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Genetic modification of western wheatgrass (*Pascopyrum smithii*) for the phytoremediation of RDX and TNT

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Main Conclusion: Transgenic western wheatgrass degrades the explosive RDX and detoxifies TNT

ABSTRACT

Contamination, from the explosives, hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and 2, 4, 6-trinitrotoluene (TNT), especially on live-fire training ranges, threatens environmental and human health. Phytoremediation is an approach that could be used to clean-up explosive pollution, but it is hindered by inherently low *in planta* RDX degradation rates, and the high phytotoxicity of TNT. The bacterial genes, *xplA*, and *xplB*, confer the ability to degrade RDX in plants, and a bacterial nitroreductase gene *nfsI*, enhances the capacity of plants to withstand and detoxify TNT. While previous studies have used model plant species to demonstrate the efficacy of this technology, trials using plant species able to thrive in the challenging environments found on military training ranges are now urgently needed. Perennial western wheatgrass (*Pascopyrum smithii*) is a United States native species that is broadly distributed across North America, well-suited for phytoremediation, and used by the US military to re-vegetate military ranges. Here we present the first report of the genetic transformation of western wheatgrass. Plant lines transformed with *xplA*, *xplB* and *nfsI* removed significantly more RDX from hydroponic solutions and retained much lower, or undetectable, levels of RDX in their leaf tissues when compared to wild-type plants. Furthermore, these plants were also more resistant to TNT toxicity, and detoxified more TNT than wild-type plants. This is the first study to engineer a field-applicable grass species capable of both RDX degradation and TNT detoxification. Together, these findings present a promising biotechnological approach to sustainably contain, and remove, RDX and TNT from training range soil and prevent groundwater contamination.

Keywords: phytoremediation; RDX; TNT; western wheatgrass; transformation, monocot promoters; stacked genes.

INTRODUCTION

The explosives hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT) are used extensively by the US military. These compounds are damaging to the environment and human health: RDX targets the nervous system and can cause seizures in humans and animals (Ramasahayam et al. 2017) while TNT is considered to be highly toxic and mutagenic (Bolt et al. 2006; Honeycutt et al. 1996; Travis et al. 2008). The US Environmental Protection Agency has classified both RDX and TNT as possible human carcinogens (USEPA, 2014), and during explosives manufacture, transportation, storage and disposal, these toxic pollutants are released into the environment. Additionally, live-fire training at military bases has resulted in the contamination of soils around targets with particulates of munitions compounds, which have leached RDX into the underlying groundwater. The total area of operational ranges in the United States contaminated with munitions constituents is estimated to be more than 16 million acres (United States General Accounting, 2004), with clean-up of active ranges estimated by the US Department of Defense to cost between US$16 billion and US$165 billion (United States General Accounting, 2004). Chemical-based methods to remediate explosives, such as alkaline hydrolysis (Sviatenko et al. 2017), redox reactions using iron-bearing materials (Oh et al. 2016), and permeable reaction barriers (Ahmad et al. 2007) have proven too cost-intensive for the scale of the contaminated areas. Phytoremediation of RDX and TNT has been developed as a potentially more cost-efficient, and sustainable method to clean up contaminated soil.

Plants readily take up and translocate RDX from the soil to the aerial tissues (Vila et al. 2007; Brentner et al. 2010), with studies of poplar (*Populus deltoides* x *nigra* DN-34) showing that RDX can be 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) (Van Aken et al. 2004). However, despite high uptake rates, plants have relatively low rates of RDX degradation (Mezzari et al. 2004; Best et al. 1999; Winfield 2004) with RDX accumulated in living plant tissues returning to the environment following plant senescence (Yoon et al. 2006).

In contrast to RDX, TNT remains predominantly in root tissues with less than 25 % translocated to aerial tissues (Vila et al. 2007; Brentner et al. 2010). TNT is also more phytotoxic (Pavlostathis and Jackson 2002; Hannink et al. 2007; Johnston et al. 2015) than RDX, and symptoms of toxicity such as stunted growth and chlorosis have been observed at concentrations comparable to those found on military training ranges (Pichtel 2012). Plants have a limited ability to detoxify TNT. Endogenous nitroreductases, including oxophytodienoate reductases (Beynon et al. 2009) reduce TNT to hydroxyl-amino and then amino-dinitrotoluenes (HADNTs and ADNTs). These transformed intermediates are then conjugated to hydrophilic molecules such as sugars (Gandia-Herrero et al. 2008) and glutathione (Gunning et al. 2014), then appear to be incorporated into cell wall materials (Rylott and Bruce 2009).

While plants are limited in their ability to remediate these explosives, bacteria able to degrade RDX and detoxify TNT have been isolated from explosives-contaminated soils, and the genes responsible characterized: a fused flavodoxin-cytochrome P450 XplA and associated flavodoxin reductase XplB from *Rhodococcus rhodocous* strain 11Y confer aerobic biodegradation of RDX (Seth-Smith et al. 2002) and from *Enterobacter cloacae*, *nfsI* encodes a nitroreductase (NR), which transforms TNT, favoring the production of the 4-ADNT isomer (Hannink et al. 2001). Numerous species of bacteria containing *xplA* and *xplB* have since been isolated from RDX-contaminated locations around the world (Seth-Smith et al. 2008; Rylott et al. 2011b; Andeer et al. 2009; Andeer et al. 2013; Bernstein et al. 2011; Thompson et al. 2005; Binks et al. 1995; Coleman 1998; Adrian and Arnett 2004). Yet despite the presence of RDX-degrading bacteria, RDX contamination in these regions persists, suggesting that other limitations are preventing bacterial degradation of RDX.

