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Screening of Mutant Arabidopsis Thaliana and Chlamydomonas Reinhardtii for their Potential Use as Phytosensors in 2,4,6 Trinitrotoluene (TNT) Contaminated Environments

Nrupali Patel
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To the Graduate Council:

I am submitting herewith a thesis written by Nrupali Patel entitled "Screening of Mutant Arabidopsis Thaliana and Chlamydomonas Reinhardtii for their Potential Use as Phytosensors in 2,4,6 Trinitrotoluene (TNT) Contaminated Environments." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

Neal Stewart, Major Professor

We have read this thesis and recommend its acceptance:

Carl Sams, Andreas Nebenführ

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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We have read this thesis
and recommend its acceptance:

Carl Sams

Andreas Nebenführ

Acceptance for the Council:

Anne Mayhew

Vice Provost and Dean of Graduate Studies

(Original signatures are on file with official student records.)

SCREENING OF MUTANT *ARABIDOPSIS THALIANA* AND *CHLAMYDOMONAS REINHARDTII* FOR THEIR POTENTIAL USE AS PHYTOSENSORS IN 2,4,6-TRINITROTOLUENE (TNT) CONTAMINATED ENVIRONMENTS

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Abstract

Plant biotechnology is a diverse field that is expanding from agricultural research towards environmental applications. The focus of this project was to exploit vegetative effects, such as photosynthesis and growth in genomic model organisms *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* to 2,4,6-trinitrotoluene (TNT) with a goal to develop biomonitoring systems. Plants and algae have evolved with various biochemical pathways that have the potential to be exploited for the use of sensing explosives and chemical warfare agents in soil, water and air. The first part of the project involved characterizing the effects of TNT on germination and early seedling development of wild-type *Arabidopsis thaliana*. It was determined that 10 μM TNT was the tolerance level for *Arabidopsis* and was used to screen fast neutron irradiated mutant *Arabidopsis* to evaluate the phenotypic stress responses in the seedlings. TNT responsive mutant lines (lines 1, 2, 3, and 4) were selected on a basis of a leaf color change from dark green to pale green. The second part of the project was to determine the growth response of wild-type and mutant *Chlamydomonas reinhardtii* to TNT. Growth response studies of wild-type *Chlamydomonas* revealed that 3 μg/ml of TNT was the maximum TNT concentration that allowed growth. Insertional mutant lines were screened on 3 μg/ml TNT where one mutant (CL48) was selected on the basis of a color change from green to white. Growth response of CL48 in TNT indicated that this mutant line was hypersensitive to TNT compared with transformation recipient line and wild-type *Chlamydomonas*. The third part of the project involved using microarray technology to determine the differential gene expression of *Chlamydomonas* in response to TNT. Approximately 158 responsive genes were differentially expressed. Genes involved in

photosynthesis and energy metabolism were up-regulated in the presence of TNT. TNT may cause oxidative stress since many oxidative stress related genes were up-regulated. Among the down-regulated genes, the expression of cell wall-related genes was repressed. Several unidentified genes were also induced or repressed. The overall study promotes future work involving the identification of the genes that are involved in TNT response.

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NOMENCLATURE

TNT	2,4,6-trinitrotoluene
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
NaCl	Sodium chloride
2,4-DNT	2,4-dinitrotoluene
DNA	deoxyribose nucleic acid
Gy	Grays of irradiation dosage
GFP	Green florescent protein
TDG	Thiodiglycol
TAP	Tris-acetate-phosphate
ASL	Arginosuccinate lyase
CL	Cell line
EST	Expressed sequence tags
ROS	Reactive oxygen species
ANOVA	Analysis of variance
WT	Wild-type

CHAPTER ONE

Literature Review

Introduction

Explosives such as 2,4,6-trinitrotoluene (TNT) and other nitrated compounds are widely used in military ammunition. Their combustion and decomposition products can enter the environment from manufacturing activities, field usage and improper disposal (Best *et al*., 2001; Halasz *et al.,* 2002). The presence of this compound in contaminated sites is a major concern. It is highly toxic to many organisms including humans by manifesting as aplastic anemia and hepatitis (Rosenblatt, 1980). One of the major field usages of TNT is in the use of anti-personnel landmines (APLs) in warfare. Landmines are small explosive devices that are placed underground and detonate upon activation. APLs can thus kill or injure combatants and non-combatants alike. Recently United Nations estimates placed the burden of landmine clearance at 33 billion dollars and 1100 years using current detection technologies (Sylvia *et. al.,* 2000). Humanitarian groups and several nations have called for the potential ban on landmines and thus, cost effective, and environmentally accepted detection systems are required.

Anti-personnel mines come in various shapes and sizes and can be encased in metal, plastic, or wood containers. Typically they are shaped in the form of a disk with diameters from 20 to 125 mm, length from 50 to 100 mm and weighing only 30 g (Hussein & Waller, 2000). For TNT based landmines, TNT and its derivative, 2,4-dinitrotoluene (2,4-DNT) permeate through the landmine components as well as cracks

and pores through the mine casing (Sylvia *et al.,* 2000). Studies on the environmental effects of explosives and their transport through the soil indicate that a detectable amount of explosive reaches the surface soil (George *et al.,* 1999). At the surface, some of the TNT degrades to 4-amino-2, 6-dinitrotoluene, but considering greater than 99% of the explosive material is TNT (Sylvia *et al.,* 2000), the most effective sensor that samples the surface soil would detect a TNT signature.

The broader concept of this project involves the development of biosensors based on using whole plant systems (phytosensors). The seeds can be sown over a minefield in a manner that would result in uniform coverage. The plants would germinate and those located over a landmine would exhibit a distinctive phenotypic change. This would allow the detection of TNT in the soil thereby locating the landmine areas.

Phytotoxicity TNT and the transformation of TNT by plants

There are three main classes of high explosives that include the nitroaromatics (TNT), nitroamines (hexahydro-1, 3, 5,- trinitro- 1, 3, 5- triazine (RDX)) and nitrate esters (glyceroltrinitrite (GTN)). Historically, TNT is the most frequently used explosive and is associated with widescale soil contamination. It belongs to the nitroaromatic group which are characterized by an aromatic ring and three nitro groups (Esteve-Nunez *et al.,* 2001).

The potential for a particular plant species to be a phytosensor is largely dependant on its ability to tolerate the contaminant; consequently many research groups have studied phytotoxicity thresholds for explosives (Burken *et al.,* 2000). Research in cell culture, germination, and more recently mature plants has been explored.

Phytotoxicity appears to be species-dependent and is also affected by factors such as growth stage and the bioavailability of the contaminant (Scheidemann *et al.,* 1998). Recent studies conducted by Peterson *et al.,* (1996) have reported the effect of TNT on the germination and seedling development of tall fescue (*Festuca arundinacea*) demonstrating a linear effect of TNT concentration on germination. Studies on two other species, switchgrass (*Panicum virgatum*) and smooth bromegrass (*Bromus inermis*) show differing results. Concentrations of up to 15 mg/L (66 μM) did not affect switchgrass germination rate but at lower concentrations (7.5 mg/L TNT (33 μM)) stunted root growth of smooth bromegrass (Peterson *et al.,* 1998). These data suggest that phytotoxicity of TNT is species-dependant.

The uptake of TNT has been documented for many plant species. Once the explosive compound has been uptaken and metabolized by the plant, both oxidation and reduction products are produced. Overall, studies have reported aerobic reduction products of TNT with the major products being monoaminated TNT metabolites (4-amino-2, 6-dinitrotoluene, 2-amino-4, 6-dinitrotoluene) (Burken *et al.,* 2000). Type I nitroreductase enzymes have also been proposed to catalyze the reduction of TNT (Medina & McCutcheon, 1996). Goheen *et al.*, (1999) isolated a ferrodoxin $NADP^+$ that is 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 (Miskiniene *et al.,* 1998).

Oxidation processes in the metabolism of TNT in plants have also been observed. Bhadra *et al*., (1999) isolated six metabolites that were distinct from reduction products. These included 2,4-dinitro-6-hydroxy-benzyl alcohol, 2-amino-4, 6, dinitrobenzoic acid

and 2,4-dinitro-6-hydroxytoluene. These products may occur in plants because oxidation is the primary reaction in the detoxification of pesticides and herbicides (Bolwell *et al.,* 1994).

It is well known that plants uptake and metabolize TNT into various compounds via enzymes and co-factors. Plants have many biochemical pathways that are affected by abiotic stress responses. Exploring gene expression in the presence of TNT has a potential to evaluate gene expression regulation and the corresponding biochemical pathways that are involved in TNT metabolism.

Phytotoxicicty and plant metabolism of RDX and HMX

Toxicity of these compounds is considerably less than that for TNT however, in general, nitroamines are toxic (Harvey *et al.,* 1997, Hawari J, 2000) and are considered possible carcinogens. RDX is also known to have adverse effects on the central nervous system (Burken *et al.,* 2000). The effects of RDX on plants have indicated that it is less toxic to plants than TNT. Research conducted by Lucero *et al.,* 1999 determined the toxicity of both RDX and HMX to *Datura innoxia*. The cell cultures tolerated supersaturating concentrations up to 270 μM RDX and 17 μM HMX indicating that soils contaminated with HMX or RDX will not likely inhibit plant growth (Lucero *et al.,* 1999).

As predicted, the uptake and metabolism of RDX is different from that of TNT in plants. Best et al, 1999 treated aquatic plants with [U-^{14}C]-RDX in hydroponic incubations. The uptake of [U-^{14}C]-RDX derived ^{14}C in plants was slower than that of TNT and transport was significant where 23 % (sweet-flag, *Acorus calamus*) and 81 %

(parrot feather, *Myriophyllum aqaticum*) of the total ^{14}C located in the shoots. Higher concentration of RDX occurred at sites where new plant material was synthesized (Best *et al.,* 1999). Concentration of up to 21 mg/L of RDX did not affect the growth or the transpiration rate of hybrid poplars in a hydroponic study (Thompson *et al.,* 1999). Accumulation of RDX has also been studied in garden vegetables and agricultural crops (Larson, 1997) such as maize, lettuce and tomato. Uptake of RDX by maize (*Zea mays*), soybean (*Glycine max*), sorghum (*Sorghum sudanese*), and wheat (*Triticum aestivum*) was similar to other crops species (Burken *et al*, 2000). Concentrations of RDX in plant species were directly proportional to the RDX levels in the hydroponic solutions. Furthermore, properties of the soils have a considerable effect on plant uptake, limiting the uptake of RDX to the shoots.

HMX is another high explosive that has replaced TNT and RDX in numerous military applications because of its higher chemical yield and stability. Uptake and translocation of HMX in aquatic plants and hairy root cultures of *Catharanthus roseus* was studies by Bhadra *et al.,* (2001). Plants were exposed to 5 mg/L of HMX where aquatic plants showed no transformation of HMX and minimal biological activity by axenic roots (Bhadra *et al.,* 2001). Studies on the long-term fate of HMX in intact plant tissue were determined in hybrid poplar trees. HMX was not toxic to the hybrid poplar cutting at saturated concentrations. Radiolabelled [U-^{14}C] HMX was translocated and accumulated in leaves and no metabolites were observed (Yoon *et al.,* 2002).

Model organisms

Arabidopsis thaliana

Arabidopsis thaliana is a small weed that belongs to the Brassicaceae family. It occurs throughout the temperate regions of Europe, Asia and North Africa and has been introduced to other areas including North America and Australia. *Arabidopsis thaliana* grows vegetatively as a small rosette of about 2-5 cm in diameter from which a flowering stem is produced. Flowers are typical of crucifers and produce four sepals, four petals, six stamens and a single ovary consisting of fused carpels. *Arabidopsis thaliana* is self-fertile, producing several hundreds siliques that contain about 50 seeds and shatter upon ripening for seed dispersal (Anderson and Wilson, 2000).

Arabidopsis is an attractive experimental model. In comparison to other angiosperms, *Arabidopsis* has a relatively small genome of 125 MB in total (Arabidopsis Genome Initiative, 2000). Its genome has relatively little repetitive DNA, with over 60 % of nuclear DNA having a protein coding function (Meyerowitz, 1994). *Arabidopsis* has a relatively short life cycle of six to eight weeks.

One of the advantages of *Arabidopsis thaliana* is the availability of mutants that have been generated via a range of mutagenesis strategies. Mutants are particularly important in the analysis of physiological and developmental specific pathways. Many genes are identified through generating abnormalities in gene function that results in a change in phenotype. Classical methods of mutagenesis have included the use of chemicals such as ethylmethane sulfonate, nitrosomethyl urea and irradiation with X-rays, fast neutrons, or heavy charged particles (Anderson and Wilson, 2000).

Fast neutron ionizing irradiation has been shown to be an effective mutagen for *Arabidopsis*. It induces chromosome breaks such as deletions and rearrangements (Dellaert, 1981). Roughly two thousand five hundred lines treated with fast neutrons at a dose of approximately 60 Gy (Grays of irradiation dose) are required to inactivate a particular gene. The *Arabidopsis* genome contains about 25 000 genes, therefore it is expected that about 10 genes are randomly disrupted in each line (Li *et al*., 2001).

