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Comparative physiological and leaf proteome analysis between drought-tolerant chickpea *Cicer reticulatum* and drought-sensitive chickpea *C. arietinum*

Sertan Çevik[1]*, Gürler Akpinar[2], Aytunç Yildizli[3], Murat Kasap[2], Kübra Karaosmanoğlu[4] and Serpil Ünyayar[3]

[1]*Vocational School of Mut, Mersin University, Mersin, Turkey*

[2]*Medical Biology Department/Dekart Proteomics Laboratory, Kocaeli University, Kocaeli, Turkey*

[3]*Biology Department, Art and Science Faculty, Mersin University, Mersin, Turkey*

[4]*Biomedical Engineering Department, Technology Faculty, Kocaeli, Turkey*

**Corresponding author (Email, srtncvk@gmail.com)*

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Comparative physiological and proteomic analysis were performed to understand the stress responses of two chickpea species (*C. reticulatum* and *C. arietinum*) against drought. Our study revealed that drought stress reduced root length, leaf water content, and enhanced free proline content in both species. Effect of drought stress appeared to be greater in *C. arietinum* compared to *C. reticulatum*. A total of 24 differently expressed proteins were identified by using MALDI-TOF/TOF-MS/MS in response to drought. The proteins involved in photosynthesis and energy mechanisms were up-regulated in *C. reticulatum* and down-regulated in *C. arietinum* under drought. Our results suggest that the photosynthesis capacity of *C. reticulatum* is greater than that of *C. arietinum* under drought stress. Abundance of proline and sucrose biosynthesis related proteins, glutamine synthetase and cyctosolic fructose-bisphosphate aldolase, respectively, also increased in *C. reticulatum* under drought stress. The findings of this proteome analysis will help in understanding the mechanism of drought resistance in chickpea and may be also helpful in developing drought-resistant transgenic plants.

Keywords. Chickpea; drought stress; physiological analysis; proteomics

Abbreviations: APX, ascorbate peroxidase; COX, cytochrome c oxidase; FBA, fructose-bisphosphate aldolase; FNR, ferredoxin-NADP reductase; GME, GDP-mannose-epimerase; GS, glutamine synthetase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; IFR, ısoflavone reductase; LHCB, light-harvesting chlorophyll a/b-binding protein; LWP, leaf water potential; OEE1, oxygen evolving enhancer protein 1; OEE2, oxygen evolving enhancer protein 2; PGK, phosphoglycerate kinase; ROS, reactive oxygen species; RWC, relative water content; SBPase, sedoheptulose-1,7-bisphosphatase; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis

1. Introduction

Chickpea (*Cicer arietinum* L.) is a valuable agricultural crop, used as a nutrient source for human diet and animal feed (Pandey *et al.* 2008). It is one of the most important legumes in the world and yields a total of 11.6 million tonnes/annually. Although, chickpea has a high yield potential (4000 kg/ha), actual yields are quite low due to biotic and abiotic stresses (Canci and Toker 2009).

Drought stress affects various morphological, physiological and biochemical processes that causes loss of yields in plants. Developing tolerant varieties is one of the most important tolerance strategy to improve productivity in drought stressed condition but there are not enough adequate selection criteria for stress tolerance in chickpea (Toker and Cagirgan 1998).

The effects of drought stress on chickpea growth have been revealed by some researchers with morphological (Sabaghpour *et al.* 2006), physiological (Turner *et al.* 2007; Rahbarian *et al.* 2011), biochemical (Gunes *et al.* 2006; Mafakheri *et al.* 2010) and molecular parameters (Mantri *et al.* 2007; Garg *et al.* 2016) Although the effects of drought

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stress on plant growth are well known in general terms, the main effects of drought stress at the biochemical and molecular levels have not considerably been figured out yet (Lisar *et al.* 2012). Understanding the response mechanisms of plants to drought stress is important to develop strategies that improve drought tolerance in crops (Roy 2014).

At molecular level, plants react to stress conditions by changing gene expression levels. Several drought-inducible genes have been identified by transcriptome analysis in various plants (Zhou *et al.* 2007; Dong *et al.* 2014; Chung *et al.* 2016; Bai *et al.* 2017). Identified genes by these analyses do not always represent the actual dynamics of final gene products, the proteins (Jaiswal *et al.* 2014), because the levels of specific mRNAs do not always correlate well with the levels of proteins (Budak *et al.* 2013; Jedmowski *et al.* 2014). Proteins are key functional effectors of cellular processes, therefore to understand their biological system, the molecular characterisation of proteome should be figured out.

Proteomic studies offer an occasion to categorise patterns of protein accumulation during stress perception, adaptation and cell defense (Pawlowski 2009). Identifying new proteins, determining their expression patterns in drought response and considering their functions would provide the basis of effective engineering strategies to improve crop stress tolerance (Jaiswal *et al.* 2014). Faghani *et al.* (2015) reported that the integrated physiology and proteomic analysis provided a better insight into the molecular responses of plants during drought.

In this study, we carried out physiological, biochemical and proteomic analyses on the drought stress response of *C. arietinum* (ILC482, cultivar) and *C. reticulatum* (AWC611, wild type). The aims of the present study were (1) to evaluate the level of oxidative stress by measuring the physiological and biochemical traits (2), and to identify and compare changes in the leaf proteome of *C. arietinum* (cultivar) and *C. reticulatum* (wild) during drought stress to identify candidate proteins which may have important roles in stress tolerance mechanisms.

2. Materials and methods

2.1 *Plant material, growth conditions and drought treatment*

The seeds of *C. arietinum* L. ILC482 (cultivated chickpea) were obtained from GAP International Agricultural Research and Training Center and seeds of *C. reticulatum* Ladiz. AWC611 (wild type) were obtained from Akdeniz University Faculty of Agriculture. Seeds have been imbibed in aerated water for the first day at 22 °C. Then they were transferred to plastic pots (2 L) and filled with a soil: peat: manure mixture (2/1/1, 2000 g). All seedlings were grown at 24±2°C, 16/8 h photoperiod, irradiance 480 µmol $m^{-2}s^{-1}$, 65 ± 5 relative humidity up to twenty first days by irrigating (in Versalite Environmental Test Chamber, MLR-352H, Sanyo). Then half of the pots were well-watered (control) and other pots were exposed to drought stress by withholding irrigation for seven days. After that leaves of plants were harvested and immediately put into liquid nitrogen and stored at −80 °C for further analyses.

