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Genomic analysis of the response of *Arabidopsis thaliana* to trinitrotoluene as revealed by cDNA microarrays

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Abstract

2,4,6-trinitrotoluene (TNT) is a nitro-substituted xenobiotic explosive that is toxic to plants and animals. Plants absorb and metabolize TNT, but the pathways are uncertain and plant responses at the molecular level have not been adequately characterized. We analyzed gene expression in response to relatively long-term exposure to TNT at low and high concentration in *Arabidopsis* through the use of cDNA microarrays. *Arabidopsis* seedlings were grown on media containing 1 and 10 μM TNT, concentrations that were empirically determined by plant growth analysis. Microarray analysis revealed that a total of 52 genes were significantly upregulated, and 47 genes were downregulated in response to TNT at a 1.7-fold cut-off for differential gene expression. A substantial number of these genes have predicted functions in cell defense and detoxification. Conserved motifs were discovered in the promoter region of co-regulated genes, some of which are potentially novel *cis*-regulatory elements. With follow-up real time RT-PCR, we confirmed findings from the microarray experiments and examined the regulation of selected genes to two other xenobiotic substances: the explosive RDX and thiodiglycol. Results showed that increased transcription of At5g61600 encoding for a DNA-binding protein in shoots is specific to TNT and increased transcription of At5g42530 encoding for an unknown protein in shoots is specific to both TNT and RDX.

Keywords: Microarrays; *Arabidopsis thaliana*; TNT; RDX; Xenobiotic; Promoter; Motif

1. Introduction

The production and use of trinitrotoluene (TNT), since the early part of the last century has resulted in the widespread contamination of military bases, manufacturing facilities and their wastewater. TNT is a xenobiotic nitroaromatic compound, synthesized and only recently released into the biosphere by humans. Very few natural nitro-substituted compounds are known to exist and most are broadly toxic [1]. Upon ingestion, TNT can be oxidized by the intestinal microflora and mammalian cytochrome-P450 resulting in very reactive toxic and carcinogenic derivates [2]. TNT toxicity in humans causes anemia and hepatitis [3] and toxicity to plants is manifested as stunted growth and chlorosis [4].

Phytoremediation is currently considered the most suitable and cost-effective method to clean-up contaminated land and water [5]. Transgenic plants expressing bacterial reductases showed increased tolerance and removal of TNT [6,7]. However, to fully take advantage of phytoremediation strategies, it is necessary to characterize genes and pathways involved in TNT transformation by plants. Metabolic studies have established the ability of plants to absorb and transform TNT [8]. The most commonly observed initial step of TNT transformation in plants consists of the reduction of the nitro groups to form 4-amino-2,6-dinitrotoluene, and 2-amino-4,6-dinitrotoluene (ADNTs) [8]. Further reduction of ADNTs to diamino-nitrotoluenes (DANTs) has also been reported in *Myriophyllum aquaticum* [9,10]. Oxidative pathways for the biodegradation of TNT were evidenced by Bhadra et al. [11] who identified six products derived from the oxidation of the ring substituted methyl group or aromatic dihydroxylation.

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Nevertheless, products identified following the initial steps of TNT reduction or oxidation do not account for the majority of the TNT transformed by plants. Studies tracing TNT with ^{14}C labeling in plants generally show a very low level of mineralization, up to 0.2% and conversion of 8–33% of the TNT to unidentified polar products by gas chromatography-mass spectrometry analysis [8]. The remaining ^{14}C is in a biomass-associated fraction in an unextractable form that is likely incorporated in lignin.

Plants could also be used as reporters (phytosensors) for the detection of explosives. Currently, the detection of TNT in the environment relies on extensive sampling followed by analytical methods such as chromatography. All these methods are time consuming and costly. The phytosensor that could be envisioned is an inducible promoter fused to a reporter gene such as that encoding green fluorescent protein (GFP) in transgenic plants. Such a designer phytosensor for explosives could also be deployed in minefields to pinpoint mine location. The key feature of this concept is the specificity of inducibility of the promoter. Plant promoters are modular units of several *cis*-acting elements and often exhibit a complex expression profile. To overcome this limitation, synthetic promoters could be designed with defined regulatory elements.

To identify genes and potential *cis*-regulatory elements responsive to TNT, microarray analysis was performed in *Arabidopsis*. Experiments were designed to reveal genes induced during relatively long-term exposure to TNT at low (1 μM) and high (10 μM) concentrations. A long-term exposure is appropriate real-life approach to study the sustained level of transcription over time. This fact is important, since we are interested in engineering plants to detect and report the presence of explosives. For a subset of genes (hence promoters) of interest, we confirmed microarray results by real-time RT-PCR and assess their specificity to TNT by examining their response to two other xenobiotic substances: the royal demolition explosive (RDX) (hexahydro-1,3,5-trinitro-1,3,5-triazine) and thiodiglycol (TDG) (1,2-ethanedithiol) a chemical resulting from the hydrolysis of sulfur mustard agent.

2. Materials and methods

2.1. Plant growth

Seeds of *Arabidopsis thaliana* ecotype Col1 were obtained from the ABRC (Ohio State University, Columbus). Seeds were plated under sterile conditions on MS media supplemented with B5 vitamins, 10% sucrose and 2% Gelrite®, pH 5.8. After incubation at 4 °C for 4 days in the dark for stratification, the plates were transferred to a tissue culture room at 25 °C with a photoperiod of 16 h. Four leaf-stage plantlets were transferred to Magenta boxes containing 50 mL of media with TNT concentrations of 0, 1, 5, 10, 20, 30 and 50 μM for growth experiments then 0, 1 and 10 μM for microarray and follow-up experiments. For experiments using RDX and TDG the media contained final concentrations of 20 μM in both cases. Ten days later, plants were harvested for RNA extraction.

2.2. Microarray analysis

Total RNA was extracted from control and TNT-grown plants using TRIzol ® reagent (Invitrogen, Carlsbad, CA), and mRNA was purified using Oligotex mRNA Mini Kit (Qiagen, Valencia, CA) according to manufacturers' instructions. Microarray hybridizations were carried out by AFGC (*Arabidopsis* Functional Genomics Consortium, Michigan State University, East Lansing) [12], using two technical replicates with reverse labeling for each TNT concentration. For microarray data analysis and identification of TNT regulated genes, results were first filtered on the basis of regression coefficient ($R^2 \geq 0.5$). Ratios were calculated without background subtraction for low abundance transcripts with both channel intensities below 1000. For the remaining transcripts, ratios were calculated after background subtraction, and then all ratios were normalized for channel intensities. Average ratios and standard deviations were calculated for the two technical replicates and a 1.7-fold up- or downregulation was retained as the threshold for significant differential expression.

2.3. Bioinformatics

For EST clones corresponding to differentially regulated genes, additional gene and chromosome locus assignments were made by BLASTN searches against the GenBank nucleotide database. Functional classification of the corresponding protein was according to automatically derived functional categories from the MIPS *Arabidopsis thaliana* database (http://mips.gsf.de/proj/thal/db/index.htm).

For each group of differentially regulated genes, the proximal 500 nucleotides from the transcription start site were retrieved from the AGI database (www.arabidopsis.org) and sequence analysis was performed using the computer programs MEME (version 3.0) [13] and MAST (version 3.0) [14]. The data of both programs were processed on the Cray T3E supercomputer at the San Diego Supercomputer Center accessed through the internet http://www.sdsc.edu/meme. For MEME, the free parameters of the analysis were set as the following: (i) the occurrences of a single motif distributed among the sequences were any number of repetitions; (ii) the maximum number of motifs to find was 10; (iii) the optimum width of each motif was from 6 to 50 nucleotides; (iv) both strands of DNA were searched. For MAST, only MEME motifs with an E-value < 1 were presented, and the reverse complement DNA strand was considered with the forward orientation in the search. The motifs discovered by MEME were scanned for the presence of known plant *cis*-regulatory elements in the PLACE database [15].

2.4. Real-time RT-PCR

Total RNA was extracted from shoots and roots of 1 g frozen *Arabidopsis* plants using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two samples were extracted for each treatment. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis through agarose gels stained with ethidium bromide. Total RNA (4 μg) was reverse transcribed in the presence of random primers using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).

Real-time quantitative RT-PCR, based on TaqMan methodology was performed using the Smart-Cycler II System (Cepheid, Sunnyvale, CA). PCR was performed in a total volume of 25 μL containing 1x TaqMan buffer; 3.5 mmol/L $MgCl_2$; 200 μmol/L each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 400 μmol/L deoxyuracil triphosphate; 300 nmol/L each primer; 300 nmol/L probe; 0.5 U of AmpErase uracil *N*-glycosilase; 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), 2 μL of cDNA equivalent to 100 ng total RNA.

