*Pak. J. Bot.*, 52(5): 1525-1535, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-5(26)

# COMPARATIVE PROTEOMICS ANALYSIS REVEALS IMPORTANT DROUGHT RESPONSIVE PROTEINS IN THE LEAVES OF A POTATO VARIETY TOLERANT TO DROUGHT STRESS

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**Abstract**

Drought stress inhibits the growth and productivity of potato. The mechanism of drought tolerance in potato remains largely unclear. Potato Dongnong 308, tolerant to drought stress, was selected to perform an identification of different abundance proteins using 2D-PAGE and LC-MS/MS accompanying with a physiological and ultrastructural observation to uncover its drought tolerant mechanism in potato leaves. Potato plants can adapt morphologically and physiologically to reduce the damage of drought stress to the plant cell. Under drought condition, the water content in potato leaves had a weak reduction and resulted in a significantly decreased root activity. The SOD activity and MDA content showed a significant increase, suggested that physiological changes occurred in potato leaves under drought stress. After inducing water deficitstress, some chloroplasts separated from cell wall and moved to cell center and grana lamella become netty with increased space between lamellas. In addition, a few chloroplasts became swelled, suggesting its function become weak. With the decreasing supply of energy from chloroplast, other energy supply metabolic pathways were active. Sixteen proteins were accumulated differentially and were involved in chloroplast structure and function, secondary metabolism activation, protein folding/processing and synthesis, glycolysis regulation, and stimulation of ATP synthesis. Their functions in potato leaves were mainly related to activate the process of metabolism adjustment and to mobilize the defense system under drought stress. The abundance of heat shock proteins (HSP) correlated with alleviating the accumulation of reactive oxygen species (ROS) against abiotic stresses, which should facilitate the increase of drought tolerance in potato. The identified physiological parameters and proteins provide new insight of the molecular mechanism to drought tolerance in potato.

**Key words:** Potato; 2D-PAGE; Proteome; Different abundance proteins; Physiological parameters; Chloroplast.

## Introduction

Potato is the fourth crop cultivated widely in the world (Zhang *et al*., 2017). Its production is considerably limited in drought climate condition (Hijmans, 2003; Obidiegwu *et al*., 2015) and drought stress result in the change of the physiological metabolism in potato (Romero *et al*., 2017). In field conditions, it have been shown that potato plants lose chlorophyll and some proteins and accumulate some harmful metabolites when they are subjected to drought condition (Obidiegwu *et al*., 2015), which is similar to its response to salt stress when potato is planted in arid and semiarid field (Heuer *et al*., 1998; Aghaei *et al*., 2008; Fahad *et al*., 2017). In addition, many results demonstrate that potato plants show similar drought sensitivity to other crops (Monneveux *et al*., 2013). Water deficit under the condition of drought stress causes harm to the ultrastructure of chloroplast (Zhang *et al*., 2015) and affects physiological parameters such as water potential, enzyme activities (Bohnert & Jensen, 1996) and C/N transition (Ashraf, 2010) and results in a low crop yield, which accelerate the researches in improving crop drought tolerance. To increase drought tolerance of crops, lots of researches have engaged in the identification of important genes underlying drought tolerance in crops including potato (Evers *et al*., 2010), soybean (Manavalan *et al*., 2009), wheat (Fleury *et al*., 2010), and rice (Swain *et al*., 2017). Significant progresses have been made about drought tolerance in crops at gene expression level (Krannich *et al*., 2015), which facilitate the elucidation of the molecular mechanism in response to drought stress and how the plant adapts to the water-deficit environment and what strategy should be adopted to increase the drought stress tolerance in field crops. However, drought-tolerant researches about gene expression related to water deficit in a gene level do not provide us a fully understanding of regulation mechanism to drought tolerance (Manavalan *et al*., 2009; Pinheiro & Chaves, 2011). Furthermore, protein abundance is usually not in accordance with gene expression level and many proteins are involved in post-translational modifications (Greenbaum *et al*., 2003) with an important physiological function. So, it is necessary to analyze the drought stress response at protein level and many researches have been reported (Ghatak *et al*., 2016; Ghatak *et al*., 2017). For example, more than 1000 protein spots are detected in three-week old leaves of rice with a 23-days water deficit and 42 proteins showed a significant change in protein abundance under drought stress (Salekdeh *et al*., 2002). Two-dimensional difference gel electrophoresis coupled with a MS identification is also used to analyze wheat flag leaves and developing grains under drought stress, and the identified differentially accumulated proteins in flag leaves are mainly involved in photosynthesis (Deng *et al*., 2018). In addition, 20 differential abundance proteins in shoot tips of potato tolerant to drought are identified using 2D-IEF/SDS-PAGE coupled with a mass spectrometry and many proteins is related to chloroplast function (Bündig *et al*., 2016). Most of the identified differential abundant proteins are enzymes related to chloroplast, energy metabolism, redox regulation and protein turnover (Wang

*et al*., 2016), antioxidant defenses and signal transduction (Pan *et al*., 2017). However, only a few researches about drought response are reported in potato. More works should be done to elucidate the tolerant mechanism in potato to improve yield and quality of potato.

Potato is a primary food resource in the world and sensitive to water deficit (Obidiegwu *et al*., 2015). Under high water-deficit environment, the activity of antioxidant enzymes increases (Abd El Baky *et al*., 2016) and more proline are accumulated (Knipp & Honermeier, 2006) and the yield of microtubers reduces (Kawakami *et al*., 2006). Despite few reports mentioned above, much remains unclear regarding the molecular mechanism of responses of potato to drought stress. In the present work, a comprehensive physiological and proteomic analysis was performed to measure the relative water content, chloroplast ultrastructure and the accumulation profiles of differential abundance proteins with or without water deficit to facilitate the releasing of potato variety tolerant to drought stress.

