Journal of the Korean Society of Grassland and Forage Science 42(3): 137~145, 2022
https://doi.org/10.5333/KGFS.2022.42.3.137
ISSN 2287-5824 (Print)
ISSN 2287-5832 (Online)

Research Article

Analysis of Aluminum Stress-induced Differentially Expressed Proteins in Alfalfa Roots Using Proteomic Approach

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ABSTRACT

Aluminum (Al) is one of the major factors adversely affects crop growth and productivity in acidic soils. In this study, the effect of Al on plants in soil was investigated by comparing the protein expression profiles of alfalfa roots exposed to Al stress treatment. Two-week-old alfalfa seedlings were exposed to Al stress treatment at pH 4.0. Total protein was extracted from alfalfa root tissue and analyzed by two-dimensional gel electrophoresis combined with MALDI-TOF/TOF mass spectrometry. A total of 45 proteins differentially expressed in Al stress-treated alfalfa root tissues were identified, of which 28 were up-regulated and 17 were down-regulated. Of the differentially expressed proteins, 7 representative proteins were further confirmed for transcript accumulation by RT-PCR analysis. The identified proteins were involved in several functional categories including disease/defense (24%), energy (22%), protein destination (9%), metabolism (7%), transcription (5%), secondary metabolism (4%), and ambiguous classification (29%). The identification of key candidate genes induced by Al in alfalfa roots will be useful to elucidate the molecular mechanisms of Al stress tolerance in alfalfa plants.

(Key words: Alfalfa root, Acidic soil, Aluminum stress, Forage, Proteome)

Ⅰ. INTRODUCTION

Aluminum (Al) is the third most abundant metal in the earth crust, and one of the major factors reducing most plant growth and productivity in acidic soils. Approximately, 30% of the world's land contains acidic soil, and more than 50% of the arable land is acidic (Uexkull and Mutert, 1995). In acidic soil, Al is solubilized into the toxic form Al^{3+}, which interferes with a number of cellular processes, and inhibits water and nutrient absorption in the roots of plants (Kochian et al., 2015). It is known that the most prominent symptom of Al toxicity is inhibition of root growth. Al inhibits the cell division and elongation of root tissue, and reduces the uptake of inorganic nutrients such as P, K, Mo, Ca, Mg and N (Riaz et al., 2018). Moreover, Al stress increases the production of reactive oxygen species (ROS), which cause oxidative damage to the cellular components (He et al., 2019). Antioxidant enzymes such as SOD, CAT, POD, GST, and GR are involved in the detoxification of the ROS generated by Al stress (Wei et al., 2021).

Several studies have been reported that various aluminum tolerance mechanisms so far, but they can be grouped into external exclusion mechanisms and internal tolerance mechanisms. (Wei et al., 2021). Plant secretes several organic acids such as citrate, malate, and oxalate from root tips under acidic soils, which help to plants to confer Al stress tolerance (Kochian et al., 2015). For instance, Al-activated malate transporters (ALMT) have been identified from barley (Gruber et al., 2010) and maize (Ligaba et al., 2012). In addition, MATE, multidrug transporters, have been identified from soybean (*Glycine max*) (Zhou et al., 2019) and maize (Maron et al., 2010). As internal tolerance mechanisms, that is involved in Al uptake, translocation, and detoxification (Li et al., 2014). Studies on the Al tolerance of plants have been actively carried out using molecular mapping with SSR and RFLP markers (Eujayl et al., 2004) and the development of transgenic plants using organic acids (Barone et al., 2008), transporter proteins (Magalhaes et al., 2007), and

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antioxidant-related genes (Ghanati et al., 2005). In addition, a number of proteomic studies to reveal the mechanisms Al tolerance in plants have been reported from plants such as rice (Yang et al., 2007), soybean (Zhen et al., 2007), and tomato (Zhou et al., 2009). Differentially expressed proteins after exposure to Al stress, of which were associated with oxidative stress, detoxification, organic acid metabolism (Zheng et al., 2014). However, a proteomic analysis of the root protein of alfalfa plants has not yet been reported.

