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Degradation of trinitrotoluene by transgenic nitroreductase in *Arabidopsis* plants

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ABSTRACT

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The explosive 2,4,6-trinitrotoluene (TNT) is a highly toxic and persistent environmental pollutant. TNT is toxic to many organisms, it is known to be a potential human carcinogen, and is persistent in the environment. This study presents a system of phytoremediation by *Arabidopsis* plants developed on the basis of overexpression of NAD(P)H-flavin nitroreductase (NFSB) from the *Sulfurimonas denitrificans* DSM1251. The resulting transgenic *Arabidopsis* plants demonstrated significantly enhanced TNT tolerance and a strikingly higher capacity to remove TNT from their media. The highest specific rate constant of TNT disappearance rate was 1.219 and 2.297 mL/g fresh weight/h for wild type and transgenic plants, respectively. Meanwhile, the nitroreductase activity in transgenic plant was higher than wild type plant. All this indicates that transgenic plants show significantly enhanced tolerances to TNT; transgenic plants also exhibit strikingly higher capabilities of removing TNT from their media and high efficiencies of transformation.

Keywords: nitroaromatic compound; biodegradation; contamination; phytotoxicity; fluorescence

The 2,4,6-trinitrotoluene (TNT) explosive is one of the most important synthetic nitroaromatic compounds and its industrial production is often linked to military and civilian purposes (Ayoub et al. 2010). Extensive TNT usage resulted in some contamination of our soil and groundwater and the evidence about high levels of TNT in environment were previously reported (Spain et al. 2000, Pennington and Brannon 2002). The presence of TNT and its metabolites in the environment has been associated with mutagen and carcinogen to human (Nipper et al. 2001, Robidoux et al. 2002), therefore, it is essential to degrade or remove TNT from the contaminated environment.

Phytoremediation is a solar energy-driven, cost-effective and clean up technique with green plants for *in situ* removal, degradation or containment of contaminants in soils, sludge's, sediments, surface water and groundwater (Chatterjee et al. 2013). French et al. (1999) reported that seeds of the transgenic tobacco introduced a bacterial enzyme pentaerythritoltetranitrate (PETN) reductase and were able to germinate and grow in the 0.05 mmol TNT solution while wild type plants were inhibited in the same condition. Transgenic aspen tolerated up to 57 mg TNT/L in hydroponic media and more than 1000 mg TNT/kg soil, whereas the parental aspen could not endure in hydroponic culture with more than 11 mg TNT/L or soil with more than 500 mg TNT/kg (Van et al. 2008). Phytotoxicity is caused by reduction of TNT in the mitochondria, forming a nitro radical that reacts with atmospheric

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oxygen, generating reactive superoxide (Johnston et al. 2015). Recently, Zhang et al. (2017a) first reported TNT detoxification with transplastomic plants and expressed *nfsI* in the plastids of tobacco and examined the response of the plants to TNT. Moreover, they also focused on the expression of *xplA*, *xplB* and *nfsI* in switchgrass and creeping bentgrass for the phytoremediation of hexahydro-1,3,5-trinitro-1,3,5-trizaine (RDX) and TNT in soils (Zhang et al. 2017b).

In our previous paper, a system of TNT phytoremediation was developed by overexpressing old yellow enzyme from *Saccharomyces cerevisiae* (*OYE3*). The results showed that transgenic *Arabidopsis* plants enhanced TNT tolerance and removed TNT from their media (Zhu et al. 2012). In this report, an NFSB enzyme which is capable of decolorizing TNT from the *Sulfurimonas denitrificans* DSM1251 (GenBank ID: YP_393671.1) was synthesized through successive polymerase chain reaction. The objective of this study is to determine whether transgenic plants can degrade TNT from the environment more effectively than wild type plants and to obtain more genes that can effectively degrade TNT. *Arabidopsis* responds to TNT and this mechanism may be used as a basis to develop transgenic plants with enhanced tolerance and faster removal rates in heavily TNT-contaminated sites. Our study provided an alternative approach to TNT degradation.

MATERIAL AND METHODS

The acquisition of transgenic plants. According to the amino acid sequence, the *NFSBI* gene was synthesized by successive polymerase chain reaction (Xiong et al. 2006). The method of construction of plant expression vector and plant transformation were the same to our previous study (Zhu et al. 2012).

