

Proteome analysis of wheat embryo (*Triticum aestivum*) *Sensu stricto* germination under osmotic stress

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Abstract

Seed germination is an important growth phase in the plant life cycle that can be injured by osmotic stress. Germination is divided into three phases and many processes are initiated before radical protrusion. Many metabolic activities occur, especially in *Sensu stricto* germination phase since the seed embryo is a live organ, so differences in protein patterns are important in this phase. This study was carried out to find out the effects of osmotic stress on protein patterns in *Sensu stricto* germination phase. Effects of osmotic stress (-12 bar) on *T. aestivum* embryo (Sardari and Qods, tolerant and susceptible cultivars, respectively) proteins were evaluated. An osmotic potential of -12 bar was selected as the critical stress level, because at this treatment, germination percentage in susceptible cultivar was too low, but in tolerant cultivar was 50% as compared to control condition. Osmotic stress decreased germination percentage and caused adverse effects on protein patterns. After 2-DE proteome analysis of wheat embryo axis, 1000 spots were reproducible, although 16 spots were expressed differentially. Five protein categories were identified by MALDI-TOF/TOF in 16 differentially expressed, including energy (19%), stress/disease /defense (25%), metabolism (37%), protein synthesis/degradation (13%), and signal transduction (6%). Therefore, it should be noted that seeds were acting as intelligent systems which has an important role in response mechanism to osmotic stress during germination, especially before radical protrusion. In this study, it was demonstrated that stress and metabolism-related proteins such as HSPs, 1-Cys peroxiredoxin, alpha amylase inhibitor, and ADP-glucose pyrophosphorylase were more than other protein classes had vital roles in response to osmotic stress at 24 HAI. The results indicated that protein abundance under osmotic stress depends on the severity and amount of stress and imbibition time.

Keywords: Seed embryo, Germination, Proteomics, Wheat.

Abbreviation: *T. Aestivum*: *Triticum aestivum*, HAI: hours after imbibition, MALDI_matrix assisted laser desorption ionization; TOF_time of flight; 2-DE_two dimensional gel electrophoresis, HSP: Heat shock proteins.

Introduction

Plant responses to environmental stress depend on rapid activities of the molecular cascade signaling, including signal transport, stress perception alteration in patterns of gene expression and post-translational variation (Hossain et al., 2013). The plants have many different strategies to confront the stresses, so recent researches had paid special attention to plant responses, particularly at the molecular background (Zhu, 2003; Chaves et al., 2003; Fujita et al., 2006; Deyholos, 2010; Collins et al., 2008; Hu et al., 2010; Liu et al., 2011). Only embryo and aleurone layers in cereals stay alive, the endosperm cell undergoes programmed cell death (PCD) and does not survive (Bewley et al., 2012). Since cereal embryo has abilities to tolerant desiccation under osmotic stress, it is used as experimental models (Ingram and Bartels, 1996). Germination was defined as the ability of embryo to resume metabolic activity (Rajjou et al., 2012). During germination, seeds may experience distinct phases of water uptake, initially seeds absorb water exponentially, where this phase continue until cell contents are fully hydrated (phase I) followed by the slope of the water diminishing dramatically (phase II,) but these phases are not considered as complete germination. Water imbibition with an initiating third phase increase again, eventually leading to the emergence of the radical (Nonogaki et al., 2010).

