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ORIGINAL PAPER

Phytoremediation and phytosensing of chemical contaminants, RDX and TNT: identification of the required target genes

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Abstract High explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and 2,4,6-trinitrotoluene (TNT) are important contaminants in the environment and phytoremediation has been viewed as a cost-effective abatement. There remains, however, an insufficient knowledge-base about how plants respond to explosives, especially in the steady state. Microarray analysis was conducted on *Arabidopsis thaliana* that were grown in Murashige and Skoog media containing steady-state levels of 0.5 mM RDX or 2.0 μM TNT to study the effect of these compounds on its transcriptional profile. Our results for both RDX and TNT were consistent with the existing theory for xenobiotic metabolism in plants. Among the genes that were differentially expressed included oxidoreductases, cytochrome P450s, transferases, transporters, and several unknown expressed proteins. We discuss the potential role of upregulated genes in plant metabolism, phytoremediation, and phytosensing. Phytosensing, the detection of field contamination using plants, is an end goal of this project.

Keywords Explosives · RDX · TNT · Microarrays · Phytoremediation · Phytosensing

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Introduction

Explosives such as hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; Royal Demolition Explosive or Research Department Explosive) and 2,4,6-trinitrotoluene (TNT) are widely used in military munitions (Best et al. 2001; Hannink et al. 2002; Halasz et al. 2002). They and their breakdown products are major human-produced contaminants in the environment; manufacturing, deployment, and improper disposal contribute to contamination (Best et al. 1997, 2001; Halasz et al. 2002; Rosenblatt et al. 1991). RDX and TNT are important constituents of unexploded ordnance on many military and nonmilitary sites; landmines often contain RDX and/or TNT. Landmine detection and remediation remains an important environmental need. Landmines leak explosives from inexpensive plastic cases. RDX and TNT are generally recalcitrant to degradation and remain in the biosphere in ecological time, where they constitute a source of toxic, mutagenic, and carcinogenic effects on humans and other biota. In humans, high and prolonged exposures to TNT cause hyperplasia of the bone

marrow leading to aplastic anemia and a drastic loss of blood platelets (Rosenblatt 1980). Toxic hepatitis is also reported in humans from TNT exposure and RDX toxicity includes gastrointestinal, central nervous system (generalized convulsions), and renal effects (Rosenblatt 1980). Thus, RDX and TNT require widespread environmental abatement.

Typically, environmental abatement includes incineration, land filling, and composting (Hannink et al. 2002; Nishino et al. 2000; Peterson et al. 1998). Incineration destroys the soil structure, disturbs ecology, and costs between US $523 and $785/$m^3$ of soil, while landfilling results in displacement of contamination to another location, and composting may result in incomplete breakdown with costs between $528 and $611/$m^3$ of soil (Hannink et al. 2002; Nishino et al. 2000; Peterson et al. 1998). If effective, phytoremediation could be a more environmentally friendly alternative.

The two greatest advantages of phytoremediation compared with traditional abatement methods are (1) cost-effectiveness and (2) soils remain in place thereby causing less ecosystem disruption. Cropping systems with costs ranging between $200 and $10,000/ha would correspond to a remediation cost of $0.02–1.00/$m^3$ of soil per year, which is many orders of magnitude savings when compared to costs associated with physicochemical remediation technologies (Cunningham et al. 1995). There are several studies showing that plants in general readily take up RDX and TNT. For example, recently Vila and others reported that agronomic plants (maize, soybean, wheat, and rice) could grow on soils containing RDX and TNT and were able to uptake these compounds (Vila et al. 2007). In another recent study, it was reported that maize (*Zea mays* L.) and broad beans (*Vicia faba* L.) were able to remove TNT from soils (Van Dillewijn et al. 2007). Also, *Catharanthus roseus* (Vinca) hairy root cultures, *Myriophyllum aquaticum* (parrot feather) plants, and hybrid poplars have been reported to take up RDX (Bhadra et al. 2001; Thompson et al. 1999). Harvey and others have reported bioaccumulation of RDX in bush bean plants grown in hydroponic culture (Harvey et al. 1991). However, unmodified plants are typically not very efficient in their accumulation and degradation of explosives. Therefore, genetic engineering might help increase phytoremediation capacity and certainly would be required for phytosensing, i.e., using plants to report the presence of contaminants. In this regard, plants have been genetically engineered to phytoremediate explosives (French et al. 1999; Hannink et al. 2001, 2007; Rylott et al. 2006; Van Dillewijn et al. 2008; Rylott and Bruce 2009; Van 2009; Eapen et al. 2007), but there is no published report on phytosensors for explosives. Understanding plant transcriptional responses to explosives is thus necessary and useful for developing phytosensors or phytoremediators.