## Materials and Methods

**Materials and drought treatment:** Potato cultivar Dongnong 308 was selected to perform a drought stress observation. The healthy sprouted tubers were planted in sterilized soil and nodal sections of growing plants in a 500 mL pot were used as primary explants. Stem-cutting including a single node and leaf was selected and sterilized for 15 min in 10 % sodium hypochlorite and then washed 3 times with sterile liquid medium. The stem-cuttings were sub cultured for 21 days until the tube plantlets grew to 8 cm plant height with 6 or 7 leaves in the stem. The temperature of growth chamber was 25°C with 16/8 h light/dark photoperiod and 150 μmol $m^{-1}s^{-1}$ light intensity. Finally, three single nodes in each container were cultivated in full-strength MS liquid medium for 3 days (Wang *et al*., 2018) to conduct an induction of drought tolerance for further analysis.

**Ultrastructure observation of chloroplast:** Three plantlets were selected randomly. 1 mm × 3 mm samples of the fully expanded uppermost leaves from water-deficit and control plantlets of Dongnong 308 were fixed in 100 mM sodium cacodylate buffer (pH 6.7, 2.5% glutaraldehyde) (Sabatini *et al*., 1963) and post-fixed in 2 % (w/v) $OsO_4$ for 1.5 h. The samples were then dehydrated through a graded ethanol series and embedded in Spurr's epoxy resin (Spurr, 1969). Furthermore, the samples were sliced with a diamond cutter (ULPRACUT EXINXIN, Leica, German) and then stained with uranyl acetate and lead phosphate. The ultrastructure of chloroplast was generated and three sections per treatment were observed with a transmission electron microscope (H-7650 Hitachi, Japan).

**Water content, root activity, POD, SOD and MDA measurement:** Water potential in potato leaves and stems of tube plantlets sub cultured for 21 days was determined according to the procedure described by Kosmala *et al*., (2012). Root activity was measured with the triphenyl tetrazolium chloride (TTC) method (Wang *et al*., 2017). Peroxidase (POD) activity was measured using a spectrophotometric method (Yu *et al*., 2010). Superoxide dismutase (SOD) activities in potato leaves and stems of tube plantlets sub cultured for 21 days were measured following to the ultraviolet absorption method (Giannopotitis & Ries, 1977). Lipid peroxidation was evaluated by measuring malondialdehyde compound (MDA content) in potato leaves of tube plantlets sub cultured for 21 days was quantified according to the ninhydrin-based colorimetric metric method (Delauney, 1992). All the measurements were performed in five biological replicates.

**Protein extraction:** Leaves in the same growth condition from vary beakers were excised using sterilized bladeand 1 g leaves were weighed and immediately ground to powder in liquid nitrogen using a MM301 mixer (Retsch GmbH, Germany). Protein extraction buffer (30 ml) (10% TCA in cold acetone, 0.07% beta-mercaptoethanol) was added in it. The homogenate was centrifuged at 15000 g for 10 min at 4°C. Supernatant was discarded and the precipitate was suspended in 30 ml 80% cold acetone (0.01% DTT and 0.07% beta-mercaptoethanol) and the precipitate was suspended repeatedly for 3 times. The resulting precipitate was washed for three times with cold ethanol and dried in vacuum freezer before homogenizing on ice with lysis buffer (7 M urea, 2 mol/L thiourea, 4% CHAPS, 1% DTT). The homogenate was centrifuged for 15 min at 12000 g at 4°C. About 4 volume cold acetone with 1% DTT was added to the supernatant in a clean tube and kept at -20°C for 2 h and then centrifuged at 4°C for 10 min at 12000 g. After purified with GE 2D clean-up kit and quantified with BSA method, the supernatant was subjected to 2D-PAGE analysis.

### Two-dimenional polyacrylamide gel electrophoresis (2D-PAGE)

**First dimensional isoelectric focusing (IEF):** IEF analysis was performed according to Aghaei *et al*., (2008) with minor modification. In brief, the resuspended protein sample was loaded to immobiline drystrip (7 cm, pH 4-6) in an Ettan IPGphor II isoelectric focusing (Amersham Biosciences, USA) for isoelectric focusing. According to the manufacturer's instruction, IPG strip was run in 6 steps. After isoelectric focusing, IPG strip was equilibrated and transferred to 5 mL equilibration solution (50.0 mmol/L pH 8.8 Tris-HCl, 6.0 mol/L urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue) with 0.125g iodoacetamide to soak for 15 min.

**Sodium dodedyl suphate polyacrylamide gel electrophoresis (SDS-PAGE):** SDS-PAGE analysis was followed the guide of General Electric Company (https://www.gelifesciences.com). After equilibration, a 10% SDS-PAGE was used to conduct the second-dimension experiment. The SDS-gel was run at 10 mA per gel for 30 min, subsequently increased to 20 mA per gel for 6 h.

**Image acquisition and data analysis:** SDS gel was stained with 0.12% CBB G-250 and scanned with a high-resolution scanner (GS-800 Calibrated Imaging Densitometer, Bio-Rad) to obtain a high quality of gel image. The positions of individual proteins in 2D-PAGE image were evaluated automatically with PDQuest software version 7.4. Three replicates for control and drought treatment were used for image analysis, respectively. The amount of a protein was determined with the ratio of the pixel for the protein spot to that of the total protein spots in the gel. The result data from image analysis were transferred to PDQuest software for identifying the differential abundance protein. Statistical analysis of the data was carried out using Microsoft Excel 2016.

**Protein identification by MALDI-TOF-MS:** After image analyses, the differential abundance proteins were excised and digested with trypsin and then identified with MALDI-TOF-MS (Webster & Oxley, 2012). The database search of resultant peptide mass fingerprint against the NCBInr protein sequence databases was performed with the Mascot search engine (Cottrell & London, 1999). Tryptic digest was selected for the searching of enzyme specificity. Fixed modification was carbamidomethylation on cysteines and variable modification was methionine oxidation. Furthermore, one miscleavage was tolerant for each peptide and peptide mass tolerance was ±100 ppm. The statistically significance for a mascot score was set as $p<0.05$ (Koenig *et al*., 2008).

