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Differential gene expression of *Chlamydomonas reinhardtii* in response to 2,4,6-trinitrotoluene (TNT) using microarray analysis

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Abstract

The exposure of *Chlamydomonas reinhardtii* to environmental stress, such as that caused by the explosive 2,4,6-trinitrotoluene (TNT) can alter its gene expression. Expression analysis was conducted using a microarray composed of 3079 *Chlamydomonas* ESTs to characterize the broad range of responses of gene expression exposed to this common ordnance compound. TNT treatment conditions were determined by growth analysis of *Chlamydomonas* in 0–5 μg/mL TNT. One and 3 μg/mL were used for microarray analysis since 1 μg/mL of TNT did not decrease the cell count after 7 days of treatment, whereas 3 μg/mL of TNT was the maximum TNT concentration that allowed growth, respectively. Transcriptional profiling revealed that approximately 158 responsive genes were differentially expressed representing several functional categories. Genes responsible for photosynthesis, energy metabolism and oxidative stress were upregulated in the presence of TNT, while the expression of cell wall related genes were downregulated. Several unidentified genes were also affected. The microarray results were validated using real-time RT-PCR for a subset of genes. Information from the microarray analysis can be used to engineer algae-based sensors to signal TNT exposure in addition to potential explosives cleanup applications.

Keywords: Chlamydomonas; Microarrays; TNT (2,4,6-trinitrotoluene); Gene expression; Xenobiotics

1. Introduction

Trinitrotoluene (TNT) has been extensively used as an explosive since 1902 [1]. Its use in military bases and in the production, purification and loading of ammunition has resulted in large amounts of wastes being generated. As a result of its improper disposal, TNT has entered the environment and contaminated both soil and groundwater systems. TNT and its degradative products are known to be toxic to many organisms such as algae [1,2], bacteria [3], plants [4–6] and invertebrates [7,8]. In addition, a major concern about the effect of TNT is its ability to potentially harm humans. Aside from unexpected and unintended explosions of landmines filled with TNT that can cause injury to humans, the ingestion of this compound can result in the formation of carcinogenic derivatives [9]. As a result, research in the field of detection and remediation has been driven by the need to clean up contaminated environments on a global scale.

The extent of the toxicity of TNT varies among organisms and consequently certain organisms should be useful in biomonitoring and bioremediation. Current detection systems monitoring surface and sub-surface contamination rely on active environmental sampling using chemical and bioanalytical analysis of contaminated samples to assess the level of pollution, which are costly [10]. Traditional methods of controlling TNT pollution include costly incineration processes [11]. An attractive alternative technology involves the use of plants and algae that can be developed as phytosen-

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sors and phytoremediation systems. Phytosensors are plants and algae that produce a phenotypic response to specific environmental stimuli while phytoremediation is characterized by the use of plants for in situ treatment of contaminated areas polluted by a variety of hazardous substances [12,13].

The potential use of these organisms for the phytoremediation of TNT contaminated sites has lead to the study of TNT uptake and the metabolic mechanism of TNT transformation in plants. Once the explosive compound has been taken up and metabolized by the plant, both oxidation and reduction products are generated. Overall, studies have reported aerobic reduction products of TNT with the major product being monoaminated TNT metabolites (4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene) [14]. Type I nitroreductase enzymes have also been proposed to catalyze the reduction of TNT [15]. Goheen et al. [16] isolated a ferrodoxin $NADP^+$ that was responsible for the conversion of TNT to 4-hydroxylamino-2,6-dinitrotoluene. Thioredoxin reductase in *Arabidopsis thaliana* was shown to catalyze the redox cycling of TNT via a single electron reduction [17]. Oxidation processes in the metabolism of TNT in plants have also been observed, which are similar to those transforming agricultural xenobiotics such as herbicides and pesticides. These reactions are often catalyzed by cytochrome P450 [18]. Bhadra et al. [19] investigated TNT oxidative pathways in *Myriophyllum aquaticum* (parrot feather) and found six metabolites were isolated after exposure to TNT. These metabolites included 2,4-dinitro-6-hydroxy-benzyl alcohol, 2-amino-4,6-dinitrobenzoic acid, 2-*N*-acetoxyamino-4,6-dinitrobenzaldehyde and two binuclear metabolites. Oxidative pathways are currently not well investigated and the formation of the products is yet to be elucidated.

In order to further investigate the fate of TNT in plants, changes in gene expression can be informative. Ekman et al. [20] have described the gene expression pattern of *Arabidopsis* seedling roots in response to TNT using serial analysis of gene expression (SAGE). TNT responsive genes may be useful in developing transgenic plants that respond to explosives for phytosensing or phytoremediation. Likewise, genetic engineering of plants possessing the capabilities of other bioremediating organisms such as bacteria and yeast may constitute an efficient tool for removing contaminants in soil [21]. For example, Hannink et al. [22] have developed transgenic tobacco that remediates explosive contaminants such as TNT. The tobacco plants expressed nitroreductase enzyme from the bacteria, *Enterobacter cloacae.* In addition, plants expressing bacterial pentaerythritol tetranitrate also possessed the ability to degrade TNT more effectively than wildtype [23].

The use of plants and algae as a cleanup technology for contaminated soils and water is both low-tech and cost effective. The limitation of using certain plant species to remediate pollutants is their relatively low biomass compared to other crops. Furthermore, some plants acclimatize poorly to particular climates and soil conditions [12,24]. These restrictions may be evaded by the use of molecular techniques that may reveal the functions of certain genes, which may be transferred to other plants to enhance the remediation process. In addition, promoters that are induced by the contaminant may be revealed. These promoters may be fused with marker genes such as one encoding green fluorescent protein that can be used as biological sensors that detect the pollutant (phytosensors).

In order to better understand gene regulation patterns in response to TNT, we have used *Chlamydomonas reinhardtii* (*Chlamydomonas*), a unicellular green alga as a model organism. With the completion of the *Chlamydomonas* genome project and the recent availability of microarray chips, several genes that are involved in the response to TNT may be identified. *Chlamydomonas* has several advantages as a model organism for stress response. Growth is rapid with cells attaining logarithmic growth phase in 2–3 days. They are also sensitive and respond to small changes in the environment by regulating transcription by the activation or repression of genes [25]. Genes identified in *Chlamydomonas* may also be transformable into common green algae ubiquitous to the environment [26].