Chlamydomonas reinhardtii

The green alga *Chlamydomonas reinhardtii* is an attractive model species for research because it is unicellular and has generation time of 5 hours. Growth can take place in liquid culture or on solid media where it forms single colonies. Cells can grow on simple medium of organic salts, using photosynthesis to provide energy. They can also thrive in the absence of light if acetate is provided as an alternative source of carbon (Harris, 1988). *Chlamydomonas* has a relatively small genome size of 100MB and has 17 chromosomes. One major advantage of *Chlamydomonas* in research is the ease of creating mutants that allow identification of gene functions. The most common method used to create such mutants involves using plasmids to create gene knock-out insertional mutants. The plasmid pArg7.8 contains the arginosuccinate lyase (ASL) gene, which is the last enzyme of arginine biosynthetic pathway that converts arginosuccinate into arginine (Debuchy *et al.,* 1989) which is required in cell growth. Insertional mutagenesis is used to generate mutants by the nuclear transformation of arg^- cells with the pArg7.8 vector that contains the ASL gene, thus, rescuing the arginine requirement. Since the introduced DNA integrates randomly into the nuclear genome, each event potentially

disrupts a gene that may alter the phenotype. These mutants can be rescued on arginine free media and further analyzed to determine the disrupted gene function.

Many studies have shown that algae display a response to environmental stress stimuli. The fluctuation of a number of different environmental factors including levels of specific nutrient or the presence of a xenobiotic will alter the growth rate of the photosynthetic organisms. The metabolic state of the organism alters the rate of growth. Under extreme conditions, when essentially all growth stops, significant changes may be observed in pigmentation, activities of various metabolic processes and cell morphology. The growth response and toxicity effects of green algae in contaminated TNT water are important in designing algae-based detection systems. In addition to terrestrial pollution, TNT can also pollute water. Several studies have been reported on TNT toxicity to alga. *Selenastrum capricornutum* showed no effect on growth at concentrations up to 3 mg/l TNT but toxic effects were noticed at higher concentration (Smock *et al.* 1976, Tadros *et al*., 2000) The effect of TNT on *Microcystis aeruginosa* was much less pronounced than was on *S. capricornutum*. Cells growth was uniform through 15 mg/l of TNT but at 25 mg/l TNT the cell culture growth was completely inhibited (Smock *et al.* 1976).

Microarray analysis

Microarray technology is a powerful tool that allows the analysis of genome-scale gene expression (Wiseman and Ohlrogge, 2000). It consists of a microarray chip on a glass slide that contain cDNA sequences or oligonucleotides representing individual genes. A series of arrays will thus contain all genes of a genome. These arrayed sequences are hybridized simultaneously to a two-color fluorescently labeled cDNA

probe pair prepared from mRNA samples of different tissue type or under differing conditions to allow direct comparative analysis of gene expression (Seki *et al,* 2001). Traditional methods of determining gene functions generally work on single gene analysis per experiment, which means that the throughput is very limited and the inter-relation of gene function is hard to obtain. Alternatively, DNA microarrays can monitor the whole genome on a single chip which gives a better understanding of the interactions among thousands of genes simultaneously (Schena *et al*., 1998). The underlying principle of DNA microarray is hybridization. The array has orderly arranged ESTs (expressed sequence tag) and provides a medium for matching known and unknown DNA samples based on base-pairing. Samples are labeled with a reporter molecule that identifies their presence. The reporters currently used in comparative hybridization are fluorescent dyes such as rhodamine and fluorescein or Cy3 and Cy5. To observe the colors the fluors are stimulated with a specific frequency of light by a laser. The wavelength of emitted light is then used to measured by a detector which measures fluorescence (Schena *et al.,* 1998, Seki *et al.,* 2001)

Comparative hybridization experiments can reveal genes whose transcription changes in response to an environmental stimulus, such as TNT. In the simplest experiment, the sample is subjected to the stimulus, and allowed to reach a steady state of transcription. Transcription levels in the subjected samples can be compared to the controlled sample. Samples can also be subjected to a stimulus with removal of samples at successive points in time. This would reveal gene transcription patterns change from the old to the new steady state. Temporal studies can also identify the order of gene

expression providing evidence about which genes control the response directly and which genes are only indirectly affected (Schena *et al.,* 1998).

CHAPTER TWO

Phytotoxicity studies of wild-type *Arabidopsis thaliana* and the screening for leaf color response of mutant lines in TNT

Abstract

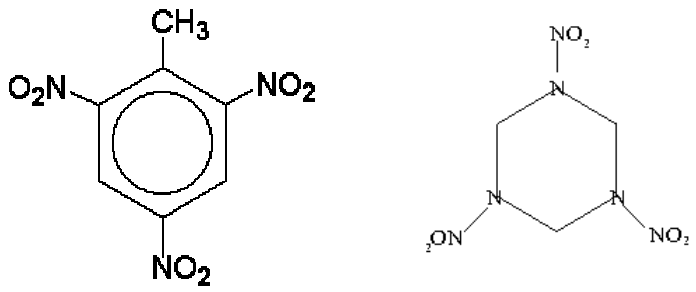
The present study involves the development of a model plant-based biomonitoring system that responds to 2,4,6-trinitroluene (TNT) contaminated environments. This goal was advanced by determining the growth response of wild-type (Col 1) and mutant *Arabidopsis thaliana* plants to TNT. Growth analysis of wild-type *Arabidopsis* in TNT revealed that 10 μM TNT was the phytotoxicity tolerance threshold of *Arabidopsis.* Germination frequency was less sensitive to TNT than seedling development. Rosette diameter and the dark green leaf color of seedlings in 10 μM TNT was similar to the control seedlings growing in 0 μM TNT. Growth at concentrations higher than 10 μM TNT resulted in yellowing of the leaves, smaller rosette diameters and short root lengths. In order to select for TNT responsive mutants that exhibit a difference in leaf color, mutant lines from fast neutron irradiated seeds were screened on 10 μM TNT. Four putative TNT responsive mutant lines were isolated on the basis of a leaf color change from dark to pale green. Mutant line 1 did not have a distinct leaf color response in the subsequent generation, however lines 2, 3, and 4 exhibited pale green leaf color phenotype. TNT specificity of the leaf color response in the subsequent generation was determined by growth in hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and high salinity

media. Results indicated that mutant line 4 exhibited a pale green leaf color in response to TNT but not in RDX or NaCl, while lines 1, 2, and 3 exhibited similar pale green leaf color phenotypes in RDX and TNT. The isolation of these mutant lines indicate that mutations in the genes may be involved in TNT responses.

Introduction

Explosive compounds such as 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) are major components in military ammunition (see Figure 1). These compounds are disseminated into the environment through ammunition production plants, field usage and improper disposal (Best *et al.,* 1997; Harvey *et al.,* 1997, Halasz *et al.,* 2002). An estimated 0.82 million cubic meters of soil at former military installations throughout the US are contaminated with TNT (Peterson *et al.,* 1998). TNT is toxic and carcinogenic to many organisms including humans and it has been shown to cause liver injury and anemia (Won *et al.,* 1974, Gong *et al.,* 1999).

A major source of TNT contamination in soil occurs through the existence of buried explosive devices such as anti-personnel landmines. Over time, the explosive material, TNT and its derivative 2,4-dinitrotoluene (2,4-DNT) permeates through the cracks and pores of the mine casing, thereby contaminating the soil and ground water (Sylvia *et al.,* 2000). Landmines themselves are also a major threat as they can kill or injure combatants and non-combatants alike. It is estimated that there are more than 80 million landmines buried worldwide and cause 15,000-20,000 casualties per year (Stephnié *et al.,* 2003). Clearance of these landmines remains dangerous and underfunded, therefore the development of a cost effective and environmentally accepted



TNT **RDX**

Figure 1: The structures of RDX and TNT.

detection system is vital. In addition, low-cost remediation systems need to be developed in order to clean landmine sites that are contaminated with TNT.

A rapidly developing technology is phytoremediation, which is characterized by the use of vegetative species for *in situ* treatment of land areas polluted by a variety of hazardous substances (Cobbett and Meagher, 2002, Bizily *et al.,* 2003). One of the first steps in developing a phytoremediation system involves phytoxicity studies of the desired plant species (Wang *et al.,* 1995). Phytotoxicity studies are common methods used to evaluate the toxic range and tolerance threshold of the contaminant to the plant. It is one of several parameters established in order to determine the remediation potential of a particular plant species (Peterson *et al.,* 1998). Phytotoxicity tests are also important tools in defining the criteria or reference values for ecological risk assessment and characterizing processes monitoring contaminated soils (Robidoux *et al.,* 2003; Gong *et al.,* 1999).

Genetically engineered plants possessing the capabilities of other bioremediating organisms such as bacteria and yeast may constitute an efficient tool for removing contaminants in soil (Moffat, 1995; Thomas *et al.,* 2003). For example, a mammalian

gene metallothionein (Gleba *et al.,* 1995) and the bacterial gene organomercurial lyase (Bizily *et al.,* 2003) have been engineered into *Arabidopsis thaliana* to assess the effect on the uptake of harmful heavy metals such as mercury by the plant. Studies conducted by Hannink *et al* (2001) have developed transgenic tobacco that remediate explosive contaminants such as TNT. The tobacco plants expressed nitroreductase enzyme from the bacteria, *Enterobacter cloacae.*

In addition to phytotoxicity studies, research to understand the genetic basis for phenotypic responses to toxic substances may lead to the development of phytosensors. Phytosensors are plants that produce a phenotypic response to specific environmental stimuli. Responsive DNA elements described in phytotoxicity studies may be used in developing transgenic plants that respond to specific contaminants. The first step would involve conducting phytotoxicity studies to determine the tolerance of a contaminant to the plant and more importantly it would involve the isolation of plant genes that are responsive to TNT. Manipulations of these genes may result in a visible phenotype that would occur only in the presence of the contaminant.

In this study, *Arabidopsis thaliana* (*Arabidopsis*) was used as a model system. It has a small size and short life cycle (Page and Grossniklaus, 2002) and the genome has been sequenced. Determination of function of all sequenced genes is the focus of current research (Scholl *et al.,* 2000, Page and Grossniklaus, 2002). The success of *Arabidopsis* as a model organism is largely the result of its amenability to forward genetics screens. Forward genetics begins with the identification of a mutant phenotype, and follows the isolation of the gene involved in the mutation (Krysan *et al.,* 1999). Endogenous genes may be disrupted by chemical treatments, irradiation or insertional mutagenesis.

Knockout techniques are powerful tools that allow the characterization of gene functions and the analysis of physiological and developmental pathways.

In this study, commercially available mutagenized *Arabidopsis* seeds were used. The seeds had been mutagenized with fast neutrons, which is a highly efficient mutagen that produces deletion mutations or chromosomal rearrangements (Li and Zhang, 2002; Li *et al.,* 2001). Optimal dosage with the fast neutron treatment (60 Gy) was used to saturate the *Arabidopsis* genome. According to Li *et al.,* 2001, approximately 2500 mutant lines should be screened in order to saturate 80 % of the genome. At a single dosage of 60 Gy, it is estimated that 10 genes are randomly deleted in each line.

In this report the growth response of wild-type *Arabidopsis* to TNT was studied. Phytotoxicity studies determined the TNT tolerance concentration of *Arabidopsis*. This concentration was used to screen mutants that exhibited a change in leaf color in the presence of TNT. The subsequent generation of the putative TNT responsive mutants were further characterized for their stability and specificity to the leaf color response by comparing leaf color response in other abiotic stress conditions.

Materials and Methods

Phytotoxicity studies of wild-type *Arabidopsis*

Plant material and TNT cultivation conditions

Wild-type *Arabidopsis thaliana* (ecotype Columbia) were used for the phytotoxicity studies. Seeds were surface sterilized by treatment for 5 min in 20 % (v/v) commercial bleach (NaHClO) containing 2 % tween. Seed were periodically agitated and rinsed with sterile water. TNT (Chemical services, West Chester, PA) concentrations of

0, 1, 5, 10, 20 and 30 μM in MS media (Murashige and Skoog, 1962) solidified with agar was prepared from a 100 μM TNT/MS stock solution. The solutions were autoclaved at 120 °C for 25 min and poured into sterile 10 cm diameter sterile polystyrene Petri dishes. Seeds were sown in a water suspension using a Pasteur pipette at density of 30 regularly spaced seeds per plate. Primary root length was determined by arranging seeds in a row in TNT supplemented growth media on a Petri dish, orienting this in a vertical position. Seed dormancy was broken by cold treatment at 4 °C for 2 days. Growth was encouraged at optimal *Arabidopsis* growth of 25 °C day and 23 °C night temperatures, under cool fluorescent lighting with an 18-hour photoperiod.

Growth parameter measurements

Germination studies were conducted for each TNT treatment. Germination was quantified by recording the number of newly emerged radicles and converted to germination frequency (percentage of seed capable of completing germination in 14 days). The rosette diameter was measured for each seedling 14-days post incubation and root lengths were measured 10 days post-incubation using a standard metric ruler (cm). In addition, leaf color was visually observed and recorded. Each TNT concentration was replicated in triplicate. The data was statistically analyzed by ANOVA, and the means were compared using Student-Newman-Keuls multiple comparison test at $P<0.05$ (Graphpad Instat, San Diego, CA).

Mutant screening analysis

Plant material and growth condition

Mutant seeds of *Arabidopsis* were derived from mutagenesis with fast neutron irradiation (55Gy), carried out by a commercial supplier (Lehle Seeds, Round Rock, TX). Five screening experiments were conducted. Five parental groups that contained M_2 (second generation of seeds of a mutant line) seeds derived from approximately 1,358 M_1 parental lines were the seed source for each screening experiment. The mutant seeds were sown (equally spaced) by Pasteur pipette in a water suspension, in 15-mm Petri plates containing MS solid culture media supplemented with TNT to a final concentration of 10 μM TNT. After a cold treatment (2 days at 4 °C) to break dormancy, seeds were grown in a controlled environment growth chamber at 25 °C day and 23 °C night temperatures, under cool fluorescent lighting with an 18-hour photoperiod. The change in the leaf color phenotype was observed 14 days after sowing. Putative TNT responsive mutants were identified on the basis of a difference in leaf color from the wild-type individuals. In addition, the number of non-germinating seeds, albino seedlings, sterile, and lethal mutants that exhibited a different color from the wild-type seedling were recorded for each experimental screen. All putative TNT responsive plants were transplanted to soil for M_3 seed collection.

