Bangladesh J. Bot. **46**(4): 1241-1248, 2017 (December)

CHARACTERIZATION OF HORMONE LEVELS AND ACCUMULATION OF FREE AMINO ACIDS IN *CLPC1* AND *CLPC2 ARABIDOPSIS* HEYNH. IN HOLL & HEYNH. MUTANTS

MD SARAFAT ALI, DONG-WON BAE[1], JAE GYU PARK[2] AND KWANG-HYUN BAEK[3*]

Department of Biotechnology and Genetic Engineering, Bangabandhu Sheikh Mujibur Rahman Science & Technology University, Gopalgonj-8100, Bangladesh

Keywords: *ClpC1, ClpC2, Clp* protease, Free amino acids, Phytohormones

Abstract

ClpC1 and *ClpC2* comprise the chaperonic part of the Clp protease complex, which is the central protein degradation machinery in the plastid of a plant cell. In *Arabidopsis*, *ClpC1* and *ClpC2* shared 93% similarity with each other at the nucleotide sequence level. Mutation and dysfunction of *ClpC1* led to chlorosis and growth retardation phenotype, whereas *ClpC2* mutation did not show any phenotypic change relative to wild type. When the roles of *ClpC1* and *ClpC2* were investigated based on the presence of the T-DNA insertion mutant, significant alteration of the amount of IAA was detected in the *clpc1* and *clpc2* mutants, with no significant difference in the amounts of GA_3. Many free amino acids were significantly altered in *clpc1* mutants relative to wild type, whereas no changes in individual amino acids were found in *clpc2* mutants. In response to external application of NAA, GA_3 and BAP, *clpc1* mutant accumulated more free amino acids, while those of the wild type were unchanged. These findings provide new insight into the role of the chaperonic part of *ClpC1* in the *Clp* protease complex regulating the content of free amino acids via different amounts of hormone levels.

Introduction

The *Clp* protease is located in plastids as a special proteolytic system similar to the proteasome in the cytoplasm and nucleus. *Clp* protease, which is the principle constitutive house keeping protease in the stroma, is essential to plant viability. The structure of the *Clp* protease can be divided into two major components, a barrel-shaped tetradecameric protease core with catalytic sites sequestered inside the complex and a hexameric ring-like ATP-dependent chaperone. In *Arabidopsis thaliana*, the *Clp* protease is composed of more than 15 proteins, including three *Clp* AAA+ chaperones (*ClpC1*, *ClpC2* and *ClpD*), five serine type *Clp* proteolytic subunits (*ClpP1*, *ClpP3*, *ClpP4*, *ClpP5* and *ClpP6*), two core proteins (*ClpS1* and *ClpS2*), four non-proteolytic regulatory subunits (*ClpR1*, *ClpR2*, *ClpR3* and *ClpR4*) and two other proteins (*ClpT1* and *ClpT2*) (Adam *et al.* 2006).

As chaperonic parts in the *Clp* protease, *ClpC1* and *ClpC2* play important roles in protein quality control (Sjogren *et al.* 2014). Over expression of the *ClpC2* gene complements the *clpc1* mutant to the wild type of *Arabidopsis* (Kovacheva *et al.* 2007). A previous study reported that knock-out of the *ClpC2* gene by T-DNA insertion did not induce distinct phenotypic changes (Kovacheva *et al.* 2007), indicating that the role of *ClpC2* in *Clp* protease function is limited and can even be negligible. However, studies of mutant *clpc1* indicate important roles of the *ClpC1* protein (Sjogren *et al.* 2014) and of controlling chlorophyll b biosynthesis (Nakagawara *et al.* 2007). Moreover, a mutant line absent both *ClpC1* and *ClpC2* showed no embryogenesis or seed formation (Kovacheva *et al.* 2007).

[*]Author for correspondence: <khbaek@ynu.ac.kr>. [1]Central Instrument Facility, Gyeongsang National University, Jinju 52828, Korea. [2]Pohang Center for Evaluation Biomaterials (POCEB), Pohang Technopark Foundation, Gyeongbuk 37668, Korea. [3]Department of Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 38541, Korea.

Phytohormones are involved in reducing the adverse effects of stresses through modulation of physiological processes and biochemical mechanisms (Fatma *et al.* 2013). The role of phytohormones is critical to modulation of physiological responses that lead to adaptation of plants to unfavorable environments. Foliar application of GA_3 increased amino acids content, which counteracted some of the adverse effects of stresses by maintaining membrane permeability and increasing macro- and micronutrient levels (Tuna *et al.* 2008).