2.2 *Plant-water relations (leaf water potential, relative water content)*

The leaf water potential was measured by using pressure chamber (PMS Instrument Co., Model 1000). Leaf relative water content (RWC) was determined according to Smart and Bingham (1974), based on the following equation: $RWC = \frac{FW-DW}{TW-DW} \times 100$ where FW is leaf fresh weight, DW is dry weight of leaves after drying at 85°C for third days, and TW is the turgid weight of leaves after soaking in water for 4 h at room temperature. All measurements of water potential and relative water content were replicated five times per plot, and for each measurement a different plant was used.

2.3 *Determination of free proline content*

Free proline accumulation was determined by using the method of Bates *et al.* (1973). 0.5 g fresh leaf sample was homogenized with 3% sulfosalicylic acid (10 ml). After that the homogenate was centrifuged at 3000 rpm for 15 min, then 2 ml supernatant was mixed well with 2 ml acetic acid, 2 ml acid ninhydrin and boiled for 1h. After cooling of the tubes in ice, the products were extracted with 4 ml of toluene by vortex mixing and the toluene phase was decanted into a glass cuvette and its absorbance was measured at 520 nm and was determined by UV-visible spectrophotometer (Jena, Specord 210Plus). The proline concentration was calculated by using a calibration curve and expressed as µmol proline g^{-1} FW.

2.4 *Protein extraction*

Protein extraction was performed according to Kim *et al.* (2001) with minor modifications. Leaves were grounded into a fine powder in liquid nitrogen and transferred to pre-cooled Mg/NP-40 extraction buffer. After that, the homogenate was mixed with 15 % PEG4000 and centrifuged. The supernatant was precipitated by adding cold acetone and put at –20 °C overnight. The total proteins were collected through centrifugation and washed five times with ice-cold aceton. Then pellets were collected and resuspended in 2 ml suspension buffer. Protein concentration was determined using Bradford assay with the BSA standard (Bio-Rad, USA).

2.4.1 *Two-dimensional polyacrylamide gel electrophoresis-DIGE:* Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was performed according to Görg *et al.* (1988) with minor modifications. Isoelectric focusing (IEF) was performed at 20 °C by using Protean IEF system (Bio-rad,

USA). After IEF, the second dimension-SDS-PAGE was performed according to Laemmli (1970). Proteins on gels were visualized by using Colloidal Coomassie stain (Kera-Fast, USA).

For DIGE experiments, protein samples of each group and an internal standard were labelled prior to 2D-PAGE with CyDyes using the CyDye DIGE Fluor Dye Labelling Kit (GE Healthcare, USA) according to the manufacturer's protocol. After labelling, the labelled protein samples were mixed and the volume of the mixture was completed to 250 µl using 2DE buffer. First and second dimension separations were performed similar to the 2DE experiments (Unlu *et al.* 1997). To ensure the reproducibility of the gels, 2DE was repeated at least three times for each group of plants.

2.4.2 *Image analysis and spot cutting:* VersaDoc4000 MP (BioRad, USA) system was used to display the gels and to compare protein spot profiles PDQuest Advance (BioRad, USA) 2DE analysis software was used. For automated spot detection, parameters used were sensitivity (13.8), spot size scale (3) and minimum peak intensity (258). Automated analyses were performed to detect total spot numbers and volumes within the normalized area. A manual editing tool was used to inspect the determined protein spots detected by the software. Spots were cut by using automated spot cutting tool, ExQuest spot cutter (BioRad, USA), and disposed into 96 well plates for protein identification.

2.4.3 *Identification of proteins:* Protein identification experiments were performed at Kocaeli University DEKART proteomics laboratory by using ABSCIEX MALDI-TOF/TOF 5800 system. In-gel tryptic digestion was performed by using an in-gel digestion kit following the recommended protocol of the manufacturer (Pierce, USA). Zip-Tip (Millipore, USA) cleaning was performed for each digested sample before deposition onto a MALDI plate. Peak data were analysed with MASCOT by using a streamline software, Protein Pilot (ABSCIEX, USA). The parameters for searching were; enzyme of trypsin, 1 missed cleavage, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (M), peptide mass tolerance: 50 ppm, fragment mass tolerance: ±0.4 Da, peptide charge of 1+ and monoisotopic. Only significant hits, as defined by the MASCOT probability analysis ($p < 0.05$) were accepted. Classification of the proteins was performed by using a freely available classification system, PANTHER.

2.5 *Statistical analysis*

Statistical analysis was performed on five biological replicates for morphological and physiological biochemical analyses. Proteome analysis were carried out by using three biological replicates. Data were given as means ± standard deviation (SD). Significant differences between control and drought-stressed samples for all measurements were analysed by Student's t test.

3. Results

3.1 *Cicer reticulatum is more drought tolerant than Cicer arietinum*

Drought stress significantly reduced root length, leaf water potential (LWP) and relative water content but it did not affect stem length in both species, compared with control groups. On the other hand, content of free proline increased in both species under drought conditions (figures 1 and 2).

3.2 *Proteomic analysis*

To determine the proteins whose abundance changed during drought stress, a proteomic study using 2D-DIGE followed by MALDI-TOF/TOF-MS/MS was performed. Preliminary 2DE analysis of leaf protein extracts in the 3–10 pH range showed that the majority of the protein spots were clustered between pH gradient of 5–8 (Supplementary data). Therefore, a pH range of 5 to 8 was selected for further experiments.

To assess the adaptation mechanism at molecular level of chickpea plants to drought stress, the temporal changes in the chickpea proteome were monitored by using 2D-DIGE. 675 and 685 protein spots were detected on 2D-DIGE maps for *C. arietinum* and *C. reticulatum*, respectively. When compared 2D-DIGE maps of control and drought stressed samples, 11 protein (table 1) spots were identified to be differentially expressed in *C. arietinum* (figure 3E) and 13 protein (table 2) spots were identified to be differentially expressed in *C. reticulatum* (figure 4E). Fold change ratio of identified proteins have been shown for *C. arietinum* on figure 3F, for *C. reticulatum* on figure 4F.