To increase the ability of plants to degrade RDX, *xplA* and *xplB* have been transformed into a range of plant species. The co-pollutant TNT is phytotoxic, and also inhibits XplA activity (Jackson et al. 2007). To overcome these hindering factors, the TNT detoxifying nitroreductase has been expressed in tobacco (*Nicotiana tabaccum*; Hannink et al. 2001), the RDX-metabolizing *xplA* and *xplB* in Arabidopsis (*Arabidopsis thaliana*; Rylott et al. 2006); and all three genes have since been simultaneously expressed

in Arabidopsis (Rylott et al. 2011a), and creeping bentgrass (*Agrostis stolonifera*; Zhang et al. 2017a). These transgenic plants were all able to degrade RDX and resist high levels of TNT when compared to wild-type plants. In our recent report, *nfsI* transformed into the plastid genome of tobacco conferred enhanced resistance to TNT (Zhang et al. 2017b). However, Arabidopsis, tobacco, and creeping bentgrass are not well-adapted to thriving in the challenging environments found in military training ranges. Switchgrass (*Panicum virgatum*), is a United States native species, with additional attributes that make it suitable for phytoremediation of explosives on military training ranges, and recently plants have been transformed with *xplA*, *xplB* and *nsfI* and shown to remediate RDX (Zhang et al. 2017a). However, since the range of the lowland switchgrass species is limited, and planting a single species in the field is likely to result in vulnerability to pests, diseases and other environmental stresses; additional grass species are needed. Western wheatgrass (*Pascopyrum smithii*) is a long-lived, cool season, United States native species that is broadly distributed across North America, with characteristics that make it a desirable plant for the phytoremediation of RDX and TNT on live-fire training ranges. Its low maintenance requirements and vigorously spreading rhizomes give it the ability to recover quickly after damage caused by military vehicle traffic, explosive fire or the range fires that occur on training ranges (Palazzo et al. 2005). Furthermore, cultivars, which have improved performance over existing commercially available cultivars, have been developed by the US military specifically for the re-vegetation of ranges including western wheatgrass (Palazzo et al. 2005). However, until now wheatgrass species have proven recalcitrant to transformation. This study uses *xplA*, *xplB*, and *nfsI* to demonstrate an effective transformation protocol, and validate the ability of this species to remediate RDX and TNT.

MATERIALS AND METHODS

Plasmid construction

The binary vector pEDLZ2014 (Figure 1) was constructed using a protocol similar to that used to transform switchgrass, prcs2-NABNR (Zhang et al. 2017a) with the following modifications. The *Os*act sequence was amplified from pANIC 5A (Mann et al. 2012) and used to replace the 35S promoter in

pSAT4a (Tzfira et al. 2005) to produce pNSAT4a. The *xplA, xplB* genes (GenBank accession number AF449421 for both), and *nfsI* gene (M63808) were cloned by PCR from the vectors pMLBart-*xplA*, pART27-*xplB*, and pART27-*nfsI* (Rylott et al. 2011a) and inserted into pNSAT3a, pNSAT4a, and pNSAT6a, respectively, to produce pNSAT3a-*xplA*, pNSAT4a-*nfsI*, and pNSAT6a-*xplB* (Zhang et al. 2017a). The hygromycin resistance gene, hygromycin B phosphotransferase (*hpt*), was cloned by PCR from pCambia1301 and inserted into pNSAT1a to produce pNSAT1a-*hpt*. The expression cassettes of *Os*Actin-*hpt*-35s T, *Zm*Ubi-*xplA*-mas T, *Os*Actin-*nfsI*-35S T, and *Pv*Ubi-*xplB*-rbc T were sequentially inserted into the pRCS2 binary vector at these respective restriction sites *Asc*I-*Asc*I, I-*Ppo*I-I-*Ppo*I, I-*Sce*I-I-*Sce*I, PI-*Psp*I-PI-*Psp*I to produce pEDLZ2014. All the primers used in this research are listed in Supplement Table 1 and the sequences of all the elements in the vector were listed in Supplement Table 2.

Culture condition for plant materials

Western wheatgrass plants (including callus regeneration, RDX and TNT uptake experiments) were cultured at 25°C under 50 μE m^{-2} sec^{-1} constant light with a 16 h day/8 h night photoperiod.

Transformation of western wheatgrass

The grain husks of western wheatgrass were removed and the seeds surface-sterilized in 10 % bleach for 30 min, then rinsed four times with sterile water. Sterile seeds were cultured on callus induction (CI) medium (Murashige and Skoog's (MS) basic medium (Murashige and Skoog 1962) with 0.5 g/L proline, 0.5 g/L N-Z amine, 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L 6-benzylaminopurine (6-BAP), 30 g/L sucrose, pH 5.8) in the dark for embryogenic callus induction. The embryogenic calli were sub-cultured onto CI medium at one-month intervals.

