Molecular mechanism of sensitive and tolerant barley cultivars under different times of salinity stress exposure using proteome analysis

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Abstract

To evaluate the protein response of barley cultivars under salinity stress, leaf samples were prepared 0, 3, 6, 9, 12, and 15 days after the tillering stage. Following two-dimensional electrophoresis, 153 and 141 protein spots were identified in tolerant (Afzal) and sensitive cultivars (Macouei), respectively. In total, 21 and 17 spots with significant induction factor (IF) were revealed in Afzal and Macouei, respectively. The most common proteins between two cultivars were involved in the removal of antioxidants (five proteins), and for each protein group including heat shock, proton transfer, Calvin cycle and photosynthesis optical reaction proteins, a protein was detected. Also, 12 protein spots were exclusively present in the tolerant cultivar (Afzal), most of them being involved in the elimination of antioxidants, and eight protein spots were found exclusively in the sensitive cultivar (Macouei), which was also largely involved in the removal of antioxidants. Lower expression of these proteins in the susceptible cultivar compared to the tolerant one resulted in a decreased homeostasis in susceptible cultivar under salinity stress. Also, for most proteins, the highest and lowest protein expression levels occurred in tolerant and susceptible cultivars, 12 and 9 days after initiation of salinity stress.

Keywords: barley, proteomics, responsive proteins to stress, salinity stress tolerance

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Introduction

Barley (*Hordeum vulgare*) is one of the most tolerant cereals to salinity stress (Abdi et al., 2016; Zellerhoff et `al., 2010) and is widely grown in arid and semi-arid regions for forage and as a grain crop (Al-Karaki, 2001). It is classified as a relatively tolerant forage crop and a very tolerant grain crop. Salinity and drought stresses are the most important agricultural problems in semi-arid and arid regions. These conditions cause a significant reduction in yield in cultivated lands and cause a wide range of disorders at the cellular level and the whole plant, which leads to reduced production and ultimately plant death at high salinity. However, plants that grow naturally in arid and semi-arid regions have been shown to be more adaptable to salinity stress (Kidou et al., 1993). The ability to maintain cellular inflammation despite water deficiency due to salinity stress may maintain metabolic processes

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and thus disrupt plant cycle growth (McEvoy and Brudvig 2006). During salinity stress in a plant, most processes such as photosynthesis, protein and energy synthesis and fat metabolism are affected. The first response can also be a decrease in leaf area growth rate and subsequent cessation of growth. A good understanding of the molecular mechanisms involved in plants' response to salinity stress is extremely important. A proper understanding of these mechanisms is also essential for the continued development of breeding strategies as well as the creation of transgenic plants to improve stress tolerance in crops. Significant changes in gene expression, biomembrane lipid composition, and small molecular aggregation have been shown to be closely related to these processes (Hashiguchi et al., 2010).

Proteomics analysis is a tool used to compare the pattern of proteins, which provide a wealth of information about individual proteins involved in specific biological responses. So far, a number of plant proteomic studies have been published to identify proteins in response to salinity (Hashiguchi et al., 2010). Advances in proteomics technology and separation and identification of proteins with the identification of proteins based on mass spectrometry have a great impact on the study of plant response to salt stress. Protein isolation, detection, and identification based on mass spectrometry have had an increasing impact on the study of plant responses to salt stress (Caruso et al., 2008). A type of proteomics that compares the composition of different proteomes is differential-expression proteomics. In research on plants under abiotic stress, the most common proteome comparison is the that of proteomes isolated from non-stress (control) plants and related proteomes under stress. In this regard, the characteristics of the molecular response of salinity stress in plants indicate the involvement of several genes responding to salinity stress. In fact, proteins play an important role in the response to plant stress as they are directly involved in the processes of increasing stress tolerance. Similar to other stresses, salinity stress results in several adaptations in plants, including increased level of stress-related proteins (dehydration-inducing proteins, ion transporters, and ROS scavenging enzymes), changes in cell signaling, gene expression, cellular metabolism, and inducing regulatory processes (Komatsu et al., 2014). Studies suggest that saline tolerant species show higher levels of stress-related protein scavenging enzymes (ROS), salt ion transporters (SOS1, V-ATPase). They also have relatively higher levels of anabolism-related proteins (such as RubisCO activase and other proteins related to photosynthesis such as OEE proteins) as compared with salinity susceptible species under stress condition. On the other hands, salinity-susceptible species show relatively higher levels of catabolism-related proteins such as glycolytic and respiratory enzymes (Peng et al., 2009; Joseph and Jini, 2010). Several proteomics studies have been used to identify proteins in response to salinity stress. In these experiments, many proteins have been identified that were involved in various processes including photosynthesis, light respiration, transduction, metabolism, defense against oxidative stress, ion channels and folding of proteins (Saqib et al., 2006; Joseph and Jini, 2010)

This study compared proteome pattern, protein expression changing, and the role of differential proteins of sensitive and tolerant barley cultivars during different times of salinity stress.

Materials and Methods

In this experiment, two cultivars of spring barley, namely Afzal (tolerant) and Macouei (susceptible) were investigated under different time of salinity stress at the seedling stage in hydroponic culture greenhouse condition at University of Mahabad, Iran. Samples were collected on days 0 (control), 3, 6, 9, 12, and 15 after 250 mM (NaCl) salinity was imposed at the tillering stage. Four replications were considered for proteome analysis. Total proteins were extracted from 0.5 g frozen leaf for every biological replicate, which was suspended as fine powder in cold acetone containing 10% TCA and 0.07% 2-Mercapthoetanol. The resultant powder was dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 60 mM DDT, and 1% ampholyte (pH: 3-10). Also, protein concentration was determined using Bradford assay (Bradford, 1976). The first dimension electrophoresis was performed using IPG strips.

