Proteomic Response of Alfalfa Subjected to Aluminum (Al) Stress at Low pH Soil

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ABSTRACT

In order to reveal the aluminum (Al) stress tolerance mechanisms in alfalfa plant at low pH soil, a proteomic approach has been conducted. Alfalfa plants were exposed to Al stress for 5 days. The plant growth and total chlorophyll content are greatly affected by Al stress. The malondialdehyde (MDA) and H_2O_2 contents were increased in a low amount but free proline and soluble sugar contents, and the DPPH-radical scavenging activity were highly increased. These results indicate that antioxidant activity (DPPH activity) and osmoprotectants (proline and sugar) may involve in ROS (H_2O_2) homeostasis under Al stress. In proteomic analysis, over 500 protein spots were detected by 2-dimentional gel electrophoresis analysis. Total 17 Al stress-induced proteins were identified, of which 8 protein spots were up-regulated and 9 were down-regulated. The differential expression patterns of protein spots were selected and analyzed by the peptide mass fingerprinting (PMF) using MALDI-TOF MS analysis. Three protein spots corresponding to Rubisco were significantly down-regulated whereas peroxiredoxin and glutamine synthetase were up-regulated in response to Al stress. The different regulation patterns of identified proteins were involved in energy metabolism and antioxidant / ROS detoxification during Al stress in alfalfa. Taken together, these results provide new insight to understand the molecular mechanisms of alfalfa plant in terms of Al stress tolerance.

(**Key words :** Alfalfa, Aluminum stress, Proteome)

Ⅰ. INTRODUCTION

Aluminum (Al) is the third most abundant component that limits crop production on acid soils. Approximately 40% of world's total arable lands are acidic, and excessive Al containing soil may lose about 25~80% yield of various crop plants (Narasimhamoorthy et al., 2007). In acidic soil, when pH drops below five (<5.0), Al^{3+} is solubilized in to the soil which is extremely toxic to plant growth. Therefore, Al toxicity is one of the most serious agricultural problems for sustainable crop production. Al toxicity limits water and mineral nutrient uptake; it has been reported (Kochian et al., 2004), many potential sites are injured including cell wall, plasma membrane surface, the cytoskeleton, and the nucleus.

Proteomics is the most powerful tool that represents the study of the expression of all proteins in cells, organs or tissues (Wilkins et al., 1996). It has several advantages over other mRNA-based approaches to study cellular processes at the molecular level. It has been applied to the field of crop

abiotic stress-tolerance research for comparative analyses of different proteomes. Recent proteomic studies (Nunes-Nesi et al., 2014; Sha Valli Khan et al., 2014; Zheng et al., 2014) were conducted mostly on field based food crops but there have been few reports of forage legume proteome study under Al stress on acidic soil.

Stress response of alfalfa is important for model legume forage systems. Farmer's select the alfalfa due to high yielding forage quality and N_2 benefits for the soil. However, alfalfa growth and development is greatly affected by soil acidity and Al toxicity. Therefore, it is imperative to identify genes or enzymes those are involved in Al stress response under Al stress on acidic soil. In this study, we carried out an integrated physiological and proteomic analysis of alfalfa plant response to Al stress. Plants were exposed to Al stress and potential changes were observed at physiological and molecular level. The objective of this study was to identify potential proteins/genes in alfalfa that provide new insight for the plant improvement with enhanced Al stress tolerance.

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Ⅱ. MATERIALS AND METHODS

1. Plant growth and treatment

Alfalfa seedlings were grown in a growth chamber maintained at 25°C under white florescent light (80 μmol $m^{-1}s^{-1}$) with 16 h photoperiod. Soil pH was maintain and Al was treated according to method describe previously (Duressa et al., 2011). Three days old seedlings were transferred to soil pot and maintained the growth up to 2 weeks. The pH of the $AlCl_3$ was adjusted to 4.0 and pots were irrigated everyday. A group of seedling was exposed to Al (200 μM) and without Al (control) up to five days; leaves were collected and immediately frozen in liquid nitrogen and stored at −80°C until use. Treated plants (three independent repeats) were sampled at the same growth stage and used for the experiment.

2. Determination of shoot length, chlorophyll content, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content

The chlorophyll content was measured according to Lichtenthaler (1987). The MDA content of alfalfa leaf tissue was determined as described previously (Ezzine and Ghorbel, 2006). The H_2O_2 content was measured spectrophotometrically as described previously (Lin and Kao, 2001).

