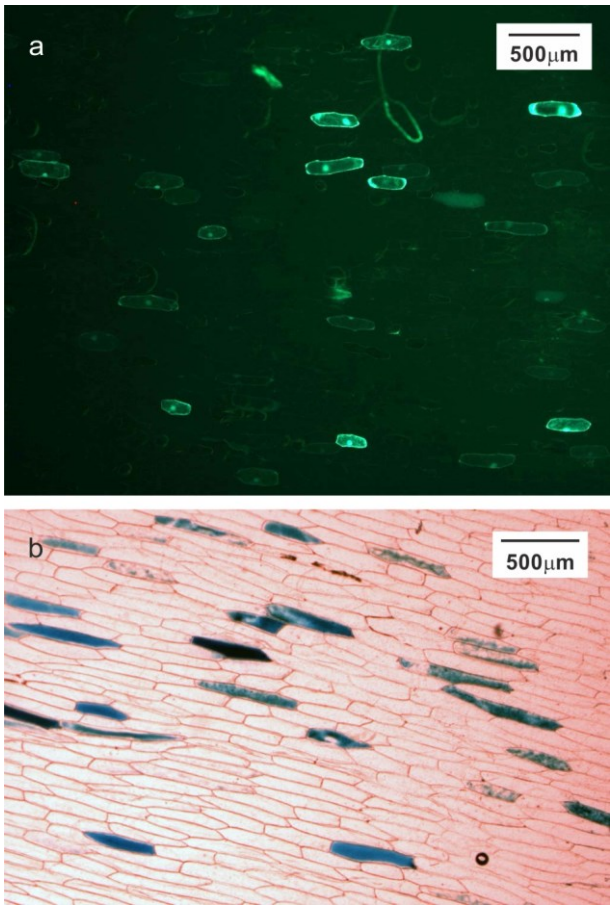
572

573

574

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 GPF expression following particle bombardment with
578 pNSAT1a-GFP (*Os*Act-GPF-35S). b) Histochemical staining of GUS expression following particle
579 bombardment with pNSAT3a/6a-GUS (*Zm*Ubi-GUS-Mas; *Pv*Ubi-GUS-rbc).



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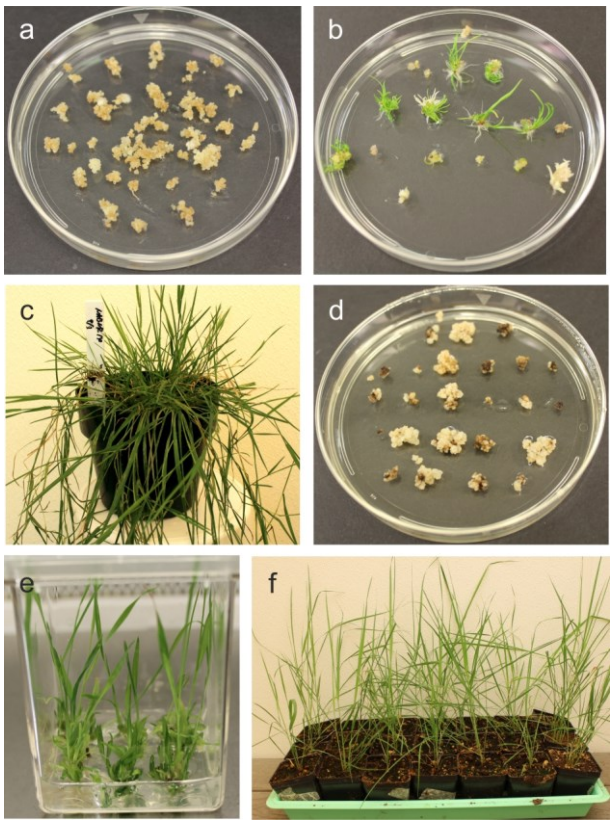
581

