Physiological and proteomic responses to drought stress in leaves of *Amygdalus Mira (Koehne)* Yü et Lu

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Research article

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Abstract

Background:Plant development is strongly influenced by various stresses, such as drought and salinity. Drought is a serious threat which can reduce agricultural productivity and obstruct plant growth. Although the mechanism of plants adapted to drought stress has been studied extensively, the adaptive strategies of Amygdalus Mira (Koehne) Yü et Lu grown in drought and re-watered habitats remain undefined. In this paper, A. Mira from the Tibetan Plateau have outstanding environmental, economic, nutritional and medicinal values, and can thrive in extreme drought.

Results:This paper investigated physiological and proteomic responses in leaves of A. Mira during the period of drought stress and recovery, to understand their strategies mechanism. The changes of plant growth, photosynthesis, enzymes and non-enzymatic antioxidant during drought and re-watering were analyzed in leaves. Compared with controls, A. Mira showed stronger adaptive and resistant characteristics to drought stress. Proteomic technique was also be used to study mechanisms of drought tolerance in A. Mira leaves. Differentially expressed proteins were identified using mass spectrometry. Accordingly, 103 proteins involved in 10 functional categories: Cytoskeleton dynamics, Energy metabolism, Carbohydrate metabolism, Photosynthesis, Transcription and translation, Transport, Stress and defense, Molecular chaperones, Other materials metabolism, and Unknown function were identified. These results showed that increase of stress-defense-related proteins in leaves after drought treatment were contributed to cope with drought stress. Importantly, A. Mira developed adaptive mechanism to scavenge reactive oxygen species (ROS), including enhancement of antioxidant enzymes activities and non-enzymatic low molecular, reduction of energy, and efficiency of adjusting gas exchanges.

Conclusions:These results may help improve understanding concerning the adaptation of A. Mira to drought.

Introduction

Drought can decrease photosynthetic rate in leaves for preventing water loss or affected the capacity of quenching antioxidant and osmotic adjustment of plants (Ahuja et al., 2010; Danquah et al., 2014; Wang et al., 2016; Wei et al., 2018; Xiong et al., 2018). At the protein levels, proteins related to defense and energy generation were increased, suggesting that plant defenses and energy consumption were essential for regulations to against drought stress. For instance, as molecular chaperones, heat shock proteins (HSPs) in plant play critical roles for preserve target protein and help the recovery of denatured proteins assembly, degradation and translocation of damaged proteins, protein folding during protecting plants against stress (Wang et al., 2004; Sato et al 2008; Timperio et al., 2009). Additionally, inducible defense-related proteins have been described in a host of plant species upon environmental stress including drought, salinity, low temperatures (Lee et al., 2011; Zhou et al., 2016;). These proteins serve essential functions in plant life, whether in defense or not. In summary there are complex responsive mechanisms in plants under drought stress.

In the Tibetan Plateau, alpine plants are exposed to strong ultraviolet (UV) radiation, cold, drought and low oxygen concentration (Gou et al., 2007; Ni 2000). According to previous studies, to adapt this severe situation, alpine plants have evolved various strategies including changes of morphology, physiological and molecular features (Hughes and Atchison, 2015; Rosina et al., 2018 Zong et al., 2018). In particular, changes in leaves characteristics, such as high enzymatic and non-enzymatic antioxidants, increase of photosynthetic efficiency and accumulation of pigment have been previously reported. However, this precise physiological and molecular mechanism of this adaptation are still unclear for alpine plants. Here, we report the alpine plants for drought tolerance.

*Amygdalus Mira (Koehne)*Yü et Lu, which is a deciduous fruit tree, is widespread in mixed forests on hillsides or along valleys of Yunan Province, Sichun Province and the Tibetan Plateau, which ranges from 2000–3400 m (Zhang et al., 2013; Peng et al., 2015; Cao et al., 2017). It has outstanding environmental, economic, nutritional and medicinal values. It can thrive in extreme drought and present a higher tolerance to drought (Fig. 1). Accordingly, *A. Mira* can be used as an excellent resource for drought tolerance-related gene discovery. For this, an understanding of the physiological and molecular mechanisms of *A. Mira* adaption to drought stress is fundamental. Thus, to obtain the responsive mechanisms in details in *A. Mira,* the systemic experiments should be carried out.

The responses of plants to drought stress have been reported intensively. To withstand water deficiency, plants have developed complex mechanisms: reduction of growth, stomatal closure, osmotic adjustment, posttranslational modifications and protein-protein interactions (Chen et al., 2010; Urano et al. 2010; Meyer et al. 2014; Swarbreck et al. 2011). For this reason, to better understand tolerance mechanisms, proteomic method is being increasingly performed. Previous investigations into the proteome profiles of plant species under drought stress have mainly focused on model plant but no alpine plants. *A. Mira* as wild peach species, unlike cultivated peach, presents a higher tolerance to drought (Cao et al., 2017). The previous studies showed that compared to cultivated peach, *A. Mira* displayed contrasting defense characteristics against drought stress, and more sensitive activities of PS (Huang et al., 2018). Therefore, because of the complexity of mechanisms in drought response, further studies should be needed, which also invested more challenging and meaningful as ideal wild peach germplasm for improving cultivated peach plants.

Until now, an army of drought tolerance-related proteins have been identified in *Arabidopsis* and *Oryza sativa* (Cao et al., 2017; Kumar et al., 2017; Manuka et al., 2018; Nguyen et al., 2018; Sylva et al., 2018; Saddique et al., 2018). The protein regulation networks under drought in a multitude of woody plants have been reported recently (Bonhomme et al., 2009; Ko et al., 2014; Simova-Stoilova et al., 2018). Various specialized proteins are identified in plants during drought stress, where they played key and crucial roles as chaperones, signaling molecules, ion homeostasis molecules, osmolytes, reactive oxygen scavengers, and heat shock proteins (Milan et al., 2017; Wang et al., 2018). The responses of drought-inducible proteins are closely related to plant species. In particular, alpine plants may use special strategies to adapt to the extreme environment, but the underlying proteome and physiological mechanisms of the adaptation are not now better understood.

To our knowledge, unfortunately, there are few studies relating *A. Mira* to drought stress based on proteomics technologies. In this study, to reveal the responding mechanism and to identify proteins related to drought responses in *A. Mira,* we used physiological and proteomic methods. Therefore, we carried out different physiological measurements on *A. Mira* seedlings after drought treatment. Moreover, we also compared the two-dimensional electrophoresis (2-DE) leaf proteome of seedlings of *A. Mira* that demonstrate the positive responses to drought.

Materials And Method

Plant materials and experimental design

The experiments taken place at Northeast Forestry University. All seeds were obtained from College of Agriculture and Animal Husbandry, Tibet University. The seedlings (1-year-old) were planted in plastic pots containing a 1:3(v/v) mixture of meteorite and soil. Potted seedlings were grown for 20 days in a greenhouse under natural conditions. Seedlings as control treatment were watered every 4 days with a half-strength of Hoagland solution. Experiments were divided into two groups: well -watered seedings were irrigated every 4 days as control, for the water deficit treatment, the irrigation of seedlings was withdrawn for 16 consecutive days until re-watering (day 16).

