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Full Length Article

Physiological Response and Comparative Proteomic Analysis of Tobacco Seedling Roots to NH_4^+

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Abstract

Plants absorb nitrogen, an essential element for their growth and development, mainly in the form of nitrate or ammonium. These nitrogen species have different effects on crops, little is known about their precise influence at both physiological and proteomic levels. This study investigated these mechanisms, hydroponically grown tobacco seedlings treated with three different ratios of NO_3^- to NH_4^+ (10:0, 5:5 or 0:10) for 15 days. The results showed that exposure to high NH_4^+ concentration affected root morphology and inhibited root development. Moreover, malondialdehyde content and the activity of glutamine synthetase, superoxide dismutase, peroxidase and glutathione S-transferase increased with the concentration of NH_4^+ in solution. Two-dimensional protein gel analysis showed that 40 protein were differentially expressed between the three treatments. 36 of these proteins were identified using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Gene ontology analysis indicated that these proteins were involved in metabolism, energy production, cell defense or signal transduction or structural constituents related pathways. Our results suggest that tobacco seedling roots are subjected to NH_4^+ toxicity that triggers oxidative stress. Our results help understanding the responses to different nitrogen species and to NH_4^+ toxicity in roots. © 2017 Friends Science Publishers

Keywords: Nitrogen species; Proteomics; 2-ED; Roots; Tobacco
Abbreviations: GS, glutamine synthetase; SOD, superoxide dismutase; POD, peroxidase; GST, glutathione S-transferase; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; 2-ED, two-dimensional gel electrophoresis; NO_3^-, nitrate; NH_4^+, ammonium; MDA, malondialdehyde; NR, nitrate reductase; MS, mass spectrometry; ANOVA, analysis of variance

Introduction

Nitrogen is an essential element for plant growth and development not only included in many biological molecules but also involved in the regulation of metabolic processes such as nitrogen assimilation, cation-anion balance or photosynthesis (Siddiqi *et al.,* 2002). Plants absorb nitrogen from the soil mainly in the form of NO_3^- and NH_4^+. Previous studies indicate that these nitrogen species have a different influence on plant physiological processes, growth, development and in turn on plant morphology and biomass (Tschoep *et al.,* 2009).

Due to the differences in the mechanisms of nitrogen absorption, transportation and assimilation in different plants, most crops preferentially absorb NO_3^- (Miller and Cramer, 2005). When NH_4^+ as the sole nitrogen source only a few crops are suitable for growth, such as rice and conifers (Britto and Kronzucker, 2004), On the contrary, the majority of other crops display growth inhibition, reduced yield and even symptoms of toxicity, such as leaf chlorosis and a decrease in net photosynthetic rate. This is because NH_4^+ as the sole nitrogen source may result in plant toxicity (Kotsiras *et al.,* 2005).

Combined application of NO_3^- and NH_4^+ can alleviate or eliminate symptoms of NH_4^+ poisoning in many plants (Tabatabaei *et al.,* 2008), especially under abiotic stress such as salinity, low temperature or heavy metal (Matraszek, 2008; Tang *et al.,* 2011). (Garnica *et al.,* 2009) considered that this synergistic effects is closely related to changes in the endogenous content of polyamines and ethylene, while other studies proposed that this might be dependent on root cytokinin or on nitrogen uptake and assimilation (Debouba *et al.,* 2007). Thus far, previous studies investigated the effect of both NO_3^- and NH_4^+ on crops such as lettuce (El-Nemr *et al.,* 2012), rice (Silber *et al.,* 2004) and so on. However, these are mainly based on morphological, physiological or biochemical evidence. To the best of our knowledge, there are no studies on root responses to different nitrogen species at the proteomic level.

Comparative proteomics provides an ideal method for identification and analysis of proteins differentially

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expressed under biotic and abiotic stress (Xie *et al.,* 2014). In recent years, several new technologies allowed the analysis of differentially expressed proteins, such as microarray, isotope-coded affinity tag (ICAT) and isobaric tag for relative and absolute quantification (iTRAQ). However, 2-DE remains the most widely used method for comparative proteomics (Jagadish *et al.,* 2009). Additionally, tobacco is an important model organism, widely used for study on plant diseases control, miRNA function (Burklew *et al.,* 2014) and gene function (Jia *et al.,* 2016) among the others. Here, tobacco was used to investigate physiological changes and differential protein expression profiles in roots in response to NH_4^+ using 2-DE coupled with MS and bioinformatic techniques. Our analysis will be helpful to further understand the influence of nitrogen species on root development in crops.