Screening of putative TNT responsive M_3 seeds in TNT and other stress conditions

Putative TNT-responsive mutant lines (15 seeds per mutant line) were tested on 0, 1, 5, 10, 20 and 30 μM TNT in order to confirm the leaf color response and to observe

segregation. Two weeks post germination, the seedlings were observed for their leaf color phenotype. In order to determine the specificity of the putative TNT leaf color response, the selected mutants were grown in two other abiotic stress environments. The selected mutants were grown on MS growth media supplemented with 0, 1, 5, 10, 20 and 30 μM RDX (Royal Demolition Explosive). The mutant lines were also treated with various concentrations of sodium chloride (NaCl) ranging from 50 mM to 200 mM NaCl, to induce salinity stress.

Results

Germination and seedling growth of wild-type *Arabidopsis*

After analyzing the data it was apparent that germination frequency was less sensitive to TNT than was seedling development. There was no significant difference ($P<0.05$, Student-Newman-Keuls multiple comparison test) in the germination frequency between TNT treatment concentrations (0 μM – 30 μM TNT) with germination percentages ranging from 91 % - 93 % (see Figure 2). At TNT concentrations of 0, 1, and 5 μM TNT, there was no significant difference ($P<0.05$, Student-Newman-Keuls multiple comparison test) in rosette diameter 14 days after the start of the experiment and the seedlings had a rosette diameter of 1.4 ± 0.04 (means ± standard deviations), 1.3 ± 0.3, and 1.5 ± 0.2 cm respectively (see Figure 2). However, higher concentrations of TNT resulted in significant differences between rosette diameter ($P<0.05$, Student-Newman-Keuls multiple comparison test), where the diameters at 10 μM, 20 μM and 30 μM TNT

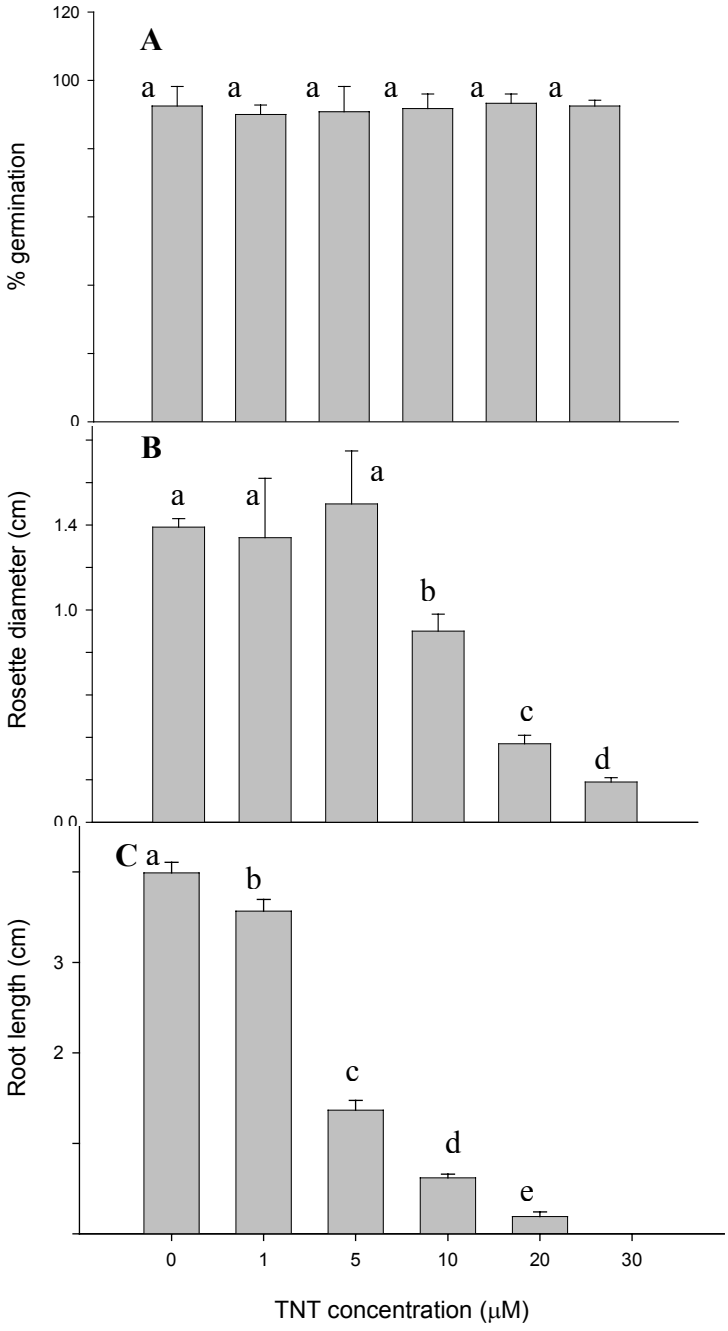


Figure 2: The growth parameters of wild-type *Arabidopsis* in TNT. **A:** The germination frequency. **B:** The effect of TNT on rosette diameter. **C:** The root length in the presence of TNT. Columns denoted by differing letters are significantly different ($P<0.05$). Vertical bars represent standard deviations.

were 0.9 ± 0.08, 0.3 ± 0.04, and 0.2 ± 0.001 cm respectively. Primary root growth of wild-type *Arabidopsis* was determined 10 days post incubation (see Figure 2). The root lengths at each TNT concentration differed significantly between each other ($P<0.05$, Student-Newman-Keuls multiple comparison test). Root lengths at 0 μM TNT and 10 μM TNT were 3.9 ± 0.1 cm and 0.6 ± 0.04 cm respectively and indicated a considerable decrease in root growth in the presence of TNT. Seedlings growing in 20 μM TNT resulted in a 95 % decrease in root length with a root length of 0.2 ± 0.11 cm compared to 4.0 ± 0.05 cm to that of control. The highest TNT concentration (30 μM TNT) resulted in excessively short roots with root length measurements of < 0.1 cm. The effect of TNT on *Arabidopsis* leaf color phenotype was also visually observed. TNT concentrations up to 10 μM TNT did not affect leaf color and were similar to the seedlings growing in 0 μM TNT, however at 20 μM and 30 μM TNT, the seedlings indicated signs of leaf necrosis and yellowing (see Figure 3).

To determine the TNT tolerance level for *Arabidopsis*, the growth parameters of the wild-type *Arabidopsis* in TNT were analyzed collectively. Growth response studies revealed that the TNT tolerance level for *Arabidopsis* was 10 μM TNT. At 10 μM TNT there was a significant difference in the rosette diameter to the seedlings growing in 0 μM TNT ($P<0.05$) however the seedlings had a green leaf color. Concentrations above this resulted in the inhibition of growth. In order to isolate TNT responsive mutants, the mutants were screened on 10 μM TNT. Wild-type seedlings growing in this concentration did not exhibit a change in leaf color to that of the seedlings growing in

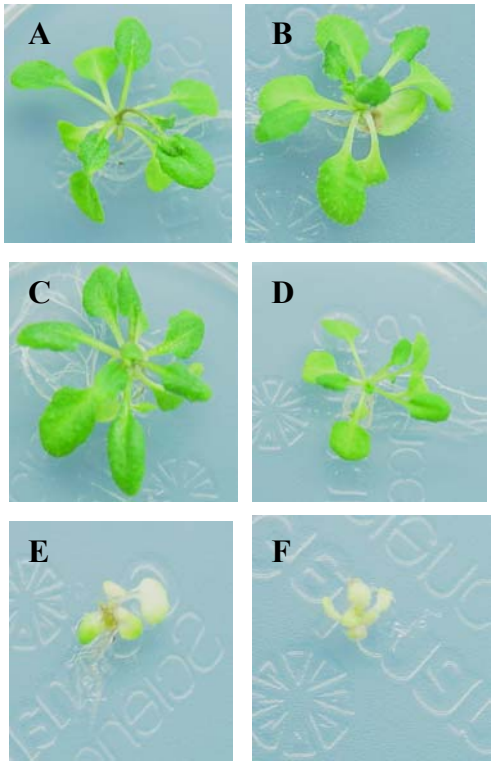


Figure 3: Wild-type *Arabidopsis* growing in MS growth media supplemented with TNT. **A:** Control, 0 μM TNT. **B, C, D:** 1. 5. 10 μM TNT respectively; seedlings had similar leaf color to the control plant. **E, F**: 20 and 30 μM TNT; seedlings growth was stunted and exhibited a yellow leaf color phenotype.

0 μM TNT and thus mutants that displayed a different leaf color at 10 μM TNT enabled the selection of TNT responsive mutants.

Screening of mutant *Arabidopsis* that respond to TNT

M_2 seeds were sown and mutants that exhibited visual differences in the leaf color phenotype to the control were selected (see Figure 4A). Five screening experiments were conducted, where mutants that showed variation in leaf color phenotype were assigned into phenotypic classes (see Table 1). The putative TNT responsive mutants were selected from these classes.

A total of 11,250 M_2 seeds originating from the five parental groups were sown on growth media supplemented with 10 μM TNT. Out of the 11,250 M_2 seeds sown,

Table 1: Quantitive profile of the screening process of fast neutron irradiated mutants grown in 10 μM TNT supplemented growth media.

Screening Experiments	Parental group	M_2 seeds sown	Early lethals		Mutants exhibiting variation in leaf color from the control		Putative TNT responsive mutants (fertile)
			No germination	Albino	Sterile	Lethal	
1	P1	3000	295	ND	1	5	2
2	P2	1500	121	39	1	2	1
3	P3	3450	288	63	2	9	1 (line 1)
4	P4	1320	139	37	1	7	1 (line 2)
5	P5	1980	198	48	1	6	2 (line 3, line 4)
Total		**11,250**	**1,041**	**187**	**21**	**22**	**7**

ND: not determined

9.25 % did not germinate and approximately 107 albino plants were isolated (see Table1). Each screening experiment yielded *Arabidopsis* mutant seedlings that did not have green leaves. These colors included white (albino), yellow and pale green. The selected mutants were further classified into fertile TNT – responsive mutants, sterile and lethal mutant seedlings. More than half of the selected mutants bore lethal-effect mutations dying before completing their life cycle, or were sterile and did not produce seeds. Putative TNT responsive mutants that exhibited a change in leaf color are shown in Figure 4C-F. Out of the seven putative TNT responsive mutants, only four were selected for further analysis. These were line 1 and line 2 which originated from M_2 lines found in parental groups 3 and 4 respectively. Line 3 and line 4 were derived from M_2 lines found in parental group 5. Putative TNT responsive mutant lines 2, 3, and 4 exhibited a pale green color in their leaves when grown in 10 μM TNT, whereas wild-type *Arabidopsis* had a dark green leaf color (see Figure 4D-F). Mutant line 1 exhibited pale green petioles with dark green leaf color similar to wild-type (see Figure 4C). This phenotypic response was not visually distinctive.

M_3 seeds from the putative TNT-responsive mutants were further analyzed for their leaf color response. In this study, these mutant lines 2, 3, and 4 did not segregate in the leaf color phenotype and the M_3 progeny exhibited pale green leaf color phenotype in response to TNT (see Figure 5). It was noted that line 1 did not have a distinct pale green stem color.

To determine the specificity in the TNT leaf color phenotype, the putative TNT responsive M_3 seedlings were screened on two other abiotic stress environments. The

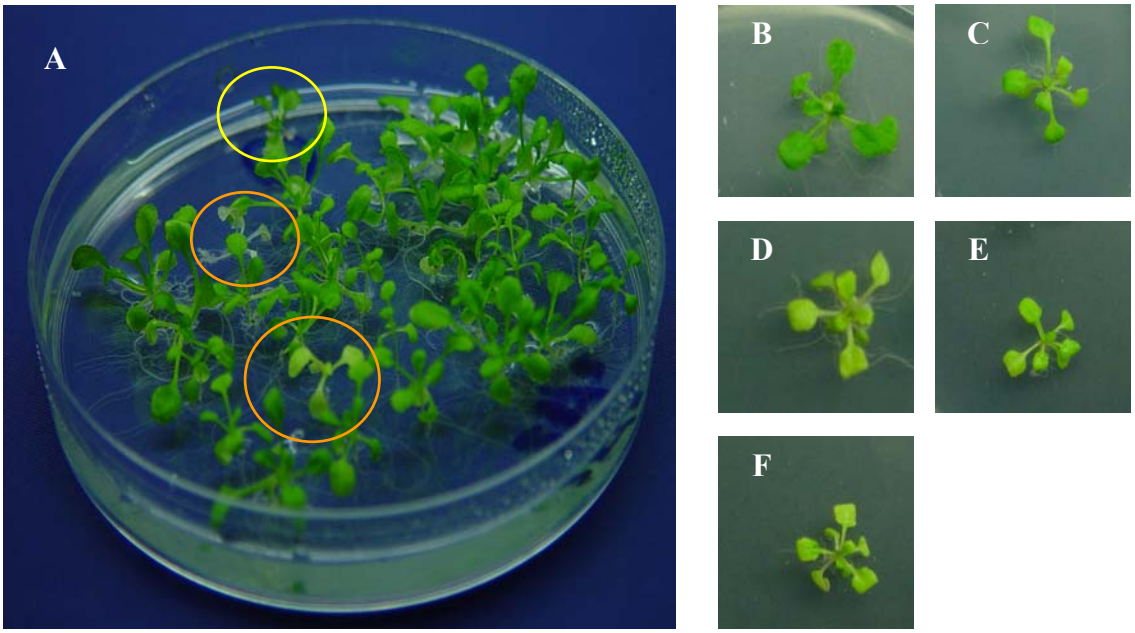


Figure 4 A: Screening of fast neutron irradiated M_2 *Arabidopsis* seeds on 10μM TNT. The circled seedlings (orange) were selected on the basis of color difference to that of the control wild-type plant (yellow circle). **B:** Wild-type *Arabidopsis* (ecotype Columbia) **C:** Putative TNT responsive mutants, Line 1 **D:** Line 2, **E:** Line 3**, F:** Line 4. These seedlings exhibited a pale green leaf color response in the presence of TNT.