For the first dimension of PROTEAN IEF focusing tray (Bio Rad) and the PROTEAN IEF cell (Bio Rad) were used. Then, balancing of the strips (equilibration) was carried out (Ifuku et al., 2005). Also, the second dimension gels were prepared as two pieces including separating and holder gel (Stacking gel). The separation gel was prepared using a combination of acrylamide for separating gel 8.5 ml, separating gel buffer (pH = 8.8) 6.3 ml, distilled water 2ml, 10% APS 120 µl, and TEMED 20 µl. The stacking gel was prepared through combining acrylamide for stacking gel 1 ml, stacking gel buffer (pH = 6.8) 3ml, distilled water 2ml, 10% APS 30µl, and TEMED 20 µl. Then, first dimension strips were placed on the second dimension gel using agarose 1%. Finally, protein loading was conducted in the second dimension with a current of 35 mA for each gel. After the second dimension electrophoresis, gel staining was performed using a solution of Coomassie blue (Ifuku et al., 2005). Gels were scanned using BioRad GS-800 scanner. Image analyses were performed with PDQuest™ software (BioRad). After determining the protein spots with significant expression and data normalization, a one-way ANOVA model was used to identify the differentially expressed protein spots between normal and stress conditions. Induction factor (IF) measurement was used for selecting between significant spots and detecting spots with more expression change during salinity stress. Then, two-stage mass spectrometry (MS/MS) and liquid chromatography combined with bioinformatics tools were used to identify target spots. One microliter of digested peptides was injected into the C18 column of PepMap nano-chromatography. The peptides were then diluted with 0.1% formic acid in acetonitrile and were separated in C18 columns by inverting phase eee movement. Subsequently, peptides were sprayed into mass spectrometers. The range of ratio of mass to load in peptides was considered between 100 and 2000. The data obtained from the spectrophotometer with Bioworks software was converted by Mascot search engine usable format.

Results

In this experiment, 153 and 141 protein spots were identified in salinity treatments of tolerant (Afzal) and susceptible (Macouei) cultivars, respectively. The spots with significant IF that were larger than 2 or smaller than 0.5 were selected. According to IF value, from 153 and 141 repeatable protein spots, 21 and 17 protein spots were identified on the gels in the tolerant and susceptible cultivars, respectively. Out of these, 9 protein spots were common between the two cultivars and 12 spots were identified in the Afzal cultivar while eight protein spots were identified in the Macouei cultivar exclusively. In other words, a total of 29 responsive protein spots were identified under stress in both cultivars (Figs. I and II, Tables 1 and 2). Two dimensional (2D) electrophoresis gel is shown in Figs. (I) and (II) for the two cultivars, i.e. Afzal and Macouei. Based on the results it can be stated that increase in protein expression in the tolerant barley under salinity was more than that in the susceptible cultivar (Fihs. IV and V) especially up to 12 days after the start of stress. In total, 20 uncommon proteins including 12 spots in Afzal, 8 spots in Macouei, and 9 common proteins were detected (Tables 1 and 2). Also Figs. (IV) and (V) suggest a changing trend

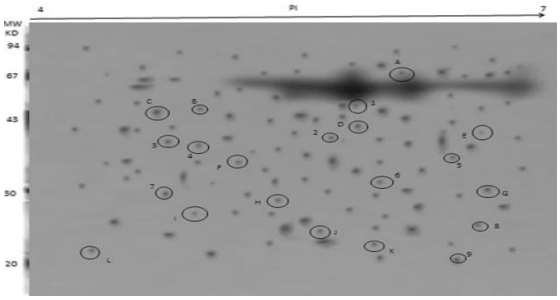

Fig. I. Two-dimensional electrophoresis reference gel of Afzal cultivar where common responsive protein spots to salt stress are shown with numbers while uncommon protein are shown with letters.

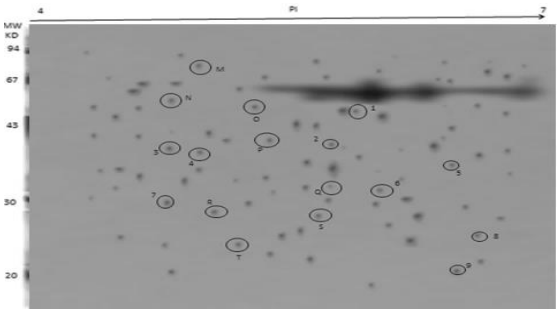

Fig. II. Two-dimensional electrophoresis reference gel of Macouei cultivar where common responsive protein spots to salt stress are shown with numbers while uncommon proteins shown with letters.

Table 1.
Characteristics of uncommon proteins in Afzal as tolerant and Macouei as sensitive cultivar under salinity

Functional group of protein	Spot code	Experimental		Theoretical		name of protein	Accession number
		MW	pI	MW	pI		
Proton transport	A	67.2	6.14	53.88	5.06	ATP synthase CF1 beta subunit	gi|14017579
Heat shock protein	B	45.3	5.03	73.72	4.9	70kDa heat shock protein	gi|254211611
Calvin cycle	C	43.5	4.77	53.4	6.2	Ribulose-1,5-bisphosphate carboxylase/oxygenase Large subunit	gi|61378609
Calvin cycle	D	41.1	5.88	47.34	8.62	ribulose 1,5-bisphosphate carboxylase activase isoform	gi|167096
Calvin cycle	E	39.5	6.67	18.80	8.83	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	gi|4038719
Remove of antioxidant	F	35.3	5.26	20.35	5.3	Cu/Zn superoxide dismutase	gi|1572627
Remove of antioxidant	G	30.9	6.69	23.39	5.4	2-Cys peroxiredoxin BAS1, chloroplastic	gi|2499477
photoreaction of photosynthesis	H	28.8	5.48	27.42	8.84	Oxygen-evolving enhancer protein 2, (OEE2) chloroplastic	gi|131394
Remove of antioxidant	I	26.3	5.01	23.39	5.4	2-Cys peroxiredoxin BAS1, chloroplastic	gi|2499477
Signal transduction	J	23.2	5.73	29.36	4.83	14-3-3 protein homologue	gi|22607
photoreaction of photosynthesis	K	22.1	6.05	15.45	9.82	Photosystem I reaction center subunit IV	gi|131176
photoreaction of photosynthesis	L	21.9	4.38	29.30	8.69	Chlorophyll a-b binding protein 8, chloroplastic	gi|474121685
Remove of antioxidant	M	88.4	5.03	45.26	8.3	Glutathione S-transferase DHAR2	gi|474023258
Remove of antioxidant	N	58.6	4.84	31.83	5.39	putative glyoxalase I	gi|7619802
Cell Transport	O	50.3	5.31	35.16	4.96	Alpha-soluble NSF attachment protein	gi|475620929
photoreaction of photosynthesis	P	40.8	5.42	46.10	4.82	Peptidyl-prolyl cis-trans isomerase CYP38, chloroplastic	gi|474219338
Starch biosynthesis	Q	33.4	5.77	21.97	5.68	adenosine diphosphate glucose pyrophosphatase	gi|21322655
Calvin cycle	R	27.8	5.17	42.55	6.04	Sedoheptulose-1,7-bisphosphatase, chloroplastic	gi|1173347
proton transport	S	26.9	5.72	53.88	5.06	ATP synthase CF1 beta subunit	gi|14017579
Remove of antioxidant	T	23.3	5.26	31.83	5.39	putative glyoxalase I	gi|7619802

