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Proteomic profiling of vetiver grass (Chrysopogon zizanioides) under 2,4,6-trinitrotoluene (TNT) stress

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Key Points:
- Proteomic profiling of vetiver exposed to TNT provides important clues to the mechanism of TNT stress response and tolerance in plants
- Several growth-related proteins were downregulated correlating well with a decline in plant growth
- Upregulation of ethylene signaling pathway and plant defense proteins at lower treatments could be involved in TNT stress tolerance

Supporting Information:
- Supporting Information S1

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Proteomic profiling of vetiver grass (*Chrysopogon zizanioides*) under 2,4,6-trinitrotoluene (TNT) stress

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Abstract Vetiver grass is an ideal plant for 2,4,6-trinitrotoluene (TNT) phytoremediation, due to its ability to tolerate and metabolize TNT as previously reported. The current study is the first attempt to investigate the changes in the proteomic profile of a plant under TNT stress. Vetiver plants were grown in nutrient media with varying concentrations of TNT (0, 25, 50, and 100 mg L^{-1}) for 10 days. Although the plants appeared healthy, significant biomass reductions ($p = 0.0008$) were observed in treated plants. Total proteins in the root decreased significantly ($p = 0.0003$). Proteomic analysis of root proteins revealed the downregulation of functional proteins involved in key cellular mechanisms such as transcription, ribosome biogenesis, nucleo-cytoplasmic transport of proteins, protein glycosylation, and translation. Growth-related proteins were downregulated; plant defense proteins were upregulated at lower TNT concentrations but downregulated at higher concentrations. Comprehensive understanding of changes in the proteomic profile provides important clues to the mechanism of TNT stress response and tolerance in vetiver.

1. Introduction

Phytotoxicity associated with 2,4,6-trinitrotoluene (TNT) has remained a major limitation to the use of plants for remediation of TNT-contaminated soil and aqueous media [*Hannink et al*., 2002]. Hence, in spite of several successful laboratory-based studies on TNT uptake and transformation by plants, no large, field-scale implementation of the phytoremediation technique to remediate contaminated military sites with high TNT concentrations has been undertaken. One of the proposed solutions is creating transgenic plants capable of tolerating the stress associated with the high TNT concentrations [*Hannink et al*., 2002; *French et al*., 1999]. Researchers have reported the development of transgenic *Arabidopsis*, tobacco, and poplar containing bacterial genes with enhanced TNT degradation capabilities [*Rylott and Bruce*, 2009]. However, regulatory restrictions will continue to hinder the widespread use of these plants in the field. On the other hand, a few plant species have been reported to exhibit remarkable tolerance to high levels of TNT [*Kiiskila et al*., 2015], but the biochemical mechanisms which make these plants innately tolerant to TNT stress still remain to be elucidated.

While most plants exhibit a range of adverse effects to TNT exposure including impaired growth and chlorosis, plants like parrot feather and vetiver grass exhibit higher TNT tolerance, indicating the presence of innate detoxification mechanisms in these plants [*Hannink et al*., 2002; *Kiiskila et al*., 2015]. In earlier reports, we have shown that vetiver grass is tolerant to TNT and is capable of taking up and degrading TNT [*Makris et al*., 2007; *Das et al*., 2015]. However, the biochemical mechanism of TNT tolerance and degradation has not been fully deciphered in vetiver.

Vetiver grass (*Chrysopogon zizanioides*) is a perennial, clump grass. The Sunshine variety of this grass, which sterile, and can only be propogated vegetatively, has been assigned a low-risk score for invasiveness potential from U.S. Department of Agriculture [*U.S. Department of Agriculture Natural Resources Conservation Service*, 2009]. It is a fast, growing, high-biomass grass with a substantial root system and is tolerant to a number of environmental contamiants, both metal and organic [*Danh et al*., 2009].

Earlier reports from our group demostrated the presence of TNT metabolites such as 2-amino-dinitrotoluene (2-ADNT) and 4-amino-dinitrotoluene (4-ADNT) in the root tissues of vetiver grass [*Das et al*., 2015], and concurrently, enhanced activity of nitroreductase enzyme in TNT-treated vetiver plants was observed [*Das et al*., 2017]. Plant proteins play major roles in controlling the stress-related mechanisms following exposure to contaminants [*Ahsan et al*., 2009]. While loss or downregulation of some functional proteins on exposure to

contaminants may interrupt various biological processes and produce phytotoxic effects in plants, increase in levels of other proteins which take part in the detoxification/degradation pathways may confer the plant tolerance to contaminants.