A biolistic particle system (Bio-Rad PDS-1000) was used for delivery of pEDLZ2014 with 7,584 kPa (1,100 psi) rupture disks, a microcarrier flight distance of 9 cm and a vacuum of 97 kPa (27 in) Hg, with all hardware and reagents produced by Bio-Rad. Microprojectile preparation was as described earlier

(Lutz et al. 2006). The embryogenic calli were incubated on osmotic medium (CI medium with 36.4 g/L sorbitol and 36.4 g/L mannitol) for 6 h prior to bombardment. After bombardment, the calli were keep in the dark overnight on osmotic medium, then transferred to CI medium for two days and then to CI medium with 75 mg/L hygromycin. After two months of growth, hygromycin-resistant calli were transferred to fresh selective CI medium for further selection and callus propagation. After three more months of growth, the hygromycin resistant calli were transferred to regeneration medium (MS medium with 1 mg/L 6-BAP, 0.1 mg/L 1-naphthaleneacetic acid (NAA), 30 g/L sucrose, pH 5.8) with 75 mg/L hygromycin. Regenerated plantlets were transferred to MS medium containing 50 mg/L hygromycin, then rooted plantlets transferred to shoot induction medium (MS medium with 2 mg/L 6-BAP, 0.2 mg/L NAA, 30 g/L sucrose, pH 5.8) with 50 mg/L hygromycin for cluster shoots induction. After two months, new branches in cluster shoots were excised and transferred to fresh MS medium with hygromycin at 50 mg/L to promote growth and rooting. The rhizomes were cut into sections with a node on each fragment and cultured on MS medium for new plantlet development. One week later, new shoots and roots developed from nodes of rhizomes fragments. Healthy plants from the cluster shoots or rhizomes were selected and transferred to soil.

Transgene expression analysis

DNA was extracted using DNeasy plant mini kits (Qiagen, Valencia, CA, USA), and PCR reactions performed using the primers listed in Table S1. For transcript analysis, mRNA was extracted from mature western wheatgrass leaf blades using RNeasy plant mini kits (Qiagen). One microgram of total RNA was treated with DNase I (Invitrogen) and used to synthesize cDNA with random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) was performed using a SensiFast SYBR No-ROX kit (Bioline). Data from RT-qPCR were normalized to the western wheatgrass 5.8S gene (GenBank: GU557082.1). Protein analysis used SDS-PAGE with 8 μg of crude protein per sample, and antibodies were used as reported previously, XplA, (Rylott et al. 2006); XplB, (Jackson et al. 2007) and nitroreductase (Rylott et al. 2011a).

RDX uptake studies

Wild-type and transformed plants of comparable biomass and developmental status were selected and cultured in 5 mL ½ MS media with 30 mg/L RDX for 12 days. Levels of RDX in the medium were assayed at regular time intervals, with volumes of medium refilled back to 5 mL with tap water each time before sampling. After 12 days, the RDX concentrations in the plant tissues were determined.

TNT uptake studies

Mature leaf blades were cut into sections about 1.5 cm length and cultured in 20 mL water containing 12.5, and 32.5 mg/L TNT. The starting biomass in each bottle was 0.572 ± 0.003 g. Samples of medium were removed at regular intervals for analysis. After 66 hours, the concentrations of TNT and 4-ADNT in the plant tissues were determined.

Extraction of RDX, TNT, and 4-ADNT from plant tissue

Plant leaf tissue (100 mg) was freeze dried and ground to a powder using a Fast Prep 24 (MP Biomedicals, LLC. Solon, USA). RDX, TNT and ADNT were extracted from leaf materials using 1 mL methanol with shaking for 12 hours, then three repeat extractions with shaking for two hours. The combined extraction products were dried using an Eppendorf Vacufuge 5301, re-dissolved in 1.5 mL methanol, centrifuged at 13,000 for ten minutes and the supernatant (1000 µL) analyzed by HPLC.

Determination of RDX, TNT and ADNT levels

RDX, TNT and ADNT concentrations were analyzed using 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 described 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.

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. Groupings differing by statistical significance are labeled by letters in the figures.

RESULTS

Creation of transgenic western wheatgrass

Following results from the expression of *xplA*, *xplB*, and *nfsI* in switchgrass and creeping bentgrass, the vector used in our former report (Zhang et al. 2017a), was modified to improve transgene expression. The 35S promoter driving expression of *nfsI* was replaced with the rice (*Oryza sativa*) actin promoter (*Os*act); and the switchgrass ubiquitin promoter (*Pv*ubi) driving *xplA* expression was replaced with the maize (*Zea mays*) ubiquitin promoter (*Zm*Ubi). The structure of the resulting vector, pEDLZ2014, is shown in Figure 1.

To successfully transform western wheatgrass, vigorously-growing embryogenic calli were needed. Figure 2a shows the white or light-yellow, friable embryogenic calli obtained after 1-2 months of growth. Following additional culturing, calli suitable for transformation were obtained (Figure 2b) and bombarded with pEDLZ2014 vector DNA. Figure 2c shows the appearance of the surviving calli two months after bombardment; after three to four months, green and healthy plantlets were obtained (Figure 2d). In total, 10 plates of embryogenic calli were bombarded, with 98 calli showing hygromycin resistance and of these, 30 produced green and healthy plantlets (Figure 2e). To produce biomass for subsequent analysis, the plantlets were propagated on cluster shoot induction medium which initiated the formation of robust shoots and rhizomes as shown in Figure 2f. The rhizomes were cut into nodal sections, as shown in Figure 2g, and cultured to regenerate new plantlets. Figure 2h shows the appearance of the fully regenerated plants in soil.