In order to specifically investigate the transcriptional profile of *Chlamydomonas* in response to TNT, an expression microarray analysis was conducted. This technique allows monitoring of changes in levels of transcripts of almost all genes in a specific organism [27,28]. The differential expression of *Chlamydomonas* genes after a 24 h treatment with 1 and 3 μg/mL of TNT revealed genes that were regulated in response to low concentrations of TNT. Further, in order to validate the microarray results, real-time RT-PCR was performed. The possible involvement of these genes in response to TNT is discussed.

2. Materials and methods

2.1. Chlamydomonas strain and culture conditions

C. reinhardtii (Utex 89, the Culture Collection of Algae at the University of Texas at Austin) were maintained on Tris-acetate-phosphate (TAP) agar media [29] at 24 °C under continuous light (65 μmol/(m^2 s^1)). Growth curves of *Chlamydomonas* were obtained for a low range of TNT concentrations (0–5 μg/mL TNT) to determine the appropriate TNT exposure treatment for the microarray experiments. A stock solution of 100 μg/mL was prepared by dissolving crystalline TNT (Chemical Services, West Chester, PA) in TAP media. To obtain the desired TNT in growth media, a serial dilution of the TNT stock solution and TAP growth media was conducted. The *Chlamydomonas* inoculum was concentrated to 40×10^7 cells and harvested by centrifugation ($5000 \times g$ for 5 min) and inoculated in the 50 mL of the various TAP/TNT growth media. Cells were counted at daily intervals for 1 week using a hemacytometer. Growth curve analysis was conducted in triplicate and statistical analysis (ANOVA) was used to compare the treatments.

For RNA extraction, 100 mL of sample culture was inoculated in 500 mL of TAP media and allowed to attain logarithmic growth (10^7 cells/mL) by growing under continuous light as on a rotary shaker (140 rpm). Approximately 10^7 cells were harvested by centrifugation (5000 × *g* for 5 min) and inoculated in 50 mL of the desired culture medium and grown for 24 h before RNA isolation. The three culture media used for RNA isolation were supplemented with 0, 1 and 3 μg/mL of TNT.

2.2. RNA extraction and preparation of fluorescent probes

For each sample (treated and control) 1.5×10^7 cells were harvested and treated with 1 mL of Tri-Reagent (Molecular Research Center, Cincinnati, OH). Cells were disrupted by three freeze–thaw reactions in liquid nitrogen and 37 °C waterbath. The total RNA was extracted with chloroform and precipitated with isopropanol. RNA pellets were washed with 75% ethanol, air dried and resuspended in 50 μl RNase-free H_2O.

Fluorescently labeled Cy3 and Cy5 cDNA probes were generated from 10 μg total RNA using direct labeling with reverse transcription that incorporates aminoallyl nucleotide analogs that label cDNAs with fluorescent dyes. Each experiment (control versus treated sample) was replicated three times and cultures were completely independent. Experimental design included a dye swap per experiment. The labeling was performed using the CyScribe post-labeling kit (Amersham Biosciences, Birmingham, UK) and the labeling procedure according to the manufacturer. Labeled cDNA was purified through PCR purification columns (Qiagen, Valencia, CA) and eluted with 100 μl of RNAse-free H_2O. Corresponding Cy3 and Cy5 samples were combined and lyophilized. Pellets were resuspended in 70 μl DIG Easy Hyb hybridization buffer (Roche, Indianapolis, IN), denatured at 65 °C for 2 min and allowed to cool at room temperature for 2 min before probe hybridization to the microarray slide.

2.3. Hybridization reaction and microarray analysis

The *Chlamydomonas* microarray slides (chip 1.1v, Carnegie Institute, CA) contain 3079 unique ESTs, each represented four times. The probe solution was applied to microarray slides under a 22 mm × 50 mm lifter slip (Erie Scientific Company, Portsmouth NH) and placed in a humidified hybridization chamber (Corning Microarray Technology, Corning, NY). Ten microliters of water was placed inside each chamber before sealing. Hybridization was performed in a 50 °C waterbath for approximately 16 h. After hybridization, the slides were removed and placed in a slide rack submerged in washing solution (2× SSC, 0.03% (w/v) SDS), with the array face of the slide tilted down so that the lifter slip would drop off without scratching the slide. Once the lifter slip was removed, the slide rack was plunged up and down for approximately 2 min and then transferred to 1× SSC for 2 min and finally to 0.05× SSC for 30 s. All washing steps were carried out at 42 °C. Slides were tapped dry before they were scanned.

Hybridized microarrays were scanned for Cy3 and Cy5-labeled probes with the GenePix microarray scanner (Axon Instruments, Union City, CA). Separate images were acquired for each fluor at a resolution of 10 μm per pixel. To normalize the two channels with respect to signal intensity the photomultiplier was adjusted such that the pixel ratio was as close to 1.0 as possible.

2.4. Data analysis

For data analysis, spot intensities from scanned slides were quantified using Scanalyze software (Version 2.32; M. Eisen, Standford University http://www.genome-ww4.stanford.edu/MicroArray/SMD/restech.html). Microarray grids were predefined and manually adjusted to ensure optimal spot recognition. Data spots with abnormal shapes or high local background were discarded manually. To ensure that only data from spots of high quality were used in the analysis, quality control measurements produced by the Scanalyze software were used. Intensity values below 1.5 times their local background were deemed non-significant and excluded from the data analysis. Each microarray image was uploaded on the University of Tennessee Microarray Database (c.f. Stanford Microarray Database; SMD) at http://www.genome.ws.utk.edu. The criteria used for selection of the upregulated genes were based on: (a) normalized channel intensities greater than 150 with greater than a 1.7-fold increase in mRNA abundance, and (b) a regression correlation of >0.5. To select for down-regulated genes, normalized channel intensities of >150 with <0.6-fold decrease in mRNA and a regression correlation of >0.5 was used. Average ratios and standard deviations were calculated for the three replicates. For *Chlamydomonas* expressed sequenced tag (EST) identification, the BLASTN program was used to generate the entire list of known or putative gene functions (http://www.biology.duke.edu/chlamy_genome/).

2.5. Real-time RT-PCR

Validation of selected genes was performed using real-time RT-PCR. Total RNA was extracted as described earlier. Two samples were extracted for each treatment. RNA was quantified spectrophotometrically, and its quality was checked by electrophoresis using 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 (Life Technologies, INVITROGEN, Carlsbad, CA).