Proteomics is a comprehensive approach to study complex biological functions of proteins in phytoremediation candidate plants, which can help identify the molecular mechanisms that play key roles in plant tolerance or detoxification of contaminants [*Ahsan et al*., 2009]. For example, *Gillet et al*. [2006] found that in algae, several proteins involved in photosynthesis were significantly decreased on exposure to cadmium, whereas proteins related to defense mechanisms such as glutathione biosynthesis, adenosine triphosphate (ATP) metabolism, and oxidative stress were significantly increased.

Global "omics" approaches, such as transcriptomics, can be applied very effectively to model organisms, because of the availability of well-characterized genomic information [*Carpentier et al*., 2008]. For nonmodel organisms such as vetiver, gene expression under the influence of stress could be understood by using the proteomic approach. Since protein sequences are more conserved, identification of nonmodel genes by comparison to well-known orthologous proteins is efficient and acceptable [*Liska and Shevchenko*, 2003]. Most of the proteomic studies conducted so far have investigated the changes in plant proteome following exposure to the toxic metals [*Bell et al*., 2014]. An examination of the impact of an organic contaminant such as TNT in bringing about changes in the proteomic profile will help in understanding the biochemical pathways engaged in TNT-related stress. The present study conducted a proteomic profiling of vetiver to identify the candidate proteins likely to play major roles in regulating biochemical and physiological responses in vetiver under varying levels of TNT. To our knowledge, this is the first attempt to investigate the proteomic profile of a plant in response to TNT stress.

2. Materials and Methods

2.1. Experimental Setup

The effect of TNT on the vetiver proteomic profile was investigated in plants grown in hydroponic media containing four TNT concentrations (0, 25, 50, and 100 mg L^{-1}). Vetiver plants were purchased from Florida Farms and Nurseries (FL, USA). Plants were first grown in plastic pots by using potting soil in a greenhouse to acclimatize them. Plants were then washed thoroughly and placed in Hoagland's solution and grown in a plant growth chamber with a 16/8 h day/night photoperiod. After 10 days, vetiver plants were removed from Hoagland's solution, washed, weighed, and placed in solutions containing various concentrations of TNT in triplicate for a further period of 10 days. Plants were then washed with deionized water, and final weights of the plants were measured to determine the growth or biomass reduction. Root and shoot tissues were separated, flash-frozen in liquid nitrogen, and stored at −80°C for further analysis.

2.2. Total Soluble Protein

Total soluble proteins from root and shoot tissues of vetiver grass grown in each of the TNT concentrations were extracted twice by using ice-cold phosphate buffer (50 mM, pH 7.8). The protein concentration was quantified by using ReadyPrepTM protein extraction kit (Bio-Rad, CA) and quantified by using bicinchoninic acid protein assay kit (Bio-Rad, CA). Protein estimations were done in triplicate.

2.3. Analysis of Plant Proteome

Two-dimensional (2-D) gel electrophoresis was conducted (according to the manufacturer's instructions, Bio-Rad, CA) to separate the proteins. Briefly, 300 μg of protein was treated with a ReadyPrep 2-D Cleanup Kit (Bio-Rad Laboratories, USA). For the first dimension, isoelectric focusing was done by using immobilized pH gradient (IPG) strips with a linear pH range (11 cm, pH 3–10) in PROTEAN™ IEF Cell (Bio-Rad Laboratories, USA). The IPG strips were equilibrated in buffer and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by using Criterion TGX gels (10–20%). For the second dimension, proteins were separated on 15% SDS polyacrylamide gels and stained with Coomassie Blue G-250. Gels were imaged with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, USA). ImageMaster™ 2D Platinum (version 7.0, GE Healthcare, WI) was used to determine the differentially expressed proteins. Significantly, differential protein spots, those exhibited fold change ≥2, were selected for mass spectrometric analysis. Protein spots of interest were excised from gels, digested with trypsin, and analyzed through matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (Bruker, WI) following the

