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Transferases and transporters mediate the detoxification and capacity to tolerate trinitrotoluene in *Arabidopsis*

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Abstract The effect of recalcitrant soil and water pollutant
2,4,6-trinitrotoluen (TNT) on gene expression in *Arabidop-
sis thaliana* rosettes and roots was studied separately for the
first time using microarrays. Seven-day exposure to TNT
resulted in 170 up- and 122 down-regulated genes in the
rosettes and 61 up- and 51 down-regulated genes in the
roots (expression difference > 1.5-fold; $p[t$ test$] < 0.05$). TNT
concentration, 5 µg/ml, was selected according to the dose
response analysis and study of TNT uptake from liquid
media. Although many TNT induced genes fell into
ontology groups annotated as response to biotic and abiotic
stresses in rosettes and roots, only a small overlap of TNT
effects on transcriptome was observed between rosettes and
roots. The rosettes exhibited induction of several genes
associated with toxin metabolism, such as UDP-
glycosyltransferases and ATP-binding cassette (ABC) fam-
ily transporters. On the other side, no genes known to be
involved in TNT transformation were found to be up-
regulated in the roots. The genes coding for enzymes
involved in the cell wall modifications were abundantly up-
regulated in roots. Microarray data indicated that after a
relatively long incubation with TNT (7 days), metabolism
of this xenobiotic proceeded mainly in aerial parts, while its
translocation into cell walls still took place in the roots.
Results obtained by microarray hybridization were validat-
ed by quantitative real-time reverse-transcription PCR.
Nitrate reductase 1, several glycosyltransferases and ABC
transporters, sucrose–proton symporter 2, thioredoxin-
dependent peroxidase 2, and gamma-glutamyltransferase
are discussed for their potential to enhance detoxification
and toleration capability of plants to TNT.

Keywords Microarrays · *Arabidopsis thaliana* · TNT ·
Phytoremediation · Toxicity · Xenobiotic · Transcriptome
comparison · Detoxification pathways

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Introduction

Trinitrotoluene (TNT) was used as an explosive since the
beginning of the twentieth century, and its production
accelerated during the Second World War. TNT represents,
together with other military explosives, a considerable
environmental ballast where manufactured, as well as near
shooting ranges. Due to TNT's extensive usage, contami-
nated areas have been widespread across the world, most of
them still waiting for cleanup (Spain 2000; Bazar et al.
2008; Travis et al. 2008). The presence of three nitro

groups, withdrawing electrons from the aromatic ring, makes TNT particularly recalcitrant to degradation by microbial oxygenases. This may be the reason for TNT's persistence in the soil or ground water for prolonged time (Spain 2000; Rylott and Bruce 2009). Negative TNT effects on mammalian, bird, reptile, or amphibian health were confirmed by toxicological tests, e.g., alterations in central nervous systems, pathological changes in spleen, liver, and kidney function, reduced production of sperm, and hemolytic anemia (Mcfarland et al. 2008; Johnson et al. 2005; Paden et al. 2008; Reddy et al. 2000; Bazar et al. 2008). Moreover, TNT has been classified according to the International Agency for Research on Cancer (1996) as carcinogenic in animals and as possibly carcinogenic in humans.

Solubility of TNT in water is limited to 130 mg/l (Seidel 1941) and depends also on temperature and pH (Ro et al. 1996). At contaminated locations, concentrations of TNT in ground and soil water range from 1 mg/l and above (Gerth et al. 2003). Phytoremediation represents a potential low-cost approach to decontamination, supposing that TNT concentrations do not exceed limits acceptable for plants. The studies of TNT metabolism by plants have been already utilized to acquire an insight into its metabolic pathways and to identify genes involved in the detoxification processes. Two possible initial steps of TNT catabolism have been described in plants. One way is reduction of nitro groups yielding subsequently 2- or 4-hydroxylaminodinitrotoluene (HADNT) and 2- or 4-aminodinitrotoluene (ADNT) (Subramanian et al. 2006). The second possibility is oxidation of methyl group or direct hydroxylation of aromatic ring. The corresponding metabolites were found by Bhadra et al. (1999) in the aquatic plant *Myriophyllum aquaticum*. In the second phase, reduction products HADNT and ADNT can be conjugated with sugars by UDP-glycosyltransferases (UGTs) (Gandia-Herrero et al. 2008), yielding conjugates with lower toxicity and higher solubility than the parent compound (Coleman et al. 1997). In the third phase, conjugates may be either sequestrated from the cytosol to vacuoles or apoplast (Sandermann 1992) or incorporated to the cell wall (Sens et al. 1998). Although TNT metabolic pathways in plants have been partially elucidated (including the corresponding proteins and intermediate products), unequivocal identification of enzymes involved in the initial reduction of nitro groups, as well as the enzymes involved in incorporation of conjugates to the cell walls, still remains to be achieved. Also, dynamics of the response reflected by differences in the expression profiles between the short (up to 24 h; Ekman et al. 2003; Gandia-Herrero et al. 2008) and long (1 week or more; Mentewab et al. 2005; Rao et al. 2009) exposure should be considered.

The above-mentioned transcriptomic studies performed on TNT used whole plants. In addition, the root transcription response after 24-h TNT treatment was studied (Ekman et al. 2003). We decided to characterize transcriptome response of *A. thaliana* separately in roots and rosettes after a 7-day treatment. The aim of this study has been to compare processes taking place in both plant parts and to identify novel candidate genes for the enhancement of plant capability to metabolize TNT.

Materials and methods

Plant growth and exposure to TNT

Seeds of *Arabidopsis thaliana* (WT, cv. Columbia) were surface sterilized with 60% ethanol and 0.5% NaClO, rinsed with sterile water, and transferred onto modified Murashige and Skoog (MS; 1962) medium supplemented with 3% sucrose and 0.8% agar, and pH was adjusted to 5.8. The seedlings were cultivated at 23°C with 16/8 h light/dark cycle, at light intensity 7,200 µmol m^{-2} s^{-1}, in the growth cabinet (MLR-350, Sanyo Electric Co, Japan). After 3 weeks, plants were moved onto the rafts in Magenta boxes (two plants per box; Osmotek, Israel) containing 50 ml of sterile liquid MS media and cultivated for another 3 weeks in the same conditions. Afterward, TNT (obtained from Institute of Energic Materials, University of Pardubice) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, Czech Republic) and added to the media to final concentrations 1, 5, 20, and 50 µg/ml. An equivalent amount of DMSO was used in control variants. Plants were harvested after 12-day TNT incubation periods, and their fresh weight was assessed. Two independent experiments with three replicates were performed. Tissue samples for transcriptome analyses were washed with water, dried with paper towels, flash frozen in liquid nitrogen, and maintained at −80°C.