To confirm the integration and expression of the transgenes in the hygromycin-resistant plantlets, PCR and qRT-PCR analysis were conducted on thirteen, independently-transformed lines. The results, presented in Figure 3a, show expression of *xplA*, *xplB* and *nfsI* in the transgenic lines, while the transgenes were not detectable in the wild-type, untransformed plants. Expression of the transgenes was highest in lines S35, N1, N9 and D22 and subsequent western blot analysis (Figure 3b) of these lines revealed the presence of 60, 45 and 24 kDa bands, which corresponded in size to the XplA, XplB and NR proteins, respectively.

RDX uptake studies

To determine the rate of RDX uptake, the plants were grown in liquid MS medium in open test tubes. Evaporation of RDX from the medium was minimal due to its low volatility (Xiong et al. 2009). Over the course of the experiment, all the transformed lines removed RDX from the medium faster than the wild-type plants (Figure 4a). After 3 days, the RDX concentration in the medium containing transformed plants was significantly lower than that of wild-type plants ($p = 2x10^{-6}$). After 6 days, the difference between wild-type and transformed lines further increased with lines N9 and D22 removing significantly more RDX than lines S35 and D45 ($p = 0.0003$). After 12 days of culture, 65 % of the RDX had been removed by wild-type plants while 86 % and 87 % by line N9 and D22 respectively. After 12 days, wild-type RDX leaf tissue levels were found to be 0.12 ± 0.03 mg/g RDX, in comparison, RDX was not detected in tissue from transformed lines tested, N9, D22, and S35, and RDX was at 0.033±0.001 mg/g in transformed line D45 (Figure 4b). To confirm that the lack of accumulation of RDX in transformed tissue was caused by degradation, rather than by dilution in growing plant tissues, leaf blades of wild-type and lines, N9 and D22 were cultured in water with 20 mg/L or 40 mg/L RDX. Figure 4c shows that while the concentration of RDX in the wild-type leaves increased from day 2 to day 8, no RDX was detected in the transformed line D22. Although RDX was detected in leaf tissue of transformed line N9, it was 28 % lower than that in wild-type ($p = 0.004$) at day 2 when cultured in RDX solution at 20 mg/L, and 53% lower ($p = 0.033$) at day 2, with 40 RDX mg/L. In contrast to wild-type, the RDX concentration in line N9 decreased after 2

230 days of culture and could not be detected on day 8. This result confirmed that both N9 and D22 are able to
231 degrade RDX.

232 **TNT detoxification studies**

233 Though the main target of this research was to engineer grasses for RDX degradation, the grasses also
234 need to be able to resist the phytotoxicity of TNT since training ranges are contaminated by both RDX
235 and TNT, and TNT can also inhibit XplA activity (Jackson et al. 2007). Thus the *xplA-xplB-nfsI*
236 transformed lines N9, and D22, which had the highest RDX degradation abilities, were tested for their
237 resistance to TNT conferred by NR activity. When leaf sections were cultured in water containing 15
238 mg/L TNT, the transformed lines removed TNT more quickly than wild-type plants ($p = 0.0019$ at 18 hr
239 incubation). Line D22 removed TNT most rapidly, with TNT undetectable in the solution after 48 hr.
240 After 66 hr, nearly all TNT had disappeared from solution from wild-type and transformed plants (Figure
241 5a). Though wild-type western wheatgrass removed TNT from the solution, when compared to the
242 transgenic lines after 66 hr, the wild-type leaf sections exhibited symptoms of toxicity, with overall
243 yellowing of the leaf surfaces and darkening of the cut edges (Supplement Figure 1a). After 66 hours of
244 culture in water containing 32 mg/L TNT, the wild-type plants were unable to remove any TNT, while the
245 transformed lines removed nearly all the TNT (0.38 ± 0.16 mg/L remaining for N9 and 0.51 ± 0.35 for
246 D22; Figure 5b). Furthermore, the wild-type plants showed signs of toxicity, as indicated by a reduction
247 in growth rate such that the biomass gain of wild-type was 24 % and 30 % of that of N9 and D22,
248 respectively after 3 days (Supplement Figure 1b).

249 While in whole plants, TNT and transformation products are localized predominantly in the roots, when
250 grown in liquid culture, leaf tissues are known to also contain these products (Hannink et al. 2007;
251 Hannink et al. 2001). To verify that TNT was transformed in the tissues of the modified plants, TNT and
252 ADNT levels, in leaf tissue were determined after 42 hours culture in TNT solution (Figure 5c). When
253 cultured in 12.5 mg/L TNT solution, TNT was not detected in the tissues of either wild-type or
254 transformed plants, while ADNT was detected in wild-type leaf tissue at a level lower than for N9 and

D22 ($p = 0.0188$). When cultured in 25 mg/L TNT solution for 42 hours, the wild-type leaf tissue accumulated 11.91±1.64 µg/g TNT, about 3 times of that in lines N9 and D22 ($p = 0.0004$). At the same time, the ADNT concentration in the wild-type plants was 6.01 ± 0.92 µg/g, about 60 % of that in leaf tissue of transformed plants ($p = 0.0012$). These results demonstrated that though wild-type plants have the ability to uptake and detoxify limited amounts of TNT, the *nfsI*-transformed western wheatgrass are able to detoxify significantly more TNT, resulting in increased tolerance, and subsequent ability to take up overall more TNT.