Based on studies published to date, a working hypothesis for how plants deal with organic chemical contaminants such as RDX and TNT is based on three phases (Best et al. 1999, 2005, 2006; Larson et al. 1999; Bhadra et al. 1999a, b, 2001; Just and Schnoor 2004; Van et al. 2004; Ekman et al. 2003, 2005; Harvey et al. 1990; Van Dillewijn et al. 2008; Hannink et al. 2002; Rylott and Bruce 2009; Sandermann 1992; Coleman et al. 1997): phase I (transformation)—a transformation phase of metabolism of the chemical, phase II (conjugation)—conjugation of the chemical contaminant to endogenous hydrophilic molecules to facilitate compartmentalization of the contaminant, and phase III (compartmentation)—movement of the contaminants and breakdown products into vacuoles to reduce their toxicity. The comprehensive *Arabidopsis thaliana* oligonucleotide microarray analysis in the current study refines this model by identifying genes that might specifically contribute to each of these phases.

Materials and methods

Plants and phytotoxicity studies

A. thaliana (ecotype Columbia) plants were grown on Murashige and Skoog (MS) medium supplemented with B5 vitamins, 1% sucrose, and 2% gelrite, pH 5.8. *Arabidopsis* seeds were sterilized using 20% bleach and 0.1% Tween-20. Surface sterilized seeds at the rate of six seeds per petri plate were placed on solid MS medium containing RDX concentrations of 0, 0.1, 0.25, 0.50, 0.75, and 1.00 mM and TNT concentrations of 0, 1, 2, 3, 4, 5, 6, 8, and 10 μM. Each concentration of RDX and TNT were replicated three times. The control plates contained MS medium with dimethyl sulfoxide (DMSO) in proportion to the concentration of RDX or TNT, since DMSO was needed to solubilize both RDX and TNT. The seeds were then cold stratified at 4°C for 3–5 days and then transferred to a growth chamber at 25°C with a photoperiod of 16 h. The growth responses and phytotoxicity tolerance threshold of wild-type *Arabidopsis* plants to RDX and TNT were analyzed by measuring the primary root length 6–7 days after germination when grown on vertically oriented plates. RDX was obtained from Restek Corporation, Bellefonte, PA, USA and TNT from Chem Service, Inc., West Chester, PA, USA.

RNA preparation

Arabidopsis seeds were germinated and plants were grown for 8–9 days on MS media containing 0.5 mM (111 mg/L) RDX or 2.0 μM (454 μg/L) TNT, or no explosives but with proportional amounts of solvent DMSO. Total RNA

was extracted from *Arabidopsis* seedlings using TRI REAGENT® (Molecular Research Center, Inc., Cincinnati, OH, USA) according to manufacturer's protocol. The total RNA isolated was purified using RNeasy ® Midi kit (Qiagen Inc., Valencia, CA, USA). RNA quality was assessed by agarose gel electrophoresis for two-color microarrays and by Agilent Bioanalyzer for the Affymetrix microarrays. mRNA was extracted from the total RNA pools using Oligotex mRNA mini kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's protocol. The mRNA isolated was used for synthesis and labeling of cDNA probes using Superscript™ Plus Direct/Indirect cDNA labeling system with Alexa Fluor® dyes. The labeled cDNA probes were used for hybridizing to microarray slides.

Microarray hybridizations and analysis

Two-color microarrays

Two-color hybridization was done only for the RDX-treated plants. The experiment included three biological replicates and a dye swap technical replicate (to avoid dye bias) for every set of replicates. *Arabidopsis* oligonucleotide microarray hybridizations were performed as described in Abercrombie et al. (2008).

Affymetrix microarrays

This experiment involved hybridization of four slides consisting of two biological replicates. Total RNA from the same biological samples that were used for two-color hybridizations were used to prepare labeled cRNA. Labeled cRNA targets were prepared according to the instructions for *Arabidopsis* ATH1 genome array (Affymetrix, Santa Clara, CA, USA) containing approximately 24,000 genes represented by more than 22,500 probe sets. Labeled cRNA that was purified and fragmented was hybridized to *Arabidopsis* ATH1 genome array at 45°C for 16 h at a setting of 60 rpm. The gene chips were further washed and stained using an Affymetrix Fluidics 450 wash station following which the gene chips were immediately scanned with a GeneChip 7G scanner. The gene chips were processed at the University of Tennessee, Knoxville Affymetrix Core Facility. Raw CEL files were created from the DAT image file of the chip using the gene chip operating software from Affymetrix. Array Assist Software (version 3.4.2152.32776; Stratagene, La Jolla, CA, USA) was used and the guanine cytosine robust multi-array analysis (GC-RMA) algorithm was applied to the CEL files for background subtraction and normalization. The GC-RMA values were then $\log_2$-transformed. The normalized and $\log_2$-transformed data from both two-color and Affymetrix microarrays were statistically analyzed according to Breitling et al. (2004) as described in Abercrombie et al. (2008).