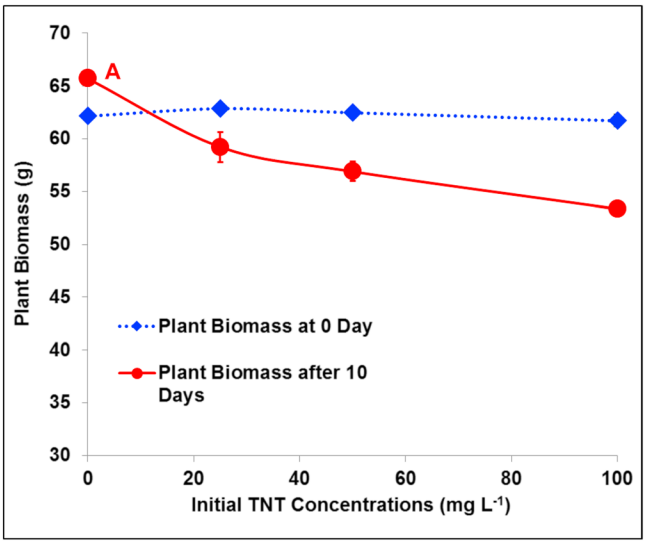

Figure 1. Effect of TNT on vetiver biomass of vetiver grass after 10 days. Data are expressed as mean ($n = 3$) + one standard deviation.

method described by *Shevchenko et al.* [2006]. Peptide mass fingerprint spectra were searched combined with Mascot program search engine (http://www.matrixscience.com) and National Center for Biotechnology Information protein database (http://www.ncbi.nlm.nih.gov). Protein identities were confirmed by matching molecular weight and isoelectric point obtained from the 2-D gel electrophoresis. Functional annotations of the identified proteins were carried out according to Uniprot database, and predicted functional partners of the identified proteins were searched by using String 10.0 database [*Jensen et al.*, 2009]. The experiment was repeated twice.

2.4. Data Analyses

Data were expressed as mean ($n = 2$) along with standard deviation. Two-way analysis of variance was carried out by using statistical software JMP IN version 8.0 (13). Significant differences among treatment means were calculated by using a Tukey-Kramer honest significant difference test. Statistical significance of protein spots' intensities was calculated by using Student's *t* test using ImageMaster™ 2D Platinum software.

3. Results and Discussion

3.1. Effect of TNT on Plant Growth

Although vetiver grass shows much higher tolerance to TNT compared to other grasses studied for phytoremediation [*Makris et al.*, 2007; *Das et al.*, 2015], toxicity symptoms such as yellowing of shoots was noted in our earlier experiments at higher TNT concentrations in soil [*Das et al.*, 2010]. The current study evaluated the effect of TNT exposure on growth of vetiver plants under hydroponic conditions, where enhanced TNT plant availability is expected. Phytotoxicity was correlated with any potential loss of functional proteins determined by the proteomic approach. After 10 days of exposure to varying TNT containing solutions, the plants appeared healthy with no visible signs of toxicity such as chlorosis of shoot, which is a common symptom associated with TNT stress [*Hannink et al.*, 2002]. However, significant decrease in plant biomass ($p = 0.0008$) was found with increasing TNT concentrations in the media, whereas the plants in TNT-free controls showed significant growth during the experimental period (Figure 1). In addition to decreasing biomass, exposure to TNT resulted in decreased total

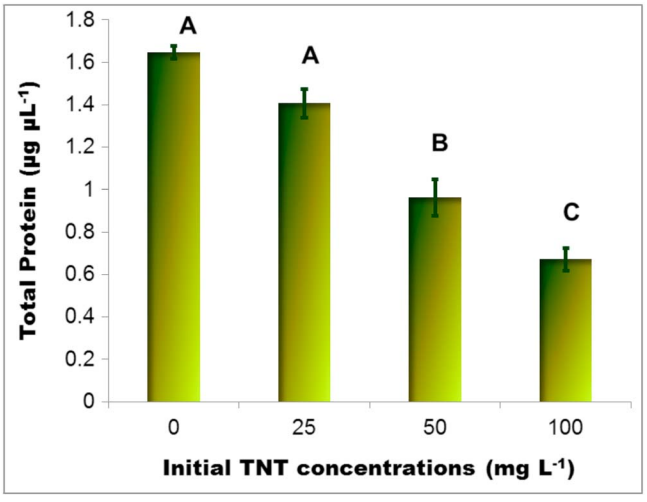

Figure 2. Effect of TNT on the total proteins in root. Data are expressed as mean ($n = 3$) + one standard deviation.