During *Sensu stricto* germination several events happen, such as reserve mobilization, *de novo* mRNA synthesis and translation, translation of stored mRNAs, activation of amino acid metabolism and respiration (Preston et al., 2009; Vander Willigen et al., 2006; Manz et al., 2005). Some proteins related to dehydration tolerance such as late embryogenesis abundant (LEA) accumulated during germination and osmotic stress, these proteins are a class of proteins that protect macromolecules from dehydration injury and gene expression of these proteins is induced by ABA hormone during maturation and germination phases (Tunnacliffe and wise, 2007) although groups of proteins such as cytosolic ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione S-transferase (GST) and glutathione peroxidase have antioxidants activity that detoxification ROS are generated during stresses (Miller et al., 2010; Rizhsky et al., 2004 ; Koussevitzky et al., 2008 ; Irar et al., 2010 ; Grigorova et al., 2011). In the past two decades, to identify proteins involved in living organisms, especially plants, numerous studies have used proteomics because one of the most important methods has been two-dimensional gel electrophoresis (2D-PAGE) (ability to separate more than 1,000 proteins in the protein pattern) (Ostergaard et al., 2004) and to help a powerful technique named mass-spectrometry capabilities for detection of proteins (Rabilloud, 2002).

Since environmental stresses can prevent the appropriate establishment, especially at the germination stages of plants, review of seeds responses at molecular levels is necessary. We investigated seed embryo proteins of *Triticum aestivum* using 2-DE by MALDI-TOF/TOF in this study for the first time to find a wide view of the patterns of regulatory proteins during the early phase of germination (*sensu stricto*) under osmotic stress.

Results and discussion

Effects of different levels of osmotic stress on germination responses

According to the results, interaction of osmotic stress and cultivar on germination was significant ($P < 0.001$) (Table 1), therefore, wheat seed germination was significantly and negatively affected by osmotic stress. As osmotic levels increased under polyethylene glycol (PEG) treatment, percentage of germination decreased, however, this reduction was higher for susceptible cultivar. Up to -9 bar, no difference was detected between the two cultivars (Fig 1). Germination phase of most plants as halophyte plants are more sensitive than other growth phases to stresses (Meyers and Cuper, 1989). Critical potential was detected at -12 bar because the germination percentage diminished significantly at this osmotic stress level in a susceptible cultivar, while was 50% in tolerant (Fig 1). These results were similar to barely seed germination analyses (Gallardo et al., 2001; Finnie et al., 2004, 2006).

Proteome analysis of wheat seed embryo germination (sensu stricto)

Proteins of tolerant and susceptible wheat embryos are shown in Fig 2. Most spots fall in 10-80 kDa molecular mass range. After analyzing spots, about 1000 reproducible spots between all treatments was detected by MALDI-TOF/TOF as part of ongoing winter wheat proteome analyses (Sarhadi et al., 2010) (Fig 2). Among all treatments, 16 spots were expressed significantly. The detected proteins of the two cultivars were placed in following categories: energy, stress/disease/defense, metabolism and protein synthesis/degradation, and signal transduction (Table 2). A major protein class in this study was metabolism-related proteins (37%) (Fig 3). Each class of proteins in wheat embryo and their roles are presented separately below. These major protein classes in categories of this research were similar to other researches (Rajjou et al., 2012; Sarhadi et al., 2010; Finnie et al., 2004).

Proteins related to energy (and carbohydrate metabolism)

Three spots related to carbohydrate metabolism and energy were identified in wheat embryo germination. Two embryo beta-amylase proteins (spots 589, 1100) increased in the tolerant cultivar, especially under stress conditions and the end of *sensu stricto* germination phase (Figs 4, 5). Beta-amylase is an external hydrolyzed enzyme that removes beta maltose residue from non-reducing end of alpha1-4 glucan chains (Scheidig et al., 2002). Many previous studies reported that this protein besides alpha amylase increased during the germination phase (Finnie et al., 2004 and Ma et al., 2001). These proteins involve in glucose degradation compare to starch biosynthesis during late grain filling, although their accumulation increased under abiotic stress (Hurkman et al., 2009; Majoul et al., 2004).