Real-time quantitative RT-PCR, based on TaqMan methodology was performed using the Smart-Cycler II

Table 1
List of primers and probes used for real-time RT-PCR

Gene (accession no.)[a]	Forward and reverse primers 5′–3′	Probe 5′–3′
Rubisco	TGGAGAGGAGTGAACAGTGG TCGGTCGTCTTACGCAGTT	TTATCCCCTGACAGGAATATACATGGT
Nitrate reductase (BM002822)	GCGTGCTGCCATACACAG CGTTAGCCCGTTTTGGTG	CCCCTTATGATTATGTATCGCATTGCATCA
Unknown protein (BE725473)	ACGTGCCCCCATCAGTAA TGGTCCATGCGTGCTAGA	AAGAATTCTTCACAGCTGCCGCGCTATT
Thioredoxin (BE453412)	GGCCGGGCTCCTACTTAT GCCGCATTGTTTCGTTTC	TTAAGGGCTTACAAAACTGCCACCCATACG
Unknown protein 1 (BE726502)	AGACTACCGCCCAACTGAAG GTCGGGTGCTGTTGTAGGTT	CCACTGCAAAACTTGTGACGAGACCACTAT
Putative lycopene β-cyclase (BM003222)	CCAGCCAAACCCAAACAC GAAGGCGTTTAGGCGTCA	CACAAGCCCACATGCAAGTCGAGAG

[a] Sequences available to GenBank.

System (Cepheid, Sunnyvale, CA). PCR was performed in a total volume of 25 μL containing 1× 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); and 2 μL of cDNA equivalent to 100 ng total RNA.

Primers and probes were chosen using the Primer 3 program [30] to have melting temperatures of 58–60 and 70 °C, respectively. The amplified product size was about 100 bp. Probes were labeled in 5′ with TET and in 3′ with TAMRA. To normalize the amount of total RNA present in each reaction, the housekeeping gene RUBISCO was co-amplified. The RUBISCO probe was labeled with FAM instead of TET. Primers and probes are listed in Table 1.

All amplification reactions consisted of one cycle of an initial incubation of 50 °C for 2 min followed by a hot start of 95 °C for 10 min and 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 58 °C for 1 min. Gene expression changes were quantified by calculating the average values of three runs each of two independent samples. PCR efficiencies for each amplicon were calculated using serial dilutions from a reference sample, which was a mix of equal amounts of cDNA from 1 μg/mL TNT and 3 μg/mL TNT treated samples. Specific mRNA transcript levels were expressed relative to the reference sample using calculations described by Pfaffl [31].

3. Results

3.1. *Chlamydomonas TNT treatment conditions*

TNT growth response studies were conducted in order to determine the *Chlamydomonas* treatment conditions for the microarray experiments. The growth response of *Chlamydomonas* was determined by conducting a time-based growth study on a range of TNT concentrations (Fig. 1). When compared to the control (0 μg/mL TNT) culture, there was no significant difference ($P > 0.05$, Student–Newman–Keuls multiple comparison test) in cell concentration at 1 μg/mL TNT on the final day of cell counts ($t = 168$ h). The cell concentration in 0 μg/mL TNT was 730 ± 22 (×10^4 cells/mL; all *Chlamydomonas* cell concentrations) and cell concentration at 1 μg/mL TNT was 739 ± 17 cells/mL. Data indicated that 3 μg/mL TNT was the maximum concentration of TNT supporting *Chlamydomonas* growth. The cell concentrations of *Chlamydomonas* growing in 4 and 5 μg/mL TNT were considerably lower than those exposed to lower concentrations, where *Chlamydomonas* cell counts after 168 h reached 28 ± 3 cells/mL, while control cultures had final cell counts of 730 ± 22 cells/mL.

The TNT concentrations used for the microarray experiments were 1 and 3 μg/mL TNT. At 1 μg/mL the growth response in terms of cell counts was not apparent since there was no significant difference in cell counts from the control cultures. Thus, in order to determine the response of TNT at the transcription level, *Chlamydomonas* cells were treated with 1 μg/mL TNT. The 3 μg/mL TNT concentration was used because it was the maximum TNT tolerance threshold concentration for apparently healthy *Chlamydomonas* growth.

3.2. *Microarray analysis*

The Carnegie Institute microarray contains 3079 unique ESTs representing approximately 30% of the genome. A global representation of the changes in expression of all the expressed sequence tags (ESTs) on the microarray is illustrated in Fig. 2. For the majority of the transcripts, expression appeared unchanged with TNT treatment (Fig. 2). Using the selection criteria outlined in Section 2, and accounting for the ESTs that correspond to similar genes in the

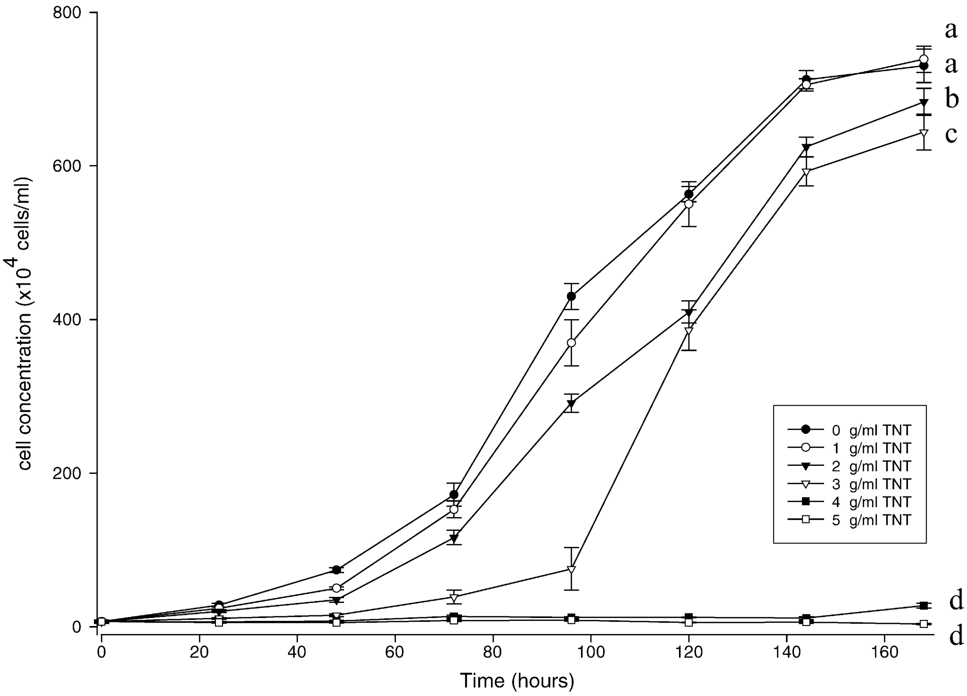

Fig. 1. The growth response of wild-type *Chlamydomonas* to TNT. Cell concentrations were measured every 24 h. Differing letters indicate significant difference ($P < 0.05$) at 160 h. Vertical bars represent standard deviations.