Table 1. Bivariate Correlation and Regression Parameters of Initial TNT Treatments With the Growth of Vetiver Grass and Total Soluble Protein Content of the Vetiver Root

Parameters	Correlation (r)	Regression (R^2)	P value
Growth	−0.91	0.82	0.0019
Total protein in root	−0.97	0.94	<0.0001

chlorophyll content (data not shown). Loss of chlorophyll as a function of TNT concentration is a common TNT stress symptom [*Hannink et al.*, 2002]. The total soluble protein content in the vetiver root showed significant ($p = 0.0003$) continual decrease as a consequence of increasing TNT concentrations (Figure 2). In the root tissues of vetiver grass, the total soluble protein content decreased by 15%, 42%, and 59% in plants grown in 25, 50, and 100 mg L^{-1} initial TNT concentrations, respectively. However, similar results were not observed in the shoot tissue of the vetiver grass. The total soluble protein content in shoot did not show any significant change ($p > 0.05$) (data not shown).

A number of earlier reports indicate that TNT is primarily metabolized in root tissues, with very little translocation to the shoot [*Ouyang et al.*, 2005; *Adamia et al.*, 2006; *Brentner et al.*, 2010]. In an earlier greenhouse study, we reported the presence of high concentrations (140 mg/kg) of TNT and trace amounts of the TNT degradation metabolite 4-amino-2,6-dinitrotoluene (4-ADNT) in root when vetiver plants were grown in soil contaminated with 100 mg kg^{-1} of TNT. Shoot tissue had no TNT, but the presence of metabolites 1,3,5-trinitrobenzene, 4-ADNT, and small amounts of 2-amino-4,6-dinitrotoluene (2-ADNT) was observed [*Das et al.*, 2015]. Moreover, nitroreductase enzyme activity, which is likely to be responsible for TNT degradation, was 250-fold higher in shoot tissue compared to the roots [*Das et al.*, 2015]. Hence, we performed proteomic profiling of root tissue to understand the biochemical pathways impacted by TNT stress in vetiver, as no significant degradation of TNT seems to be occurring in the root.

3.2. Proteomic Profiling of Root Tissue

Root proteins showed a significant ($p < 0.0001$) negative correlation ($r = -0.97$) with TNT and followed a linear ($R^2 = 0.94$) decrease with increasing TNT concentrations in solution (Table 1), suggesting significant loss of functional proteins in the root tissues of vetiver grass as results of TNT stress. Figure 2 shows decreasing levels of total proteins, with increase in TNT concentration in plant growth media. At the highest TNT concentration of 100 mg L^{-1}, a 2.5-fold decrease in total proteins compared to control plants grown in TNT free media was observed. Figure 3 shows gel images of the root samples after 2-D gel electrophoresis. Twenty most prominent protein spots in the gels containing samples exposed to TNT were found to have a minimum twofold

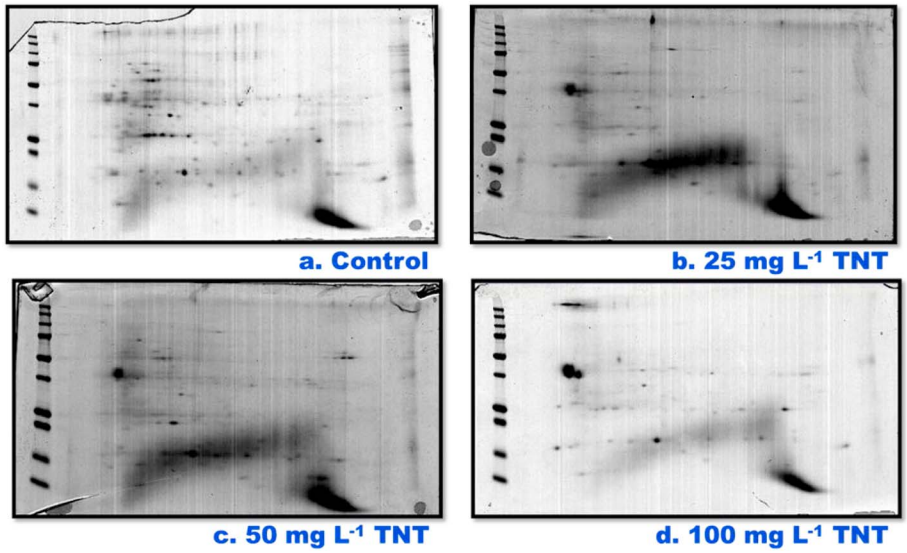

Figure 3. Gel images showing the protein spots in root tissues treated with different initial TNT concentrations.