Primers and probes were chosen using the Primer 3 program [16] to have melting temperatures of 58–60 and 70 °C, respectively. The primers, whenever possible were placed in different exons and amplified products of about 100 bp. Probes were labeled in 5′ with FAM and in 3′ with TAMRA. To normalize the amount of total RNA present in each reaction, we co-amplified the housekeeping gene encoding Elongation Factor 1α (EF1α). The EF1α probe was labeled with TET instead of FAM. Primers and probes are listed in Table 1.

All amplification reactions were carried out at 60 °C for 1 min followed by a denaturation step of 15 s at 95 °C for 40 cycles after an initial hot start of 10 min at 95 °C. Each sample was run in duplicate. PCR efficiencies for each amplicon were calculated using serial dilutions from a reference sample that was a mix of equal amounts of cDNA from roots and shoots of different samples pooled together. Relative amounts of specific mRNAs in the reference sample and the test samples were calculated as described by Pfaffl [17] and expressed as percentages.

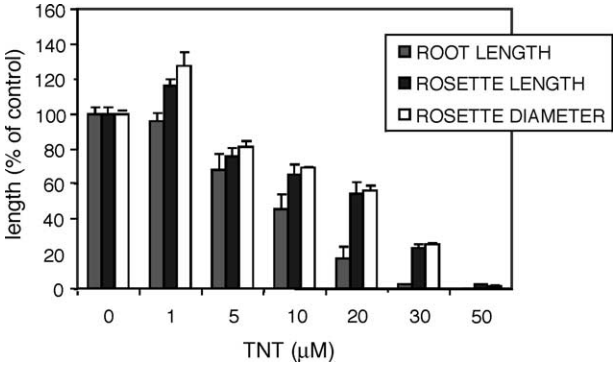

Fig. 1. Growth of *Arabidopsis* ecotype Col1 plants exposed to TNT. Plants were grown on media supplemented with 0–50 μM TNT then plants were measured (roots, rosette length and diameter) after 21 days. Bars indicate S.D. from $n = 6$.

3. Results

3.1. Microarray analysis

A. thaliana ecotype Col1 plants were grown from 0 to 50 μM TNT added to sterile media to assess the toxicity of TNT and to determine the appropriate concentrations for microarray experiments. Growth was reduced at increasing concentrations of TNT except at 1 μM TNT, where a slight hormetic increase in growth was observed (Fig. 1). At 10 μM, root length was significantly reduced by about 50% compared to the control. Given the differing effects of TNT at low and high concentrations, we chose to examine the transcription profiles at 1 and 10 μM TNT and examine overlaps indicative of TNT specific induction at relatively low and high concentrations. Plants were exposed to TNT for 10 days before mRNA from control and treated plants was extracted. It was estimated that more than 50% of the TNT was present in the media at the end of the experiment, given the very slow rate of TNT clearance by *Arabidopsis* [18]. cDNAs were hybridized to *Arabidopsis* slides containing about 14,000 unique clones. The list of EST clones and microarray data are publicly available at the Stanford Microarray Database (http://genome-www5.stanford.edu/MicroArray/SMD). Clones that are differentially expressed were ranked by the fold induction or repression ratio and are

Table 1
Primers and probes used in TaqMan RT-PCR assays

	Forward and reverse primers 5′–3′	Probe 5′–3′
EF1α	GAGCACGCTCTCCTTGCT TCGTACCTGGCCTTGGAG	TCTGCTGTTGTAACAAGATGGATGCCACTA
PR1 (At4g33720)	GCAGCGGTAGCATGACAG CACACTGTTTGTCCCATGC	TGGGTGGACGAGCAATTTGACTACGATTAT
Unknown protein (At5g48850)	ATGGCTTTCCAAAACCACAC TCCCTGTGGAGACACTCCTT	TCTCTATCAACTGAGCATGTTTGGCTCTGA
DNA-binding protein-like (At5g61600)	ACCGCCCTTACCGAATCTAT CCACGGTCTTCGTCTCACTC	CTCGAGAACGGTTTCAACAAAGACAGAGAA
Unknown protein (At5g42530)	GCGGAGGAGAAGGTAGGTTT CTTGGCGCACAAGAGGAA	CATCTGAAACGAGGAATCCTTTGTTTCTGG
ABC transporter-like (At3g55130)	GAGGCAGGGACACAAGAGAG TGTCCCCGTTTTTATCCAAG	ATGTTCTGTACGCGTTGTGTGTTGTCTCTC

listed in Table 2 for 1 μM TNT and in Table 3 for 10 μM TNT. At 1 μM TNT, 14 clones were upregulated, and 19 clones are downregulated. The latter 19 clones mapped to 13 loci, showing substantial redundancy of ESTs on the array as well as consistency of microarray results. More genes were differentially regulated at 10 μM TNT. Forty-three clones (39 loci) were upregulated, and 43 other clones (34 loci) were downregulated.

There was very little observed overlap between genes that were differentially expressed at 1 and 10 μM TNT. Two downregulated transcripts at 1 μM TNT corresponded to a putative protein (At5g24030), and the small nuclear ribonucleoprotein U1A were also downregulated at 10 μM TNT. Three transcripts that were downregulated at 1 μM (PR1-like, osmotin precursor and unknown protein At3g22240) were upregulated at 10 μM.

3.2. Genes differentially expressed at 1 μM TNT

Low concentrations of TNT predominantly affected genes putatively involved in signaling pathways: transcription factors, kinases and ABA synthesis. Two genes encoding ethylene-response element binding transcription factors were upregulated at 1 μM TNT. Ethylene responsive factors have conserved DNA binding domains and interact with the GCC box in the ethylene-responsive element that is necessary and sufficient for the regulation of transcription by ethylene [19]. While most ethylene responsive factors including EREBP-4 transactivate gene expression, some function as repressors of transcription [20,21].

At 1 μM TNT, the transcript corresponding to neoxanthin cleavage enzyme is upregulated. This enzyme catalyzes the first committed step in the synthesis of the plant hormone ABA, which, in turn, is involved in the adaptation to various environmental stresses such as drought, salinity and cold [22]. Yet, low levels of TNT appear to suppress defense related genes such as PR-1 like and an osmotin precursor. Osmotin and osmotin-like proteins are members of the thaumatin-like, PR-5 family of the PR proteins. Additionally a putative aminotransferase gene similar to L-2,4-diaminobutyrate-2-ketoglutarate 4-aminotransferase from *Acinetobacter baumannii* was downregulated. This enzyme leads to the formation of one of the unusual amino acid, diaminobutyrate found in peptide antibiotics in bacteria and plants [23].

Among other genes whose expression level was affected at low levels of TNT was that encoding fructose bisphosphate aldolase, an essential glycolytic enzyme found in all vertebrates and higher plants catalyzing the cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP). It is involved in the pentose phosphate pathway whose function in the cell is the formation of ribose-5-phosphate for nucleic acid synthesis and the generation of NADPH, a compound required for various biosynthetic pathways as well as for the stability of catalase and the preservation and regeneration of the reduced form of glutathione (GSH).

Upregulation of a gene coding a pectin methylesterase like protein might be indicative of the effects TNT on the cell wall. The function of plant pectin methylesterases is to dimethylesterify homogalacturonans in the plant cell wall. Physiological roles attributed to pectin methylesterases are cell wall stiffening, maintenance of extracellular pH, elongation of the cells within the root tip, cell-wall degradation leading to border cell separation and stem elongation [24–26].