in protein spots in both cultivars. The most responsive protein in Afzal was up-regulated while the most responsive protein in Macouei was down-regulated. Also, for some proteins the changing trends during stress were unfixable (Figs. IV and V). In general, 9 protein spots were identified as common between tolerant and susceptible cultivar under salinity stress, which were different (Table 2 and Figs. IV and V). According to Table 2, most of these proteins were involved in removing antioxidants inside cells (5 proteins) and one protein for photoreaction of photosynthesis was identified for each group of heat shock proteins, proton transport, and Calvin cycle. In addition, 12 protein spots were present only in the tolerant cultivar (Afzal), most of them being involved in the removal of antioxidants, photoreaction of photosynthesis, and Calvin cycle, and due to the increased expression of these proteins, this cultivar had stronger biosynthesis

Table 2
Characteristics of common proteins in Afzal and Macouei cultivars under salinity stress

Functional group of protein	Spot number	Experimental		Theoretical		name of protein	ccession number
		MW	pI	MW	pI		
Heat shock protein	1	45.8	5.88	73.72	4.9	70kDa heat shock protein	gi|254211611
Removing antioxidant	2	39.3	5.78	17	5.37	Type 2 peroxiredoxin	gi|473787383
Removing antioxidant	3	38.8	4.81	20.35	5.3	Cu/Zn superoxide dismutase	gi|1572627
Removing antioxidant	4	36.7	5.04	23.6	5.8	Glotathione S-transferase	gi|5923877
Proton transport	5	35.9	6.49	17.72	4.49	ATP synthase delta chain, chloroplastic	gi|475627717
Removing antioxidant	6	32.2	6.10	29.5	10.2	Harpin binding protein 1	gi|38679331
Calvin cycle	7	29.9	4.79	51.24	6.9	Ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic	gi|474153435
photoreaction of photosynthesis	8	24.8	6.66	27.42	8.84	Oxygen-evolving enhancer protein 2, chloroplastic	gi|131394
Removing antioxidant	9	21.3	6.51	45.26	8.3	Glutathione S-transferase DHAR2	gi|474023258

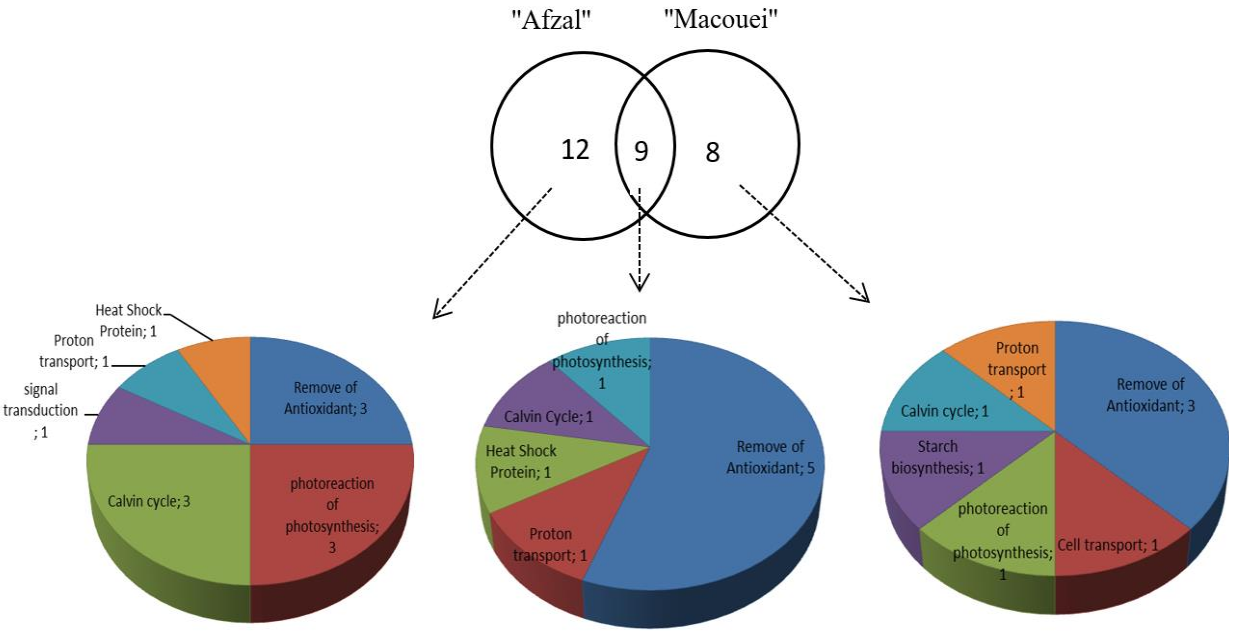

Fig. III. The numbers and grouping of common and uncommon proteins in Afzal and Macouei cultivars under salinity stress

and cellular detoxification (Table 2, Figs. III and IV). Only eight protein spots were observed in the sensitive cultivar (Macouei) and most of the proteins were related to removal of antioxidants (Table 2, Figs. III and V). According to findings, the less increase in the expression of these proteins in sensitive cultivar as compared with the tolerant cultivar led to a reduction in the performance of homeostasis in susceptible cultivar under salinity stress. The changing trends in these protein spots during stress time were not equal and for most of the proteins, the highest level of expression was related to 12 days after the start of stress treatment in the Afzal cultivar (Fig. IV) while the lowest level of protein expression was related to 9

days after the start of stress treatment in Macouei cultivar (Fig. V).