Table 2. Downregulated Proteins in TNT-Treated Vetiver Root, Showing a Differential Expression Compared to Control, Annotated Using UniProt Database

UniProt Accession Numbers	Entry Names	Protein Names	Gene Names
Q9LM60	B3GT5_ARATH	Probable beta-1,3-galactosyltransferase 5 (EC 2.4.1.-)	B3GALT5 At1g22015 F2E2.6
P51119	GLNA2_VITVI	Glutamine synthetase cytosolic isozyme 2 (EC 6.3.1.2) (glutamate-ammonia ligase)	GS1-2 GS1;2
A4KAG7	KSL5_ORYSI	Ent-isokaur-15-ene synthase (EC 4.2.3.103) (ent-kaurene synthase-like 5) (OsKSL5) (isokaurene synthase)	KSL5 OsI_007599 OsI_07744
A3BN26	PUS6_ORYSJ	RNA pseudouridine synthase 6, chloroplastic (EC 5.4.99.-) (RNA pseudouridylate synthase 6) (RNA-uridine isomerase 6)	Os07g0660400 LOC_Os07g46600 OsJ_024448 P0496C02.118
Q0DVX2	RH50_ORYSJ	DEAD-box ATP-dependent RNA helicase 50 (EC 3.6.4.13)	Os03g0108600 LOC_Os03g01830 OJ1384D03.13 OsJ_09113
P69243	RPO3A_TOBAC	DNA-directed RNA polymerase 3A, chloroplastic (EC 2.7.7.6) (NictaRpoT3-syl) (T7 bacteriophage-type single subunit RNA polymerase 3A)	RPOT3-SYL
Q9SSY6	ETR1_CUCSA	Ethylene receptor 1 (EC 2.7.13.3) (CS-ETR1)	ETR1
Q43312	H2A7_WHEAT	Protein H2A.7 (wcH2A-10) (wcH2A-4)	H2A-4; H2A-10
A2YEQ6	RAN3_ORYSI	GTP-binding nuclear protein Ran-3 (OsRan3) (Ras-related nuclear protein 3)	RAN3 OsI_022799
A2YEQ6	RAN3_ORYSI	GTP-binding nuclear protein Ran-3 (OsRan3) (Ras-related nuclear protein 3)	RAN3 OsI_022799

change in their intensities compared to the control gel containing proteins extracted from root tissue of plants grown in TNT-free solution (Table S1 in the supporting information). Among them, 14 protein spots were significantly ($p < 0.05$) downregulated with increasing initial TNT treatments.

A total of six protein spots were upregulated at lower initial TNT treatments (25 mg L^{-1}) but downregulated at higher initial TNT concentrations. The proteins exhibiting these trends in response to TNT exposure were identified by using MALDI-TOF-mass spectrometry, and functional annotation analyses were carried out.

3.2.1. Downregulated Proteins

Nine out of 14 root proteins showed continuous downregulation in response to the exposure to increasing levels of TNT (Table 2 and Figure 4). Functional annotation analysis using UniProt database revealed the major functions of these proteins and the biochemical pathways they are involved in. The results show that TNT majorly affects key functional cellular mechanisms such as transcription, ribosome biogenesis, nucleocytoplasmic transport of proteins, and the protein glycosylation pathway. Table S2 shows the Pearson correlation of the negative relationship between downregulated protein expressions with initial TNT treatments. Histone H24A, a subunit of histone protein and a core component of the nucleosome which wrap and compact DNA into chromatin, was downregulated by increasing TNT concentration (Figure 4). Histones play a principal role in transcription regulation, DNA repair, DNA replication, and chromosomal stability by limiting DNA accessibility to the cellular machineries that need DNA as a template [*Pawlak and Deckert*, 2007]. Various environmental stressors such as heavy metals and radiation have been reported to cause histone modifications leading to chromatin remodeling and changes in gene expression [*Pawlak and Deckert*, 2007].