Free amino acids act as compatible solutes and accumulate in plants under unfavorable environmental conditions. The accumulation of compatible solutes is related to improvement of plant tolerance to various stresses because of its ability to overcome osmotic and water stress and maintain nutrient homeostasis and ion compartmentalization (Khan *et al.* 2012).

Previously, co-suppression of the chaperonic part *ClpC1* and *ClpC2* in the *Clp* protease resulted in cholorotic appearance of leaves with retarded growth in *Nicotiana benthamiana* (Ali *et al.* 2015). Analysis of free amino acids in co-suppressed leaves revealed accumulation of significantly high contents of free amino acids (Ali *et al.* 2015). These results indicate that the chaperonic part composed of *ClpC1* and *ClpC2* exerts significant roles in plant development and metabolome changes. However, no studies have investigated the roles of the each gene in the levels of hormones and free amino acids. Therefore, authors acquired *Arabidopsis* lines with mutated forms of *ClpC1* and *ClpC2* and analyzed the levels of hormones and free amino acids. Present authors also measured the differential accumulation of free amino acids in response to external hormone treatment of the *clpc1* and *clpc2* mutants. In this paper, we report the results of our studies conducted with *Arabidopsis* lines.

Materials and Methods

Arabidopsis ecotype Columbia-0 was used as the wild type. *Arabidopsis* mutant seeds of *ClpC1* (SALK_014058C) and *ClpC2* (SALK_011603C) with the T-DNA inserted in the *ClpC1* and *ClpC2* genes (Fig. 1a) were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH, USA). All seeds, which had previously been vernalized at 4°C for 72 hrs to break the dormancy, were sown in soil composed of 70% coco peat, 17% peat moss, 5% zeolite and 8% perlite in plastic pots. The plants were watered regularly and grown under fluorescent lights at 120 µ Einstein/m^2/s under a 16/8 hrs light/dark cycle at 22 ± 2°C in a controlled walk-in chamber.

Confirmation of the T-DNA insertion in the *ClpC1* was conducted by genomic PCR using a set of *ClpC1* specific primers. The T-DNA left border primer LBa1.3 (forward; 5'-ATTTTGCCGATTTCGGAACC-3') and the *ClpC1*-specific primer AraC1RP (reverse; 5'-CTTCGTTCATTCTCAACACATGC-3') that binds to the fourth intron of the *ClpC1* gene were used (Fig. 1b). The homozygous and heterozygous *clpc1* T-DNA insertion mutant lines were confirmed by genomic PCR using the *ClpC1*-specific primer AraC1LP (forward; 5'-CAACTTGGTAACTCCCTTCTCT-3'), which is designed to bind to the first exon of the *ClpC1* gene, and the AraC1RP primer. The same protocol was applied for confirmation of T-DNA insertion and homogeneity testing in the *clpc2* mutant under the same conditions using the forward primers, LBa1.3 or AraC2LP (5'-GAGATTGTGGCCAAGTAATTGAAG-3'), and the reverse primer, AraC2RP (5'-AATCGAAACGCGAATACACAAC-3').

Extraction and quantification of plant hormones were conducted according to the method described by Pan *et al.* (2010). To investigate the effects of exogenous hormones on amino acid accumulation in *clpc1* mutant, 5 µM NAA, 5 µM BAP and 100 µM GA_3 purchased from Duchefa Biochemie (Netherlands) were sprayed on 21-day-old *Arabidopsis* two times for one week. The leaves of *Arabidopsis* were analyzed for free amino acids and total protein at day 28 using the method described by Ali *et al.* (2015).

There were three replicates in all experiments, and they were each conducted three times. The data are presented as the mean ± standard deviation of the three replicates. Differences among groups were evaluated by ANOVA using the Statistical Analysis Software (SAS) version 9.2 (SAS Inc., Cary, USA).