Quantification of TNT in cultivation medium

The media harvested from control and TNT-treated variants were applied on SEP-PAK™ C_{18} cartridges (Waters, USA). TNT was eluted with 10 ml methanol. The eluate was evaporated at 40°C in vacuo, and the solid residue was dissolved in 1 ml methanol. The samples were analyzed by HPLC (pump Chrom SDS 15 s, Watrex, Czech Republic; Thermo Separation Products AS300 Autosampler, Thermo Separation Products, USA) with reverse-phase (Biosphere Si C_{18}, 7 µm; Labio, Czech Republic) packed stainless steel column (250×4 mm) using a linear gradient of methanol in water (10–100%) for 40 min. TNT was detected using a PDA detector (PDA Jasco MD 1510, Jasco Inc., USA) at 230 nm. The concentration of TNT was determined using multipoint calibration curve, calculated by linear approxi-

mation. Statistical significance was tested by Student's two-tailed *t* test (comparison of TNT in media with and without plants) and ANOVA test followed with Tukey's HSD and Scheffe's tests (growth on different TNT concentrations). All the statistical analyses were carried out by STATISTICA 8.0 (StatSoft, Inc., OK, USA) software.

RNA isolation, labeling, hybridization, and data processing

Total RNA was isolated from rosettes and roots from individual plants harvested after 7-day treatment with 5 μg/ml TNT or DMSO (control plants) from two independent experiments with Agilent Plant RNA Isolation Mini Kit. The integrity of RNA was checked by the Agilent 2100 Bioanalyzer. RNA was labeled using a two-color Low RNA Linear Amplification Kit PLUS. Cy3- and Cy5-labeled complementary ribonucleic acid (cRNA) was purified by a RNeasy Plant Mini Kit (Qiagen, Germany), fragmented and hybridized on Agilent Arabidopsis 2 Oligo Microarray according to manufacturer's protocol. After 17-h hybridization at 65°C, slides were washed in GE Wash Buffers (unless otherwise indicated, all reagents were obtained from Agilent Technologies, USA) and scanned on a GenePix 4000A scanner (Axon Instruments, USA). Experiments were repeated five times with leaf and five times with root cRNA prepared independently from individual plants for each slide with a dye swap design to avoid dye-based bias. Acquisition and processing of the microarray data was achieved with TM4 software (Saeed et al. 2003). LOWESS normalization was used to balance the mean fluorescence intensities between the green and red channels. Only the spots with signal intensity higher than the mean plus two standard deviations of negative control spots and those presented on all slides were selected for further analysis. Student's *t* tests (five $\log_2$ expression ratios for root and rosette arrays) were used for statistical evaluation. Genes showing ≥1.5-fold change in gene expression and *p* value<0.05 were selected. Gene identification and ontology annotations were downloaded from the *Arabidopsis* information resource (TAIR) website (http://arabidopsis.org/tools/bulk/go/index.jsp) for up- and down-regulated genes (Rhee et al. 2003). Normalization of functional classification was performed using the Classification SuperViewer tool (Provart and Zhu 2003). Significance analysis of microarrays (one class analysis; Tusher et al. 2001) was applied to calculate *q* values for genes with *p* value >0.05 included into further analyses.

Quantitative real-time reverse-transcription PCR analysis

RNA samples from five biological replicates which were analyzed in microarray experiments were used for quantitative real-time reverse-transcription PCR (q-RT-PCR) assays. RNA was treated with DNAseI DNA-free kit (Ambion, USA) to eliminate the traces of genomic DNA. Complementary DNA (cDNA) was prepared from the total RNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany) according to the manufacturer's instructions. PCR was performed using a Light Cycler 480 Probes Master kit (Roche). The LightCycler 480 (Roche) was programmed as follows: initial denaturation at 95°C for 10 min, 45 cycles of 10 s at 95°C for melting, 30 s at 60°C for annealing and extension, and 1 s at 72°C for data acquirement. For each sample, two runs were performed, and the mean expression value was calculated. Primers and probes were selected according to the online Roche Universal ProbeLibrary Assay Design Center (Table 1). The primers were placed in different exons, whenever possible. Probes from Universal ProbeLibrary Set (Roche) were used. To normalize the amount of total RNA present in each reaction, we amplified two housekeeping transcripts encoding EF1-α (*At1g07920*) and CYP20-2 (*At5g13120*). PCR efficiencies were estimated from calibration curves generated from serial dilution of cDNAs. The cDNA derived from calibrator RNA was included in each LightCycler run to correct for run-to-run differences. The calibrated normalized ratios of the relative amount of the target and reference gene were calculated as follows:

$$E_{\mathrm{T}}{}^{\mathrm{CpT(C)-CpT(S)}} \times E_{\mathrm{R}}{}^{\mathrm{CpR(S)-CpR(C)}}$$

where E_{T} and E_{R} are the efficiency of target or reference gene amplification, respectively; $C_{\mathrm{p}}T$ and $C_{\mathrm{p}}R$ are cycle numbers at target or reference gene detection threshold (crossing point); S is the sample; C is the calibrator.

Results

Growth of *A. thaliana* exposed to TNT

Dose response analysis was performed to select a suitable concentration of TNT for microarray experiments. At TNT concentrations of 1 and 5 μg/ml, no significant difference was observed in fresh weight of the plants which was reduced approximately to 70% of the control. At higher TNT concentrations, 20 and 50 μg/ml, the growth suppression was more extreme, to 32–24% (Fig. 1). However, even the highest concentration tested was not acutely lethal.