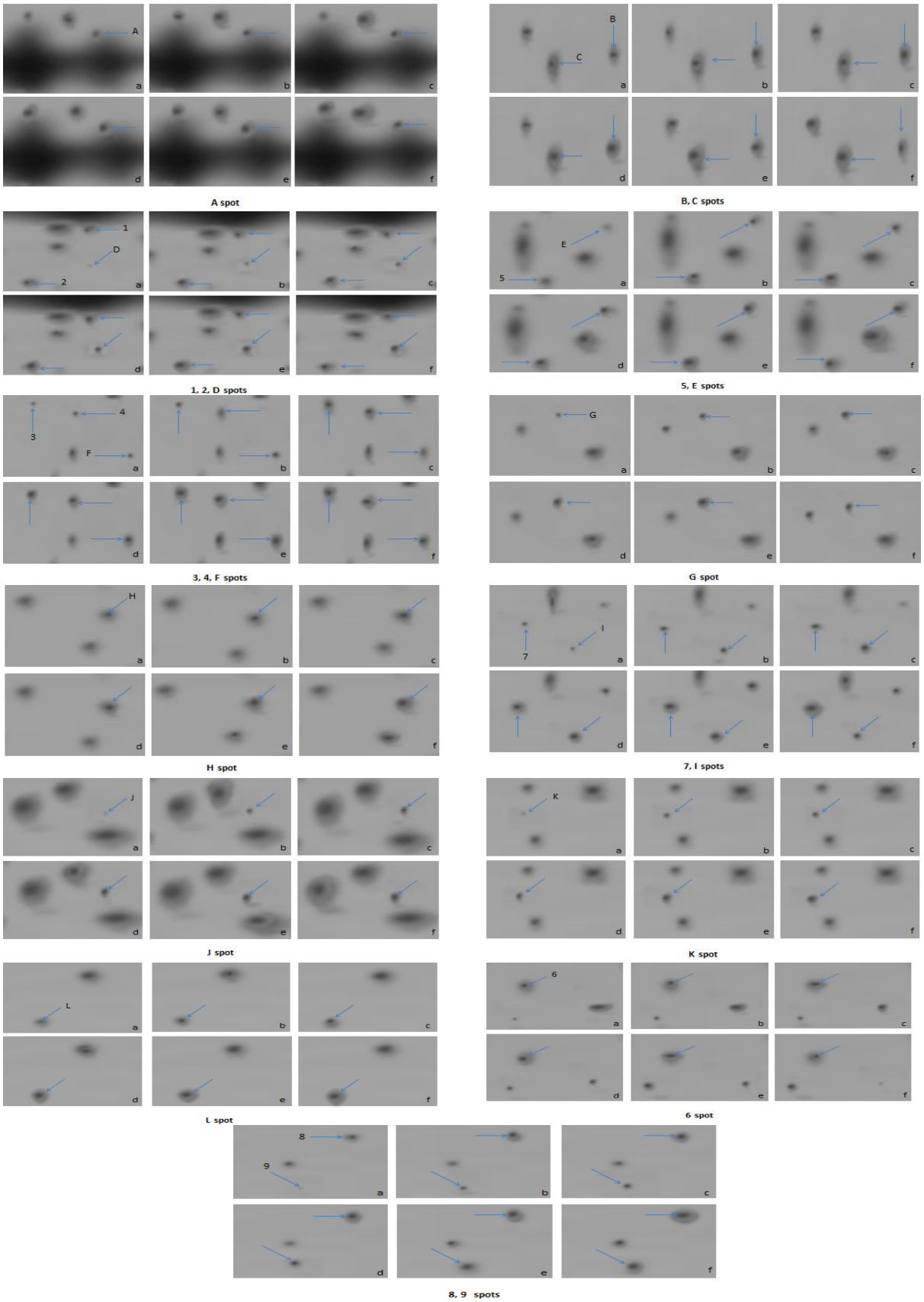

Fig. IV. The trend change of protein spots common and uncommon in Afzal (as tolerant cultivar); a: control; b: 3 days after start of salinity; c: 6 days after start of salinity; d: 9 days after start of salinity; e: 12 days after start of salinity; f: 15 days after start of salinity