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

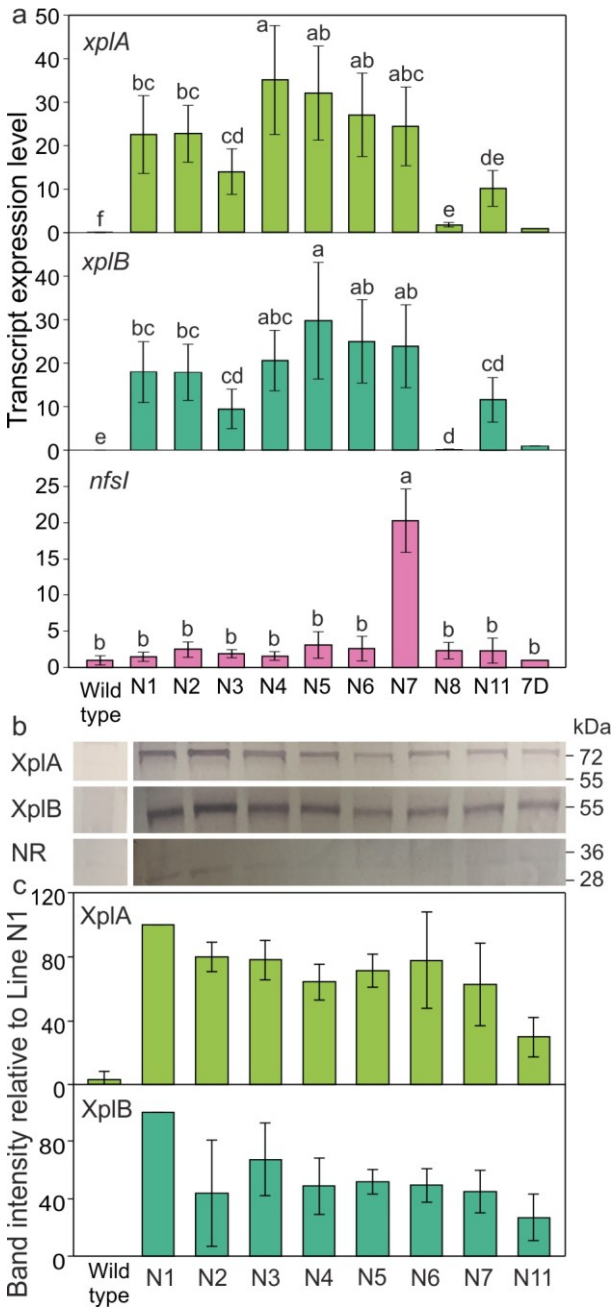


591

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

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

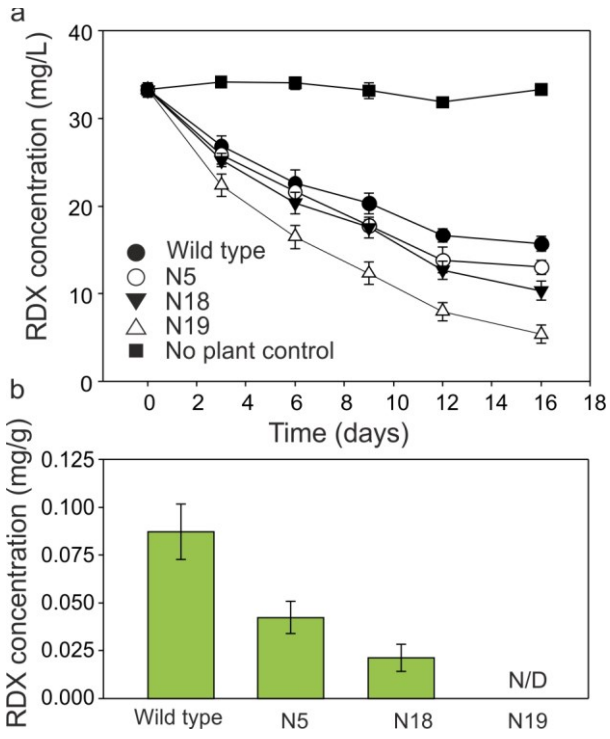
b) Western blot analysis on leaf blades of switchgrass lines expressing XplA, XplB and nitroreductase (NR) protein. c) Band intensities were quantified for XplA and XplB expression. Levels were normalized to the Coomasie-stained RUBSICO large subunit, results are from three replicate blots ± SE.


a
xplA
xplB
nfsI
Transcript expression level
Wild type
N1
N2
N3
N4
N5
N6
N7
N8
N11
7D
b
kDa
XplA
XplB
NR
72
55
55
36
28
c
Band intensity relative to Line N1
XplA
XplB
Wild type
N1
N2
N3
N4
N5
N6
N7
N11