BLASTN search, 158 ESTs were differentially expressed in response to TNT. Of these, expression of 38 ESTs were upregulated and 43 ESTs were downregulated at 1 μg/mL TNT. At 3 μg/mL TNT, 35 genes were upregulated and 42 genes were downregulated. There was an overlap of very few genes at exposure to 1 and 3 μg/mL TNT. The expression data based on EST description and BLAST homologies for 1 μg/mL TNT-responsive ESTs are described in Tables 2 and 3. Differentially expressed genes in 3 μg/mL TNT are represented in Tables 4 and 5.

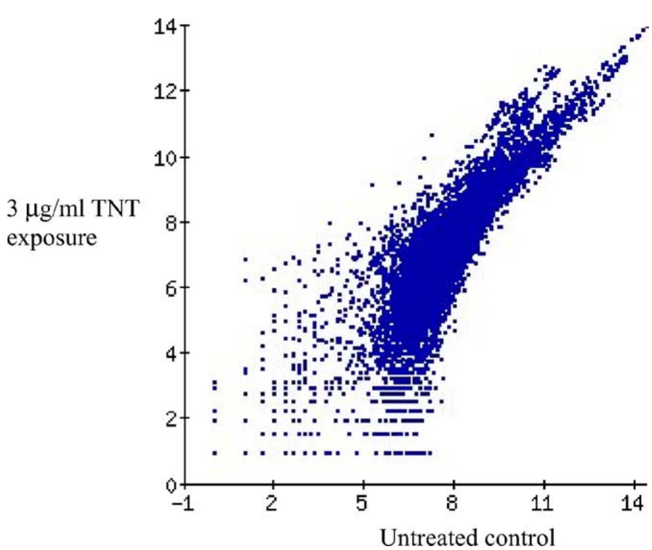

Fig. 2. Scatter plot of signal intensities for all ESTs on the microarray for the 3 μg/mL TNT experiment. A similar pattern was observed for the 1 μg/mL TNT microarray experiment. Normalized log-channel intensities for each clone on the microarray are plotted with signals from the control and the TNT-treated on the *x* and *y*-axis, respectively. Most values fall near the line $x = y$ indicating that most of the genes are unaffected by the treatment conditions. Values that fall outside the general $x = y$ line are the differentially expressed genes; values above $x = y$ line indicate upregulated genes and those below are downregulated. The genes we focused on are listed in Tables 2–6.

3.3. Functional classification of upregulated genes

Genes involved in several processes are differentially expressed in the presence of TNT. One of these functional processes is photosynthesis and energy metabolism: Photosystem I, Photosystem II, plastocyanin, cytochrome b_6f, and the light-harvesting complex genes are upregulated after 24 h of TNT treatment (Tables 2 and 4). These complexes constitute the photosynthetic electron transport chain, which primarily generates NADPH and ATP, required for the reduction of carbon and other chloroplast activities [32].

In addition to the upregulation of photosynthetic genes, many ribosomal proteins were upregulated by TNT. Ribosomal proteins are involved in protein synthesis. Both small and large subunit ribosomal proteins that are found in the chloroplast and cytosol were identified. There was approximately 2-fold increase in the expression of 50S and 30S chloroplast ribosomal genes in 1 μg/mL TNT (Table 2). At 3 μg/mL TNT the expression level of the large 50S subunit was 1.89 ± 0.12-fold induction, while the small 30S subunit expression was approximately 2.14 ± 0.15-fold higher (Table 4).

Table 2
Genes upregulated by a treatment with 1 μg/mL TNT

Accession no. or *Chlamydomonas* EST clone[a]	Gene description[b]	Putative functional category	Fold ratio ± S.D.
89409D12*	Unknown	Unclassified	2.68 ± 0.86
BE726502	Unknown	Unclassified	2.66 ± 0.2
BI722534	Succinoglycan biosynthesis like-protein	Exopolysaccharide used as reserve material	2.62 ± 1.63
BE12221	Phosphatase like protein	Involved in dephosphorylation of protein	2.51 ± 0.55
BU648787	Unknown	Unclassified	2.58 ± 1.67
BE453626	Unknown	Unclassified	2.29 ± 0.17
BE452532	Photosystem I polypeptide precursor	Photosynthetic electron transport chain	2.26 ± 0.06
BU654212	Putative ubiquitin specific protease	Involved in the removal of abnormal protein using the Ub/26S proteasome pathway	2.26 ± 0.0
BQ816253	Unknown	Unclassified	2.20 ± 0.09
AV642759	Putative chaperone protein	Involved in protection against heat induced protein aggregates	2.19 ± 0.37
BE452532	Polypeptide 35 precursor	Unclassified	2.17 ± 0.25
BE453562	Unknown	Unclassified	2.12 ± 0.51
BG848114	Unknown	Unclassified	2.11 ± 0.22
BE725903	30S ribosomal protein	Chloroplast located protein	2.07 ± 0.24
BE352272	Unknown	Unclassified	2.06 ± 0.29
BE724272	Chloroplast 50S ribosomal protein	Chloroplast located protein	2.01 ± 0.15
BE725909	Light-harvesting complex of Photosystem I	Involved in gathering light energy during photosynthesis	1.97 ± 0.14
BF862205	50S ribosomal like protein	Chloroplast located protein	1.95 ± 0.22
BF860102	Expressed protein	Unclassified	1.93 ± 0.18
BU654085	Agglutinin	Hydroxyproline-rich glycoprotein found in cell wall of *C. reinhardtii*	1.93 ± 0.14
BE122147	27S ribosomal protein	Cystolic located protein	1.93 ± 0.08
BM003222	Putative lycopene β-cyclase	Carotenoid production associated with photosynthesis and antioxidant agent	
BU651578	NADH malate dehydrogenase	Regulatory enzyme involved in an energy-dependant assimilation of carbon dioxide.	1.92 ± 0.17
BE351986	Unknown	Unclassified	1.92 ± 0.19
BE122147	Putative zinc finger protein	Transcription factor	1.90 ± 0.12
BF864612	Cytochrome b_6f	Electron transfer and proton-translocating enzyme	1.90 ± 0.11
BE453268	Plastocyanin	Photosynthetic electron transport; small copper binding protein that accepts electrons from cytrochrome b_6f.	1.87 ± 0.11
BI727105	Disulphide isomerase like protein	Assists in protein folding by formation of disulphide bridge	1.86 ± 0.12
BE237654	25S ribosomal like protein	Cystolic located protein	1.84 ± 0.01
BF860102	60S ribosomal like proteins	Cystolic located protein	1.83 ± 0.11
BE212030	Putative component of vesicle-mediated transport	Transport of proteins in vesicles to compartments in the cells; putative transport protein containing proteins which fuse to membrane.	1.81 ± 0.05
BE024336	40S ribosomal like protein	Involved in protein synthesis	1.81 ± 0.03
BE237659	Putative chloroplast 50S ribosomal protein	Chloroplast located protein	1.80 ±0.03
BE726790	Putative acyl carrier protein	Small acidic proteins that carry acyl chains during lipid synthesis	1.80 ± 0.08
BE352263	Unknown	Unclassified	1.76 ± 0.06
BM518983	60S ribosomal protein L12	Cystolic translation protein	1.75 ± 002
BE761412	Cytochrome b_6f-associated phospoprotein precursor	Electron transfer and proton-translocating enzyme	1.75 ± 0.05