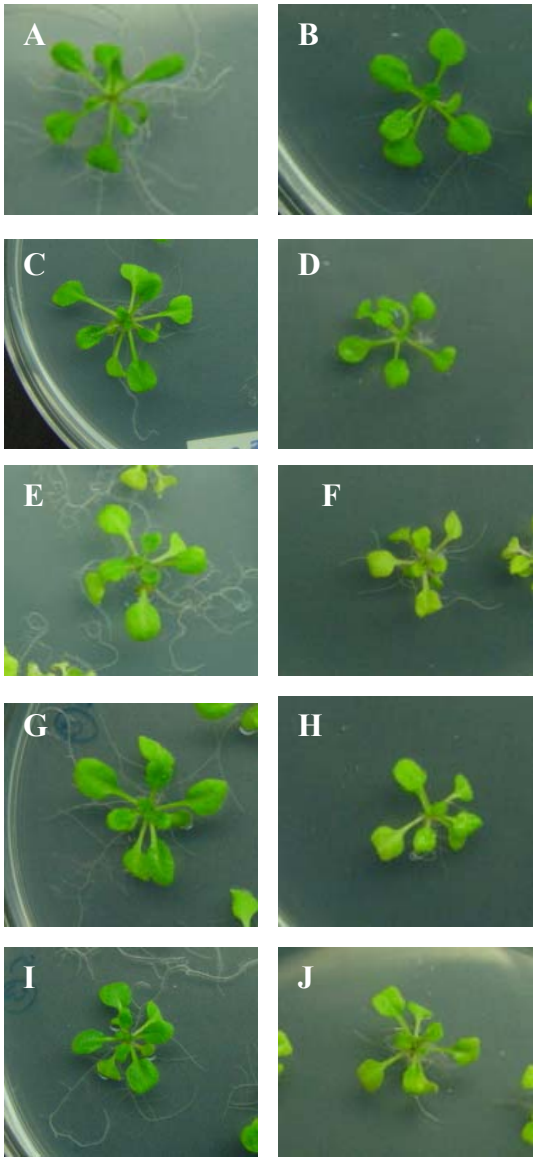


Figure 5: M_3 seedlings of the putative TNT – responsive mutant lines selected from the screening experiments. Panel A, C, E, G, I are seedlings growing in 0 μM TNT and panel B, D, F, H, J are seedlings growing in 10 μM TNT. **A and B:** Wild-type *Arabidopsis* exhibit dark green leaf color phenotype in both TNT environments. **C and D**: Line 1 seedlings did not exhibit a distinct leaf color change from wild-type. **E and F:** Line 2, **G and H:** Line 3 and **I and J:** Line 4, all seedlings exhibited a pale green leaf color response in the presence of TNT.

response of the mutant seedlings to RDX and NaCl is illustrated in Figure 6. At 10 μM RDX line 1, 2, and 3 exhibited a pale green leaf color phenotype, which was similar to the response of wild-type *Arabidopsis*. Both line 2 and 3 had similar leaf color phenotypes in TNT and RDX. Seedlings growing in these conditions resulted in a pale green leaf color.

It was interesting to note that line 4 displayed a pale green leaf color growing in TNT, however in RDX the leaf color was similar to the seedlings growing in 0 μM TNT and RDX, indicating some signs of specificity. At 50 mM NaCl the mutant lines and wild-type exhibited a dark green leaf color phenotype similar to wild-type.

Discussion

The initial experiments conducted in this study described the phytotoxicity of wild-type *Arabidopsis* to TNT. It was determined that 10 μM was the phytotoxicity threshold concentration that allowed growth of seedlings. Screening mutant *Arabidopsis* seeds on this concentration allowed the isolation of TNT responsive mutants that have a difference in leaf color from the dark green wild-type seedlings. Four mutant lines were isolated that exhibited a pale green leaf color in response to TNT. Specificity studies indicate that line 4 exhibits a TNT specific leaf color response.

The germination frequency of wild-type *Arabidopsis* in TNT environments was notably less sensitive than was seedling growth. A high percentage of germination was achieved at the highest concentration of TNT. The process of seed germination depends largely on the energy reserves in the cotyledons, and this may make germination less

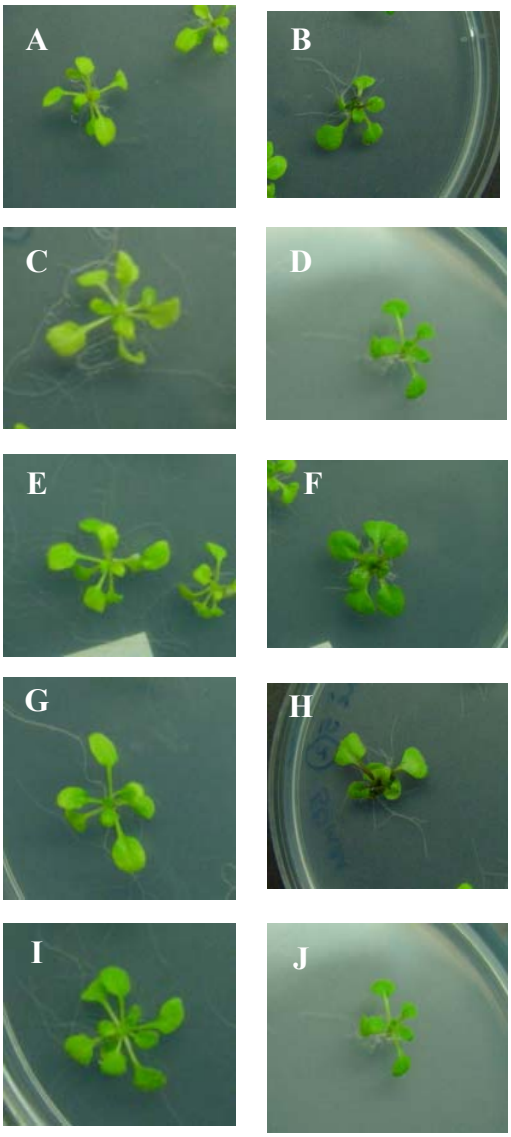


Figure 6: Specificity studies conducted on M_3 putative TNT - responsive mutant lines. Lines were grown in RDX and NaCl stress environments. Panels A, C, E, G, I are seedlings growing on 10 μM RDX and panel B, D, F, H, J are seedlings growing on 50 mM NaCl. **A:** Wild-type *Arabidopsis* **C**: Line 1, **E:** Line 2, **G:** Line 3, exhibited a pale green leaf color phenotype in RDX. **I:** Line 4 exhibits a dark green leaf color response similar to wild-type seedlings grown in 0 μM TNT. **B, D, F, H and J:** wild-type and all mutant lines exhibited dark green leaf color phenotype.

sensitive to environmental pollution (Gong *et al.,* 1999). As a result, seed germination was not a good indicator of phytotoxicity in this study. However, damage that occurred during seed germination in TNT environments could be accumulative and may become apparent during seedling growth (Gong *et al.,* 1999). Studies conducted by other researchers have indicated that TNT does indeed affect germination of certain plants species. According to Peterson *et al.,* 1996 the germination of tall fescue (*Festuca arundinacea*) decreased with increasing TNT concentrations, with substantial reductions at 45 and 60 mg/l TNT.

Smooth bromegrass (*Bromus inermis*) germination decreased with an increase in TNT, however switchgrass (*Panicum virgatum*) germination was unaffected by TNT (Peterson *et al.,* 1998). These results indicate that the effect of TNT on germination is species dependent.

The effects of TNT on *Arabidopsis* seedling development allowed the phytotoxicity tolerance threshold to be determined. *Arabidopsis* seedlings were able to tolerate 10 μM TNT. It was noted that root growth was more sensitive to TNT than rosette size and leaf color response. Root length exhibited a negative growth relationship with TNT where exposure to a low concentration of TNT such as1 μM TNT resulted in a considerable decrease in root growth. Rosette diameter decreased at TNT concentrations greater than 5 μM TNT.

According to Peterson *et al.,* (1996), early seedling development (roots and shoots) of tall fescue (*F. arundinacea*) decreased linearly with the increase of TNT concentration when grown in sterile growth media. Plants were able to maintain at

concentrations of 30 mg/l TNT (44 μM TNT). Concentrations up to 15 mg/l (22 μM) of TNT did not affect the root growth of switchgrass, however, the root growth of bromegrass was reduced at concentrations above 7.5 mg/l TNT (11 μM TNT). Gong *et al.,* (1999), reported the phytotoxicity of two monocotyledons, *Avena sativa* (oat) and *Triticum aestivum* (wheat) and two dicotyledons, *Lepidium sativum* (cress) and *Brassica rapa*, (turnip) growing in soil spiked with TNT. They concluded that oat and wheat were more tolerant of TNT than the dicotyledons. Oat was capable of tolerating 1600 mg TNT $kg^{-1}$of soil. Earlier studies, however, showed differing trends of phytotoxicity to monocotyledons. Görge *et al.,* (1994) reported that the dicotyledon alfalfa growing in hydroponic cultures was more tolerant to TNT than the monocotyledon chives (*Allium schoenoprasum*). In more recent studies, Robidoux *et al.,* (2003)*,* studied the toxicity of TNT using two terrestrial plant species, lettuce (*Lactuca sativa*) and barley (*Hordeum vulgare*) growing in forest soil and silica. The results indicated that TNT was toxic to both lettuce and barley at concentrations greater than 55.9 mg/kg however TNT is more toxic to barley seeds in forest soil than in silica. The precise mechanism of TNT toxicity is currently not well characterized (Spain *et al.,* 2000), but species dependant phytotoxicity on wild-type species is well known.

There are no previous reports on screening mutagenized plants in TNT growth conditions. If these mutant plants that have mutations in genes involved in TNT responses cause viable phenotypes, distinguishable from the wild-type by leaf color traits, a search for such mutants based on observation may be worthwhile. The advantage of this study is that mutants are readily available for screening, however the screening process can be very slow, since plants are studied one at a time under conditions that do not limit

or prevent wild-type seedlings. In addition, lethal mutants and mutations that affect genes of redundant functions would be excluded in the search for viable and visible mutants (Berná *et al.,* 1999). In this study, the result of screening mutant *Arabidopsis* in TNT isolated seedlings that possess a different leaf color to the wild-type. These mutant seedlings fall into three mutational classes. One class of mutants that may exhibit a different leaf color in the screening process would include the lethal mutants. The lethal alleles would probably include null mutations and hypomorphic mutants that have mutations in housekeeping genes or those involved in developmental pathways (Berná *et al.,* 1999). The second class of mutants corresponds to mutations in genes of redundant functions (Berná *et al.,* 1999; Page and Grossniklaus, 2002). These mutations would not cause phenotypes distinguishable from the control wild-type. The third class may involve mutations in genes that exhibited a difference their leaf color phenotype. The mutations in these seedlings may have genes that are involved TNT stress response, which may be associated with pigment development, however this class also includes mutations that affect the leaf color (Budziszeski *et al.,* 2001) but have no response to TNT.

From the present study, the three selected mutant lines had a difference in leaf color in response to TNT. Segregation of the leaf color phenotype was not observed in the M_3 generation. Since *Arabidopsis* are diploid, the mutant phenotypes are rarely seen in the M_1 generation. Allowing the M_1 *Arabidopsis* to self fertilize, 25 % of the M_2 seed generation are homozygous for the mutant allele and thus express the mutant phenotype (Berná *et al.,* 1999). These mutants are recessive**.** It was assumed that the selected mutants were homozygous recessive for the mutant allele and therefore segregation was not expected. Line 2 and line 3 exhibited distinct pale green leaf color in the M_3

generation of the plants, however line 1 did not have a distinct leaf color differences in the M_3 generation in the presence of TNT. In particular line 4 had a pale green leaf color growing in TNT, however in RDX the leaf color was similar to the seedlings growing in 0 μM TNT and RDX. This indicates that the leaf color response to TNT may be specific but further specificity experiments using other stress environments need to be conducted in order to extend the result.

The cause of the leaf color in response to TNT in this study was not determined. However, studies conducted with other xenobiotic compounds have revealed that herbicides affect the production of carotenoids by inhibiting desaturase enzymes (Devine *et al.,* 1993). In this way, the leaves of the plants appeared yellow and white. At high concentrations of TNT (30 μM TNT) *Arabidopsis* seedlings appear white and show signs of chlorosis. TNT may be responding to the plants in a similar manner as herbicides. The mutants growing on lower concentrations of TNT may have mutations in the carotenoid related genes thereby resulting in light green leaf color in response to TNT. The leaf color response could also be a result of the disturbance in the electron transport chain. Similar results have been reported where the mechanisms of herbicide actions affect the photosynthetic electron transport chain (Devine *et al.,* 1993). Certain herbicides such as atrazine block the electron transport chain. The inhibition of the electron flow leads to excessive radiative excitation in the blocked photosynthetic pigment. The result of this includes photooxidation and phytotoxicity at the organelle, cell and tissue level (Pallett and Dodge, 1980).