603

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

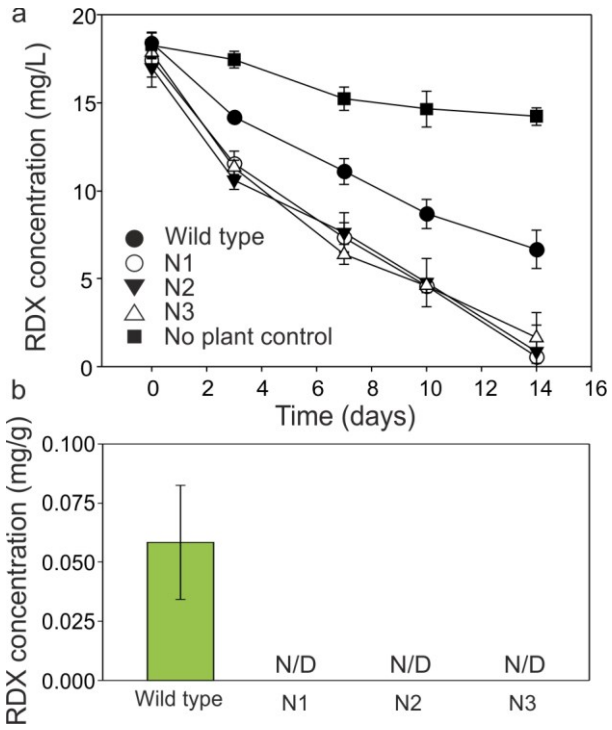
a) Concentration of RDX in culture medium over the course of the experiment. After three days, the medium from line N19 contained significantly (p=0.010) less RDX than medium from wild type plants and after nine days, lines N5 and N18 had also removed significantly more RDX from the media than wild type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



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 ± SE, N/D = none detected).

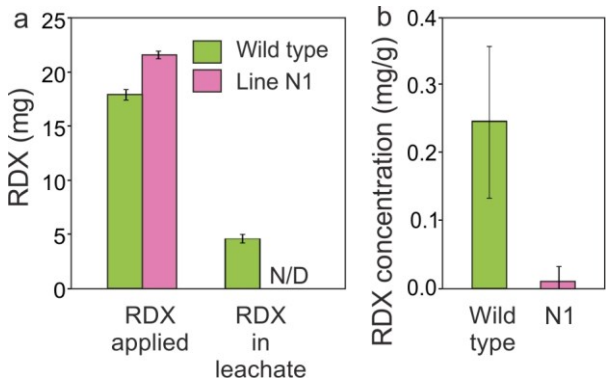
618



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 ± SE, N/D = none detected).



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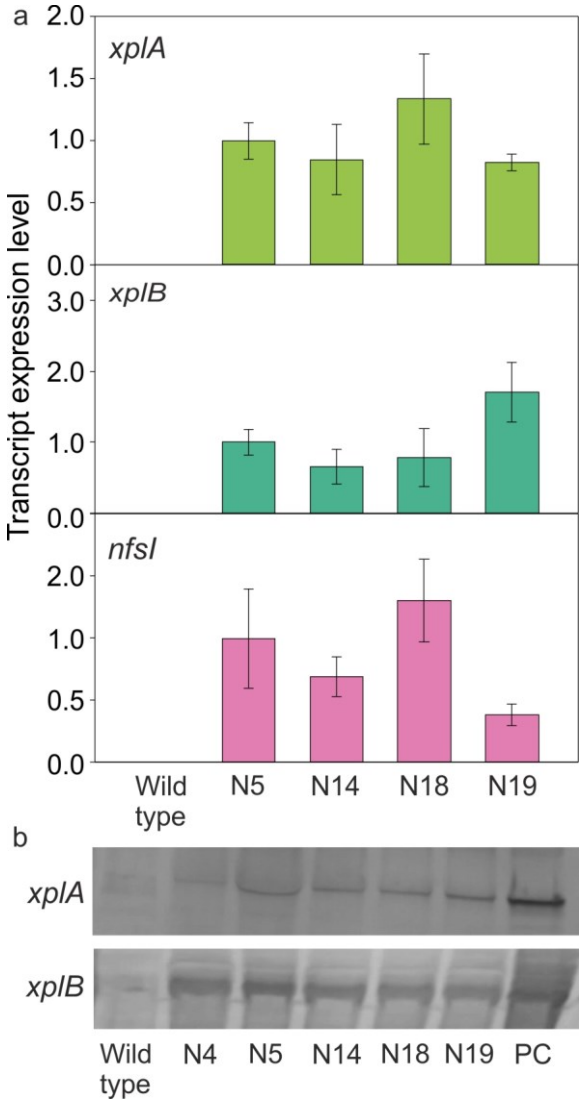
Supplementary Materials