4. Discussion

In this study, drought-tolerant chickpea *C. reticulatum* and drought-sensitive chickpea *C. arietinum* were compared by the morphological, physiological and proteomic analyses under drought stress conditions. Results of morphological and physiological analysis showed that effect of drought stress appeared to be greater in *C. arietinum* compared to *C. reticulatum*. We also identified the differentially expressed proteins in chickpea leaves in response to drought using a proteomic approach. Proteomic analysis is required to understand cellular processes that associated with drought stress (Wang *et al.* 2015a, b). In total, 24 proteins had significant differential abundance as a result of drought, and they were associated with photosynthesis mechanism,

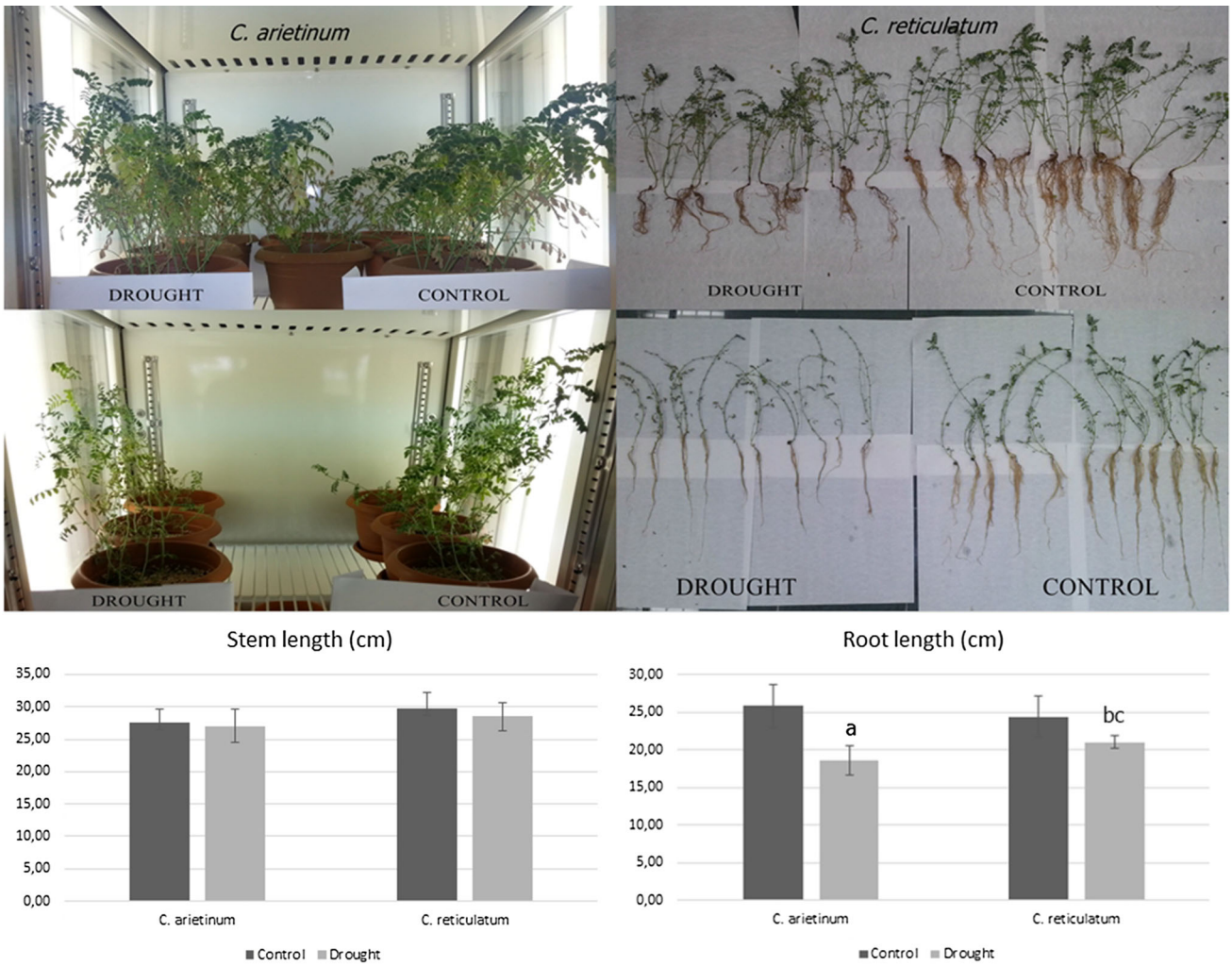

Figure 1. Stem and root lenth of *Cicer arietinum* and *C. reticulatum* grown under control and drought stressed conditions. Letters indicates statistical differences as determined by the Student's *t*-test ($p < 0.05$). a and b indicate difference between control and drought-stressed groups, c indicates difference between *C. arietinum* and *C. reticulatum*.

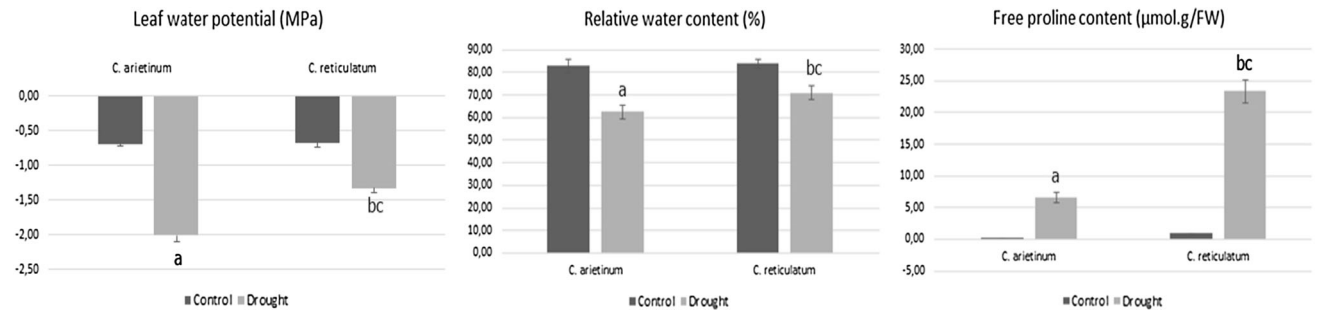

Figure 2. Leaf water potential, Relative water content and Free proline content of *Cicer arietinum* and *C. reticulatum* grown under control and drought stressed conditions. Letters indicates statistical differences as determined by the Student's *t*-test ($p < 0.05$). a and b indicate difference between control and drought-stressed groups, c indicates difference between *C. arietinum* and *C. reticulatum*.

biosynthesis, energy metabolism and antioxidant system. The findings of this proteome analysis will help in understanding the mechanism of drought resistance in chickpea and may be also helpful in developing drought-resistant transgenic plants.