Phenotypic leaf color response of TNT in *Arabidopsis* has the potential of being useful in developing plant-based TNT biomonitoring systems. The eventual goal of the

phytosensor project would be to develop phytosensors that detect landmines by responding to TNT. In this study three-candidate mutants lines that possess leaf color responses to TNT were isolated. The isolation of these mutant lines, forms the basis for future work involving the identification of the genes that are involved in the leaf color response.

CHAPTER THREE

Differential growth response of wild-type and mutant strains of *Chlamydomonas reinhardtii* to TNT

Abstract

In order to develop an aquatic biomonitoring model system for explosives, the growth response of wild-type and mutant strains of *Chlamydomonas reinhardtii* (*Chlamydomonas*) to 2,4,6-trinitrotoluene (TNT) was studied. TNT is a commonly used explosive compound in military stockpiles. Contamination of groundwater by TNT is widespread and is caused by various activities such as manufacturing, military testing, and training. Growth analysis of wild-type *Chlamydomonas* in 0 to 5 μg/ml TNT revealed that 3 μg/ml of TNT was the maximum TNT concentration that allowed growth. Mutant lines were generated via insertional mutagenesis by the nuclear transformation of arginine deficient cells with the pArg7.8 plasmid. This plasmid contains the arginosuccinate lyase gene, thus, rescuing the arginine requirement in the transformants. Out of 1000 transformants analyzed on 3 μg/ml TNT, one mutant cell line (CL48), was selected on the basis of a color change from green to white. Growth analysis revealed that cell line 48 exhibited a negative concentration dependent growth response. Specifically, growth of CL48 at 3 μg/ml TNT was 146 ± 21 ($x10^4$) cells/ml compared to the cell concentrations of the arg^- transformation recipient which was 588 ± 30 ($x10^4$) cells/ml. The growth response of CL48 in TNT indicated that the mutant CL48 is hypersensitive to

TNT compared with the transformation recipient line and wild-type *Chlamydomonas*. In order to determine the specificity of TNT growth response, the growth response of CL48 in thiodiglycol (TDG) was determined. CL48 was not sensitive to TDG and the growth response was similar to the wild-type and the transformation recipient arg^- line.

Introduction

Polluted water can disrupt the ecology of normal aquatic environments and can be hazardous to many organisms. Heavy metal (Rubinelli *et al.,* 2002, Prasad *et al.,* 1998) and chemical pollution (Shehata *et al.,* 1993) enter ground water systems through industrial waste effluents. One of the major sources of groundwater contamination results from the production and military use of 2,4,6-trinitrotouene (TNT). TNT is a well-known explosive compound that has been used as ammunition since the early part of the 20^{th} century (Tadros *et al.,* 2002). It is persistent and resistant to degradation, and constitutes a major environmental pollutant (Snellinx, 2002). In the past few decades, wastes from TNT manufacturing, loading and packing facilities were routinely released into artificial holding lagoons, natural ponds, or freshwater streams (Smock *et al.,* 1976; Boopathy *et al.,* 1994). As a result, significant amounts of TNT and their breakdown products now persist at these sites and pose a serious environmental hazard to a wide variety of organisms (Hwang *et al.,* 2000).

In order to monitor pollutants in contaminated water, both field and laboratory phytotoxicity tests have been conducted using algal species (Boyle, 1984; Whitton, 1984). The growth response of many algal species including *Chlamydomonas reinhardtii* (*Chlamydomonas*), on various abiotic stresses has been extensively studied (Dodard *et*

al., 1999; Rioboo *et al.,* 2001; Hanikenne *et al.,* 2001). These algae have been subjected to a myriad of different types of laboratory studies and have been measured by using different biological responses. For example, the effect of pH on the growth of *C. acidophila* was assessed by the measuring chlorophyll content (Visviki and Palladino, 2001). Suzuki *et al.,* 2000, determined the stimulatory effects of the local anesthetic, procaine, on the growth of *Danaliella primolecta* by measuring cell concentration over a period of time. Metal ions and other common abiotic stress on the growth of algae have been studied. Toxicity studies of cadmium and copper were determined for *Chlamydomonas* by measuring the growth as a function of cell density (Prasad *et al.,* 1998).

In this study, *Chlamydomonas* was used as the model organism. It is a commonly used laboratory algal species and is amenable to biochemical, genetic and molecular analysis. (Lefebvre and Silflow, 1999; Debuchy *et al.,* 1989). In nature, the *Chlamydomonas* genus is distributed worldwide and is found in a diversity of habitats. *Chlamydomonas* has been isolated from freshwater ponds, lakes, sewage ponds, marine waters, snow, garden and agricultural soil and forests (Harris, 1988). The use of *Chlamydomonas* as a model system in stress response studies is useful because of the vast genetic information that is publicly available (Lefebvre and Silflow, 1999). The isolation of distinct phenotypes in mutant *Chlamydomonas* can be used for the identification of gene function under certain stress conditions and can be possibly linked to the uptake and metabolism of the contaminant. Further, genes that may be involved in these processes may be cloned and engineered into other organisms and used for biomonitoring and bioremediation systems.

The most commonly used method for assaying the growth of algae over a period of time is the batch culture system (Madigan *et al.,* 1997). This is a closed-system, which contains an algal culture in a fixed volume of growth media. This method was developed to detect nutrient status of culture media in response to cell growth (Merril and Walsh, 1984). Growth of cells in this system may be divided into three phases; the lag phase, exponential phase, and stationary phase. When an inoculum is first transferred into the growth medium, the lag phase begins. During this period the cells must first adjust metabolically and establish their cellular synthesis machinery (Madigan *et al.,* 1997) in preparation for the next phase. The exponential phase involves cellular division, where cell number doubles during each unit time period. In batch culture, exponential growth cannot occur indefinitely due to the limitation of nutrients and the accumulation of metabolic wastes in the growth media. The cells enter into stationary phase where there is no net increase in cell number. Increasing accumulation of toxic wastes and nutrient availability can cause cell death (Madigan *et al.,* 1997).

In addition to studying the growth response of wild-type strains to abiotic stresses, the study of the growth response of mutant lines is useful in isolating mutants that are sensitive to these stresses. In addition, mutational studies also allow opportunities for forward genetic analysis. Classical forward genetics involves two steps, the first is the isolation of a distinct phenotype and the second is determining the genotype of the corresponding phenotype. Forward genetics begins with the phenotypic screening of mutant populations. These mutants can be generated by random insertional mutagenesis. This method involves the disruption of gene functions by the insertion of a foreign DNA into the genome. The isolation of a distinct phenotype through the mutated gene can then

facilitate the identification and isolation of the disrupted gene (Grossniklaus and Page, 2002).

In *Chlamydomonas*, DNA can be introduced into the nuclear genome by glass bead agitating method (Kindle, 1990), via the biolistic delivery system (Blowers *et al.,* 1989) and electroporation delivery system (Tang *et al.,* 1995). The most commonly used method of transformation of *Chlamydomonas* involves using a strain (arg^- cells) that has a mutation in the arginosuccinate lyase gene. In the arginine biosynthetic pathway arginosuccinate lyase is the last enzyme needed to convert arginosuccinate into arginine. To generate mutant lines, the auxotrophic arg^- cells are transformed with plasmid pArg7.8, which encodes arginosuccinate lyase. The auxotrophic mutants are unable to survive in $arginine^-$ deficient medium. As a result only those cells that have successfully integrated the plasmid into the host genome, and thus express the arginosuccinate lyase gene, would grow and survive on the minimal media (Kindle, 1990).

In the following report, a model algal-based TNT biomonitoring system was developed using *Chlamydomonas*. TNT growth response was studied on wild-type strains to determine the maximum TNT concentration that allowed growth. Mutant strains were generated via insertional mutagenesis using the arg^- deficient strains. TNT sensitive mutants were further characterized for their growth response in 1, 5, 10, 20 and 30 μM TNT concentrations.

Materials and Methods

Chlamydomonas strains and culture conditions

Wild-type *Chlamydomonas reinhardtii* (Utex 89, The Culture Collection of Algae at the University of Texas, Austin) were maintained on Tris-acetate-phosphate (TAP) agar (Harris, 1988). *Chlamydomonas* strain CC-1617 (*cw-15 arg-2 mt-*; cell wall-less, The Chlamydomonas Genetics Center, Duke University, Durham, NC), were used for transformation and were maintained on TAP medium supplemented with 100mg/l arginine. The *arg-2* mutation affects the *ARG2* gene encoding arginosuccinate lyase. Cells were maintained at 24°C under continuous light (65 μmol $m^{-2}s^{-1}$).

TNT growth analysis

Growth curves of *Chlamydomonas* were obtained for a range of TNT concentrations (0, 1, 2, 3, 4, 5 μg/ml TNT) to determine the effects of TNT on growth rates. The stock solution of 500 μg/ml was prepared by dissolving crystalline TNT (Chemical Services, West Chester, PA) in TAP medium. A serial dilution of the TNT stock solution into TAP growth medium was conducted to obtain the range of TNT concentrations for growth analysis. The *Chlamydomonas* inoculum was concentrated to 6.8 $x10^4$ cells and harvested by centrifugation (5000x g for 5 min) and inoculated in 50 ml of the 0, 1, 2, 3, 4, 5 μg/ml TAP/TNT growth media. Growth was analyzed by determining cell concentrations (cells/ml). Cells were counted at seven time points (24, 48, 72, 96, 120, 144, 168 hours) using a hemacytometer. Each TNT concentration was replicated in triplicate and data was analyzed statistically by ANOVA (Graphpad Instat

San Diego, CA). The means per harvest and time period were compared using the Student-Newman-Keuls multiple comparison test at $P<0.05$.

Transformation of arg$^-$ *Chlamydomonas* strain

Nuclear transformation of a *Chlamydomonas* strain lacking a cell wall was performed by the glass-bead method (Kindle, 1990). Cells were transformed with 2 μg pArg7.8 plasmid. This plasmid is composed of pUC19 and of a 7.8-kb *Chlamydomonas* nuclear DNA fragment bearing the wild-type gene coding arginosuccinate lyase (*ASL*) (Debuchy *et al*, 1989). Transformants contained the *ASL* gene to correct for arginine auxotrophs. The transformed colonies were selected on TAP agar plates, while untransformed lines were unable to grow without the endogenous expression of *ASL* gene.

Phenotypic analysis and growth response assays

To select sensitive mutants, the arg$^+$ clones were transferred onto fresh TAP agar medium (0 μg/ml TNT), and onto TAP media containing 3 μg/ml TNT (~80 colonies per plate). Colonies were exposed to TNT for 14 days before observation for phenotypic comparison. TNT sensitive colonies were selected on the basis of a difference in color change to that of the control. Colonies that grew with a different phenotype were selected and further analyzed using liquid culture phytotoxicity studies. To evaluate the growth response of TNT putative mutants that demonstrate a response to TNT, liquid cultures were prepared from the colonies grown on the control plates. For the phytotoxicity studies, $6.7x10^4$ cells were harvested by centrifugation and inoculated into 50 ml of TAP

supplemented with a range of TNT. Cell cultures were grown for 7 days and final cell concentrations were determined using a hemacytometer.

To compare the level of sensitivity of the putative TNT mutant, the cell growth was also determined for WT, the arg^- cells, and two unresponsive arg^+ lines. The cell growth was determined in a range of TNT concentrations from 0 μg/ml to 5 μg/ml TNT. Comparison of the final cell concentration in each *Chlamydomonas* line was used to evaluate the sensitivity to TNT of the putative mutants. Each TNT concentration was replicated in triplicate. The data was statistically analyzed by ANOVA and the means per harvest was compared using the Student-Newman-Keuls multiple comparison test. The one tail t-test was used to compare two groups and assess which group had the larger mean (Graphpad Instat). In order to determine the specificity of the TNT growth response, the selected mutants cell lines were exposed to another xenobiotic chemical, 2,2-thiodiethanol (thiodiglycol, TDG) and similar growth assays were conducted.

Results

The growth response of wild-type *Chlamydomonas* in TNT

The growth response of *Chlamydomonas* was determined by conducting a time-based growth study on a range of TNT concentrations (see Figure 7). There was a negative relationship of the growth of *Chlamydomonas* and the concentration of TNT. 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 hours).