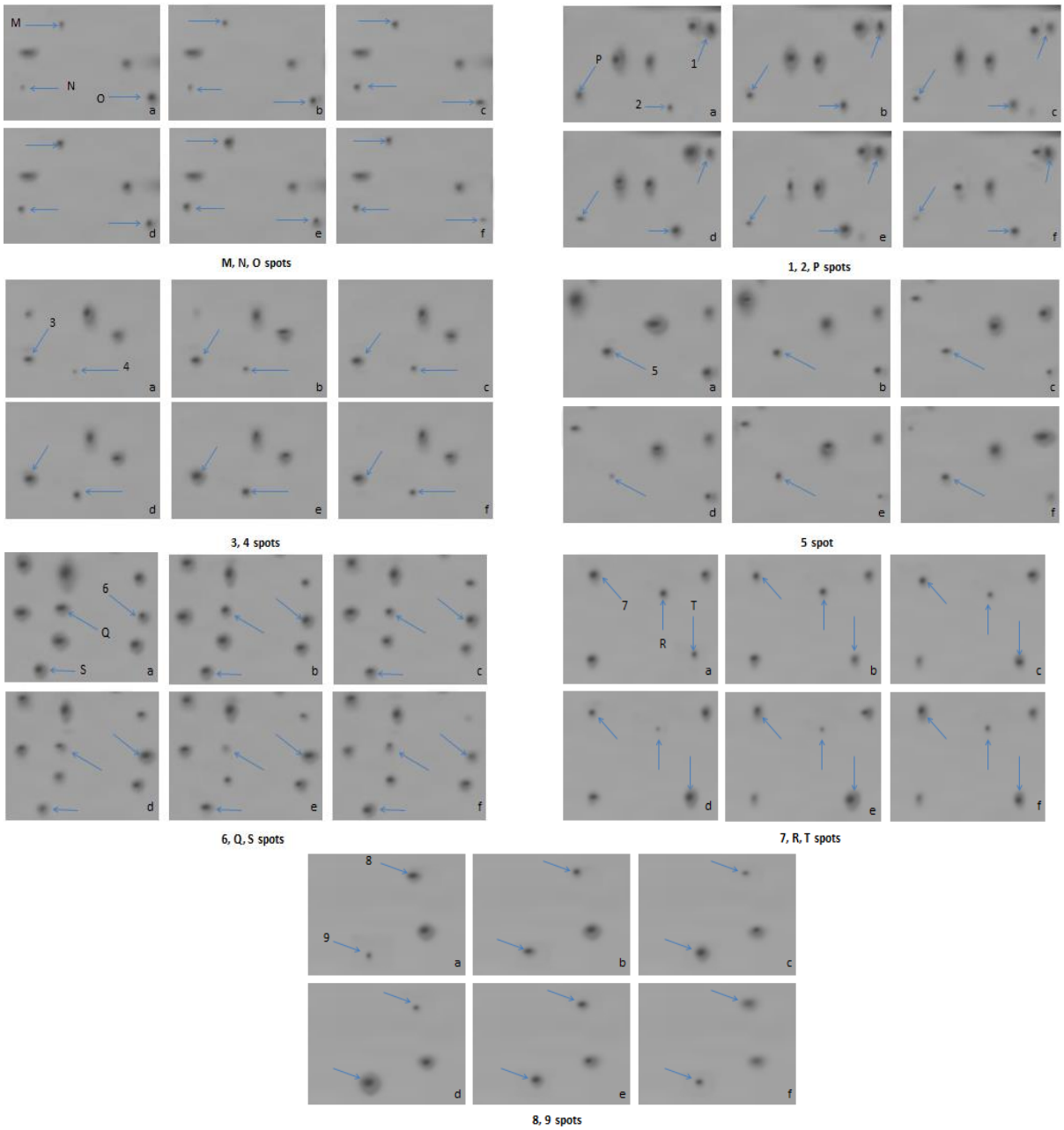

Fig. V. The trend change of protein spots common and uncommon in Macouei (as susceptible cultivar); a: control; b: 3 days after start of salinity; c: 6 days after start of salinity; d: 9 days after start of salinity; e: 12 days after start of salinity; f: 15 days after start of salinity

Discussion

The present study characterizes proteome barley cultivars to elucidate time-dependent and cultivar-specific modulation of leaf in response to salinity stress. Previously, it has been demonstrated that the two barley cultivars, Afzal and Macouei, show contrasting levels of salinity tolerance and some candidate proteins underlying this difference have been identified in phenotype and growth (Khalili et al., 2016). The current study both extended the comparison of their leaf proteomes and tracked the expression of these proteins over a period of salinity stress. In this way, to recognize responsive proteins during salinity stress according to the results, common and uncommon proteins between two cultivars were divided in several functional grouping (Fig. III) and the most important of them were antioxidant removal proteins.

Totally, the numbers of common and uncommon proteins related to the removal of antioxidants were the highest, i.e. 11 proteins including Cu/Zn superoxide dismutase (spots No. 3 and F code), 2-Cys peroxiredoxin BAS1, chloroplastic (spots No. 2 and G, I code), Glotathione S-transferase (spots No. 4, 9 and M code), putative glyoxalase I (N, T code in Macouei), and Harpin binding protein 1 (spot No. 6) (Table 1, 2, Figs. I, II, III, IV, and V). Higher plants have active oxygen-scavenging systems consisting of multiple defense enzymes that can modulate the steady state level of reactive oxygen species (ROS) (Khalili et al., 2013). We found that several antioxidant enzymes were accumulated (11 protein spots) (Tables 1 and 2) under salinity showing upregulation during stress up to 12 days after start of treatment in both cultivars, but these showed less increase in the susceptible cultivar (Macouei) (Figs. IV and V). Moreover, it was suggested that key metabolic pathways including osmolytes and ROS scavenging enzymes might act as an integrated strategy to curtail oxidative burden caused by salt stress and other abiotic factors (Borland et al., 2006). Finally, the functions described above appear to be closely connected with defense mechanism and regulation of cellular state in order to defend cells against harmful salt stress conditions. Protein group of superoxide dismutase (SOD) in the first line of defense against ROS are superoxide and hydrogen peroxide molecules that convert are less toxic or convert ROS to less toxic compounds. In the absence of enough carbon dioxide as a final acceptor of electrons, electrons from the photosynthetic membrane flow through Mehler reaction to the oxygen molecules and create superoxide ions (Cakmak, 2005). In response to salt stress, one chloroplast Cu-Zn SOD (spots No. 3 and F code) in susceptible (Macouei) showed less increased expression compared to Afzal and the highest expression in both cultivars were in 12 days after treatment (Table 1, 2, Fig. IV and V). In line with these results, the accumulation of SOD in tolerant rice and lower expression and their frequency has been reported in the susceptible cultivar in response to salt stress (Komatsu and Tanaka, 2004). Lower levels of this protein in the leaves of Macouei cultivar under stress denotes high levels of hydroxyl radicals produced in the chloroplasts of the cultivar (Sun et al., 2006). On the other hand, peroxiredoxin proteins are widely expressed in tissues and mitochondria, peroxisomes, and cytosol. Cellular location of these proteins suggests that antioxidants play a very important role in cell organelles whose main source is ROS. In addition to the antioxidant activity, this protein is activated in the control signals. The end of this protein molecules is N containing cysteine, which in turn oxidizes polyethylene solphinic acid to react with peroxide. In fact, by the activity of copper/zinc superoxide dismutase enzymes ROS is converted into H_2O_2 and type 2 peroxiredoxin revives H_2O_2 molecules. In many studies peroxiredoxin response to stress has been reported as a responsive protein under stress (Hashimoto et al., 2009). More active presence of this protein in Afzal cultivar (spots No. 2 and G, I code) shows the role of this protein in salinity stress tolerance specially 12 days after start of the treatment (Figs. IV and V, and Tables 1 and 2). In general, due to the protein changes under salinity stress, reaction of Afzal was better than Macouei in terms of the removal of antioxidant proteins. On the other hand, Glutathione S transferase is a joint stress protein expressed in plants under diverse stresses (Salekdeh and Komatsu, 2007; Zhang et al., 2011). Functionally, GSTs are glutathione-dependent detoxifying enzymes that bind glutathione to an extensive type of natural products, environmental toxins (such as herbicides), and oxidative stress products. Glutathione compounds are then forwarded to vacuoles for next metabolism within a types of sulfur-containing metabolites. The observation of a GST protein spots (spots No. 4, 9 and M code) in the present study is evidence that these enzymes are present in plant cells even under non-stress conditions, and expression of these proteins increases during stress and tends to the maximum value 12 days after start of the treatment (salinity stress) and then decreases except for spot no. 9, showing an increase in all levels of stress for Afzal and upregulation for Macouei up to 9 days after treatment with salinity stress when it showed a decrease in expression (Figs. IV and V). Although their exact functions in natural cellular processes are not yet clearly understood, it has been suggested that GSTs may be effective in detoxifying endogenous oxidative damage