DISCUSSION

On live-fire training ranges, deposition of RDX and TNT onto soils around targets is ongoing and thus a continuing source of soil and groundwater contamination. Phytoremediation could be a relatively low-cost method to contain and clean-up these sites but many plant species grow poorly in RDX and TNT contaminated soil (Vila et al. 2007; Via and Zinnert 2016; Panz and Miksch 2012; Via et al. 2015) and are not able to degrade significant amounts of RDX (Best et al. 1999; Yoon et al. 2006; Best et al. 2006).

Studies in model plant species have demonstrated that genetically modifying plants with *xplA*, *xplB* and *nfsI* conferred the ability to degrade RDX and detoxify TNT (Hannink et al. 2007; Rylott et al. 2011a). The subsequent development of genetically modified switchgrass and creeping bentgrass confirmed that this system for RDX degradation worked efficiently in monocot grasses (Zhang et al. 2017a). However, expression of the *nfsI* gene, under the control of the 35S promoter, in these lines was poor, and accordingly, the transformed switchgrass did not show enhanced resistance to TNT. Expression of the 35S promoter has been reported to be lower in monocots (Jang et al. 2002; Christensen et al. 1992). In this research, the 35S promoter driving the expression of *nfsI* was replaced with the monocot *Os*Act promoter. The resulting western wheatgrass plants showed both enhanced RDX degradation and enhanced TNT detoxification.

Western wheatgrass has many desirable attributes that make it suitable for deployment on training ranges. It is native to the US, growing across a broad geographical location with includes military lands (USDA, 2002). Furthermore, its robust leaves and rhizomes make it able to withstand stresses associated with military ranges such as fire, military vehicular traffic, and nutrient poor soils. Some of these attributes also make western wheatgrass valuable as a forage grass, so advances in its genetic modification are important in agriculture. Here we describe a method for reliably transforming western wheatgrass. This method could also be adapted to other forage grasses, such as slender wheatgrass (*Elymus trachycaulus*) and Siberian wheatgrass (*Agropyron fragile*). In our experience, a prerequisite for successful development of transformed callus is the selection of the friable white callus morphology (Figure 2b).

In conclusion, this is the first report on the genetic transformation of western wheatgrass. This protocol will contribute to wider plant biotechnology uses, here, we utilize it to show the efficacy of *xplA*, *xplB*, *nfsI*-expressing plants to degrade RDX and detoxify TNT. These species neatly complement transgenic switchgrass lines, enabling this technology to be used across a broader range of environmental, and geographic conditions for the containment and detoxification of explosives on military training ranges.

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443

444 **Legends**

445 **Figure 1** Construction of vector pEDLZ2014 for transformation of western wheatgrass.

446 T-DNA region of the binary vector plasmid pEDLZ2014 used to transform western wheatgrass. The RDX
447 degradation gene *xplA*, flavodoxin reductase gene *xplB*, and TNT detoxifying nitroreductase gene *nfsI*
448 were constructed into versatile cloning vector pNSATs. Arrows show the direction of transcription.

Abbreviations: *Os*act, *Oryza sativa* actin promoter; *hpt*, selection marker gene, *hygromycin B phosphotransferase* (*hpt*); 35s T, terminator of CaMV 35s gene; *Zm*ubi, *Zea mays* ubiquitin promoter; mas T, terminator of *manopine synthase* gene of Agrobacterium; *Pv*ubi, *Panicum virgatum* (switchgrass) ubiquitin promoter; rbc T, terminator of rubisco small subunit of tobacco; RB left border; RB right border

Figure 2 Tissue culture, transformation and propagation of western wheatgrass.
(**a**) Two month old calli induced from mature seeds. (**b**) Embryogenic calli that were used for bombardment with vector DNA. (**c**) Calli screened on Callus Induction medium with 75 mg/L hygromycin for two months after bombardment. (**d**) Hygromycin resistant plantlets from calli that were transferred to regeneration medium for 1-2 month. (**e**) Regenerated plantlets growing on MS medium with 75 mg/L hygromycin. (**f**) PCR and qRT-PCR positive plants were transferred to cluster shoot induction medium to induce cluster shoots and rhizomes. (**g**) Shoot induced on rhizomes sections with nods on MS medium. (**h**) Transgenic plants growing in soil.

Figure 3 Molecular characterization of *xplA-xplB-nfsI* transformed western wheatgrass.
a) Transcript abundance measured using quantitative RT- PCR on plant lines transformed with *xplA*, *xplB* and *nfsI*. Values were normalized to the 5.8s rRNA gene and relative to expression level of the transformed line D22. Data are the means ± SE, n = 3.
b) Western blot analysis on leaf blades of western wheatgrass lines expressing XplA, XplB and nitroreductase (NR) protein. WT, wild type; PC, positive control, *xplA-xplB-nfsI* transformed Arabidopsis (Rylott et al. 2011a).