[a] Sequences available to GenBank or *Chlamydomonas* EST clones (*) available to *Chlamydomonas* EST database. (http://www.biology.duke.edu/chlamy_genome/cgp.html).
[b] Gene description annotated with BLASTN homology search.

Table 3
Down-regulated genes at 1 μg/mL TNT

Accession no. or *Chlamydomonas* EST clone[a]	Gene description[b]	Putative functional category	Fold ratio ± S.D.
BI529617	Unknown	Unclassified	0.28 ± 0.12
BG848114	Unknown	Unclassified	0.30 ± 0.10
894058C1*	Unknown	Unclassified	0.31 ± 0.13
BE212109	Expressed protein	Protein similar to a *Arabidopsis thaliana* protein	0.31 ± 0.09
BE129394	Unknown	Unclassified	0.33 ± 0.15
BU646281	Unknown	Unclassified	0.33 ± 0.12
Olivier/Clp**	Unknown	Unclassified	0.33 ± 0.10
BM519195	Unknown	Unclassified	0.34 ± 0.14
BE129407	Unknown	Unclassified	0.34 ± 0.14
BE122216	Unknown	Unclassified	0.34 ± 0.15
BE352103	Unknown	Unclassified	0.35 ± 0.16
894030A0**	Unknown	Unclassified	0.35 ± 0.16
894044A0**	Hypothetical protein	Unclassified	0.36 ± 0.11
BE23786	Unknown	Unclassified	0.37 ± 0.02
BE452608	Unknown	Unclassified	0.38 ± 0.14
BE724681	Porphorin I precursor	Chlorophyll structural component	0.38 ± 0.12
BE12210	Unknown	Unclassified	0.39 ± 0.15
BE227716	Unknown	Unclassified	0.40 ± 0.09
BE726019	Unknown	Unclassified	0.40 ± 0.06
Stern:C12**	Unknown	Unclassified	0.41 ± 0.09
894004H1	Unknown	Unclassified	0.44 ± 0.15
BE337577	Putative membrane protein	Unclassified; component of cell membrane	0.44 ± 0.03
BE726560	Putative protein	Third enzyme in the porphyrin biosynthetic pathway	0.46 ± 0.05
963082D0*	Unknown	Unclassified	0.46 ± 0.07
BF860436	Hypothetical protein	Unclassified	0.46 ± 0.06
BM518930	Unknown	Unclassified	0.47 ± 0.08
BI725674	Multicopper ferroxidase	Involved in iron uptake	0.47 ± 0.07
BI999281	Unknown	Unclassified	0.49 ± 0.05
BM003014	p60 katanin	Protein that binds to microtubules and severs then in an ATP-dependant manner	0.51 ± 0.07
BI722399	Gametolysin		0.52 ± 0.09
BE453108	Putative selenoprotein	Protein family that contain selenium	0.53 ± 0.05
BE351855	Unknown	Unclassified	0.55 ± 0.04
BE725812	Unknown	Unclassified	0.55 ± 0.02
BM518939	Unknown	Unclassified	0.55 ± 0.03
BF865887	Unknown	Unclassified	0.55 ± 0.02
BI723489	Putative ζ-carotene desaturase precursor	Involved in carotene biosynthetic pathway	0.55 ± 0.04
BE725245	Expressed protein	Similar to a *Arabidopsis thaliana* protein	0.55 ± 0.03
BE724263	Unknown	Unclassified	0.57 ± 0.02
BE726116	Unknown	Unclassified	0.57 ± 0.03
BM518836	Putative indole-3-glycerol phosphates synthase	Metabolic enzyme in the production of indol-3- glycerol phosphate	0.58 ± 0.01
BE725843	Putative sterol-methyltransferase	Involved in the sterol biosynthetic pathway	0.59 ± 0.01
BE453183	Unknown	Unclassified	0.59 ± 0.01

[a] Sequences available to GenBank or *Chlamydomonas* EST clones (*) available to *Chlamydomonas* EST database. (http://www.biology.duke.edu/chlamy_genome/cgp.html). (**) Indicates EST clones made available to microarray chip 1.1v; sequences not available to *Chlamydomonas* EST database at time of study.
[b] Gene description annotated with BLASTN homology search.

Another major category of differentially regulated genes encode for cell defense proteins, which include anti-oxidative stress proteins and heat shock proteins. The majority of these transcripts were upregulated in 3 μg/mL TNT. The anti-oxidative stress proteins include peroxiredoxin-like proteins, DegP protease-like protein, thioredoxin and glutathione *S*-transferase (GST). The genes encoding the peroxiredoxin-like protein, DegP protease-like protein and thioredoxin were upregulated at least 2-fold in treated cells. Peroxiredoxins form a group of peroxi-