4.1 *Effect of drought stress on morphological parameters*

In our study, while drought stress decreased root length, it did not lead to statistically significant change on stem length in both species. The similar results were observed in different plants

Table 1. Differentially expressed proteins in *C. arietinum,* drought-treated leaves compared to control

Spot No.	Identified protein-species	Swissprot accession number	Best Protein Score	Matched peptides	% sequence coverage	Best protein mass	pI	Sub-cellular localisation	Fold change
1208	Oxygen-evolving enhancer protein 1 (*Pisum sativum*)	P14226	666	16	30	34872	6.25	Chloroplast	11.24
1209	Chlorophyll a-b binding protein 8 (*Pisum sativum*)	P27490	113	7	15	28508	5.16	Chloroplast	3.11
3210	Oxygen-evolving enhancer protein 2 (*Pisum sativum*)	P16059	204	10	22	28030	8.29	Chloroplast	−11.16
5210	Probable plastid-lipid-associated protein 6 (*Arabidopsis thaliana*)	Q9LW57	80	2	3	30436	5.82	Chloroplast	1.93
6506	Putative cytochrome c oxidase subunit II (*Pinus strobus*)	P84733	82	4	100	1707	9.63	Mitochondria	1.74
7112	Enolase (*Ricinus communis*)	P42896	396	16	23	47883	5.56	Cytosol	9.45
8216	Carbonic anhydrase (*Pisum sativum*)	P17067	453	15	25	35355	7.01	Chloroplast	−9.16
8217	L-ascorbate peroxidase 2 (*Oryza sativa subsp. japonica*)	Q9FE01	149	3	11	27101	5.21	Cytosol	6.08
8323	Isoflavone reductase (*Nicotiana tabacum*)	P52579	93	4	9	34632	5.57	Cytosol	−19.88
8326	Ferredoxin-NADP reductase (*Pisum sativum*)	P10933	455	21	26	40169	8.56	Chloroplast	−2.01
8328	Fructose-bisphosphate aldolase 1 (*Pisum sativum*)	Q01516	619	18	33	38633	5.83	Chloroplast	−2.04

under drought conditions (Ashraf and O'leary 1996; Jaleel *et al.* 2009; Nedunchezhiyan *et al.* 2012). The plant growth depends on cell division, enlargement and differentiation and all of these events are affected by drought stress (Sankar *et al.* 2007). The reduction of *C. arietinum*'s root length was much more than that of *C. reticulatum*'s root length under drought stress conditions (approximately 25% in *C. arietinum* and approximately 13% in *C. reticulatum*). Reduction in root growth is a good indicator of drought susceptibility of cultivars (Macar *et al.* 2009). Rooting depth is very important for the avoidance of water stress and this may be an advantage for *C. reticulatum* compared with *C. arietinum* under drought stress.

4.2 *Effect of drought stress on leaf water potential and relative water content*

It is believed that leaf water potential (LWP) and relative water content (RWC) are reliable parameters for quantifying the plant drought stress response (Siddique *et al.* 2000; Yan *et al.* 2016) and utilizations of LWP and RWC as an indicator of plant water status are usual (Lawlor and Cornic 2002). In this study, drought stress decreased the LWP and RWC in both species. The decrease in LWP (approximately 66 % in *C. arietinum* and approximately 50% in *C. reticulatum*) and RWC (approximately 25% in *C. arietinum* and approximately 15 % in *C. reticulatum*) for *C. arietinum* was more severe than that of *C. reticulatum* under drought stress. Some researchers have also found similar results for LWP (Leport *et al.* 1999; Basu *et al.* 2007; Fang *et al.* 2010; Krouma 2010) and for RWC (Nayyar and Chander 2004; Rahbarian *et al.* 2011; Talebi *et al.* 2013) under drought stress in chickpea. The tolerance or sensitivity of chickpea to drought is related to its capability to maintain good leaf water status (Krouma 2010). Talebi *et al.* (2013) also indicated that cultivars which have high LWP and RWC are more resistant to drought stress. These results showed that *C. reticulatum* is better than *C. arietinum* in preserving water under drought stress and this is very important for plant growth and development under drought stress.

4.3 *Effect of drought stress on free proline content*

Drought stress increased proline concentration in both species (approximately 13 times in *C. arietinum* and approximately 26 times in *C. reticulatum*). The accumulation of