Parallel to dose response analysis, uptake of TNT from the medium was measured as shown in Fig. 2. Concentration of TNT was compared in media after 12-day incubation with or without plants (control variant). The presence of plants led to significant decrease of TNT content in case of

Table 1 Primers and probes designed by online Universal ProbeLibrary Assay Design Center

Gene	Forward and reverse primers 5′–3′	Probe number
TCH4 (At5g57560)	gctcaacaaaggatgagatgg cctcttcgcatccgtacaat	6
PP2C (At3g16800)	cggttcagctctcggtagac cgtccatccgattgtttatc	38
ZF1 (At3g55980)	tcttctcaatttcattccaggat tgttaaggttcttgaagagcagag	86
GSTF3 (At2g02930)	caaagacggtgagcacaaga ggctggaacttgaccaaaag	55
MYB 59 (At5g59780)	ttttgtagcgaaagtttcaggtt cttcttgtggagtcatcttaccac	54
ATAMT1;2 (At1g64780)	acaacttatgccgtcgacaa tgcatggcaaagacaaggta	35
NIT2 (At3g44300)	cgtgatgagttccgcaagta gttcttcccggccaactc	72
EXT4 (At1g76930)	accaaccctacctttacaaatcac ttgtttctttgtgtatcgtcgtc	63
PRP1 (At4g33720)	ggggtgggacccttaagat cgctggttagcatagttacgg	83
SEN1 (At4g35770)	gaaactcaaacttccgatggag ggaattttaactgcctctgctg	6
FER1 (At5g01600)	ctgaaaaaggagatgctttatatgc tgaggccactttgtgaacg	21
EF1-α (At1g07920)	gagcgttgttctcgtaattgg aaaacaggaagaatgtgtgtgtaga	10
CYP20-2 (At5g13120)	cattccatcgtgtcatcagag ggccatatacactttacctcca	52
NR1 (At1g77760)	aagccgtacacattaaaaggcta tcacctcaaccctcgttacc	65
TPX2 (At1g65970)	gcctggattcattgggaaa catcacaaatggatcattcaca	17
UGT72E1 (At3G50740)	gagatcgaggcgttggtg ctttctcatctcagcaccttcc	22

low concentrations (1 and 5 μg/ml). No significant change was imposed by plants at a TNT concentration of 20 μg/ml. At the highest concentration tested (50 μg/ml), the variant with plants showed even slightly higher TNT content than the control one. This paradoxical effect is caused by limited solubility of TNT in the medium. Exudates released by plants to the media probably allow TNT to dissolve more than in pure medium without plants. Based on the above described results, the final concentration of 5 μg/ml of TNT was chosen for expression analysis. At this TNT concentration, the plants were still able to grow relatively well (68% of the weight of control plants) and metabolize this xenobiotic, unlike the situation at higher concentrations, when they seemed to suffer from severe stress (32% and 24% of the weight of control plants).

Microarray-based expression profiling

The effects of TNT exposure on the transcriptome of *A. thaliana* roots and rosettes were studied using microarrays.

From the total amount of 21,495 unique genes spotted on the microarray, 13,908 and 15,470 genes were identified in rosettes and roots, respectively. Statistical analysis and subsequent filtering out of genes with expression change
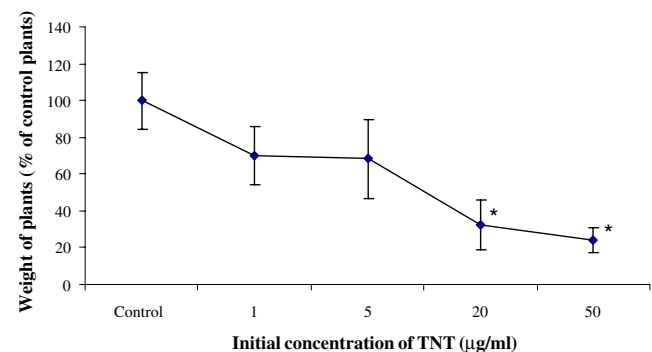

Fig. 1 Weight of *Arabidopsis* plants after 12 days of exposition to TNT. *Bars* indicate SD. * indicates that weight was significantly different from control at concentrations 20 and 50 μg/ml according to analysis of variance with Tukey's HSD and Scheffe's multiple comparison tests ($n=12$; $p<0.01$)
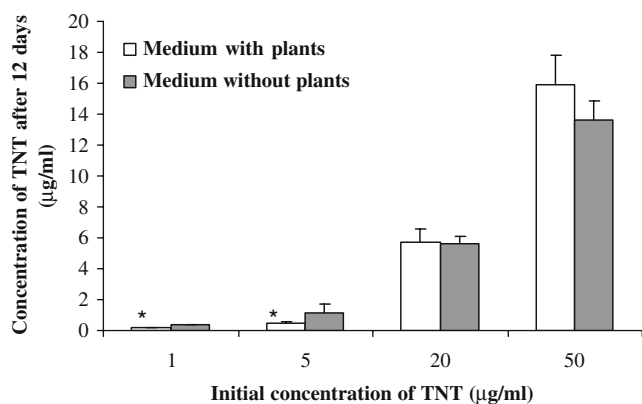
Fig. 2 Uptake of TNT from medium after 12 days of exposition to TNT. *Bars* indicate SD. * indicates significantly different TNT concentrations between medium with and without plants according Student's two tailed *t* test ($n=6$; $p<0.05$)

lower than 1.5-fold revealed 170 up- (Supplementary Table 1 in the Electronic Supplementary Materials) and 122 down-regulated (Supplementary Table 2 in the Electronic Supplementary Materials) transcripts in the rosettes, as well as 61 up- (Supplementary Table 3 in the Electronic Supplementary Materials) and 51 down-regulated (Supplementary Table 4 in the Electronic Supplementary Materials) transcripts in the roots. Additional seven up-regulated rosette transcripts and two up- and one down-regulated root transcripts, which did not pass the *t* test, were included into further analyses (Table 3) because the mean value of their expression was markedly changed and they seemed either to have function in xenobiotic detoxification or to participate in TNT metabolism. Comparison between rosettes and roots revealed only a small overlap, as only three induced transcripts were common for both tissues (1.3% of all up-regulated genes from both parts) and another five (2.9% of all down-regulated genes from both parts) were repressed in aerial as well as in root parts. Two genes were simultaneously up-regulated in rosettes and down-regulated in roots (0.9% of rosette up-regulated and root down-regulated genes).

Genes differentially expressed in rosettes

The first main group of transcripts that were up-regulated in aerial part included genes coding for the enzymes potentially involved in TNT metabolism and transportation (Table 2). Among the 20 most up-regulated genes, nitrate reductase 1 (*At1g77760*) was detected. The corresponding enzyme is involved in reduction of nitrate to nitrite (Hwang et al. 1997). Further up-regulated genes included five members coding for the glycosyltransferases with different substrate specificity (*At3g50740*, *At1g60140*, *At3g62660*, *At3g62720*, *At2g41640*), which might be involved in TNT conjugation. Moreover five cytochromes P450 (*At5g45340*, *At3g26220*,

At3g26280, *At5g05690*, *At2g26710*) and four glutathione transferases (*At1g02930*, *At1g02920*, *At4g02520*, *At2g02930*) were up-regulated. We also recorded stimulation of transcription activity of three ATP-binding cassette (ABC) transporter members, P-glycoprotein 21 (*At3g62150*), P-glycoprotein 19 (*At3g28860*), and ATABC1 (*At4g04770*).