Materials and Methods

Plant Cultivation

A hydroponic experiment was conducted in greenhouse at Hunan Agriculture University, Changsha, China. Plants were with a modified version of the method of literature (Xie *et al., 2*014). Briefly, seeds of *Nicotiana tabacum* cultivar k326 were surface sterilized with 5% H_2O_2 for 15 min, rinsed thoroughly with distilled water, soaked with deionized water for 1 day at 25°C and germinated in sterilized moist vermiculite in a greenhouse with a 12/12 h day/night photoperiod (light intensity of 450 Me m^{-2} s^{-1}), a 28/23°C light/dark temperature regime and 70% relative humidity.

After germination and at five-leaf stage, uniform seedlings were transplanted into 5 L rectangular plastic pot (37×23×10 cm). Each pot was covered with polystyrol plates with 15 evenly distributed holes (1 plant per hole). After transplanting, seedlings were precultured for 5 days with a half-strength Standardized Hoagland's solution (Hoagland, 1950) and then divided into three treatment groups on the basis of NO_3^- to NH_4^+ ratios (10:0, 5:5 or 0:10) with a constant total nitrogen concentration (5 mM). Nitrogen was supplied with either KNO_3 or $(NH_4)_2SO_4$. The concentration of potassium was maintained at the same level in all treatments using K_2SO_4, and other nutrient elements and their contents of nutrient solution applied were formulated according to Standardized Hoagland's solution. Moreover, the nutrient solution was renewed completely every 3 days. Every treatment was repeated three times.

Determination of Root Biomass and Morphology

After 15 d of treatment, 15 tobacco seedlings with uniform growth were harvested and washed clean with distilled water before separating shoots and roots. Root fresh weight was immediately determined. Root dry weigh was drying at 75°C in drying oven. The fresh root morphology was scanned with EPSON Perfection V700 Photo and their pictures were analyzed with WinRHIZO software (Regent Instruments Ltd, Ontario, Canada).

Determination of the Content of NO_3^-, NH_4^+, MDA and Soluble Protein

NO_3^- content in root was determined by the salicylic acid sulfuric acid method (Li *et al.,* 2000), while NH_4^+content was measured by ninhydrin and ascorbic acid respectively with UV-visible spectrophotometer (UV-2000, Shanghai, China) (Li *et al.,* 2000). Based on the chemical reaction of MDA and thiobarbituric acid the MDA content was measured by commercial MDA assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The soluble protein content in root was determined by the Coomassie Blue method taking bovine serum albumin (sigma) as standard (Bradford, 1976).

Determination of the Activity of NR, GS, SOD and POD

NR, GS was extracted and NR activity was determined referred to literature (Horchani *et al*., 2010). GS activity was measured by using hydroxylamine as substrate and quantifying the formation of γ-glutamyl hydroxamate (γ-GHM) (Wallsgrove *et al.,* 1979).

SOD activity was quantified using the nitroblue tetrazolium (NBT) method and POD activity was measured using the guaiacol method according to reference (Li *et al*., 2000).

Protein Extraction, 2-DE and Image Analysis

Total protein extraction was performed using phenol and methanolic ammonium acetate precipitation (Hurkman and Tanaka, 1988). Protein concentration was measured using the Bradford method (Bradford, 1976). 2-DE was performed as described by literature (Sharifi *et al*., 2012). Briefly, IPG strips (pH 4–7, 24 cm length, Bio-Rad) were rehydrated at room temperature overnight in immobile DryStrip Reswelling tray (GE healthcare). Isoelectric focusing (IEF) was conducted using the Ettan IPGphor3 (GE Healthcare) and the 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using an Ettan DALT six electrophoresis unit (GE Healthcar). 100 μg and 1200 μg protein extract were loaded in-gel rehydratation used for analytical and preparative gels, respectively. Analytical gels were stained suing silver nitrate, preparative gels with Coomassie Brilliant Blue G-250. 2-DE analyses were carried out triple replicate for each treatment.

In order to analyze protein spots, PowerLook1100 scanner (UMAX) was used to scan the silver-stained gels and then the GE HealthCare Software (Amersham Biosciences) was employed to analyze the images. Only spots with significant (ANOVA, $p<0.05$) increases of more than 1.5-fold in abundance were considered as

differentially expressed protein spots and were chosen as target protein spots for mass spectrometric analysis.

Protein Identification and Functional Classification

Target protein spots were manually excised from gels stained with Coomassie Brilliant Blue G-250 stained and digested with trypsin (Gharechahi *et al.,* 2013). Extracted peptides were analyzed with tandem MS (ABI 4800 MALDI-TOF-TOF proteomics analyzer, USA). Data explorer software version 4.5 (Applied Biosystems) was used to analyze data in MASCOT version 2.1 (Matrix Science, London, U.K) and against the NCBInr database. Proteins successfully identified were categorized by searching against Uniprot knowledgebase (http://www.uniprot.org/), NCBI database (https://www.ncbi.nlm.nih.gov/) and Gene Ontology (Go) (http://www.geneontology.org/) on the basis of their molecular function, biological processes and the metabolic pathways.