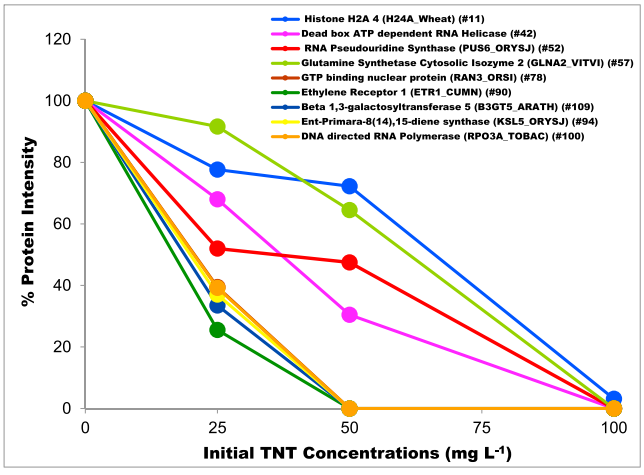

Figure 4. Identified proteins that showed continued downregulation with each increased TNT treatment. Relative intensity of protein spots extracted from TNT-treated root samples as compared to that of proteins extracted from root samples grown in TNT-free control vetiver plants expressed as percentage.

DNA-dependent RNA polymerase was another downregulated protein that plays a major role in transcription. Dead box ATP-dependent RNA helicase is ubiquitous, preferentially

expressed in the root, which was also downregulated [*Mingam et al*., 2007]. It is involved in ribosome biogenesis through rRNA processing and decaying nonsense-mediated mRNA [*Mingam et al*., 2007]. TNT stress also resulted in downregulation of GTP-binding protein, which is required for regulating the transport of RNA and proteins across the nuclear membrane [*Zang et al*., 2010], and is also involved in the assembly of mitotic spindle and nuclear envelope. Abiotic stress such as salt and osmotic stress has been reported to reduce transcription of these proteins in rice and Arabidopsis [*Mingam et al*., 2007]. β-1,3-galactosyltransferase is involved in cell wall synthesis and was reported to be downregulated in Arabidopsis due to water stress [*Bray*, 2004]. It transfers galactose from uridine diphosphate (UDP) galactose to substrates with a terminal glycosyl residue. The current study revealed a continuous, significant downregulation of the above proteins in response to increasing levels of TNT exposure.

Downregulation of growth-related proteins were also observed due to increasing TNT stress. Glutamine synthetase cytosolic isozyme 2 is involved in glutamine biosynthesis process, through which ammonia assimilation into glutamine and glutamate occurs. Glutamine and glutamate are precursors for almost all N compounds and thus play important role in plant growth [*Teixeira and Fidalgo*, 2009]. RNA pseudouridine synthase 6 is another protein which was affected by TNT exposure. This enzyme catalyzes the synthesis of pseudouridine, the most abundant, ubiquitous yet enigmatic constituent of structural RNAs [*Charette and Gray*, 2000]. Normal growth is severely compromised in the absence of pseudouridine synthase. Earlier research works also showed that genetic mutants lacking specific psi residues in tRNA or rRNA exhibited difficulties in translation, displayed slow growth rates in an *Escherichia coli* mutant deficient in a pseudouridine synthase [*Charette and Gray*, 2000].

Interestingly, exposure to TNT also affects proteins related to plant defense. Ent-pimara-8(14),15-diene synthase, a plant defense protein that is reported to be highly expressed in plant root [*Margis-Pinheiro et al*., 2005], was also significantly downregulated as consequence of TNT treatments. Ent-pimara-8(14),15-diene synthase belongs to the terpene synthase family, which produces phytoalexins to defend plants against microbial infection [*Chen et al*., 2011]. Ethylene receptor 1, a membrane component which binds ethylene, is also downregulated by TNT exposure in vetiver root. It acts in the ethylene signal transduction pathway, as an ethylene receptor, or as a redundant negative regulator of ethylene signaling [*He et al*., 2005]. Ethylene is responsible for the regulation of plant growth and development as well as plant response to stress and is considered a plant defense hormone [*Wang et al*., 2002]. Factors involved in the ethylene signaling pathway include ethylene receptors (ETRs); five ETRs have been characterized in Arabidopsis. ETR1 has histidine kinase activity, whereas the other four receptors mainly have serine kinase activities [*Xie et al*., 2002]. Several types of stresses can induce ethylene, but the role of ethylene receptors in plant stress responses still remains to be fully understood. Dehydration, salt stress, and wounding have been reported to enhance expression of ethylene receptor [*Xie et al*., 2002]. Since ethylene receptors are negative regulators of ethylene response, downregulation of ethylene receptor protein is expected to increase response of ethylene.

Predicted functional partners of the above downregulated proteins were investigated by using the String database (Figures S1–S6 in the supporting information). These downregulated proteins have been either predicted to or experimentally found to interact with proteins related to ribosomal RNA processing, transcription factors involved in cell differentiation, mRNA processing, and ribosome recycling during translation.