The most abundant group of genes up-regulated in rosettes involved transcripts associated with responses to various biotic and abiotic stresses (Fig. 3a), such as pathogens, wounding, extreme temperatures, salt, and oxidative stress. The stress-induced response genes involved mainly those implicated in ethylene biosynthesis and signaling, namely ethylene-forming enzyme (*At1g05010*). Further induced transcripts coded for enzymes were involved in salicylic acid or jasmonic acid signaling cascades. Simultaneously, however, transcription of a gene for hydroperoxide lyase 1 (*At4g15440*), which might be involved in jasmonic acid biosynthesis, was repressed. Other up-regulated genes coding for antioxidant enzymes were thioredoxin-dependent peroxidase (*At1g65970*) and γ-glutamyltransferase (*At4g39640*).

The third group of up-regulated genes included those coding for the enzymes involved in cell wall modifications: expansin 1 (*At1g69530*), α-xylosidase 1 (*At1g68560*), touch 4 (*At5g57560*), and UDP-glucosyltransferase 72E1 (*At3g50740*).

Transcripts repressed in aerial parts by TNT included specific transcription factors and especially genes involved in lipid metabolism. From the ten most repressed transcripts in the aerial part were three members of basic/helix-loop-helix superfamily of transcription factors (*At2g41240*, *At5g04150*, *At3g47640*; Supplementary Table 2 in the Electronic Supplementary Materials). Apart from ammonium transmembrane transporter, ATAMT1;2 (*At1g64780*), two lipid transporters, *At1g55260* and *At5g64080*, were down-regulated. Also other genes coding for enzymes of lipid metabolism were down-regulated concretely: three GDSL-motif lipase/hydrolase family proteins (*At3g48460*, *At5g45950*, *At1g28600*), putative lipase *At1g28600*, fatty acid desaturase 2, and lipid-associated family protein *At2g22170*. Interestingly, although some cell wall loosening transcripts, e.g., expansin 1, were induced, other transcripts, like expansin 5 (*At3g29030*), were down-regulated. Functional classification of down-regulated genes revealed that the most often repressed transcripts fall into plasma membrane (4% of total estimated gene number), response to abiotic and biotic stimulus (3.7%), response to stress (3%), and endoplasmic reticulum (3%; Fig. 3b).

Genes differentially expressed in roots

Transcripts abundantly up-regulated in the roots involved many genes engaged in the response to biotic and abiotic

Table 2 Up-regulated transcripts potentially involved in TNT metabolism (7 days, concentration of TNT 5 μg/ml)

Locus identifier	Fold change	Gene name	Gene description
Phase I (rosettes)			
At1g77760*	2.52	Nitrate reductase 1 (NR1)	Cytosolic minor isoform of nitrate reductase; involved in the first step of nitrate assimilation
Phase II (rosettes)			
At3g50740*	3.15	UDP-glucosyltransferase 72E1 (UGT72E1)	UDPG:coniferyl alcohol glucosyltransferase; thought to be involved in lignin metabolism
At1g60140	1.72	Trehalose phosphate synthase (ATTPS10)	Putatively involved in trehalose biosynthesis
At3g62660	1.57	Galacturonosyltransferase-like 7 (GATL7)	Putative galacturonosyltransferase activity.
At3g62720	1.60	Xylosyltransferase 1 (XT1)	Xylosyltransferase activity, which is specific for UDP-xylose as donor substrate and for oligosaccharides with a degree of polymerization >4
At2g41640	1.79		Similar to glycosyltransferase (*Medicago truncatula*)
Phase III (rosettes)			
At3g62150	2.02	P-glycoprotein 21 (PGP21)	ATPase, coupled to transmembrane movement of substances; identical to Multidrug resistance protein 17 (MDR17; *Arabidopsis thaliana*)
At3g28860	1.66	P-glycoprotein 19 (PGP19)	Belongs to the family of ABC transporters; also known as AtMDR11 and PGP19
At4g04770	1.52	ATP binding cassette protein 1 (ATABC1)	Member of the NAP subfamily of ABC transporters; involved in Fe–S cluster assembly; similar to SufB
At1g69530	1.69	Expansin A1 (ATEXPA1)	Member of α-expansin gene family; involved in the formation of nematode-induced syncytia in roots of *Arabidopsis thaliana*
At1g68560	2.15	α-Xylosidase 1 (XYL1)	Bifunctional α-L-arabinofuranosidase/β-D-xylosidase that belongs to family 3 of glycoside hydrolases
At5g57560	2.26	Touch 4 (TCH4)	Encodes a cell wall-modifying enzyme, rapidly up-regulated in response to environmental stimuli
Phase III (roots)			
At1g22710	1.51	Sucrose–proton symporter 2 (SUC2)	High-affinity transporter essential for phloem loading and long-distance transport; major sucrose transporter, can also transport a wide range of physiological and synthetic glucose conjugates with both α- or β-linkage
At1g02640	2.14	β-Xylosidase 2 (BXL2)	Protein similar to a β-xylosidase located in the extracellular matrix; member of glycosylhydrolase family 3 and has six other closely related members
At4g30270	1.51	Meristem-5 (MERI5B)	Protein similar to endoxyloglucan transferase in sequence; also very similar to BRU1 in soybean, which is involved in brassinosteroid response
At4g03210*	2.21	Xyloglucan endotransglucosylase/ hydrolase 9 (XTH9)	Member of xyloglucan endotransglucosylase/hydrolases (XTHs) that catalyze the cleavage and molecular grafting of xyloglucan chains function in loosening and rearrangement of the cell wall
Antioxidative activity genes (rosettes)			
At1g65970*	3.17	Thioredoxin-dependent peroxidase 2 (TPX2)	Thioredoxin-dependent peroxidase
At4g39640	1.65	Gamma-glutamyltransferase	Involved in the degradation of glutathione. Also mitigates oxidative stress by metabolizing GSSG (oxidized form of GSH—glutathione) in the apoplast

Complete list of TNT up- and down-regulated genes in rosettes and roots is in Supplementary Tables 1, 2, 3, 4 in the Electronic Supplementary Materials

*$p > 0.05$

stresses. Nevertheless, only three stress response genes, SEN1 (*At4g35770*), ferretin 1 (*At5g01600*), and UDP-glucose 4-epimerase 1 (*At1g12780*), were simultaneously induced in rosettes as well as in roots.

Only in roots, three peroxidases were induced (*At2g37130*, *At4g36430*, *At5g05340*).