Figure 4 Uptake and degradation of RDX by *xplA-xplB-nfsI* transformed western wheatgrass. (**a**) Concentration of RDX in culture medium over the course of the experiment. (**b**) Concentration of RDX in plant tissue after 12 days culture. (**c**) Accumulation of RDX in leaf tissue during 8 days culture in 20 mg/L

475 and 40 mg/L RDX solution. Letters indicate that RDX concentration in tissue were significantly different
476 ($p<0.05$) from other lines. Data are the means ± SE, n = 3; ND = None Detected; NPC, No Plant Control
477
478 **Figure 5** Uptake of TNT *by xplA-xplB-nfsI* transformed western wheatgrass. Plants were grown in water
479 with TNT at (A) 15 mg/L, and (b) 32 mg/L. (C) The concentration of TNT and 4-ADNT in the leaf tissue
480 after 42 hours culture in 12.5 and 25 mg/L TNT solution. The lowercase letters and uppercase letters
481 indicate that TNT and 4-ADNT concentration in tissue were significantly different from other lines at
482 0.05 or 0.01 level (ND = None Detected). Data are the means ± SE, n = 3.
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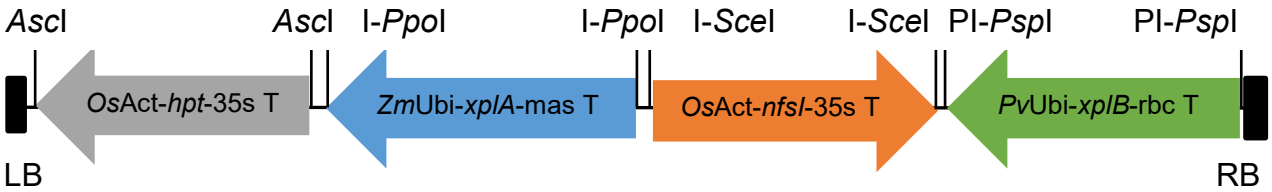

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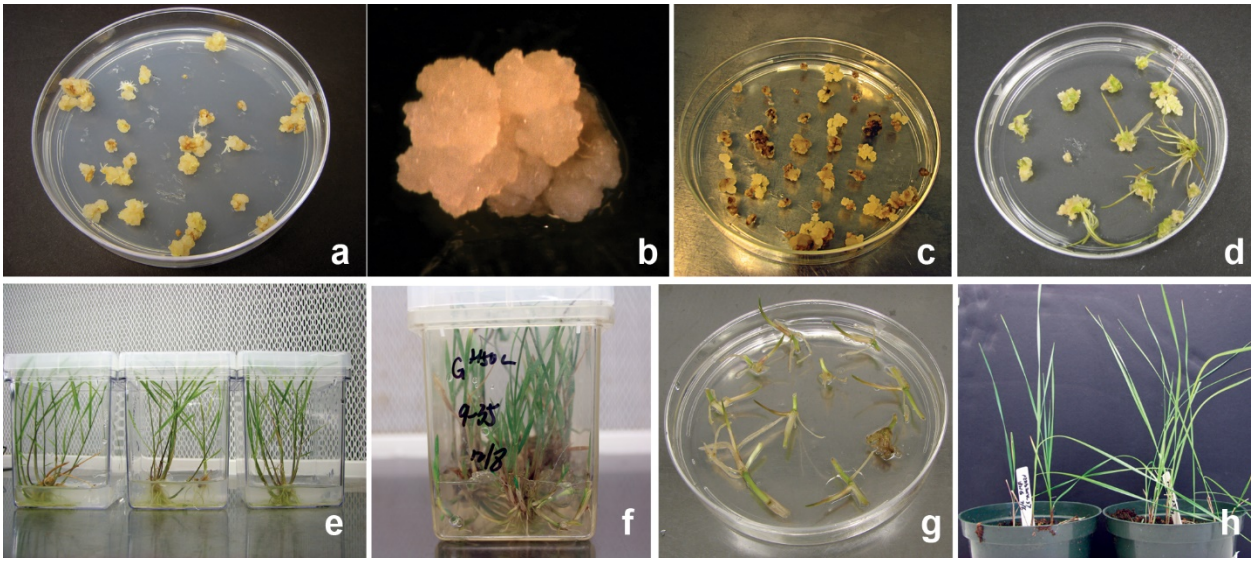