Results and Discussion

The growth and development of *clpc1* and *clpc2* mutants generated by T-DNA insertion were analyzed. The *clpc1* mutant grew more slowly than the wild type and *clpc2* mutant. Additionally, the *clpc1* mutant was smaller than the wild type, while the *clpc2* mutant showed development very similar to the wild type (Fig. 1a). *ClpC1* expression was several-folds higher than that of *ClpC2* (Kovacheva *et al.* 2007); therefore, *ClpC1* gene expression in the *clpc2* mutant might exert a compensatory response for *ClpC2*, resulting in the phenotype of *clpc2* mutant being similar to that

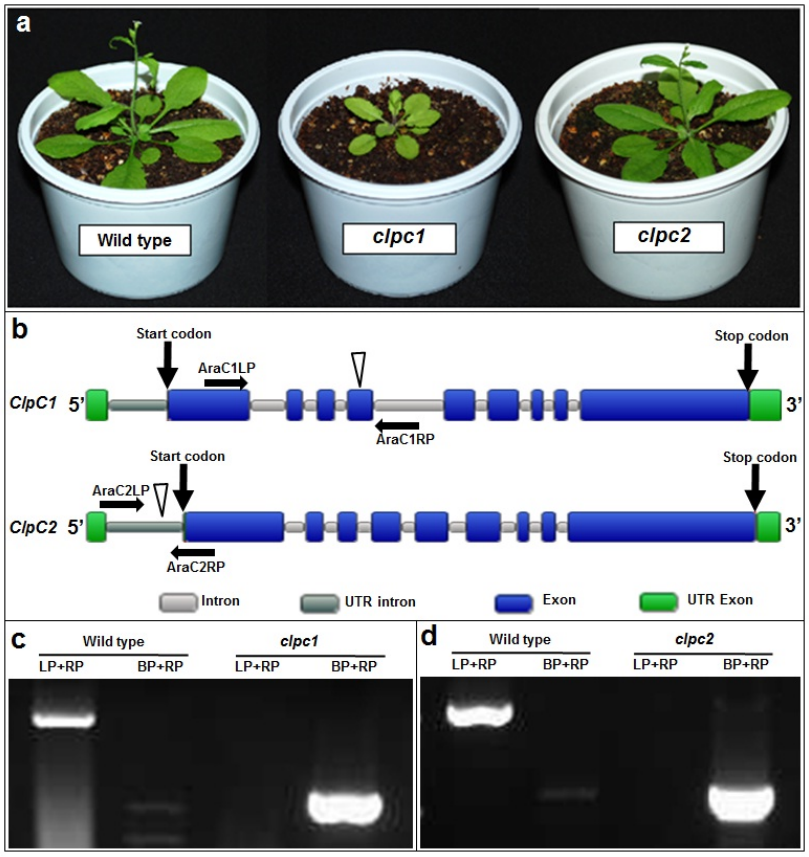

Fig. 1. Phenotypes and identification of homozygous mutant lines of *ClpC1* and *ClpC2*. (a) Phenotypes of wild type, *clpc1* and *clpc2* mutants. Photographs were taken at 27 days. (b) Schematic pictures of the genomic *ClpC1* and *ClpC2* genes in *Arabidopsis*, respectively. The triangle indicates T-DNA insertion sites for *clpc1* and *clpc2* mutants. (c) PCR analysis of the *ClpC1* gene to confirm T-DNA insertion and screening of homozygous mutant. (d) PCR analysis of *ClpC2* gene for confirmation of T-DNA insertion and screening of homozygous mutant. LP, left genomic primer. RP, right genomic primer. BP, T-DNA border primer.

of the wild type. Wild type and *clpc2* mutant started bolting earlier than the *clpc1* mutant, indicating that the *clpc1* mutant could have the advantage of a life cycle approximately two weeks longer than that of the wild type and *clpc2* mutant. Throughout the vegetative growth period, *clpc1* mutant exhibited a chlorotic appearance in various organs, including the influorescences, cauline leaves and siliques, as well as the vegetative leaves, suggesting that ClpC1 plays important roles in the development of chloroplasts and all organs throughout the life cycle.

Due to the unique phenotype of the *clpc* mutant, the content of IAA and GA_3 was analyzed (Fig. 2a, b). Auxin is primarily produced in the young expanding leaves of growing shoot apices and actively transported basipetally through the polar auxin transport stream; therefore, we expected the level of auxin in *clpc1* mutant to be lower than that in wild type and *clpc2* mutant. However, the IAA content of the *clpc1* mutant was significantly higher than those of the wild type, while these levels were significantly lower in the *clpc2* mutant than the wild type (Fig. 2a). An unknown mechanism resulted in higher levels of auxin in *clpc1* mutant, suggesting that undifferentiated leaves due to mutation in the *clpc1* gene might interrupt the transport of auxin to roots and cause accumulation of high levels of auxin. The GA_3 content did not differ significantly among wild type, *clpc1* and *clpc2* mutants (Fig. 2b).