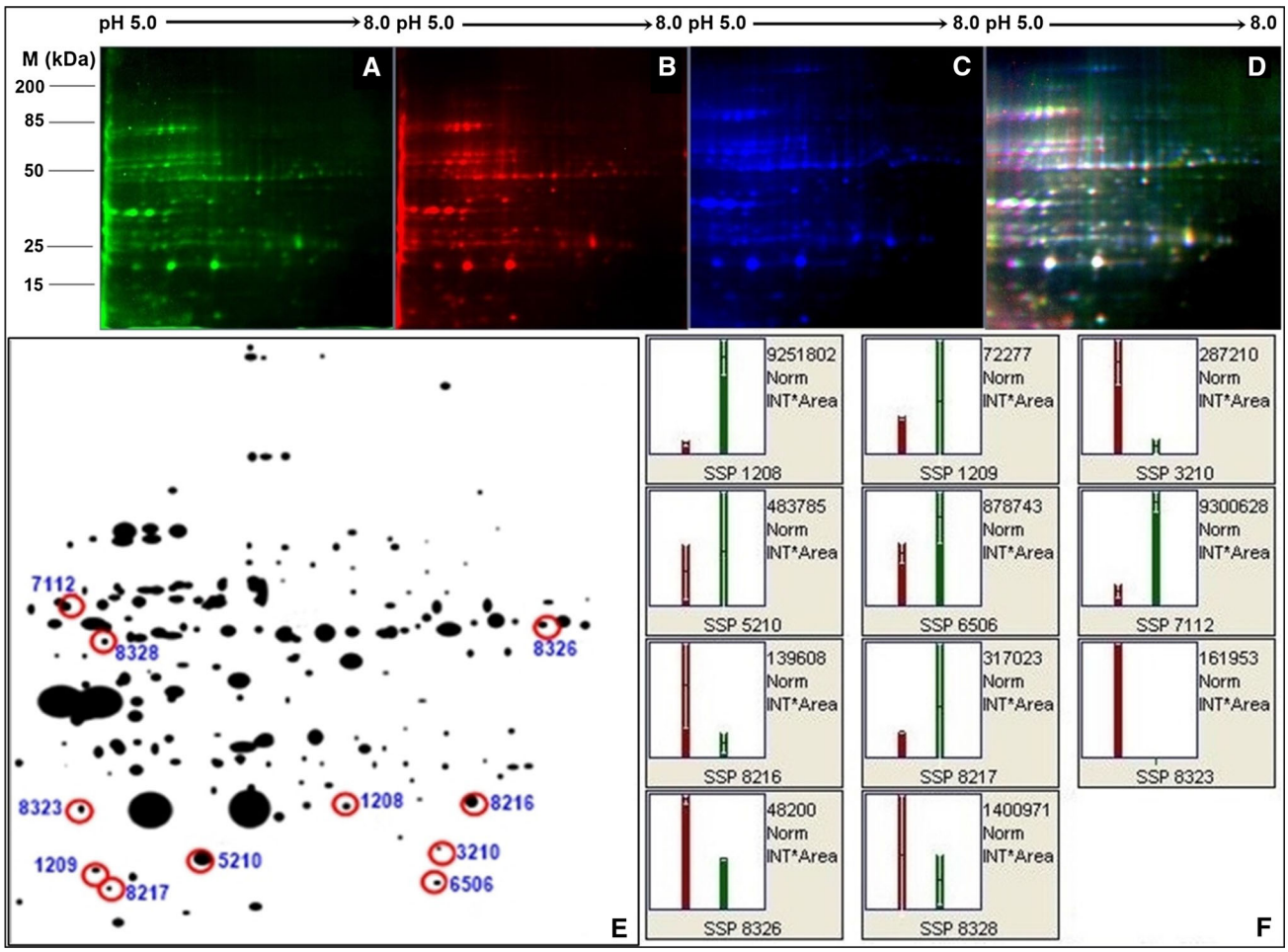

Figure 3. Images from 2D-DIGE analysis of *C. arietinum*. (**A**) Cy3, for the protein sample of well watered (control) group. (**B**) Cy5, for the protein sample of drought stress group. (**C**) Cy2, mixing equal amounts of all of the proteins as the internal standard. (**D**) The labelled proteins were visualised for all of the fluorophores. (**E**) PDQuest-generated master gel image showing the general spot pattern of matched protein spots and selected protein spots from the total proteome of *C. Arietinum.* (**F**) Fold change ratio of selected proteins from the total proteome of *C. arietinum*, red bar is control, green bar is drought stressed group.

osmolytes such as proline during stress is well documented (Vendruscolo *et al.* 2007; Lum *et al.* 2014) and, it is known for a long time that the concentration of proline increases in a large variety of plants under drought stress. In addition to its role as osmolyte, proline may also protect protein structure and membranes from damage, and reduce enzyme denaturation (Lhout *et al.* 2001). Several studies have also attributed an antioxidant feature to proline, suggesting reactive oxygen species (ROS) scavenging activity (Szabados and Savoure 2010). As well as having scavenging features, proline can regulate cytosolic pH and NAD/NADH ratio under drought stress (Vendruscolo *et al.* 2007). Under stress conditions, the rate of the Calvin cycle is diminished, which prevents oxidation of NADPH and restoration of $NADP^+$ and this can lead to accumulation of NADPH (Szabados and Savoure 2010). As a result of the accumulation of NADPH, the electron flow from the light reactions is slow, and this may cause the formation of ROS. Therefore, regulation of $NADP^+$/NADPH ratio is an important mechanism to decrease generation of ROS in cells under stress conditions. During proline synthesis, NADPH in chloroplast is converted to $NADP^+$, and this conversion is important for the maintenance of electron flow from photosystem to $NADP^+$. Higher proline accumulation under drought stress may provide an advantage to *C. reticulatum* in terms of reducing ROS production.

4.4 *Proteomic analysis*

4.4.1 *Proteins involved in photosynthesis:* Photosynthesis, provides energy as well as organic molecules for plant growth and development (Nouri *et al.* 2015), is a key mechanism and this mechanism is severely affected by drought stress (Çevik *et al.* 2014). In this study drought stress has changed abundance of four light reactions related proteins; oxygen evolving enhancer protein 1 (OEE1), oxygen evolving enhancer protein 2 (OEE2), ferredoxin-

Table 2. Differentially expressed proteins in *C. reticulatum,* drought-treated leaves compared to control

Spot No.	Identified protein-species	Swissprot accession number	Best protein score	Matched peptides	% sequence coverage	Best protein mass	pI	Sub-cellular localisation	Fold change
108	Oxygen-evolving enhancer protein 1 (*Pisum sativum*)	P14226	673	22	40	34872	6.25	Chloroplast	1.78
301	Sedoheptulose-1,7-bisphosphatase (*Triticum aestivum*)	P46285	107	13	26	42034	6.04	Chloroplast	1.46
1204	Chloroplast stem-loop binding protein (*Arabidopsis thaliana*)	Q9YLA9	166	8	9	43903	8.54	Chloroplast	10.71
3202	Putative cytochrome c oxidase subunit II (*Pinus strobus*)	P84733	74	4	100	1707	9.63	Mitochondria	−1.54
3401	Glutamine synthetase (*Medicago sativa*)	Q9XQ94	384	15	22	47086	6.29	Chloroplast	5.79
3603	Enolase (*Ricinus communis*)	P42896	315	13	22	47883	5.56	Cytosol	5.42
5202	Ferredoxin-NADP reductase (*Pisum sativum*)	P00455	207	13	25	41162	8.67	Chloroplast	−1.33
5303	Fructose-bisphosphate aldolase 1 (*Pisum sativum*)	Q01516	563	16	30	38633	5.83	Chloroplast	2.56
5401	Phosphoglycerate kinase (*Nicotiana tabacum*)	Q42961	704	19	36	50146	8.48	Chloroplast	2.41
5503	GDP-mannose 3,5-epimerase 2 (*Oryza sativa subsp. japonica*)	Q2R1V8	186	10	19	42105	5.75	Cytosol	2.68
6001	Carbonic anhydrase (*Pisum sativum*)	P17067	482	15	28	35355	7.01	Chloroplast	1.54
6404	Glyceraldehyde-3-phosphate dehydrogenase (Pisum sativum)	P12859	535	21	23	48067	7.57	Chloroplast	1.77
8301	Fructose-bisphosphate aldolase 1 (*Pisum sativum*)	O65735	555	22	44	38428	6.21	Cytosol	3.02

NADP reductase (FNR) and light-harvesting chlorophyll a/b binding protein.