Table 2
Differentially expressed clones at 1 μM TNT

Clone ID	Description	Putative functional category	Locus	Ratio	S.D.
1.7-fold upregulated by 1 μM TNT					
F21J6_103	Ethylene-responsive element-like protein; ethylene-responsive element binding protein homolog, *Stylosanthes hamata*	Transcriptional control/unspecified signal transduction	At5g25190	2.35	0.65
T18B16_140	Neoxanthin cleavage enzyme-like protein; neoxanthin cleavage enzyme, *Lycopersicon esculentum*	Metabolism/C-compound, carbohydrate catabolism	At4g19170	2.30	1.24
M49C7STM	*Arabidopsis* chloroplast genome		AP000423	2.26	1.86
K24G6_19	Putative protein	Unclassified	At5g48850	2.04	0.52
M22B6STM	Putative protein	Unclassified	At5g64190	1.91	0.43
93M23T7	GDSL-motif lipase/hydrolase family protein	Metabolism/biosynthesis of derivatives of homoisopentenyl pyrophosphate/breakdown of lipids, fatty acids and isoprenoids	At5g33370	1.86	0.46
M44A4STM	DNA-binding protein-like similar to EREBP-4, *Nicotiana tabacum*	Transcriptional control	At5g61600	1.84	0.81
MNJ7_9	Pectin methylesterase-like protein	Cell wall/C-compound, carbohydrate catabolism	At5g47500	1.83	0.21

Table 2 (*Continued*)

Clone ID	Description	Putative functional category	Locus	Ratio	S.D.
F4P13.34	Hypothetical protein	Photosynthesis/ transport/protein modification, folding and destination	At3g01580	1.83	1.67
178E18T7	Fructose-bisphosphate aldolase-like protein	C-compound metabolism/glycolysis and gluconeogenesis/pentose-phosphate pathway	At4g26530	1.83	0.42
M42E3STM	Phosphatidylinositol 4-kinase-like protein	Unspecified signal transduction/second messenger mediated signal transduction/cell division	At5g09350	1.81	1.32
C18G5_30	Putative protein, similar to disease resistance protein RPP1-WsA	Resistance proteins/ defense related proteins	At4g08450	1.79	1.60
MVA3_20	Unknown protein	Unclassified	At5g17670	1.74	0.95
F25I24_120	Hypothetical protein	Unclassified	At4g10910	1.72	0.28
>1.7-fold downregulated by 1 μMTNT					
111G9T7	Patatin-like protein, similar to latex allergen from *Hevea brasiliensis*	Storage protein	At2g26560	2.76	0.81
PR-1	Pathogenesis-related PR-1-like protein	Disease, virulence and defense	At2g14610	2.63	1.14
198G3T7	Unknown protein, similar to mitochondrial processing peptidase 55K protein precursor (potato)	Unclassified	At3g22240	2.52	0.24
196H13RTM	Unknown protein, similar to mitochondrial processing peptidase 55K protein precursor (potato)	Unclassified	At3g22240	2.43	0.17
245H8T7	Unknown protein, similar to mitochondrial processing peptidase 55K protein precursor (potato)	Unclassified	At3g22240	2.24	0.21
220O22T7	Myo-inositol oxygenase-like protein	Unclassified	At2g19800	2.23	1.06
186C24T7	Putative aminotransferase, similar to L-2,4-diaminobutyrate-2-ketoglutarate 4-aminotransferase related protein *T. acidophilum*	Amino acid metabolism	At3g22220	2.19	0.53
163B24T7	Unknown protein	Unclassified	At3g22240	2.17	0.09
60E3T7	Nodulin-like protein; similar to nodulin, *Glycine max*	Unclassified	At5g25250	2.14	0.52
T4M8.14	EST Not found		2.14	0.91	
247C14T7	Osmotin precursor	Unspecified signal transduction/disease, virulence and defense	At4g11650	2.10	1.11
222H13T7	Unknown protein, similar to mitochondrial processing peptidase 55K protein precursor (potato)	Unclassified	At3g22240	2.06	0.36
97O17T7	Putative protein; similar to various predicted proteins	Unclassified	At5g26290	2.05	1.58
127P22T7	Putative aminotransferase, similar to L-2,4-diaminobutyrate-2-ketoglutarate 4-aminotransferase related protein (*T. acidophilum*)	Amino acid metabolism	At3g22220	1.99	0.07
204I18T7	Putative protein, similar to naringenin-chalcone synthase whp1	Unclassified	At2g14560	1.78	0.17
158P16T7	Phi-1-like phosphate-induced protein	Unclassified	At5g64260	1.77	0.07
MZF18_9	C4-dicarboxylate transporter/malic acid transport family protein, weak similarity to Tellurite resistance protein tehA. (*Escherichia coli*)	Unclassified	At5g24030	1.76	0.23
38C6T7	Small nuclear ribonucleoprotein U1A	mRNA processing	At2g47580	1.74	0.15
142E11T7	Band 7 family protein, similar to hypersensitive-induced response protein (Zea mays)	Stress response	At3g01290	1.73	0.03

Four leaf stage *Arabidopsis* plants were transferred to media containing either 0 or1 μM TNT and grown for 10 days. Ratio for differential gene expression was calculated as transcript abundance in TNT treated plants divided by transcript abundance in control plants for upregulated genes (and the inverse for downregulated genes). S.D.: standard deviation of ratio.

Table 3
Differentially expressed clones at 10 μM TNT

Clone ID	Description	Putative functional category	Locus	Ratio	S.D.
1.7-fold upregulated by 10 μM TNT					
196A24M4	Pathogenesis-related protein 1 precursor, 19.3K	Disease, virulence and defense	At4g33720	8.58	0.31
103C7T7	Unknown protein	Unclassified	At5g42530	2.41	0.39
222M12T7	RING-H2 finger protein RHF2a	Unclassified	At5g22000	2.36	1.88
161P11T7	Scarecrow transcription factor family protein; similar to lateral suppressor protein (*Lycopersicon esculentum*)	Transcriptional control/plant development	At3g49950	2.30	0.04
F7K2_80	Putative protein, similar to glycine-rich cell wall protein precursor	Unclassified	At4g22500	2.26	0.01
CORI1	Coronatine-induced protein 1, similar to carboxymethylenebutenolidase (*Synechocystis* sp.)	Unclassified	At1g19670	2.19	0.84
G5F5T7	Subtilisin-like serine protease	Protein degradation	At5g59090	2.18	0.32
M25E7XTM	Putative protein	Unclassified	At4g26850	2.16	0.51
88C3T7	Putative peroxidase, similar to cationic peroxidase (gi 1232069)	Disease, virulence and defense/detoxification	At1g30870	2.16	0.04
140F6T7	Root cap protein 2-like protein	Plant development	At5g54370	2.14	0.94
244K16T7	Unknown protein, weak similarity to ubiquitin-specific protease 12 (*Arabidopsis thaliana*)	Unclassified	At3g20380	2.14	0.04
218C6T7	Putative expansin	Cell wall	At2g40610	2.13	0.15
37F10T7	Antifungal protein-like (PDF1.2)	Unclassified	At5g44420	2.13	0.06
123O13T7	Photoassimilate-responsive protein PAR-1b-like protein	Unclassified	At3g54040	2.12	0.30
P1178	Ribonuclease 3 precursor	Polynucleotide degradation	At1g26820	2.09	1.40
134M11T7	Inorganic phosphate transporter (PHT2)	Phospate transport	At5g43370	2.08	0.09
157E12T7	Hypothetical protein	Unclassified	At1g75360	2.08	0.08
G10B1T7	Unknown protein	Unclassified	At4g26850	2.03	0.05
200L10T7	Inorganic phosphate transporter (PHT1)	Phospate transport	At5g43350	2.02	0.20
G8H1T7	ABC transporter-like protein, strong similarity to breast cancer resistance protein 1 BCRP1 (*Mus musculus*)	ABC transporters	At3g55130	1.96	0.00
124L13T7	Anthranilate *N*-benzoyltransferase	Defense related proteins/secondary metabolism	At5g48930	1.95	0.14
M40G10STM	Unknown protein; similar to harpin-induced protein hin1 (*Nicotiana tabacum*)	Resistance proteins	At3g11660	1.94	0.23
141F5T7	Putative protein; contains similarity to lipase	Breakdown of lipids, fatty acids and isoprenoids	At5g14180	1.92	0.19
PR-1	Pathogenesis-related PR-1-like protein	Disease, virulence and defense	At2g14610	1.91	0.63
165M6T7	Proline-rich protein, similar to proline-rich protein precursor (*Glycine max*)	Unclassified	At3g62680	1.90	0.40
241F8T7	Protein kinase -like protein; similar to protein kinase APK1	Unspecified signal transduction	At3g54030	1.87	0.57
169C12T7	Putative peroxidase ATP12a, strong similarity to *Arabidopsis* peroxidase ATP11A	Disease, virulence and defense/detoxification	At1g05240	1.85	0.29
104K7T7	Unknown protein	Unclassified	At2g25510	1.84	0.39
47F10T7	Unknown protein	Unclassified	At5g55620	1.79	0.16
230I23T7	Nitrate transporter (NTP3)	Anion transport/peptide transport	At3g21670	1.78	0.04
204N5XP	Putative peroxidase, strong similarity to peroxidase ATP13a	Disease, virulence and defense/detoxification	At4g26010	1.77	0.10
186F14T7	Ribonucleotide reductase small subunit, putative	Deoxyribonucleotide metabolism	At3g27060	1.77	0.28
F12L6.15	F-box family protein, similar to SKP1 interacting partner 2 (SKIP2)	Unclassified	At2g39490	1.77	0.29
M25B5XTM	Anthranilate *N*-benzoyltransferase	Defense related proteins/secondary metabolism	At5g48930	1.76	0.34
247C14T7	Osmotin precursor	Unspecified signal transduction/disease, virulence and defense	At4g11650	1.75	0.54
84B4T7	Late embryonic abundant protein-like; similar to late embryonic abundant protein EMB7, white spruce	Development	At5g60530	1.74	0.55