At each time point (day 4, 8, 12, 16 and 20), the leaves of control and treatment seedlings were harvested. Then all leaves were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Each treatment group was conducted with three independent biological replicates.

Determination of physiological parameters Leaf water content (LWC) and soil water content (SWC)

Four fresh leaves from each treatment were collected from three randomly selected seedlings, measured fresh weighed (FW)immedia, then dried at 70ºC till constant weight (DW). Leaf water content (LWC) was estimated as follows (Wang et al., 2013): LWC (%) = (FW−DW)×100/ FW.

Soil (10g) from each plastic pot were collected from three randomly selected seedlings, weighed (FWC), then dried at 105ºC till constant weight (CWC). Soil water content (SWC) was estimated as follows (Wang et al., 2013): SWC (%) = (FWC−CWC)×100/ FWC.

Measurement of gas exchanges of leaves

Gas exchange measurements were made on clear and cloudless weather days at 9:00-11:00, non-detached fully expanded leaves with a Portable Photosynthesis System (LI-6400, LI-COR Inc., Lincoln, NE, USA). The artificial light source was set to 1200 μmol/m^2/s, CO_2 concentration was the atmospheric CO_2 concentration, relative humidity was 60%–70%, air flow rate was 400 μmol/s, and leaf chamber temperature was 28 ± 2°C. Five seedlings per treatment were selected to measure net photosynthesis rate (Pn), stomatal conductance (*G*s), transpiration rate (*T*r) and intercellular CO_2 concentration (*C*i).

Determination of relative conductivity, $O_2^{\cdot-}$ and H_2O_2

The relative conductivity (REC, %) of leaves was accessed according to the method of (Cavalcanti et al., 2007). Four leaf discs (1 cm^2 or so) were placed into the tubes containing distilled deionized water about 20ml. The tubes were incubated at 30 ºC for 5 h and the initial electrical conductivity (EC_{min}) was determined, and then the tubes were boiled at 100ºC for 30 min. After the tubes were cooled to room temperature, the final electrical conductivity (EC_{max}) was measured. The relative conductivity was calculated: REC (%) = EC_{max}/EC_{min} ×100.

For measurement of $O_2^{\cdot-}$ production rate, 0.5 g fresh leaves were grinded and mixed in solution containing EDTA (0.1 mM), PVP (1%, *w*/*v*), PMSF (0.1 mM), and Triton X-100 (0.2%, *v*/*v*). Then, the mixture was centrifuged at 12 000 rpm for 15 min at 4 °C on the supernatant (1 mL) was mixed with hydroxylamine hydrochloride (1 mL), β-aminobenzene sulfonic acid (1 mL), and 1 mL *α*-naphthylamine. Finally, the solution was incubated at 25 °C for 20 min. The absorbance of the mixture at 530 nm using a $NaNO_2$ standard curve was measured to determine the concentration of superoxide radical (Wang et al., 2013).

H_2O_2 content was determined by the modified method of (KawAsAki et al., 2000). 0.5 g leaves were homogenized in 0.1% cold trichloroacetic acid (TCA) (4 ml) and centrifuged at 12 000 rpm for 10 min. Then the supernatant (0.5 ml) was put into the mix containing 1 M potassium (1 ml) and potassium phosphate buffer (0.5 ml, 50 mM, pH = 6.8). After reaction for 5 min, absorbance values were calculated to standard curve at 560 nm.

Assays of SOD, POD, APX, GR, DHAR and MDHAR

0.5 g leaves were ground into a fine powder with a mortar and pestle in liquid nitrogen and dissolved in potassium phosphate buffer (10 mM, PH = 7.0) containing Ethylenediaminetetraacetic acid (EDTA, 1mM) and polyvinylpyrrolidone (1%). Then, the mixture was centrifuged at 12000 rpm at 4ºC for 30 min and the supernatant was used to analyze enzyme.

SOD (EC1.15.1.1) activity was estimated following the method of Hernández et al (1993). The reaction mixture contained 50 mM potassium phosphate buffer (pH = 7.8), 195 mM methionine, 0.3 mM ethylenediaminetetraacetic acid, 1.125 mM NBT and 60 μM riboflavin. The SOD activity was detected at 560 nm (Beauchamp et al., 1971). The SOD amount which is required to inhibit 50% epinephrine oxidation is considered as one unit of the enzyme activity.

POD (EC1.11.1.7) activity was assayed as previous method with minor modifications. The assay mixture contained 2 ml potassium phosphate buffer (50 mM, pH = 7.8), 25 μl extraction enzyme, 14 μl guaiacol and 19 μl H_2O_2 (30%,v/v). POD activity was measured at 470 nm., and the POD activity was indicated as H_2O_2 reduced.

APX (EC1.11.1.11) activity assay was measured using the method of Nakano et al (1981). The reaction mixture contained potassium phosphate buffer (50 mM, pH = 7.8) including 2 mM H_2O_2 and 200 µl ascorbic acid (AsA). APX activity was measured at 290 nm. The enzyme activity of APX was expressed as degree of oxidation of AsA.

GR (EC1.6.4.2) activity was determined by the method of Carlberg *et al.* (1975). The reaction mixture contained potassium phosphate buffer (100 mM, pH = 7.8), EDTA (2 mM), NADPH (0.2 mM), and glutathione (0.5 mM). GR activity was determined by NADPH oxidation at 340 nm.

For measurement of DHAR activity (EC 1.8.5.1), the reaction solution contained DHA (0.5 mM) and reduced glutathione (GSH, 5 mM). DHAR activity determined at 265 nm, following the method of Dalton et al (1986).

MDHAR (EC 1.6.5.4) activity was determined according to the method of Hossain et al (1984). The reaction mixture contained NADH (0.2 mM), AsA (1 mM), and AsA oxidase (1 U). MDHAR activity was measured at 340 nm. One unit of AsA oxidase is equal to the amount of the enzyme that oxidizes 1 mM of AsA to monodehydroascorbate acid per min.

Measurements of ascorbate acid (AsA)and Glutathione (GSH)

AsA content was determined using the method of Law et al (1983). AsA supplies electrons to AsA peroxidase scavenging H_2O_2, and is oxidized to monodehydroascorbate. Monodehydroascorbate is regenerated to AsA by the reduction of monodehydroascorbate reductase (MDHAR), while the escaped monodehydroascorbate from this reduction disproportionates to oxidized AsA (dehydroascorbic acid, DHA). Then, dehydroascorbate reductase (DHAR) reduces DHA to AsA with the reducing power supplied by the oxidation of GSH to oxidized GSH (GSSG). The calibration curve prepared from L-ascorbate (0–100 µg) was used for calculation of total and reduced ascorbate. The reaction mixture contained phosphate buffer (0.5 ml, 150 mM, pH7.4), double distilled water (0.2 ml), α'-dipyridyl (0.4 ml) and $FeCl_3$ (0.2 ml, 3%). The mixtures were incubated at 40ºC for 40 min and centrifuged at 12 000 rpm for 20 min. The supernatant was collected and the absorbance was monitored at 525 nm.