Another protein related to energy category that increased during osmotic stress, especially 24 HAI in tolerant cultivar compared to susceptible cultivar was the ATP synthase beta subunit (spot 603) (Fig 4). This protein up-regulated in rice seed germinating during copper treatment, therefore, it seems that these proteins are involved in stresses related responses (Ahsan et al., 2007), but analysis of the endosperm cap of cress seed indicated that the abundance of this protein decreased toward endosperm rupture (post-germination) (Muller et al., 2010). A number of ATPase in chickpea indicated differential response during dehydration stress (Pandey et al., 2008). Since desiccation diminished CO₂ assimilation via reduction in the amount of ATP, it was recommended that induction of ATP synthesis helps tolerance traits in abiotic stresses (Zhang et al., 2008).

Proteins related to stress/disease/defense mechanism

Heat-shock proteins (HSPs) are associated with desiccation and expression of their genes often coincides with LEAs during seed development and expressed in the wheat embryo, especially under stress (Crosatti et al., 1999; Wehmeyer and Vierling, 2000). Analysis of 2-DE gels indicated the abundance of two HSPs proteins, (HSP70 (spot 622) and heat shock cognate 70 kDa protein 3 (spot 631)) increased greatly after 24 HAI under stress conditions in tolerant cultivar (Fig 4). HSPs is thought to function by restoring protein structure and prohibiting protein denaturation (Wang et al., 2004) and was acting as molecular chaperones (Hong and Vierling, 2000). In *Arabidopsis*, their abundant (HSP70) decreased, but remained constant at the end of the germination process (Gallardo et al., 2001). The abundance of these proteins increased (Georgopoulos and Welch, 1993), however, the frequency of these proteins in creeping bent grass cultivars (Xu and Huang, 2010) declined during desiccation and water stress and elevated temperatures (Sano et al., 2013). Reduction in HSP70 expression resulted in increased reactive oxygen species generation, therefore, recommended that this protein might play a crucial role in cellular redox status regulation (Guo et al., 2007). In *sensu stricto* germination of *Medicago sativa* seed was reported that HSP70 and GroEL-like chaperone abundance increased as molecular chaperone regulation (Yacoubi et al., 2011). 1-Cys peroxiredoxin located in the chloroplast and mitochondrion and are abundant low-efficiency peroxidases (Dietz, 2003). These proteins have many functions like intracellular signaling molecules and antioxidants regulators (Jang et al., 2004). After proteome analysis of wheat seed embryo germination, 1-Cys peroxiredoxin proteins (spots 218) in both tolerant and sensitive cultivars increased, but it was higher in tolerant cultivar (Fig 4). Gene involved in expression of 1-Cys-peroxiredoxin in *Arabidopsis thaliana* is related to ABI3 transcriptional activator, was also up regulated during oxidative stress and ABA treatment (Stacy et al., 1996). 1-Cys peroxiredoxin increased and decreased during mature drying and desiccation of maize seed, respectively (Huang et al., 2012). The abundance of this protein as antioxidant enzyme in endosperm cap increased during germination of cress seed (Muller et al., 2010). High expression of this protein was reported in late seed germination and 1-Cys peroxiredoxin transcript only observed in embryo of seed that survived during desiccation (Dietz, 2003; Haslekas et al., 2003). In rice seed, it was suggested that it has many antioxidant enzymes such as peroxiredoxin frequency toward to increase at the beginning of dehydration (Sano et al.,

Table 1. Variance analysis of osmotic stress treatments on germination percentage in two wheat cultivar, Sardari (Tolerant) and Qods (susceptible).

Variable and source	df	Mean square
Block	3	265/961*
Cultivar	1	1346/941**
Drought stress	16	10052/191**
Cultivar * Drought stress	16	1087/441**
Error	99	70/246
Total	135	