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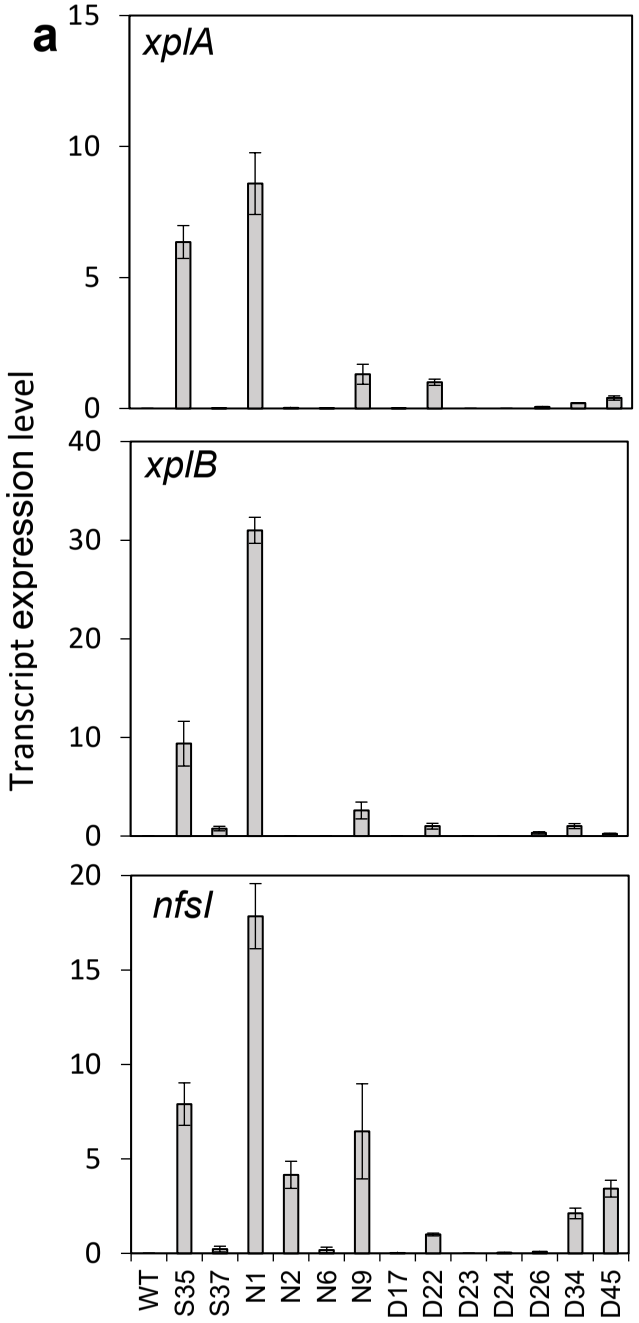
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xplA
xplB
nfsI
Transcript expression level
0
5
10
15
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30
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WT
S35
S37
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N6
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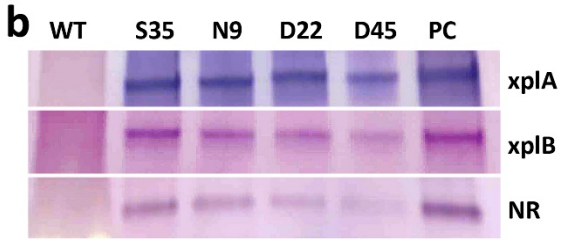

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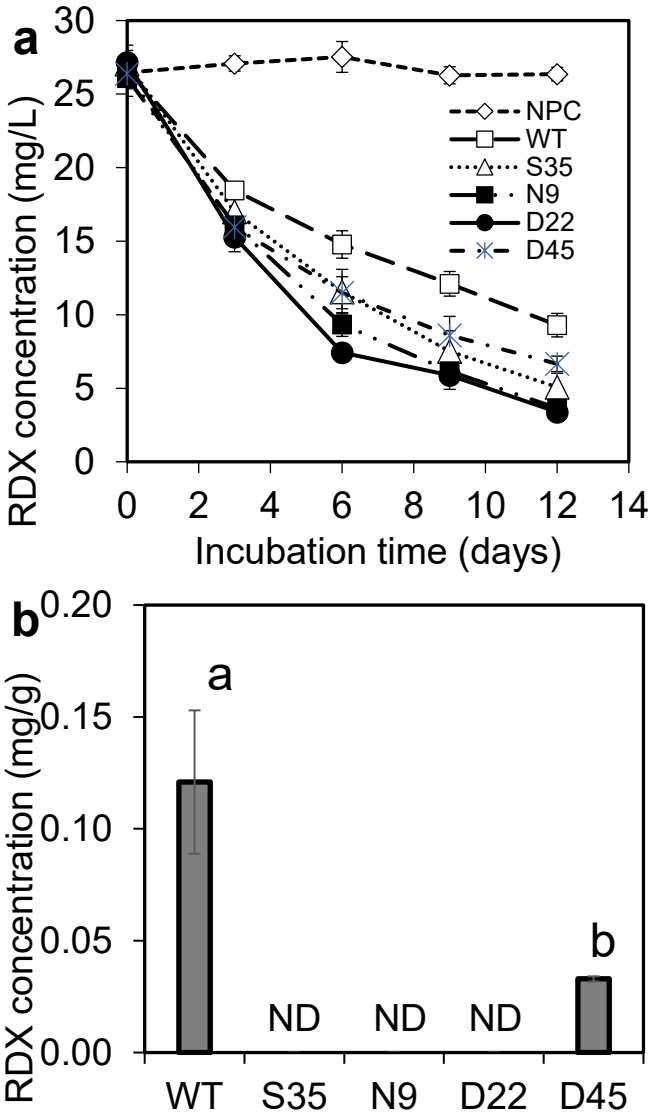
a
RDX concentration (mg/L)
Incubation time (days)
NPC
WT
S35
N9
D22
D45
b
RDX concentration (mg/g)
a
b
ND
ND
ND
WT
S35
N9
D22
D45