Alfalfa is one of the most widely cultivated forage legume plants in the world. It is a perennial forage legume that contains high nutritive values, and has excellent nitrogen-fixing ability. In Korea, alfalfa is cultivated in a small scale, and mostly is imported from the USA or other countries. The soil structure and low soil pH in the mountainous grassland or cropland are the major constraints decreasing the productivity of alfalfa in Korea. Therefore, the development of alfalfa varieties tolerant to acidic soil along with the improvement of soil acidity by liming is important for improving the forage productivity and sustainability of mountainous grasslands in Korea. Therefore, understanding of Al-tolerance mechanisms is critical to generate Al-tolerant alfalfa variety in the future. In this study, we identified key proteins involved in Al stress response in alfalfa root tissues, which would be useful for the understanding of the Al stress tolerance mechanisms.

Ⅱ. MATERIALS AND METHODS

1. Plant growth and aluminum treatments

Alfalfa (*Medicago sativa.* cv. Vernal) seeds were surface sterilized using 70% ethanol following 30% (v/v) sodium hypochlorite solution, and washed three times with distilled water. Seeds were germinated in the dark for 3 days at 25℃ and then transferred to a hydroponic system containing Hoagland solution (pH 7.0) at 25℃ under white florescent light (480 μmol $m^{-1}s^{-1}$) with 16 h photoperiod. After 15 days, seedlings were treated with 0, 200, 800 and 2000 μM $AlCl_3$ (pH 4.0) for 3 days, and root samples were collected and stored at -80℃ for protein extraction.

2. Protein extraction, 2DE and gel image analysis

Proteins were extracted from alfalfa root tissue using a phenol extraction method according to the previous report (Lee et al., 2007). The two-dimensional gel electrophoresis (2-DE) was performed following the method of Lee et al (2007). After SDS-PAGE, The CBB-stained gels were scanned by scanner, the densitometric analysis of 2-DE gel spots were analyzed using PDQuest software (version 7.2; Bio-Rad, Hercules, CA, USA).

3. In-gel digestion and MALDI-TOF/TOF MS

The in-gel digestion and MALDI-TOF/TOF MS were performed according to the protocol described previously (Lee et al., 2007). Briefly, differentially expressed protein spots were manually excised from the CBB-stained gels and gel slices were alkylated, digested with trypsin, and the digested peptides were extracted and finally 1 μl of the homogenate was loaded onto the MALDI plate. Peptides were analyzed using a MALDI-TOF/TOF MS (SCIEX-4800 TOF/TOF Mass). The peptide mass fingerprintings (PMFs) obtained from each digested protein were compared with PMFs in the non-redundant National Center for Biotechnology Information (NCBI) database using the ProFound program. The search was performed within green plants (Viridiplantae) (Rahman et al., 2014).

4. RNA extraction and RT-PCR

Root samples were collected from alfalfa seedlings that had been treated with Al stress for 0, 6, 12, 24, 48 and 72 h. Total RNA was extracted from the root tissues using a Plant RNeasy mini kit (Qiagen, CA, USA). After isolation, total RNA samples were treated with RNase-free DNase I (Promega, U.K). To determine of quantitative expression of Al-responsive genes, RT-PCR amplification was performed using an RT-PCR kit (TOYOBO, Japan), in accordance with the manufacturer's instruction. The gene-specific primer sequences were used for RT-PCR are shown in Table 2.

5. Statistical analyses

The results of the gel spot intensities were statistically analyzed using on analysis of variance (ANOVA) or Student's t-test. The values were considered significant at the $p \leq 0.05$

level. All data were shown as means ± S.E. of at least three independent experiments.

Ⅲ. RESULTS AND DISCUSSION

1. Al stress-induced proteins in alfalfa roots tissues

To investigate the response of the differentially expressed proteins in alfalfa roots under Al stress, 3 days-old Al stress-treated alfalfa roots were used for proteome analysis. Total proteins were separated one-dimensionally using strips with isoelectric points p*I* 4 to 7, and separated two-dimensionally according to molecular weight. The 2-DE gel was observed with CBB R-250 staining and spots were analyzed by PDQuest software. About 1,200 proteins spots were found and were compared that the differences in expression of each protein spots between the Al-untreated control and Al-treated samples. It was confirmed that 49 protein spots were differently expressed in