The other genes, highly up-regulated in roots, encoded enzymes involved in hydrolysis and cell wall

Table 3 Genes with *p* value >0.05 included into further analyses or discussed in the text

Locus identifier	Gene name	Fold change	*t* test *p* value	FDR *q* value (%)
Rosettes				
At1g77760	Nitrate reductase 1 (NR1)	2.52	0.385	48.37
At3g50740	UDP-glucosyltransferase 72E1 (UGT72E1)	3.15	0.061	10.62
At3g44300	Nitrilase 2 (NIT2)	6.55	0.217	29.37
At1g02930	Glutathione *S*-transferase (ATGSTF6)	3.27	0.055	8.99
At1g65970	Thioredoxin-dependent peroxidase 2 (TPX2)	3.17	0.139	17.47
At3g16530	Legume lectin family protein	7.81	0.091	12.56
At4g02520	Glutathione *S*-transferase PHI 2 (ATGSTF2)	2.11	0.144	20.54
Roots				
At4g35770	Senescence 1 (SEN1)	2.26	0.085	28.59
At4g03210	Xyloglucan endotransglucosylase/hydrolases 9 (XTH9)	2.21	0.051	23.01
At4g33720	Putative pathogenesis-related protein	−2.88	0.194	83.93

modifications (Fig. 3c), such as extensin 4 (*At1g76930*), *β*-xylosidase 2 (*At1g02640*), and two xyloglucan endo-transglucosylase/hydrolases MERI5B (*At4g30270*) and XTH9 (*At4g03210*).

Similarly to rosettes, stimulation of the expression of genes coding for specific transporters was detected; however, unlike transporters from ABC family enhanced in rosettes, roots exhibited induction of sucrose–proton symporter 2 AtSUC2 (*At1g22710*).

The most down-regulated genes in roots (Supplementary Table 4 in the Electronic Supplementary Materials) included light-responsive genes, namely two genes coding for early light-induced proteins ELIP1 (*At3g22840*) and ELIP2 (*At4g14690*) and one gene for sigma factor E (*At5g24120*).

Another large group of down-regulated transcripts consisted of six members engaged in flavonoid biosynthesis (especially chalcone synthase (*At5g13930*), a key enzyme of flavonoid biosynthesis). Other down-regulated genes were also involved in the production of secondary metabolites, concretely two genes from the strictosidine synthase family (*At5g22020*, *At3g57020*), engaged in alkaloid biosynthesis.

In contrast to aerial part, no transcript stimulation was found for nitroreductase. Also no enhanced activity of UGTs and GSTs was found. In fact, the second and third most repressed transcripts in roots were two UGTs (*At3g21560*, *At2g22590*).

Transcripts with decreased activity in roots as well as in the aerial parts were ammonium transporter 1;2 (ATAMT1;2) which contributes to ammonium uptake in the roots (Neuhauser et al. 2007), chalcone-flavanone isomerase (*At5g05270*), ferredoxin 1 (*At1g10960*), evolutionarily conserved C-terminal region 8 (*At1g79270*) with unknown function, and finally a member of glutaredoxin

family *At1g64500*. Functional categories of the most important groups suppressed in roots fall to extracellular (7.8% of the total estimated gene number) and endoplasmic reticulum (7%) category (Fig. 3d).

Validation of microarray results

Results of microarray expression analysis were verified using quantitative RT-PCR. Selected group of genes (Table 4) covered the whole range of expression profiles, from the highly stimulated genes to the strongly suppressed. The expression levels of 14 genes analyzed by q-RT-PCR showed general agreement with results obtained by microarrays. However, the absolute values of the expression level ratio differed between q-RT-PCR and microarrays, being usually higher in case of q-RT-PCR, which reflects different sensitivity of both methods (Manthey et al. 2004).

Discussion

Microarray evaluation of the effect of 7-day exposure of *A. thaliana* seedlings to 5 μg/ml TNT allowed us to pin-point the transcripts affected by this xenobiotic separately in the roots and rosettes. Induction of several genes which may have been involved in TNT metabolism was observed in the rosettes. Gene products potentially participating in TNT metabolism included nitrate reductase 1, several glycosyltransferases, and hydrolases. Simultaneously, transcripts associated with cell wall rebuilding were up-regulated. In the roots, only transcripts coding for hydrolases and cell wall-modifying enzymes were stimulated. Genes potentially involved in TNT metabolism are included in Table 2.

Fig. 3 Proportion of up- and down-regulated genes from the total number of genes in the respective category as calculated with the Classification Super-Viewer (Provart and Zhu 2003) and expressed in percents (*horizontal axis*). **a** Up-regulated transcripts in the rosettes; **b** down-regulated transcripts in the rosettes; **c** up-regulated transcripts in the roots; **d** down-regulated transcripts in the roots. Only proportions above 1% are shown. *Error bars* indicate bootstrap standard deviation
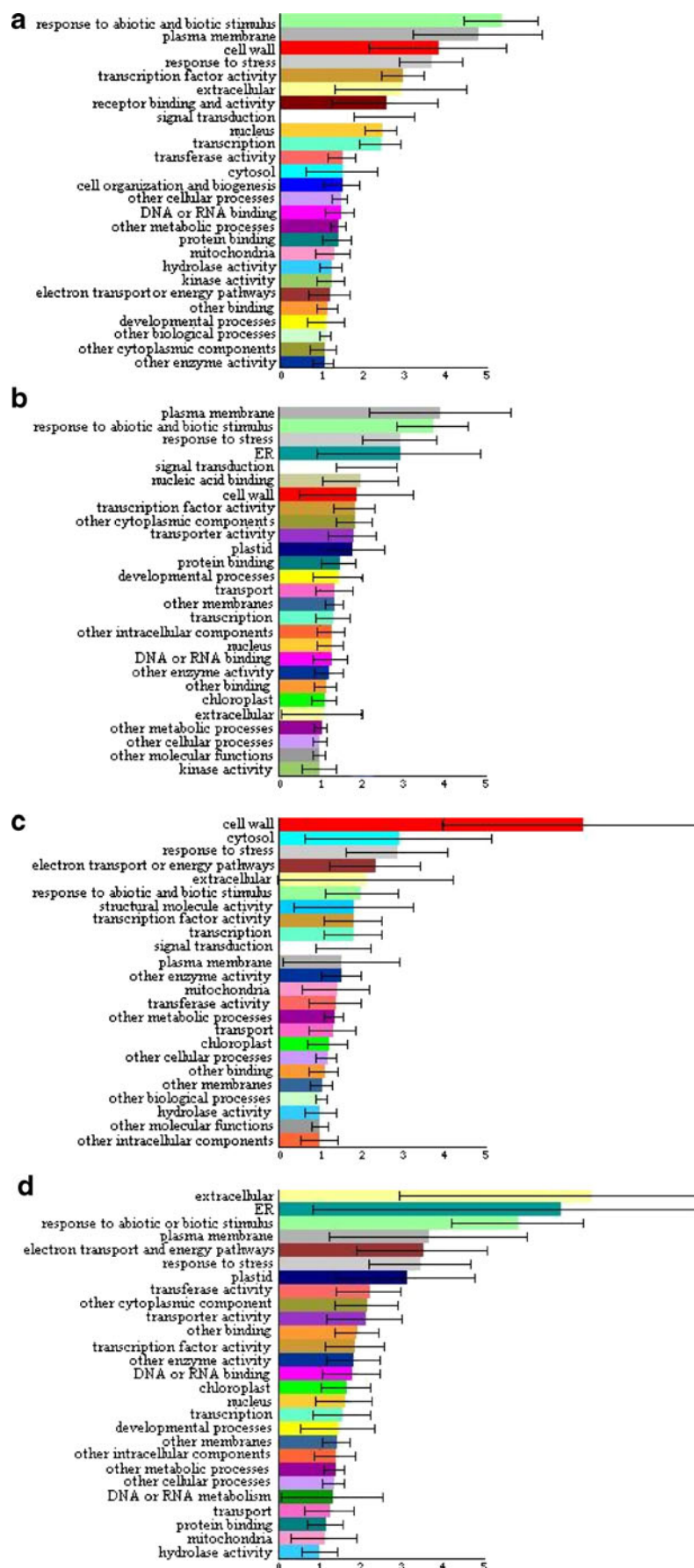
Table 4 Validation of transcription profile by q-RT-PCR