GSH content was carried out following the method of Law et al (1983). The calibration curve prepared from GSH (0–100 µg) was used for calculation. GSH content was determined according to the absorbance of reduced chromogen and DTNA (5,5'-Dithiobis, 2-nitrobenzoic acid) at 412 nm.

Protein Extraction and Quantification

0.5 g leaves were ground in a fine powder with a mortar and pestle in liquid nitrogen and transferred into the tube contained 10% acetone and 1% Polyethylene pyrrolidone, then the mixture was stored at -20 °C overnight. After centrifugation at 13000 rpm at 4°C for 20 min, the precipitate was washed with ice-cold 80% then 100% cold acetone and centrifuged at 13000 rpm at 4°C for 20 min. After centrifugation, the precipitate was vacuum dried. Then, the dried powder was dissolved in lysis buffer (7 M urea, 2 M

thiourea, 4% CHAPS, 40 μM DTT and 0.2% 2% (v/v) pH 4–7 IPG buffer). Protein concentration was determined using Bradford assay using BSA as the standard (Bradford, 1976).

Gel electrophoresis, gel staining and image analysis

Two-dimensional gel electrophoresis(2-DE) was carried out according to the method of Wang et al. 50 mg protein samples were used for isoelectric focusing (IEF). The IEF procedure consisted of the application of 30 V for 14 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 0.5 h and 8000 V for 5 h. After IEF, gels were equilibrated in 10 ml equilibration buffer containing 0.1 g DTT for 15 min. Then, gels were incubated in equilibration buffer containing 1.5 g iodoacetamide instead of DTT for 15 min. Second dimension SDS-PAGE was carried out in a 12.5% (w/v) polyacrylamide gel. After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) R-250 solution.

Gel images were scanned using Image Scanner system (GE Healthcare, Bio-Sciences, Uppsala, Sweden) and analyzed by software (Amersham Biosciences, Piscataway, NJ, USA, 2011). The average volume % values were calculated from three technical replicates to represent the final volume % of each biological replicate.

Matrix-Assisted Time of Flight Mass Spectroscopy (MALDI-TOF-MS) Analysis

Selected protein spots from gels were excised, washed with 50% (v/v) acetonitrile in 0.1 M NH_4HCO_3 twice, and dried at room temperature. Proteins were reduced with 1 mM DTT and 2 mM NH_4HCO_3 at 55 °C for 1 h and alkylated with 55 mM iodoacetamide in 25 mM NH_4HCO_3 in the dark at room temperature for 45 min. The gel pieces were thoroughly washed with 25 mM NH_4HCO_3, 50% acetonitrile, and 100% acetonitrile, and then dried. The proteins were digested in 10 ml modified trypsin (Promega, Madison, WI, USA) solution (1 ng/ml in 25 mM NH_4HCO_3) during an overnight incubation at 37 °C. Digests were immediately spotted onto 600 mm anchorchips (Bruker Daltonics, Bremen, Germany). Spotting was achieved by pipetting 1 ml analyte onto the MALDI target plate in duplicate and then adding 0.05 ml 20 mg/ml α-CHCA in 0.1% TFA/33% (v/v) ACN, which contained 2 mM ammonium phosphate. All samples were analyzed in the positive-ion reflection mode on a TOF Ultraflex II mass spectrometer (Bruker Daltonics, Billerica, United states). Each acquired mass spectra (a m/z range of 700–4000 and a resolution of 15,000-20,000) was processed using Flex Analysis v2.4 software (Bruker Daltonics, Bremen, Germeny,2004). Proteins were identified with Mascot software (http://www.matrixscience.com) based on the mass signals used to search for proteins in the Swiss-Prot, NCBI, and MSDB databases.

Protein Classification and Hierarchical Cluster analysis

Each protein motifs were classified based on the BLAST alignment, Gene Ontology, and information from the literature. The hierarchical clustering of all proteins was performed according to fold change values of protein spots.

RNA isolation and Quantitative RT-PCR analysis

Quantitative real-time PCR (qRT-PCR) was performed to confirm the differential expression of DEPs. Total RNA was isolated from the leaves using plant RNA extraction kit (Biotecke, China) and reverse-transcribed by PrimeScript Reverse Transcriptase (Takara, Japan). The cDNA was amplified in Lightlycler480 system (Roche, USA). The gene expression level was detected by SYBR Green Realtime PCR Master Mix (Toyobo, Japan). Protein code and forward and reverse primer sequences were listed in Table S3. ACTIN was used as the family gene (Table S3). The relative expression level of target genes was calculated using $_{\triangle\triangle}$Ct method.

Statistical analysis

Statistical analyses were performed with SPSS 17.0 software (SPSS Inc. Chicago, IL, USA, 2009). All parameters are presented as mean ± standard error and were obtained from at least three replicates. Parameters were analyzed using Duncan's multiple range test or Student's t-test. A *p*-value <0.05 was considered significant.

Results

Effect of drought and re-watering on plant growth

The leaves of seedlings did not exhibit any obvious phenotype differences on day 4, 8 and 12 of drought stress (Fig. 2A a, b, c), however, because of continuous drought, plants showed wilting on day 16 of drought stress (Fig. 2A d). Additionally, re-watering on day 20 could remarkably improve the morphology of drought-stressed plants (Fig. 2A e).

Leaf water content was maintained at approximately 75% in leaves under controls but gradually decreased to 20% or so on day 16 of drought stress and rapidly increased on day 20 of re-watering (Fig. 2B). Similarly, soil water content did not differ significantly under controls. Drought treatment significantly decreased soil water content on day 16 (Fig. 2C).

Effect of drought and re-watering on Relative conductivity, $O_2^{\cdot-}$ and H_2O_2

Relative conductivity of leaves on seedlings subjected to drought stress did not differ significantly compared with control seedlings on day 4, 8 and 12 of treatment, only showed a single peak on day 16 of drought stress, and restored back to control levels after re-watering on day 20 (Fig. 2D). Similarly, $O_2^{\cdot-}$ content was significantly higher under drought stress (on day 12 and 16) than it was under control conditions, and restored back to control levels after re-watering (Fig. 2E). The highest value of H_2O_2 of leaves on drought treated seedlings was observed on day 16 when drought stress was severe compared to control seedlings, whereas this difference was not observed on day 4, 8 and 12 of drought stress and day 4 of re-watering (day 20) (Fig. 2F).

Effect of drought and re-watering on Photosynthesis

Four main photosynthetic parameters were measured to determine drought severity: *P*n, *Ci*, *G*s and *T*r. *P*n, *G*s and *T*r of the drought treated seedlings gradually decreased on day 12 and 16 in comparison with control seedlings, and increased to controls levels on the fourth day after re-watering. In contrast, *C*i remained higher value on day 12 and 16 of drought stress before declining after re-watering (Fig. 3).

Effect of drought and re-watering on antioxidant enzymes activities

The activities of SOD, APX, DHAR, GR and MDHAR did not manifest obvious variation between drought treated seedlings and control seedlings at 4, 8 and 12 days, but they were significantly increased by drought treatment compared with controls (day 16) (Fig. 4). After re-watering, the parameters were recovered to controls levels, and the activities of APX, DHAR and MDHAR of drought treated seedlings were higher those in control conditions (Fig. 4). POD activity declined rapidly by drought treatment, and the highest value was recorded on day 16 when drought stress was severe, whereas it decreased upon re-watering (Fig. 4).

