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Protein pattern analysis in tolerant and susceptible wheat cultivars under salinity stress conditions

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ABSTRACT

To investigate proteome pattern of wheat cultivars, young leaves were collected from tillering stage of seedlings two weeks after development of the salinity stress. The extraction of proteins from leaf tissue was done and two dimensional electrophoresis using IPG strips and SDS-PAGE in the control and salinity treatments were performed. In total, 198 and 203 protein spots were identified in tolerant ('Moghan3') and susceptible ('Pishtaz') cultivars respectively. Also, among these, spots number 21 and 22 were detected with significant IF in 'Moghan3' and 'Pishtaz' respectively. Two-stage mass spectrometry (MS/MS) was used to identify protein spots. Common identified proteins, including proteins involved in removal of oxidants, Calvin cycle proteins, proteins involved in light reaction of photosynthesis and proton transfer, and heat shock protein were identified on basis of the functional groups and their frequency. In total, 'Moghan3' maintained the stability of the structure and performance of carbon metabolism under stress better than susceptible cultivar. In addition, defense against oxidative stress induced by salinity stress was performed by 2-cys peroxiredoxin BAS1 and Cu-Zn SOD proteins that tolerant cultivar defended against oxidative stress better than the susceptible cultivar. The greatest strength of 'Moghan3' and major weakness in 'Pishtaz' are relying on the unique proteins formed under salinity stress for the removal of oxidants and to maintain the activity of the photosynthetic light reactions, respectively.

Key words: proteomics analysis; salt tolerance; stress response proteins; two-dimensional electrophoresis; wheat

IZVLEČEK

ANLIZA VZORCA BELJAKOVIN V ODPORNI IN OBČUTLJIVI SORTI PŠENICE V RAZMERAH SLANOSTNEGA STRESA

Za analizo proteomskega vzorca v dveh sortah pšenice so bili vzorčeni mladi listi v fazi bilčenja dva tedna po izpostavitvi slanostnemu stresu. Izvleček beljakovin iz listnih tkiv je bil narejen z dvodimenzionalno elektroforezo z uporabo IPG trakov in SDS-PAGE, za rastline iz kontrole in tiste v slanostnem stresu. Celokupno je bilo evidentiranih 198 beljakovinskih točk za odporno sorto ('Moghan3') in 203 beljakovinskih točk za občutljivo sorto ('Pishtaz'). Med temi sta bili ugotovljeni beljakovinski točki št. 21 in 22 z značilnimi vrednostmi IF za 'Moghan3' in 'Pishtaz'. Za določitev beljakovin v točkah je bila uporabljena dvofazna masna spektrometrija (MS/MS). Določene beljakovine so obsegale encime, ki so vključeni pri odstranjevanju oksidantov, encime Kalvinovega cikla, beljakovine, ki so udeležene v svetlobnih reakcijah fotosinteze in v protonskem transportu ter beljakovine vročinskega udara. Beljakovine so bile določene na osnovi funkcionalnih skupin in njihove frekvence. V splošnem je v stresnih razmerah odporna sorta 'Moghan3' ohranjala stabilnost zgradbe in poteka presnove ogljika bolje kot občutljiva sorta. Dodatno sta se za obrambo proti oksidacijskem stresu v razmerah slanosti inducirala dva proteina, 2-cis peroksiredoksin BAS1 in Cu-Zn SOD protein, ki sta odporno vrsto ščitila bolje kot občutljivo. Odpornost sorte 'Moghan3' in občutljivost sorte 'Pishtaz' na slanostni stres temelji na edinstvenem vorcu beljakovin, ki se tvorijo v razmerah slanosti za odpravljanje oksidantov in vzdrževanje aktivnosti svetlobnih rekacij fotosinteze.

Ključne besede: proteomska analiza; odpornost na sol; stresni proteini; dvodimenzionalna elektroforeza; pšenica

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1 INTRODUCTION

Salinity stress tolerance in plants is a complex phenomenon and it is communicated with the physiological, biochemical and molecular mechanism. In this regard, proteomic approaches are identified as one of the most important methods for understanding the molecular basis of salt stress tolerance at the protein levels (Thiellement et al., 2002). Abiotic stresses such as salinity, before any effects are detected at the production level cause the change in cytoplasmic calcium concentration and pH which is understood as the main plant response mechanism under these condition. Osmotic stress and ion toxicity (sodium and chloride) derived from salinity stress act in both the inner and outer plasma membrane by trans-membrane proteins or enzymes inside cytosol. Many osmotics raised under drought stress are understood as stress sensors (Abdul Kader and Lindberg, 2010). Salt interferes with plant growth and can lead to physiological drought and ionic toxicity. Thus, salinity and drought stresses often affects the physiological aspects of plant metabolism, creating tension (hyperionic and hyper osmotic), and eventually plant will die. Salinity and drought stresses overlap on physiological level because salt in soil decreases the amount of available water and leads to reduced water absorption (Tuteja, 2007).