Real-time RT-PCR

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis was done for six genes. This experiment was performed in three replicates using the RNA samples described above. RT-PCR was performed using the Superscript III mix (Invitrogen, Carlsbad, CA, USA) and SYBR Green mix (Qiagen Inc., Valencia, CA, USA) on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers were designed using Primer Express v. 2.0.0 (Applied Biosystems, Foster City, CA, USA) and were as follows: lipoxygenase (*At3g45140*) forward, 5′-CTGACCAGCGGATTACGGTAGA-3′ and reverse 5′-CCCGCCGGGTAATTTAAGCT-3′; leucine-rich repeat family protein (*At4g33970*) forward, 5′-TTGCCAGTTGCCTAATTTGGTG-3′ and reverse, 5′-ACGCAATCTCCTTGCGACTACC-3′; expressed protein (*At4g35720*) forward, 5′-GGGAAGCTCGTTGTGATGATGA-3′ and reverse, 5′-TTCCATGGCTGCCTCTACACC-3′; pseudoresponse regulator 9 (APRR9; *At2g46790*) forward, 5′-TGTATGCTGAGAGGTGCTGCTG and reverse, 5′-TCACGCAAAGTCAGTCTTCTCCA-3′; myb-related transcription factor (CCA1; *At2g46830*) forward, 5′-CACGGGAAGAGGGAAGTCAGAAT and reverse, 5′-TGAGCTCCCCAATGGCACTAG-3′; and DNA topoisomerase-related (*At3g15950*) forward, 5′-GCCTGCAGATGGTGTATGTGGT and reverse, 5′-GATGTGGTGAGCCGAGAGGTC-3′. The *Arabidopsis* β-actin-7 (*At5g09810*) was used as the reference gene and the primer sequences (forward—AGTGGTCGTACAACCGGTATTGT; reverse—GAGGAAGAGCATTCCCCTCGTA) for this gene were taken from Campbell et al. (2003). The amplification conditions for the RT-PCR were as follows: enzyme activation at 55°C for 2 min, which was followed by denaturation step at 95°C for 15 min and then 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. A negative control without reverse transcriptase was included for all the reactions to ensure that there was no genomic DNA contamination. The PCR products were confirmed for size and sequence by agarose gel electrophoresis and by sequencing the PCR products respectively. Data were analyzed according to Pfaffl (2001).

Gene ontology

Gene ontology annotations available on the *Arabidopsis* information resource (TAIR) website (http://arabidopsis.org/tools/bulk/go/index.jsp) were used to functionally characterize the differentially regulated genes (Rhee et al.

2003). The GO tool available on the TAIR website was used to render the functional categorization graphics.

Meta-analysis to identify unique genes

The following databases were searched for microarray experiments involving nitrogenous compounds in *Arabidopsis* to better characterize explosives-specific responses: Gene Expression Omnibus, ArrayExpress, and the Stanford Microarray Database. Three relevant datasets were found of which two were from Gene Expression Omnibus database (GSE6824 and GSE9148) and one from ArrayExpress database (E-MEXP-828). These datasets were downloaded and were also analyzed using rank-product statistics similar to the analysis of our datasets and the gene list from our experiment was compared with these five datasets to find redundant differentially regulated genes, i.e., those not specifically regulated by RDX or TNT.

Results

Growth of plants on RDX and TNT media

Based on the primary root growth, 0.5 mM (111 mg/L) of RDX and 2.0 μM (454 μg/L) of TNT were considered as sublethal concentrations and were used for the subsequent microarray experiments (Fig. 1; Figs. S1 and S2).

Microarray analysis of RDX-treated plants

Two-color platform

A false discovery rate (FDR) value cutoff of 10% and 1.5-fold change criteria resulted in the determination that 173 genes were differentially regulated. The complete lists of these genes can be found in the supplemental data Tables S1 and S2, respectively (see supplementary file 1). The most highly upregulated gene in this experiment was a leucine-rich repeat family protein (*At4g33970*) with a twofold change. Genes from this family are cell wall constituents involved in protein–protein interactions in plants as well as transducing pathogen recognition signals (Baumberger et al. 2003; Kobe and Deisenhofer 1994; Li and Chory 1997) and the upregulation of this gene in response to RDX might only indicate a general stress response. Other genes that were upregulated included a protease inhibitor/lipid transfer protein (*At4g12500*) that is involved in lipid transport and lipid binding (Rhee et al. 2003), a putative mannitol transporter (*At4g36670*), which is located in the membrane and involved in carbohydrate transporter activity, a putative xyloglucan/xyloglucosyl transferase (*At4g14130*), multicopper oxidase type I family protein (*At1g21850*) which has oxidoreductase activity, and lipoxygenase (*LOX2*; *At3g45140*), which is targeted to chloroplast and is known to be involved in wound induced jasmonic acid accumulation in *Arabidopsis* (Bell et al. 1995). Many other genes that were upregulated were either unknown or hypothetical proteins. The downregulated genes included a putative cysteine protease (*At4g11320*), a putative protease inhibitor (*At1g73330*), which is responsive to drought (Rhee et al. 2003), phosphoribulokinase (*At1g32060*), an ABC transporter family protein (*At5g64840*), and a xyloglucan/xyloglucosyl transferase (*At5g57560*) that is linked to cold tolerance (Purugganan et al. 1997).