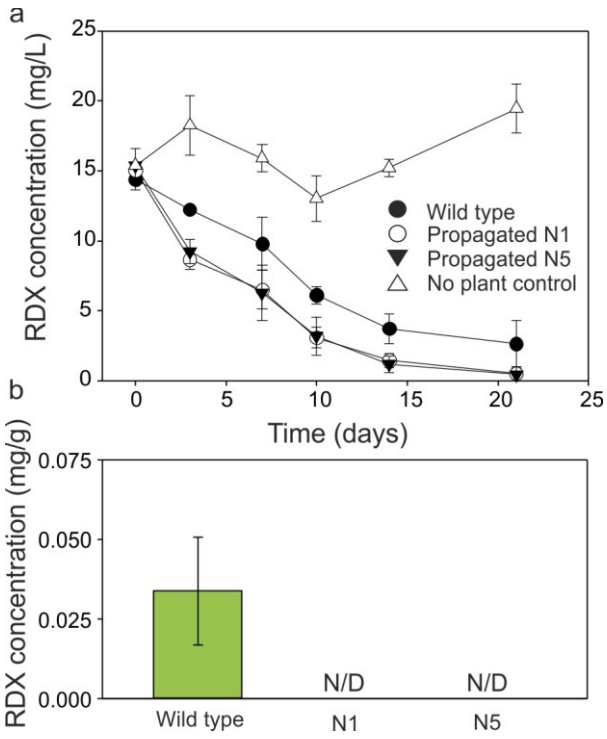
Supplemental Figure 1. Molecular characterization of transgene creeping bentgrass**.**

a) Transcript abundance measured using quantitative RT- PCR on plant lines transformed with *xplA*, *xplB* and *nfsI*. Values were normalized to the creeping bentgras reference gene 5.8SrRNA and are relative to the expression levels of line N5 (n = 3 ± SE).
b) Western blot analysis on leaf blades of creping bentgrass lines expressing XplA, XplB and nitroreductase (NR) protein (PC, positive control; total soluble protein isolated from *xplA-xplB-nfsI* expressing *Arabidopsis* line 7D (Rylott et al., 2011).



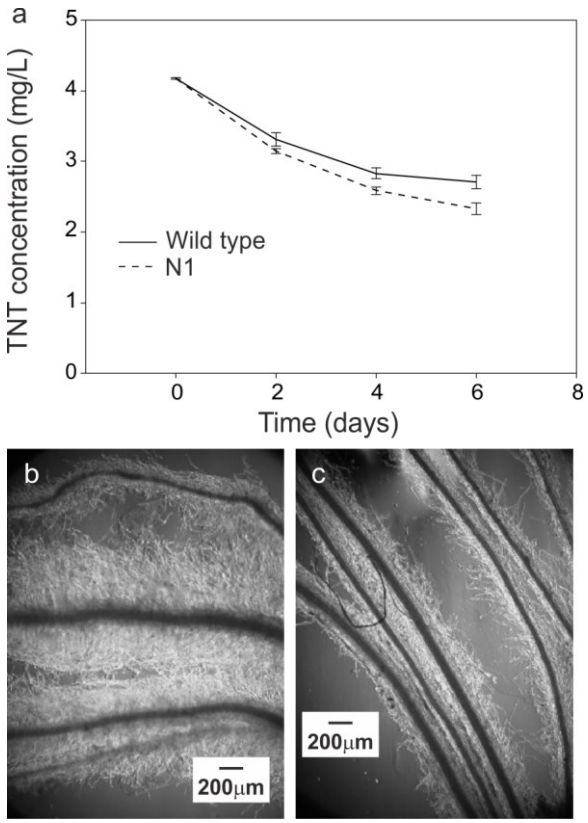
Supplemental Figure 2. Uptake of RDX by **propagated plants from** *xplA-xplB-nfsI* transformed switchgrass grown in liquid culture

a) Concentration of RDX in culture medium over the course of the experiment and b) concentration of RDX in switchgrass tissue after 16 days (n = 3 ± SE, N/D = none detected).