PCR analysis. Reverse transcription PCR (RT-PCR) and quantity RT-PCR were performed to verify the expression of *SdNFSBI*. A 250 bp fragment was amplified by two specific primers (*SdNFSBI*Z1: 5'-TGT GAT GAC AAC ATC TAC TTC-3'; *SdNFSBI*F1: 5'-GTC GAT GAA CTC GAC AAC TTC-3') according to the sequence of the *SdNFSBI* gene. The internal standard gene and PCR reaction were the same to Zhu et al. (2012).

The toxicity experiments in plates. Wild type and transgenic seeds were surface sterilized with bleaching powder (5%, w/v) for 20 min, washed with sterile water three times, and placed in Petri dishes that contained the Murashige and Skoog (MS) medium with 2% sucrose, 0.8% agar and MS complete medium plates contained 0, 0.025, 0.050 and 0.075 mmol TNT, respectively. The seeds were then cold-stratified at 4°C for 3 days and incubated vertically under long-day conditions (16/8 h photoperiod day and night 23 ± 1°C) for 2 weeks.

The damage of TNT on the growth of wild type and transgenic plant. One hundred seeds of wild type and transgenic lines were placed on the MS medium with 0, 0.05, 0.10 or 0.15 mmol TNT predissolved in acetone. The survival rate was scored after seedlings grown for 10 days and then transferred to sterile flasks with 20 mL MS liquid medium, recovered for 48 h and transferred aseptically to 20 mL conical flasks in sterile MS liquid medium with the same concentration TNT as in the previous experiment. The wet weight of seedlings was then determined for further 14 days. Four-week-old plants in soil were soaked with 0, 0.25, 0.50 and 0.75 mmol TNT for a week. Chlorophyll was extracted from leaves with 95% ethanol and its content was determined spectrophotometrically at 470, 649 and 665 nm. Photochemical efficiency of photosystem II (PS II) determined by variable fluorescence (F_v)/maximum fluorescence (F_m) and chlorophyll II fluorescence emission from the upper surface of the leaves was measured with a Model PAM-2100 plant efficiency analyser (Heinz Walz GmbH, Effreltrich, Germany). The F_v and F_m parameters were determined after 30 min in dark, and the light-adapted values (F_v' and F_m') were measured after 30 min of illumination with 500 μmol/m^2/s.

Rate constant of transgenic plants for TNT uptake. All plants were grown in a growth room (16 h light: 8 h dark, 120 μmol photons/m^2/s light intensity). TNT uptake by the 30-day old transgenic and wild-type *Arabidopsis thaliana* plants was modelled by a 15 mL hydroponic system containing 0, 0.05, 0.10, 0.15 mmol TNT, respectively and the plants were weighed for the fresh weight (FW). In the liquid medium, TNT concentration decreased with time, which was described by the equation during the hydroponics:

$$C_{TNT}(t) = C_{(TNT,0)}e^{-kX_{FW}t}$$

Where: C_{TNT} – TNT concentration (mg/L) in the liquid medium; $C_{(TNT,0)}$ – initial concentration of TNT in the liquid medium (mg/L); *e* – constant, e = 2.718 ... and *t* rep-

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resents time (h) of the transgenic and WT plants exposed to the liquid medium containing TNT; X_{FW} – *A. thaliana* biomass density of the medium suspension (g FW/mL) and *k* – rate constant for TNT uptake (mL/g FW/h) and it varies with the TNT concentration and degradation time. Values for *k* were estimated from the least-squares slope of $\ln(C_{TNT})$ vs. time data.

The nitroreductase activity determination. The nitroreductase activity was measured according to the Villanueva's method (1964) and the leaves from four-week-old plants were used as the material. The difference with Villanueva's is that the reaction solution contained 0.18 mmol ρ-dinitrobenzene, 0.5 mmol L-cysteine, 1.08 mmol $NADH_2$, 50 μL enzyme preparation and 0.067 mol/L phosphate buffer pH 7.6 up to 200 μL.

Relative TNT degradation for plants *in vivo*. The leaves (0.1 g) from 4-week-old transgenic and wild type plants were used for the extraction of crude protein. The reaction mixtures of activity assay *in vitro* (100 μL) contained 50 mmol Tris-HCl (pH 7.4), 0.02 mmol NADH, 0.02 mmol FMN, 0.02 mmol TNT, 50 mg of crude enzyme. The extraction protein and detection method of TNT were also the same to Zhu et al. (2012).