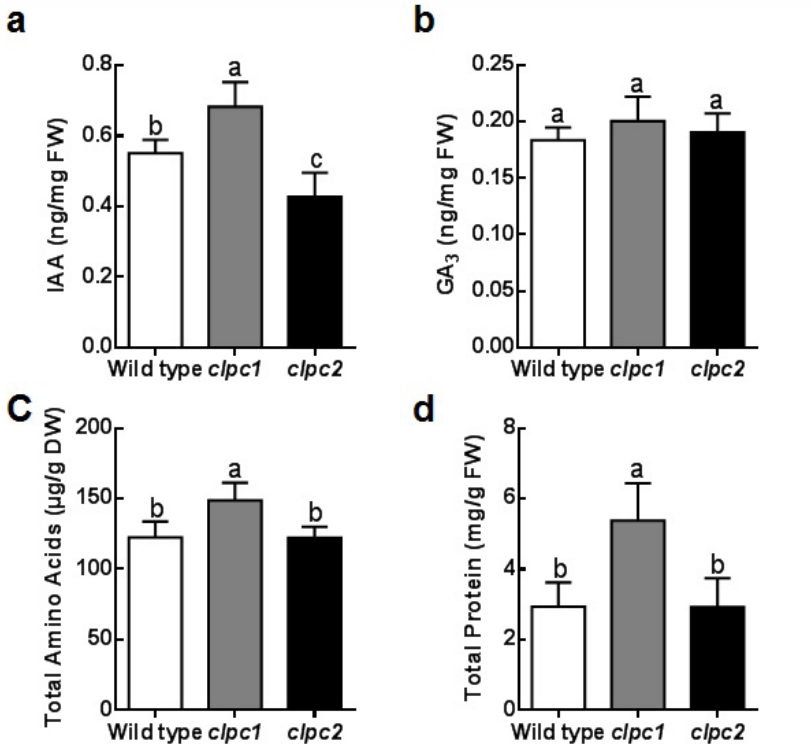

Fig. 2. Content of hormones, free amino acids and total proteins in the leaves of wild type, *clpc1* and *clpc2* mutants. (a) IAA; (b) GA_3; (c) Total free amino acids; (d) Total proteins. IAA and GA_3 contents were measured using high-performance liquid chromatography-mass spectrometry. Different letters on bars indicate significant differences at $p < 0.05$.

The levels of 16 free amino acids were measured in the wild type, *clpc1* and *clpc2* mutants. Coincided with the phenotypes in *clpc1* and in *clpc2* mutants compared with that in wild type, the *clpc1* mutant contained significantly different profile of the free amino acids contents compared with wild type but the *clpc2* mutant was not different in the content of free amino acids from the wild type (Table 1). The *clpc1* mutant contained 1.22-folds higher levels of total free amino acids than the wild type (Table 1). In addition to the high accumulation of free amino acids, the *clpc1* mutant contained 1.83-folds higher levels of total protein than the wild type (Fig. 2c, d).

Table 1. Levels of 16 selected amino acids in wild type *Arabidopsis*, as well as *clpc1* and *clpc2* mutants.

Amino acids	Concentration (µg/g dw)		
	Wild type	*clpc1* mutant	*clpc2* mutant
Nonpolar aliphatic			
Glycine	0.9 ± 0.4a[z]	1.0 ± 0.1a	1.1 ± 0.4a
Alanine	13.3 ± 1.6a	12.9 ± 0.5a	13.8 ± 0.7a
Proline	3.1 ± 0.7a	1.0 ± 2.0b	3.4 ± 0.6a
Valine	7.3 ± 1.7b	9.8 ± 0.6a	6.3 ± 0.3b
Leucine	2.8 ± 0.5a	2.7 ± 0.4a	2.4 ± 0.3a
Isoleucine	1.7 ± 0.3a	1.9 ± 0.1a	1.6 ± 0.3a
Methionine	0.8 ± 0.4a	0.4 ± 0.0a	1.0 ± 0.5a
Nonpolar aromatic			
Phenylalanine	1.3 ± 0.3a	1.3 ± 0.0a	1.3 ± 0.3a
Tyrosine	1.0 ± 0.2a	0.3 ± 0.4b	0.9 ± 0.1a
Polar uncharged			
Serine	16.4 ± 5.6b	25.7 ± 2.0a	17.3 ± 7.0ab
Threonine	8.6 ± 0.8b	16.8 ± 3.8a	9.9 ± 0.9b
Polar positively charged			
Lysine	2.8 ± 0.7a	1.8 ± 0.3b	2.4 ± 0.5ab
Histidine	0.6 ± 0.2a	0.7 ± 0.1a	0.6 ± 0.1a
Arginine	5.0 ± 2.9a	3.9 ± 1.2a	3.7 ± 1.3a
Polar negatively charged			
Aspartate	26.0 ± 2.3a	29.3 ± 5.1a	26.0 ± 2.8a
Glutamate	30.6 ± 3.6b	39.4 ± 3.1a	30.4 ± 1.3b
Total	122.4 ± 11.4b	148.8 ± 12.5a	122.1 ± 7.9b