NS, **, * nonsignificant or significant at P= 0.05, 0.01 respectively

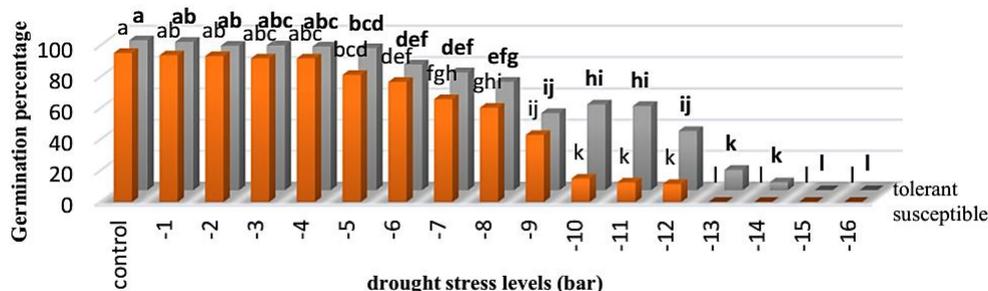


Fig 1. Mean comparison germination percentage of two wheat cultivar under different drought stress for determination of critical potential was shown. Potential -12 bar was selected since in this potential, germination percentage in susceptible cultivar was low and in tolerant cultivar equally with half of maximum index

2013). This protein was down-regulated during *sensu stricto* germination of alfalfa and Arabidopsis seeds and up-regulated in final Arabidopsis seed maturation (Yacoubi et al., 2011; Haslekas et al., 1998). Since seeds generate ROS during imbibition time, it seems that one of the important antioxidant enzymes for ROS detoxification is peroxiredoxin (Mustafiz et al., 2010) and synthesis of this protein initiated from 16 hours after imbibition of Arabidopsis seed (Galland et al., 2014).

Proteins related to metabolism

Accumulation of starch in embryo axis occurred during seed development and reached a minimum level in the final stages of maturation (Black et al., 1996). Alpha Amylase inhibitor was observed in many of cereals seeds (Chougule et al., 2004). One of the proteins in seed extracts which caused variation in several cultivars of barley has been identified as alpha-amylase/trypsin inhibitor (Ostergaard et al., 2002). Four alpha amylase inhibitors (Alpha-amylase/trypsin inhibitor CMB (spot 841), α -amylase inhibitor 0.19 (spot 143), endogenous α -amylase/subtilisin inhibitor (spot 194) and α -amylase inhibitor 0.53 (spot 140)) were detected after 2-DE analysis (Table 2). Three spots (841, 143 and 140) increased by increasing in imbibition time in susceptible cultivar, but there was one exception for spot 194 with higher level in tolerant cultivar, although it was suggested that higher abundance in tolerant cultivar may be due to resistance of this cultivar to insects onset (Figs 4, 5). In barley, this protein (α -amylase/ subtilisin inhibitor) act as an inhibitor of the endogenous α -amylase isoform AMY2 function (Abe et al., 1993). Many studies showed that these proteins had a resistance role, although they provide resistance to digestion from insects which used alpha amylase activity for carbohydrates (starch) digestion (Franco et al., 2002). It is recommended that they play a major role in keeping starchy seed versus endogenous amylase activity and

declined during the end of germination phases besides these roles (Avigad and Dey, 1997). A single isoform of this protein was detected by three spots that one of them was identified during desiccation (Finnie et al., 2006). Another protein involved in metabolic activity is an ADP-glucose pyrophosphorylase large subunit (spot 579) (Table 2), which was observed in wheat embryo protein. The abundance of this protein in tolerant and susceptible cultivars was similar, although with increasing in imbibition time, abundance of protein increased and the highest abundance was observed after 24 HAI in tolerant cultivar under stress (Fig 4). This protein is the first enzyme in the pathway of starch synthesis (Kotting et al., 2010). Transcripts of this protein accumulated in the lemma and palea of barley seed, which exposed to water stress (Abebe et al., 2010). After analysis of the expression profile of the maize embryonic axis, it was reported that ADP-glucose pyrophosphorylase genes up-regulated during germination (7.00-fold) (Jimenez-Lopez et al., 2011). Metabolism-related proteins such as fructose-bisphosphate aldolase (spot 56) were identified after 2-DE gel analysis (Table 2). This enzyme catalyzed Fru-1,6-bisP to glyceraldehyde 3-phosphate and dihydroxyl acetone phosphate, and a cytosolic Fru-1,6-bisP expressed in soybean and rapeseed differentially (Houston et al., 2009). In our study, it was detected as abundant protein in tolerant more than susceptible cultivar. Moreover, the highest abundance of this protein was detected during osmotic stress, especially in tolerant cultivar (Fig 4). In other studies, it was recognized that this protein was involved in glycolysis and gluconeogenesis/the calvin cycle and their frequency were up regulated in two cultivars of peanut during storage (Kottapalli et al., 2008). This highly abundant protein was observed in the rice embryo (Wang et al., 2008). This proteins reported increased highly in the endosperm cap during germination of cress seed after 18 h of imbibition (before endosperm rupture and ABA increased) (Muller et al., 2010).