Effect of drought and re-watering on AsA and GSH

No differences were observed in AsA between drought treated seedlings and control seedlings at 4, 8 and 12 days, and it were higher under drought stress compared to those of control seedlings on day 16 of drought stress (Fig. 5A). Compared with control seedlings, after drought treatment, GSG content of drought treated seedlings was significantly higher than that of control seedlings, but it largely decreased upon re-watering (Fig. 5B).

Identification of Differentially Expressed Proteins under drought stress and re-watering

The image analysis of 2D gels revealed that approximately 1,000 protein spots were detected (Fig. 6A), while 103 protein spots were detected as differentially expressed spots (DEPs) ($p < 0.05$). All these DEPs were submitted for protein identification. Based on the BLAST alignment, Gene Ontology, and information from the literature, 103 DEPs in leaves were classified into 10 functional categories: Cytoskeleton dynamics, Energy metabolism, Carbohydrate metabolism, Photosynthesis, Transcription and translation, Transport, Stress and defense, Molecular chaperones, Other materials metabolism, and Unknown function. The distributions of proteins with different functions expressed in the proteome of leaves is illustrated in Fig.6C and detailed information of functional classification of all DEPs were listed in Tables S1 and S2, respectively. The most represented DEPs in leaves were associated with stress and defense (28.2%), most of which showed increased levels on day 16 of drought stress. Moreover, a total of 21 Photosynthesis (20.4%) related DEPs in leaves were identified.

The hierarchical clustering analysis was carried out to analyze protein expression characteristics in each functional category (Fig. 6B). All DEPs were clustered into two main groups. A larger number of proteins involved in photosynthesis and stress/defense in Group Ⅰwere up-regulated on day 16 of drought and recovered to control levels after re-watering, whereas main proteins in Group Ⅱ were down-regulated clusters on day 16 of drought and recovered to control levels after re-watering (Fig. 6B). These proteins

having same function were clustered in the same group, suggesting that they might play key roles in different stages of drought.

Leaf Proteomic Characteristics under Drought Stress and Re-watering

21 proteins involved in photosynthesis were identified. Chlorophyll a/b binding protein (spot 36 and 37) was induced on day 16 of drought stress and declined to controls levels on day 4 after re-watering. CO_2 assimilation related proteins including carbonic anhydrase (Spot 41), ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Spot 11) and Ribonucleo protein (Spot 43) were increased on day 16 of drought stress. In addition, Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic (Spot 98), Photosystem II stability/assembly factor HCF136 (Spot 100) and carbonic anhydrase 2, chloroplastic-like isoform X1 (Spot 102) were detected only on day 16 of drought stress.

29 stress-defense-related proteins were identified. A slice of enzymatic antioxidants, e.g., glutathione S-transferase (GST) (Spots 39 and 75), peroxiredoxin (Prx) (Spots 103 and 104), catalase (CAT) (Spots 32) and abscisic acid stress ripening protein homolog (Asr1) (Spots 38), were up-regulated in expression on day 16 of drought stress. Several proteins, such as arginase (Spot 18 and 26), plastid-lipid-associated protein (Spot 30) were also induced after drought treatment (day 16). In contrast, putative glycine-rich RNA-binding protein (Spot 66) and putative luminal binding protein (Spot 89) showed up-regulated on day 4 and down-regulated after drought treatment (day 16), respectively.

We also identified 16 proteins involved in carbohydrate metabolism, of which 11 proteins were up-regulated after drought treatment (day 16). Interestingly, on day 16 after drought treatment the changes in the abundance of prunasin hydrolase isoform PH B precursor were not uniform: two proteins (Spot 76 and 81) increased, but one (spot 90) decreased in expression.

11 proteins involved in energy metabolism mainly included ATP synthases (Spot 76 and 81) and NADH dehydrogenase (Spot 51, 63). Most of these proteins were reduced in drought-treated seedlings on day 16, while two proteins (Spot 22 and 72) increased in expression.

8 proteins involved in molecular chaperones in leaves, including heat shock protein (Spot 1. 106 and 109), Calreticulin (Spot 5), endoplasmin homolog (Spot 2), Endoplasmin-like protein (Spot 3) and so on, were found to be remarkably increased by drought treatment (on day 16). The abundance of two proteins related to transport (Spot 35 and 107) increased by drought treatment (on day 16). Similarly, three proteins (Spot 47, 78 and 79) involved in cytoskeleton dynamics were also induced by drought treatment (on day 16). In addition, three proteins involved in cytoskeleton dynamics reached maximal accumulation on day 16 of drought treatment.

Validation of DEPs at transcript levels by qRT-PCR

To confirm the differential expression of DEPs, quantitative real-time PCR (qRT-PCR) was carried out (Fig. 7; Fig. S1). We selected 12 candidate genes representing a variety of functional categories including

cytoskeleton dynamics (Spot 84), carbohydrate metabolism (Spot 80), transcription and translation (Spot 49), stress and defense (Spot 28, 34, 38, 86, 87, 91, 104), and molecular chaperones (Spot 1). Overall, we found weak correlation of transcript levels between proteome and qRT-PCR. Only protein spot (Spot 28) involved in stress and defense had consistent expression at transcript and protein levels (Table S2; Fig. 7).

Discussions

Two mechanism including to drought avoidance and drought tolerance were used to adapt to drought stress (Kooyers 2015). By drought avoidance, plants often reduce growth to save energy cost and reduce water losses (Zenda et al., 2018). Compared to controls seedlings, growth of *A. Mira* seedlings was inhibited after drought treatment, demonstrating the suppression of drought on growth of *A. Mira.* However, a significant recovery of growth can be observed after re-watering, which is also demonstrated that *A. Mira* seedlings can rapidly adjust water balance to adapt water losses. In addition, our proteomics results also revealed that most proteins involved in energy were decreased after drought treatment. This implies that low levels expression of energy protein contributed to the decline of growth.

To reduce water losses, stoma might be rapidly closed in response to severe drought (Mcadam et al., 2013; Li et al., 2015). Accordingly, net photosynthetic rate (*P*n), stomatal conductace (*G*s), transpiration rates (*T*r) would be inhibited due to stoma closure. When the pores are closed, the carbon dioxide required for photosynthesis cannot enter the tissue where light and action occur, so photosynthesis is weakened; the increase in carbon dioxide concentration (*Ci)* is due to the increase in carbon dioxide produced by cellular respiration and cannot be discharged from the pores. In the present study, photosynthetic parameters including *P*n, *G*s and *T*r of leaves of *A. Mira* seedlings decreased after drought treatment. In contrast to the decline of these parameters upon drought, a significant recovery was detected after re-watering, suggesting that *A. Mira* seedlings can enhance growth recovery after drought treatment by adjusting gas exchanges.