Statistical Analysis

The data are given as mean ± standard error (SE) of three replicates. ANOVA was performed using SPSS (21.0) and Microsoft Excel.

Results

Changes in Root Biomass and Morphology

With the increase of NH_4^+ concentration in solution, the decrease in fresh mass, dry mass, total length and total volume was observed ($p \leq 0.01$, Fig. 1 and Table 1). Root total surface area decreased ($p \leq 0.05$, Fig. 1 and Table 1) as well. Therefore, the results indicated that the presence of NH_4^+ inhibited the development of tobacco seedling roots.

Root Physiological Responses

With the increase of NH_4^+ concentration in solution, NH_4^+ content and GS activity of roots increased, on the contrary, NO_3^- content and NR activity decreased (Fig. 2A and B). ANOVA indicated a significant difference in values of NH_4^+ content and NO_3^- content ($P \leq 0.01$) between NO_3^- to NH_4^+ ratios of 10:0 to 0:10 and in values of GS activity and NR activity ($P \leq 0.01$) between NO_3^- to NH_4^+ ratios of 10:0 and 0:10.

The content of MDA and the activity of SOD and POD in roots increased with the increase of NH_4^+ concentration in solution (Fig. 2C and D). ANOVA indicated a significant difference in these values ($P \leq 0.05$) between NO_3^- to NH_4^+ ratios of 10:0 and 5:5 ($P \leq 0.05$), as well as between NO_3^- to NH_4^+ ratios of 5:5 and 0:10 ($P \leq 0.01$).

There wereno differences observed in soluble protein content in roots treated with different NO_3^-: NH_4^+ ratios of 10:0 and 5:5 ($P \geq 0.05$) (Fig. 2C). However, soluble protein content significantly decreased when NH_4^+ used as the sole nitrogen source in solution ($P \leq 0.01$) (Fig. 2C).

Root Proteins Responses to Nitrogen Species

The differences of protein expression of tobacco seedling roots exposed to different NO_3^- to NH_4^+ ratios were investigated using 2-ED coupled with MS. In total, approximately 960, 1123, 1171 spots were detected after treatments with the different NO_3^- to NH_4^+ ratios (Fig. 3). pI values and Mr of these protein spots ranged from 4 to 7 and from 10 to 170 kDa. The intensities of 40 protein spots had significant variations across treatments. 36 out of these 40 protein spots were successfully identified by tandem MS (Table 2).

Comparing samples from plants treated with a ratio of 0:10 to those treated with a NO_3^- to NH_4^+ ratio of 10:0, the intensity of seven spots (spots 2, 3, 5, 6, 8, 11 and 12) decreased (Table 2 and Fig. 4), while of seventeen spots (spots 14, 16, 17, 19–27, 30–33 and 35) increased (Table 2 and Fig. 4). The intensity of one spot (spot 13) (Table 2 and Fig. 4) increased in plants treated with a ratio of 5:5 and decreased in treated with a ratio of 0:10 contrasted treated with a ratio of 10:0. Four spots (spots 1, 4, 34 and 36) were not detectable in treated with a ratio of 5:5 and spots 9, 18 and 28 were presented only in 5:5, 0:10, 10:0 (NO_3^-: NH_4^+) treatments (Table 2 and Fig. 4), respectively. Spots 10 and 15 were not detectable in plants treated with a 10:0 NO_3^- to NH_4^+ ratio, while spots 7 and 29 were not visible in plants treated with a 0:10 NO_3^-: NH_4^+ ratio. 36 protein spots identified successfully were classified into six functional categories (Fig. 5): (1) metabolism (50.00%), (2) stress and defense (27.28%), (3) signal transduction (8.34%), (4) energy (5.56%), (5) structural constituent (5.56%), (6) unclear classification (2.78%).

Discussion

The existing literature suggests that excessive NH_4^+ induces stress in plants, leading to their inhibition of growth and development (Wang *et al.,* 2008a). Our research clearly indicated that an increase in the concentration of NH_4^+ in the hydroponic solution changed the morphology and reduced the biomass of tobacco seedling roots (Fig. 1 and Table 1). A similar phenomenon is also present in other crops such as cucumber, barley and bean (Yan *et al.,* 2013). ROS accumulation, resulting from exposition to biotic and abiotic stress, causes oxidative damage to DNA, lipids and other cellular components (Møller *et al.,* 2007). In order to deal with ROS-induced damage, plants deploy various protective enzymes such as SOD or POD (Mittler, 2002). MDA is the final product of lipid peroxidation induced by free radical oxidation (Chen *et al.,* 2003). SOD, POD and MDA are important physiological indexes for plant stress, as the level of SOD and POD activity and MDA content specifically reflected the degree of cellular damage (Huang *et al.,* 2007).