**RNA extraction and quantitative real-time PCR:** RT-PCR was conducted to follow the procedure described by Zhao *et al*., (2013). Briefly, RNA was extracted with the RNAiso reagent (Takara) and total RNA was digested with DnaseI to remove genomic DNA contamination. A total reaction of 20 µl for each sample was performed including 1 µl cDNA, 0.2 µM primer and 2 × SYBR Green PCR Master Mix. In addition, gene-specific primers were searched using primer premier 5.0 (Table 1). The expression level analysis for each identified gene was followed the manufacturer's instruction on Lightcycler480 Real-Time PCR Detection System (Roche).

**Data analysis**

Parameter data were presented as means with standard deviations. Data were subjected to One-Way ANOVA and Duncan's multiple range for each parameter at $p<0.05$ using excel 2016 and SPSS 19.0.

**Results**

**Effect of drought stress on the ultrastructure of chloroplasts:** The chloroplasts of the tube plantlets with normal water supply were in close proximity/attached to the cell wall closely and thylakoids were compact. Well compartmentalized grana stacks with grana lamellaes were observed in mesophyll cells (Fig. 1A). When exposed to drought stress resulted from full-strength MS liquid medium for 3 days, some chloroplasts get separated from cell wall and moved to cell center and grana lamella become netty with increased space between lamellas. In addition, a few chloroplasts became swelled (Fig. 1B). The change of the ultrastructure of chloroplast suggests its corresponding physiological function also changed.

**Effects of drought stress on water content, root activity, MDA content, POD activity and SOD activity in tube plantlets:** Root activity gives a reflection of root respiration and oxidizing activity under abio-stress. Determination of water potential and root activity can indicate how the plants response to drought. The water content in leaves decreased from 90.87% to 65.1% and resulted in a significant low root activity (Fig. 2A and B). The activity of some enzymes also showed response to the drought stress. The POD activity in leaves showed a warm increase from 161.33 U/g to178.66 U/g after water deficit, while a significant increase was observed from 279.66 U/g to 324 U/g for SOD activity and from 0.727 umol/L to 1.083 umol/L for MDA content (Fig. 2C and D), suggested that physiological changes also occurred in potato leaves under drought stress.

**Identification of different expression proteins (DEPs) using 2D-PAGE coupled with LC-MS/MS:** To further understand the regulated mechanism of potato leaves to drought stress in a protein level, DEPs were identified using a 2-DE strategy. A total of 16 proteins were detected to express differentially with at least 2-fold abundance difference compared between control and drought treatment in the polyacrylamide gel (Fig. 3, Table 2). Among the 16 DEPs identified in the potato leaves, the protein relative abundance of spot 1 to spot 14 under drought stress showed an up-regulated in comparison to the control with normal water supply. While that of spot 15 and spot 16 under water deficit showed a down-regulated compared to the control. After a LC-MS/MS identification, the function of 16 differentially expressed proteins were identified to cluster in several metabolism pathways. Spot 1 was a phosphoglycerate mutase; Spot 5 was an ATP synthase (Predicted); Spot 6 was a soluble inorganic pyrophosphatase 1 (Predicted); Spot 7 was a ribulose-1,5-bisphosphate carboxylase/oxygenase; Spot 14 was a D-Ribulose-5-Phosphate 3-Epimerase. Spot 15 was an isocitrate dehydrogenase (Predicted). All these six proteins were involved in energy metabolism. In addition, spot 2 was a haloacid dehalogenase (Predicted), a protein related to water absorption. Spot 3, spot 4 and spot 8 were heat shock protein and related proteins. Spot 10 to spot 13 were identified as functional proteins related to chloroplast and photosystem stability. Spot 16 was disulfide-isomerase precursor-like protein related to protein stability. Spot 9 was a glycine-rich protein 2 (Predicted) related to abiotic stress. The annotation above of the 16 DEPs suggested they were all related to environmental stress response.

**Table 1. List of the primers used for the verification of differential expressed genes.**

| Spot | Protein name | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|---|
| 1. | Phosphoglycerate mutase | aggttgggatgcacaagttc | acaacagcatcaccatccaa |
| 2. | PREDICTED: haloacid dehalogenase-like hydrolasedomain-containing protein At3g48420-like | ttgcagtatgcagcacttcc | ggtgtcggctgctaacagat |
| 3. | PREDICTED: heat shock cognate 70 kDa protein 2-like | ggcaaatttgagctctctgg | ttgtcgttggtgatggtgat |
| 4. | PREDICTED: heat shock protein STI-like | gctcccaccaatcatgttct | gtaagcagaaaccgcatcgt |
| 5. | PREDICTED: ATP synthase subunit beta, mitochondrial-like | ggtctctgctttgcttggtc | caagtcatcagcaggcacat |
| 6. | PREDICTED: soluble inorganic pyrophosphatase 1, chloroplastic-like | atctacaatccacagattcag | gctcatctgtaggcaacttcc |
| 7. | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | gagatcatattcactctggtac | atacctcctcctgaagccacaggta |
| 8. | PREDICTED: heat shock protein STI-like | gctcccaccaatcatgttct | gtaagcagaaaccgcatcgt |
| 9. | PREDICTED: glycine-rich protein 2-like | tgatggagacagtggctacg | gccaccactctgggtacact |
| 10. | dehydroascorbate reductase | gattctagtgacggtactgag | tcaggcacactccacttcttga |
| 11. | Chloroplast manganese stabilizing protein | atgtactgatgctgccaagat | tagggcactggttagcagtt |
| 12. | PREDICTED: glyceraldehyde-3-phosphatedehydrogenase B, chloroplastic-like | cggtgtgtttgttgatggtc | tgcaagaggcattgcttatg |
| 13. | PREDICTED: 50S ribosomal protein L4, chloroplastic-like | gccactattccaattctgtcct | gtaagggtggaagcagtaccg |
| 14. | Chain B, D-Ribulose-5-Phosphate 3-Epimerase from Solanum Tuberosum Chloroplasts | aggcaggctgtgactggattca | gtgaacactgacaatatcagcac |
| 15. | PREDICTED: isocitrate dehydrogenase [NAD] regulatory subunit 1, mitochondrial -like | cagtccgatccgtcacctat | cacatccggaggcatactct |
| 16. | Disulfide-isomerase precursor-like protein | gcgggtatcatcgaatcact | ttggcgtaatcagctccttt |