Salinity stress causes ion stress through the changes in potassium and sodium ion ratios. External sodium ions can have a negative effect on the absorption of calcium ions. Salinity resulted increases in the concentration of sodium and chloride ions in cytosol could be detrimental to the cells. Sodium ion can eliminate membrane potential, thus facilitates the absorption of chloride. High concentration of sodium ions (up to 100 mM), is toxic for the cell metabolism and can prevent activity of many essential enzymes, cell division and expansion, causing membrane damage and osmotic imbalance and thus stops the growth. High concentration of sodium ions can lead to the production of reactive oxygen species and reduction of photosynthesis. Potassium is one of the most essential elements required for plant growth. The concentration of potassium ions (due to severe salinity stress) causes osmotic imbalance problem in stomata functions and action of enzymes. Salinity damages cells, reduces leaf transpiration, resulting in the prevention of growth and causing cell intoxication. Salts can accumulate in older leaves and causing cell death (Tuteja, 2007).

Several studies involved in identification of proteins' response to salinity stress have used proteomics approaches. Most of the proteins affected under stress were involved in process of photosynthesis, photorespiration, transduction, metabolism, defense against oxidative stress, ion channels control and folding of proteins (Joseph and Jini, 2010). For example, changes in wheat proteome 30 days after exposure to 125 mM NaCl in the culture chamber were evaluated and a significant negative correlation between tolerance to salt and sodium concentration in wheat stems were observed. Protein expression change was more than 5 %, but the difference between the different groups of protein modifications (over expression, knockdown, disappearance and appearance) was variable from 1 to 8 % under salinity stress (Saqib et al., 2006). In order to better understand the development of wheat roots, Song et al. (2007) established a reference map of the major soluble proteins using a combination of 2-DE and MALDI TOF MS and a total of 450 protein spots were detected with silver staining in a pH ranges of 4 - 7, in which 282 protein spots were identified. These identified proteins grouped into diverse functional categories. In comparison with wheat leave proteome, in root, proteins involved in metabolism and transport increased expression, whereas proteins involved in energy, disease and defense, transcription, and signal transduction were of reduced expression. They also showed that hybridization between two parental lines could be different in protein expression in the offspring in comparison with their parents. Proteomic approach was used to identify the salt stress-responsive proteins in an elite Chinese wheat cultivar, 'Zhengmai 9023', which exhibits a high yield, superior gluten quality and better biotic resistance. Three-week-old seedlings were treated with NaCl of four different concentrations (1.0 %, 1.5 %, 2.0 %, and 2.5 %). The total proteins from the leaves of untreated and NaCl-treated plants were extracted and separated by two-dimensional gel electrophoresis (2D-DIGE). A total of 2358 protein spots were detected on the gels, among which 125 spots showed a significant change in protein abundance, and 83 differentially expressed spots were localised on preparative gels. A total, 52 salt-responsive spots were identified, which were classified into six functional categories that included transport-associated proteins, detoxifying enzymes, ATP synthase, carbon metabolism proteins, protein folding proteins, and proteins with unknown biological functions. Of the 52 differentially expressed proteins, 26 were upregulated, 21 were downregulated, and five spots showed multi-expression patterns. In particular, some important proteins for salt tolerance were found to be upregulated in this cultivar under salt stress, such as H^+-ATPases, glutathione S-transferase, ferritin and triose phosphate isomerase (Gao et al., 2011). On the other hands, proteomic investigation have been conducted to further understand the mechanism of plant responses to salinity in a salt-tolerant ('Afzal') and a salt-sensitive ('Line 527') genotype of barley. At the 4-leaf stage, plants

were exposed to 0 (control) or 300 mM NaCl. Salt treatment was maintained for 3 weeks. Total proteins of leaf were extracted and separated by two-dimensional gel electrophoresis. More than 500 protein spots were reproducibly detected. Of these, 44 spots showed significant changes to salt treatment compared to the control: 43 spots were upregulated and 1 spot was downregulated. Using MALDI-TOF-TOF MS, 44 cellular proteins were identified , which represented 18 different proteins and were classified into seven categories and a group with unknown biological function. These proteins were involved in various many cellular functions. Upregulation of proteins which were involved in reactive oxygen species scavenging, signal transduction, protein processing and cell wall may increase plant adaptation to salt stress (Fatehi et al., 2012).

This study compares two-dimensional electrophoresis pattern of salinity susceptible and tolerant wheat cultivars and ultimately, identification of expression changes and evaluation role of identified proteins under salinity stress.

2 MATERIALS AND METHODS

2.1 Plant material and experimental design

In this study, two cultivars of spring wheat namely 'Moghan3' (tolerant) and 'Pishtaz' (susceptible) at seedling stage in hydroponic culture system under salinity stress were evaluated in the greenhouse at University of Mahabad, Iran in 2015. Hoagland solution specifications used in wheat culture was mixture of NO_3 = 15 mM, K = 6 mM, Mg = 2 mM, Zn = 8 mM, B = 100 mM, Mn = 8 mM, Cu = 2 mM, Mo = 2 mM, Ca = 5 mM and Fe as Fe-EDTA = 4 mg l^{-1}. Experimental plots were tubes which were filled with sandy loam soil and were connected with nutrition source. The plant material was evaluated with factorial experiment based on Randomized Complete Block Design (RCBD) with four replications. The first factor included two levels of salinity exposed with chloride sodium such as control (non-stress) and 250 mM (NaCl) (as severe stress) and the second factor was wheat, above mentioned cultivars. Salinity started from tillering stage for two weeks and then treatments were sampled for proteome analysis. The rest of leaf samples were used for measuring of leaf water potential with pressure chamber, leaf relative water content by Morant-Manceau et al. (2004) method, osmotic potential by osmometer. Also, chlorophyll content (SPAD) and chlorophyll fluorescence was identified by chlorophyll meter and fluorometer respectively. In final, plant height and plant dry mass were measured.