Drought stress increased the expression of OEE1 in both species. Some researchers also indicated similar result (Bo-geat-Triboulot *et al.* 2007; Ngamhui *et al.* 2012; Wang *et al.* 2015a, b). It is believed that oxygen-evolving enhancer proteins have two important roles; optimize the manganese cluster during water photolysis (Heide *et al.* 2004) and protect reaction centre proteins from ROS as an antioxidant (Kim *et al.* 2015) for PSII core stability. Although drought stress decreased OEE2 expression in *C. arietinum*, it did not affect the expression of this enzyme in *C. reticulatum*. This decline may be disadvantageous for *C. arietinum* to the stability of PSII under drought.

FNR, has important roles for balancing electron transport and redox homeostasis, transfers electrons from ferredoxin to $NADP^+$ to generate NADPH. The NADPH is subsequently utilized as reducing power in several biosynthetic pathways including carbon fixation (Faghani *et al.* 2015). FNR also an important ROS scavenging enzyme and has a critical role to maintain balance between NADPH/$NADP^+$ (Xiao *et al.* 2009). In our study, drought decreased expression of FNR in *C. arietinum* but it did not lead to significant change in *C. reticulatum*. Decline in abundance of FNR after drought stress have also been reported by some researchers in different plants (Sanda *et al.* 2011; Zadražnik *et al.* 2013; Budak *et al.* 2013; Gharechahi *et al.* 2015). The reaction catalyzed by this enzyme is thought to be a rate-limiting step in photosynthesis (Faghani *et al.* 2015). Hajirezaei *et al.* (2002) indicated that reduced FNR activity resulted in decreased photosynthetic activity. Therefore, the reducing in amount of this enzyme may decrease photosynthesis rate in *C. arietinum*. Preservation of enzyme activity may be important for sustainability of photosynthesis in *C. reticulatum* under drought stress condition.

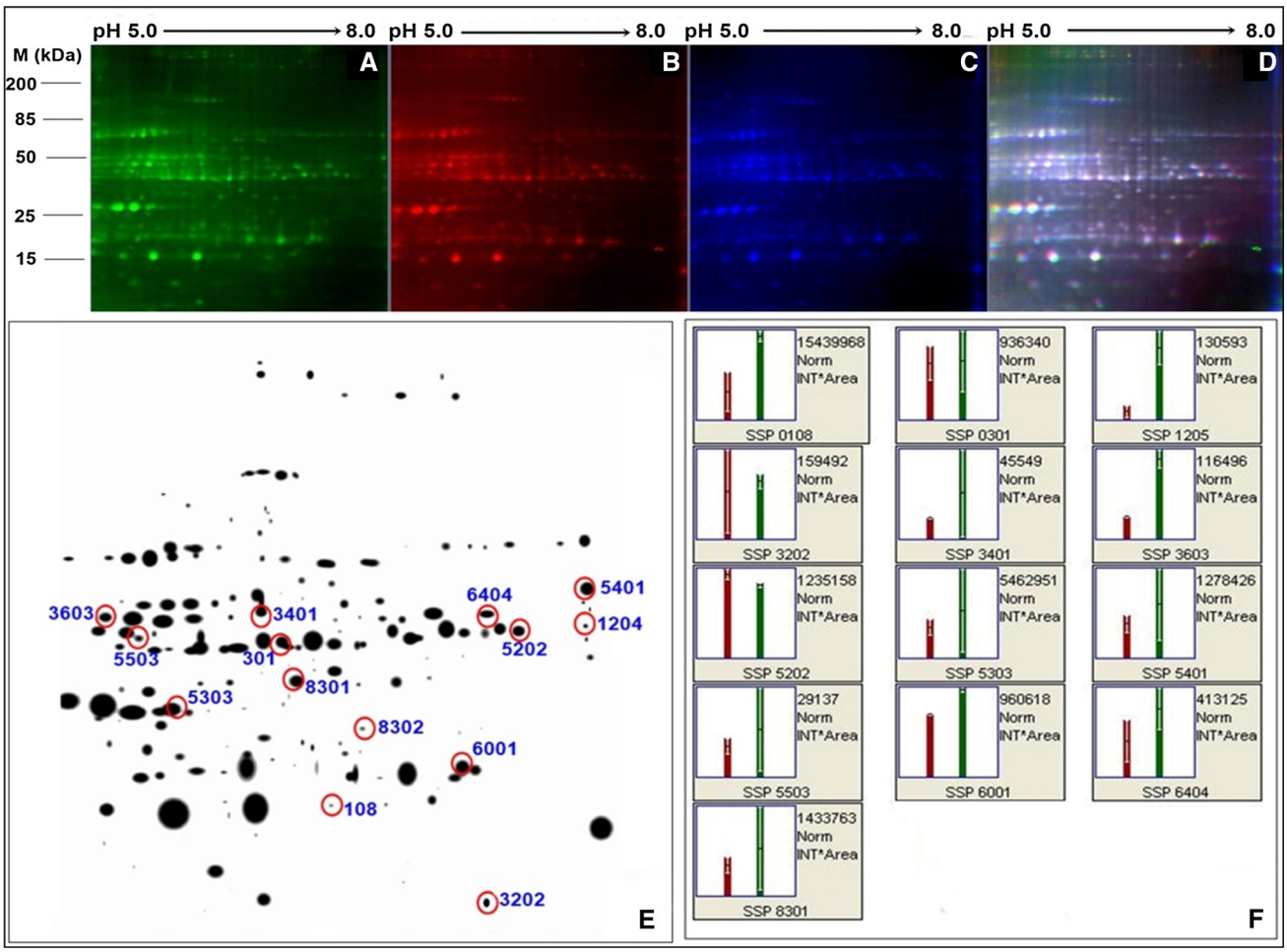

Figure 4. Images from 2D-DIGE analysis of *C. reticulatum.* (**A**) Cy3, for the protein sample of well watered (control) group. (**B**) Cy5, for the protein sample of drought stress group. (**C**) Cy2, mixing equal amounts of all of the proteins as the internal standard. (**D**) The labelled proteins were visualised for all of the fluorophores. (**E**) PDQuest-generated master gel image showing the general spot pattern of matched protein spots and selected protein spots from the total proteome of *C. Reticulatum.* (**F**) Fold change ratio of selected proteins from the total proteome of *C. reticulatum*, red bar is control, green bar is drought stressed group.