**Table 2. Identification results of proteins differentially expressed from potato under water stress.**

| Spot | Protein name | Accession No. | Protein score | Protein MW/PI | Pep. count | MS Ion intensity | Enzyme number | Up/down regulated |
|---|---|---|---|---|---|---|---|---|
| 1. | Phosphoglycerate mutase | gi\|4582924 | 339 | 61219.2/5.42 | 10 | 151968.27 | EC:5.4.2.1 | Up |
| 2. | PREDICTED: haloacid dehalogenase-like hydrolasedomain-containing protein At3g48420-like | gi\|565369655 | 134 | 34060.6/5.9 | 3 | 217107.89 | | Up |
| 3. | PREDICTED: heat shock cognate 70 kDa protein 2-like | gi\|565348072 | 117 | 71193.2/5.14 | 9 | 85998.64 | | Up |
| 4. | PREDICTED: heat shock protein STI-like | gi\|565378093 | 155 | 65090.8/5.9 | 4 | 21527.19 | | Up |
| 5. | PREDICTED: ATP synthase subunit beta, mitochondrial-like | gi\|565354610 | 779 | 59531.9/5.65 | 18 | 947468.88 | EC:3.6.3.14 | Up |
| 6. | PREDICTED: soluble inorganic pyrophosphatase 1, chloroplastic-like | gi\|565392594 | 157 | 32454.7/6.02 | 4 | 194643.64 | | Up |
| 7. | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | gi\|108773138 | 347 | 52910.8/6.55 | 14 | 782543.94 | EC:4.1.1.39 | Up |
| 8. | PREDICTED: heat shock protein STI-like | gi\|565378093 | 165 | 65090.8/5.9 | 6 | 117980.15 | | Up |
| 9. | PREDICTED: glycine-rich protein 2-like | gi\|565387787 | 449 | 19715.6/5.42 | 6 | 456453.75 | | Up |
| 10. | Dehydroascorbate reductase | gi\|160347100 | 516 | 23441.2/6.09 | 13 | 860882.38 | | Up |
| 11. | Chloroplast manganese stabilizing protein | gi\|313586398 | 441 | 34096.5/6.73 | 8 | 253786.08 | | Up |
| 12. | PREDICTED: glyceraldehyde-3-phosphatedehydrogenase B, chloroplastic-like | gi\|565350398 | 294 | 47976.6/6.52 | 8 | 229014.92 | EC:1.2.1.- | Up |
| 13. | PREDICTED: 50S ribosomal protein L4, chloroplastic-like | gi\|565363267 | 274 | 31852.3/6.88 | 10 | 409865.97 | | Up |
| 14. | Chain B, D-Ribulose-5-Phosphate 3-Epimerase FromSolanum Tuberosum Chloroplasts | gi\|4930131 | 225 | 24601/5.75 | 4 | 238454.55 | EC:5.1.3.1 | Up |
| 15. | PREDICTED: isocitrate dehydrogenase [NAD] regulatory subunit 1, mitochondrial-like | gi\|565399535 | 303 | 40388.6/6.8 | 6 | 275851.47 | | Down |
| 16. | Disulfide-isomerase precursor-like protein | gi\|77745442 | 143 | 39466.9/5.62 | 5 | 65068.63 | | Down |