2.2 Proteome analysis

2.2.1 Protein extraction

Total protein were extracted from 0.5 g frozen leaf for each biological replicate and it 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). In addition, protein concentration was determined by Bradford assay (Bradford, 1976).

2.2.2 The first and second dimension electrophoresis

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) was used. Then balancing of strips (equilibration) was carried out ((Herbert, 1999). Also, the second dimension gels as two pieces (including separator gel (separating gel) and holder gel (stacking gel)) were prepared. The separation gel, 8.5 ml, was prepared from the combination of acrylamide , 6.3 ml separating gel buffer (pH = 8.8), 2 ml distilled water, 120 µl 10 % APS and 20 µl TEMED. While stacking gel was prepared from combining of acrylamide for 1 ml stacking gel, l. 3 ml stacking gel buffer (pH = 6.8), 2 ml distilled water, 30 µl 10 % APS, and 20 µl TEMED). Then first dimension strips were put on the second dimension gel using agarose 1 %. Finally protein loading in second dimension with a current of 35 mA for each gel was conducted. After the second dimension electrophoresis, gel staining was performed using a solution of Coomassie blue (Herbert, 1999).

2.2.3 Gel imaging and protein spots analysis

Gels were scanned using BioRad GS-800 scanner. Images 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. Also it was used IF (Induction Factor) measurement for selection among significantly different spots for detecting spots with more expression change during salinity stress. Then the 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 separated in C18 columns by inverting phase movement. Subsequently,

peptides were sprayed into mass spectrometers. The range of ratio of mass to load in peptides was considered to be between 100 and 2000. The data obtained from the spectrophotometer with Bioworks software (ver. 3.3.1, Thermo Fisher) were converted into a usable format by Mascot search engine.

3 RESULTS AND DISCUSSION

3.1 Analysis of variance and Mean comparisons

Variance analysis is shown in Table 1. According to the results, between stress levels and cultivars were significant differences for all studied traits. The cultivar × stress interaction was not significant for any studied traits. The minimum and maximum coefficients of variation were related to SPAD (6.67) and chlorophyll fluorescence (10.18), respectively. Table 2 shows the comparison of the mean of stress levels and cultivars. According to results, quantitative mean of 'Moghan3' cultivar was better than 'Pishtaz' cultivar for all studied traits under salinity stress.

Table 1: Analysis of variance for morphological and physiological traits in wheat under salt stress

S.O.V	degree of freedom	Mean of squares						
		Plant Dry Mass	Plant Height	Fluorescence	SPAD	Osmotic Potential	RWC	LWP
Replication	3	0.01^{ns}	36.65^{ns}	0.003^{ns}	1.33^{ns}	0.008^{ns}	3.67^{ns}	0.005^{ns}
Stress (S)	1	0.98^{**}	95.56^{**}	0.010^{**}	19.25^{**}	0.34^{**}	153.01^{**}	0.38^{*}
Cultivar (C)	1	0.96^{**}	98.88^{**}	0.011^{**}	20.28^{**}	0.43^{**}	169.44^{**}	0.42^{**}
C × S	1	0.04^{ns}	33.70^{ns}	0.001^{ns}	3.88^{ns}	0.005^{ns}	2.65^{ns}	0.003^{ns}
Error	9	0.05	45.40	0.005	5.87	0.009	3.97	0.22
CV (%)		7.34	9.66	10.18	6.67	10.08	8.50	7.53

ns ,* and ** are non-significant and significantly in 5 % and 1 % probability levels respectively.

Table 2: Comparison of of the means for the stress levels and cultivars for studied traits in wheat

	Plant Dry Mass (g)	Plant Height (cm)	Fluorescence	SPAD	Osmotic Potential (MPa)	RWC (%)	LWP (MPa)
Control	1.73±0.02	29.3±0.7	0.8325±0.0023	42.1±0.3	-0.65±0.02	75.33±1.40	-1.36±0.03
Salt stress	1.08±0.04	21.1±0.9	0.7812±0.0034	39.8±0.5	-1.02±0.02	64.25±1.23	-1.89±0.01
Difference	$\pm0.65^{**}$	$\pm8.2^{**}$	$\pm0.0513^{**}$	$\pm2.3^{**}$	$\pm0.37^{**}$	$\pm11.08^{**}$	$\pm0.53^{*}$
Moghan3	1.92±0.02	31.4±1.0	0.8992±0.0020	45.3±0.4	-1.14±0.03	79.69±1.95	-1.06±0.02
Pishtaz	0.88±0.01	19.5±0.8	0.7761±0.0009	38.9±0.9	-0.53±0.01	61.04±0.89	-1.92±0.03
Difference	$\pm1.04^{**}$	$\pm11.9^{**}$	$\pm0.1231^{**}$	$\pm6.4^{**}$	$\pm0.61^{**}$	$\pm18.65^{**}$	$\pm0.86^{**}$

*, ** are significantly in 5 % and 1 % probability levels, respectively

3.2 Proteomics results

3.2.1 2Dimensional Gel Electrophoresis

Protein analysis discovered 198 and 203 protein spots in tolerant (''Moghan3) and susceptible ('Pishtaz') cultivars, respectively. Following, among significant spots, protein spots with significantly larger IF than 2 or smaller IF than 0.5 were picked (IF more than 1 points the increased protein expression under salt stress). According to IF value, out of 198 and 203 protein spots, 21 and 22 protein spots in 'Moghan' and 'Pishtaz' cultivars were detected, respectively. Out of these protein spots, 16 protein spots between the two cultivars were in common while five and six protein spots were unique for the 'Maghan3' and 'Pishtaz' cultivars, respectively. In other words, a total of 27 responsive protein spots under stress in both cultivars were identified. Gel image for both cultivars,'Moghan3' and 'Pishtaz' are shown in Figures 1 and 2.