Gene/plant part	Annotation	Microarray fold change±SD	q-RT-PCR fold change±SD
TCH4 (At5g57560) rosettes	Xyloglucan endotransglycosylase	2.26±0.63	2.70±1.88
PP2C (At3g16800) rosettes	Protein phosphatase	2.01±0.62	1.99±0.31
ZF1 (At3g55980) rosettes	Zinc finger family protein	1.99±0.38	2.39±0.48
GSTF3 (At2g02930) rosettes	Glutathione S-transferase	1.67±0.53	3.40±4.33
MYB 59 (At5g59780) rosettes	Transcription factor	1.78±0.65	2.30±1.39
ATAMT1;2 (At1g64780) rosettes	Ammonium transporter	−1.56±0.16	−1.56±0.26
ATAMT1;2 (At1g64780) roots	Ammonium transporter	−1.98±0.72	−2.39±1.28
NIT2 (At3g44300) rosettes	Nitrilase	6.55±10.56	49.63±111.29
EXT4 (At1g76930) rosettes	Extensin	1.93±0.66	2.10±0.88
PRP1 (At4g33720) roots	Pathogenesis-related protein	−2.88±3.63	−3.58±4.27
SEN1 (At4g35770) rosettes	Senescence-associated protein	4.86±3.93	4.78±3.25
SEN1 (At4g35770) roots	Senescence-associated protein	2.26±1.49	5.40±8.55
FER1 (At5g01600) rosettes	Ferritin	5.36±3.98	4.23±3.22
FER1 (At5g01600) roots	Ferritin	1.65±0.49	1.61±.0.27
NR1 (At1g77760) rosettes	Nitrate reductase	2.52±5.04	2.27±6.24
TPX2 (At1g65970) rosettes	Thioredoxin-dependent peroxidase	3.17±3.71	13.42±26.59
UGT72E1 (At3G50740) rosettes	UDP-glucosyltransferase	3.15±2.38	4.37±3.52

Genes coding for enzymes involved in phase I of TNT catabolism

One of the most up-regulated transcripts in the rosettes coded for nitrate reductase 1 (At1g77760). The corresponding enzyme is similar to molybdopterin oxidoreductases at the N terminus and to FAD/NAD-binding cytochrome reductases at the C terminus. Its primary function is reduction of nitrate to nitrite (Hwang et al. 1997). It might be, however, involved also in reduction of nitro groups on TNT ring, which is considered as the first step in the TNT detoxification pathway. Our results are in accordance with the microarray data of Patel et al. (2004), who observed induction of nitrate reductase in green algae, Chlamydomonas reinhardtii, in response to TNT. They also accord with those of Medina et al. (2004), who suggested that TNT reduction by spinach crude extract could be achieved by nitroreductase or nitrate and nitrite reductase activities.

The transcriptomic studies of early responses of A. thaliana to TNT (Ekman et al. 2003; Gandia-Herrero et al. 2008) showed induction of 12-oxophytodienoate reductases (OPR1 and OPR2). Products of these genes were suggested to be potential nitroreductases related to bacterial pentaerythritol tetranitrate reductase, which has been shown to increase TNT tolerance when expressed in transgenic tobacco or Arabidopsis plants (French et al. 1999; Kurumata et al. 2005). Also, Podlipna et al. (2007) identified oxidoreductase protein from Saponaria officinalis with high homology to OPR1 from A. thaliana participates in the degradation of TNT. Recently, Beynon et al. (2009)

confirmed that OPR1, OPR2, and OPR3 are directly involved in TNT transformation. Nitrate reductase 1 may play a similar role in TNT metabolism as OPRs.

In our study, we did not find enhanced expression of OPR genes. Our data are in accordance with the results of Rao et al. (2009), who did not find induction of any OPR transcript after 8–9 days exposure of A. thaliana to TNT. The achieved data indicate that expression of OPR genes might be down-regulated after prolonged TNT treatment of A. thaliana, reaching the basal level.

Genes coding for enzymes involved in phase II of TNT catabolism

Further, an important group of transcripts stimulated in the aerial part was coding glycosyltransferases. These enzymes may play a role in the second phase of TNT metabolism, when the reduced TNT products are conjugated with various sugars. We observed up-regulation of the following genes with glycosyltransferase activity: UDP-glucosyltransferase 72E1 (At3g50740), trehalose phosphate synthase (At1g60140), galacturonosyltransferase-like 7 (At3g62660), xylosyltransferase 1 (At3g62720), and potential glycosyltranferase (At2g41640). Our results correspond to findings of Gandia-Herrero et al. (2008), who confirmed that HADNT and ADNT are conjugated in the second phase of TNT transformation by UGTs with six-carbon sugars via hydroxyl or amino groups, respectively (Fig. 4a).