Table 3 (*Continued*)

Clone ID	Description	Putative functional category	Locus	Ratio	S.D.
130M20T7	Beta-1,3-glucanase class I precursor	C-compound, carbohydrate catabolism/defense related proteins	At4g16260	1.72	0.26
278B7T7	Anthranilate *N*-benzoyltransferase	Defense related proteins/secondary metabolism	At5g48930	1.72	0.12
94E10XP	Putative UDP-glucose glucosyltransferase	Biosynthesis of glycosinolates and derivatives/biosynthesis of phenylpropanoids/d etoxification	At3g21760	1.71	0.36
G8E10T7	ABC transporter-like protein; strong similarity to breast cancer resistance protein 1 BCRP1 (*Mus musculus*),	ABC transporters	At3g55130	1.71	0.02
163B24T7	Unknown protein, similar to mitochondrial processing peptidase 55K protein precursor (potato)	Unclassified	At3g22240	1.71	0.09
171M2T7	Arabinogalactan-protein (AGP3)	Unclassified	At4g40090	1.71	0.16
139B18XP	Pectinesterase	C-compound, carbohydrate metabolism/ cell wall	At5g04960	1.70	0.23
>1.7-fold downregulated 10 μM					
122C15T7	Glutamine-dependent asparagine synthetase	Amino acid biosynthesis	At3g47340	4.24	0.03
207C15T7	Glutamine-dependent asparagine synthetase	Amino acid biosynthesis	At3g47340	3.73	0.43
38C6T7	Small nuclear ribonucleoprotein U1A	mRNA processing	At2g47580	3.46	0.27
108O17XP	Expansin-like protein (AtEXLA1), similar to cim1 induced allergen, (*Glycine max*)	Cell wall	At3g45970	2.84	0.00
123A7T7	Unknown protein	Unclassified	At1g50040	2.79	0.09
146L14XP	Xyloglucan endotransglycosylase TCH4 protein	C-compound, carbohydrate metabolism/cell wall	At5g57560	2.74	0.08
171P10T7	Glucose transporter (STP1)	C-compound, carbohydrate transport	At1g11260	2.73	0.02
184O7T7	Putative phosphate-induced (phi-1) protein, similar to phi-1 (phosphate-induced gene) (*Nicotiana tabacum*)	Unclassified	At1g35140	2.69	0.13
MZF18_9	C4-dicarboxylate transporter/malic acid transport family protein, weak similarity to Tellurite resistance protein tehA. (*Escherichia coli*)	Unclassified	At5g24030	2.56	0.20
203K11T7	Beta-xylosidase, putative, similar to beta-xylosidase GI:2102655 from (*Aspergillus niger*)	C-compound, carbohydrate catabolism/cell wall	At1g02640	2.29	0.23
144L3XP	Purine permease-related protein	Unclassified	At1g57990	2.17	1.02
182A9T7	Glucose transporter (STP1)	C-compound, carbohydrate transport	At1g11260	2.16	0.06
193J13T7	Unknown protein	Unclassified	At1g11380	2.12	0.51
M15F6STM	Xylosidase	C-compound, carbohydrate catabolism/cell wall	At5g49360	2.10	0.80
T6B20.5	Unknown protein	Unclassified	At2g30600	2.05	0.07
125L9T7	Imbibition protein homolog, strong similarity to probable imbibition protein (*Brassica oleracea*)	C-compound, carbohydrate anabolism	At3g57520	2.03	0.13
166K5T7	Putative protein	Unclassified	At5g63160	2.01	0.17
40B7T7	Putative protein, similar to MTD1 (*Medicago trunculata*)	Unclassified	At5g21940	2.00	0.74
94L12T7	Seed imbitition protein-like; similar to seed imbitition protein Sip1 (*Hordeum vulgare*)	C-compound, carbohydrate anabolism	At5g20250	1.98	0.22
MMG15.4	UV hypersensitive protein (UVH3)	DNA repair	At3g28030	1.98	0.42
165G22T7	Xylosidase	C-compound, carbohydrate catabolism/cell wall	At5g49360	1.97	0.10
182A10T7	Glucose transporter (STP1)	C-compound, carbohydrate transport	At1g11260	1.93	0.40
T31E10.6	Hypothetical protein	Unclassified	At2g34600	1.92	0.17
187F3XP	Putative protein, similar to several hypothetical proteins (*Arabidopsis thaliana*)	Unclassified	At3g62540	1.90	0.32
F24J7_90	TMV resistance protein N-like; TMV resistance protein N (*Nicotiana glutinosa*)	Defense related proteins/stress response	At4g19530	1.88	0.41
M30A10STM	Unknown protein	Unclassified	At2g30600	1.88	0.08
222P23T7	Nodulin-like protein	Unclassified	At5g14120	1.86	0.15
124C21XP	Putative sugar transport protein, ERD6	C-compound, carbohydrate transport	At1g08930	1.85	0.37
191D5T7	Unknown protein	Unclassified	At3g19680	1.82	0.05
167G20XP	Purine permease-related protein	Unclassified	At1g57990	1.81	0.19
245O8T7	Xylose isomerase	C-compound, carbohydrate anabolism	At5g57655	1.81	0.08

Table 3 (*Continued*)

Clone ID	Description	Putative functional category	Locus	Ratio	S.D.
96D6T7	Unknown protein, similar to chitinase precursor yeast (*Saccharomyces cerevisiae*)	Unclassified	At2g30930	1.80	0.10
172P5T7	Protein kinase-like protein, strong similarity to Pto kinase interactor 1 (*Lycopersicon esculentum*)	Signal transduction, key kinases	At3g59350	1.76	0.09
123N22T7	Blue copper binding protein	Transcriptional control	At5g20230	1.74	0.31
F1O11.20	Putative receptor-like protein kinase	Transmembrane signal transduction	At2g36570	1.73	0.46
172B21T7	Receptor-protein kinase-like protein	Transmembrane signal transduction	At5g54380	1.77	0.01
M21D9XTM	Imbibition protein homolog, strong similarity with probable imbibition protein (*Brassica oleracea*)	C-compound, Carbohydrate anabolism	At3g57520	1.79	0.01
122I20XP	Putative protein, similar to NPR1-like protein (*A.thaliana*)	Unclassified	At5g67480	1.72	0.15
282B11T7	Xylose isomerase	C-compound, carbohydrate anabolism	At5g57655	1.77	0.28
F18O22_240	Early nodule-specific protein-like, strong similarity to early nodule-specific protein (*Medicago trunculata*)	Biosynthesis of derivatives of homoisopentenyl pyrophosphate	At5g14450	1.71	0.55
172C23T7	Serine carboxypeptidase II-like protein	Protein degradation	At5g23210	1.71	0.31
41G10T7	Unknown protein, similar to TOM71 protein with similarity to Tom70p/Mas70p (*S.cerevisiae*)	Unclassified	At2g42580	1.79	0.14
M56E10STM	Seed imbitition protein-like, seed imbitition protein Sip1 (*Hordeum vulgare*)	C-compound, carbohydrate anabolism	At5g20250	1.73	0.10

Four leaf stage *Arabidopsis* plants were transferred to media containing either 0 or10 μM TNT and grown for 10 days. Ratio for differential gene expression was calculated as transcript abundance in TNT treated plants divided by transcript abundance in control plants for upregulated genes (and the inverse for downregulated genes); S.D.: standard deviation of ratio.

3.3. Genes differentially expressed at 10 μM TNT

TNT induced a large number of defense related genes at 10 μM. The most notable is PR-1 with more than eight-fold induction. PR-1 is strongly induced by diverse biotic and abiotic stresses including ethylene, wounding and SA (salicylic acid). Other pathogenesis-related (PR) proteins induced were a PR-1 like protein, an antifungal protein-like (PDF1.2), a class I beta-1,3-glucanase, a protein similar to hin1 and an osmotin precursor. In this category, there were three peroxidases, enzymes whose function is related to oxidative metabolism.