RESULTS AND DISCUSSION

The reduction of the seedlings growth in plates. The 250 bp band was detected in BH222 lines, whereas no signal was detected in wild type plants (Figure 1a). The quantity RT-PCR analysis also revealed that *SdNFSBI* was expressed with the different transcript levels in transgenic lines (Figure 1b). All plants grew at almost the same

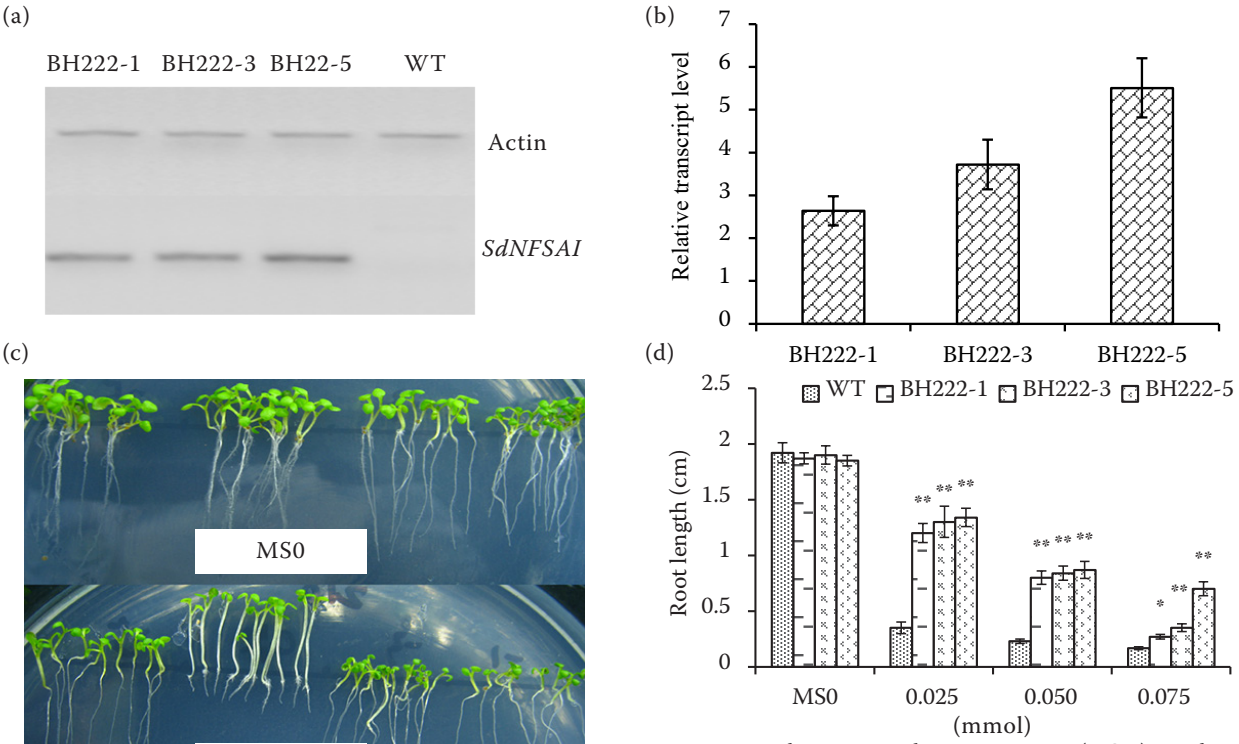

Figure 1. Polymerase chain reaction (PCR) analysis of the *SdNFSBI* gene fragment and enhanced TNT (2,4,6-trinitrotoluene) tolerances of plants on plates (a) RT-PCR analysis of the *SdNFSBI* gene fragment; (b) relative transcript levels analyses of different transgenic *Arabidopsis* lines; (c) the growth of wild type and transgenic plants, and (d) root length of wild type and transgenic plants. Data are the mean ± standard deviation of three replicates. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after the TNT treatment