Generally, inhibition of photosynthesis would induce the production of reactive oxygen species (ROS), which can lead to oxidative damage in cells (Møller et al., 2001). To enhance drought tolerance, plants can counteract or scavenge ROS accumulation by antioxidant enzymes and non-enzymatic molecular (Bowler et al., 1992; Smirnoff et al., 2000; Mittler et al., 2002). In the current study, the contents of relative conductivity, $O_2^{\cdot-}$and H_2O_2 of leaves on *A. Mira* seedlings were significantly decreased after 16

days of drought (Fig.2). All these changes indicated that drought stress led to membrane damage. After re-watering, the level of these parameters returned to normal levels. For *A. Mira* seedlings, the activities of SOD, APX, DHAR, GR and MDHAR elevated after drought treatment (16 days), whereas they rose after re-watering. Furthermore, our proteomics results also revealed that the expression levels of three antioxidative enzymes (CAT, GST, and Prx) were increased after drought treatment (Table S2). In addition, non-enzymatic low molecular, such as AsA + DHA and GSH +GSSG, was consistently up-regulated after drought treatment. These results demonstrated that sensitive switch of ROS and active antioxidant

capacity between drought and re-watering might be the major mechanism for *A. Mira* seedlings to tolerate drought efficiently.

To prevent damage and maintain a balance of metabolites, energy is required by plants. In this study, most proteins involved in energy showed less accumulation after drought treatment (on day 16). These results suggest *A. Mira* seedlings were able to deal with drought stress through low energy metabolism. Various processes, such as transporting ion, synthesis of osmolytes and scavenging of ROS are regulated by energy. Comparative proteomic analyses also showed that when some plant species were subjected to environmental stress, some DEPs are enriched among those involved in the processes of energy metabolism (Wang et al., 2016)., our results are not like this, This may be due to that *A. Mira* seedlings had different the strategies of energy metabolism under drought stress.

Drought can lead to oxidative damage and ROS production. Accordingly, plants balance oxidative stress by inducing various stress-defense-related proteins. In our experiment, some stress-defense-related proteins were significantly induced by drought stress (on day 16) (Table S2). Especially, two 2-Cys peroxiredoxin proteins (Prx) (Spot 103 and 105) were detected on day 16 of drought stress. It is widely known that peroxiredoxins possess antioxidant capabilities (Rhee et al., 2012). 2-Cys Prx is a member of the peroxiredoxin family of peroxidases and plays an important role in cell protection against oxidative stress by detoxifying peroxides and as sensor of hydrogen peroxide-mediated signaling events (Dietz et al., 2006; Jang et al., 2010). Here, the up-regulated expression of 2-Cys Prx suggested that an antioxidant system was induced in leaves of *A. Mira* seedlings in response to drought stress. Two Glutathione S-transferase (GSTs) (Spot 39 and 75) were also detected after drought treatment. GSTs are multifunctional proteins involved in response to the oxidative stress including drought, heavy metals, salt, and so on (Marrs et al., 1996; Wagner et al.,2002). It was suggested that GSTs are important to A. Mira seedlings in stress tolerance. In addition to GSTs, here, two stress related protein catalase (CAT) (Spot 13 and 91) was responsive to the drought treatment. Interestingly, one was decreased and another was increased in expression on day 16 of drought stress. Its function in drought tolerance of *A. Mira* seedlings is not clear. Previous researches showed that stress-defense-related proteins were significantly regulated in various plant species under drought stress. Therefore, stress-defense-related proteins should play key roles in the response to drought stress.

To avoid drought damage, plants can close stoma or/and reduce stomatal density (Chaves et al. 2003). Accordingly, photosynthesis and growth of plants will be reduced due to decreased carbon uptake. In this study, CO_2 assimilation related proteins (Spot 11, 41 and 43) were up-regulated after drought stress (on day 16). However, it should be noted that some photosynthesis-related proteins (Spot 98, 100 and 102) were actually down-regulated. Thus, the physiological significance of these protein different changes need be studied further. We also identified 11 up-regulated proteins involved in carbohydrate metabolism after drought treatment (16 days), suggesting the positive adaptation in carbohydrate metabolism of leaves on *A. Mira* seedlings to drought stress. Enhancement of Glucose–6-phosphate isomerase (Spot 14) and fructokinase (Spot 32 and 81) implied that the TCA cycle was increased under drought stress. Or

ATP will be produced by more carbon substrates. Indeed, our results are consistent with previous proteomic studies.

Here we found that in *A. Mira* seedlings two heat shock-related (Hsps) proteins were responsive to drought stress (Fox et al., 2018; Gka et al., 2018). Three Hsp proteins (Spots 1, 106 and 109) were increased in abundance after drought treatment. Members of Heat shock proteins can help to fold an ocean of proteins and assisted folding involves repeated cycles of substrate binding and release according to regulating osmotic stress tolerance (Waters et a., 1996). The increased abundance of Hsps after drought treatment suggests a increase in the transportation of newly synthesized peptides, which may facilitate intercellular transportation of vital cellular enzymes or maintain the integrity of quiet a few proteins under drought stress.

To interpret protein function and stress responses, the relationship between protein abundance and gene expression should be analyzed. Here, we found weak most proteins abundance is not consistent with mRNA levels except for Spot 28. Generally, the relationship between protein and mRNA expression levels attribute to mRNA stability and gene expression regulation. Therefore, low degree of correlation between transcription and translation to *A. Mira* seedlings under drought stress and re-watering can due to post translational regulation. These comparative results also indicated the importance of employing proteomics to reveal tolerance mechanisms to drought.

Conclusion

In conclusion, the reduction in growth, photosynthesis and transpiration are the foremost tactics of *A. Mira* for surviving under drought condition. At the physiological level, drought stress reduced leaf water content and soil water content, and *Amygdalus mira (Koehne)* Yü et Lu responded to drought by changed the levels of antioxidant enzymes activities, non-enzymatic, the relative conductivity and so on. By analyzing proteins in the treatment and control groups over time, we support quantitative evidence regarding how biological processes are regulated during drought and re-watering and confirmed the differential expression of DEPs. Compared with their controls, *A. Mira* showed stronger adaptive and resistant characteristics to drought stress. In summary, a conclusion can be drawn from the experiment that Physiological and proteomic responses to drought stress in leaves of *Amygdalus Mira (Koehne)* Yü et Lu.

Additional File

Additional file 1: Fig. S1. Semi-quantitative PCR analysis of twelve genes and ACTIN of Amygdalus mira (Koehne) Yü et Lu leaves after drought treatment and re-watering, respectively.(DOCX 145 kb)

Additional file 2:Table S1. Differentially expressed proteins in leaves of Amygdalus mira (Koehne) Yü et Lu by mass spectrometry (MS) analysis.(DOCX 184 kb)

Additional file 3:Table S2. Expression levels of differentially expressed proteins in leaves of Amygdalus mira (Koehne) Yü et Lu after drought treatment and re-watering.(DOCX 34 kb)

Additional file4:Table S3. The primer sequences for Real-time PCR.(DOCX 14 kb)

Declarations

Acknowledgment

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Authors' contributions

Liping Xu and Guangze Jin did all experiment, interpreted the results, and wrote the manuscript. Qiuxiang Luo and Fanjuan Meng designed the experiment and performed technical guidance. Fachun Guan provided assistance in the data analysis. Liping Xu and Guangze Jin contributed equally to this work. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing financial interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Figures