3.2.2. Upregulated Proteins

Proteins that were initially upregulated at 25 mg L^{-1} TNT concentration, possibly indicating the kicking-in of mechanisms that result in the tolerance of vetiver plants to TNT stress at lower concentrations, are shown in Table 3. These proteins were downregulated at higher TNT treatments (Figure 5). One of such protein is S-adenosylmethionine synthase (SAM), which again indicates the role of ethylene biosynthesis pathway as one of the biochemical defense mechanisms against TNT stress. This enzyme catalyzes the synthesis of S-adenosylmethionine, which is a precursor in ethylene biosynthesis. It is also required for biosynthesis of the phenylpropanoid constituents of the cell wall, which are produced as a response to stress, causing cell wall lignification. In Arabidopsis, the expression of SAM gene has been correlated with tissues undergoing lignification. Moreover, Arabidopsis *mto3* mutant, which lacks a functional SAM3 protein, exhibits decreased AdoMet levels and lignin content [*Shen et al*., 2002].

UDP-*N*-acetylglucosamine-peptide-*N*-acetylglucosaminyl transferase (SEC) was upregulated in 25 mg L^{-1} concentration of TNT but downregulated at higher concentrations (Figure 5). Posttranslational modification

Table 3. Upregulated Proteins in 25 mg L^{-1} TNT-Treated Vetiver Root, Showing a Differential Expression Compared to Control, Annotated Using UniProt Database

UniProt Accession Numbers	Entry Names	Protein Names	Gene Names
B6U8R7	CSPL9_MAIZE	CASP-like protein 2C4 (ZmCASPL2C4)	
P24274	DRP90_SOYBN	DNA-binding protein DRP90	DRP90
A9PHC5	METK4_POPTR	S-adenosylmethionine synthase 4 (AdoMet synthase 4) (EC 2.5.1.6) (methionine adenosyltransferase 4) (MAT 4)	METK4 POPTR_0006s12510g
Q9M8Y0	SEC_ARATH	Probable UDP-N-acetylglucosamine-peptide *N*-acetylglucosaminyltransferase SEC (EC 2.4.1.-) (protein Secret Agent)	SEC At3g04240 T6K12.14
Q96301	SPY_ARATH	Probable UDP-*N*-acetylglucosamine--peptide *N*-acetylglucosaminyltransferase SPINDLY (EC 2.4.1.-)	SPY At3g11540 F24K9.29

of proteins with *O*-linked *N*-acetylglucosamine, catalyzed by *O*-GlcNAc transferases (OGT), is very important and is catalyzed by two OGTs, SPY-like and SEC-like OGTs, which seem to have both unique and overlapping roles, affecting plant processes such as response to hormones and environmental signals, circadian rhythms, development, intercellular transport, and virus infection [*Olszewski et al*., 2010]. While the SPY protein negatively regulates gibberellin signaling [*Iyer and Hart*, 2003], the Secret Agent (SEC) protein, which was identified in vetiver root, is associated with plant defense mechanism. SEC deficiency impairs infection by plum pox virus in Arabidopsis. SEC modifies the virion capsid protein, and its absence in mutated SEC plants that makes the virus movement is slower [*Olszewski et al*., 2010].

Another protein that was initially upregulated at 25 mg L^{-1} TNT and then downregulated at higher TNT concentrations was pentatricopeptide repeat (PPR) protein (Figure 5). PPR proteins are targeted to mitochondria or chloroplasts. They bind one or several organellar transcripts and influence their expression by altering RNA sequence, turnover, processing, or translation [*Barkan and Small*, 2014], impacting various crucial plant functions including photosynthesis, respiration, plant development, and environmental responses [*Barkan and Small*, 2014]. Arabidopsis mitochondrial pentatricopeptide repeat domain protein PPR40 mutant has impaired mitochondrial electron transport [*Zsigmonda et al*., 2012] and overexpression of the PPR40 gene in Arabidopsis resulted in better salt tolerance, with increased germination and better plant growth under saline conditions. Increased respiration, reduced reactive oxygen species, diminished lipid peroxidation, lower ascorbate peroxidase, and superoxide dismutase activity were reported under salt stress [*Zsigmonda et al*., 2012].