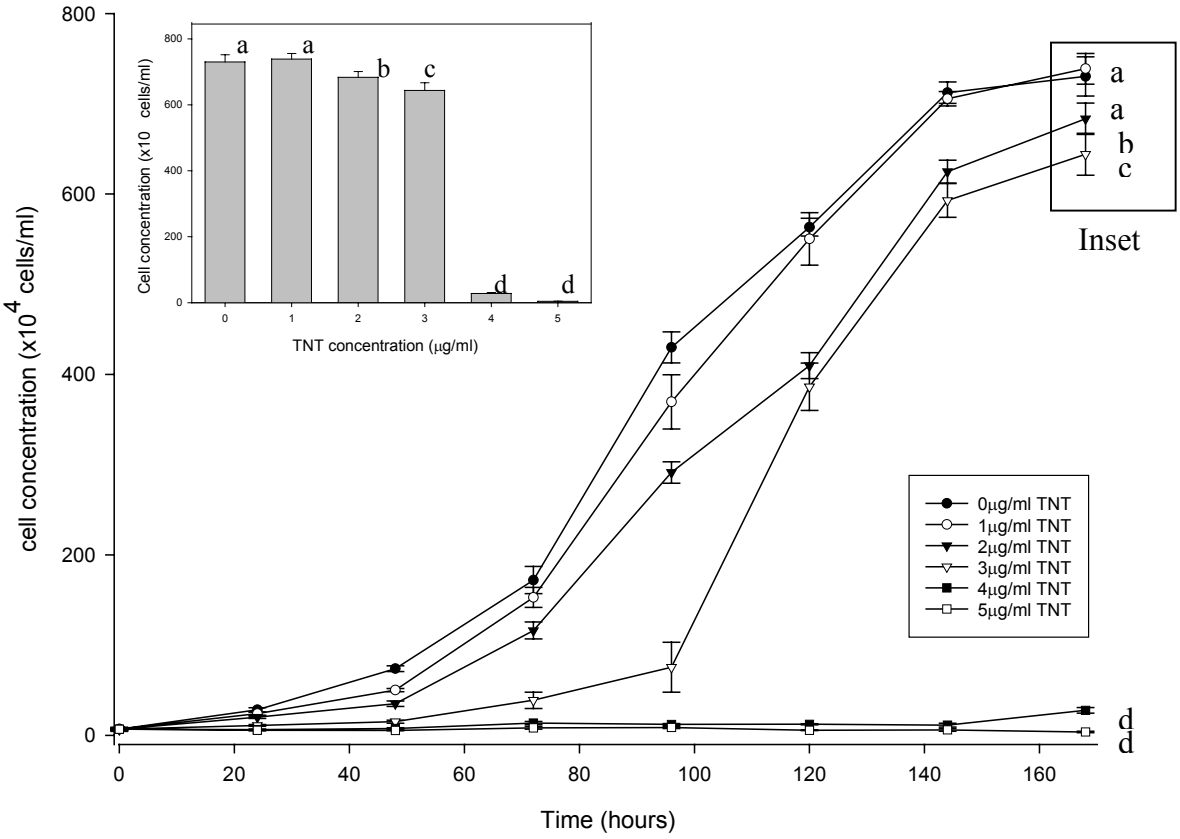


Figure 7: Time course analysis of wild-type *Chlamydomonas* growing in TAP media supplemented with TNT. Cell concentrations were measured every 24 hours. Final counts were determined at day seven for phytotoxicity comparison (inset, bar graph). Columns denoted by differing letters are significantly different ($P<0.05$). Vertical bars represent standard deviations.

The cell concentration in 0 μg/ml TNT was 730 ± 22 (x 10^4 cells/ml; all *Chlamydomona*s cell concentrations) and cell concentration at 1 μg/ml TNT was 739 ± 17 cells/ml. At 168 hours (day 7) post inoculation, there was no significant difference between the cell concentration in growth medium containing 2 and 3 μg/ml TNT, but were significantly difference to the cell counts at 0, 1, 4, and 5 μg/ml TNT concentrations ($P< 0.05$, Student-Newman-Keuls multiple comparison test). At 72 hours (3 days) post inoculation, significant toxicity to *Chlamydomonas* was observed at 3 μg/ml TNT, where the cell concentration was 38 ± 9 cells/ml. Growth of cells at 2 μg/ml TNT, however was 116 ± 9 cells/ml. The concentration of *Chlamydomonas* growing in 4 and 5 μg/ml TNT was significantly less than the growth in the lower TNT concentrations, where *Chlamydomonas* cell counts after 168 hours reached 28 ± 3 cells/ml, while control cultures had final cell counts of 730 ± 22 cells/ml.

TNT treatments from 0 μg/ml TNT to 3 μg/ml TNT resulted in growth population trends that were relatively sigmoidal, indicating the three growth phases present in batch culture systems. However, at higher concentrations growth was inhibited indicating TNT concentrations above *Chlamydomonas* tolerance threshold levels (see Figure 7). Thus, 3 μg/ml TNT was considered the growth threshold concentration of *Chlamydomonas* and was used to screen for TNT responsive *Chlamydomonas* mutants in further experiments.

Isolation of TNT-responsive mutants

Approximately 1000 arg^+ transgenic events were isolated following transformation of the arg^- cells with the pArg7.8. These lines were analyzed for their

ability to grow on TNT containing media. After 10 days, colonies were screened for phenotypic changes to that of the control plates. One mutant cell line (CL48) sensitive to TNT was isolated on the bases of its phenotypic discoloration to white at 3 μg/ml TNT (see Figure 8). Cells from the same mutant line appeared green on TAP media containing 0 μg/ml TNT.

The putative TNT-sensitive transformed mutant (CL48), together with wild-type *Chlamydomonas,* the arg^- cells, and two other transformed mutants that did not exhibit phenotypic responses to TNT (CL42 and CL800), were grown in liquid culture to quantify cell growth (cells/ml). Liquid cultures were used to determine *Chlamydomonas* growth response for a range of TNT concentrations (0 to 5 μg/ml TNT) (see Figure 9). Transformant CL48 exhibited a negative TNT concentration dependant growth response. There was a significant difference between the cell growth at 3 μg/ml TNT of CL48 and arg^- cells ($P<0.05$). At 3 μg/ml TNT, the cell concentrations of CL48 was 146 ± 21 cells/ml compared to the cell concentrations of the arg^- cells which was 588 ± 30 cells/ml ($P<0.05$, one tailed t-test). At concentrations lower than 3 μg/ml TNT, growth of CL48 was higher (793 ± 21 cells/ml at 1μg/ml TNT) compared to the arg^- cells (673 ± 23 cells/ml at 1μg/ml TNT).

To determine the specificity of the TNT response for CL48, the selected cell lines were treated with a hydrolysis product of sulfur mustard, thiodiglycol (TDG) (see Figure 10). For each cell line there was a decrease in cell growth with an increase in TDG concentration. In particular, CL48 did not exhibit cell growth trends as did in TNT. These results indicate that the growth response of CL48 may be specific to TNT.

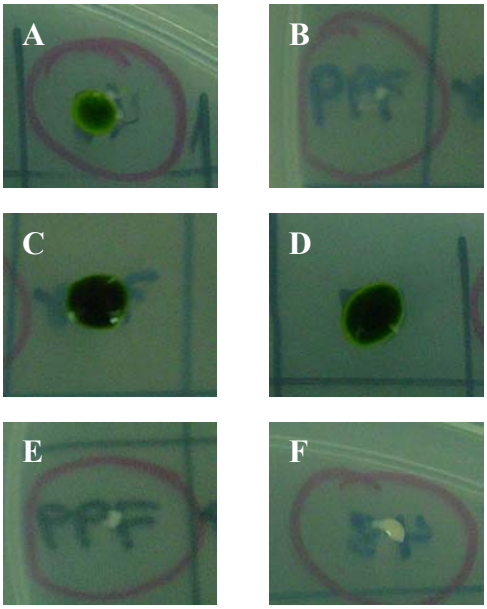


Figure 8: *Chlamydomonas* arg^{+} transformants. Panels A, C and E contain colonies growing in 0μg/ml TNT and panel B, D, F are the same cell lines growing on 3 μg/ml TNT. **A** and **B:** CL48 exhibited a phenotypic discoloration from green to white after 14 days of exposure to TNT. **C and D**: CL800 exhibited no response when exposed to TNT. **E and F:** Non-responsive transformants exhibiting a white phenotype in both 0 μg/ml TNT and 3 μg/ml TNT.

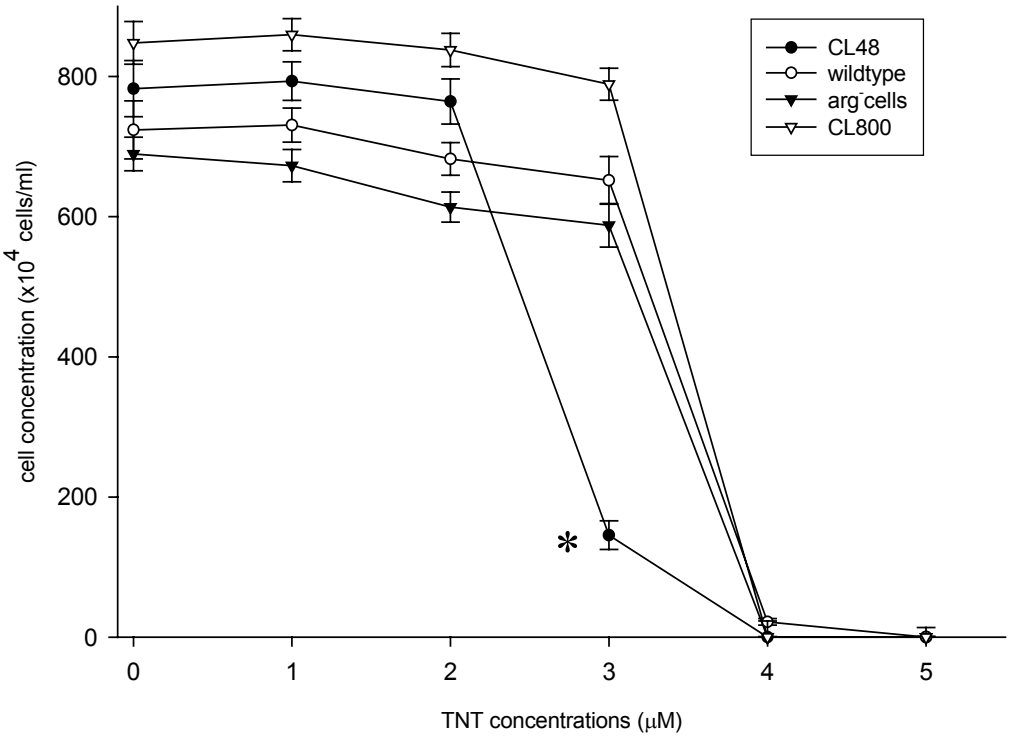


Figure 9: TNT phytotoxicity studies of *Chlamydomonas* cell lines (wild-type, arg^- cells, and transformed arg^+ mutant lines (CL48, CL800). Cell concentrations were determined seven days post inoculations. Asterisc represents a significant difference in growth ($p<0.05$). The growth of CL48 at 3μg/ml TNT is significantly different from all the cell lines tested.

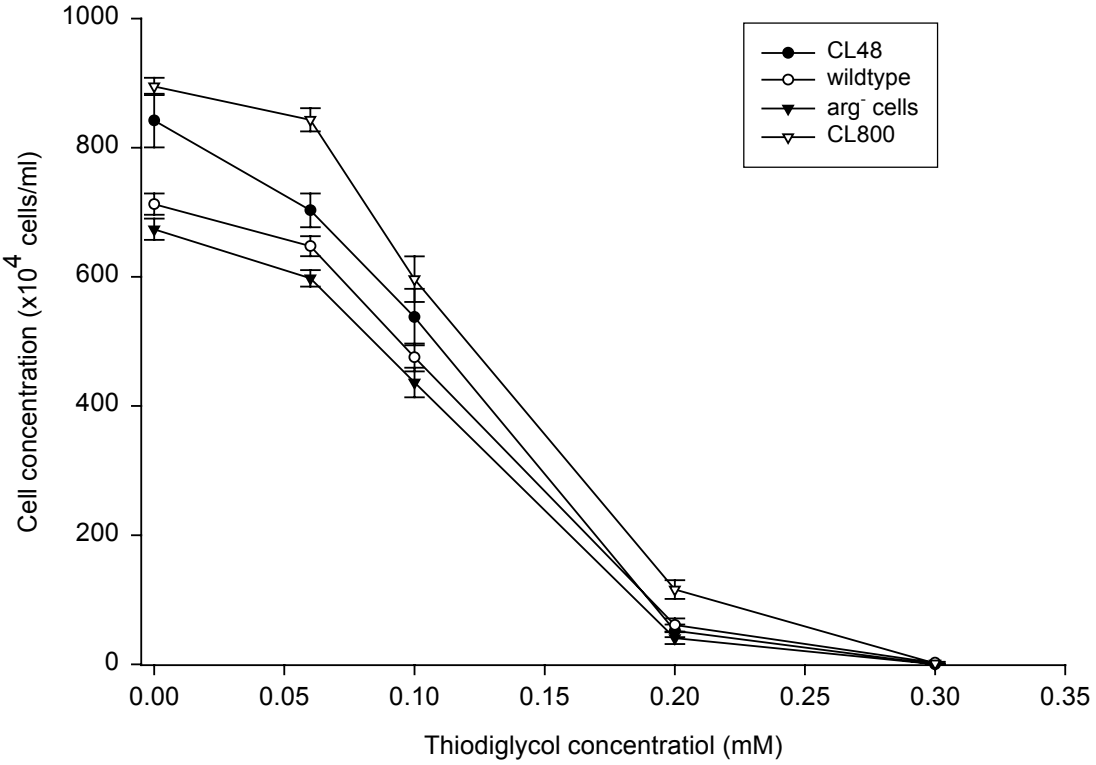


Figure 10: Thiodiglycol phytotoxicity studies of the *Chlamydomonas* cell lines. Cell concentrations were determined seven days post inoculations. CL48 did not display hypersensitivity response as did in the TNT phytotoxicity studies.

Further specificity experiments using other stress compounds are needed to extend the results. Transformants CL42 (data not shown) and CL800 were selected to compare growth between TNT responsive (CL48) and non-responsive transgenic lines. These cell lines appeared as green colonies in both 3 μg/ml TNT and 0 μg/ml TNT. It should be noted that the transformed cell lines exhibited the highest cell counts than all the cell lines after seven days in the absence and presence of TNT and TDG.