Table 4
Genes that are upregulated with a treatment with 3 μg/mL TNT

Accession no. or *Chlamydomonas* EST clone[a]	Gene description[b]	Putative functional category	Fold ratio ± S.D.
BE725473	Unknown	Unclassified	6.09 ± 3.84
BU 648787	Hypothetical protein	Unclassified protein; similar to an expressed protein in *Arabidopsis thaliana*	3.05 ± 0.67
			2.69 ±0.87
AV643891	Heat shock protein	Cell defense	2.52 ± 0.48
BI728129	Sulfate transport system permease protein	Sulfate transport into cells	2.3 ±0.48
	Peroxiredoxin like protein	Antioxidative enzyme catalyze the reduction	2.26 ± 0.12
AV642759	Putative chaperone protein	Involved in protection against heat induced protein aggregates	2.22 ± 0.52
BE453199	Plastid ribosomal like protein	Chloroplast located	2.19 ± 0.12
BF864539	Light-harvesting complex protein precursor	Involved in gathering light energy during photosynthesis	2.16 ± 0.22
BE121746	30S ribosomal like protein	Chloroplast located protein	2.14 ± 0.15
BE237902	Unknown	Unclassified	2.12 ± 0.21
BM002900	Unknown	Unclassified	2.10 ± 0.24
BF862306	DegP protease like protein	Involved in thermal and oxidative tolerance; degrades misfolded and aggregated proteins in the periplasm	2.10 ± 0.36
Stern: A03**	Unknown	Unclassified	2.05 ± 0.2
BF864539	Light-harvesting complex II precursor protein	Intercept light energy in photosynthesis	2.04 ± 0.21
BE453412	Thioredoxin	A disulphide-reducing redox protein involved in antioxidant functions.	2.03 ± 0.26
BE337707	Unknown	Unclassified	2.00 ± 0.20
BF863557	Unknown	Unclassified	1.99 ± 0.11
Olivier/ClpC2**	Unknown	Unclassified	1.98 ± 0.21
BI726314	Sulfotransferase	Involved in sulfur metabolism	1.94 ± 0.05
BM003222	Putative lycopene β-cyclase	Carotenoid production associated with photosynthesis and antioxidant agent	1.93 ± 0.16
BE024621	Unknown	Unclassified	1.92 ± 0.35
BE212144	Unknown	Unclassified	1.92 ± 0.19
BM002822	Nitrate reductase	Primary enzyme that catalyzes reduction of nitrate to nitrite	1.90 ± 0.16
BE724272	Chloroplast 50 S ribosomal like protein	Chloroplast located ribosomal protein involved in translation	1.89 ± 0.12
BE129393	Glutathione *S*-transferase like protein	Primary enzyme in oxygen detoxification (oxidative stress)	1.89 ± 0.12
BI529617	Putative purple acid phosphatase	Primary enzyme of cell walls and involves the mobilization of phospohorus from organic compounds in soil	1.89 ± 0.09
BE352248	16S ribosomal like protein	Chloroplast located protein	1.86 ± 0.13
BE024560	Putative phenlyalanine t-RNA synthetase	Protein synthesis	1.85 ± 0.11
BM519278	Expressed protein	Unclassified	1.83 ± 0.06
BE453407	50S ribosomal protein	Chloroplast located	1.80 ± 0.13
BE056399	Unknown	Unclassified	1.80 ± 0.12
BF862787	QM family protein	Involved in cell growth and differentiation	1.80 ± 0.06
BE024692	Unknown	Unclassified	1.77 ± 0.07

[a] Sequences available to GenBank or *Chlamydomonas* EST clones (*) available to *Chlamydomonas* EST database. (http://www.biology.duke.edu/chlamy_genome/cgp.html). (**) Indicates EST clones made available to microarray chip 1.1v; sequences not available to *Chlamydomonas* EST database at time of study.

[b] Gene description annotated with BLASTN homology search.

Table 5
Down-regulated genes at 3 μg/mL TNT

Accession no. or *Chlamydomonas* EST clone[a]	Gene description[b]	Putative functional category	Fold ratio ± S.D.
BE453282	Unknown	Unclassified	0.35 ± 0.10
BF863625	Putative porphorin precursor	Chlorophyll structural component	0.35 ± 0.04
BF761376	Unknown	Unclassified	0.35 ± 0.04
BF863773	Unknown	Unclassified	0.37 ± 0.05
Stern: B10**	Unknown	Unclassified	0.37 ± 0.06
BE724871	Unknown	Unclassified	0.38 ± 0.13
BE452945	D-β-Hydroxybutyrate dehydrogenase	Enzyme found in the mitochondria membrane	0.41 ± 0.09
BF863761	α-Tubulin like protein	Microtubule protein	0.43 ± 0.09
BM519086	Unknown	Unclassified	0.44 ± 0.11
BE227503	Putative α-2-chain		0.45 ± 0.07
BE352141	Unknown	Unclassified	0.45 ± 0.07
BF863819	Putative sulfated surface glycoprotein	Surface protein	0.46 ± 0.07
BE024783	Unknown	Unclassified	0.48 ± 0.04
BF860856	Unknown	Unclassified	0.48 ± 0.1
BE725330	Putative hydroxyproline rich glycoprotein	Component of cell wall proteins	0.48 ± 0.12
BE238331	ATP synthase	Energy evolving enzyme	0.48 ± 0.04
BE725207	Unknown	Unclassified	0.49 ± 0.05
BE453150	Expressed protein	Similar to *Arabidopsis thaliana* expressed protein	0.49 ± 0.07
BE352179	Unknown	Unclassified	0.49 ± 0.07
BE725344	Putative transketolase	Enzyme that catalyzes the transfer of two carbon fragment from a ketose to a aldose	0.49 ± 0.09
BE238314	Unknown	Unclassified	0.49 ± 0.07
BE725502	Unknown	Unclassified	0.50 ± 0.04
BE237914	Translation elongation factor like protein	Involved in translation	0.50 ± 0.09
963104B1*	ATP dependant protease	Energy related enzyme	0.50 ± 0.06
BE726129	Inorganic pyrophosphatase precursor	Vacuolar proton translocating protein	0.51 ± 0.05
BF860319	Unknown	Unclassified	0.51 ± 0.05
BF863295	Putative vegetative cell wall protein	Component of the cell wall	0.51 ± 0.05
BE725158	Putative ATP synthase alpha chain	Energy evolving protein	0.51 ± 0.06
BF860406	Hypothetical protein	Similar to protein in Desulfovibrio desulfuricans	0.51 ± 0.06
BF861408	Unknown	Unclassified	0.52 ± 0.08
BE725556	Unknown	Unclassified	0.52 ± 0.03
BE724687	Unknown	Unclassified	0.52 ± 0.04
BE122081	Unknown protein	Protein similar to *Arabidopsis thaliana*	0.53 ± 0.02
BF860682	Unknown	Unclassified	0.56 ± 0.02
BE351718	Unknown	Unclassified	0.56 ± 0.05
BI999544	Putative ABC transporter subunit	Involved in the active movement in a wide variety of substrates across cell membranes.	0.57 ± 0.02
BM518842	14-3-3 Protein (G-box binding factor)	Signal transduction	0.57 ± 0.01
BM518842	Unknown	Unclassified	0.57 ± 0.01
BE725268	*S*-Adenosylmethionine decarboxylase proenzyme	Enzyme involved in the polyamine synthetic pathway	0.58 ± 0.02
BE726480	BBC1-like protein	Involved in activation of transcription	0.58 ± 0.02
BF859990	Unknown	Unclassified	0.59 ± 0.02

[a] Sequences available to GenBank or *Chlamydomonas* EST clones (*) available to *Chlamydomonas* EST database. (http://www.biology.duke.edu/chlamy_genome/cgp.html). (**) Indicates EST clones made available to microarray chip 1.1v; sequences not available to *Chlamydomonas* EST database at time of study.