[z]Different letters in the same row indicate a significant difference ($p < 0.05$).

Treatment of wild type plants with NAA, GA_3 and BAP did not lead to significant changes in the free amino acids contents (Table 2). However, the levels of free amino acids in the *clpc1* mutant increased 1.25-, 1.10- and 1.29-folds in response to treatment with NAA, GA_3 and BAP, respectively (Table 3). All hormonal treatments significantly increased the free amino acids contents in the *clpc1* mutant relative to those in the wild type (Fig. 3a). Evaluation of the total protein contents revealed that treatment of *clpc1* mutant with NAA and BAP significantly increased the total protein content, while treatment with GA_3 led to a significant decrease in protein (Fig. 3b).

The total free amino acids content was higher in *clpc1* mutant than the wild type and *clpc2* mutant. This may have occurred owing to stimulated synthesis or inhibited degradation of amino acids, impaired synthesis of certain proteins and/or less conversion of certain amino acids into biologically active substances. Loss of *ClpC1* gene function in *clpc1* mutant has been reported to lead to increases in all subunits of the Clp protease except ClpD (Sjogren *et al.* 2014). When protein profiling of chloroplasts was investigated in the *clpc1* mutant, the most striking outcome

was the increased level of most other stromal chaperones and several RNA binding proteins (Sjogren *et al.* 2014). These findings indicate that *clpc1* mutant may up-regulate the chaperonic proteins that help renature or remove the denatured proteins. Therefore, the induction of increased total proteins is likely a compensatory response to the lower ClpC content and inability to form sufficient active Clp protease.

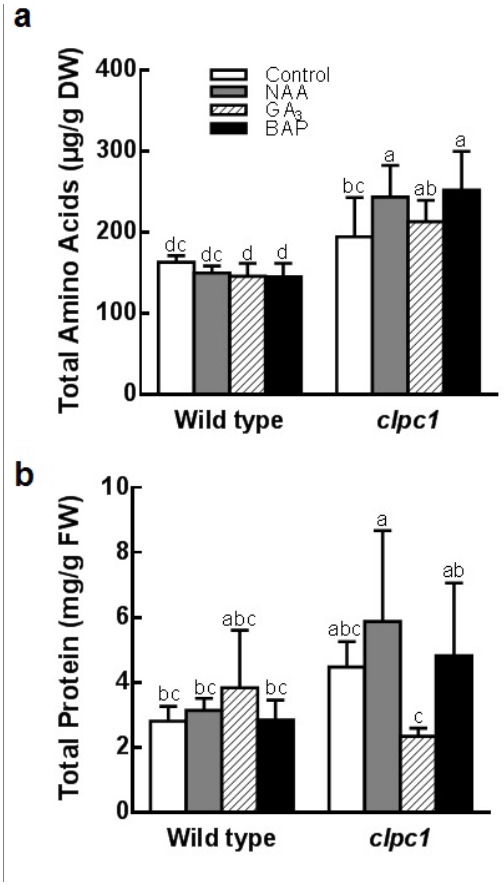

Fig. 3. Contents of total free amino acids and total proteins in leaves of *Arabidopsis* wild type and *clpc1* mutant in response to NAA, GA_3 and BAP treatment. (a) Total free amino acids. (b) Total proteins. Different letters on bars indicate significant differences at $p < 0.05$.