Table 2. Protein identification by MALDI-TOF-TOF analysis from wheat embryo of two cultivar (susceptible and tolerant)

Spot no	Protein	Accession	Score	Sequence	Pi		Mw (kDa)		OS	Protein function
					theo	exp	theo	exp		
589	β -amylase (EC 3.2.1.2) 1,4- α -D-glucan maltohydrolase	gi 157830459	357	Snmllynyvpv ...g	5.4	5.41	55.6	55.63	<i>Glycine max</i>	E
603	ATP synthase beta subunit	gi 525291	147	Matrralssl ...ena	5.56	5.61	59.2	59.1	<i>Triticum aestivum</i>	E
1100	β -amylase	gi 223930617	359	Mevnvkgnvy ...lpatm	5.49	4.53	56.1	56	<i>Hordeum vulgare</i>	E
622	HSP70	gi 2827002	170	Makgepaig ...gpkievd	5.13	5.12	71	60.95	<i>Triticum aestivum</i>	S
631	Heat shock cognate 70 kDa protein 3	gi 108711797	405	Maptkgegpa ...agpkievd	5.09	5.07	71.3	70.41	<i>Oryza sativa subsp. Japonica</i>	S
218	1-Cys peroxiredoxin	gi 34539782	233	Mpgltigtv ...gylrftkv	6.08	6.1	23.96	24.02	<i>Triticum aestivum</i>	S
847	1-Cys peroxiredoxin	gi 1710077	182	Mpgltigtv ...gylrftkv	6.31	6.28	23.1	22.97	<i>Hordeum vulgare</i>	S
56	fructose-bisphosphate aldolase	gi 226316441	94	Msaycgkykd ...lhvdkyky	6.85	6.89	38.7	38.67	<i>Triticum aestivum</i>	M
841	Alpha-amylase/trypsin inhibitor CMB	gi 585290	172	Masksscdll ...qwnrfcss	5.6	5.62	16.52	16.5	<i>Hordeum vulgare</i>	M
143	α -Amylase inhibitor 0.19	gi 66841026	111	Cypgqafqvp ...kdvaaypda	6.7	6.69	12.7	12.73	<i>Triticum aestivum</i>	M
194	Endogenous α -amylase/subtilisin inhibitor	gi 123975	217	Dpppvhdtdg ...vvvfkkappa	6.88	6.79	19.6	19.64	<i>Triticum aestivum</i>	M
140	α -Amylase inhibitor 0.53	gi 123968	219	Sgpwmcypgq ...ypda	5.23	5.21	13.1	13.27	<i>Triticum aestivum</i>	M
579	ADP-glucose pyrophosphorylase large subunit	gi 32812836	360	Mssmqfssvl ...vv	5.89	5.81	57.7	57.64	<i>Triticum aestivum</i>	M
471	Eukaryotic initiation factor 4A	gi 1170509	357	Magmapesq ...adll	6.31	6.34	46.9	46.86	<i>Triticum aestivum</i>	P
738	Protein disulfide-isomerase	gi 508975	413	Maiskvwisl ...lkdel	4.99	4.92	56.4	56.39	<i>Triticum aestivum</i>	P
151	Calmodulin-3	gi 152013376	209	Madqltddq ...efvkvmmak	4.1	4.14	16.7	16.71	<i>Oryza sativa subsp. indica</i>	SI

Accession: accession number in database, Protein function (E: Energy, S: Stress/disease/defense, M: Metabolism, P: Protein synthesis/degradation, SI: Signal transduction).