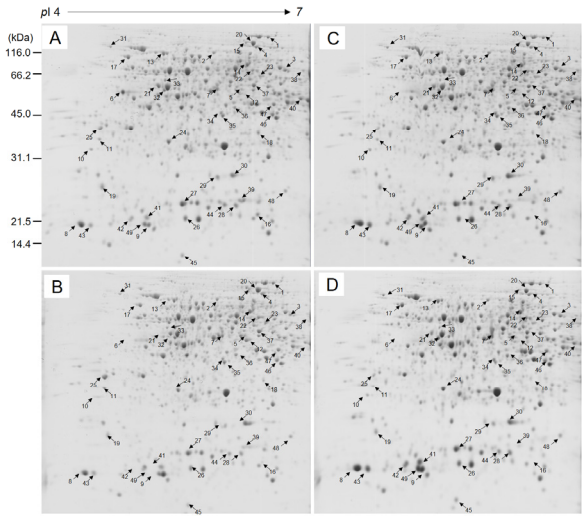

Fig. 1. Two-DE gel profiles of differentially expressed proteins in alfalfa roots after Al stress treatment. A, no Al with pH4.0 ; B, 200 uM Al with pH4.0; C, 800 mM Al with pH4.0; D, 2,000 mM Al with pH4.0.

Fig. 2. Expression levels of the Al stress-induced identified proteins in alfalfa roots under Al stress. Spot intensities were measured densitometer (PDQuest, Bio-Rad) and compared.

response to Al treatment (Fig. 1). These protein spots were excised from the 2-DE gels and digested with trypsin, and peptides were identified by PMF using MALDI-TOF/TOF MS. Finally 45 proteins were identified (Table 1). Among these, 28 were up-regulated, and 17 spots were down-regulated (Fig. 2). These results demonstrate that Al stress treatment under acidic (pH 4.0) condition is toxic to the root cells of alfalfa plants, which would induce expression of Al-responsive proteins in root cells.

2. Functional categorization of Al stress-induced root protein

Identified proteins were classified into the following categories including disease/defense (24%), energy (22%), protein destination (9%), metabolism (7%), transcription (5%), secondary metabolism (4%) and miscellaneous (29%) (Fig. 3).

2.1. Change in disease/defense-related proteins

Disease/defense-related protein spots, 7, 9, 12, 26, 34 and 41 were increased, and 3, 6, 39 and 45 were decreased. Spots 7 and 12 were identified as alcohol dehydrogenase, which is involved in acetaldehyde detoxification and anaerobic stress resistance (Dolferus et al., 1985). Superoxide dismutase (spot 26) plays a role of defense against ROS generated under stress conditions (Alscher et al., 2002). Isoflavonoids (spot 34) are important secondary metabolites produced primarily in leguminous plants. It plays important roles in plant defense (Wang et al., 2006). The glutathione reductase (spot 3) is an enzyme that catalyzes the conversion of glutathione disulfide (GSSG) to glutathione (GSH) by NADPH-dependent reduction. It is the essential reaction for the keep of glutathione levels. The major role of glutathione is reductant in oxidation-reduction processes, serves in detoxication, and great importance in several cellular functions (Carlberg and Mannervik, 1985). Dehydrin (spot 6) is a protein associated with ABA-related response, low temperature, and salinity stresses (Nylander et al., 2001). Glutathione peroxidase (spot 39) is an enzyme that protects cells from oxidative damage caused by ROS produced by stress (Rodriguez et al., 2003). Thioredoxin (spot 45) and glutathione are major regulators of redox homeostasis, and are essential for postembryonic meristematic activities (Bashandy et al., 2010). Most disease and defense-related genes are thought to play an important role in detoxifying toxins such as ROS.

Table 1. Aluminum stress-responsive proteins in alfalfa roots identified by MALDI-TOF/TOF MS analysis