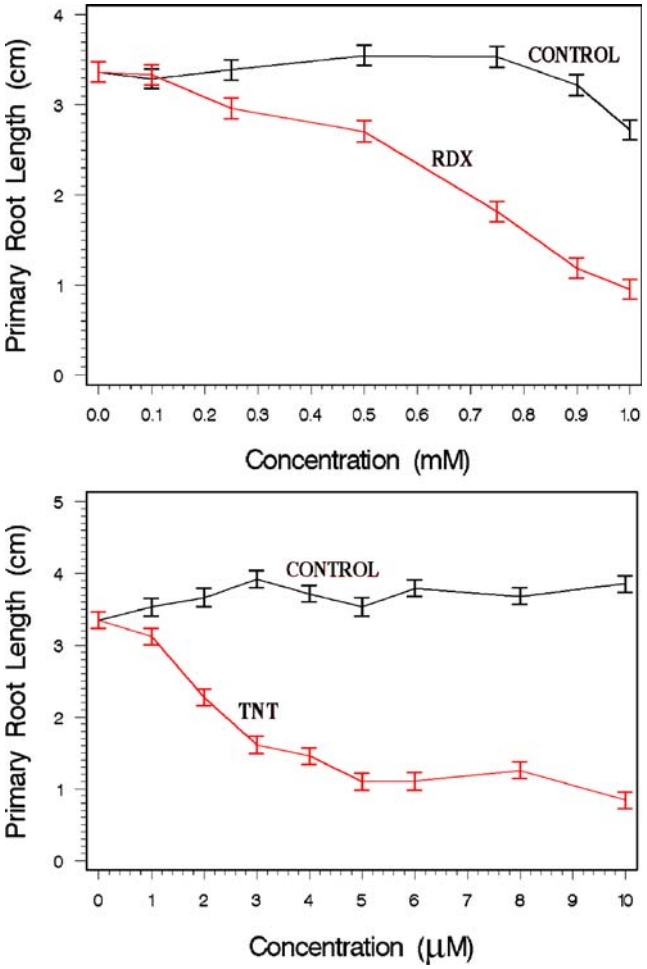

Fig. 1 Primary root length of *Arabidopsis* plants exposed to RDX and TNT (6–7 days after germination). On the *Y*-axis is the primary root length in centimeters and on the *X*-axis is the concentration in millimolar for RDX and micromolar for TNT. The control plants were grown on MS media supplemented with proportional amounts of solvent DMSO without RDX or TNT. RDX concentration of 0.5 mM (111 mg/L) and TNT concentration of 2.0 μM (454 μg/L) was used for microarray analysis

Affymetrix platform

An FDR cutoff value of 10% and fold change of 2.0 indicated that there were 217 differentially expressed genes

in this experiment. The complete lists of these genes can be found in the supplemental data Table S3 and S4, respectively (see supplementary file 1). Among the upregulated genes, lipoxygenase (*LOX2*; *At3g45140*) was the most upregulated gene with approximately sevenfold change in expression. Genes that were also upregulated included an ABC transporter (*At2g39350*), which was upregulated 4.8-fold. It is expressed in roots and is responsive to nematodes (Rhee et al. 2003). In addition, a uridine diphosphate (UDP)-glucoronosyl/UDP-glucosyl transferase family protein (*At5g49690*), a putative peroxidase (*At5g39580*), a glutaredoxin family protein (*At1g03020*), which has arsenate reductase (glutaredoxin) activity (Rhee et al. 2003), a sugar transporter family protein (*At1g73220*), and several genes with unknown biological function were also upregulated. The most highly downregulated gene (20-fold) was an unknown expressed protein (*At1g13650*). Other genes exhibiting repressed transcription in response to RDX stress included genes encoding for a neurofilament protein-related (*At3g05900*), a cytochrome p450 family protein (*At5g47990*), a putative myrcene/ocimene synthase (*At3g25820*), and a putative pathogen-responsive alpha-dioxygenase (*At3g01420*).