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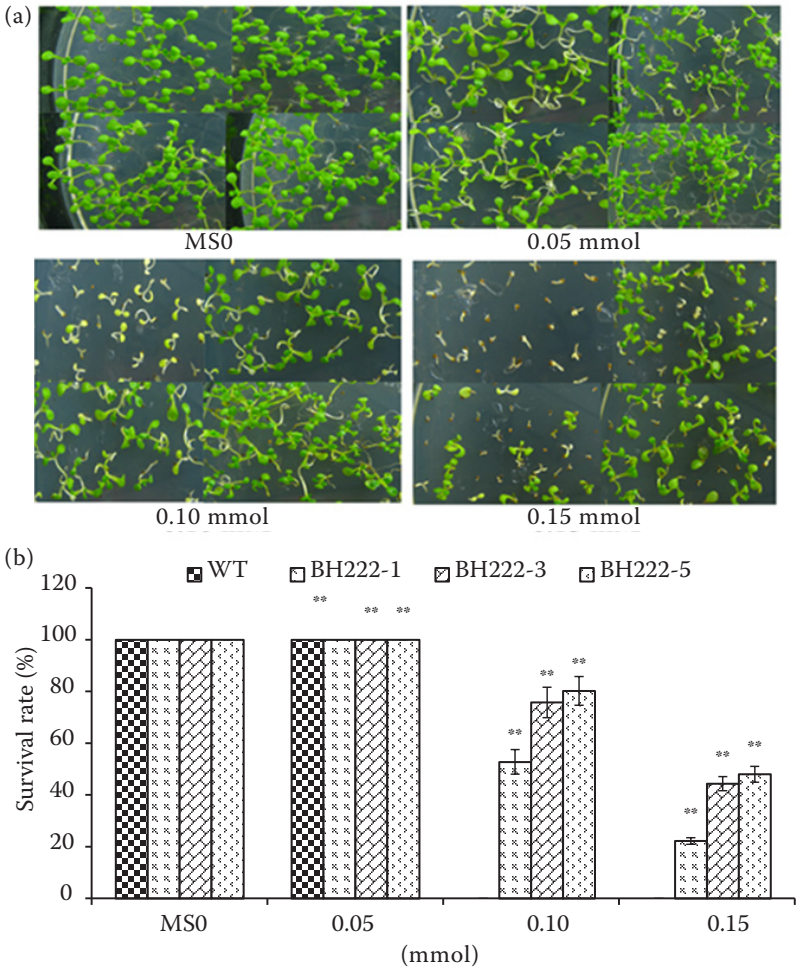
rate in MS medium (Figure 1c). The transgenic seedlings, especially BH222-3 or -5, exhibited a normal extensive root branches in the TNT medium (Figure 1d). The TNT degradation of transgenic plant was better than that of wild type. It was noteworthy that the root growth of wild type was obviously related with their average root length being about 4 times shorter than those of the transgenic plants, but the wild type shoots were longer and stronger than transformed lines (Figure 1c). According to Johnston et al. (2015), TNT pollution in soil negatively affects the growth of *Arabidopsis* roots. Many scholars reported that TNT and its metabolites have an inhibitory effect on plant growth, resulting in the stunting of both root and shoot development (Pavlostathis et al. 1998, French et al. 1999). However, the shoot growth was different from those previously reported on this point.

The presence of TNT significantly inhibited the growth of all plants (Figure 2a). All seedlings exhibited stress symptoms including inhibition germination, leaf loss and colour at 0.1 or 0.15 mmol. However, the TNT damage in wild type plants was more harmful and almost all of them were killed and the survival rate was zero. As opposed to that, many transgenic plants were green and more than 40% plants survived (Figure 2b). Combined with Figure 1, it could be considered that different expression level of *SdNFSBI* maybe induce different degree tolerance of transgenic lines. The degrading effect of transgenic plants on TNT was better than that of wild type plants.

Higher TNT removal efficiencies in transgenic *A. thaliana*. The wild type and BH222-5 seedlings grown appeared healthy and the wet weight was nearly the same without TNT. This indicated that the toxic effects observed were only due to TNT. They showed considerable phytotoxic effects and the root growth was distinctly inhibited and the yellow leaves appeared at 0.05 mmol TNT (Figure 3a). However, BH222-5 showed less phytotoxic effects and the increased growth biomass was 1.48 times higher than in the wild type. At 0.10 mmol TNT,



WT	BH222-3
BH222-1	BH222-5

Figure 2. Seedlings growth of wild type and transgenic plants in different TNT (2,4,6-trinitrotoluene) plates. (a) Effects of TNT on the seedling growth on MS agar plates containing 0, 0.05, 0.10, or 0.15 mmol TNT, and (b) seedlings survival rate above the experiment. Counting the seedlings with leaf as survival seedlings in this experiment. Data are the mean ± standard deviation of three replicates. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after TNT treatment