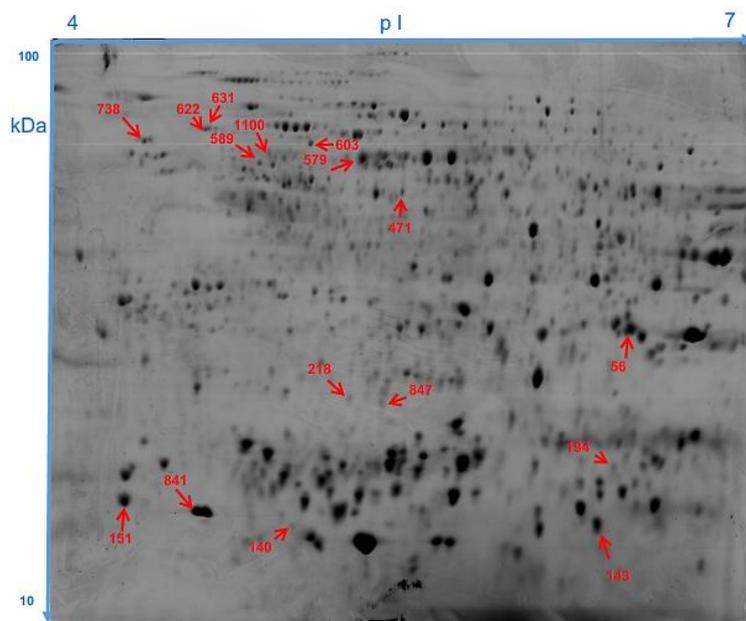


Fig 2. The two dimensions gel image stained with Coomassie Brilliant Blue dye. Points with significant differences were shown.

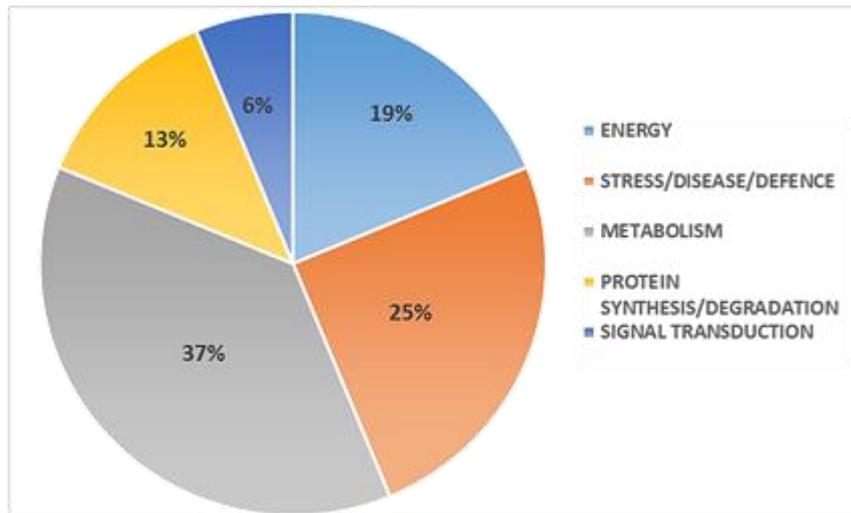


Fig 3. Classification of proteins identified by MALDI-TOF/TOF in 16 differentially expressed during wheat embryo germination under osmotic stress.