Functional categorization (gene bar charts) of differentially regulated genes by loci (molecular function) for both two-color, as well as Affymetrix microarrays, is shown in supplemental Fig. S3 (see supplementary file 2). For categorization by loci for biological process and cellular component please refer to Supplemental Fig. S4 for two color and Fig. S5 for Affymetrix (see supplementary file 2). Briefly, functional categorization (by loci for molecular function) for the upregulated genes revealed that most of the genes (36% for two-color and 26% for Affymetrix) had unknown molecular function, while other interesting GO categories were transcription factor activity (14% for two-color and 11% for Affymetrix), transferase activity (8% for two-color and 9% for Affymetrix), and transporter activity (2% for two-color and 10% for Affymetrix).

Correlation between the Affymetrix and the two-color platforms

A simple correlation analysis indicated a positive moderate relationship between Affymetrix and two-color microarray platforms using $\log_2$ ratios of the signal intensities for all the genes (Fig. 2). The Pearson correlation coefficient (r) between Affymetrix and two-color $\log_2$ ratios was 0.38 (p value <0.0001).

Real-time RT-PCR analysis

Real-time RT-PCR analysis was carried out for confirming the microarray results. The total RNA that was used for the microarray experiment was also used for the RT-PCR analysis. Six genes were selected for this experiment, three of which were upregulated (*At3g45140*, *At4g33970*, *At4g35720*) and three of which were downregulated (*At2g46790*, *At2g46830*, *At3g15950*) in the RDX microarrays. The *Arabidopsis* β-actin-7 gene (*At5g09810*) was used as the reference gene. The results obtained from this analysis corresponded with the microarray analysis (Table S7 in supplementary file 1).

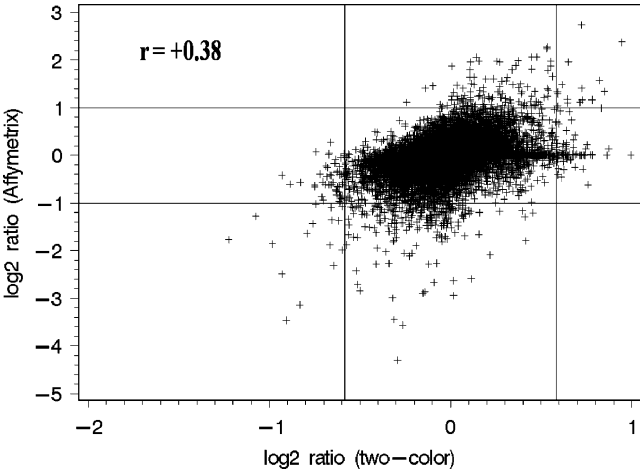

Fig. 2 Log_2 ratios of signal intensities for RDX from the two platforms (two-color and Affymetrix) plotted against each other. On the y-axis are the $\log_2$ ratios from Affymetrix and on x-axis are $\log_2$ ratios from two-color. The *horizontal lines* on the y-axis at values +1 and −1 represents the cutoff value of +2.0 and −2.0 linear fold change, respectively, for Affymetrix and the *vertical lines* at values +0.58 and −0.58 on the x-axis represents the cutoff values of +1.5 and −1.5 linear fold change, respectively, for two-color. The value for the Pearson correlation coefficient is represented as r on the graph

Affymetrix microarray analysis of TNT-treated plants

Two hundred ninety-seven genes were differentially expressed at an FDR cutoff value of 10% and fold change cutoff of 2.0. The complete lists of these genes are in Supplemental Table S5 and S6 (see supplementary file 1). In this experiment, the most highly upregulated gene was an expressed protein with unknown biological function (*At3g15310*) with a fold change of 17. Other upregulated genes included an *O*-methyltransferase N terminus domain containing protein (*At5g42760*), a putative pathogenesis-related protein (*At4g33720*), a putative cysteine proteinase (*At2g27420*), a myb family transcription factor (*At1g01520*), and many other expressed proteins with unknown functions. Among the downregulated genes, a male sterility MS5 family protein (*At5g48850*) was the most highly downregulated gene, and other downregulated genes included a putative CTP synthase (*At1g30820*), a putative glycine hydroxymethyltransferase (*At1g36370*), a

glycosyl transferase family 20 protein (*At2g18700*), a glutaredoxin family protein (*At3g62950*), and a protease inhibitor (*At4g12500*).

Functional categorization by molecular function of differentially expressed genes in response to TNT was apparently different from RDX experiments (Figs. S3 and S6 in supplementary file 2). Functional categorization by loci (molecular function) for upregulated genes revealed that maximum number of upregulated genes had unknown molecular function (28%), which was closely followed by genes with transcription factor activity (27%). Other interesting gene categories included transferase activity (12%), transporter activity (8%), and protein binding (11%).