We did not find stimulation of glucosyltransferases or glutathione transferases in roots, which seems to contradict
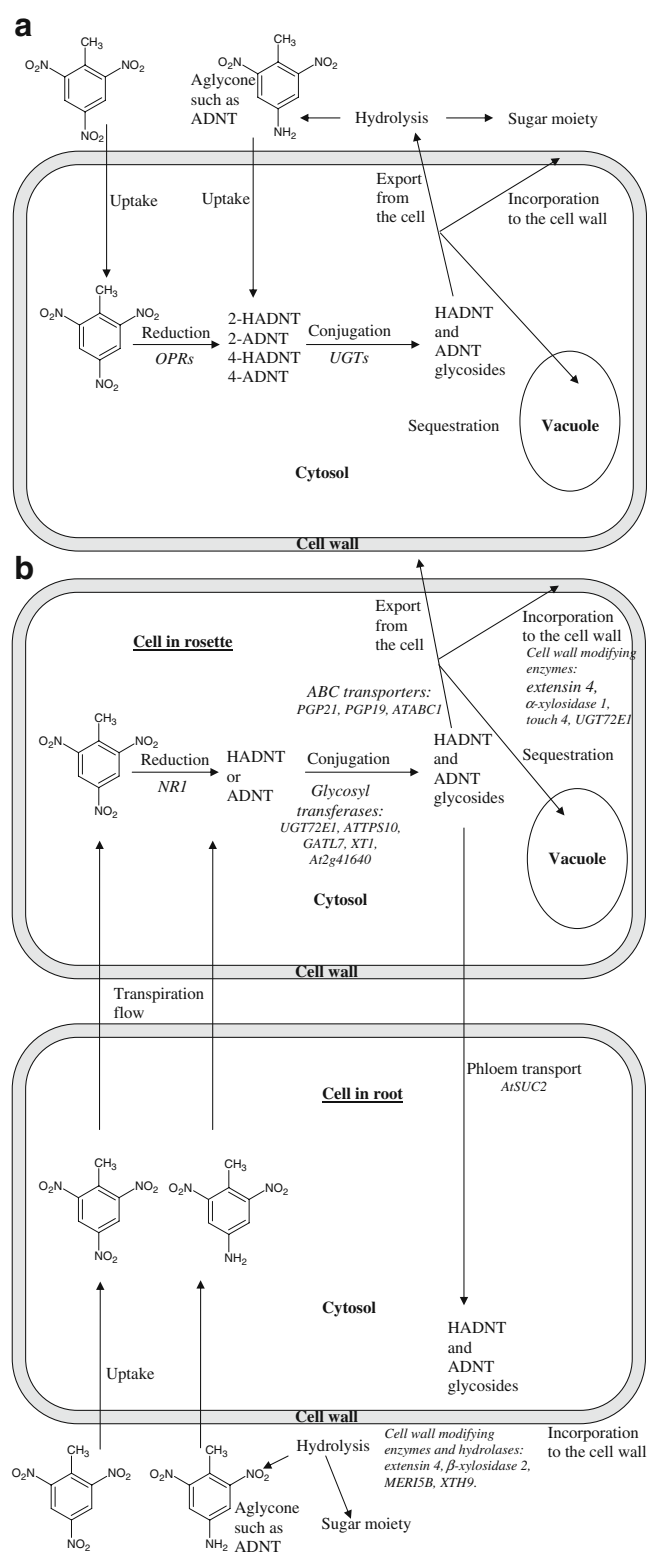
Fig. 4 a Proposed metabolism of TNT in *Arabidopsis* plants. ▶
Involved proteins are stated in *italics*. In the first phase, TNT is
reduced in the cytosol to hydroxylaminodinitrotoluenes (*HADNT*) and
aminodinitrotoluenes (*ADNT*). These products are in the second phase
conjugated with six-carbon sugars. Conjugation enables sequestration
to vacuoles or export to extracellular space or incorporation to the cell
wall in the third phase of TNT metabolism. Scheme is adopted from
Gandia-Herrero et al. (2008) and Brazier-Hicks et al. (2007). **b**
Proposed metabolism of TNT in *Arabidopsis* after 7-day exposition to
TNT, based on the data obtained by microarray analysis of rosettes
and roots. Potentially involved genes are stated in *italics*. From these
genes, only the genes coding for cell wall modifying enzymes,
hydrolases and phloem-specific transporter AtSUC2 were up-
regulated in the roots. Their activities of corresponding enzymes
could be associated with the incorporation of TNT glycosides into the
cell walls or with their hydrolysis. The activity of AtSUC2 might be
associated with phloem transport of TNT glycosides. In the rosettes,
TNT metabolism is manifested by increased expression of several
glycosyltransferases and ABC transporters. Nitrate reductase 1 could
be involved in the initial TNT reduction step. The genes coding for
cell wall modifying enzymes were up-regulated in both the rosettes
and the roots

the results of Ekman et al. (2003), who observed induction of
several UGT, GST, and OPR transcripts after 24-h treatment
with TNT in the roots. Another three transcriptome studies,
Mentewab et al. (2005), Gandia-Herrero et al. (2008), and
Rao et al. (2009), analyzed whole *A. thaliana* plants, which
did not allow discrimination between the rosettes and the
roots.

Genes coding for enzymes involved in phase III of TNT catabolism

Once TNT is conjugated, it can exit the cytoplasm using
specific transporters such as ABC transporters (Mentewab
et al. 2005). The up-regulated transporter genes included
three members of ABC family transporters: P-glycoprotein
21 (*At3g62150*), P-glycoprotein 19 (*At3g28860*), and
ATABC1 (*At4g04770*), which might be involved in
transport of TNT metabolites. Another up-regulated
phloem-specific transporter was sucrose–proton symporter
2 AtSUC2 (*At1g22710*) which is able to carry a wide range of
physiological and synthetic glucose conjugates (Chandran
et al. 2003). The induction of AtSUC2, a transporter coding
for a protein essential for phloem loading, involved in long-
distance transport and able to carry a wide range of
physiological and synthetic glucose conjugates (Srivastava
et al. 2008; Imlau et al. 1999; Chandran et al. 2003),
indicates that these transporters could participate in long-
distance movement of TNT conjugates between roots and
leaves (Fig. 4b).