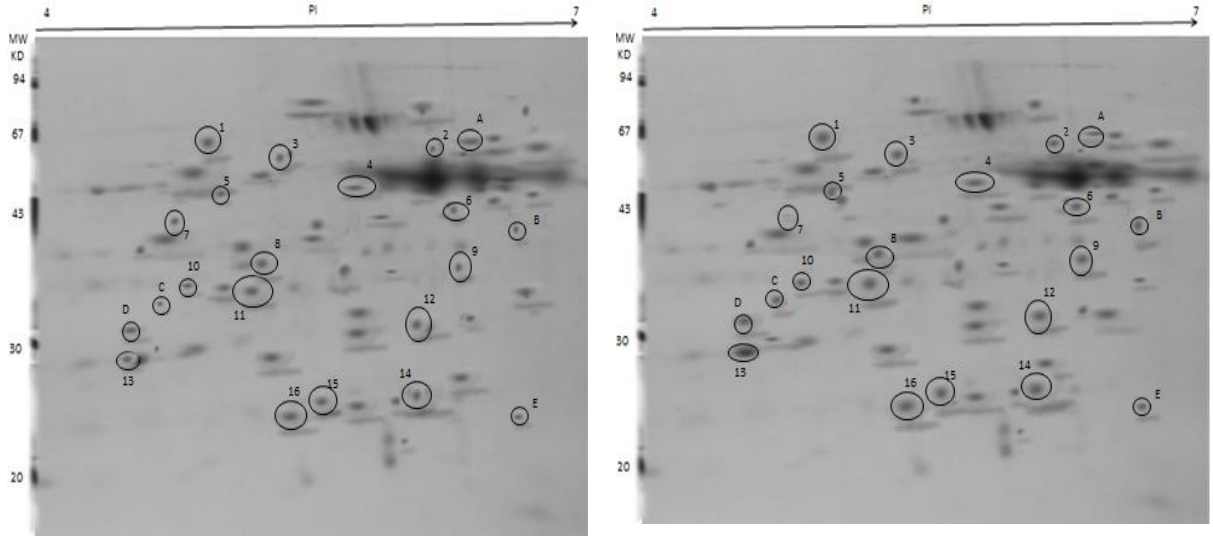

Figure 1: Comparison of 2D gel electrophoresis of 'Moghan3' under control (left) and salinity stress (right). Responsive common protein spots for salt stress are shown with numbers and uncommon protein with letters

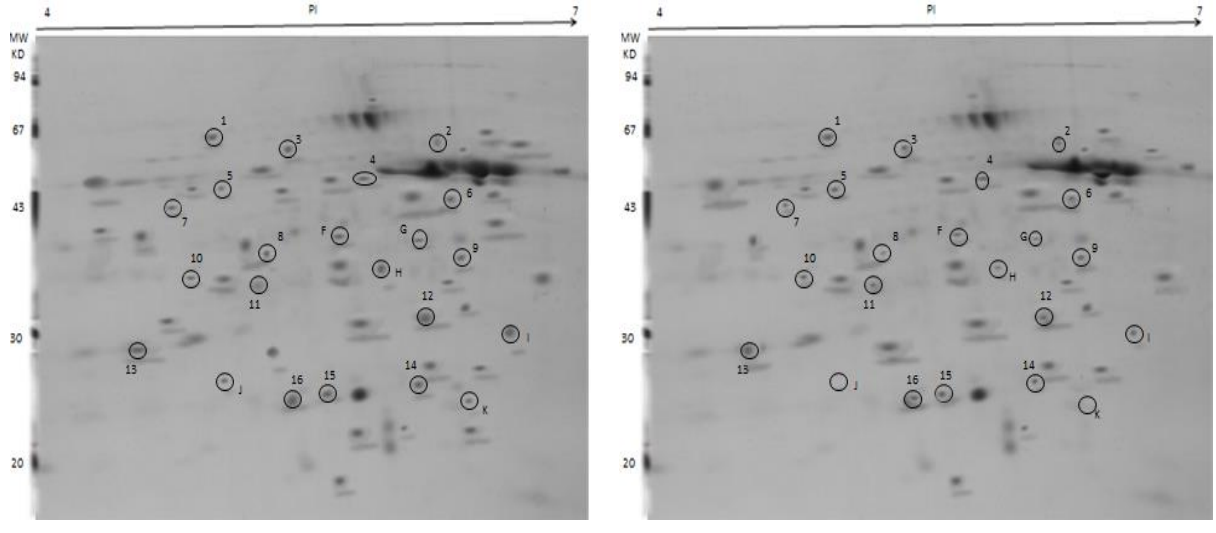

Figure 2: Comparison of 2D gel electrophoresis of 'Pishtaz' under control (left) and salinity stress (right). Responsive common protein spots for salt stress are shown with numbers and uncommon protein with letters

Based on the results it can be stated that increased expression of proteins in tolerant wheat cultivar under salinity stress was bigger than in susceptible cultivar (Tables 3 and 4). Also tolerant cultivar reaction to maintain its growth was better than in susceptible cultivar under salinity stress. These results are similar to results reported by Hosseini Salekdeh et al. (2002) and Naghavi (2014).