3. DPPH-radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) activity was measured by the method described earlier (Kang and Saltveit, 2001). The DPPH was used as a substrate to evaluate anti-oxidative activity of leaf extract. For this, 0.5 g of grinded leaf sample was taken in a falcon tube and homogenized in 5 ml of absolute ethanol. The homogenate was centrifuged at 12,000 g for 15 min, then 0.5 ml supernatant was taken and mixed with 0.25 ml DPPH (0.5 mM) in 0.5 ml acetate buffer (100 mM, pH 5.5). The supernatant was kept in room temperature for 30 min, and then absorbance was measured at A_{517} nm.

4. Analysis of free proline and soluble sugar

Free proline was estimated according to the method as described previously (Bates et al., 1973). Briefly, 0.5 g leaf tissue was homogenized in 10 ml of 3% sulfosalicylic acid then the homogenate was filtrated. The filtrate (2 ml) was treated with 2 ml acid ninhydrin and 2 ml of glacial acetic acid, and then the reaction mixture was extracted with 4 ml toluene. The absorbance of the chromophore was measured at A_{520} nm and compared to a toluene blank. The proline content was calculated using L-proline as a standard. Total soluble sugar content was measured according to previous method (Hansen and Moller, 1975).

5. Protein extraction, 2-DE PAGE, in-gel digestion and MALDI-TOF MS analysis

Proteins were extracted with Mg/NP-40 buffer essentially following the method described earlier (Hurkman and Tanaka, 1986). The protein content was quantified according to Lowry method (Lowry et al., 1951). Two-dimensional electrophoresis (2-DE) was performed as described previously (Lee et al., 2007), and selected spots were excised from the representative CBB-stained gel. After tryptic digestion, peptides were extracted according to the protocol described previously (Lee et al., 2007). Peptide samples were analyzed using a Voyager-DE STR MALDITOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). The peptide mass finger printings (PMFs) obtained from each digested protein were compared with PMFs in the non-redundant National Center for Biotechnology Information database using the profound program (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). Only significant hits, as defined by the ProFound 'expectation value' of 5e-2 (i.e., P≤0.05) were chosen for protein identification.

6. Statistical analyses

The results of the physiological parameters and spot intensities were statistically analyzed using on analysis of variance (ANOVA) or Student's t-test. The values were considered significant at the P≤0.05 level. All data were shown as means ± S.E. of at least three independent experiments.

Ⅲ. RESULTS AND DISCUSSION

1. Effect of Al stress on alfalfa growth and chlorophyll content

The shoot length of non-treated (control) alfalfa plants were increased that was approximately 1.2 fold compared to Al-treated plant (Fig. 1A). The total chlorophyll level was decreased in Al-treated plants that were about 0.8 fold relative to the control (Fig. 1B). Inhibition of shoot growth and chlorophyll level are typical symptoms of Al toxicity. Results in our study demonstrated that the adverse effects of Al on shoot elongation and chlorophyll content. The

toxic effect may cause due to distribution of Al ions in plant tissue. Report in earlier (Haider et al., 2007), soybean plant growth was severely inhibited at low pH by Al stress compared to Cd stress that supports to our findings.

2. Reactive oxygen species (ROS), lipid peroxidation and DPPH-activity under Al stress

The leaf MDA and H_2O_2 contents are the indication of cellular damage. After Al treatment, both MDA and H_2O_2 level were parallel that increased approximately 1.2 fold higher compared control plants (Fig. 2A and B). The MDA and H_2O_2 accumulation were increased but not in considerable level, possibly this is due to the activation of detoxifying enzyme (peroxiredoxin; spot 27). As a consequence of our results, we checked the DPPH-radical scavenging activity that was about 2.0 fold higher compared to control plant (Fig. 2C). High DPPH-activity is supported or an indication of ROS homeostasis in Al treated alfalfa plant. According to previous study (Kang and Saltveit, 2001), DPPH-activity was significantly increased in rice plant under chill and heat stresses that enhanced stress tolerance.