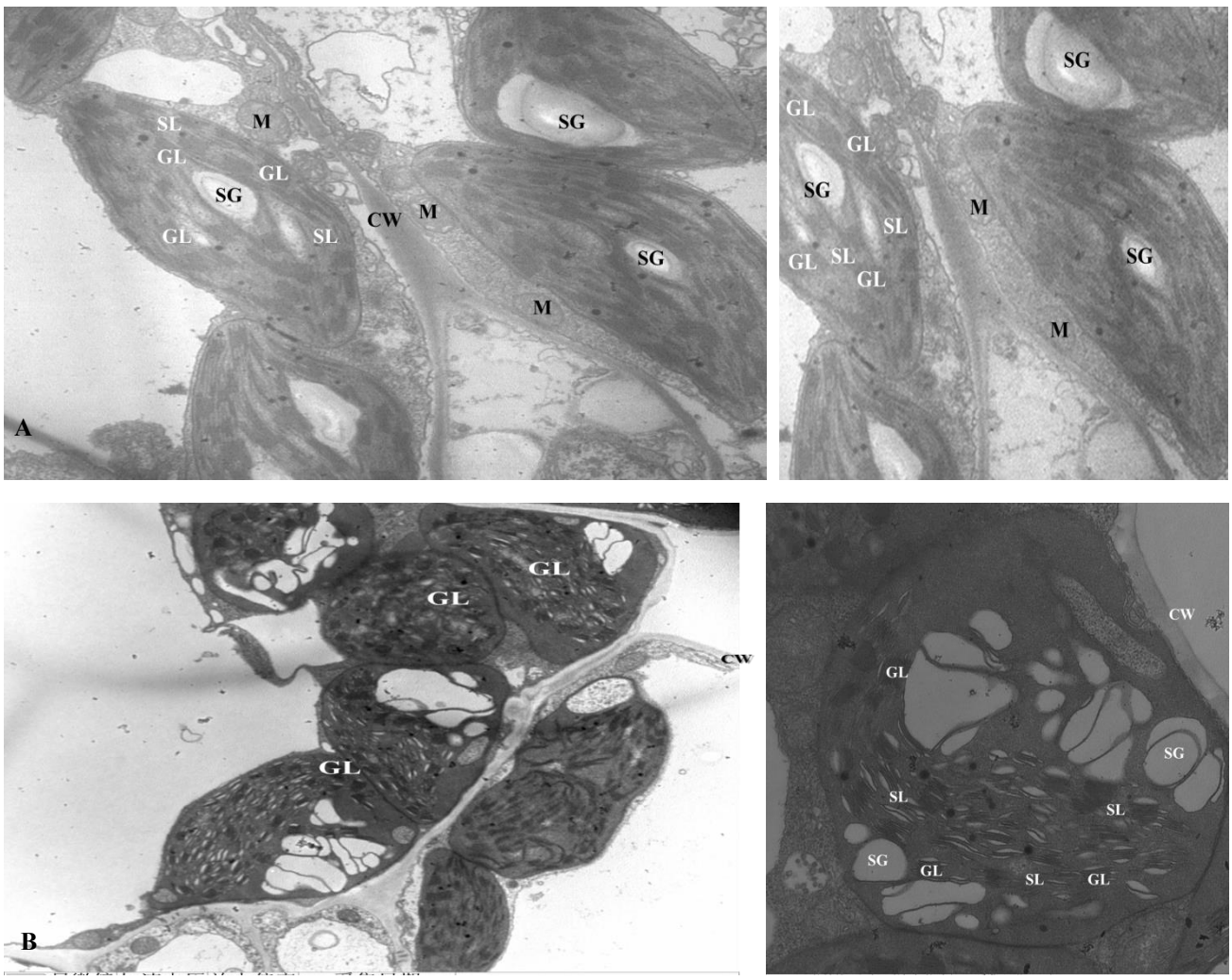


Fig. 1. Normal and PEG disrupted chloroplast
A: Normal chloroplast with enough water-supply (Control); B: Disrupted chloroplast under drought. GL, granum lamella; M, mitochondrion; SG; starch grain; SL, stroma lamellae; CW, cell wall.

**Validation of differentially expressed proteins by RT-PCR:** To verify the identified DEPs in potato leaves with 2-DE strategy, the primers for amplifying the genes corresponding to the sixteen DEPs were searched with primier primer 5 and synthesized in Sangon Biotech (Shanghai) to identify the gene expression level. Most of the gene expression level except for spot 2, 3, 11, 12 and 16 were followed similar trends to the protein abundance identified by proteomic analysis (Fig. 4), thereby suggesting that the proteomic analysis results were reliable, and some post-translated modification should be occurred to the five proteins (spot 2, 3, 11, 12 and 16). Among these different expression genes, spot 1(Phosphoglycerate mutase) and 14 (D-Ribulose-5-Phosphate 3-Epimerase from Solanum Tuberosum Chloroplasts) increased 1.28 fold and 1.31 fold in comparison to normal water supply. While more genes showed a significant increase from 1.28 fold to 6.82 fold in gene expression level compared to normal water supply, such as spot 4 (PREDICTED: heat shock protein STI-like) was 4.50 fold, and spot 5 (PREDICTED: ATP synthase subunit beta, mitochondrial-like) was 2.05 fold, and spot 6 (PREDICTED: soluble inorganic pyrophosphatase 1, chloroplastic-like) was 3.10 fold, and spot 7 (Ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit) was 6.82 fold, and spot 8 (PREDICTED: heat shock protein STI-like) was 4.68 fold, and spot 9 (PREDICTED: glycine-rich protein 2-like) was 1.76 fold and spot 10 (dehydroascorbate reductase) was 2.17 fold, and spot 13 (PREDICTED: 50S ribosomal protein L4, chloroplastic-like) was 3.62 fold, and spot 15 (PREDICTED: isocitrate dehydrogenase [NAD] regulatory subunit 1, mitochondrial-like) was 1.72 fold, respectively. Moreover, spot 2 (PREDICTED: haloacid dehalogenase-like hydrolasedomain-containing protein At3g48420-like), spot 3 (PREDICTED: heat shock cognate 70 kDa protein 2-like), spot 11 (Chloroplast manganese stabilizing protein), 12(PREDICTED: glyceraldehyde-3-phosphatedehydrogenase B, chloroplastic-like) decreased from 0.003 fold to 0.408 fold in contrast to normal water supply. Lastly, spot 16 (Disulfide-isomerase precursor-like protein) showed similar gene expression level with that of normal water supply.