3.2.2 Protein identification by mass spectrometry

The responsive proteins to salinity stress on the staining gel were isolated and were identified using mass spectrometry. 16 common proteins and 11 uncommon proteins such as 5 spots in 'Moghan3' and 6 spots in 'Pishtaz' were detected (Tables 3 and 4). Also in Figure 3 numbers of downregulated, upregulated and absent/present proteins in two cultivars are shown The majority of responsive proteins in 'Moghan3' were upregulated while the majority of them in 'Pishtaz' were downregulated by salinity.

Table 3: Characteristics of 16 known common protein spots from all of significantly different accessions of both wheat cultivar under salinity stress

Functional group of protein	Spot number	Experimental		Theoretical		name of protein	Accession number	Expression in 'Moghan3'	Expression in 'Pishtaz'
		MW	pI	MW	pI				
proton transport	1	65.1	4.9	53.88	5.06	ATP synthase CF1 beta subunit	gi|14017579	upregulated	downregulate
proton transport	2	64.2	6.1	53.88	5.06	ATP synthase CF1 beta subunit	gi|14017579	upregulated	downregulate d
heat shock protein	3	63.4	5.4	73.72	4.9	70 kDa heat shock protein	gi|254211611	upregulated	downregulate d
Calvin cycle	4	50.3	5.7 5	53.4	6.2	Ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit	gi|61378609	upregulated	downregulate
Calvin cycle	5	45.1	5.1	47.34	8.62	ribulose 1,5-bisphosphate carboxylase activase isoform	gi|167096	further increase	increased less
protein synthesis/degradation	6	44.4	6.1	39.9	6.5	Triticain gamma	gi|111073719	upregulated	downregulate d
photoreaction of photosynthesis	7	42.9	4.7	37.01	5.4	photosystem II stability/assembly factor HCF136, chloroplastic-like	gi|357117071	decrease less	further decrease
removal of oxidants	8	38.3	5.3	27.9	5.6	acidic endochitinase	gi|116346	upregulated	downregulate d
Calvin cycle	9	38.1	6.1 5	18.80	8.83	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	gi|4038719	upregulated	downregulate d
removal of oxidants	10	37.3	4.8	29.5	10.2	HrPB1	gi|38679331	further increase	increased less
removal of oxidants	11	36.5	5.2	20.35	5.3	Cu/Zn superoxide dismutase	gi|1572627	upregulated	downregulate d
Calvin cycle	12	33.1	5.9 5	42.21	5.9	chloroplast fructose-bisphosphate aldolase	gi|223018643	upregulated	downregulate d
removal of ioxidants	13	28.9	4.6	23.39	5.4	2-cys peroxiredoxin BAS1, chloroplastic	gi|2499477	further increase	increased less
removal of oxidants	14	26.0	5.9	23.39	5.4	2-cys peroxiredoxin BAS1, chloroplastic	gi|2499477	upregulated	downregulate d
photoreaction of photosynthesis	15	25.2	5.6	27.42	8.84	oxygen-evolving enhancer protein 2, (OEE2) chloroplastic	gi|131394	upregulated	downregulate d
Remove of antioxidant	16	24.1	5.5	23.39	5.4	2-cys peroxiredoxin BAS1, chloroplastic	gi|2499477	upregulated	downregulate d

Table 4: Characteristics of five uncommon protein spots in ‘Moghan3’ and six uncommon spots in ‘Pishtaz’ under salinity stress

Functional group of protein	Spot code	Experimental		Theoretical		name of protein	Accession number	Expression in‘Moghan3’’	Expression in ‘Pishtaz’
		MW	pI	MW	pI				
heat shock protein	A	65.2	6.18	73.72	4.9	70 kDa heat shock protein	gi|254211611	downregulated	-
protein synthesis/degradation	B	39.9	6.3	24.4	10.1	50S ribosomal protein L10	gi|218192573	upregulated	-
removal of oxidants	C	34.1	4.68	29.52	9.51	HrPB1	gi|38679331	upregulated	-
removal of oxidants	D	32.0	4.61	17	5.37	Type 2 peroxiredoxin	gi|473787383	upregulated	-
removal of oxidants	E	24.3	6.35	20.35	5.3	Cu/Zn superoxide dismutase	gi|1572627	upregulated	-
photoreaction of photosynthesis	F	39.9	5.7	24.44	8.69	light-harvesting complex I, partial (chloroplast)	gi|544700	-	downregulated
photoreaction of photosynthesis	G	39.5	5.9	28.6	7.7	thylakoid lumenal 29.8 kDa protein	gi|195656049	-	downregulated
removal of oxidants	H	37.2	5.85	23.6	5.8	glutathione S-transferase	gi|5923877	-	downregulated
removal of oxidants	I	31.1	6.28	27.96	5.0	ascorbate peroxidase	gi|15808779	-	downregulated
proton transport	J	27.0	5.1	17.72	4.49	ATP synthase delta chain, chloroplastic	gi|475627717	-	absent under stress
photoreaction of photosynthesis	K	23.5	6.2	27.42	8.84	oxygen-evolving enhancer protein 2, chloroplastic	gi|131394	-	absent under stress