Discussion

The present study describes the differential growth response of wild-type and mutant strains of *Chlamydomonas* to TNT. The growth response of wild-type *Chlamydomonas* revealed that 3 μg/ml TNT is the maximum dosage of TNT. Screening the mutants on 3 μg/ml TNT isolated one mutant that was sensitive to TNT.
The study of algal growth response in TNT is limited to a few species such as *Selenastrum capricornutum* (Dodard *et al.,* 1999; Tadros *et al.,* 2000) and *Microcystis aeruginosa* (Smock *et al.,* 1976). In this study growth response analysis of wild-type *Chlamydomonas* revealed that concentrations up to 1 μg/ml of TNT did not decrease the overall cell concentrations after seven days of TNT exposure. In similar studies, Smock *et al.,* (1976), reported that *S. capricornutum* exhibited no growth effects at concentrations up to 3 μg/ml TNT. In further studies, Smock *et al.,* (1976) also reported that *M. aeruginosa* was able to tolerate concentrations up to 15 μg/ml TNT. These comparisons indicate that *Chlamydomonas* is more sensitive to TNT than these two species. *Chlamydomonas* can tolerate up to 1 μg/ml TNT and toxic effects were

noticeable at 2 μg/ml and 3 μg/ml TNT. However, growth inhibition was not permanent at the latter concentrations and the growth of the cells in batch culture produced a typical growth curve resulting in lag, log and stationary phases.

There have been no previous reports on the uptake and metabolism of TNT in *Chlamydomonas*, however several reports have used other organisms to evaluate the phytotoxicity of TNT derivatives, which are aerobic metabolites of microbial degradation. Tadros *et al.,* (2000) reported the toxic effects of hydroxylamino intermediates from microbial degradation of TNT on *S. capricornum* but concluded that the hydroxylamino intermediates were much less toxic than the parent compound (TNT). Recent ecotoxicity studies of TNT derivatives indicate that many metabolites of TNT reduction are less toxic to the bacteria *Vibrio fischeri* than TNT and suggest that TNT degradation by microbial reduction may be associated with the detoxification process (Dodard *et al.,* 1999). For several years, many plant species have been studied for their capacity to uptake and metabolise explosives. Mueller *et al.,* (1993) observed that plant cells (*Datura innoxia*) in cell suspension culture can remove TNT from solution through actively internalizing the TNT and breaking it down into a variety of TNT derivative products. Studies of whole plants of *Datura quercofolia* and *Lycopersion peruvianum* in soil contaminated with ^{14}C-labeled TNT and analysis of plants extracts show that most of the stored radioactivity is in the form of TNT metabolites.

The growth response study of *Chlamydomonas* revealed 3 μg/ml as the maximum TNT threshold concentration that still allowed growth. This concentration was used in screening mutant lines for their sensitivity to TNT. The use of mutant *Chlamydomonas* lines in past research has led to the isolation of mutant lines that are sensitive or resistant

to specific heavy metals. Hanikenne *et al.,* (2001) isolated *Chlamydomonas* mutant lines that were hypersensitive to heavy metals and to oxidative stress. Research studies conducted by Collard and Matagne, (1994) revealed cadmium resistant mutant strains of *Chlamydomonas,* and McHugh and Spanier, (1993) isolated cadmium sensitive mutants. Adam *et al.,* (1993) used insertional mutagenesis to isolate acetate-requiring *Chlamydomonas* mutants.

The variation in the response of TNT between the wild-type *Chlamydomonas* and arg^- deficient mutants may be due to the lack of a cell wall barrier in the transformation recipient. Many studies have indicated that the presence of a cell wall can play a role in conferring tolerance to toxic metals (Macfie and Welbourn, 2000; Prasad *et al.,* 1998). Macfice and Welbourn, (2000) suggested that metal tolerance may be due to the ions binding onto cell wall surfaces, however they also stated that tolerance to specific ion may also involve other mechanism. This was indicated by the significant amounts of cadmium and copper removed from the surface of the wall-less cells. Thus, it is interesting to note that in this study, CL800 and CL42 were also cell wall deficient but were able to grow notably better than all the other cell lines, indicating that tolerance may not be limited only to the cell wall barrier. The reason transformants grew significantly higher than the non-transformed *Chlamydomonas* cells is not clear. Although Debuchy *et al.,* (1989) reported that transformants generally contain multiple inserts of the arginosuccinate lyase gene, the specific activity of the enzyme is not increased relative to the wild-type. The regulation of arginine synthesis occurs primarily at two steps and is catalyzed by *N*-acetylglutamate-5-phosphotransferase and ASL respectively. N-acetylglutamate-5-phosphotransferase is inhibited by arginine (Harris, 1988) and ASL

appears to be feedback regulated by other arginine products (Debuchy *et al.,* 1989). This feedback regulation should prevent a significant increase of ASL activity in the transformants.

The study of algal growth responses to chemical stress pollutants has the potential to be useful in developing biomonitoring and phytoremediation systems. The development of algal-based TNT biosensors through mutational screening has isolated at least one candidate *Chlamydomonas* cell line that atypically responds to 3 μg/ml TNT. The isolation of this mutant lays the foundation of further work in the cloning of the knocked out gene to determine how this gene product is involved in TNT response. Using this model system, genetic tools can be used to develop algae based biosensors, which include promoters of TNT sensitive genes fused to biomarkers such as green fluorescent protein.

CHAPTER FOUR

Differential gene expression of *Chlamydomonas reinhardtii* in response to TNT using microarray analysis

Abstract

The acclimatization of *Chlamydomonas reinhardtii* to environmental stress, such as the exposure to 2,4,6-trinitrotoluene (TNT) is related to expression of various genes. To provide a broad range of gene expression in response to this common ordnance compound, microarray analysis was conducted on 3079 *Chlamydomonas* EST clones. To determine TNT treatment conditions, growth analysis of *Chlamydomonas* in 0 to 5 μg/ml TNT was conducted. One microgram per milliliter of TNT did not decrease the cell count after seven days of treatment, while 3 μg/ml of TNT was the maximum TNT concentration that allowed normal growth, thus cells were treated with both 1 and 3 μg/ml of TNT for microarray analysis. Transcriptional profiling revealed approximately 158 responsive genes that were differentially expressed. Many of the up-regulated genes were further classified into functional categories. Genes responsible for photosynthesis and energy metabolism genes were induced in the presence of TNT. TNT may result in oxidative stress since many oxidative stress related genes, such as glutathione-S-transferase were up-regulated. Among the down-regulated genes, the expression of cell wall related genes were repressed. Several unidentified genes were also induced or repressed. The possible involvement of the differentially expressed genes in the respone to TNT is discussed.

Introduction

Trinitrotoluene (TNT) has been extensively used as an explosive since 1902 (Tadros *et al.,* 2000). Its use in military bases and in the production, purification and loading of ammunitions has resulted in large amounts of wastes being generated. As a result of improper disposal, it 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 (Smock *et al.,* 1976, Nipper et al., 2001, Tadros *et al.,* 2000), bacteria (Gorontzy *et al.,* 1993), plants (Gong *et al.,* 1999, Lucero *et al.,* 1999, Paterson *et al.,* 1996, Peterson *et al.,* 1998, Scheidemann et al., 1998) and invertebrates (Robidoux *et al.,* 2002, Dodard *et al.,* 2003). One of the major concerns about TNT's environmental presence is its potential risk to 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 and can cause anemia and hepatitis (Won *et al*., 1974). 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 from organism to organism and consequently they can be exploited for their use in biomonitoring and bioremediating contaminated sites. Current detection systems monitoring surface and sub-surface contamination rely on frequent soil and water sampling which is expensive and labor intensive. Other traditional methods include chemical and bioanalytical analysis of contaminated samples to assess the level of pollution (Halasz *et al.,* 2002). Traditional methods of controlling TNT pollution include costly incineration processes (Bradley *et al.,* 1995). An attractive alternative technology, may involve organisms such

as plants and algae that can be developed as phytosensors and phytoremediation systems, which is a perfect marriage where the organisms perform both functions.

The potential use of plants to attenuate 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 produced. Overall, studies have reported aerobic reduction products of TNT with the major product being monoaminated TNT metabolites (4-amino-2, 6-dinitrotoluene, 2-amino-4, 6-dinitrotoluene) (Burken *et al.,* 2000). Type I nitroreductase enzymes have also been proposed to catalyze the reduction of TNT (Medina & McCutcheon, 1996). Goheen *et al.*, (1999) 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 (Miskiniene *et al.,* 1998). Oxidation processes in the metabolism of TNT in plants have also been observed. Bhadra *et al*., (1999) isolated six metabolites that were distinct from reduction products. These included 2,4-dinitro-6-hydroxy-benzyl alcohol, 2-amino-4, 6, dinitrobenzoic acid and 2,4-dinitro-6-hydroxytoluene. These products may occur in plants because oxidation is the primary reaction in the detoxification of other xenobiotics such as pesticides and herbicides (Bolwell *et al.,* 1994).

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. Further some plants acclimatize poorly to particular climates and soil conditions (Rubinelli *et al.,*

2002, Cobbett *et al.,* 2002). These restrictions may be evaded by the used 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 green fluorescent proteins that can be used as biological sensors that detect the pollutant.

In order to better understand gene regulation patterns in response to TNT, we have investigated the use of *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 could 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 (Kanesaki *et al.,* 2002). Genes identified in *Chlamydomonas* may also be transformable to common green algae ubiquitous to the environment.

In order to specifically investigate the transcriptional profile of *Chlamydomonas* in response to TNT, microarray analysis was conducted. This technique allows monitoring of changes in levels of transcripts of almost all genes in a specific organism (Watson *et al.,* 1998, Richmond and Somerville, 2000, Schena *et al.,* 1995). The differential expression of *Chlamydomonas* after a 24 hour treatment with 1 μg/ml and 3 μg/ml of TNT has been determined and the possible involvement of these genes in TNT response has been discussed.

Materials and Methods

Chlamydomonas strain and culture conditions

Chlamydomonas reinhardtii (Utex 89, The Culture Collection of Algae at the University of Texas at Austin) were maintained on tris-acetate-phosphate (TAP) agar (Harris, 1989) at 24 °C under continuous light (65 μmol $m^{-2}s^{-1}$). Growth curves of *Chlamydomonas* were obtained for a range of TNT concentrations (0, 1, 2, 3, 4, 5 μg/ml TNT) to determine the TNT treatment concentrations for the microarray experiments. The 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 growth media, a serial dilution of the TNT stock solution and TAP growth media was conducted. The *Chlamydomonas* inoculum was concentrated to 40 x 10^7 cells and harvested by centrifugation (5000x g for 5 min) and inoculated in the 50 ml of the various TAP/TNT growth media. Cells were counted at seven time points (24, 48, 72, 96, 120, 144, 168 hours) using a hemacytometer. Growth curve analysis was conducted in triplicate and statistical analysis (ANOVA) was used to determine the multiple comparisons between the treatments.

For RNA extraction, 100 ml of sample culture was inoculated in 500 ml of TAP media and allowed to attain logarithmic growth (1x10^7 cells/ml) by growing under continuous light as on a rotary shaker (140 rpm). Approximately 10x10^7 cells were harvested by centrifugation (5000x g for 5 min) and inoculated in 50 ml of the desired culture medium and grown for 24 hours before RNA isolation. The three culture media used for RNA isolation were supplemented with 0 μg/ml, 1 μg/ml and 3 μg/ml of TNT.

RNA extraction and preparation of fluorescent probes

For each sample (treated and control) $1.5x10^7$ 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_20. After 10 min incubation at 55 °C the isolated RNA was stored at –70 °C until further use. 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. This allows direct labeling of cDNA molecules by reacting the aminoallyl group with the fluorescent dyes. Each experiment (control versus treated sample) was replicated three times and each starting material was completely independent, including 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 adding the probe to the microarray slide.

Hybridization reaction and microarray analysis

The *Chlamydomonas* microarray slides (chip 1.1v, Carnegie Institute, CA) contained 3079 unique ESTs each represented four times. The probes solution was applied to the microarray slides under a 22 mm x 50 mm lifter slip (Erie Scientific Company, Portsmouth NH) and placed in a humidified hybridization chambers (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 hours. After hybridization, the slides were removed and placed in a slide rack submerged in washing solution (2x SSC, 0.03 % (w/v) SDS), with the array face of the slide tilted down so that the lifterslip would drop off without scratching the slide. Once the lifterslip was removed, the slide rack was plunged up and down for approximately 2 min and then transferred to 1x SSC for 2 min and finally to 0.05x 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 sequentially for Cy3 and Cy5- labeled probes with the GenePix microarray scanner (Axon Instruments, Union City, CA). Separate images were acquired for each flour 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.

Data analysis

For data analysis, spot intensities from scanned slides were quantified using Scanalyze software (version 2.32; M. Eisen, Standford University http://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 UT Microarray Database (c.f. Stanford Microarray Database; SMD) at genome.ws.utk.edu. The criteria used for selection of the up-regulated 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 greater than 0.5. To select for down-regulated genes, normalized channel intensities of greater than 150 with less than 0.6-fold decrease in mRNA and a regression correlation of greater than 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 (www.duke.edu/chlamy/_genome/).

Results

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 (see Figure 11). There was a negative correlation between growth of *Chlamydomonas* and the concentration of TNT. 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 hours). The cell concentration in 0 μg/ml TNT was 730 ± 22 (x 10^4 cells/ml; all *Chlamydomona*s cell concentrations) and cell concentration at 1 μg/ml TNT was 739 ± 17 cells/ml. Growth response studies indicated that 3 μg/ml TNT was the maximum TNT tolerance threshold concentrations. The concentration of *Chlamydomonas* growing in 4 and 5 μg/ml TNT was considerably less than the growth in the lower TNT concentrations, where *Chlamydomonas* cell counts after 168 hours 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 μg/ml TNT 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 a genetic level *Chlamydomonas* cells were treated with 1 μg/ml TNT. The second TNT concentrations were used because it was the maximum TNT tolerance threshold concentration for *Chlamydomonas*.