Table 1: Effect of different NO_3^- to NH_4^+ ratios on root biomass

NO_3^-: NH_4^+	Root fresh mass (g/plant)	Root dry mass (g/plant)	Total root Length (cm)	Total surface area (cm^2)	Total volume (cm^3)
10:0	1.44±0.039A	0.107±0.001A	410.06±19.45A	82.30±2.03Aa	1.90±0.03A
5:5	1.19±0.013B	0.078±0.001B	276.59±6.77B	73.34±1.26Ab	1.14±0.07B
0:10	0.71±0.017C	0.058±0.002C	190.18±10.78C	41.31±1.60Bc	0.72±0.01C

Note: Date are presented as mean ± SE (n=3) and different capital, small letters in the same column indicate significant differences between treatments at $p \leq 0.01$, or $p \leq 0.05$ (Duncan test), respectively

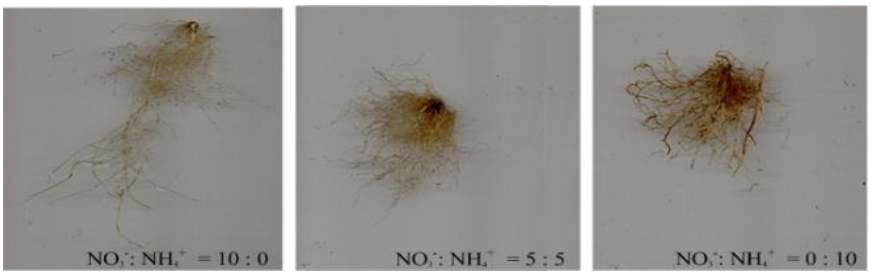

Fig. 1: Morphological changes in tobacco seedling roots exposed to different NO_3^- to NH_4^+ ratios 15 days after treatments

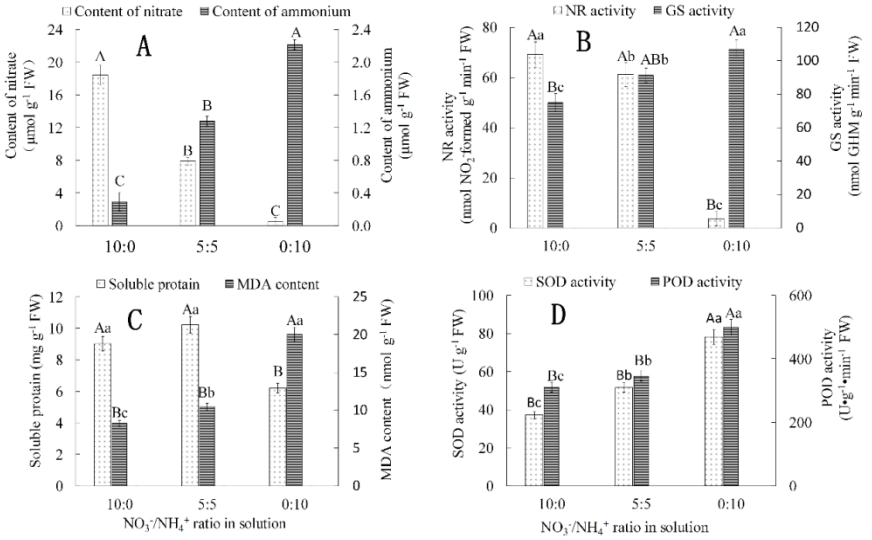

Fig. 2: Effects of different NO_3^- to NH_4^+ ratios on the content of NO_3^-, NH_4^+, MDA, soluble protein and on the activity of NR, GS, SOD, POD in roots. Bars represent means ± SE (n = 3). Different capital, small letters indicate significant differences between treatments at $p \leq 0.01$ or $p \leq 0.05$ (Duncan test), respectively

The results of present study suggested that NH_4^+ increase SOD and POD activity and MDA content of tobacco seedling roots (Fig. 2). These are consistent with previous reports (Wang *et al.,* 2009). In addition, high NH_4^+ concentration decreased soluble protein content, likely due to ROS-dependent protein degradation or fragmentation (John *et al.,* 2008). Similar findings have been reported in *Potamogeton crispus* (Yin *et al.,* 2016) and in *Egeria densa* (Su *et al.,* 2012).

GS is not only a key enzyme for NH_4^+ assimilation in the GS/glutamate synthase (GOGAT) pathway (Skopelitis and Nikolaos, 2006) but also participates in the synthesis of glutathione, a compound with antioxidant function and in signal transduction (Kamachi *et al.,* 1991). Our results clearly suggested that GS activity in roots increased with the increase of NH_4^+ concentration in solution, in agreement with previous reports (Horchani *et al.,* 2010). Therefore, it showed that NH_4^+ was toxic to the growth and development of the tobacco seedling roots.