Two upregulated transcripts encoded enzymes potentially involved in TNT transformation. Glucosyltransferases catalyze the transfer of sugar from nucleoside diphosphate donors to a wide range of acceptor molecules in processes that include detoxification of xenobiotics [27]. Anthranilate benzoyltransferases catalyze the transfert of a wide variety of aromatic acyl-CoA donors to anthranilate [28]. Both enzymes may have a direct role in conjugation of TNT derived metabolites.

A large group of differentially regulated genes encode transport proteins. Two phosphate transporters (PHT1, PHT2) and a nitrate transporter (NTP3) were upregulated, while a glucose transporter (STP1) and a putative sugar transport protein (ERD6) were downregulated. Transporters can also mediate tolerance to a wide diversity of cytotoxic agents. Multiple drug resistance proteins from mammals and yeast decrease the toxicity of a variety of anti-tumorigenic drugs. The ABC transporter At3g55130 induced by TNT showed strong similarity to breast cancer resistance protein 1 (BCRP1) from mouse that mediate substantial resistance to mitoxanthrone [29].

The effect of TNT on plant growth and development is reflected by the differential regulation of genes such as root cap protein 2-like and lateral root suppressor, a transcription factor involved in the initiation of lateral root development. A number of proteins whose functions are associated with the cell wall were also differentially regulated. Genes encoding a glycine-rich cell wall protein and an α-expansin were upregulated. Initial work on α-expansins established several close correlations between growth and α-expansin transcripts and proteins [30]. In semi-aquatic plants, expansins are induced by hypoxia, submergence and ethylene [31–33]. More recent in vivo experiments suggest an important role in growth and cell wall modifications that involve the hydrolysis of cell wall components. It is likely that expansins facilitate the access of hydrolytic enzymes to cell wall polymers. On the other hand, a protein similar to cim1 (expansin), a xyloglucan endotransglycosylase (TCH4), a β-xylosidase, a xylosidase and a xylose isomerase are downregulated.

3.4. Promoter analysis

Our ultimate interest is the exploitation of inducible promoters for phytosensor applications [34]. Thus, promoter analysis is interesting for both characterized and uncharacterized genes. In *Arabidopsis*, the average intergenic distance is about 500 bp and for in silico analysis, the relevant length of promoter is often considered to be about 300–500 bp [35]. Therefore, search of conserved motifs was carried out on the proximal 500 nucleotides from the transcription start site for each group of co-regulated genes. The MEME analysis generated a total of 18 conserved motifs for genes differentially regulated by TNT (Table 4). The

Table 4
Motifs found using the MEME program for each group of co-regulated transcripts and *cis*-regularory elements (underlined and/or bold character) found within the motif in the PLACE database

Motif	Width	Best possible match sequence 5′–3′	*Cis*-regulatory elements
Upregulated 1 μM TNT			
1	37	CCCCTACGTGGTAAGAAGTCTCCAAGTG**ACGT**GGCAG	ACGT core (2), ARE[a]
2	27	CTGGCTCCAATCCCCCCCTCTCCCTCC	HSE[b]
3	20	ATGGGTACAAGAACATCGCA	'CAANNNNATC motif'
4	15	CACGTTTTGCTCGTT	ACGT core
5	29	AAAAAA***GAAA***AAAGCTA***GGAAAA***GCATGA	DOF core (3), GT-1 (2), 'AGAAA motif'
6	19	CTTGGGCCTGTGGAGATGG	No match
7	47	ATAAGGTTTCGCTGCATCCGATTTTCGCTAAAGTATGTGGGAGAGGT	GT element, DOF core, Box-C
Downregulated 1 μM TNT			
8	11	CTCTTCTCTTC	No match
9	21	GCCCTACCGAACCCGGCCACC	Myb homolog maize P core
Upregulated 10 μM TNT			
10	15	AAGAGACA**AAAG**AGA	SEBF[c], DOF core
11	15	GAGGGGGGCGGAGGG	No match
12	21	GCCTATCGCTAGACTCTCCAC	'GATA box'
13	16	TCACATCTCTCGGGCC	'GTGA motif'
Downregulated 10 μM TNT			
14	21	CCGTCTCTTCTTCTCCCTCTC	No match
15	21	GCCGGGGGCTTGGGGGTTTGG	No match
16	21	CGTGGCAACACCT**CGACGG**TG	RAV1 binding domain'CGACG motif', 'CCGTCG motif'
17	15	ACACACACACAGACA	No match
18	15	T**AGAAAG**AAAAAAAA	'AGAAA motif', DOF core

[a] ARE: antioxident response element.
[b] HSE: heat shock element.
[c] SEBF: silencing element binding factor.

motifs found using the MEME program were searched for known regulatory elements in the PLACE database [15] to elucidate more biologically relevant motifs. In most cases, the identified motifs matched known *cis*-regulatory elements.

The largest number of motifs, 7, was obtained among the group of genes upregulated at 1 μM TNT. Motif 1 contains two ACGT sequences representing the core binding sequences of plant basic leucine zipper (bZIP) family of transcription factors [36]. The sequences adjacent to the ACGT core, which provide specificity to a particular bZIP transcription factor are different. Thus, the first ACGT core does not match any known *cis*-regulatory element in the PLACE database, whereas the second matches response elements induced by various stimuli, including light [37], xenobiotic stress [38], abiotic and biotic stresses [39]. Additionally, the latter overlaps with a consensus sequence 'RGTGACNNNGC' corresponding to an ARE (antioxidant response element) necessary for activation by oxidative stress of rat glutathione *S*-transferase Ya subunit and rat NAD(P)H: quinone reductase genes [40]. As shown in Fig. 2, motif 1 occurs three times, each time adjacent to motif 4 and only in the promoter region of gene At5g48850 encoding for a putative protein. Motif 4 also contains a core ACGT sequence, for which, no match was found in the PLACE database. This conserved and repeated pattern of motifs 1 and 4 in the promoter region of At5g48850 suggests a regulatory role for those motifs. In particular, the ACGT core sequences both in motif 1 and 4 most probably correspond to specific binding sites for bZIP transcription factors yet to be identified.

Two other motifs, 3 and 7, occur twice in the promoter region of At5g48850. Motif 3 has a 'CAANNNNATC' sequence conserved in the 5′-upstream region of light harvesting complex (Lhc) genes [41]. This motif is also found in the promoter of fructose-biphosphate aldolase-like gene (At4g26530) a key enzyme of the pentose phosphate pathway. These findings suggest that the 'CAANNNNATC' sequence regulates oxidative stress responses, as in green tissues, reductant primarily originates from photosynthetic electron transport and in non-green tissues, reductant arises from the oxidative pentose pathway.

Motif 7 contains several known *cis*-regulatory elements. It includes an 'AAAG' sequence corresponding to the core DNA binding site of the Dof family of transcription factors unique to plants [42]. The Dof domain of these transcription factors is not only involved in sequence specific binding to DNA but is also thought to play a role in protein-protein interactions, such as promoting the binding of other transcription factors [43]. In particular, the promoter of the salicylic acid and H_2O_2-inducible gene glutathione *S*-transferase GST6 from *Arabidopsis* contains closely linked bZIP factor binding sites and Dof protein binding sites [44]. The Dof core sequence is flanked by a box-C, an element found in the promoter of the gene encoding for asparagine

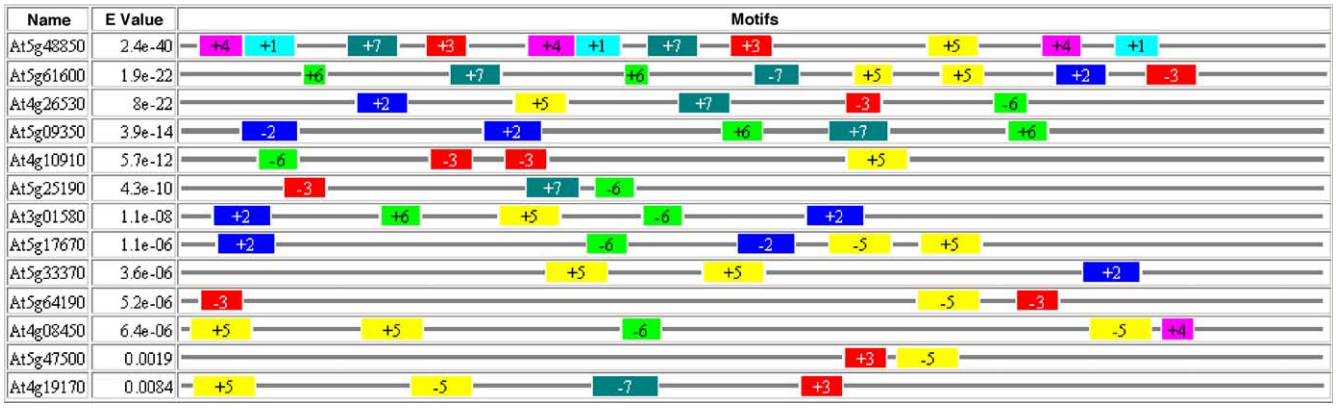

Fig. 2. Occurrence of conserved motifs in the 500 bp proximal region of the promoter of genes upregulated at 1 μM TNT. Motifs discovered with MEME program having an *E* value > 1 were presented for MAST analysis. Genes are ranked according to the combined *E* value obtained after MAST analysis. Motifs are described in Table 4. The +/− signs in front of motif numbers indicate presence in forward and reverse complementary DNA strands, respectively.

synthase (AS1) of pea [45]. Although the corresponding trans factor is not known, box-C was shown to bind to nuclear proteins and was competed by a putative repressor. Motif 7 also contains an 'AGAAA' sequence, one of two co-dependent motifs required for high level pollen specific expression of *lat* 52, a late pollen gene [46]. Overall, the nature of the elements found in motif 7 indicates a role as a modulator of other nearby elements.