products such as membrane lipid peroxides and oxidative DNA damage products. Glutathione S transferases may also be involved as non-enzymatic carrier proteins (ligands) that attach and forward plant hormones such as indoleacetic acid (Martinez et al., 2004). Glyoxalase I or Lactoylglutathione lyase (N, T code in Macouei with upregulated up to 12 days after start of the treatment) (Fig. V) is involved in the glutathione-based detoxification of methylglyoxal (MG), a toxic product of carbohydrate and amino acid metabolism. The agglomeration of MG is indicative of types of stresses such as cold, drought, and salinity (Yadav et al., 2005). The toxic effect of MG varies from mutagenization of nucleic acids to modification with next demolition of proteins. Two enzymes of glyoxalase I and glyoxalase II are active in the detoxification of MG. Glyoxalase I catalyzes the alteration to S-D-lactoylglutathione and glyoxalase II catalyzes the hydrolysis to D-lactate under the secretion of glutathione. Recently, it has been shown that tobacco plants with upregulation of glyoxalase I are more salinity tolerant than wild-type. This an was highlighted in plants converted to glyoxalase I and II. Plants that overexpress both glyoxalase enzymes were able to retain a higher reduction in the ratio of oxidized glutathione under salinity stress condition (Yadav et al., 2005). One of the most important mechanisms in tolerance to salinity stress is detoxification of ROS and maintaining protein redox balance. Higher expression of proteins involved in the scavenging of glutathione-based ROS in the more tolerant cultivar, Afzal, could indicate a pre-constructed tolerance mechanism. On the other hand, the proof of a general decline in defense mechanisms on stress in susceptible cultivar seedlings is further recognized by the detection of a decrease in the abundance of harpin binding protein-1 (spot No. 6 with upregulated up to 12 days after the start of the treatment) (Figs. IV and V), causing hypersensitivity reactions in plants. Over-expression of the harpin-encoding gene *hrf1* in rice plants showed that drought tolerance improved with increased stomatal closure and ABA, proline, and soluble sugar levels (Zhang et al., 2011). Numerous independent reports on the improvement of abiotic stress tolerance by pathogenic-related genes suggest a regulatory cascade of overlap between biotic and abiotic stresses (Sharma et al., 2013). Generally, Afzal cultivar has shown better performance against salinity stress up to 12 days after treatment in comparison with Macouei cultivar, then, 15 days after the start of the treatment decrease in the expression of these proteins shows that it is the cause for the negative effect of salinity stress on enzymes efficiency and performance of proteins biosynthesis in two cultivars.

As precursor molecules in the cytoplasm, chlorophyll-binding proteins, which have a variety of functions including light absorption, energy dissipation, and pigment storage, are synthesized and entered in the chloroplast and are deposited in the thylakoid membrane (Cakmak, 2005). As components of light-absorbing assemblies in plants, the main function of chlorophyll a/b binding proteins (spot with L code in Afzal) is to absorb light and transfer excitation energy to photochemical reaction centers (Ganeteg et al., 2001; Cakmak, 2005). In some cases, plants are exposed to more light than they need to photosynthesize. Therefore, to prevent energy inhibition and damage to the photosynthesis machine, extra energy is wasted by these light-harvesting proteins. Furthermore, researchers believe that chlorophyll a/b binding proteins are involved in pigment storage (Cakmak, 2005). The absorbed light energy by the proteins binding to chlorophyll a/b is directed for light-dependent oxidation of water and releasing molecular oxygen; thus, increasing the expression in this protein leads to a better performance of the cultivar during salinity stress (Table 1, Fig. IV). Water photolysis happens in the oxygen-evolving complex (OEC) of the reaction centers of photosystem II (PSII). The OEC is consisting of four manganese ions, calcium ions, and possibly chloride ions, which bind to external proteins (McEvoy and Brudvig, 2006). Photosystem II OEC proteins plays a role in the retention of calcium and chloride ions as two inorganic agents for the water-dissociation reaction (Ifuku et al., 2005). It is believed that the oxygen evolving enhancing protein has a dual action: (i) it correctly adjusts manganese clusters during photolysis and (ii) prevents reaction center proteins from damage of radical oxygen formed by light (Heide et al., 2004).