About 50% of all identified proteins their levels were regulated by NH_4^+ are involved in metabolism, which indicates that the metabolism of tobacco seedling roots was highly affected in this study.

Enolase (spot 21 and 35) (Table 2 and Fig. 4) also referred to as phosphopyruvate hydratase and pyruvate dehydrogenase E1 component subunit beta-1 (spot 25), a component of the pyruvate dehydrogenase complex, take part in glycolysis and TCA cycle, respectively, two of the main energy metabolisms. The abundance of these two proteins in this study increased when NH_4^+ was the sole nitrogen. This is because the high concentration of NH_4^+ caused abiotic stress on tobacco seedlings.

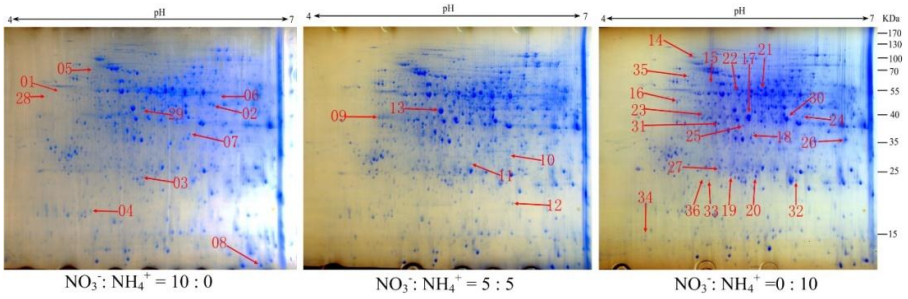

Fig. 3: 2-ED of total protein of tobacco seedling roots exposed to different NO_3^- to NH_4^+ ratios 15 days after treatments. Proteins (100 µg) were separated on 24 cm IPG strips in pI range from 4 to 7 in the first dimension and by 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gels in the second dimension. Gels were stained with silver nitrate

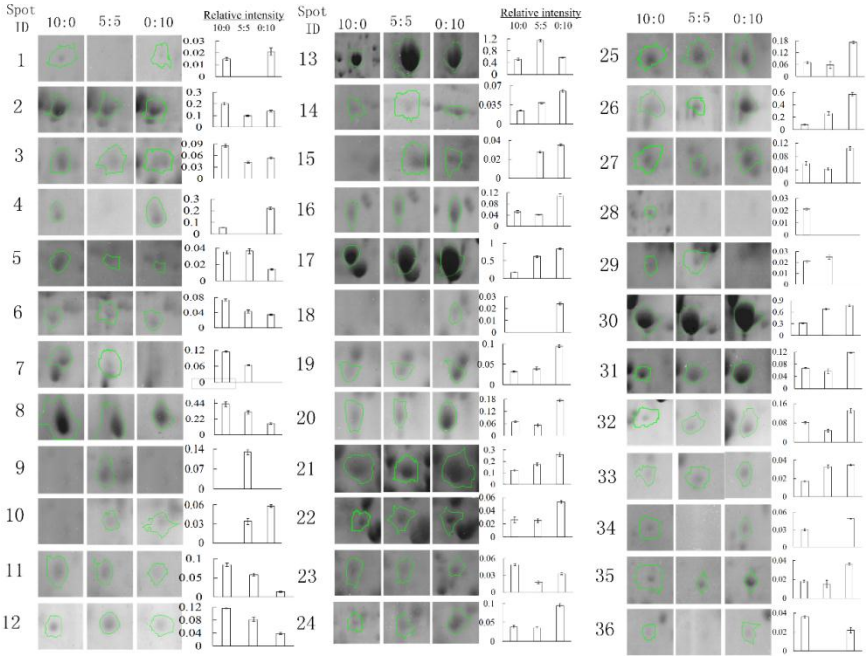

Fig.4: Magnified views and relative expression of differentially expressed protein spots. Bar graph indicates the relative expression of three biologic replicates (means ± SE). Spot are numbered as those presented in Fig. 3 and Table 2

Additionally, previous research also indicates that enolase content in maize leaf and tomato root increases under low nitrogen stress and the pyruvate dehydrogenase content in rice leaves increases under heat stress (Lee *et al.*, 2007). This indicates that enolase are widely involved in plant responses to stress.