3. Osmoregulation due to Al stress

The free proline content in Al-treated alfalfa leaf was increased approximately double compared to control (Fig. 2D). The accumulation of proline indicates that alfalfa seedling had the ability to regulate the osmotic under Al
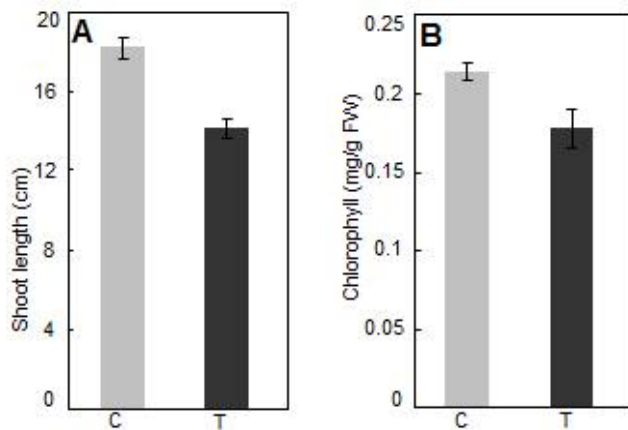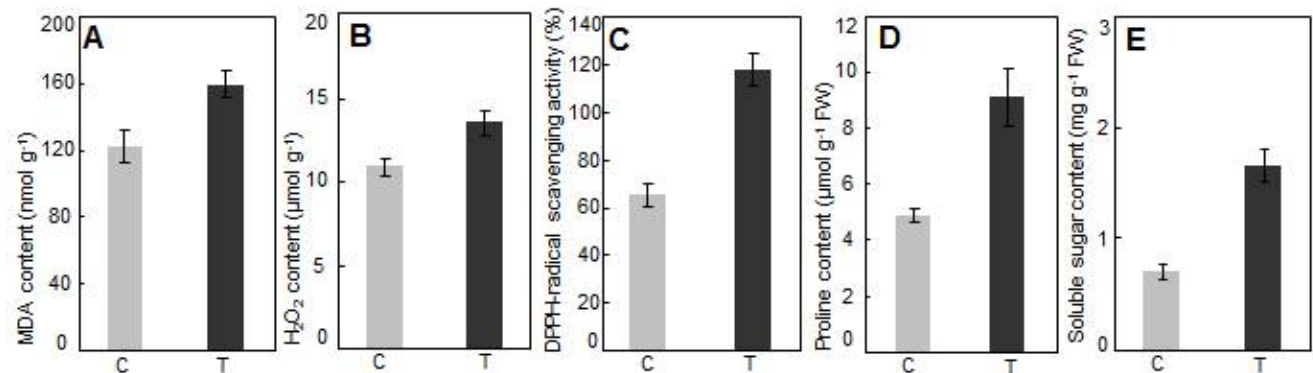

Fig. 1. Effects of Al stress on plant growth and pigment content. (A) Shoot length, (B) chlorophyll content of control (C), and Al-treated (T) plants. Data represent the mean values and SE of three independent experiments.

Fig. 2. Physiological responses of alfalfa leaf subjected to Al stress. (A) MDA, (B) H_2O_2, (C) DPPH-radical scavenging activity, (D) free proline accumulation, (E) soluble sugar in control (C), and Al treated (T) plants. Data represent the mean values and SE of three independent experiments.

stress. The soluble sugar content was also increased in Al-treated plant compared to control (Fig. 2E). Note, the proline content was parallel to the soluble sugar content. Previous study (Liu et al., 2006) suggests that soluble sugar and proline contents were increased in herbaceous plants in response to Al stress. Thus, high accumulation of sugar and proline in alfalfa leaf play an important role in osmoregulation under Al stress in alfalfa.

4. 2-D analysis of proteins

To investigate the Al stress response in alfalfa at molecular level, we analyzed the expression patterns of Al-responsive proteins using a proteomic approach. On CBB-stained 2-DE gels, over 500 protein spots were reproducibly detected. The representative 2-DE maps are presented (Fig. 3A and B). However, the expression levels of 48 proteins were altered by at least 1.5 fold after Al

treatment. Among these, 17 proteins were identified by MALDI-TOF MS (Table 1). Comparison of the differentially expression of proteins revealed that 8 proteins were up-regulated and 9 proteins were down-regulated (Fig. 4B). In the following section we discussed up-regulated and down-regulated proteins in the focus of previous molecular studies in various plant systems. Discussion on earlier studies with present results provides new insights of molecular mechanisms in alfalfa in response to Al stress.

4.1. Up-regulated proteins

In this study, peroxiredoxin (Fig. 4A, spot 27) was up-regulated under Al stress. Peroxiredoxin (Prx) is involved in ubiquitous family that catalyzes the reduction of hydrogen peroxide (H_2O_2). The up-regulation trend of Prx in response to Al stress indicated that it may protect oxidative injury of cell during stress conditions. Previous report (Vidigal et al., 2013), suggests that Prx genes are induced highly by

Table 1. Aluminum (Al)-induced proteins in alfalfa leaf identified by MALDI-TOF-MS analysis