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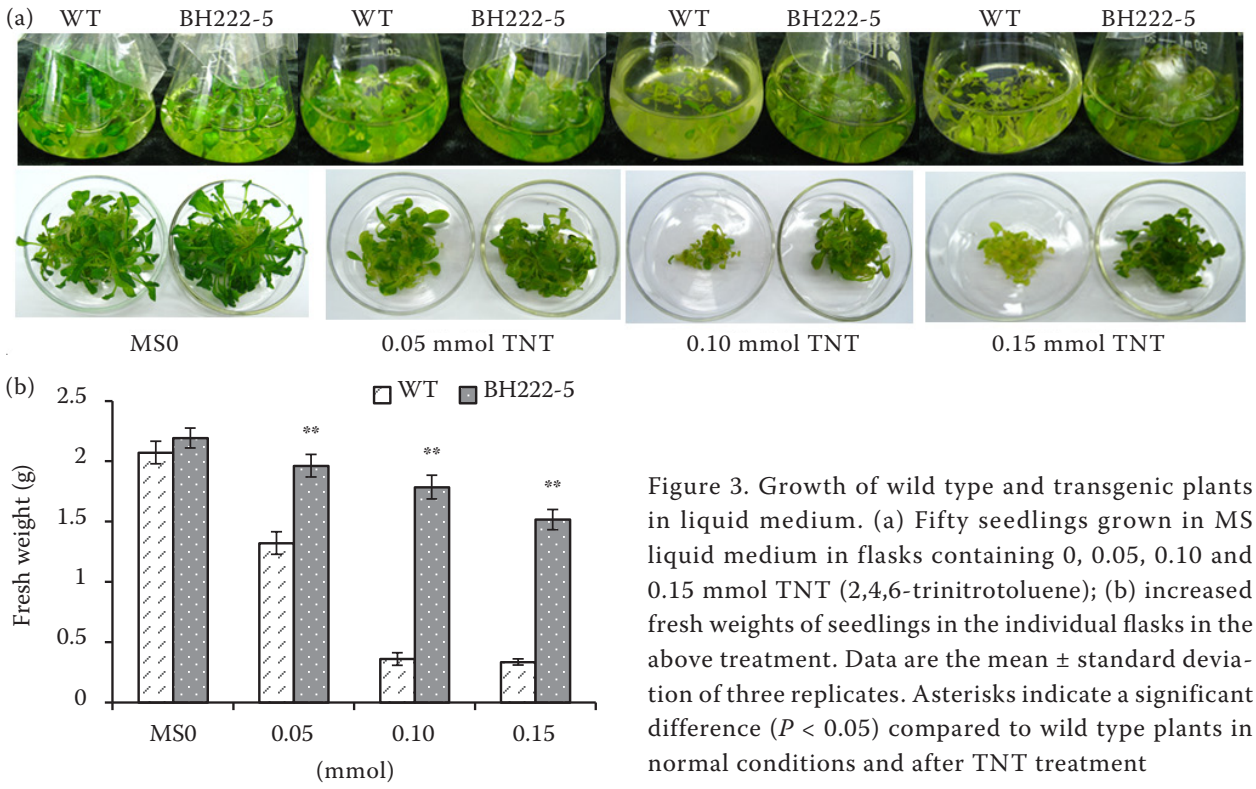

Figure 3. Growth of wild type and transgenic plants in liquid medium. (a) Fifty seedlings grown in MS liquid medium in flasks containing 0, 0.05, 0.10 and 0.15 mmol TNT (2,4,6-trinitrotoluene); (b) increased fresh weights of seedlings in the individual flasks in the above treatment. Data are the mean ± standard deviation of three replicates. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after TNT treatment

the wild type was badly inhibitory with yellow leaves, shorter roots and 0.361 g wet weight. The BH222-5 appeared healthy and gained 1.786 g in wet weight (Figure 3b). The leaves of wild type almost turned yellow but the majority leaves were green for BH222-5 at 0.15 mmol TNT. The biomass of transgenic plants was higher than that of wild plants after the TNT treatment. Over all, the seed germination, shoot and root growth reduction, leaf size and number reduction, and efficient root and shoot formation are crucial indicators of the appraisal of potential applications of phy-