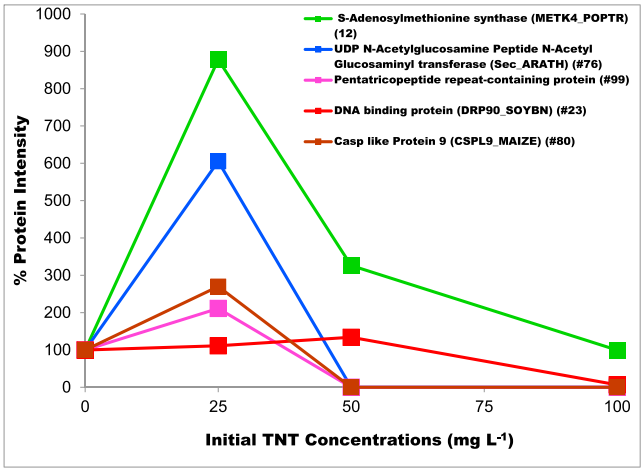

Figure 5. Identified proteins that showed upregulation at lower TNT treatments but downregulation at higher TNT concentrations. Relative intensity of protein spots extracted from TNT-treated root samples as compared to that of proteins extracted from root samples grown in TNT-free control vetiver plants expressed as percentage.

Two other proteins, namely, a protein matching DNA-binding protein DRP90 of soybean and a protein matching Casparian strip membrane domain protein (CASP)-like protein 9 of maize, show a similar trend, i.e., increased levels at 25 mg L^{-1} followed by a decline at higher TNT levels. DNA-binding protein DRP90 has motifs that strongly resemble functional motifs in other DNA-binding proteins. Casparian strip membrane domain proteins (CASPs) are four-membrane-span proteins involved in deposition of Casparian strips in the endodermis of plants by lignin polymerization [*Roppolo et al*., 2014]. CASP-like (CASPL) proteins, when ectopically expressed in the endodermis, were able to integrate the CASP membrane domain,

which suggests that CASPLs and CASPs perform the function of forming transmembrane scaffolds. In Arabidopsis, CASPL is expressed in a variety of cells, such as trichomes, abscission zone cells, peripheral root cap cells, and xylem pole pericycle cells [*Roppolo et al*., 2014]. CASPLs and CASPs likely perform similar functions, i.e., form membrane scaffolds and recruit cell wall-modifying enzymes, resulting in cell wall thickening in the root.

Predicted functional partners of the above proteins that are upregulated at low TNT concentration, followed by downregulation at higher TNT concentrations, were investigated by using the String database (Figures S6 and S7). These proteins have been either predicted to or experimentally found to interact with proteins related to vitamin B12-independent methionine synthase, homocysteine S-methyl transferase, serine/threonine protein phosphatase I, ubiquitin carboxyl-terminal hydrolase L5, and vacuolar protein sorting (VPS) 41. The enzymes methionine synthase and homocysteine S- methyl transferase synthesize or regenerate methionine and have been reported to be induced as well as downregulated under stress in various reports [*Narita et al*., 2004; *Sengupta et al*., 2011]. Increased levels of these enzymes may be useful in maintaining methionine levels, since it plays an important role in plant development.

Serine/threonine protein phosphatases are involved in regulation of various pathways during stress. Similarly, ubiquitin carboxyl-terminal hydrolase is involved in recycling of ubiquitin from small ubiquitinated peptides, which is implicated to play a role in various abiotic stress responses as well as in viral infection in plants. VPS41 is part of a group of proteins that function in vacuolar trafficking by facilitating vacuolar fusion.

To the best of our knowledge, the current study is the first attempt that reported the proteomic profiling of plant system under TNT stress. Exposure to varying levels of TNT affected the growth and the total soluble protein content in the root of vetiver grass. Proteomic profiling and functional annotation analysis of the root proteins that exhibited a minimum two fold change revealed that TNT stress majorly affects the key cellular pathways such as transcription, ribosome biogenesis, nucleocytoplasmic transport of protein, and protein glycosylation pathway. Downregulation of growth-related proteins was observed, which correlates with plant growth inhibition observed under TNT stress. Ethylene signaling pathway and plant defense-related proteins were initially upregulated at lower TNT treatments, possibly aiding the plant with tolerance to TNT stress. However, at higher concentrations, downregulation of these proteins probably contributes to phytotoxic symptoms in response to TNT. This study provides new insights into vetiver's response to TNT-induced stress. Ongoing studies in our laboratory on comparative evaluation of proteomic and metabolomic data in vetiver and a susceptible plant will help further illustrate the basis of vetiver's unique response to abiotic stress.

Acknowledgments
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