The other motifs found for genes upregulated at 1 μM TNT are more widely distributed among all promoter regions. Motif 2 contains a 'CCAAT box' or HSE (heat shock element) often found in the promoter of heat shock genes and is considered to function as an enhancer of other *cis*-regulatory regions [47]. Motif 5 was the most frequently occurring motif among this group of promoter regions. It contains Dof core sequences that overlap with GT-1 consensus sequences 'GRWAAW'. GT-1 sequences are believed to interact with the transcription complex [48] and increase inducible levels of transcription [49].

Two motifs were found in the promoter region of genes downregulated at 1 μM TNT. The first, motif 8, did not match any element in the PLACE database. As shown in Fig. 3, it is the most frequently occurring motif in this group of promoters. Its biological significance, if any, remains obscure. The second, motif 9, contains the core consensus maize P (Myb homolog)-binding site [50]. Members of the Myb family regulate the biosynthesis of phenylpropanoids, including anthocyanin and phlobaphene pigments, in several plant species. The role of this element among this group of promoters is not clear as the corresponding genes in which it is found are either unknown or are not associated to phenylpropanoid biosynthesis.

Four motifs were found in the promoters of genes upregulated at 10 μM TNT. The most common is motif 10 (Fig. 4). It contains a Dof core sequence next to a binding site for the silencing element-binding factor (SEBF) that is commonly found in PR gene promoters [51]. Motif 11 is also widespread, however, its significance remains to be investigated, as it does not match any known *cis*-regulatory element.

Motif 12 contains a GATA box, an element present in light responsive genes of all type I light-harvesting

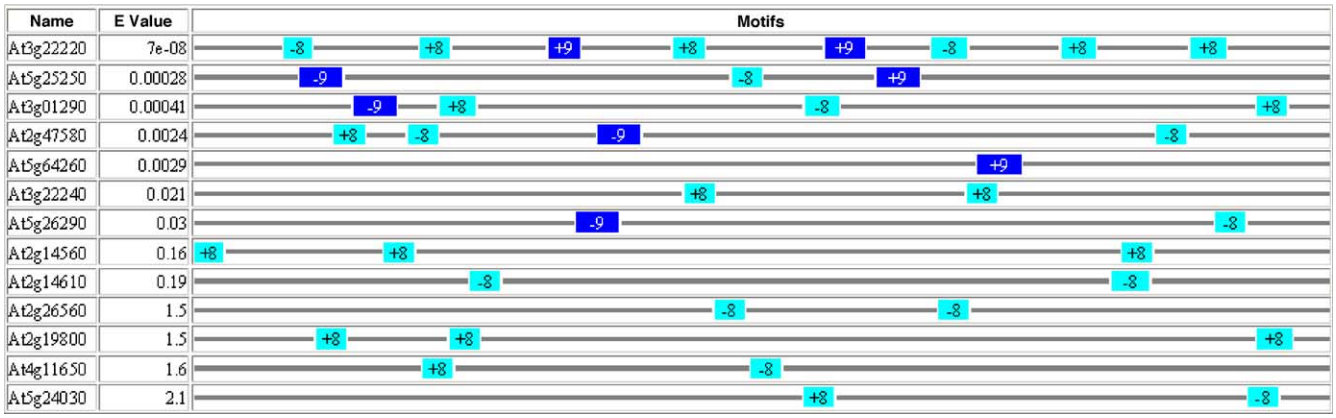

Fig. 3. Occurrence of conserved motifs in the 500 bp proximal region of the promoter of genes downregulated at 1 μM TNT. Motifs discovered with MEME program having an *E* value > 1 were presented for MAST analysis. Genes are ranked according to the combined *E* value obtained after MAST analysis. Motifs are described in Table 4. The +/− signs in front of motif numbers indicate presence in forward and reverse complementary DNA strands, respectively.

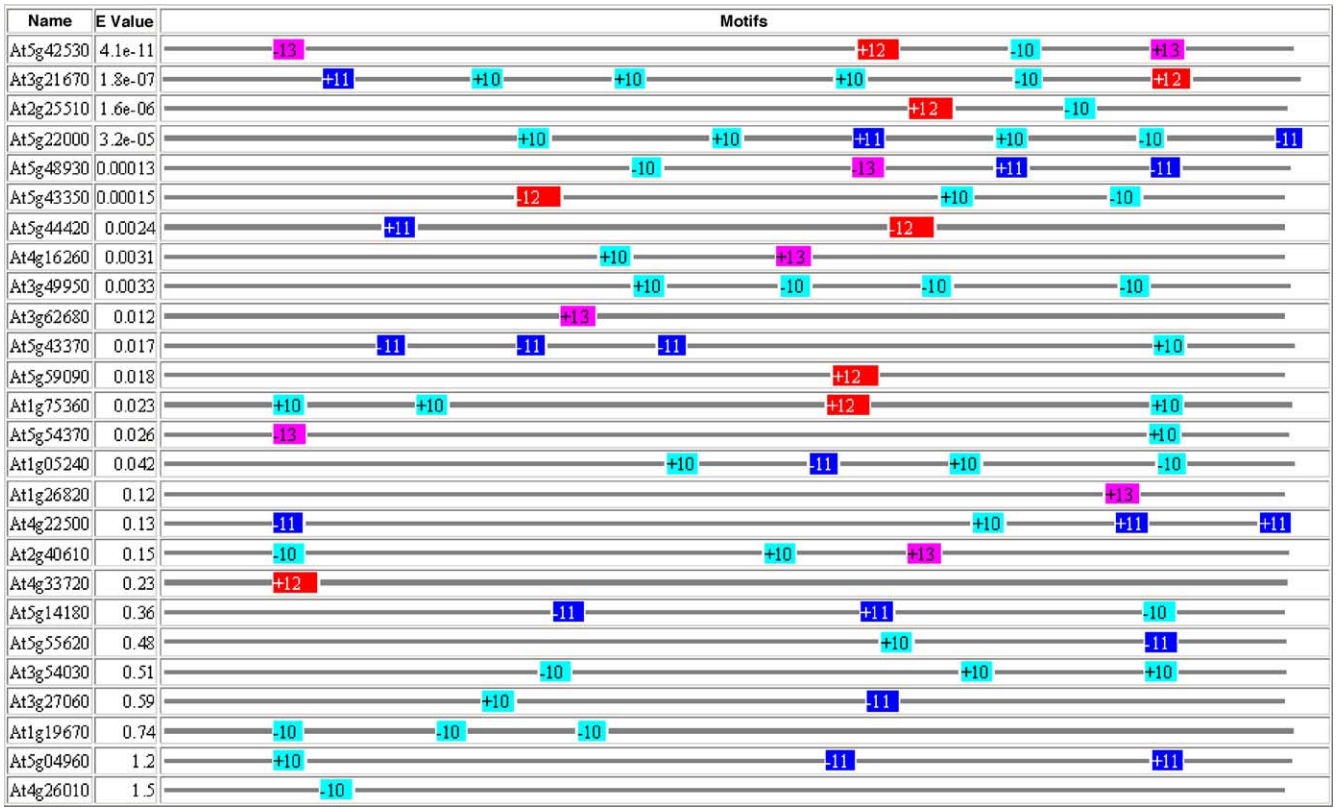

Fig. 4. Occurrence of conserved motifs in the 500 bp proximal region of the promoter of genes upregulated at 10 μM TNT. Motifs discovered with MEME program having an *E* value > 1 were presented for MAST analysis. Genes are ranked according to the combined *E* value obtained after MAST analysis. Motifs are described in Table 4. The +/− signs in front of motif numbers indicate presence in forward and reverse complementary DNA strands, respectively.

chlorophyll *a/b*-binding proteins [52]. However, in this study, it is found in the promoter of genes such as those encoding for a nitrate transporter (At3g21670), an antifungal protein-like (At5g44420) and a PR1 precursor (At4g33720). As the 'CAANNNNATC' sequence in motif 3 common to light harvesting complex gene promoters, the GATA box is likely to be associated with oxidative stress responses.