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

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



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

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Primer name	**Sequence (5' - 3')**	**Flanking Restriction site**	**Details**
HptF3	TTGAATTCATTATGAAAAAGCCTGAACTC	EcoRI	Clone and Insert *hpt* gene into pSAT1a-35S to produce pSAT35S-*hpt*
HptR3	ATTGGATCCCTATTTCTTTGCC	BamHI	
XplaF5	ttAAGCTTACCATGGccgacgtaactgtcctg	HindIII	Clone and insert *xplA* gene into pSAT6a and pnSAT6a
XplaR5	TTAAGCTTTCAGGACAGGACGATCGGC	HindIII	
XplbF1	ACGGTACCATGGACATCATGAGTGAAGT	KpnI	Insert *xplB* into pSAT4a and pnSAT3a
XplbR1	ttGGATCCTCAGCAGACCGATTCGGCCGGC	BamHI	
NrF1	TCGAATTCAACAATGGATATCATTTCTGTCG	EcoRI	Insert *nfsI* gene into pSAT7a and pSAT4a
NrR1	TTGGATCCTCAGCACTCGGTCACAATCG	BamHI	
OsActinF1	TTACCGGTCTCGAGGTCATTCATAT	AgeI	Clone and insert *Os*actin promoter into pSAT1a to produce pnSAT1a
OsActinR1	TTAAGCTTTCTACCTACAAAAAAGCTCC	HindIII	
ZmUbiF1	TTACCGGTTGCAGTGCAGCGTG	AgeI	Clone and insert *Zm*Ubi promoter into pSAT3a to produce pnSAT3a
ZmUbiR1	CCAAGCTTTGCAGAAGTAACACC	HindIII	
PvUbiF1	TTACCGGTCCACTGGAGAGGG	AgeI	Clone and insert *Pv*Ubi 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 *hpt* cassette of prcs2-abnr-hr vector in grass genome DNA.
Sat4seqr1	CCTTATCTGGGAACTACTCAC		
nSAT1aseqf1	GCTGCTTCGTCAGGCTTAGAT		Work Together with

			SAT4aseqr1 to do PCR to confirm the insertion of *hpt* cassette of prcs2-NABNR vector in grass genome DNA.
NSAT3aseqf1	CTTGATATACTTGGATGATGGC		PCR to confirm the insertion of *xplB* cassette of prcs2-NABNR vector in grass genome DNA.
Sat3aseqr1	AGCCACGCACATTTAGGA		
NSAT6aseqf1	TGCTGTGATGCTGTTTGTTG		PCR to confirm the insertion of *xplA* cassette of prcs2-NABNR vector in grass genome DNA.
Sat6aseqr1	CCGGAAACAAACAACGA		
Xpla300f	CAACAACGCGATCGACATCC		qRT-PCR of *xplA* gene
Xpla959r	TCGAACATCGCCTCCATCAC		
XplB511F	GAATTCACCGGTTCCGATCT		qRT-PCRR of *xplB* gene
XplB1143R	GACTGTCCGGTCGATCACTT		
Nfs1322f	TTCAACACGCCAGAAGCCA		qRT-PCR of *nfs1* gene
Nfs1652r	AGCACTCGGTCACAATCGT		
HptF2	TCTTAGCCAGACGAGCGGGTTC		qRT-PCR of *hpt* gene
HptR2	TACTTCTACACAGCCATCGGTCCAG		
eIF-4aF	TGATGTCATTCAGCAAGCACAA		eukaryotic initiation factor-4A (*eIFa*)
eIF-4aR	GGCATTCAACCAGGCCATAG		

Supplemental methods

Biolistic bombardment of epidermal onion cells

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

GUS assay

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

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.

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