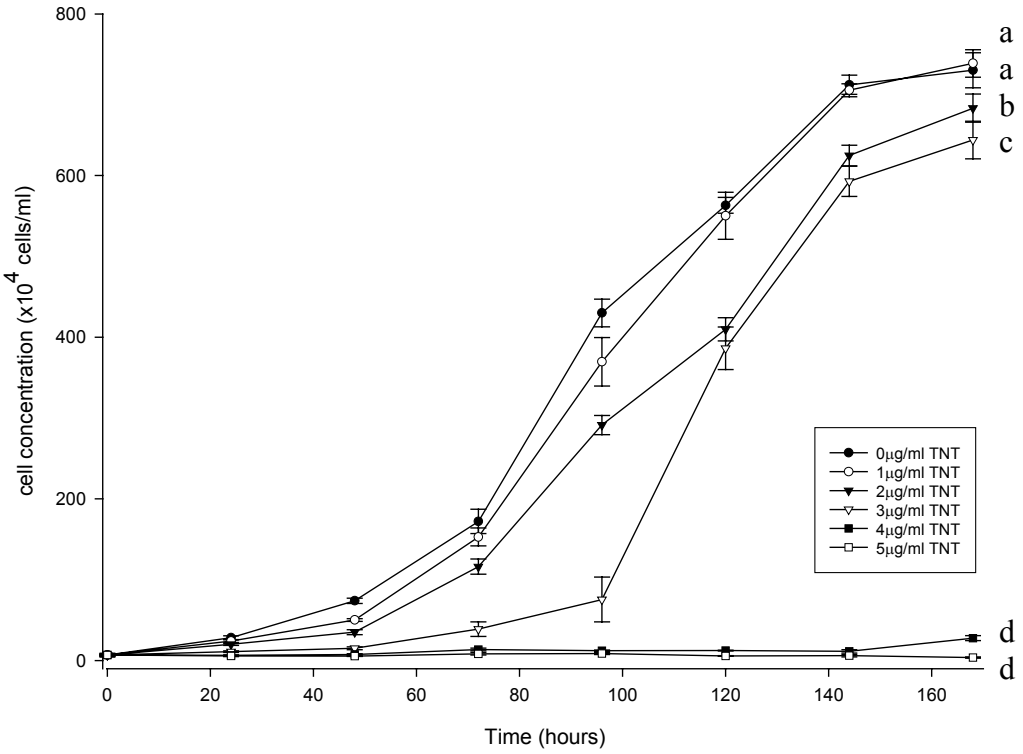


Figure 11: The growth response of wild-type *Chlamydomonas* to TNT. Cell concentrations were measured every 24 hours. Differing letters indicate significantly different ($P<0.05$). Vertical bars represent standard deviations.

Microarray analysis

The Carnegie institute microarray contains unique 3079 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 Figure 12. For the majority of the transcripts, expression appeared unchanged with TNT treatment (see Figure 12). Using the selection criteria outlined in "Materials and Methods," and accounting for the ESTs that correspond to similar genes in the BLASTN search, 158 ESTs were differentially expressed in response to TNT. Of these, expression of 38 ESTs were up-regulated and 43 ESTs were down-regulated at 1 μg/ml TNT. At 3 μg/ml TNT, 35 genes were up-regulated and 42 genes were down-regulated. The expression data based on EST description and BLAST homologies for 1 μg/ml TNT-responsive ESTs are described in Table 2 and 4. Differentially expressed genes in 3 μg/ml TNT are represented in Table 3 and 5.

Functional classification of up-regulated genes

Genes involved in several processes are differentially expressed in the presence of TNT. One of these functional processes is photosynthesis and energy metabolism. Microarray studies revealed that genes encoding photosystem I, photosystem II, plastocyanin, cytochrome b_6f, and the light-harvesting complex genes are up-regulated after 24 hours of TNT treatment (see Table 2 and 3). These complexes constitute the photosynthetic electron transport chain, which primarily generates NADPH and ATP, required for the reduction of carbon and other chloroplast activities (Hopkins, 1999).

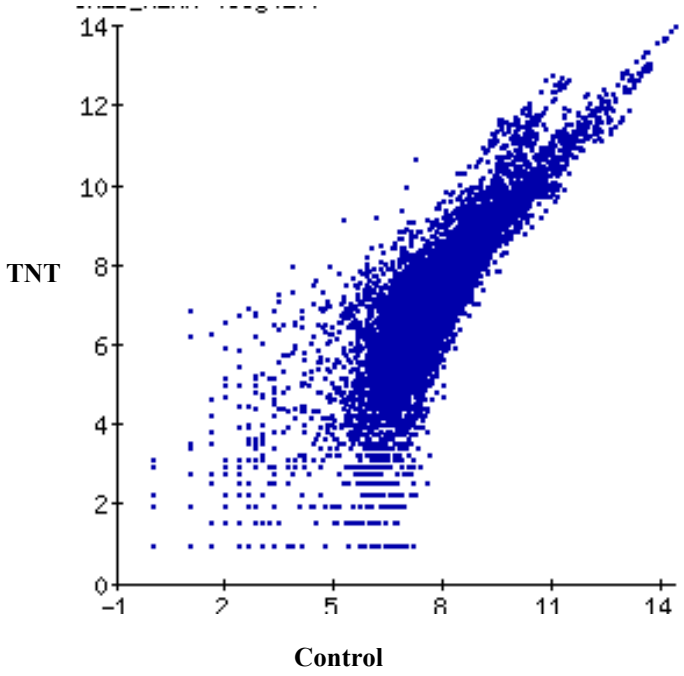


Figure 12: Scatter plot of signal intensities for all ESTs on the microarray. Normalized log channel intensities for each clone on the microarray are plotted with signals from the control and the TNT-treated on the x and y-axis, respectively. In general the values fall near the line x=y. This indicates that most of the genes are unaffected by the treatment conditions. Values that fall outside the general x=y lines are the differentially expressed genes.

Table 2: Genes up-regulated by 1 μg/ml TNT.

EST Genbank or clone ID	Gene description	Putative functional category	Fold ratio ± SD
894092D12	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 beta-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

Table 2: Continued

EST Genbank or clone ID	Gene description	Putative functional category	Fold ratio ± SD
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

Table 3: Genes that are up-regulated at 3 μg/ml TNT

EST genebank or Clone id	Gene description	Putative functional category	Fold ratio ± SD
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 beta-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

Table 3: Continued

EST genebank or Clone id	Gene description	Putative functional category	Fold ratio ± SD
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

Table 4: Down-regulated genes at 1 μg/ml TNT.

EST genbank or clone ID	Gene description	Putative functional category	Fold ratio ± SD
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 zeta-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

Table 4: Continued

EST genbank or clone ID	Gene description	Putative functional category	Fold ratio ± SD
BM518836	Putative indole-3-glycerol phosphates synthase	Metabolic enzyme in the productionof 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

Table 5: Down-regulated genes at 3 μg/ml TNT.

EST genbank or clone ID	Gene description	Putative functional category	Fold ratio ± SD
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-beta-hydroxybutyrate dehydrogenase	Enzyme found in the mitochondria membrane	0.41 ± 0.09
BF863761	Alpha-tubulin like protein	Microtubule protein	0.43 ± 0.09
BM519086	Unknown	Unclassified	0.44 ± 0.11
BE227503	Putative alpha-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 Arabidospis thaliana	0.53 ± 0.02

Table 5: Continued

EST genbank or clone ID	Gene description	Putative functional category	Fold ratio ± SD
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	BBC1like protein	Involved in activation of transcription	0.58 ± 0.02
BF859990	Unknown	Unclassified	0.59 ± 0.02

In addition to the up-regulation of photosynthetic genes, many ribosomal proteins were up-regulated 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 proteins in 1 μg/ml TNT (see Table 2). In 3 μg/ml TNT the expression level of the large 50S subunit was 1.89 ± 0.12-fold induction, while the small 30S subunit protein expression was approximately 2.14 ± 0.15-fold higher (see Table 3).

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 proteins were up-regulated in 3 μg/ml TNT. The anti-oxidative stress proteins include peroxiredoxin like proteins, DegP protease like protein, thioredoxin and glutathione-S-transferase. Peroxiredoxin-like protein, DegP protease and thioredoxin were up-regulated at least 2-fold in TNT treatment. Peroxiredoxins form a group of peroxidases found in bacteria (Tartaglia *et al.,* 1990), yeast (Chae *et al.,* 1993), animals and higher plants (Goyer *et al.,* 2002).

At the lower concentration of TNT very few known cell defense genes were over-expressed. Both TNT treatment conditions resulted in the up-regulation of a putative lycopene beta cyclase. This enzyme is involved in the synthesis of carotenoid compounds. Often these compounds are associated with photosynthesis and many also act as antioxidant agents (Hemmi *et al.,* 2003). 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 is up-regulated at 3 μg/ml TNT is the nitrate reductase gene. There is a 1.9 ± 0.16-fold increase in gene expression. Nitrate reductase is the primary enzyme that catalyzes the reduction of nitrate to nitrite (Llamas *et al.,* 2002). Other metabolic genes that are up-regulated in TNT are the sulfotranferase gene and the sulfate transport system gene. These genes are involved in sulfur assimilation in *Chlamydomonas* (Harris, 1988).

The final classifications of upregulated genes are those whose functions are not yet known. Some of these genes include hypothetical proteins and expressed protein 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 amount of up-regulation was observed for a gene whose functional category was unclassified. Among the up-regulated genes at 3 μg/ml TNT treatment is 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 is an unknown protein which had a 2.68 ± 0.86 fold ratio increase.

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 down-regulated by TNT, however it was determined that a few genes had reduced mRNA levels. Compared to the up-regulated genes the majority of the down-regulated genes

were unknown. At 1 μg/ml TNT approximately 74 % of the unknown genes were repressed and 3 μg/ml TNT 50 % of the unknown genes were down-regulated

Among the down-regulated genes, many genes associated with cell wall components of *Chlamydomonas* were repressed in the presence of 3 μg/ml TNT. Hydroxyproline rich proteins constitute a major structural component of the *Chlamydomonas* cell wall. Another set of genes that were down–regulated were the ATP related genes. ATP is involved in the expenditure of energy that drives various cellular processes in the cell (Hopkins, 1999).

Discussion

The focus of this study was identifying *Chlamydomonas* genes that were up-regulated in the presence of TNT. Among these up-regulated genes several functional categories were analyzed for their putative role in TNT response.

The data suggest that TNT regulates genes involved in photosynthesis and therefore, may affect the redox regulation of *Chlamydomonas*. It has been reported that in green algae and higher plants, transcription (Pfannsschmidt *et al.,* 1999), mRNA stability (Alexciev and Tullberg, 1997), translation (Dannon and Mayfield, 1994) and protein phosphorylation (Rintamäki, 2000) are regulated by the redox state of the photosynthetic electron transport chain. In addition, other reports suggest that the thioredoxin gene (Navarro *et al.,* 2000), some nitrogen-related genes (Alfonso *et al.,* 2001) and heat shock genes (Glatz *et al.,* 1997) were under the control of the photosynthetic electron transport. In this study thioredoxin, nitrate reductase and putative heat shock proteins were up-regulated in TNT indicating that TNT is affecting the regulation of the electron transport

chain and thereby affecting genes under the control of the electron transport chain. Studies conducted by Nocter and Foyer (1998) have characterized the antioxidant defense network in plants where they suggest that the 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, (Méndez-Álvarez *et al.,* 2000). During aerobic metabolism under these stress conditions, 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 (Desikan *et al.,* 2001). There are many research studies that have indicated that the generation of ROS during oxidative burst is one of the first cellular responses to potential pathogens and elicitor molecules (Lamb and Dixon, 1997). These ROS induce the expression of defense-related genes such as glutathione S-transferase (Desikan *et al.,* 1998), peroxidases such as ascorbate peroxidase and superoxide dismutase (Méndez-Álvarez *et al.,* 2000). In this study, both glutathione S-transferase, and peroxidase enzyme were overproduced in the presence of 3 μg/ml TNT. Glutathione acts as a redox sensor and is involved in the multiple regulatory systems coordinating the expression of defense genes (Wagner *et al.,* 2002). Arisi *et al.,* 1998 and Zhue *et al.,* 1999, 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 (Foyer *et al.,* 1995).

The up-regulation 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 the chloroplast. Studies conducted by Mendez-Alvarez *et al.,* (2000) 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 (Bohne and Linden, 2002). A majority of carotenoids are synthesized from lycopene. Beta-carotene is synthesized directly from lycopene and catalyzed by lycopene-beta-cyclase. A putative lycopene-beta-cyclase was up-regulated 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 (Bouvier *et al.,* 1998).

The up-regulation of nitrate reductase at 3 μg/ml TNT indicates that this enzyme may be associated with TNT metabolism. Hannink *et al.,* (2001) 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 hydroxyaminodinitoluene, 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 (Macfie and Welbourn, 2000). 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 (Crist *et al.,* 1994). 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 down-regulated.

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.

In order to extend the microarray data, the expression of selected genes would need to be confirmed. Many studies have used various techniques to confirm the data such as northern blot analysis (Hihara *et al.,* 2001) or real-time PCR (Sebert *et al.,* 2002). When considering the relative expression of the 3079 clones 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 (Gygi *et al.,* 1999). 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 were induced in the presence of TNT can be fused to reporter genes such as GFP to serve as biomonitoring systems.

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