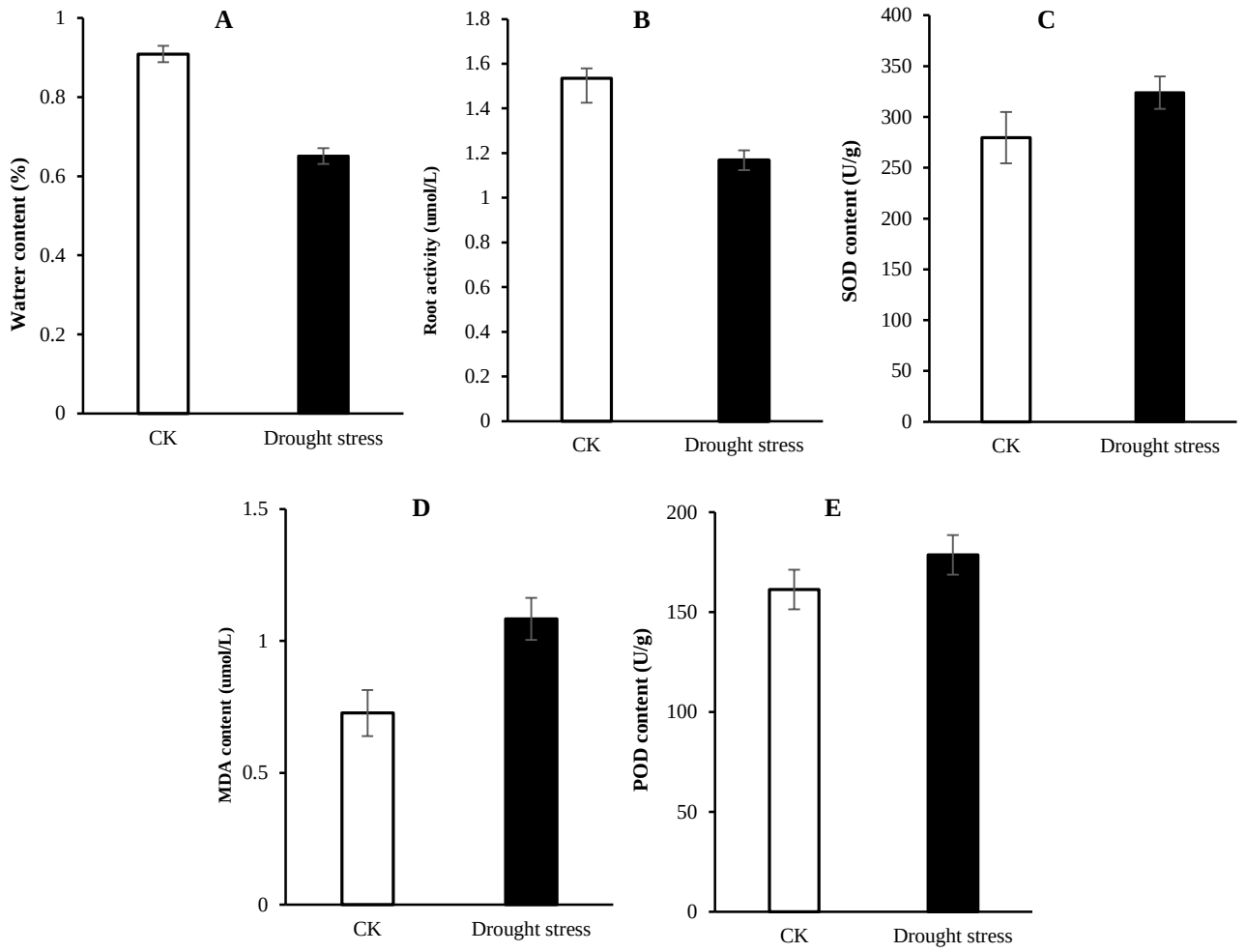


Fig. 2. Effects of drought stress on water content, root activity, MDA content, POD activity and SOD activity in tube plantlets of Dongnong 308. A: Water content decreased under drought condition; B: Root activity decreased under drought condition; C: SOD activity increased under drought condition; D: MDA content increased under drought condition; E: POD activity increased under drought condition.

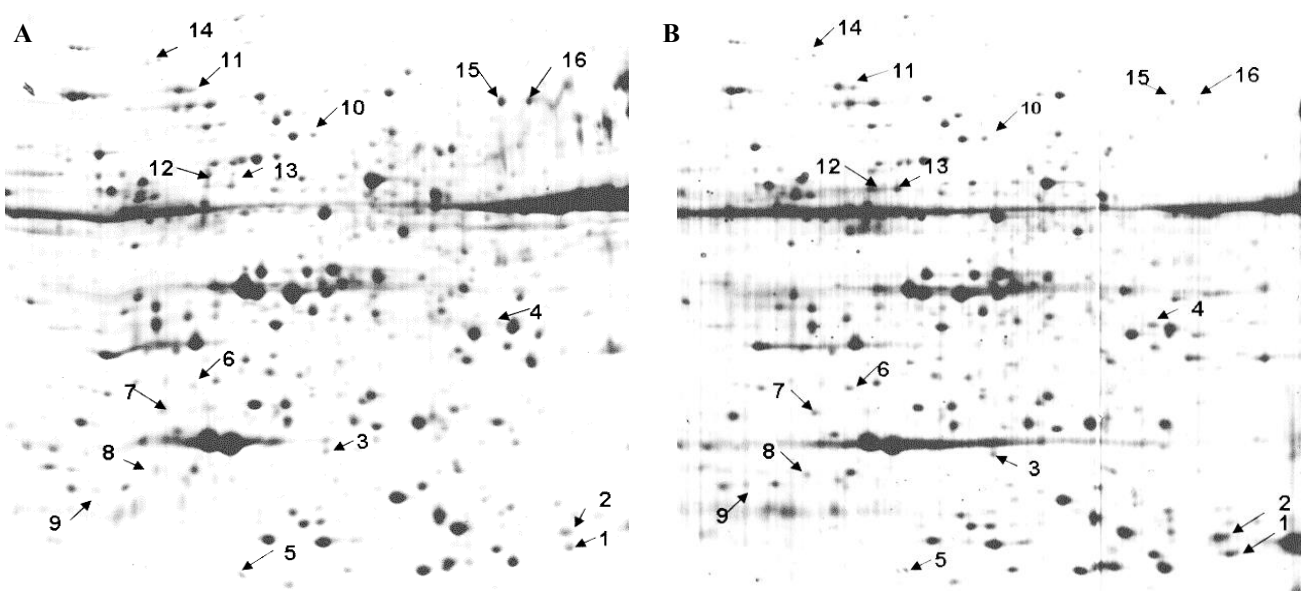


Fig. 3. Representative 2-DE maps comparing two leave proteins of Dongnong 308 under normal water supply (A) and drought stress (B). Total leave proteins were extracted and separated by 2-DE. In IEF, 90 μg of proteins were loaded onto pH 4-7 IPG strips (7 cm, linear). SDS-PAGE was performed with 10% gels. The spots were visualized by CBB G-250 staining. Differentially accumulated protein spots are indicated by arrowheads. Fourteen higher expressed spots (spot 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14) and two suppressed spots (spot 15 and 16) are indicated on the map of Dongnong308.