Spot no.	Protein	Organism	Acc. NO.	Score	M.P	pI/Mr.	Expect	SC
1↓	Aconitate hydratase	*Medicago truncatula*	357483921	355	17	6.1/98329	1.10E-28	25
2↑	Succinate dehydrogenase	*Medicago truncatula*	357483399	158	12	6.1/68922	5.40E-09	25
3↓	cytosolic glutathione reductase	*Medicago truncatula*	388523103	434	13	6.3/53791	1.30E-36	30
4↓	Transketolase	*Medicago truncatula*	357469711	77	11	6.4/79850	0.64	26
5↑	12-oxophytodienoate reductase	*Medicago truncatula*	357480957	71	6	5.9/41193	2.9	13
6↓	Dehydrin	*Medicago truncatula*	357467405	100	9	5.5/23528	0.0035	27
7↑	Alcohol dehydrogenase	*Medicago truncatula*	357463695	224	13	5.9/41097	1.30E-15	29
8↓	Pathogenesis-related protein PR10	*Medicago truncatula*	357449105	460	9	4.6/16656	3.40E-39	59
9↑	harvest-induced protein	*Medicago truncatula*	283831548	435	11	5.1/16636	1.10E-36	54
10↑	unknown	*Medicago truncatula*	217075286	175	12	4.7/24288	1.10E-10	59
11↓	20S proteasome subunit alpha	*Medicago truncatula*	388510594	472	19	4.7/25978	2.10E-40	51
12↑	Alcohol dehydrogenase	*Medicago truncatula*	357463695	312	22	5.97/41097	2.10E-24	43
13↑	PREDICTED: V-type proton ATPase catalytic subunit A-like	*Cicer arietinum*	502149512	644	38	5.2/68688	1.30E-57	53
14↑	PREDICTED: probable mitochondrial-processing peptidase subunit beta-like	*Cicer arietinum*	502103363	609	28	6.2/59486	4.30E-54	32
15↓	Methionine synthase	*Medicago truncatula*	357508781	695	38	5.9/83185	1.10E-62	54

Spot no.	Protein	Organism	Acc. NO.	Score	M.P	pI/Mr.	Expect	SC
17↑	putative endomembrane protein precursor	*Medicago sativa*	166418	1090	41	4.9/57078	3.4E-102	72
18↓	Albumin-2	*Medicago truncatula*	357440455	136	10	6.2/24961	8.50E-07	37
19↓	unknown	*Medicago truncatula*	388515413	516	12	5.5/23014	8.50E-45	37
20↓	Methionine synthase	*Medicago truncatula*	357508781	1010	41	5.9/83185	3.40E-94	61
21↓	Actin	*Medicago truncatula*	357503463	859	24	5.6/40057	4.30E-79	50
22↑	Ribulose bisphosphate carboxylase large chain	*Medicago truncatula*	357471525	637	35	6.2/50317	6.70E-57	52
23↑	unknown	*Medicago truncatula*	388513787	462	18	7.0/36179	2.10E-39	56
24↑	ATP synthase	*Medicago truncatula*	357519665	260	17	7.7/27688	3.40E-19	37
25↑	general regulatory factor 2	*Medicago truncatula*	357489745	56	7	4.7/29209		30
26↑	Superoxide dismutase	*Medicago truncatula*	357512147	110	2	5.4/15226	0.00034	23
27↓	unknown	*Medicago truncatula*	388504476	227	6	5.3/17971	6.70E-16	41
28↑	MLP-like protein	*Medicago truncatula*	357515827	224	16	6.0/18227	1.3e-015	45
29↓	unknown	*Medicago truncatula*	388500632	138	2	5.1/13423	5.40E-07	20
30↑	Full=Thaumatin-like protein	*Medicago truncatula*	68064400	212	2	8.3/3221	2.10E-14	76
31↑	PREDICTED: stromal 70 kDa heat shock-related protein, chloroplastic	*Glycine max*	356541856	266	8	5.5/48021	8.50E-20	19
32↑	RecName: Full=Glutamine synthetase leaf isozyme, chloroplastic	*Medicago sativa*	17367236	326	13	6.3/47115	8.50E-26	36
33↓	Eukaryotic initiation factor 4A(dead-box atp-dependent RNA helicase)	*Medicago truncatula*	357449913	811	27	5.3/46789	2.70E-74	55
34↑	Isoflavone reductase-like protein(phenylcoumaran benzylic ether reductase-like protein Fi1)	*Medicago truncatula*	357483525	546	20	5.6/33818	8.50E-48	53
35↑	unknown	*Medicago truncatula*	217072106	452	21	5.7/35174	2.10E-38	47
36↑	malate dehydrogenase precursor	*Medicago sativa*	2827084	1220	26	8.1/43182	3.4E-115	55
37↑	RecName: Full=Isocitrate dehydrogenase [NADP], chloroplastic	*Medicago truncatula*	2497259	563	29	6.1/48383	1.70E-68	46
38↓	unknown	*Medicago truncatula*	217074764	322	22	7.2/59475	2.10E-25	40
39↓	Glutathione peroxidase	*Medicago sativa*	357520467	101	7	9.2/24730	0.0013	34
40↓	Fructose-bisphosphate aldolase	*Medicago truncatula*	357490465	633	22	5.8/78349	1.70E-56	37
41↑	Disease resistance response protein Pi49	*Medicago truncatula*	357449119	361	17	5.1/17204	2.7e-029	56
43↑	Pathogenesis-related protein PR10	*Medicago truncatula*	357449105	100	6	4.6/16656	0.0035	46
45↓	thioredoxin h	*Medicago sativa*	71534922	224	15	5.6/12802	1.3e-015	59
46↑	chalcone reductase	*Medicago sativa*	563536	209	20	6.2/34921	4.3e-014	47
47↑	Malate dehydrogenase	Fragaria vesca subsp. vesca	470120564	310	18	6.0/35603	3.4e-024	40
48↑	Nascent polypeptide-associated complex subunit beta	*Medicago truncatula*	355508046	72	10	5.7/16501	2	41