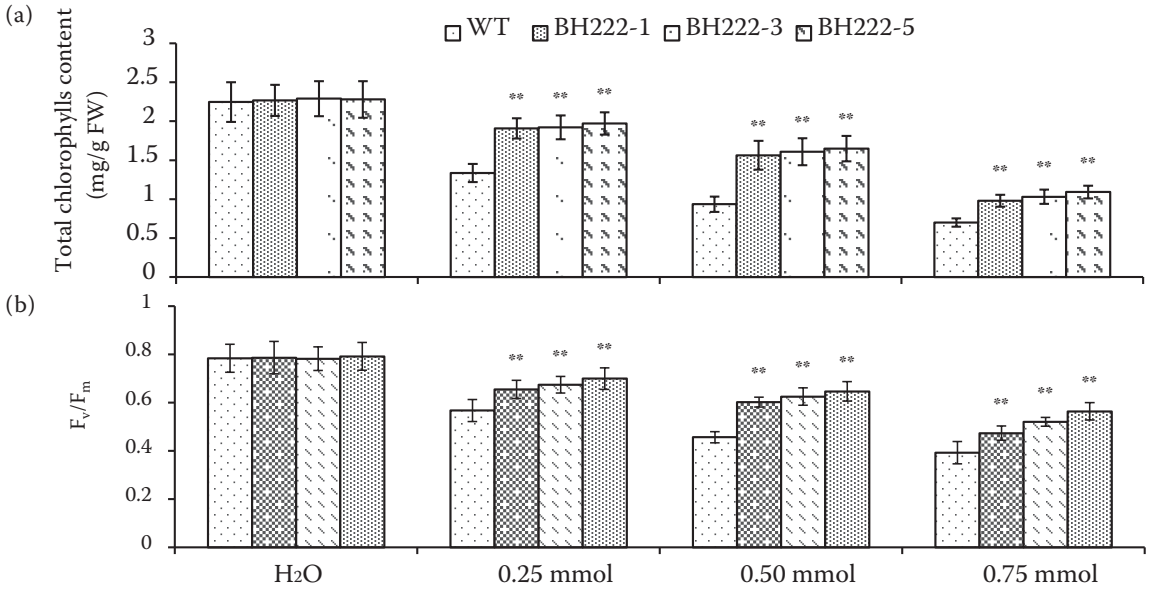

Figure 4. Effect of TNT (2,4,6-trinitrotoluene) on photosynthetic activities. (a) Chlorophyll changes in wild type and transgenic plants; (b) fluorescence (F_v)/maximum fluorescence (F_m) changes in wild type and transgenic plants. Data are the mean ± standard deviation of three replicates. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after TNT treatment. FW – fresh weight

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Table 1. The kinetics of TNT (2,4,6-trinitrotoluene) degradation from liquid medium at three initial TNT concentrations

Initial TNT concentration (mmol)	Plant	Plant normalized rate constant at different incubation time, k (mL/g FW/h)					
		24 (h)	48 (h)	72 (h)	96 (h)	120 (h)	144 (h)
0.049 ± 0.003	WT	1.219 ± 0.085	0.981 ± 0.046	0.903 ± 0.062	0.772 ± 0.036		
	BH222-1	1.993 ± 0.175 **	1.456 ± 0.103**	1.340 ± 0.108**			
	BH222-3	1.989 ± 0.184**	1.540 ± 0.107**	1.371 ± 0.085**			
	BH222-5	2.297 ± 0.169**	1.638 ± 0.114**	1.651 ± 0.123**			
0.099 ± 0.008	WT	0.835 ± 0.068	0.727 ± 0.062	0.564 ± 0.025	0.493 ± 0.044	0.421 ± 0.031	
	BH222-1	1.479 ± 0.087**	1.148 ± 0.104**	1.057 ± 0.079**	1.030 ± 0.092**		
	BH222-3	1.521 ± 0.102**	1.181 ± 0.107**	1.187 ± 0.068**	1.111 ± 0.104**		
	BH222-5	1.625 ± 0.093**	1.293 ± 0.103**	1.272 ± 0.092**	1.238 ± 0.098**		
0.148 ± 0.017	WT	0.647 ± 0.059	0.546 ± 0.047	0.485 ± 0.036	0.422 ± 0.032	0.396 ± 0.022	0.346 ± 0.016
	BH222-1	1.065 ± 0.104**	0.863 ± 0.072**	0.732 ± 0.041**	0.715 ± 0.056**	0.681 ± 0.048**	0.687 ± 0.048**
	BH222-3	1.259 ± 0.107**	1.021 ± 0.084**	0.919 ± 0.088**	0.857 ± 0.055**	0.912 ± 0.074**	
	BH222-5	1.328 ± 0.095**	1.077 ± 0.094**	0.952 ± 0.078**	0.958 ± 0.043**	1.035 ± 0.102**	

Data are the mean ± standard deviation of three replicates. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after TNT treatment. FW – fresh weight

toremediation. These characteristics are similar to those in previous reports (Hannink et al. 2001, Zhu et al. 2012).