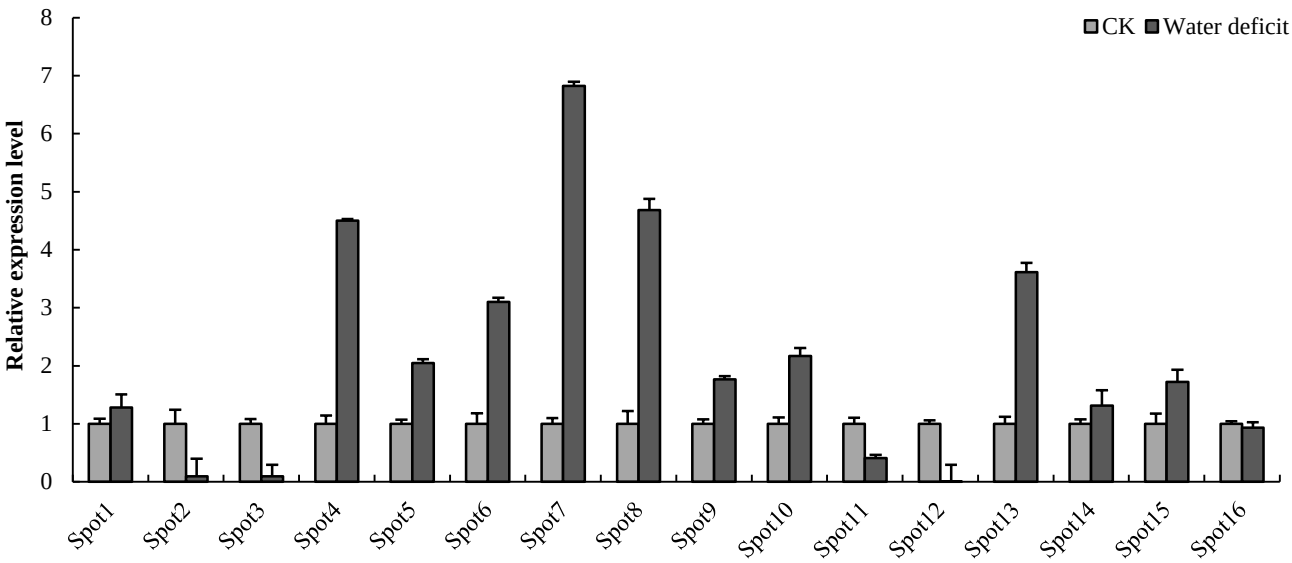


Fig. 4. RT-PCR results of differential expressed genes. Differentially expressed genes are calculated. Eleven higher expressed genes (Spot 1. Phosphoglycerate mutase; Spot 4. PREDICTED: heat shock protein STI-like; Spot 5. PREDICTED: ATP synthase subunit beta; mitochondrial-like; Spot 6. PREDICTED: soluble inorganic pyrophosphatase 1, chloroplastic-like; Spot 7. Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; Spot 8. PREDICTED: heat shock protein STI-like; Spot 9. PREDICTED: glycine-rich protein 2-like; Spot 10. Dehydroascorbate reductase, PREDICTED: 50S ribosomal protein L4, chloroplastic-like; Spot 13. Chain B, D-Ribulose-5-Phosphate 3-Epimerase FromSolanum Tuberosum Chloroplasts; Spot 14. PREDICTED: isocitrate dehydrogenase [NAD] regulatory subunit 1, mitochondrial-like;) and four suppressed genes (Spot 2. PREDICTED: haloacid dehalogenase-like hydrolasedomain-containing protein At3g48420-like; Spot 3. PREDICTED: heat shock cognate 70 kDa protein 2-like; Spot 11. Chloroplast manganese stabilizing protein; Spot 12. PREDICTED: glyceraldehyde-3-phosphatedehydrogenase B, chloroplastic-like) and one gene without expression difference (Spot 16. Disulfide-isomerase precursor-like protein) are indicated on the map of Dongnong 308.

## Discussion

**Physiological changes in potato leaves under water limitation:** Potato is the fourth crop cultivated widely in the world (Zhang *et al*., 2017) and provides thousands of people as foods on the earth. Its production is considerably limited in drought climate condition (Hijmans, 2003) and drought stress can result in the change of the physiological metabolism in potato (Romero *et al*., 2017). However, the molecular mechanism in response to drought stress remains largely unknown in potato. Drought treated results show that Dongnong 308 is a drought tolerant variety (data not shown). Therefore, the response of Dongnong 308 to drought stress can give us a clue about the drought tolerance in potato.

Plants can regulate its own physiological function to respond to biotic stress such as drought, high temperature and salt stress (Hirayama & Shinozaki, 2010; Qin *et al*., 2011). POD and SOD activity all increased under drought stress when compared with control plants (Fig. 2C and E). Similar results have been also observed in maize (Jiang & Zhang, 2002), soybean (Zhang *et al*., 2006), rice (Wang *et al*., 2005) under drought stress. Moreover, several reports have showed that POD and SOD can remove $O_2^-$ in plants to prevent the cell membrane from oxidation by $O_2^-$ (Jaleel *et al*., 2008; Abogadallah, 2010). Therefore, the enhancement of POD and SOD activity (Fig. 2C and E) could effectively resist the oxidative stress caused by drought stress, thereby improving the drought tolerance in potato in some degree.

Under drought stress, over accumulated $O_2^-$ causes the oxidization of membrane lipid and the assembling of membrane protein in cells which results in the destroy of ultrastructure in plants (Apel & Hirt, 2004). MDA content responses the oxidization level in plant cells (Hodges *et al*., 1999). With the decrease of water content in leaves (Fig. 2A), increased MDA in the potato seedlings (Fig. 2D) suggested that PEG stresses inflicted severe damage to the membranes and the cell ultrastructure (Fig. 1) and may have a negative role to increase the drought tolerance in potato (Li *et al*., 2017). Moreover, it is a common response for the accumulation of MDA in a wide range of abiotic and biotic stresses in plants (Davey *et al*., 2005), suggesting that MDA might act as an indicator of potato response to drought stress (Hodges *et al*., 1999).

The results of relative abundance of protein spots in 2D-PAGE gels of potato leaves showed a high similarity in protein expression level between control and drought treatment (Fig. 3, Table 2). Only 16 proteins were identified to express differentially out of the total detected proteins (Fig. 3, Table 2) and were involved in different important physiological and metabolic pathways (Table 2) including chloroplast and photosystem stability, energy metabolism, protein stability and heat shock protein related metabolism pathway.

Drought stress shows a serious impact on plant photosynthesis (Chaves *et al*., 2009) and its component chloroplasts can respond to this change (Goulas *et al*., 2006). Chloroplast and photosystem can transfer light energy to chemical energy for maintaining the growth and development of plants (Alberts *et al*., 2002). Water deficit

destroyed the ultrastructure of chloroplast (Fig. 1) and decreased the photosynthesis efficient in field condition (data not shown) (Pinheiro & Chaves, 2011) resulted in the reduced supply of energy. Plant can rescue this kind of destroy by a series of ways. The differentially expressed protein manganese-stabilizing protein (spot 11; Table 2) is reported as a key protein to keep photoautotrophy and photosystem II assembly/stability in higher plants (Yi *et al*., 2005) and its stabilization of the manganese cluster respond to environmental stress (Wyman & Yocum, 2005), suggesting it is a key protein to maintain chloroplast stability as poor stability of chloroplast was observed under water deficit condition (Fig. 1). In plant, ascorbic acid can maintain the photosynthetic function by its role of antioxidant (Akram *et al*., 2017). The identified differential expressed protein, dehydroascorbate reductase (DHAR) (spot 10; Table 2), can keep ascorbic acid in a low redox state in plant cell and result in chloroplast stability (Chen & Gallie, 2006). All these results suggested chloroplast stability and function have a close relationship to water stress in potato leaves. Moreover, such molecular analyses could provide an insight into mechanisms of plant adaptation to environmental water deficit.