Photosynthetic organisms have light-harvesting antenna protein complexes that bind chlorophylls and carotenoids, they use these complexes to harvest solar energy efficiently (Andersson *et al.* 2001). The light-harvesting chlorophyll a/b-binding protein (LHCB) 8 is the apoprotein of the light-harvesting complex of photosystem II (PSII) (Xu *et al.* 2012). In our study the abundance of light-harvesting chlorophyll a/b binding protein 8 increased under drought in *C. arietinum* but did not change in *C. reticulatum*. Xu *et al.* (2012) identified LHCB proteins as new players in ABA signalling in stomatal movement. Stomatal closure is the earliest response to drought that cause a decrease in CO_2 diffusion. Decreasing of CO_2 diffusion is the major reason for loss of yield under drought (Nouri *et al.* 2015). LHCBs are also modulated by ROS homeostasis (Xu *et al.* 2012). At cellular level, drought stress often leads to the accumulation of ROS (Çevik and Unyayar 2015). The enhanced amount of ROS can be viewed as a threat to the cell, but they can also act as secondary messengers involved in the stress signal transduction pathway (Uematsu *et al.* 2012). The high ROS level in *C. arietinum* may lead to be a signal to produce Cl a/b binding protein in order to protect chlorophyll under drought stress.

The photosynthetic carbon reduction (Calvin) cycle is the primary pathway for fixation of atmospheric CO_2. This cycle plays a central role in plant metabolism (Lefebvre *et al.* 2005). The abundances of phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (G3PDH), fructose-bisphosphate aldolase (FBA) and sedoheptulose-1,7-bisphosphatase (SBPase) changed under drought stress.

In our study, drought stress increased expressions of PGK and chloroplastic G3PDH enzymes in *C. reticulatum* but did not change in *C. arietinum*. Phosphoglycerate kinase catalyzes the ATP-dependent phosphorylation of phosphoglycerate in the Calvin cycle. This reaction is the first reduction step of the Calvin cycle, and affects the synthesis of the triose phosphates in the photosynthetic CO_2 assimilation (Wang *et al.* 2015a, b). Chloroplastic G3PDH removes

hydrogen from NADPH and adds it to the 1,3-bisphosphoglycerate to make glyceraldehyde-3-phosphate in Calvin cycle. Increased expressions of these enzymes could indicate an increase in photosynthetic carbon assimilation in *C. reticulatum* under drought stress. Bogeat-Triboulot *et al.* (2007) emphasized that increased abundance of photosynthesis-related proteins during the stress treatment may have partly counter balanced the decreased internal CO_2 concentration and contributed to the partial maintenance of photosynthesis during the first stages of water deficit. Therefore, we suggest that increased expressions of these enzymes are an advantage and important for drought-tolerant *C. reticulatum* under drought stress.

FBA has two different isoforms in higher plants: cytoplasmic and plastidic. Two isoforms catalyse the same reactions in different metabolic pathways. (Caruso *et al.* 2008) Although, drought stress decreased abundance of plastidic FBA in *C. arietinum*, it increased in *C. reticulatum*. Some researchers also indicated similar results in different plants under drought stress (Caruso *et al.* 2008; Zhao *et al.* 2011). In the chloroplast this enzyme catalyzes the linkage of dihydroxyacetonephosphate and glycerine-3-phosphate to fructose- 1,6-bisphosphate and initiates the regeneration of ribulose- 1,5-bisphosphate, the CO_2 acceptor of the Calvin cycle (Jedmowski *et al.* 2014). Therefore, the downregulation of plastidic FBA may indicate a decline of the carbon fixation. Some genetic researches showed that a small decline of the FBA activity leads to lower rates of photosynthesis (Haake *et al.* 1998). In contrast, the upregulation of this enzyme may be important for maintaining photosynthesis in *C. reticulatum* under drought stress conditions.

SBPase is also a key enzyme in Calvin cycle. It plays a large part in controlling the flux of carbon through the Calvin cycle. Drought stress increased abundace of this enzyme in *C. reticulatum* but did not change in *C. arietinum*. Lefebvre *et al.* (2005) and Uematsu *et al.* (2012) increased activity of SBP by overexpression cDNA metodology in Tobacco plants, and they found that an increase of this enzyme leads to a big increase in photosyntesis rate and growth parameters. Raines (2003) suggested this enzyme to be a key protein for photosynthesis efficiency. The increase in amount of this enzyme under drought stress in *C. reticulatum* may be important for the efficiency of photosynthesis.

In our study, another photosynthesis related protein: Carbonic anhydrase up-regulated in *C. reticulatum*, but down-regulated in *C. arietinum*. Budak *et al.* (2013) reported that the highest level of carbonic anhydrase was in drought-tolerant wheat genotype under drought stress. In contrast, some researchers reported that drought stress reduced abundance of carbonic anhydrase in different plants (Ghabooli *et al.* 2013; Gharechahi *et al.* 2015). Carbonic anhydrase, a zinc-containing metalloenzyme, catalyses the reversible reaction of $CO_2+H_2O \leftrightarrow HCO_3^- + H^+$. CA, facilitates CO_2 diffusion in chloroplasts and enhances CO_2 availability to RuBisCO (Budak *et al.* 2013). CO_2 concentration reduces under drought stress. High level of this protein may lead to better utilization of resources that were limited under drought stress conditions. Roles of CA are well documented in C4 and CAM plants but little information is known about C3 plants. According to our results, we suggest that CA has important roles particularly in photosynthesis mechanisms of C3 plants, and more detailed studies should be performed to understand role of this enzyme especially photosynthesis mechanism of C3 plants.

4.4.2 *Proteins involved in biosynthesis metabolism:* Glutamine synthetase (GS), which plays central role in nitrogen metabolism (Nagy *et al.* 2013) and proline production (Wang *et al.* 2015a, b), was found to increase in abundance after drought stress in *C. reticulatum* but did not change in *C. arietinum*. Nagy *et al.* (2013) reported that GS is a good indicator of drought stress in tolerant and sensitive wheat cultivars. Thus, the higher level of GS protein in *C. reticulatum*, compared to that of *C. arietinum,* might be an indicator of drought resistance. The proline accumulation in *C. reticulatum* was found to be higher than in *C. arietinum* in this study, which may be caused by the upregulation of GS in *C. reticulatum*. Proline is an important molecule for plants and has a lot of significant roles in maintaining the function of chloroplasts under drought stress, therefore the upregulation of GS might have a significant role in this mechanism.

In this study, while drought stress increased cytosol FBA in *C. reticulatum*, it did not lead to a change in *C. arietinum*. Cytosol FBA, a very important enzyme for living organism, is involved in gluconeogenesis and glycolysis. It also has an important role in sucrose biosynthetic pathway. Therefore, an increase in the cytosol FBA enzyme amount appears to be implicated in accumulation of water soluble carbohydrates and synthesis of ATP through the promotion of glycolytic pathway (Fan *et al.* 2009). Accumulation of solutes such as sucrose under drought stress is an important mechanism for plants to preserve water.