Motif 13 contains a 'GTGA motif' found in the promoter of a late pollen gene *g*10, important for high level and pollen specific expression [53]. However, this motif is distributed in the promoter of genes such as those encoding for an anthranilate *N*-benzoyltranferase (At5g48930) or a root cap protein 2-like (At5g54370) that are not pollen specific. It is, therefore, possible that novel *cis*-regulatory elements may be present within motif 13.

Five different motifs were discovered in the promoter region of genes downregulated at 10 μM TNT. Three of them did not correspond to any known elements. Among these is motif 14, the most frequently occurring sequence (Fig. 5). It is worth noting that the sequence of motif 14 contains motif 8, a widespread motif among promoters of genes downregulated at 1 μM TNT. The presence of this conserved sequence in the promoters of genes down-regulated at 1 and 10 μM TNT strongly suggests a regulatory role for this motif. Several *cis*-regulatory elements were found in motifs 16 and 18 that essentially function as coupling elements: one of the DNA binding sites of RAV1, a transcription factor that can independently bind to two distinct DNA-binding domains [54]; the hexamer 'CCGTCG motif' modulating the expression of *Arabidopsis* histone H4 gene [55] and a Dof core sequence.

3.5. Validation of expression analysis of selected genes

The expression of five genes was monitored by real-time RT-PCR analysis in roots and shoots to validate and extend the microarray data. We examined the response of the highly upregulated gene corresponding to the pathogenesis-related protein 1 precursor (At4g33720) and given our interest in identifying promoters that are specific to TNT and/or other nitro substituted compounds, we selected the two genes upregulated at 1 μM TNT (At5g48850 and At5g61600) and two genes upregulated at 10 μM TNT (At5g42530 and At3g55130) for further analysis. The choice of genes was influenced by the presence of potentially important *cis*-regulatory elements, the induction amplitude and potential role of the gene product in TNT transformation.

At5g48850 encodes for an unknown protein with a nuclear localization signal, thus, possibly a transcription factor. Similarly At5g61600 encodes for a DNA-binding protein-like related to *Nicotiana tabacum* EREBP-4. The promoter regions of both genes contained the most

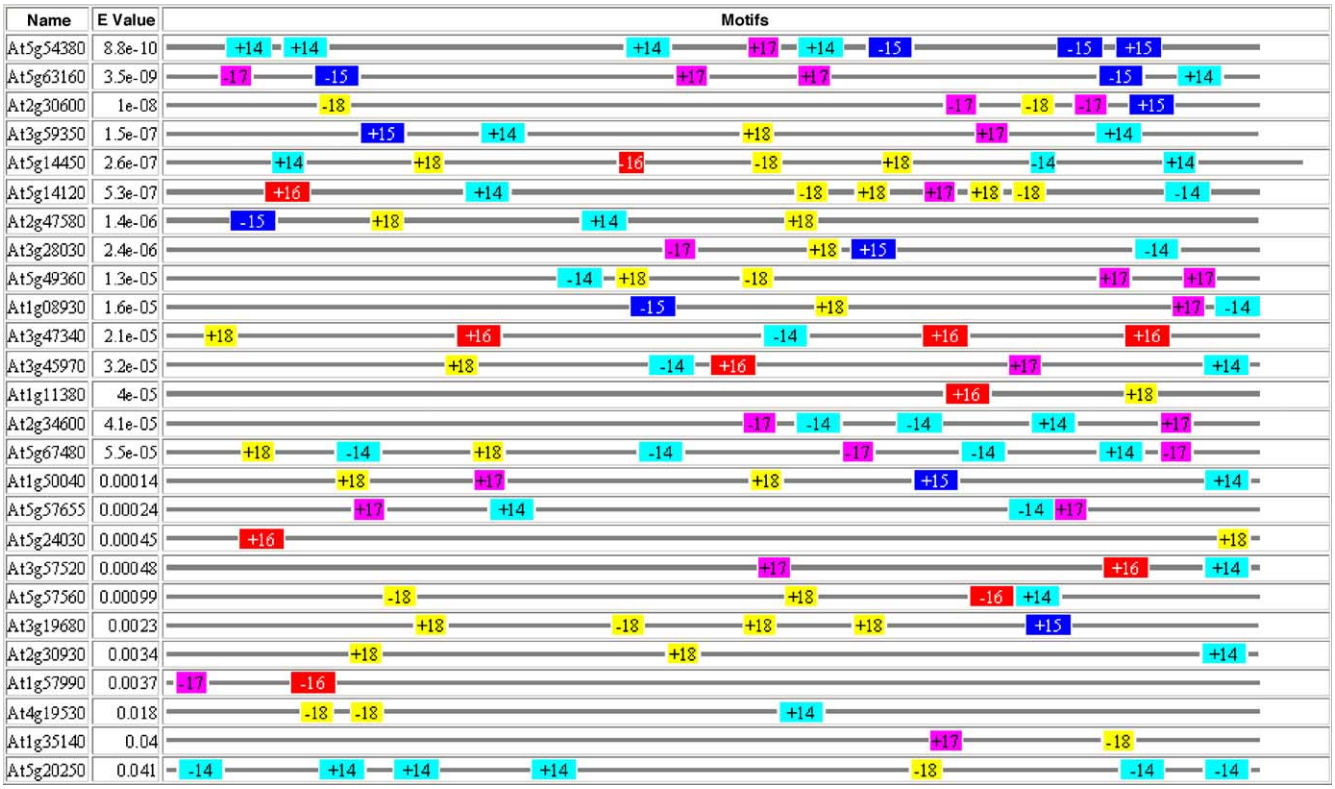

Fig. 5. Occurrence of conserved motifs in the 500 bp proximal region of the promoter of genes downregulated at 10 μM TNT. Motifs discovered with MEME program having an *E* value > 1 were presented for MAST analysis. Genes are ranked according to the combined *E* value obtained after MAST analysis. Motifs are described in Table 4. The +/− signs in front of motif numbers indicate presence in forward and reverse complementary DNA strands, respectively.

conserved motifs (highest combined *E* values) and included sequences not corresponding to known *cis*-regulatory elements.

The two genes upregulated at 10 μM TNT selected were the unknown protein (At5g42530), with the second highest upregulation ratio after the PR-1 precursor and with a promoter containing motifs 10, 12 and 13 with the highest combined *E* value. Finally, the gene encoding for an ABC transporter-like protein (At3g55130) showing strong similarity to *Mus musculus* breast cancer resistance protein 1 (BCRP1) was chosen, given the role of ABC transporters in cellular detoxification processes.

In order to dissect the specificity of their induction by TNT from a more general stress response, we also investigated their response to two other xenobiotic substances: the RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) and TDG (thiodiglycol or 1,2-ethanedithiol), a chemical resulting from the hydrolysis of sulfur mustard agent. RDX is a nitroamine explosive and has the *N*-nitro groups that are xenobiotics in the strictest sense.

However, in most studies assessing its toxicity to several plant species, little or no RDX toxicity is observed [56–58] but it was found to affect the central nervous system of animals [3]. In our study, a concentration of 20 μM RDX did not cause any visible signs of stress in *Arabidopsis* plants, whereas 20 μM TDG caused shriveling of the leaves and chlorosis.

The variation of transcript levels of the selected genes across treatments is shown in Fig. 6. The PR1 precursor transcript is strongly upregulated in roots of *Arabidopsis* plants treated with 10 μM TNT, in agreement with the eight-fold induction observed from microarray analysis (Fig. 6A). Induction of the PR1 precursor transcripts in roots was even stronger, when plants were exposed to 20 μM RDX and 20 μM TDG, respectively 10- and 13-fold. The transcript could not be detected in shoot extracts, even after the amount of cDNA used for RT-PCR was increased up to four-fold (400 ng equivalent total RNA), demonstrating that the PR1 precursor is root-specific.

Conversely, transcripts of unknown protein At5g42530 were preferentially expressed in shoots. The transcripts were detectable in roots but the amounts were about 1000-fold less than in shoots. Transcripts were upregulated after TNT and RDX treatments but not after TDG treatment. Therefore, these transcripts appear to be specific to the nitro-substituted compounds, and potentially important for phytosensing and phytoremediation of explosives.