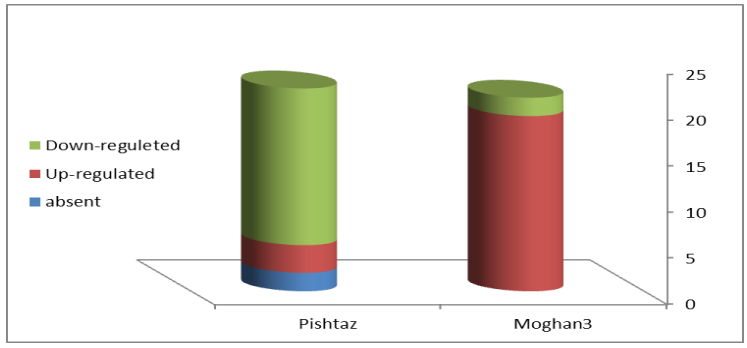

Figure 3: Number of protein spots in two cultivars of wheat under salinity stress

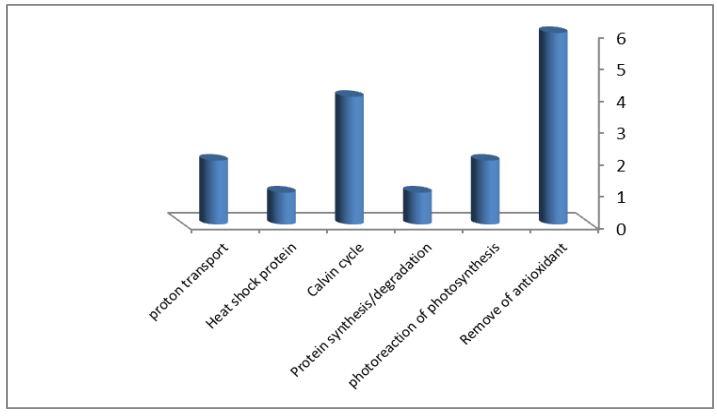

Figure 4: Number of common specific proteins in two cultivars of wheat under salinity stress

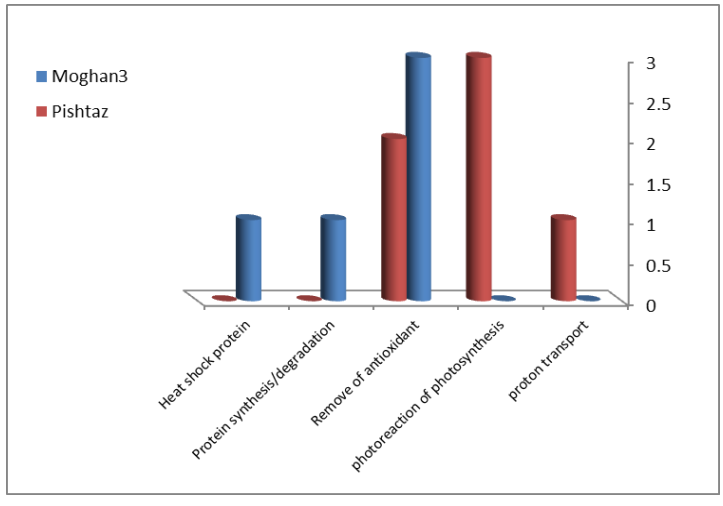

Figure 5: Number of uncommon specific proteins in two cultivars of wheat under salinity stress

3.2.3 Classification responsive proteins in two cultivars of wheat

A total 16 common protein spots were identified between tolerant and susceptible cultivars under salinity stress with difference expression (Table 3). According to Figure 4 the majority of these proteins are inside the cell, involved in removal of antioxidants (6 proteins), the Calvin cycle (4 proteins), light reaction of photosynthesis (2 proteins), proton transport (2 proteins), heat shock proteins (1 proteins) and protein involved in protein synthesis/degradation (1 protein), respectively. These results are similar to results reported

by Mittler (2002) and Noreen and Ashraf (2008). A correlation between the antioxidant enzyme activities and salinity tolerance was demonstrated by comparison of tolerant cultivar with sensitive cultivar. These activities were ascribed to increased protein expression under salinity stress and are closely related to salt tolerance in many plants (Athar et al., 2008). In addition, five protein spots were unique to the tolerant cultivar (‘Moghan3’) and had more to do with the removal of antioxidants. Due to expression increase of these proteins, this cultivar has stronger cellular detoxification system (Table 4, Figure 5). Six protein spots were seen uniquely in the sensitive cultivar (‘Pishtaz’) wherer the majority of proteins were related to photosynthetic light reaction (Table 4, Figure 5). According to results their decreased expression could be a cause for the reduction in the performance of photosynthesis under salinity stress in susceptible cultivar.

On the whole, the protein expression pattern in control (non-stress) and stress conditions were inserted into 4 groups (state) such as: a - proteins with reduced expression in both susceptible and tolerant cultivars (like protein 7) .b - proteins with increased expression in tolerant and reduce expression in susceptible cultivar (like protein 1), c - proteins present uniquely in the tolerant cultivar (such as A code protein), d - proteins only in the susceptible cultivar (such as H code protein) (Twyman, 2004).