Plant responds to drought stress in a complex readjustment to energy metabolism. With the decreasing supply of energy from chloroplast, other energy supply metabolic pathways were active. The abundance of two protein responsible for ATP production were identified to be increased under drought stress including soluble inorganic pyrophosphatase 1 (Predicted) (spot 6; Table 2) and ATP synthase (spot 5; Table 2). This process was accompanied with NAD, an isocitrate dehydrogenase related to NAD was detected to down-regulated under drought stress (Spot 15; Table 2), suggesting that energy metabolism was active as ATP energy is essential for a lot of cellular processes. Moreover, another energy metabolism pathway was also detected. The phosphoglycerate mutase (spot 1; Table 2) catalyses the reversible interconversion of 3-phosphoglycerate to 2-phosphoglycerate in glucolysis (Zhao & Assmann, 2011; Jedrzejas, 2000). Glucolysis is the center of a netty metabolic network and shares many substrates and products with other pathways (Zhao & Assmann, 2011). Under drought stress, the energy resulting from chloroplast reduced as the coli of chloroplast was destroyed (Fig. 1). So, the glucolysis has to produce more ATP to provide the energetic needs to survive the drought condition. The up-regulation of phosphoglycerate mutase (EC 5.4.2.1) (spot 5; Table 2) and ATP-synthesis synthase (EC 3.6.3.14) (spot 15; Table 2) should result from the energy need for stress protection and maintaining the functional state of tissue in water limiting condition. Moreover, cytosolic NADP-dependent isocitrate dehydrogenase (spot 15; Table 2) not only involve in redox homeostasis but also the regulation of pathogen responses (Mhamdi *et al*., 2010), suggesting that it has a versatile role in plants. In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 12; Table 2), is a key enzyme in the glycolytic pathway and catalyze the conversion reaction from glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate with the involvement of NAD+ (spot 15; Table 2) and inorganic phosphate, (Sirover, 2011). In addition to glycolysis, GAPDH a nonglycolytic functions in signal transduction cascades, DNA repair, and abiotic stress responses (Tristan *et al*., 2011; Zeng *et al*., 2016). For instance, inaspen (*Populus tremula*), GAPDH showed some increase in response to water deficit (Tristan *et al*., 2011; Zeng *et al*., 2016), suggesting that Glyceraldehyde-3-phosphate dehydrogenase is also a key enzyme for drought tolerance.

Drought stress results in metabolism disorder in plants and a readjustment is observed to respond to the abiotic stress in a C and N transition way. Ribulose-5-Phosphate 3-Epimerase (EC 5.1.3.1) (spot 14; Table 2) is involved in C and N metabolism by catalyzing the transition between Ribose-5P and Ribulose-5P (Vicente *et al*., 2015), which showed an increase under water deficit condition (Fig. 3). Moreover, ribulose-1,5-bisphosphate carboxylase (spot 7; Table 2) showed a increase under drought stress treatment (Fig. 3; Table 2), which is involved in carbon fixation in photosynthetic organisms and carbohydrate metabolism (Parry *et al*., 2002), suggesting these two proteins have important roles in photosynthesis. Furthermore, the down-regulation of these proteins shows that photosynthetic system is one of the target metabolites of drought stress effects on potato plants and results in growth decrease in Dongnong 308.

Protein disulfide isomerase (PDI) (spot 16; Table 2) is an enzyme involved in arranging correctly of disulfide bonds in proteins. The decreased abundance of protein disulfide isomerase (Fig. 3; Table 2) showed an inhibition of protein synthesis. Moreover, protein disulfide isomerase involve in the unfolded and refolded protein response (Onda & Kobori, 2014; Peng *et al*., 2017) and plays a crucial role in the stress tolerance (Gruber *et al*., 2007; Kurepa & Smalle, 2008), suggesting that protein disulfide isomerase is involving in maintaining proteins in their functional conformation and removal of non-functional and is very important for cell survival under drought stress (Vaseva *et al*., 2011).

Plants can adapt morphologically and physiologically to reduce the damage of drought stress to the plant cell. Heat-shock proteins can be induced during this process. Three heat shock proteins were identified in current study (Fig. 3; Table 2). Heat shock protein cognate 70 (spot 3; Table 2) is required for the activation of heat shock factor1 which plays an essential role in mediating the appropriate cellular response to diverse forms of physiological stress (Jacob *et al*., 2017). Another HSP protein, heat shock protein STI-like should also be involved drought response with an unclear function. Moreover, this protein is induced almost 5-fold more in leaves compared with control, suggesting it plays an important role in water deficit response in potato leaves.

The specific functions (chloroplast 50S ribosomal protein L4, RPL4) (spot 13; Table 2) was not studied in detail yet. ribosomal protein L4, which, in addition to its structural function in the 50S subunit of the plastid ribosome, may be involved in transcriptional regulation (Trifa *et al*., 1998; Trifa and Lerbs-Mache 2000) to involve in drought tolerance.

## Conclusions

In summary, the differently expressed proteins identified in potato leaves subjected to drought point at down-regulation of glycolysis and stimulation of ATP synthesis, the activation of secondary metabolism, and complex changes in protein synthesis, folding/processing and degradation processes, accumulation of some enzymes related to aminoacid and one-carbon metabolism, which emphasize the active metabolic adjustment and mobilization of the defense system in leaves to actively counteract drought stress.

## Acknowledgements

This study is financially supported by the Chinese National Natural Science Foundation No. 31601355 and National Potato Industrial Technology System No. CARS-10.

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(Received for publication 28 November 2018)