The expression of oxygen-evolving enhancer protein 2 (OEE2) (spots H in Afzal and common spot 8) increased during salinity in Afzal while it showed downregulation in Macouei (common spot 8) during salinity stress up to 9 days after treatment before it was upregulated. OEE2 is involved in oxidation of water induced through light in plant photosystem II (Tanaka et al., 2005). Increased levels of OEE2 expression may be required to repair protein damage caused by the separation and maintenance of oxygen evolution. Chemical analysis of water in photolysis with the help of OEC or oxygen-evolving complex of photosystem II has been performed (Heide et al., 2004). The subunit complex involved in PSII photosynthetic systems, known as OEC proteins, is involved in the stability of the PSII complex, so disruption of these proteins causes losses in light performance of photosystem II (Ifuku et al., 2008). Therefore, the reduced expression of this protein in sensitive Macouei cultivar up to 9 days after treatment which contained spot number 8 (Table 3, Fig. V), impairs the activity of photosystem II and eventually reduces the efficiency of light reaction. In the tolerant cultivar (Afzal) this protein (spot number 8) and H code protein showed upregulation. Results showed that salinity is severely affected a key component of photosynthesis, i.e. photosystem II complex rotation, and was involved in cellular aging of leaves and possibly their slow death. According to Komatsu and Tanaka (2004), proteome analysis of sheath in rice leaves under sodium chloride, showed an increase in the frequency of this group of proteins in response to salt stress, indicating the protective role of this protein against salinity. The proteins involved in the light-driven phase of photosynthetic reactions in plants consist of four main protein complexes that have several subunits located in the chloroplast thylakoid membrane, i.e. photosystem II (PS II), cytochrome b6f complex, photosystem I (PS I) (K code spot with upregulation in Afzal), and ATP-synthase complex (Nelson and Yocum, 2006). The two proteins of photosystem I were expressed differently, indicating the effect of salinity stress on PSI operation. Studies showed that further PS I, PS II, and OEC also suffered from stress conditions. The subunit III of photosystem I reaction center is a plastocyanin docking protein that helps to organize and transfer electrons among plastocyanin and photosystem I. The expression level of K protein spot was high under salinity stress, which may protect PS I during stress. High expression of the gene encoding this protein has already been considered in roots and leaves of some plants under salinity ad high temperature stresses (Liu et al., 2014)

On the other hands, RuBisCO is an enzyme key for fixing of carbon dioxide in photosynthesis that is formed of several large subunit catalyzers, i.e. catalytic large subunits (spot C code in Afzal) and several smaller subunits regulator, i.e. regulative small subunits (spot E code in Afzal) (Spreitzer and Salvucci, 2002). In this experiment, both spots of C and E in Afzal cultivar showed upregulation during salinity stress (Figs. I and IV). Wan and Liu (2008) and Michaletti et al., (2018) reported similar results in leaves of rice and wheat under hydrogen peroxide and drought stress, respectively. Also, Babakov et al., (2000) reported that 72 hours after PEG stress, the RuBisCO in wheat leaves increased and they argued that this increase during drought stress helps to overcome PEG stress with the increase in assimilation and efficiency of photosynthesis for use of carbon dioxide. On the other hands, Calvin cycle consists of three phases where the third phase, i.e. the cycle of re-manufactured RuBP molecules and Calvin cycle, start from the beginning. These are known by a series of enzymatic reactions that convert triose phosphate into RuBP. Some intermediary or mediator enzymes in this phase include sedoheptulose-1, 7-biphosphate (spot with R code in Macouei with downregulation up to 9 days after treatment) and fructose 1,6-biphosphate aldolase. Together, these two enzymes catalyze the reaction that eventually results in the formation of ribulose-5-phosphate. The ribulose-5-phosphate is then phosphorylated to form RuBP. Therefore, reducing the mediator enzyme in this process in susceptible cultivar (Macouei) decreases efficiency in Calvin cycle and reduces sugar production. On the other hand, it is reported that photosynthesis-related proteins such as RuBisCO activase (No. spot 7 and D code in Afzal with upregulation) (Table 1, Figs. I, II, IV, and V) showed decreased expression in susceptible

cultivar (Macouei) during salinity stress up to 9 days after the start of stress (Kausar et al., 2013).

Part of the triose phosphate that is produced during Calvin cycle is used for the biosynthesis of sucrose and starch (Tanaka et al., 2005). An essential storage polysaccharide in plants that provides an energy original for several metabolic processes is starch (Li et al., 2013). Starch synthesis consists of three enzymes: adenosine diphosphate glucose pyrophosphatase (AGPase), a starch synthase, and a branching enzyme (Guan and Keeling, 1998) only one of which was identified in the present study. A protein spot (spot with Q code in Macouei) indicating AGPase was found to have low expression up to 12 days after starting the treatment (Table 2, Fig. V). Adenosine diphosphate glucose pyrophosphatase catalyzes the constitution of ADP-glucose and mineral pyrophosphate from ATP and glucose-1-phosphate (Boehlein 2005). The final product of this reactance is ADP-glucose, which is a precursor to starch synthesis. Starch synthase then ships glucose from ADP-glucose without reducing the growing acceptor chain, thus lengthening the α-1,4-glucan chains. In the third stage, the starch branching enzyme then transports an elongated α-1,4-glucan chain and in the meantime transfers it to a receptor chain to form α-1,6 bonds (Guan and Keeling, 1998).