Figure 1

Plants grown in natural environment of the Tibetan Plateau

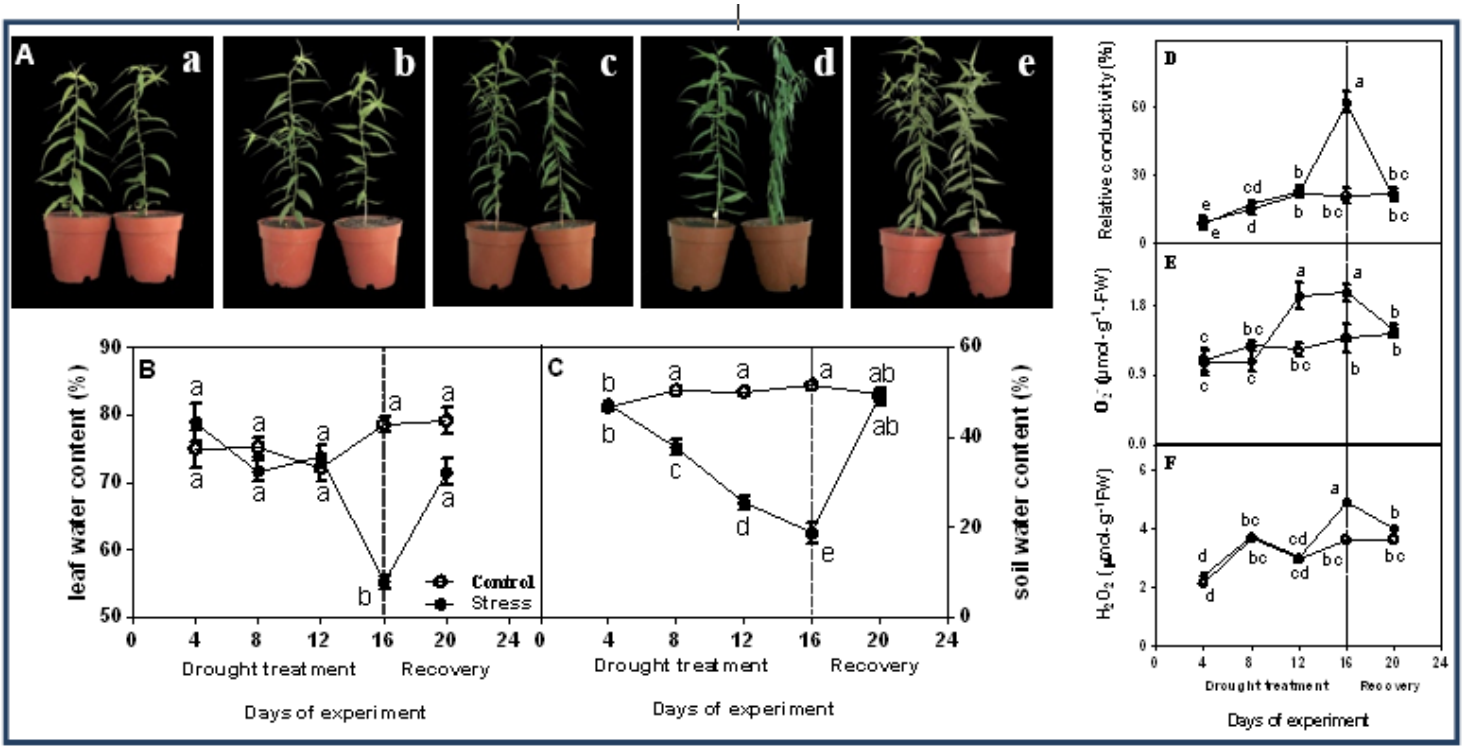

Figure 2

The morphological traits (A) , a, b, c, d and e indicted the electrophoresis gels of protein of seedlings leaves during drought treatment (4d, 8d, 12d, 16d) and recovery (4d), leaf water content (B), relative conductivity (D), O2·- (E) and H2O2 (F) of leaves on seedlings growing under control and drought stress, and soil water content (C). Data shown are the mean ± SE of three biological replicates. Different letters indicated significant differences exist at the P ≦ 0.05 level.

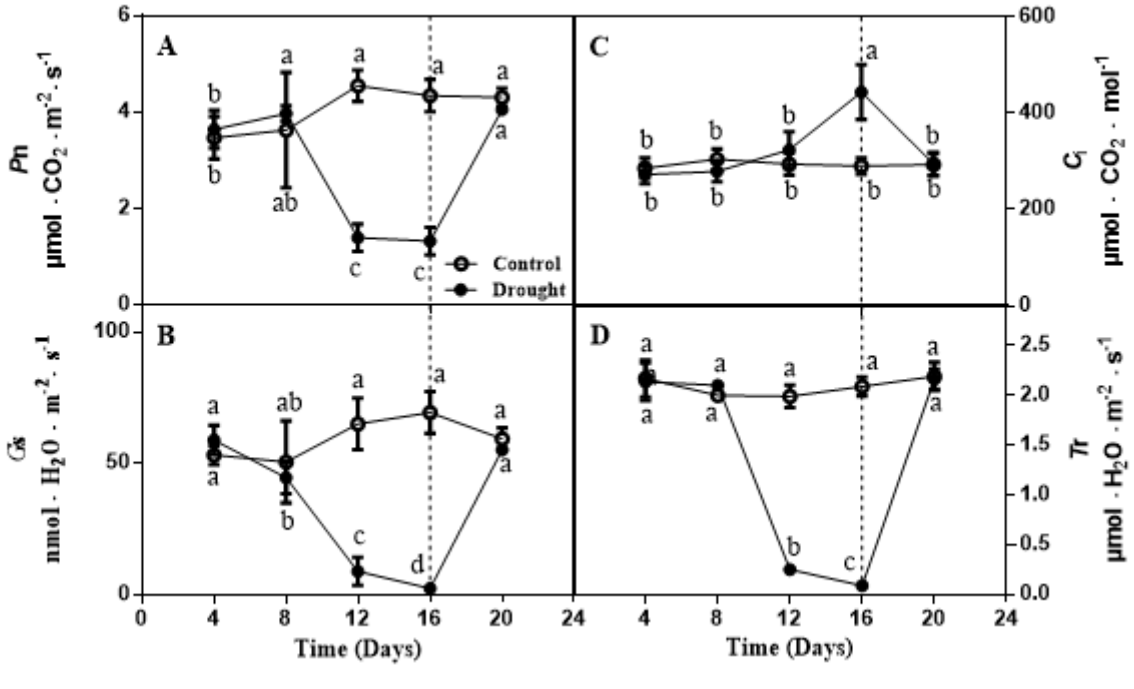

Figure 3

The changes of Pn (A), Gs (B), Ci (C) and Tr (D) of leaves on seedlings growing under control and drought stress and re-watering. Data shown are the mean ± SE of three biological replicates. Different letters indicated significant differences exist at the P ≦ 0.05 level.