Free amino acids function as osmolytes involved in intracellular osmotic adjustment, and their accumulation plays a critical role in protecting the photosynthetic activity of plants under stress (Silva-Ortega *et al.* 2008). The *clpc1* mutant was found to contain reduced amounts of chlorophylls and carotenoids with impaired photosynthetic function (Sjogren *et al.* 2014). Co-suppression of the *NbClpC1* and *NbClpC2* genes in *N. benthamiana* reduced photosynthetic capacity by altering leaf structure, impairing chloroplast function and increasing free amino acids contents (Ali *et al.* 2015). Therefore, *clpc1* mutant might accumulate free amino acids for protection of photosynthetic activity and future recovery from chlorotic phenotype, or as a result of malfunctioning chloroplasts.

Table 2. Levels of 16 selected amino acids in wild type *Arabidopsis* treated with NAA, GA_3 and BAP.

Amino acids	Concentration (µg/g dw)			
	Wild type-control	Wild type-NAA	Wild type-GA_3	Wild type-BAP
Nonpolar aliphatic				
Glycine	1.3±0.2a[z]	1.7±0.4a	1.5±0.2a	1.5±0.4a
Alanine	16.1±1.5a	15.1±1.4a	14.3±2.4a	14.9±1.4a
Proline	5.2±0.6a	6.4±0.4a	5.5±1.1a	3.9±0.7b
Valine	3.8±0.5a	3.9±0.7a	4.2±1.1a	3.1±2.2a
Leucine	3.3±1.0a	4.0±1.4a	3.3±0.5a	3.3±1.1a
Isoleucine	2.4±0.6a	2.9±1.0a	2.5±0.4a	2.6±0.8a
Methionine	1.0±0.3a	1.3±0.4a	1.0±0.2a	1.1±0.3a
Nonpolar aromatic				
Phenylalanine	2.5±1.1a	3.1±1.5a	2.5±0.5a	2.1±0.7a
Tyrosine	1.7±1.0a	2.3±1.3a	1.8±0.4a	1.5±0.8a
Polar uncharged				
Serine	18.2±1.1a	18.6±0.8a	16.8±2.2a	16.8±2.4a
Threonine	15.1±1.8a	15.1±0.9a	12.3±1.1b	12.5±1.8b
Polar positively charged				
Lysine	3.3±0.9a	4.0±1.2a	3.4±0.6a	3.4±0.9a
Histidine	1.4±0.2a	1.2±0.1ab	1.1±0.2b	1.1±0.1b
Arginine	8.2±1.4a	6.0±0.7b	6.4±1.6ab	6.4±0.9ab
Polar negatively charged				
Aspartate	35.5±2.5a	32.2±2.0a	32.0±2.9a	34.0±3.3a
Glutamate	44.2±5.5a	32.0±4.7b	37.2±4.8ab	36.9±5.4ab
Total amino acids	163.3±8.0a	149.8±8.8a	145.8±16.1a	145.1±16.8a

[z]Different letters in the same row indicate a significant difference ($p < 0.05$).

Table 3. Levels of 16 selected amino acids in *Arabidopsis clpc1* treated with NAA, GA_3 and BAP.

Amino acids	Concentration (µg/g dw)			
	clpc1-control	*clpc1*-NAA	*clpc1*-GA_3	*clpc1*-BAP
Nonpolar aliphatic				
Glycine	2.1±0.4b[z]	3.1±0.7a	3.3±0.5a	3.4±0.8a
Alanine	18.8±3.6b	22.1±3.5ab	19.8±2.6ab	25.3±5.4a
Proline	3.4±0.9b	5.0±0.8a	5.6±0.9a	4.5±0.7ab
Valine	8.9±2.1b	11.6±1.9ab	10.8±1.7ab	12.7±2.6a
Leucine	4.5±0.7b	7.4±1.4a	8.1±1.4a	8.1±1.9a
Isoleucine	3.5±0.7b	5.5±1.1a	5.8±1.3a	6.1±1.5a
Methionine	1.1±0.3b	2.6±1.1a	2.1±1.2ab	2.4±0.4ab
Nonpolar aromatic				
Phenylalanine	3.6±0.8b	6.8±1.2a	6.6±1.5a	6.3±1.8a
Tyrosine	2.2±0.5b	4.5±0.8a	4.5±1.2a	4.5±1.3a
Polar uncharged				
Serine	24.1±6.1a	31.0±4.8a	25.8±2.5a	29.3±5.0a
Threonine	24.2±7.1b	36.5±4.6a	34.8±4.0a	42.7±8.8a
Polar positively charged				
Lysine	4.0±0.5b	6.6±1.4a	7.5±1.1a	7.4±1.7a
Histidine	1.7±0.3a	2.2±0.4a	1.9±0.2a	2.2±0.4a
Arginine	5.6±0.8b	9.8±1.8[a]	7.7±0.7a	9.5±1.6a
Polar negatively charged				
Aspartate	34.8±9.4a	38.7±5.5a	32.3±3.4a	38.8±7.4a
Glutamate	52.0±15.1a	50.2±8.9a	36.7±3.9a	48.7±8.0a
Total	194.6±48.2a	243.7±38.9a	213.3±26.1a	252.0±47.7a

[z]Different letters in the same row indicate a significant difference ($p < 0.05$).