[b] Gene description annotated with BLASTN homology search.

dases found in bacteria [33], yeast [34], animals and higher plants [35]. At the lower concentration of TNT very few known cell defense genes were overexpressed. Both TNT treatment conditions resulted in the upregulation of a putative lycopene β-cyclase. Lycopene β-cyclase is involved in the synthesis of carotenoid compounds. Often these compounds are associated with photosynthesis and many also act as antioxidant agents [36]. In addition, putative chaperone proteins were expressed 2.2 ± 0.37-fold greater at 1 μg/mL TNT. In general, many of the proteins were unknown and may be involved in cell defense regulation.

One interesting gene that was upregulated at 3 μg/mL TNT was that encoding nitrate reductase with a 1.9 ± 0.16-fold increase. Nitrate reductase is the primary enzyme that catalyzes the reduction of nitrate to nitrite [37]. Other metabolic genes that were upregulated were the sulfotransferase gene and the sulfate transport system gene. These genes are involved in sulfur assimilation in *Chlamydomonas* [38].

The final category of upregulated genes comprise those whose protein functions are not yet known. Some of these genes include hypothetical proteins and expressed proteins that are similar to those found in other organisms. Approximately, 26 and 40% of responsive genes had unknown functions in 1 and 3 μg/mL TNT, respectively. For both TNT treatment concentrations, the highest upregulation was observed for a gene whose functional category was unclassified. Among the upregulated genes at 3 μg/mL TNT treatment was an unknown gene that has a 6.0 ± 3.84-fold ratio increase. In addition, the highest increase in fold ratio after 1 μg/mL TNT was for an unknown protein gene, which had a 2.68 ± 0.86-fold ratio increase.

3.4. Functional classification of the down-regulated genes

This study, which was intended to ultimately develop phytosensors and phytoremediation application, focused less on the expression of genes that were downregulated by TNT, however it was determined that a few genes had reduced mRNA levels. In contrast to the upregulated genes, the function of the majority of the down-regulated genes were unknown. At 1 μg/mL TNT, approximately 74% of the unknown function genes, and at 3 μg/mL TNT, 50% of the unknown function genes were downregulated.

Among the down-regulated genes, many genes associated with cell wall components of *Chlamydomonas* were downregulated at 3 μg/mL TNT. Hydroxyproline-rich proteins constitute a major structural component of the *Chlamydomonas* cell wall. Another set of genes that were downregulated was the ATP related genes. ATP is involved in the expenditure of energy that drives various cellular processes in the cell [32].

3.5. Real-time RT-PCR

A total of five genes from the list of upregulated genes in both in 1 and 3 μg/mL TNT treated samples were selected for confirmation based on hybridization intensities and different functional categories. Genes coding for nitrate reductase, thioredoxin and an unknown protein were upregulated in 3 μg TNT (Table 6) and an that for an unknown protein was upregulated in 1 μg/mL TNT, whereas while a lycopene gene was found to be upregulated in both 1 and 3 μg/mL TNT according to microarray results. The microarray and real-time PCR were consistent for four of the five genes analyzed (Table 6). However, real-time PCR results for the gene encoding unknown protein (BE725473) showed a very high expression level (almost 20-fold induction) and the lycopene β-cyclase gene showed a 6-fold induction in 1 μg/mL TNT. The only gene that did not correspond to the microarray result was the unknown protein (BE726502), which showed a 6-fold downregulation with real-time PCR in contrast to 1.93-fold upregulation with respect to the microarray data.

Table 6
Validation of array-based expression profile by real-time RT-PCR

Gene name	Relative expression[a]	
	Microarray	Real-time RT-PCR
Thioredoxin	2.03 ± 0.26	1.81 ± 0.29
Nitrate reductase	1.90 ± 0.16	2.31 ± 0.40
Unknown protein (BE 725473)	6.09 ± 3.84	19.94 ± 0.66
Putative lycopene β-cyclase (3 μg TNT)	1.93 ± 0.16	1.00 ± 0.64
Putative lycopene β-cyclase (1 μg TNT)	1.93 ± 0.08	6.85 ± 0.05
Unknown protein (1 μg TNT)	2.66 ± 0.2	−6.33 ± 0.52

[a] Positive value indicates upregulation and negative values indicate downregulation. The values are expressed as mean ± S.D. Each value represents a mean of two independent samples run in triplicate.

4. Discussion

The focus of this study was the identification of upregulated *Chlamydomonas* genes in the presence of microgram per milliliter amounts of TNT. The data suggest that TNT affects the regulation of genes involved in photosynthesis and therefore, may affect the redox state in *Chlamydomonas*. It has been reported that in green algae and higher plants transcription [39], mRNA stability [40], translation [41] and protein phosphorylation [42] are regulated by the redox state of the photosynthetic electron transport chain. In addition, other reports suggest that the thioredoxin gene [43], some nitrogen-related genes [44] and heat shock genes [45] were under the control of the photosynthetic electron transport. In this study, genes encoding thioredoxin, nitrate reductase and putative heat shock proteins were upregulated upon exposure to TNT, indicating that TNT can affect the electron transport chain. Studies conducted by Nocter and Foyer [46] have characterized the antioxidant defense network in plants where they suggest that disturbances of the photosynthetic electron transport chain can result in oxidative stress.