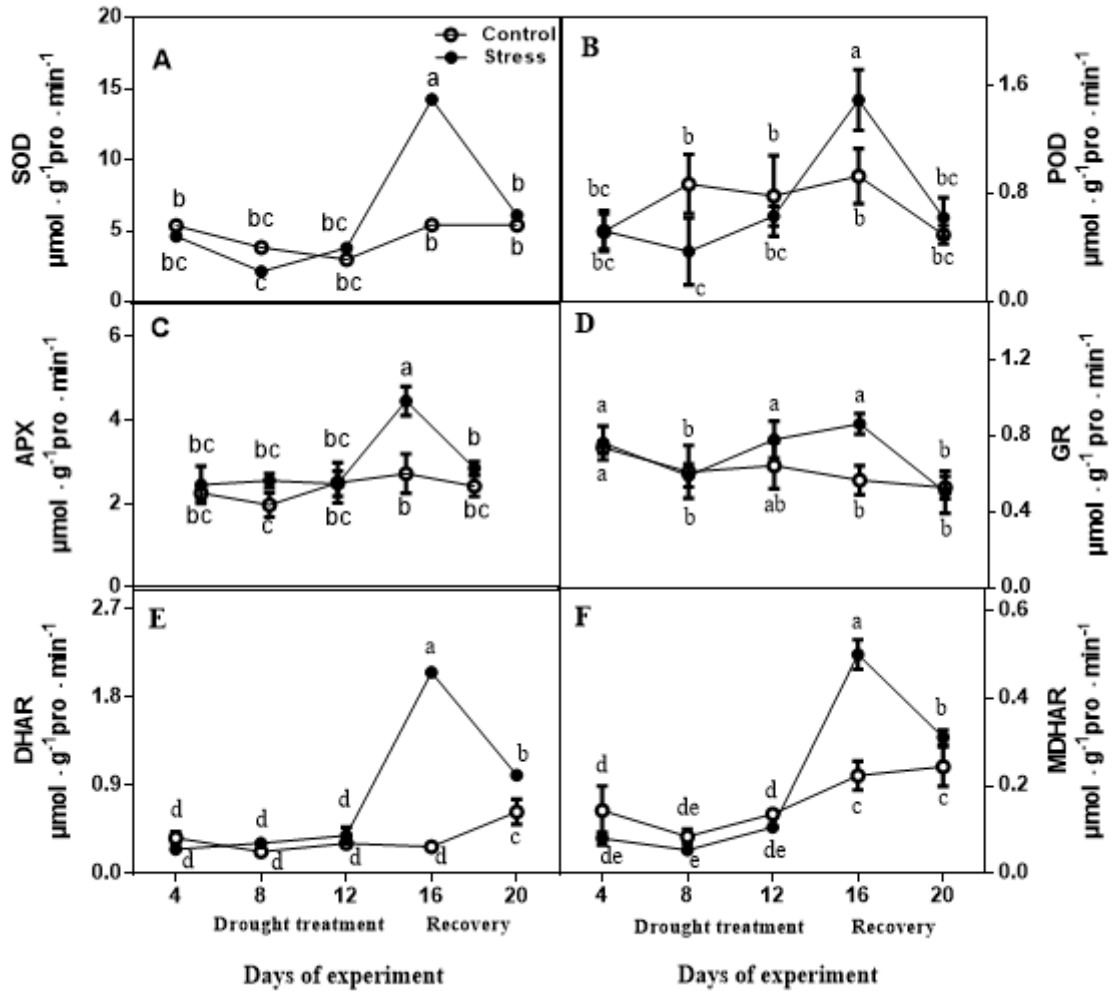

Figure 4

The changes of SOD (A), POD (B), APX (C), GR (D), DHAR (E) and MDHAR (F) of leaves on seedlings growing under control and drought stress. Data shown are the mean ± SE of three biological replicates. Different letters indicated significant differences exist at the P ≦ 0.05 level.

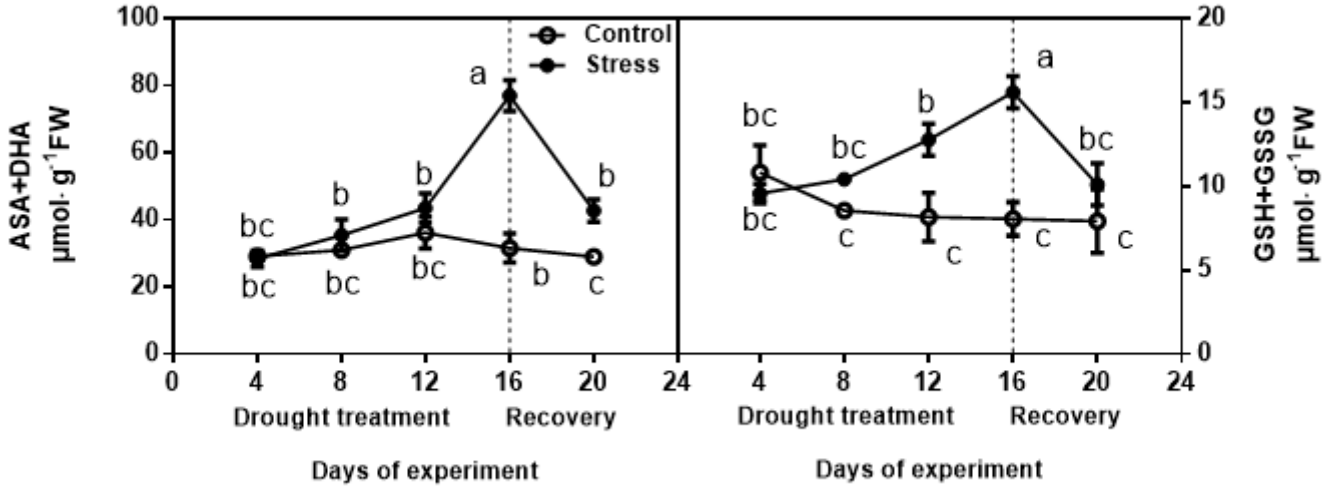

Figure 5

The changes of ASA+DHA (A), GSH+GSSG (B) of leaves on seedlings growing under control and drought stress. Data shown are the mean ± SE of three biological replicates. Different letters indicated significant differences exist at the P ≦ 0.05 level.