a) PM, number of peptides matched.
b) SC, sequence coverage by peptide mass fingerprinting using MALDI-TOF/TOF MS.
c) ACC. No., Aceeession number in NCBI database.
Arrows indicates that each protein spot was increased or decreased compare to the control.

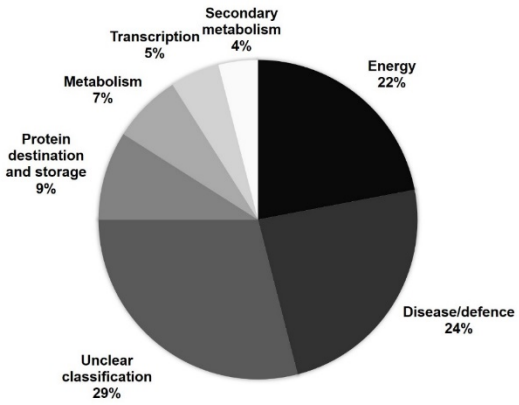

Fig. 3. Functional catalogue of Al stress-induced differentially expressed proteins in alfalfa root.

2.2. Change of energy-related proteins

Energy-related protein spots 2, 13, 14, 22, 24, 36, 37, and 47 were increased and spots 1, 4 and 40 were decreased. Aconitate hydratase (spot 1) is an enzyme that catalyzes the resolution of citric acid or isocitric acid into aconitic acid and water in the TCA cycle (Moeder et al., 2007). Plant aconitases are involved in resistance to oxidative stress, regulate gene expression regulating intracellular ROS levels, and regulate cell death (Moeder et al., 2007). Aconitate hydratase appears to protect the plant from ROS due to Al stress (Moeder et al., 2007). Ribulose bisphosphate carboxylase large chain (spot 22) catalyzes the Calvin cycle of carbon dioxide fixation (Andersson et al., 1989). Ribulose bisphosphate carboxylase large chain also catalyzes the initial oxygenation step. Malate dehydrogenase (spot 36 and 47) catalyzes the transform of oxaloacetate and malate. The main organic acids citrate, oxalate, malate are well known for directly deciphering Al toxicity (Tesfaye et al., 2001). Due to Al toxicity, alfalfa seemed to try to protect plants by producing organic acids by malate dehydrogenase to detect or detoxify it. NADP-dependent isocitrate dehydrogenase (spot 37) catalyzes the production of NADP-dependent isocitrate dehydrogenase enzyme, an essential component to maintain cell homeostasis. NADPH is a major cofactor for cellular enzymatic reactions, homeostasis of cellular redox essential in the biosynthetic pathway and detoxification processes (Leterrier et al., 2012). Therefore, NADP-dependent isocitrate dehydrogenase is thought to be helpful in detoxifying various stresses with Al stress.