Defense against oxidative stress

From total of common proteins 6 of them were related to removal of oxidants. The presence of 2-cys peroxiredoxin BAS1, chloroplastic (spots no. 13, 14 and 16), acidic endochitinase (spots no. 8), Harpin binding protein 1 (HrPB1) (spots No. 10) and Cu/Zn superoxide dismutase (spots No. 11), were induced under salinity stress in both cultivars (Table 3, Figure 1, 2, 4). On the other hands, 3 unique proteins (uncommon proteins) such as HrPB1 (C code), type 2 peroxiredoxin (D code) and Cu/Zn superoxide dismutase (E code) were identified related to remove antioxidant were upregulated in ‘Moghan3’ under salinity stress (Figure 3, Table 4). While, 2 unique proteins such as glutathione S-transferase (H code) and ascorbate peroxidase (I code) were identified related to remove of oxidants were downregulated in ‘Pishtaz’ (Figure 3, Table 4). Superoxide dismutase (SOD) proteins are in fact, the first defense line against ROS, which convert superoxide into hydrogen peroxide which is less toxic. In the absence of sufficient carbon dioxide as the final receptor of electrons, electrons migrate from the photosynthetic membrane to oxygen molecules via the Mehler reaction and generate superoxide ions (Cakmak, 2005). In response to salinity stress, one protein spot named chloroplast Cu-Zn SOD (No. 11 spot) in susceptible cultivar (‘Pishtaz’) showed decreased expression, but besides that in ‘Moghan 3’, protein spot with E code increases expression, as showed in Tables 3, 4. In line with these results, SOD accumulation in rice tolerant cultivar and its reduction in susceptible cultivars have been reported in response to salinity stress (Komatsu and Tanaka, 2004). Lower amount of this protein in stressed leaves of ‘Pishtaz’ caused a high level of hydroxyl radicals in the chloroplasts of this cultivar. In other words, under salinity stress conditions when increased production of ROS takes place, the disturbed balance between their production and elimination, especially in the tolerant genotypes is ameliorated by altering the expression of proteins associated with cellular homeostasis, the cell's balance is reestablished (Sun et al., 2006). On the other hand, peroxiredoxin proteins are expressed extensively in the tissues and are found in mitochondria and cytosols. The cell's location of these proteins, expresses the crucial role of antioxidants in the cellular organells that are the main source of ROS. These proteins, in addition to antioxidant activity, are also active in controlling signal transduction. The N-terminal of this protein, which contains cysteine, is oxidized to sulfenic acid, which acts as a bridge to react with peroxides. In fact, copper/zinc superoxide dismutase enzyme activity has been transformed ROS to H_2O_2, and the type 2 peroxiredoxin enzyme recovers H_2O_2 molecule, and in many studies, peroxirdoxin protein has been suggested as a protein responding to stress (Hashimoto et al ., 2009). The presence and activation of this protein in the ‘Moghan 3’ (spot No. 13, 14 and 16) shows the role of this protein in tolerance to salinity stress (Figure 1, 2 and Table 3). In general, due to the change of these proteins under salinity stress, reaction of ‘Moghan3’ was better than ‘Pishtaz’ as removal of antioxidant proteins.

Photosynthesis and carbon metabolism

Splitting of water by light takes place in the OEC (Oxygen-evolving complex) reaction center of photosystem II (Heide et al., 2004). The subunit of the PSII complex is the protein involved in the photosynthetic water splitting system known as OEC proteins and contributes to the stability of the PSII complex (Ifuku et al., 2008) and disruption of these proteins causes light damage to photosystem II (Takahashi and Murata, 2008). Therefore, due to reduced expression or lack of expression of this protein in the ‘Pishtaz’ (susceptible cultivar) (spot No. 15 and spot code K, Table 3, 4), the activity of photosystem II and eventually the efficiency of photosynthetic light reaction be reduced in this cultivar while this protein (Spot No. 15) showed increased expression (upregulated) in tolerant cultivar (‘Moghan3’). These

results indicate that under salinity stress, one of the important components of the photosynthesis machine, namely the oxygen swirling and the photosystem II complex has strongly affected, thus contributing to the aging of the leaves and possibly the gradual death of the cells. Komatsu and Tanaka (2004) made proteome analysis of leaf sheath in rice under salinity stress caused by sodium chloride. The frequency of this group of proteins in response to salinity stress was increased. It indicated the protective role of this protein against of salinity stress. On the other hands, HCF136 protein is a basic protein for repair, construction and stability of photosystem II complex (Plucken et al., 2002). In this experiment was found decreased expression of this protein in the 'Moghan3' (spot No.7) and further decreased expression in the sensitive 'Pishtaz'(Table 3). These results are similar to proteome analysis reported by Ford et al. (2011). In order to avoid light damage to photosynthetic apparatus, several mechanisms including adjusting absorbing antenna to light (LHC proteins) (spot code F in the 'Pishtaz') and reducing the size of antenna to reduce the absorption of light could be carried out (Eberhard et al., 2008). Our results showed that 'Pishtaz' for preventing of further damage to photosystem machine changed the expression in F code protein and these results are similar to results reported by Liu et al. (2014). Also, TL29 (thylakoid lumenal 29.8 kDa protein) (spot code G in the 'Pishtaz' with downregulated under stress) is a 29 kDa protein and is located in the thylakoid lumen (Kieselbach et al., 2000). Based on high homology with ascorbate peroxidase (APX) it was previously called also APX4 and was thought to plays a role in protecting cells against reactive oxygen species (Panchuk et al., 2005). Recently, based on testing Granlund et al. (2009) reported that this protein is associated with photosystem II and involved in prevention of photo damage to the photosystem II. So, according to reduced expression of this protein in susceptible cultivar, it could be the reason for decrease of photosystem II performance under salinity stress. In this regards, Zadraznik et al., (2013) reported that accumulation of these proteins and their isomerases in the tolerant genotypes is higher than in susceptible.