Isoflavone reductase (IFR) is also an important protein involved in biosynthesis metabolism. It is known to be a key enzyme for isoflavonoid phytoalexine biosynthesis in legume plants. The production of isoflavonoid phytoalexine accumulates in response to abiotic and biotic stresses (Kim *et al.* 2003). We found that drought stress increased IFR enzyme in *C. arietinum*, it did not change in *C. reticulatum*. The increasing amount of IFR enzyme in *C. arietinum* may protect it from oxidative stress. The quantity of IFR enzyme did not change in *C. reticulatum* under drought stress, which indicates that two species use different paths from each other to be protected against drought stress.

4.4.3 *Proteins involved in antioxidant system:* GDP-mannose-epimerase (GME) catalyses the conversion of GDP-D-mannose to GDP-L-galactose, which is an important step in the ascorbic acid (ASC) biosynthetic pathway in higher plants (Ma *et al.* 2014). ASC is a small, water-soluble

antioxidant molecule that acts as a primary substrate in the cyclic pathway of enzymatic detoxification of hydrogen peroxide (Dolatabadian *et al.* 2008) and protects plant cells against ROS. In our study, the expression of GME increased in *C. reticulatum*, but it did not change in *C. arietinum*. Higher GME amount under drought stress may lead to higher ASC level in *C. reticulatum*. In our previous study, we found that level of ASC in *C. reticulatum* was higher than that in *C. arietinum* under drought stress, and this is a big advantage for *C. reticulatum* to cope with stress conditions (Çevik and Unyayar 2015).

Ascorbate peroxidase (APX) is critical for cellular H_2O_2 homeostasis and plays an important role in growth, development and oxidative protection of cells under various abiotic stresses (Zhang *et al.* 2015). While, drought stress enhanced the APX enzyme level in *C. arietinum*, it did not lead to a change in *C. reticulatum* in our study. The high level of APX enzyme may be sign of high ROS and this enzyme might be important to scavenge ROS in *C. arietinum*. Our results may also imply that different chickpea genotypes have different response mechanisms in the antioxidative system during drought stress.

4.4.4 *Proteins involved in energy metabolism:* Enolase is responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate, which is involved in glycolysis (Yang *et al.* 2013). The present study showed that enolase was up-regulated by drought in both species. The up-regulation of the enolase has previously been shown by other researchers in different plants (Hu *et al.* 2011; Yang *et al.* 2013; Oh and Komatsu 2015). Enolase is an energy metabolism associated protein and its great abundance could be related to the need of cells for extra energy in order to deal with stress and repair damage (Zadražnik *et al.* 2013). Sufficient ATP is necessary for plants to cope with abiotic stresses (Hu *et al.* 2014). The high expression of the energy metabolism related proteins may improve the energy supply to protect chickpea from damage under drought stress conditions.

Putative cytochrome c oxidase (COX) subunit II PS17, the terminal enzyme of the respiratory chain, oxidizes cytochrome *c* and transfers electrons to molecular oxygen to form molecular water (Fambuena *et al.* 2013). While COX abundance was up-regulated by drought in *C. arietinum*, it was down-regulated in *C. reticulatum* in our study. Up-regulation of COX may probably facilitate an energy generation through the respiratory chain under drought stress in *C. arietinum*. The role of the downregulation of COX in *C. reticulatum* under drought stress remains unclear in this study. Budak *et al.* (2013) found that drought stress decreased COX abundance in wild wheat under drought stress, and they could not explain the reason of this reduction. On the other hand, José *et al.* (2013) and Tezara *et al.* (1999) observed a decrease in energy related proteins under drought stress. They explained reason of this results as a smaller amount of energy is needed by the cells during drought. These different results indicate that different metabolic pathways may be used in different species for adaptation to drought.

4.4.5 *Other proteins:* Probable plastid-lipid-associated protein 6 is a member of the fibrillin family. Fibrillin proteins have a lot of roles, such as the development of plastoglobule structure, chromoplast pigment accumulation, hormonal responses, protection of the photosynthetic apparatus from photo damage, and plant resistance to a range of biotic and abiotic stresses (Gharechahi *et al.* 2015). In the present study, drought stress enhanced Probable plastid-lipid-associated protein 6 level in *C. arietinum*, it did not change in *C. reticulatum*. Manac'h and Kuntz (1999) showed that fibrillin proteins are induced by various abiotic stresses in light but not in dark, and they suggested that an increased production of ROS under stress conditions lead to the enhance of fibrillin proteins. These findings show that ROS production of *C. arietinum* is greater than that of *C. reticulatum* under drought stress and we think that highly production of ROS induces fibrillin production, and fibrillin proteins protect cells against oxidative stress.

Chloroplast stem-loop binding protein (CSP41a) binds and cleaves RNA, particularly in stem-loops. CSP41a have a role in chloroplast ribosomal RNA metabolism, most likely acting in the final steps of 23S rRNA maturation (Bollenbach *et al.* 2003). Drought stress enhanced the amount of CSP41a in *C. reticulatum*, but did not change in *C. arietinum* under drought stress. There is no detailed information available up to date about the role of CSP41a protein in drought stress.

5. Conclusion

The present study is the first proteomic analysis which compares proteome patterns of leaves of wild and culture species of chickpea under drought stress. In this study, a comparative physiological and proteomic analysis were conducted to understand the physiological and proteomic responses of two chickpea species to drought stress. When the physiological measurements were taken into consideration, the two species clearly showed different responses against drought stress. Drought stress decreased root length, RWC and LWP, while enhancing free proline content in both species. The decreasing of root length, RWC and LWP was more severe in *C. arietinum*, on the other hand the enhancing of free proline content was much more in *C. reticulatum*. These results indicate that wild *C. reticulatum* is more resistant to drought than culture *C. ariertinum*. Proteomic analysis resulted in the identification of 11 and 13 proteins in leaves of *C. arietinum* and *C. reticulatum*, respectively. Proteins which were related to photosynthesis mechanism, biosynthesis pathway, antioxidant defense system and energy metabolism were affected by drought stress in both species. Some of the identified proteins; Carbonic anhydrase, Glutamine synthetase, cyctosolic Fructose-

bisphosphate aldolase and GDP-mannose 3,5-epimerase, may be potential candidates for enhancing drought resistance in chickpea.

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