Hydroxymethylglutaryl-CoA (HMG-CoA) synthase (spot 6) (Table 2 and Fig. 4) participates in phenylpropanoid biosynthesis and its derivatives are involved in cell wall strengthening and cell division (Mader and Hanke, 1997). Furthermore, low HMG-CoA synthase levels may lead to misregulation of growth, development and respiration (Posé *et al.*, 2009). Our result showed that high NH_4^+ concentration in the growing medium led to a decrease in the abundance of HMG-CoA synthase, implying that NH_4^+ toxicity might block the biosynthesis of cell wall components in roots. This may also explain the inhibition of growth and development of tobacco seedling root.

The plant produce protective enzymes such as SOD and POD to scavenge ROS as a response to biotic and abiotic stresses (Apel and Hirt, 2004). Additionally, GST is also an important antioxidant. In this study, the abundance of GST (spot 19, 20 and 32) (Table 2 and Fig. 4) was the highest when NH_4^+ was the sole nitrogen source in solution, implying that GST may play a key role in the resistance to NH_4^+ toxicity, as previously reported for plants under drought stress (Faghani *et al.*, 2015), salinity stress (Wei *et al.*, 2010), cold stress (Badowiec and Weidner, 2014) or pathogen attack (Han *et al.*, 2016). This suggests that GST is widely involved in plant responses to biotic and abiotic stresses.

Table 2: Differentially expressed proteins in root exposed to different NO_3^- to NH_4^+ ratios as identified by MALDI-TOF/TOF

Spot[a] ID.	Accession[b] No	Protein name[c]	protein annotation (Organism)	Functional[d] classes	Theo.Mr (kDa)/pI	Score[e]	Sequence coverage [f] (%)	Peptide[g] matched	Fold chang[h]	Fold chang[i]
1	Q56WF8	Serine carboxypeptidase-like 48	*Arabidopsis thaliana* (Mouse-ear cress)	Metabolism	56.921/4.98	173	26	15	Not found	
2	U3PJP9	GDP-mannose-3',5'-epimerase	*Nicotiana tabacum* (Common tobacco)	Stress and Defense	42.504/5.75	70	24	9	Down (-1.67)	
3	P24805	stem-specific protein TSJT1	*Nicotiana tabacum* (Common tobacco)	Metabolism	25.782/5.82	144	35	6	Down (-1.59)	
4	P13087	Miraculin	*Synsepalum dulcificum* (Miracle fruit) (*Richadella dulcifica*)	Metabolism	25.197/6.10	202	26	8	Not found	
5	gi|698554074	Glucose-6-phosphate isomerase 1	*Nicotiana sylvestris*	Metabolism	67.919/5.37	486	51	35		Down (-1.66)
6	B3F8H2	HMG-CoA synthase	*Nicotiana langsdorffii* x *Nicotiana sanderae* (Ornamental tobacco)	Metabolism	51.664/5.98	25	1	1		Down (-1.63)
7	P22195	Cationic peroxidase 1	*Arachis hypogaea* (Peanut)	Stress and Defense	35.285/5.90	150	58	14		Not found
8	C8YR32	Lipoxygenase homology domain-containing protein 1	*Mus musculus* (Mouse)	Metabolism	17.108/5.48	189	53	6		Down (-1.71)
9	Q9XJ56	Phosphoprotein ECPP44	*Daucus carota* (Wild carrot)	Stress and Defense	23.563/5.15	260	56	15	10^6	Not found
10	U3PVV5	EF1 protein	*Nicotiana tabacum* (Common tobacco)	Unclear classification	25.621/5.89	289	65	17	10^6	10^6
11	P27141	Carbonic anhydrase, chloroplastic	*Nicotiana tabacum* (Common tobacco)	Stress and Defense	29.928/5.61	304	68	20		Down (-4.93)
12	P27484	Glycine-rich protein 2	*Nicotiana sylvestris* (Wood tobacco) (South American tobacco)	Metabolism	20.493/5.93	377	62	9		Down(-1.58)
13	P53492	Actin-7	*Arabidopsis thaliana* (Mouse-ear cress)	Structural constituent	41.959/5.31	345	54	19	Up(+1.71)	
14	G9MD86	Heat shock protein 90	*Nicotiana tabacum* (Common tobacco)	Stress and Defense	96.213/4.89	119	18	15		Up(+1.88)
15	Q40577	5-epi-aristolochene synthase	*Nicotiana tabacum* (Common tobacco)	Metabolism	63.720/5.08	407	33	17	10^6	10^6
16	P12437	Suberization-associated anionic peroxidase	*Solanum tuberosum* (Potato)	Stress and Defense	38.967/5.37	50	2	1		Up(+1.71)
17	Q9SGY7	Putative proline-rich receptor-like protein kinase PERK11	*Arabidopsis thaliana* (Mouse-ear cress)	Signal transduction	26.442/7.67	41	7	1	Up(+2.85)	Up(+3.92)
18	Q9SAH9	Cinnamoyl-CoA reductase 2	*Arabidopsis thaliana* (Mouse-ear cress)	Metabolism	36.181/5.80	344	68	24	Not found	10^6
19	Q03662	Probable glutathione S-transferase	*Nicotiana tabacum* (Common tobacco)	Stress and Defense	25.102/5.27	259	42	11		Up(+2.12)
20	Q03666	Probable glutathione S-transferase	*Nicotiana tabacum* (Common tobacco)	Stress and Defense	26.000/5.67	186	33	13		Up(+2.00)
21	C5J0G6	Enolase	*Nicotiana tabacum* (Common tobacco)	Metabolism	48.083/5.40	167	26	10		Up(+1.70)
22	P17614	ATP synthase subunit beta, mitochondrial	*Nicotiana plumbaginifolia* (Leadwort-leaved tobacco) (Tex-Mex tobacco)	Energy	59.933/5.95	223	48	21		Up(+1.56)
23	P17614	ATP synthase subunit beta, mitochondrial	*Nicotiana plumbaginifolia* (Leadwort-leaved tobacco) (Tex-Mex tobacco)	Energy	59.933/5.95	188	32	12		Up(+2.09)
24	Q9FS87	IsovalerylCoA dehydrogenase, mitochondrial	*Solanum tuberosum* (Potato)	Metabolism	41.321/5.79	288	42	15		Up(+2.37)
25	Q6Z1G7	Pyruvate dehydrogenase E1 component subunit beta-1, mitochondrial	*Oryza sativa* subsp. japonica (Rice)	Metabolism	40.891/6.39	210	25	10		Up(+2.01)
26	P12437	Suberization-associated anionic peroxidase	*Solanum tuberosum* (Potato)	Stress and Defense	38.216/5.46	216	48	20		Up(+3.67)
27	P46209	carboxymethylenebutenolidase homolog	[Nicotiana sylvestris]	Metabolism	26.431/5.28	140	51	13		Up(+2.01)
28	gi|697179086	ubiquitin receptor RAD23b-like isoform X2	[*Nicotiana tomentosiformis*]	Metabolism	39.313/4.46	70	14	7	Not found	Not found
29	gi|697141200	3-isopropylmalate dehydrogenase 2, chloroplastic-like	[*Nicotiana tomentosiformis*]	Metabolism	37.863/5.62	165	21	9		Not found
30	GLNA5	Glutamine synthetase root isozyme 5	*Zea mays* (Maize)	Metabolism	39.520/5.52	32	3	1	Up(+1.90)	Up(+2.11)