Spot no.	Protein	Organism	Accession[a]	Mr/pI		SC[b]	PM[c]	Expect[d]
				Theoretical	Observed			
5 ↓	Beta adaptin-like	*Oryza sativa Japonica*	54290350	99.41/5.2	96/5.4	8	5	3.8e-3
6 ↓	DNA mismatch repair protein MutS2, putative	*Ricinus communis*	255556027	97.06/5.9	95/5.4	8	6	5.5e-3
7 ↓	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Arabidopsis thaliana*	1944432	48.02/6.1	50/6.0	11	5	7.5e-3
9 ↓	Predicted protein	*Micromonas* sp. RCC299	255074619	55.63/6.6	46/6.4	12	4	8.7-3
13 ↓	Predicted protein	*Micromonas* sp. RCC299	255074619	55.63/6.6	52/6.8	12	4	8.8e-3
17 ↓	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Arabidopsis thaliana*	1944432	53.45/5.9	50/6.2	12	5	1.1e-3
22 ↓	Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit, partial (chloroplast)	*Arabidopsis thaliana*	27752799	47.93/6.1	46/5.7	10	4	3.0e-3
23 ↑	RCD1	*Arabidopsis thaliana*	15232369	34.25/7.0	43/6.8	11	3	3.6e-3
24 ↑	F-box protein -like	*Brachypodium distachyon*	357161546	43.83/7.8	43/6.9	23	5	4.3e-3
27 ↑	Peroxiredoxin	*Medicago truncatula*	217071382	17.57/5.6	26/5.7	62	7	1.3e-3
29 ↑	Hypothetical protein SELMODRAFT_438024	*Selaginella moellendorffii*	302757633	29.11/8.7	24/6.7	20	3	2.0e-3
33 ↑	FKBP type peptidyl-prolyl cis-trans isomerase	*Pyrus communis*	18252321	17.75/5.1	18/5.6	13	3	5.5e-3
36 ↑	FKBP type peptidyl-prolyl cis-trans isomerase	*Pyrus communis*	18252321	17.75/5.1	17/5.4	13	3	4.7e-3
38 ↑	FKBP type peptidyl-prolyl cis-trans isomerase	*Pyrus communis*	18252321	17.75/5.1	17/5.8	13	3	1.6e-3
39 ↓	Seed prolamine	*Oryza sativa* Japonica	2827318	16.62/9.1	13/7.0	24	2	5.4e-3
41 ↑	Glutamine synthetase leaf isozyme chloroplastic	*Medicago sativa*	17367236	47.44/6.3	33/5.7	34	9	2.7e-3
48 ↓	CCR4-NOT transcription complex subunit 9	*Arabidopsis thaliana*	15232369	35.15/7.0	35/6.6	11	3	3.6e-3

[a] Accession number in NCBI database.
[b] SC, sequence coverage by PMF using MALDI-TOF MS.
[c] PM, number of peptides matched.
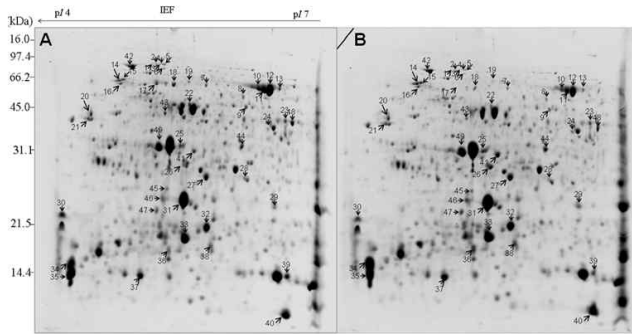[d] ProFound Expectation value; a value of <5e-2 indicates P <0.05.

Fig. 3. 2-DE analysis of alfalfa leaf proteins under Al stress. Total of 500 µg protein was separated by 2-DE and visualized with CBB staining. (A) The representative 2-DE map of control (C), and (B) Al treated (T) plants. Arrows indicate differentially expressed identified proteins in response to Al-stress.
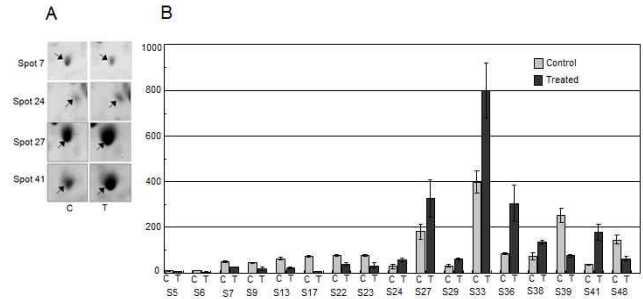

Fig. 4. Aluminum (Al) induced proteins and their relative expression level. (A) Close-up views of CBB-stained gels of the differentially expressed proteins marked at Fig. 3. (B) The expression levels of the identified proteins compared to controls (C) and Al treated (T) plants. Bars indicate the relative expression level of identified proteins.

several abiotic stresses including salt, cold, drought and extreme temperature. However, the common role of Prxs is to protect plants from oxidative damage and subsequent cellular damage.