Hydrogen ions are also released in the process, creating a transmembrane chemiosmotic potential that is utilized by ATP synthases during ATP synthesis. Chloroplastic ATP synthase subunit beta and delta in this experiment were upregulated in Afzal cultivar during salt stress to ensure adequate energy for other important processes during salt stress up to 12 days after treatment (Fig. IV) while in Macouei cultivar it showed downregulation up to 9 days after treatment, and then these showed upregulation (Fig. V). This trend has also been reported in the proteomic analysis of potato shoots under salt stress (Aghaei et al., 2008). One common spot such as spot No. 5 (with upregulation in Afzal and downregulation in Macouei) and one spot in Afzal (Spot A) and the Macouei (S code spot) with different expressions under salinity stress shows different subunits of ATP synthase complex were detected (Figs. IV and V). Characteristics of protein spots are shown in Tables 1 and 2. Proteomic study of different subunit components of this complex have been identified in canola (Albertin et al., 2009). ATP synthase is the chloroplast structure with two main components that are extrinsic CF1 and the ATP synthase CF0. Proton transfer is dumped with the help of thylakoid membrane. CF1 has five subunits, alpha, beta (common spot A in Afzal and S in Macouei), Gamma, Delta (common spots No. 5), and Epsilon while CF0 has three subunits a, b, and c (Von Ballmoos et al., 2007). One of these subunits, β subunit, which may be a catalytic and ADP-binding unit, affects the conversion of ADP to ATP, which plays a crucial role in energy metabolism through creating a proton gradient between the membranes (Zellerhoff et al., 2010). Increased expression of proteins related to ATP synthesis under abiotic stresses, including drought and salinity are reported in previous studies (Fatehi et al., 2012; Guo et al., 2012).

J code protein in Afzal (14-3-3 protein) acts as the regulator of a wide range of target proteins throughout eukaryotes and direct interaction of the protein with the other proteins. At first, interactions among 14-3-3 proteins and their targets are accomplished by phosphorylation at specific places on the target protein. Therefore, interactions with 14-3-3s are controlled by environmental conditions through signaling pathways that affect 14-3-3 junction sites. Because proteins of 14-3-3 regulate and adjust the activities of many proteins involved in signal transduction, different levels of these proteins may be involved in responses to stress in higher plants. Evidence includes stress-induced changes in 14-3-3 proteins, interaction effects among 14-3-3 and signaling proteins, and interaction effects among proteins of 14-3-3 and defensive proteins (Salekdeh and Komatsu, 2007). One of the first plant sources in which the genes encoding 14-3-3 proteins were isolated was rice calluses and seedling exposed to high concentrations of NaCl or cold (Kidou et al., 1993). Also, the redistribution of 14-3-3 protein in sugar beet cells exposed to cold or osmotic stress (Babakov et al., 2000) is another indication that these proteins act as a response to abiotic stress. In both cases, there was an increase

in 14-3-3 protein levels in the cell wall, related to an increase in the amount of H+ -ATPase /14-3-3 complexes and an increase in ATPase activity. It is now known that 14-3-3 proteins activate H+-ATPase in a phosphorylation-dependent manner by interacting with the C-terminus of its regulator. Therefore, it appears that under cold and high osmotic conditions, 14-3-3 proteins interact with the C- terminal inhibitor domain of the H+-ATPase complex of the cell wall that activates the proton pump, which is very important for plants and the protective system against destructive external effects. Therefore, the increase in the level of this protein in Afzal (as a tolerant cultivar) during salinity stress indicates the contribution of homeostasis by this protein and less reduction in plant yield (Fig. IV).

Soluble NSF attachment proteins (O code protein in Macouei) or SNAP protein, which is a member of soluble NSF attachment protein receptor (SNARE) complex, has been reported to be involved in vesicular trafficking, cell wall stability, cytokinesis (involving KNOLLE), calcium binding (involving Synaptotagmin), membrane repair, and human genetic diseases including certain cancers (El Kasmi et al., 2013). Alpha-soluble NSF (N-ethylmaleimide sensitive factor) attachment protein, which is involved in membrane trafficking was also enhanced in tolerant cultivar and reduced in the susceptible cultivar. These results indicate that in a salt sensitive cultivar like Macouei, plasma membrane (PM) damage is partly caused by reduced POD (peroxidase) level, and membrane skeleton-related proteins may play a crucial role in PM and cell membrane stabilization under salt stress (Cheng et al., 2009). Further decrease in expression of this protein was observed for every level during salinity stress (Table 1, Fig. V). In contrast, peptidyl-prolyl cis-trans isomerases (PPIases) (P code protein in Macouei) are the sole enzymes which are known to stabilize this cis-trans transition, lower the activation energy of the stabilized product, and accelerate the isomerisation process (Fischer et al.,1989). So, with reference to the decreased expression of P code protein in Macouei cultivar had poor performance in maintaining stabilization during salinity stress (Table 1, Fig. V).

Heat shock proteins (No. spot 1 and B code spot) (Tables 1 and 2) among the molecular chaperons and stability and improved kink again proteins open during various stresses and degradation in throughput have and play a decisive role within the protection of plants against stress proteins to revert to their initial shape and thus are responsible homeostasis cells (Wang et al., 2004). The trend of adjusting these proteins during salinity stress in two cultivars was different. Afzal cultivar showed upregulation up to 9 days after the start of salinity and then showed a decrease in expression while Macouei cultivar showed downregulation up to 9 days after the start of treatment and then showed upregulation. Toorchi et al. (2009) reported reduced protein in soybean under osmotic stress while Michaletti et al. (2018) reported that this protein showed less expression in susceptible cultivar while showing more expression in the tolerant cultivar of wheat under drought stress. Also, previous studies have shown that the amount of HSP70 proteins increased in glasswort under salt stress (Wang et al., 2009). These results indicate that members within the HSP family may have diverse functions in plants' tolerance to salinity.

Conclusions

Generally, 21 and 17 protein spots were identified with significantly different IF in tolerant and susceptible cultivars, respectively. A total of 9 protein spots were identified with significant changes in the expression between the tolerant and susceptible cultivars under salinity stress. Most of these proteins were involved in removing antioxidants with the maximum level of Glutathione S-transferase. Furthermore, 12 protein spots were present only in the tolerant cultivar (Afzal), which were more involved in the removal of antioxidants and thanks to increase in the expression of those proteins, there was stronger cellular detoxification. Eight protein spots were observed uniquely within the sensitive cultivar (Macouei), most of them being associated with cellular hemostasis, and due to lower expression of these proteins, cellular hemostasis performance of this cultivar is less favorable under salinity stress.

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