Table 2: Continued

Table 2: Continued

31	T1WNC9	Jasmonate ZIM-domain protein 2b.2	*Nicotiana tabacum* (Common tobacco)	Signal transduction	36.192/8.88	25	6%	1		Up(+1.83)
32	GSTF1	Glutathione S-transferase PARB	*Nicotiana tabacum* (Common tobacco)	Stress and Defense	24.650/5.54	239	77%	22		Up(+1.67)
33	H9C954	Actin	*Nicotiana benthamiana*	Structural constituent	37.620/5.47	109	34	9	Up(+1.70)	Up(+1.72)
34	P27484	Glycine-rich protein 2	*Nicotiana sylvestris*	Metabolism	20.076/5.64	74	37	5	Not found	
35	C5J0G6	Enolase	*Nicotiana tabacum* (Common tobacco)	Metabolism	48.083/5.40	22	3	1		Up(+1.83)
36	gi|698499866	acid phosphatase 1-like	[*Nicotiana sylvestris*]	Signal transduction	30.555/5.35	97	33	9	Not found	

[a]Spot no. corresponds to the no. as presented in Fig.3 and Fig. 4
[b]Accession no: Accession number in NCBI database and Uniprot database
[c,d]Protein name and function was determined according to NCBI database and Uniprot database
[e]Score of Mascot Search Results
[f]Sequence coverage of the peptides matched against the translated sequence
[g]Number of peptides that matched with the identified protein in mass analyses
[h]Fold change was calculated as R = treatment 2 (the ratios of NO_3^- to NH_4^+ was 5:5)/ treatment 1 (the ratios of NO_3^-: NH_4^+ was 10:0)
[i]Fold change was calculated as R = treatment 3 (the ratios of NO_3^- to NH_4^+ was 0:10)/treatment 1 (the ratios of NO_3^- to NH_4^+ was 10:0)

Heat shock protein 90 (Hsp90) is involved in the folding and degradation of protein, signal transduction and many other cellular processes (Wandinger *et al.*, 2008). In the present study, one protein spot (spot 14) (Table 2 and Fig. 4) was identified as Hsp90 and its abundance increased steadily with the increase of NH_4^+ concentration in solution. Previous studies suggest that some chaperone proteins are present in cells at any time, others are expressed only under stress and show protective functions (Renaut *et al.*, 2006) and HSP 90 has a protective function during NH_4^+ stress as in present study.