Meta-analysis to identify genes regulated uniquely by RDX and TNT

To ensure that the gene regulation list is specific to RDX and TNT and not generally differentially regulated by similar nitrogenous compounds, a variety of *Arabidopsis* microarray databases were searched for microarray experiments involving nitrogenous compounds. Only three relevant Affymetrix datasets involving nitrate treatment in *Arabidopsis* were found. After comparing our upregulated gene lists from the Affymetrix experiments with the gene lists from the downloaded datasets, only one gene from the Affymetrix RDX upregulated gene list (*At4g36010*) and none from the TNT gene list was filtered indicating that the explosives-responsive genes are likely not generally *N*-responsive.

Discussion

Plant metabolism of xenobiotics involves three phases: activation (transformation), conjugation, and compartmentation (elimination; Sandermann 1992; Ishikawa 1992; Ishikawa et al. 1997; Rea et al. 1998; Coleman et al. 1997; Schaffner et al. 2002). Activation generally involves oxidation or hydrolysis or reduction type of reactions, where functional groups such as hydroxyl (–OH) and carboxyl (–COOH) are added to the contaminant with enzymatic involvement of cytochrome P450 monooxygenases, esterases, reductases, dehalogenases, and dehydrogenases. The products of phase I (activation) are more hydrophilic and sometimes more toxic than the parent compound. In the phase II (conjugation), the activated contaminant undergoes deactivation by the formation of covalent linkages with endogenous hydrophilic molecules such as glucose, malonate, glutathione (GSH), or carboxylic acids using glucosyltransferase-, glutathione-*S*-transferase-, and acyltransferase-mediated reactions that result in water soluble conjugates that are less toxic compared to the parent compound. Phase III (compartmentation) involves exporting conjugates to either the vacuole or apoplast using ABC transporters or multidrug and toxic compound extrusion (MATE) transporters (Sandermann 1992; Ishikawa 1992; Ishikawa et al. 1997; Rea et al. 1998; Coleman et al. 1997; Schaffner et al. 2002).

Several genes induced by RDX treatment in this study suggest RDX detoxification via the three phases (Table 1). Functional categorization by loci of the genes upregulated in this study revealed that several genes had transferase activity and transporter activity, further supporting the notion of potential RDX detoxification in *Arabidopsis*. Also, there were nine expressed genes with unknown function from two-color experiment (greater than 1.5 fold upregulation) and 20 from Affymetrix experiment (greater than 2.0 fold upregulation) identified, some of which might be involved in RDX metabolism.

There is no earlier report on whole genome expression studies in response to RDX except a serial analysis of gene expression (SAGE) study (Ekman et al. 2005), where gene expression in *Arabidopsis* roots was characterized. These authors reported three cytochrome P450s (*At1g16400*, *At3g20940*, *At4g13310*), induced greater than fivefold in their study, to be possibly involved in phase I transformation of RDX in *Arabidopsis*. They also speculated about a putative peroxidase (*At1g49570*) and an alpha-hydroxynitrile lyase-like protein (*At5g10300*) to be involved in RDX metabolism. Incongruence of lists of differentially upregulated genes between our study and Ekman et al. (2005) can possibly be attributed to organs used in the respective studies: roots (Ekman) vs. whole plants (this study). As also suggested by Ekman et al. (2005), since RDX is readily translocated and accumulated in leaf tissues (Best et al. 1999; Harvey et al. 1991; Thompson et al. 1999), gene expression in shoots is highly relevant. We analyzed gene expression in whole plants to sample all differentially regulated genes, which is more appropriate and consistent with our objective of phytosensor engineering and phytoremediation of RDX. In another study, Mezzari et al. (2005) used semiquantitative reverse-transcription PCR to study expression of three glutathione *S*-transferases (GSTs; *At4g02520*, *At1g17170*, and *At2g29490*) and two isoforms of 12-oxophytodienoate reductases (*OPR*1 and *OPR*2) from *Arabidopsis*. These authors reported relatively low induction for all the fives genes in response to RDX and their in vivo conjugation reactions with glutathione ruled out GST-catalyzed GSH (reduced glutathione) conjugation with RDX. None of these genes were found to be upregulated in our study. This inconsistency can also be attributed to the difference in duration of exposure and the concentrations between studies. Tanaka et al. (2007) used real-time RT-PCR to study few

Table 1 List of potential genes suggesting RDX or TNT metabolism via the three phases of detoxification from RDX (two-color and Affymetrix) and TNT (Affymetrix) microarray experiments along with their fold change in linear scale