GA_3 ameliorates the adverse effects of stresses and restores normal plant growth under stress by regulating the level of other phytohormones (Hamayun *et al.* 2010). Foliar application of GA_3 increased amino acids content, which counteracted some of the adverse effects of stress by maintaining membrane permeability and increasing macro- and micronutrient levels (Tuna *et al.* 2008). In addition, amino acids production are high in GA_3 treated plants. Therefore, GA_3 application of *clpc1* mutant might increase the contents of free amino acids to restore normal growth and development following stress caused by *ClpC1* gene mutation.

Mutation of the *clpc1* gene negatively impacts plant growth, causing chlorosis, aberrant development and semi-dwarf phenotype with alterations in the content of hormones and free amino acids. Application of exogenous hormones increased the content of free amino acids in the *clpc1* mutant, while there was no change in free amino acids in wild type plants subjected to the same treatment. Present findings provide new insight into the regulation of hormone levels and free amino acid metabolism by chaperonic components, specially *ClpC1* in *Clp* protease.

Acknowledgment

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Agri-Bio industry Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA) (117044-3).

References

Adam Z, Rudella A and van Wijk KJ 2006. Recent advances in the study of Clp, FtsH and other proteases located in chloroplasts. Curr. Opin. Plant Biol. **9**: 234-240.

Ali MS, Kim KW, Dhakal R, Choi D and Baek K-H 2015. Accumulation of high contents of free amino acids in the leaves of *Nicotiana benthamiana* by the co-suppression of *NbClpC1* and *NbClpC2* genes. Plant Cell. Rep. **34**: 355-365.

Fatma M, Khan MIR, Masood A and Khan NA 2013. Coordinate changes in assimilatory sulphate reduction are correlated to salt tolerance: involvement of phytohormones. Ann. Rev. Res. Biol. **3**: 267-295.

Hamayun M, Khan SA, Khan AL, Shin JH, Ahmad B, Shin DH and Lee IJ 2010. Exogenous gibberellic acid reprograms soybean to higher growth and salt stress tolerance. J. Agric. Food Chem. **58**: 7226-7232.

Khan MIR, Iqbal N, Masood A and Khan NA 2012. Variation in salt tolerance of wheat cultivars: role of glycine betaine and ethylene. Pedosphere **22**: 746-754.

Kovacheva S, Bedard J, Wardle A, Patel R and Jarvis P 2007. Further *in vivo* studies on the role of the molecular chaperone, Hsp93, in plastid protein import. Plant J. **50**: 364-379.

Nakagawara E, Sakuraba Y, Yamasato A, Tanaka R and Tanaka A 2007. Clp protease controls chlorophyll b synthesis by regulating the level of chlorophyllide a oxygenase. Plant J. **49**: 800-809.

Pan X, Welti R and Wang X 2010. Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography-mass spectrometry. Nat. Protoc.**5:** 986-992.

Silva-Ortega CO, Ochoa-Alfaro AE, Reyes-Aguero JA, Aguado-Santacruz GA and Jimenez-Bremont JF 2008. Salt stress increases the expression of *p5cs* gene and induces proline accumulation in cactus pear. Plant Physiol. Biochem. **46**: 82-92.

Sjogren LLE, Tanabe N, Lymperopoulos P, Khan NZ, Rodermel SR, Aronsson H and Clarke AK 2014. Quantitative analysis of the chloroplast molecular chaperone ClpC/Hsp93 in *Arabidopsis* reveals new insights into its localization, interaction with the Clp proteolytic core, and functional importance. J. Biol. Chem. **289**: 11318-11330.

Tuna AL, Kaya C, Dikilitas M and Higgs D 2008. The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. Environ. Exp. Bot. **62**: 1-9.

(Manuscript received on 14 April, 2016; revised on 30 July, 2017)