In the above- as well as underground parts, several genes
involved in cell wall rebuilding were up-regulated com-
pared to control plants. Our data revealed stimulation of
transcripts involved in hydrolysis and cell wall modifica-
tions, such as extensin 4 (*At1g76930*), *β*-xylosidase 2

(*At1g02640*), and two xyloglucan endotransglucosylase/
hydrolases MERI5B (*At4g30270*) and XTH9 (*At4g03210*)
(Roberts and Shirsat 2006; Goujon et al. 2003; Campbell
and Braam 1999; Shin et al. 2006). Induction of these genes
may have been associated with polymerization of TNT

conjugates into the cell wall because conjugates of nitro aromatic molecules are partly incorporated into the cell wall (Brazier-Hicks et al. 2007). Thus, our results indicated that after 1-week incubation with TNT, conjugation was (still) active in the above-ground parts, while incorporation to the cell walls might take place in both parts. The results of Vila et al. (2007) who reported that in several species more than 70% of TNT was not extractable are in favor of our assumption. Also other studies on uptake of radio-labeled TNT by trees and various dicotyledonous and monocotyledonous plants showed that most TNT are finally bound in un-extractable form in the roots (Sens et al. 1999; Sens et al. 1998; Sun et al. 2000; Schoenmuth and Pestemer 2004; Nepovim et al. 2005).

Prevalence of TNT occurrence in the roots (more than 70%) was demonstrated, e.g., in maize, wheat, and soybean plants treated with ^{14}C-labeled TNT (Vila et al. 2007). Our transcriptomic data, however, indicated stimulation of genes for potential TNT metabolic enzymes only in aerial part. This is in contradiction with Sens et al. (1999), who detected the presence of products of TNT reduction, 2-ADNT and 4-ADNT, in the roots of axenic cultures of *Phaseolus vulgaris*. Localization of TNT metabolism mainly into the roots was also suggested by Hannink et al. (2002) in terrestrial plants; only submersed aquatic plants were supposed to accumulate TNT metabolites mainly in the leaves. A possible explanation might be that after a 7-day incubation with TNT, the physiological functions of roots are disturbed and detoxification capability of roots is exhausted. French et al. (1999) described severely stunted root systems of tobacco seedlings in a medium containing 0.025 mM TNT (5.68 µg/ml) compared with controls. Another possible reason might be the dynamics of TNT metabolism. It is possible that after 7-day incubation, TNT transported according to the concentration gradient was already metabolized or predominantly translocated to the cell walls. Thus, no necessity for enhanced transcription of genes for TNT metabolic enzymes pertained in roots. Then different expression in aerial part might be secondary reaction to TNT detoxification and stress response in the roots.

Stress response genes

Another large group of transcripts stimulated in the rosettes was related to brassinosteroid and auxin signaling. Putative reasons for their up-regulation may be their promoting effect on cell expansion and cell elongation (Clouse and Sasse 1998). However, the participation of auxins and brassinosteroids in the response to stress caused by TNT application should be also considered. Stimulation of genes associated with the auxin signaling pathway was recently found at low stress or at high CO_2 concentration (Coupe et al. 2006). We also found strong up-regulation of the gene

for nitrilase 2 (*At3g44300*), an enzyme involved in indole-3-acetic acid biosynthesis (Vorwerk et al. 2001; Quirino et al. 1999). This is in accordance with the increase of free auxin levels observed upon other stresses, e.g., after pathogen attack (Sun et al. 2009) or in drought-stressed leaves (Havlova et al. 2008).

We also observed induction of several genes encoding enzymes involved in oxidative metabolism. Five genes for cytochromes P450 (*At5g45340*, *At3g26220*, *At3g26280*, *At5g05690*, *At2g26710*) were up-regulated in aerial parts, whereas peroxidase 21 (*At2g37130*) and two putative peroxidases (*At4g36430*, *At5g05340*) were induced in roots. Although Bhadra et al. (1999) identified products of TNT oxidation in *M. aquaticum*, Subramanian et al. (2006) detected only reduced TNT intermediates but no products of oxidative pathway in TNT-treated *Arabidopsis*. The absence of reports about the presence of oxidized TNT intermediates in *Arabidopsis* indicates that these enzymes are probably not directly involved in TNT metabolism but that their induction might be associated with other processes, such as a general stress response to the pollutants (Mendez-Alvarez et al. 1999). Our microarray data also showed up-regulation of GST belonging to the phi class and of other putative GST (and of other two GSTs, which reached more than 1.5-fold stimulation of the expression but did not pass the *t* test) in concordance with observation of Ekman et al. (2003), Nepovim et al. (2004), and Gandia-Herrero et al. (2008). GSTs encode enzymes for catalyzing the conjugation of reduced glutathione with various toxic compounds (Jablonkai and Hatzios 1993). They are generally thought to confer increased solubility, decreased toxicity, and elevated transport competency of toxic compounds (Schroder and Collins 2002). However, in spite of the fact that induced levels of several GST transcripts were observed after TNT exposure, no conjugate of TNT and glutathione has yet been identified (Rylott and Bruce 2009). Moreover, confocal microscopy with fluorescent probes did not show any conjugation of reduced glutathione with TNT (Mezzari et al. 2005). These observations might indicate that up-regulation of GSTs is associated with general oxidative stress responses rather than in direct TNT conjugation.

Mentewab et al. (2005) reported strong induction (8.58-fold elevation) of the expression of pathogenesis-related protein 1 (*At4g33720*) in the roots of *Arabidopsis* exposed to TNT for 10 days. In contrast, our microarray data indicated repression of *At4g33720* after 7-day TNT treatment, similarly to another gene encoding putative pathogenesis-related protein (*At1g50060*). In order to elucidate this discrepancy in detail, we decided to include *At4g33720* to group of genes which were revalidated by q-RT-PCR. This difference in expression between two long-term incubations with TNT might be caused by different

cultivation conditions. Figure 3 shows that relatively high number of stress and biotic and abiotic stimulus genes were up- as well as down-regulated by TNT in both plant parts. It is possible that TNT stress caused different activation of stress response genes in different cultivation conditions.

Conclusions

In conclusion, we propose nitrate reductase 1 (*At1g77760*) for further testing as possible candidate enzyme involved in the first phase of TNT metabolism. After 7-day exposure to TNT, stimulation of the transcription of genes potentially involved in TNT detoxification was observed in rosettes but not in the roots suggesting that *A. thaliana* was no more able to transform TNT in roots after 7-day treatment with TNT. Based on our results, we propose to overexpress several candidate genes which could bring about increased TNT metabolism and tolerance in transgenic plants. The transcription response indicated that plants suffered from oxidative stress when exposed to TNT. Thus, besides UDP-glycosyltransferases and ABC transporters, TNT tolerance could be also enhanced by overexpression of genes coding for enzymes with antioxidative activity such as thioredoxin-dependent peroxidase 2 and gamma-glutamyltransferase l.

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