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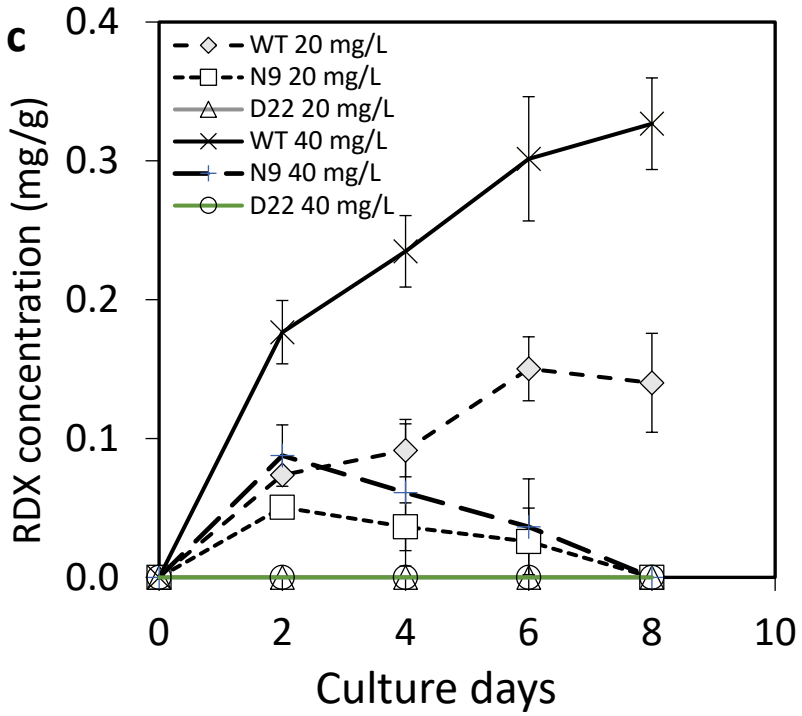

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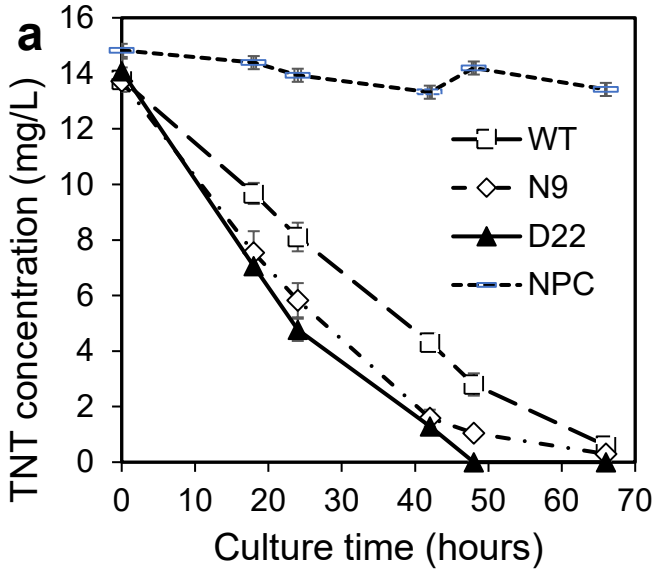

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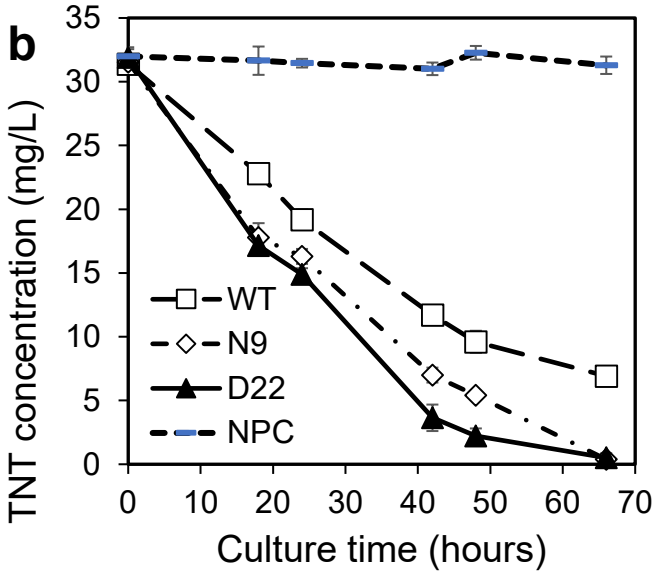

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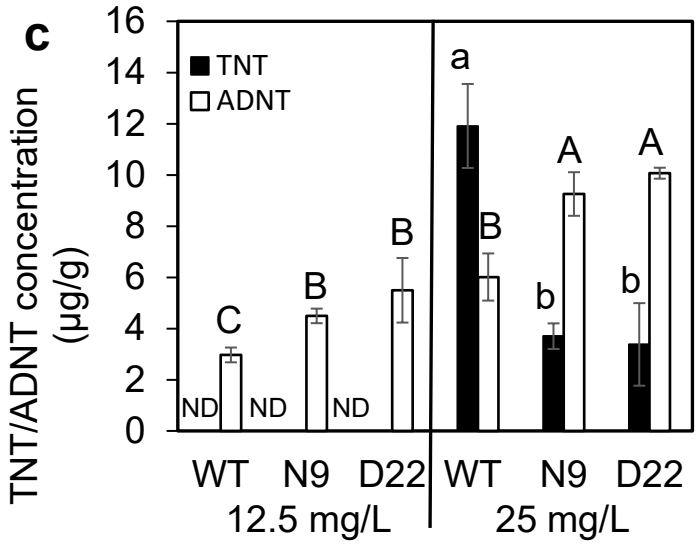

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