Phases of detoxification	RDX (two-color and Affymetrix)			TNT (Affymetrix)		
	AGI gene ID	Gene name	FC	AGI gene ID	Gene name	FC
Phase I (transformation): oxidation or hydrolysis or reduction type of reactions (cytochrome p450, esterases, oxidoreductases)	At4g16690	Esterase/lipase/thioesterase family protein	1.54	At3g15650	Phospholipase/carboxyl-esterase family protein	2.18
	At2g48080	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	1.50	At3g30180	Cytochrome P450, putative	2.18
	At3g11180	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	1.45			
	At5g05600	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	2.80			
	At5g22500	Acyl CoA reductase, putative/male-sterility protein, putative	2.15			
	At5g09970	Cytochrome P450 family protein	2.08			
	At1g64590	SDR family protein	2.04			
	At2g12190/ At1g64950/ At1g64940/ At1g64930	Cytochrome P450, putative	2.00			
Phase II (conjugation): deactivation by the formation of covalent linkages with endogenous hydrophilic molecules such as glucose, malonate, glutathione, or carboxylic acids (glucosyl transferase, glutathione *S*-transferase, acyl transferase)	At3g29590	Transferase family protein	1.56	At3g21760	UDP-glucoronosyl/UDP-glucosyl transferase family protein	3.29
	At1g78270	UDP-glucose glucosyl-transferase, putative	1.48	At3g21560	UDP-glucosyltransferase, putative	3.14
	At5g49690	UDP-glucoronosyl/UDP-glucosyl transferase family protein	4.16	At5g55380	MBOAT family protein/wax synthase-related	2.84
	At5g39050	Transferase family protein	2.68	At3g43190	Sucrose synthase, putative/sucrose–UDP glucosyl-transferase, putative	2.47
	At2g39980	Transferase family protein	2.45	At2g13290	Glycosyl transferase family 17 protein	2.42
	At5g01210	Transferase family protein	2.15	At5g17050	UDP-glucoronosyl/UDP-glucosyl transferase family protein	2.39
	At3g19710	Branched-chain amino acid aminotransferase, putative	2.07	At2g36800/ At2g36790	UDP-glucoronosyl/UDP-glucosyl transferase family protein	2.39
Phase III (compartmentation): exportation of conjugates to either the vacuole or apoplast (via transporters)	At4g36670	Mannitol transporter, putative	1.78	At1g79410	Transporter-related	3.05
	At1g73220	Sugar transporter family protein	1.57	At3g55110	ABC transporter family protein	2.97
	At2g39350	ABC transporter family protein	4.82			
	At1g73220	Sugar transporter family protein	3.88			
	At3g05400	Sugar transporter, putative	2.46			
	At1g16370	Transporter-related	2.18			
	At4g29140	MATE efflux protein-related	2.09			

SDR short-chain dehydrogenase/reductase, *MBOAT* membrane bound *O*-acyl transferase, *FC* fold change

TNT-inducible genes (chosen from the *Arabidopsis* TNT SAGE study by Ekman et al. 2003) in poplar that was exposed to RDX. Although none of these genes were found to be upregulated in our RDX study, Tanaka et al. (2007) found that all of the selected genes were upregulated in leaf tissues, whereas only one gene was upregulated in the roots. Unlike in *Arabidopsis*, which deals with RDX and TNT differently (consistent with our study and also as reported by Ekman et al. 2005), it is interesting to see upregulation of TNT-inducible genes in RDX-exposed poplar (Tanaka et al. 2007), although a thorough gene expression analysis in poplar in response to RDX and TNT would be necessary to validate this finding.

Another interesting upregulated gene found in our RDX study was lipoxygenase (*LOX2*; *At3g45140*). This gene was strongly upregulated by RDX as shown by both microarray platforms and confirmed by real-time RT-PCR. Lipoxygenases are known to be versatile catalysts (Feussner and Wasternack 2002), and there are several studies indicating that mammalian lipoxygenases are involved in the metabolism of xenobiotics and endobiotics (Kulkarni 2001). Given this background, although it might be tempting to say that *LOX2* might be involved in RDX metabolism, further investigation is warranted in this regard.

Finally, for the RDX study, the moderate correlation observed between platforms can be attributed to several factors, including array design, RNA amplification, labeling (single vs. double), hybridization, array scanning, image processing, and normalization techniques (Pylatuik and Fobert 2005). In another study, considerable differences were found to exist across three commercially available platforms (Agilent, Amersham, and Affymetrix) (Tan et al. 2003). Additionally, a lack of agreement was reported between Atlas nucleotide arrays (Clontech) and Affymetrix arrays (Rogojina et al. 2003). However, there are also studies showing reproducibility among platforms (Larkin et al. 2005; Petersen et al. 2005; Shi et al. 2006).

Despite only a moderate correlation between the two platforms here, several significant genes were commonly upregulated and downregulated between the two platforms (Table S8 in supplementary file 1). In no case did one platform indicate a gene was upregulated while the other indicated downregulation as evident by the lack of data points in the upper left and lower right corners of Fig. 2. Therefore, we conclude that either platform is adequate for screening gene candidates, while the two-color platform might be considered to be somewhat less robust.