2.3. Change in miscellaneous proteins

Several other protein spots also showed differential expression against Al stress treatment; spot 5, 17, 31, 32, 46 and 48, which were increased, and spots 11, 15, 18, 20, 21, 25 and 33, which were decreased. Glutamine synthetase (GS) (spot 32) is the major enzyme of primary nitrogen assimilation, ammonia reassimilation and detoxification (Hossain and Komatsu, 2013). Plant GS requires two magnesium ions for activity so GS can be a potential target of metal stress (Hossain and Komatsu, 2013). The increase in GS expression induces the formation of more glutathione (GSH). The increase in GSH synthesis is not only associated with high metal binding capacity, but also with an improved mechanism of cell defense against oxidative stress (Verbruggen et al., 2009). GSH is the precursor of phytochelatin (Hradilova et al., 2010) which binds to heavy metals including Al. Thus, alfalfa GS would have an important function against Al-induced oxidative stress. OPDA (12-oxophytodienoic acid, spot 5) is known as involved in the stress and defense responses in plants (Matsui et al., 2004). 20S proteasome (spot 11) is a proteolytic complex involved in abnormal cellular proteolysis (Fu et al., 1998).

2.4. Expression of key genes involved in Al stress response

Several representative proteins differentially expressed by Al stress treatment in alfalfa roots were selected for further expression analysis with transcriptional level. Among 45 identified proteins, 7 genes (alcohol dehydrogenase, PR protein, SOD, isoflavone reductase protein, general regulatory factor 2, proteasome, and GS) were chosen for mRNA expression analysis by RT-PCR. Transcripts of alcohol dehydrogenase, isoflavone reductase protein, general regulatory factor 2 and glutamine synthetase were increased in response to Al stress

Table 2. Gene specific primers were used for RT-PCR amplification

Gene Name	Sequence of PCR primer (5′ to 3′)	Gene Name	Sequence of PCR primer (5′ to 3′)
Alcohol dehydrogenase	F : TCCAGCCGTTTCGAATTAGCT R : ACCAGATTGAGAACATAGTCCCCT	Isoflavone reductase-like protein	F : GGCTCAACTCGACGTCACTG R : TCCGGGATGACAAACTTAGACA
General regulatory factor2	F : GCTGCTTCCGGTGATTCCAA R : GCAACAAGAACCTTCTTCCTTCA	Phathogenesis -related (PR) protein	F : CCCAAAGGTTATTGATGCCATCC R : TGGCAACCAAATCAAGCCCA
Superoxide dismutase	F : CCTGATGCCACCAAGGGTTC R : CCAATGTCGTGAACCCGCA	Proteasome	F : TTTGTGACCTAGCCTTGCGC R : CCCGCCAATGTGCCAAGTAT
Glutamine synthetase	F : CCCATTTAGCACGGAAGAAGCA R : GGTGTTGTGCTCACCCTTGA		

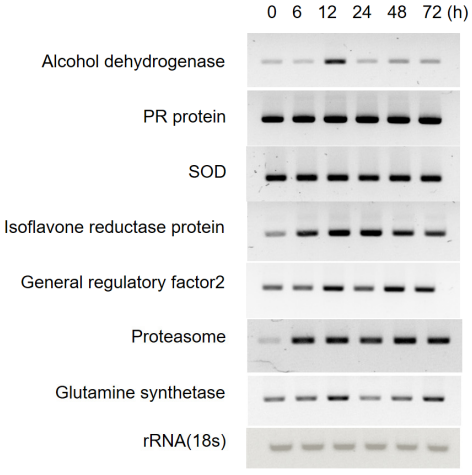

Fig. 4. Expression of Al-responsive genes by aluminum stress treatment at pH 4 with 200 μM Al.

treatment and showed similar expression pattern with protein expression (Fig. 4). On the other hand, the expression of proteasome, PR protein and SOD genes showed a different pattern from protein expression. These results suggest that the expression of each gene is differently regulated, such as transcriptional regulation or translational regulation, by Al stress treatment.

Ⅳ. CONCLUSION

In this research, data provided an understanding of proteomic responses of alfalfa root under Al stress conditions. Proteomic analysis of Al stress-responsive protein revealed that total 45 proteins were differentially expressed. Proteins were classified as disease/defense, energy, protein destination, metabolism, transcription, secondary metabolism, and unclear classification. These data indicate that most of altered proteins in the Al-stressed alfalfa root involved in different cellular functions under Al stress. Results provide better insights on adaptive mechanisms of alfalfa to Al stress under acidic soil. Further research is needed to define molecular function of each gene under Al stress.

Ⅴ. ACKNOWLEDGEMENT

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1D 1A1A01060495).

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(Received : September 14, 2022 | Revised : September 23, 2022 | Accepted : September 24, 2022)