RuBisCO is a key enzyme for fixation of carbon dioxide in photosynthesis. It is formed from several catalyzing large subunits (catalytic large subunits) (spot No. 4) and several regulatory smaller subunits (regulative small subunits) (spot No. 9) (Spreitzer and Salvucci, 2002). In this experiment, both spots of 4 and 9 in 'Maghan3' showed increase expression under stress while these protein spots showed reduced expression in 'Pishtaz' (Table 3). Wan and Liu (2008), Naghavi (2010) and Naghavi (2014), found similar results in leaves of rice, canola and wheat under hydrogen peroxide, osmotic and drought stresses, respectively. Also, Ye et al. (2013) reported that 72 hours after stress imposed by PEG, the increased expression of RuBisCO took place in wheat leaves. This increase helped plants during drought stress with the increase of assimilation and better efficiency of photosynthesis in using of carbon dioxide and enables plants to overcome the stress. On the other hands, Calvin cycle consists of three phases. The third phase of the cycle is regeneration of RuBP molecules and Calvin cycle starts from the beginning. This phase is known by a series of enzymatic reactions in which triose-phosphate is converted to RuBP. Enzymes intermediary or mediatory in this phase include sedoheptulose-1,7-biphosphate and fructose 1,6-biphosphate aldolase (spot No. 12). Together these two enzymes catalyze a reaction that eventually results in the formation of ribulose-5-phosphate. Then ribulose-5-phosphate is phosphorylated and forms RuBP (Tamoi et al., 2005). Thereby reducing the mediator enzyme in this process (spots 12) in susceptible cultivar ('Pishtaz') causes reduction of efficiency of Calvin cycle and reduces sugar production. On the other hand, it is reported that photosynthesis-related proteins such as RuBisCO activaze (spot no. 5) (Table 3) showed decreased expression in susceptible cultivar of barley under stress condition (Kausar et al., 2013).

Other protein groups

A total two common spots such as 1 and 2 (with up-regulated in 'Moghan3' and down-regulated in 'Pishtaz') and one spots in the 'Pishtaz' (spot code J with absent expression under stress condition) were detected as different subunits of ATP synthase complex (Tables 3, 4). In previous experiments different subunit components of this complex in canola (Albertin et al., 2009) and leaves of corn (Porubleva et al., 2001) have been identified. Structurally, ATP synthase in chloroplast has two main components which include extrinsic CF1 and CF0.With their help protons are transmitted over the thylakoid membrane. CF1 has five subunits, alpha, beta (common spots No. 1 and 2), gamma, delta (spot code J in the 'Pishtaz') and epsilon while CF0 has three subunits a, b and c (von Ballmoos and Dimroth, 2007). One of these subunits is the β subunit, which is a catalytic and ADP-binding unit.It plays an important role in energy conversion by converting ADP to ATP when there is a proton-slope between the membranes (Ye et al., 2013). Increased expression of ATP synthesis-associated proteins under abiotic stress conditions, including drought and salinity, have been reported in previous studies (Guo et al., 2012).

Heat shock proteins (spot No. 3 and spot code A) (Tables 3 and 4) are molecular chaperones. These proteins help to stabilize and regenerate the proteins that have been opened and decomposed during various stresses. These proteins play a decisive role in plant

protection against stress to restore proteins to their original natural form and are involved in the establishment of cell homeostasis (Wang et al., 2004). Toorchi et al. (2009) reported on reduced expression of these proteins in soybean under osmotic stress. While, Naghavi (2010) working on canola tolerant cultivar under drought stress noticed increased expression of these proteins. In other study Naghavi (2014) reported that these proteins showed decreased expression in wheat under the drought stress, in susceptible cultivar a decreased expression was bigger than in tolerant one.

4 CONCLUSIONS

According to the results, 198 and 203 protein spots in tolerant and susceptible cultivars were identified, among them 21 and 22 protein spots with significantly different IF were identified, respectively. A total of 16 protein spots were identified common for both, tolerant and susceptible cultivars under salinity stress. The majority of these proteins were involved in removal of oxidants where the highest activity had 2-cys peroxiredoxin BAS1, chloroplast protein and the Calvin cycle proteins (among them were the most common subunits of Rubisco). In addition, five protein spots were present only in the tolerant cultivar. Majority of them were involved in the removal of oxidants. Due to increased expression of these proteins in tolerant cultivar it had better performance of cellular detoxification. Six protein spots were found uniquely in the sensitive cultivar. Majority of these proteins were related to light reactions of photosynthesis. According to the decreased expression of these proteins a reduction of photosynthesis performance under salinity stress appeared in susceptible cultivar.

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