With respect to TNT, plants readily take up and accumulate TNT in roots (Burken et al. 2000; Harvey et al. 1990; Hughes et al. 1997; Larson et al. 1999). Several studies in plants have been reported supporting the three phase detoxification of TNT in plants (as reviewed by Rylott and Bruce 2009). Consistent with the suggested three phase xenobiotic detoxification system in plants, our experiment also revealed enzymes that could potentially be involved in TNT metabolism (Table 1). This notion of potential TNT detoxification in *Arabidopsis* is further supported by the functional categorization by loci that indicated many genes being involved in transferase and transporter activities. Also, there were in total 30 expressed proteins with unknown function upregulated greater than 2.0 fold in this study, some of which might play a role in TNT metabolism.

Three other plant genome level gene expression TNT studies have been reported. The Mentewab et al. (2005) study using a microarray containing only half of the *Arabidopsis* genome found that a total of 52 genes were upregulated and 47 genes were downregulated when *Arabidopsis* was exposed to TNT concentrations of 1 and 10 μM. A large fraction of these genes had predicted roles in cellular detoxification and defense. Although consistent with the three phase detoxification systems, they found genes such as UDP-glucose glucosyltrasferase isoforms that could potentially be involved in the transformation phase. The SAGE study of Ekman et al. (2003) also was consistent with the three phase detoxification system and revealed upregulated genes such as monodehydroascorbate reductase, GSH-dependent dehydroascorbate reductase, and GST. A number of cytochrome P450s, enzymes involved in detoxification of xenobiotic compounds, were also upregulated by TNT. The obvious difference among the significantly differentially upregulated genes between our study and Ekman et al. (2003) can possibly be the result of the difference in the plant material (roots vs. whole plants) that was used in these experiments. In another report, Mezzari et al. (2005) focused on the TNT-induced expression of only five genes from *Arabidopsis* that included GSTs and OPRs and they observed relatively high induction for GSTs and one of the OPRs. These researchers also performed confocal microscopy on *Arabidopsis* root cells showing that GST-catalyzed GSH (reduced glutathione) conjugation did not occur for RDX or TNT, thereby refuting the proposed glutathione conjugation of RDX or TNT. In our study, neither the OPRs nor the GSTs were significantly differentially regulated. Recently, Gandia-Herrero et al. (2008) did a microarray study that examined transcriptional responses to TNT in *Arabidopsis* and demonstrated that UDP glucosyltransferases are involved in TNT detoxification. Only two genes were commonly upregulated between our study and Gandia-Herrero et al. (2008) study, one of which is a UDP glucosyltransferase (*At2g36790*). One reason for this could be that both of the aforementioned studies analyzed plants after short-term exposure to TNT, whereas our experimental design was similar to that of Mentewab et al. (2005); transcriptional responses were measured after relatively long-term steady-

state exposure to explosives, which is the most plausible scenario for phytoremediation or phytosensing biology.

Arabidopsis had apparent differences in transcriptional regulation from RDX and TNT treatments. Few significant genes were commonly up- or downregulated among RDX- and TNT-treated plants suggesting that plants cope with these compounds differently. This lack of overlap was also observed by Ekman et al. (2005) who studied the transcriptional responses to RDX in *Arabidopsis* roots and compared it to transcriptional responses to TNT in *Arabidopsis* roots studied earlier by Ekman et al. (2003). One possible explanation could be that these two compounds differ chemically and in their metabolic pathways (Rylott and Bruce 2009; Hannink et al. 2002; Hawari et al. 2000). TNT belongs to the nitroaromatics group and consists of an aromatic ring with three nitro groups. TNT in plants is probably detoxified using phase I reductive transformation to 2- and 4-hydroxydinitrotoluene isomers by means of nitrosodinitrotoluene, followed by phase II conjugation with endogenous plant compounds such as sugars or glutathione, and finally phase III sequestration into the apoplast or vacuole (Hannink et al. 2002; Rylott and Bruce 2009). RDX is classified as a cyclic nitramine explosive and consists of *N*-nitro groups (Hannink et al. 2002; Hawari et al. 2000). The RDX detoxification mechanism, as proposed in poplar, involves reduction of RDX to hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX) followed by light-mediated cleavage of heterocyclic ring of RDX, MNX, and DNX generating formaldehyde and methanol and a final light-independent plant cell-mediated mineralization to carbon dioxide (Van et al. 2004). Therefore, common phytoremediation or phytosensing strategies between these two explosives are likely not feasible. While TNT and RDX are often used together in landmines, phytoremediation would require consideration of both compounds, but phytosensing for landmine detection might be accomplished by detection of either TNT or RDX. The characterization of differential gene regulation in this study provides relevant background data to help choose phytosensor design components. Further studies, characterizing the genes identified in this study are necessary to understand the roles of these genes in the metabolism of RDX and TNT and the development of phytosensors would involve an extensive analysis of the promoter regions of these potential gene targets for identifying novel *cis*-regulatory elements responsive to RDX and TNT.

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