Glutamine synthetase (Fig. 4A, spot 41) identified as a leaf chloroplastic enzyme. It plays a major role in nitrogen metabolism that catalyzes glutamate and ammonia to form the amino acid glutamine. It has been recommended that the assimilation of nitrate or nitrite as amino acids may serve as an alternative electron acceptor to oxygen in disposing of reducing power generated by glycolysis (Weger and Turpin, 1989). Along with the earlier evidence, up-regulation of glutamine synthetase might play an essential role in Al stress by contributing to H^+ homeostasis and maintaining osmotic potential in leaf.

The FKBP type peptidyl-prolyl cis-trans isomerase (PPIases; spots 33, 36, 38) belongs to the cyclophilins proteins family. In plants, the PPIase catalyses the isomerization of the peptide bond between a proline and the peptide residue. It has been evidenced that the cis-trans inter-conversion accelerated by PPIases is significant for the final protein structure (Dwivedi et al., 2003). In sorghum, PPIase activity was induced significantly in by drought stress (Sharma and Singh, 2003). To date, little is known about the function of PPIase under abiotic stress in plant system. However, additional experiments are needed to elucidate the specific role of PPIase in alfalfa leaf under toxic condition.

We identified F-box protein (spot 24) belongs to the member of ubiquitin protein family. Generally, F-box protein contains at least one F-box domain. In plants, F-box protein elucidates in gene networks that broadly regulated by microRNA-mediated gene silencing via RNA interference. It has been documented that the F-box protein play essential role in plant growth and development in Arabidopsis (Zhang et al., 2008). We proposed that the up-regulation patterns of F-box protein under Al stress may provide pivotal role in stress tolerance. This result supports to previous study of transcriptomic analysis in wheat in response to Al stress (Houde and Diallo, 2008).

The radical-induced cell death1 (RCD1; spot 23) identified as a key regulator of ROS- and abiotic stress responses in *Arabidopsis*. The up-regulation trend of RCD1 at protein level suggests ROS may induce in alfalfa leaf during Al stress. Our physiological data was supported to this statement (Fig. 2B). However, additional studies are needed to address the detail contribution of RCD1 the response to abiotic stress in plant system.

4.2. Down-regulated proteins

Nine proteins exhibited down-regulation patterns after exposure to Al stress (Fig. 4B, Table 1). In the present investigation, three proteins (spot 7, 17, and 22) corresponding to the large subunit of Rubisco was significantly down-regulated in response to Al stress. Similar to our investigation, down-regulation of Rubisco was documented in reed plants under Cd stress (Pietrini et al., 2003). This result suggests

that a degradation of Rubisco may be a due to cellular response of declined chlorophyll content and the net photosynthesis rate under oxidative stress.

DNA mismatch repair protein MutS2 (spot 6) identified as a key enzyme of DNA mismatch repair (MMR) system, it has been reported that MutS play an essential role to repair of oxidative DNA damage at stationary stage. According to earlier report (Fukui et al., 2011), the down-regulation of MutS2 indicates the damage DNA repair is affected during oxidative stress. In our investigation, spot 5 was identified as the β-adaptin, down-regulated expression of this enzyme was observed under Al stress. According to previous report (Manzano-León et al., 2006) the β-adaptin is a key molecule that induced under oxidative stress and act as microglial scavenger. The decline abundance of β-adaptin suggests that it may not able to maintain oxidative stress. However, additional studies are needed to address the essential role of MutS2 and β-adaptin in the response Al stress in plant system.

IV. CONCLUSION

This study provides better understanding of physiological, biochemical and proteomic responses of alfalfa leaf under Al stress. The chlorophyll content and plant's growth were affected by Al stress. Analysis of Al stress-responsive proteins revealed annotation of eight up-regulated and nine down-regulated proteins. The peroxiredoxin and glutamine synthetase leaf isozyme chloroplastic were greatly induced by Al stress in leaf, where as Rubisco large subunit was down-regulated. Different regulation of these leaf proteins involved in different cellular functions including energy metabolism, and antioxidant/detoxification processes. Results together provide molecular mechanisms for short term Al stress tolerance of alfalfa at low pH soil. Further research is needed to address the performance of alfalfa plant for long term Al stress tolerance.

V. ACKNOWLEDGEMENTS

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