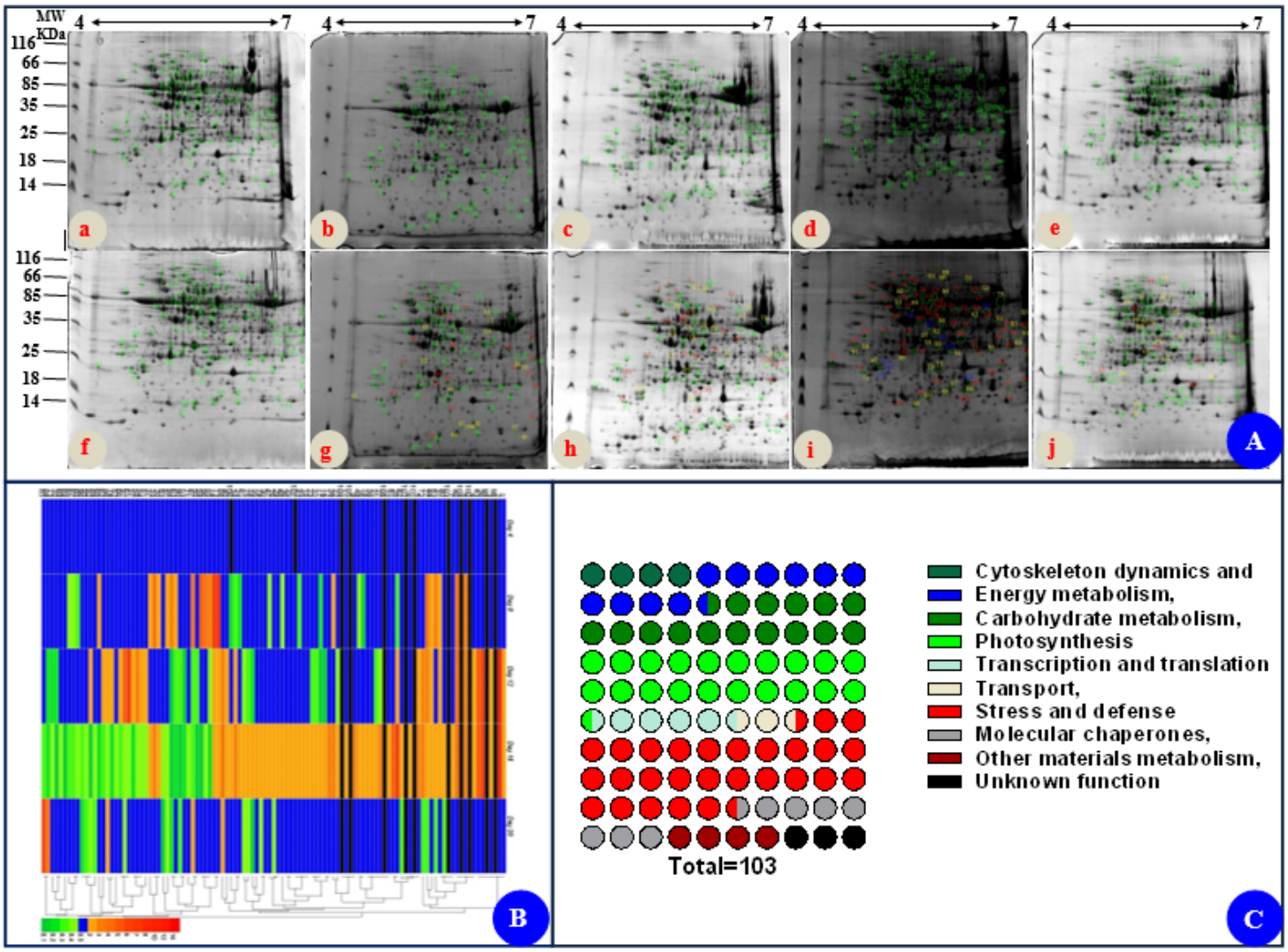

Figure 6

Coomassie Brilliant Blue stained two-dimensional electrophoresis gels of proteins (A), hierarchical clustering (B) and functional classification (C) of the identified proteins. a, b, c, d and e indicted the electrophoresis gels of protein of seedlings leaves during drought treatment (4d, 8d, 12d, 16d) and recovery (4d). f, g, h, i and j indicted the electrophoresis gels of protein of seedlings leaves under well-water.

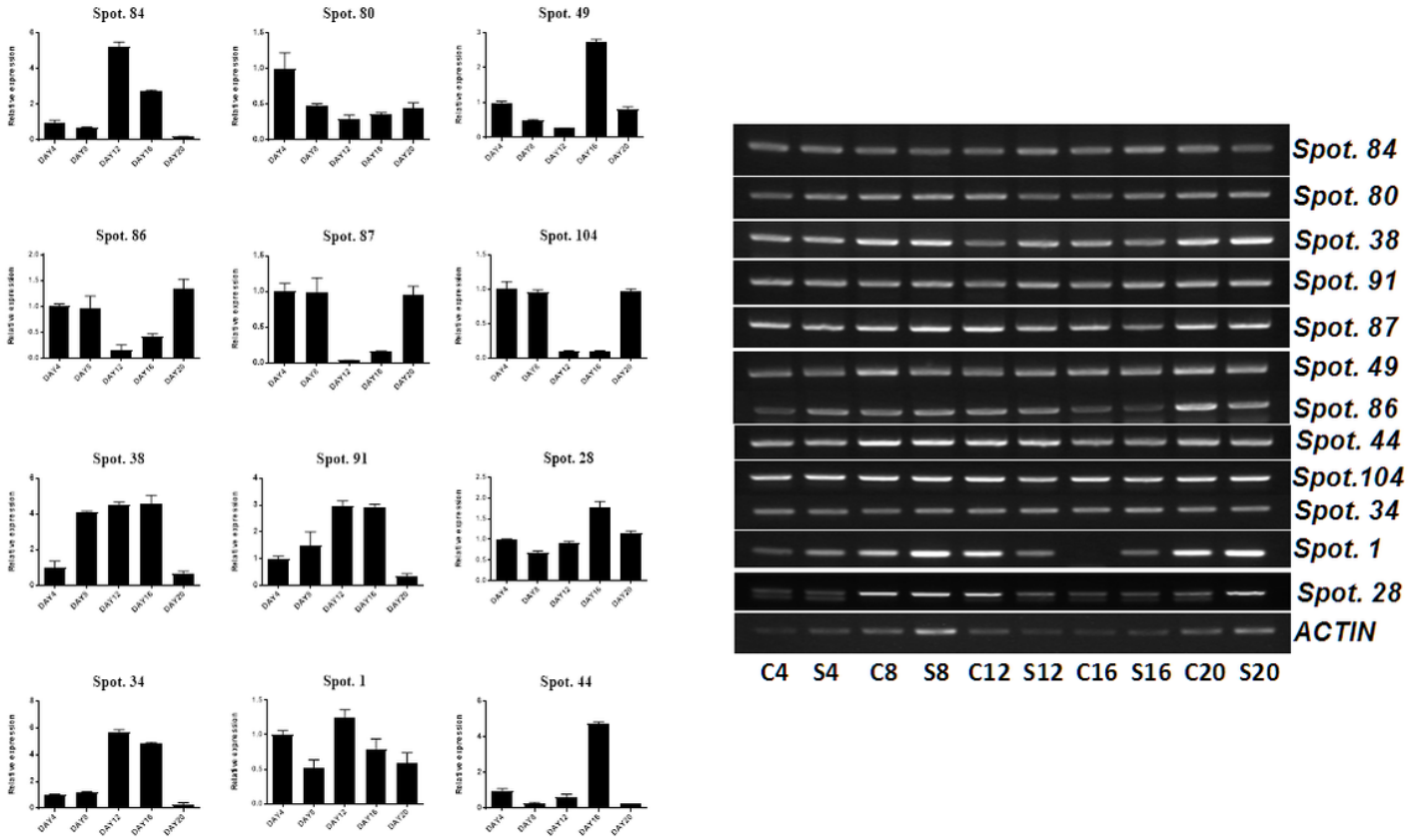

Figure 7

Relative gene expression levels of differentially expressed proteins by quantitative real-time PCR

Supplementary Files

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