Oxidative stress can occur as a result of a number of abiotic and biotic stresses. These stress environments include drought stress, osmotic stress, ionic stress, the presence of pollutants, and intense light [47]. During aerobic metabolism under stress, reactive oxygen species (ROS) are produced as a result of partial reduction of oxygen. ROS were originally considered to be detrimental to cells, but recently it has been shown that it is involved in redox regulation by adjusting cellular activities [48]. It is clear that the generation of ROS during oxidative burst is one of the first cellular responses to potential pathogens and elicitor molecules [49]. These ROS induce the expression of defense-related genes such as those encoding glutathione *S*-transferase [50], peroxidases such as ascorbate peroxidase and superoxide dismutase [47]. In this study, the GST- and peroxidase enzyme-encoding genes were both upregulated in the 3 μg/mL TNT treatment. Glutathione acts as a redox sensor and is involved in the multiple regulatory systems coordinating the expression of defense genes [51]. The GST gene was also shown to be upregulated in *Arabidopsis thaliana* when exposed to TNT [20]. Arisi et al. [52] and Zhu et al. [53] suggested that the increasing glutathione biosynthetic capacity could enhance resistance to oxidative stress. Transgenic plants that overexpress glutathione gene were found to grow better under salinity and chilling stress [54].

The upregulation of ribosomal proteins in the presence of TNT is apparent by the identification of several putative and confirmed ribosomal proteins. Ribosomes regulate the protein synthesis in the cytosol and in plastids. Studies conducted by Mendez-Alvarez et al. [47] suggest that certain ribosomal proteins may be involved in oxidative stress. Cloning and engineering the *Chlamydomonas* 60S ribosomal protein cDNA into oxidative stress sensitive *Saccharomyces cerevisiae* resulted in restoration of the oxidative stress resistance capacity of *S. cerevisiae*. This oxidative resistance capacity was induced by the synthesis of carotenoids. The increased carotenoid production may be the result of the overproduction of the *Chlamydomonas* 60S ribosome which regulates the translation of proteins. Carotenoids are a group of polyene pigments produced by photosynthetic organism and some types of fungi and bacteria [55]. A majority of carotenoids are synthesized from lycopene. β-Carotene is synthesized directly from lycopene and catalyzed by lycopene β-cyclase. A gene encoding lycopene β-cyclase was upregulated in *Chlamydomonas* after the treatment of TNT, indicating that this protein may play a role in oxidative stress resistance. ROS produced during oxidative stress have been demonstrated to act as a novel class of second messengers mediating high carotenoid synthesis during chromoplast differentiation in pepper [56].

The upregulation of nitrate reductase at 3 μg/mL TNT indicates that this enzyme may be associated with TNT metabolism. Hannink et al. [22] engineered plants that express the nitrate reductase enzyme from *Enterobacter cloacae* and described the phytodetoxification of TNT. Nitrate reductase utilizes NADPH as a source of reducing equivalents to catalyze a two-electron reduction of TNT to hydroxyaminodinitrotoluene, which is subsequently reduced to aminodinitrotoluene derivatives.

Among the down-regulated genes cell wall related genes were repressed. It is interesting to note that in some research studies hydroxy-proline rich glycoproteins aid in the resistance to metal ions [57]. The cell walls of algae have the capacity to bind metal ions in negatively charged sites. The anion carboxylate groups of pectin and glycoprotein have a strong binding affinity for metal ions [58]. In this study, the repression of cell wall genes indicates that TNT resistance may not be cell wall related but may interfere with cell wall maintenance. TNT may affect the expression of hydroxyproline rich proteins, hence the genes are downregulated.

Several of the genes discussed here were not analyzed because their functions have not yet been described. Many hypothetical genes are indeed transcribed and some of them responded strongly to TNT treatment suggesting that they have a significant role, yet to be unraveled in further studies.

Real-time RT-PCR was used to confirm the microarray data because of its high sensitivity. The real-time PCR results validated the microarray results and were consistent for four of the five genes analyzed. However, the real-time PCR showed a 20-fold induction of the unknown protein BE725473 that was upregulated in 3 μg/mL TNT compared to the microarray experiment where it was up-regulated 6-fold. The only gene that did not correspond to the microarray result was the unknown protein (BE726502), which showed a 6-fold downregulation with real-time RT-PCR in contrast to 1.93-fold upregulation with respect to the microarray data. Differences in expression levels in microarray results as compared to real-time PCR results has been reported by Rajeevan et al. [59] and Yoshida et al. [60]. This may be the result of sample-to-sample variation or underestimation of expression levels by microarray analysis.

When considering the relative expression of the 3079 genes represented on the array, it is important to realize that the expression profile by itself does not define critical genes required for stress response. In some instances changes in mRNA may not correlate with changes in protein or enzyme activity level [61]. Genes measured at low amounts of expression may have measurements that are less reliable [62] and therefore microarray analysis may not identify these genes, albeit their potential use in TNT response. Expression profiles however, do provide a useful starting point for a more in depth analysis of stress response in a particular organism. For example candidate gene lists can be created to assign putative functions to genes in response to a particular stress. In this study, TNT responsive genes were identified. Candidate genes can be further analyzed for their response in the resistance to TNT. These genes can be cloned and overexpressed into other organisms to assess tolerance to TNT. In addition, promoters that are induced in the presence of TNT may be fused to reporter genes such as *GFP* to serve as inducible biomonitoring systems. The unknown protein, which shows a 20-fold upregulation seems to be a promising candidate for further analysis of promoters. In addition, its further characterization might illuminate its function. Although microarray technology provides insight into all the genes that are expressed in response to various developmental and environmental factors, our study had to be constrained to arrays that had only 3079 ESTs. There are now arrays available with more ESTs and further studies with these arrays would probably reveal additional genes of interest. Our study would serve as a platform for all further investigation of TNT inducible genes in *C. reinhardtii*.

5. Perspectives

Whereas, a TNT-inducible algal phytosensor would be suitable for standoff detection, and algal-based phytoremediation attractive for use in engineering a self-contained decontamination of aquatic ecosystems, eukaryotic cells and tissues are not the most sensitive or effective platforms. Shriver-Lake et al. [63] have developed a continuous flow biosensor device using immunology-based technologies that employs antibodies raised against the explosives TNT and RDX that is effective at ppb to ppt levels. In addition, several bacterial species inherently possess pathways that degrade explosives [22,23,64,65]. Nonetheless, characterizing plant and algal responses to xenobiotics using genomics should complement the prokaryotic systems that are probably more adapted, as a whole, to xenobiotics than are plants. Engineered plants and algae could be more suitable than hand-held biosensors or microbes in many circumstances. Nonetheless, the problem of contamination of explosives, unexploded ordnance, and mines is great and the world needs advanced chemistry, biochemistry, biology and physics research to have a chance at providing solutions for monitoring and cleaning up the mess that mankind has created for itself and the planet.

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