In this study, spot 17 (Table 2 and Fig. 4) were identified as proline-rich receptor-like protein kinase PERK11 (AtPERK11), which belongs to the proline-rich, extensin-like receptor kinase (PERK) family that plays a fundamental role in signal transduction from the cell surface (Nakhamchik *et al.*, 2005). These results showed that the levels of PERK11 increased with the increase of NH_4^+ concentration in solution. Previous studies indicate that the PERK family is involved in the signal transduction in the defense from insects and pathogens (Silva and Goring, 2002). This implies that PERK family is also involved in the signal transduction induced by biotic and abiotic stresses.

In this study, spot 31 (Table 2 and Fig. 4) was identified as Jasmonate ZIM-domain protein 2b.2, belongs to Jasmonate ZIM-domain (JAZ) protein family, a key regulator of jasmonate (JA) signaling. Our result indicate that NH_4^+ as the sole nitrogen source sharply increased the abundance of this protein. This suggested that JA might be involved in signal transduction induced by NH_4^+ toxicity stress. JA was involved in the response to pathogen attack, wounding and ultraviolet radiation (Rakwal *et al.*, 2002). This indicates that Jasmonate, as a plant hormone, plays a role in the response against biotic and abiotic stresses (Wasternack, 2013). There is no doubt that the plant response to stress is an energy-consuming process. Plants accelerate their energy production to use defense-related pathways. ATP is the energy currency of the cell and it is synthesized by ATP synthase.

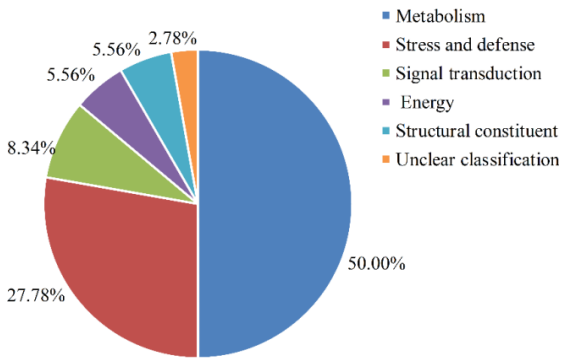

Fig. 5: Functional classification of the identified proteins

Our results identified two protein spots (spot 22 and 23) (Table 2 and Fig. 4) as ATP synthase subunit beta. The abundance of this protein was the highest when NH_4^+ was the sole nitrogen source in the growth medium. This indicated that ATP synthesis activity is enhanced, likely because plants need an increased energy production to deal with NH_4^+ toxicity. Similar observations have been reported in wheat (Wang *et al.*, 2008b) and potato (Aghaei *et al.*, 2008) under saline stress and Masson's pine under acid rain stress (Wang *et al.*, 2013), indicating that sustained ATP synthesis is a common strategy for as plants under stress.

Actin is a major component of microfilaments and cytoskeleton, participating in many biological functions including cell division, organelle movement and cell signaling transduction (Nazir *et al.*, 2016). Two protein spots (spot 13 and 33) (Table 2 and Fig. 4) in this study were identified as Actin-7 and Actin, respectively. The abundance of both proteins was higher when NH_4^+ present in the growth medium. Therefore, our result suggested that actin is involved in the response to NH_4^+ toxicity.

One protein spot (spot 10) (Table 2 and Fig. 4) was identified as EF1 but its biological functions are not recorded in detail in the Uniprot knowledgebase, NCBI or Gene Ontology (Go) database. Our results indicates that

EF1 was only expressed when NH_4^+ was present in the growth medium and that the abundance of this protein increased with the concentration of NH_4^+. More detail on the function of this protein may help us in understanding the mechanism behind the influence of different nitrogen species on the root development in tobacco seedlings.

Conclusion

Physiological analysis showed that using NO_3^- to NH_4^+ ratios ranging from 10:0 to 0:10 in solution, the morphology and development of roots was significantly changed with the increase in NH_4^+ concentration, which reflected in the decrease of root fresh mass, root dry mass, root total length and total volume. Moreover, NH_4^+ triggered oxidative stress and the antioxidant responses, as reflected by higher MDA content and enhanced activity of GS, SOD and POD in roots. Comparative proteomic analysis identified 36 proteins differentially expressed in different nitrogen form treatments. Gene ontology analysis of the 36 identified proteins suggested that those proteins involved in metabolism, energy production, cell defense and signal transduction pathways or that were structural constituents. Our results contribute to a better understanding of the physiological and molecular responses of tobacco seedling roots to NH_4^+ and provide an insight into the mechanisms underlying the tolerance to NH_4^+ toxicity.

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