The influence of TNT on plant photosynthesis. Photosynthesis is the most significant physiological process and, in all its phases, is affected by stress factors (Hniličková et al. 2017). The decrease of chlorophyll II content in the transgenic plants was significantly lower than that in wild type after TNT stress. There were significant differences between transgenic and wild-type plants from 0.25 to 0.75 mmol TNT (Figure 4a). The F_v/F_m value for wild type was dramatically lower than that of transgenic plants, not only in normal conditions but also after TNT stress. It was observed that high TNT concentrations caused a decrease of F_v/F_m, but the value of transgenic was higher than that of wild type (Figure 4b). The damage of TNT to photosynthetic characteristics of transgenic plants was less than that of wild plants.

Degradation rate of TNT within time. Table 1 showed the specific rate constant for TNT uptake. Higher initial TNT concentrations and longer incubation time resulted in a lower rate constant. At 0.05 mmol TNT, specific rate constants for TNT uptake by wild type plants ranged from 1.219 to 0.772 mL/g FW/h, with the highest rate at 24 h and the lowest rate at 96 h, while all transgenic plants ranged from 1.340 to 2.297 mL/g FW/h. It is noteworthy that the specific rate constant was decreased along with the incubation time longer. At 0.1 or 0.15 mmol TNT, the kinetics was lower along with the TNT concentration increased.

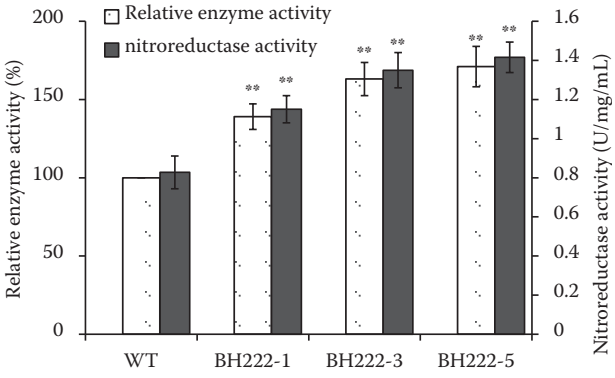

Figure 5. Nitroreductase activity of wild type and transgenic plants. Data are the mean ± standard deviation of three replicates. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after TNT (2,4,6-trinitrotoluene) treatment

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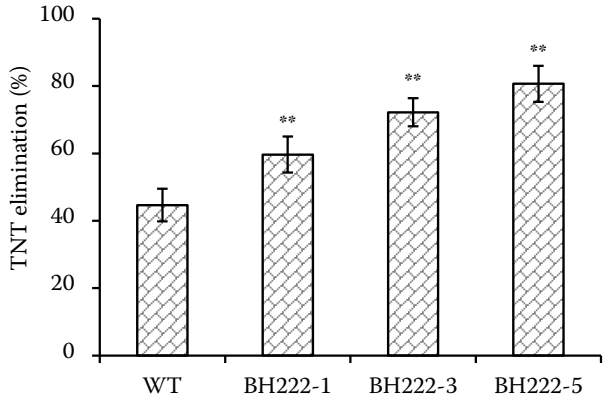

Figure 6. Relative TNT (2,4,6-trinitrotoluene) degradation rates *in vivo* for wild type and transgenic plants. Data are the mean ± standard deviation of three replicates. Asterisks indicate a significant difference ($P < 0.05$) compared to wild type plants in normal conditions and after TNT treatment

Nitroreductase activity and TNT degradation for plants *in vivo*. Nitroreductase activities are showed in Figure 5. All three transgenic lines showed higher nitroreductase activities than wild type plants. Moreover, transgenic lines showed higher TNT elimination than wild type plants. The highest degradation rate of transgenic plants was almost twice higher than wild type plants from Figure 6. This indicated that the transgenic plants over-expressing *SdNFSBI* gene could degrade TNT more efficiently than wild type plants.

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