The transcripts of both the unknown protein (At5g48850) and the DNA-binding protein-like (At5g61600) were upregulated at 1 μM TNT in shoots, as expected (Fig. 6B and C). Furthermore, the DNA binding-like protein (At5g61600) was upregulated by 10 μM TNT in shoots, but not by RDX and TDG, making it another candidate promoter phytosensing applications.

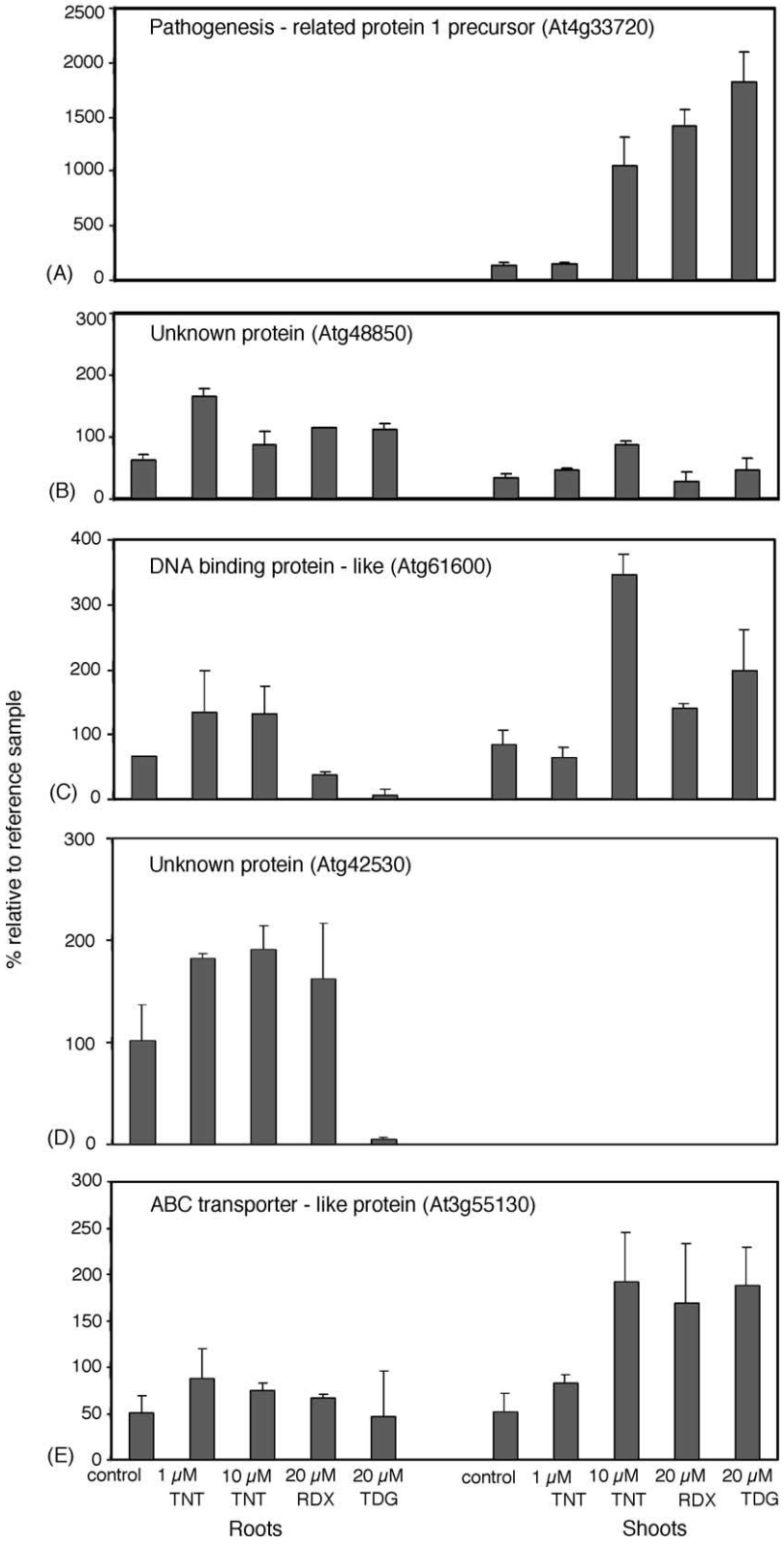

Fig. 6. Transcript abundance of selected genes as measured by TaqMan RT-PCR assays of shoot and root RNA extracts from *Arabidopsis* plants exposed to TNT, RDX and TDG (concentrations as indicated): (A) pathogenesis-related protein 1 precursor (At4g33720); (B) unknown protein (At5g48850); (C) DNA-binding protein-like (At5g61600); (D) unknown protein (At5g42530); (E) ABC transporter-like protein (At3g55130). Transcript abundance is expressed as percentage from a reference sample that was a mix of equal amounts of cDNA from roots and shoots of different samples pooled together. Bars indicate S.D. from $n = 4$ (two RNA extracts × two runs each).

The transcripts of the ABC transporter-like protein At3g55130 were equally expressed in roots and shoots of control plants. While little variation is observed in its induction levels in shoots of TNT-, RDX-, and TDG-treated plants, transcripts were upregulated more than three-fold in roots. Therefore, the 1.7-fold induction level obtained for whole plants after microarray analysis represents the combination of different levels of upregulation in roots and shoots.

4. Discussion

Xenobiotic metabolism in plants resembles that of the mammalian liver whereby the foreign compounds are metabolized following three phases [59]. An initial transformation reaction introduces functional groups by oxidation or reduction (phase I), which allows the compound to be conjugated to a hydrophilic molecule such as glutathione (phase II). Then, the product is either transported and sequestered in the vacuole or is incorporated into biopolymers such as lignin, the result of which is its compartmentation (phase III).

In this study, genes encoding for redox enzymes such as glutathione reductase and cytochrome P-450s were not found to be significantly upregulated. This finding is in contrast to results of Ekman et al. [60], who used SAGE analysis to identify *Arabidopsis* genes responsive to TNT and a microarray analysis carried out in our laboratory on *Chlamydomonas reinhardtii* [34]. The first reason that may explain the differences is that both studies used a short term exposure to TNT (24 h) in contrast to the long exposure here. Transcript levels of these primary response genes quickly go back to normal and thus, were not captured in an experiment designed for long-term exposure transcription profiling. Many redox enzymes are believed to be readily available in the cell, since TNT transformation begins within seconds after plants exposure; well before the time required for gene induction and protein synthesis [59]. Sun et al. [61] have also shown that in axenic cultures of bromegrass, prior exposure to TNT did not increase TNT metabolism, implying no induction. Another reason that could explain the discrepancies is that transcription profiling by Ekman et al. [60] was carried out on root tissue, whereas in our experiments, focused on whole plants. Roots are where TNT redox reactions occur. TNT derived metabolites found in shoots are downstream transformation products, with high molecular weights [8].

Nevertheless, similar genes potentially involved in phase II transformation of TNT were identified in this study and that of Ekman et al. [60]. Transcripts corresponding to UDP-glucose glucosyltransferase isoforms and the same anthranilate *N*-benzoyltransferase were upregulated. Unlike transcripts corresponding to redox enzymes, these transcripts obviously have a sustained upregulation over time and/or are induced in shoots.

The final fate of TNT transformation products is their incorporation in cell wall-associated fractions and to a lesser extent sequesteration in the vacuole. This assertion is supported by experiments tracing ^{14}C-labeled TNT, in which most of the radioactivity is associated with non-soluble cell fractions [62]. Once conjugated, the acceptor can exit the cytoplasm through recognition by transporters. One such transporter could be the ABC transporter At3g55130.

Overall, this study has identified a number of candidate genes and their respective promoters that can have a wide range of applications with regard to environmental problems caused by TNT contamination. Genes that favor productive, non-toxic pathways in the TNT degradation or increase overall tolerance such as UDP-glucose glucosyltransferase (At3g21760), anthranilate *N*-benzoyltransferase (At5g48930), ABC transporter (At3g55130) could be used to enhance phytoremediation capabilities. Whole promoters or defined *cis*-regulatory elements from genes specifically induced by explosives as those encoding for the unknown protein At5g42530 or the DNA-binding protein-like (At5g61600) might be fused to a reporter gene such as *gfp* to monitor explosives contamination and detect landmines using phytosensors. Coupled with phytosensors to detect TNT in aquatic systems [34], transgenic land plants could be valuable in monitoring TNT in the environment.

Acknowledgements

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