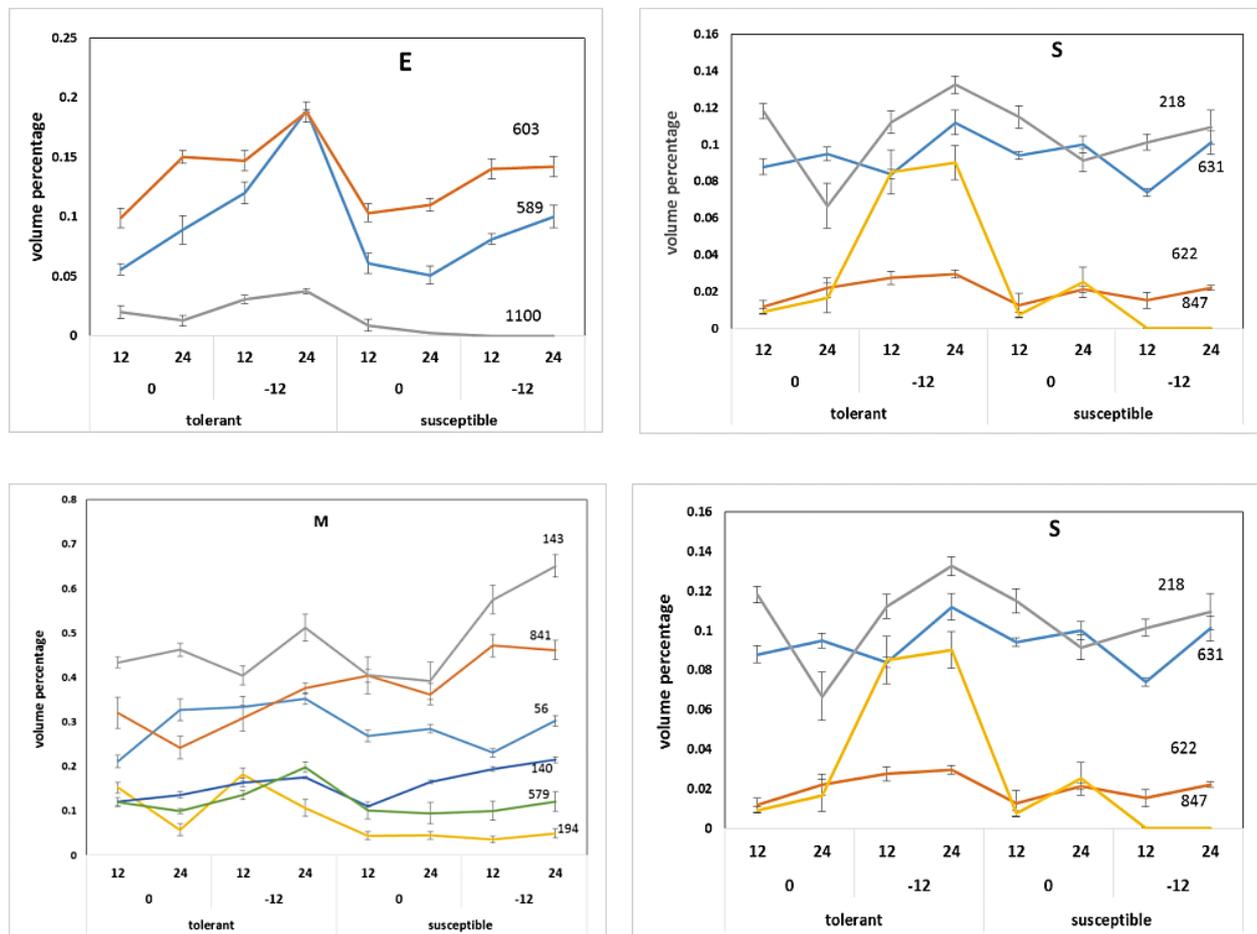


Fig 4. Volume percentage of spots according to classification of proteins identified during wheat embryo germination in two cultivar under drought stress. S: Stress/disease/defense, M: Metabolism, P: Protein synthesis/degradation, Si: Signal transduction.

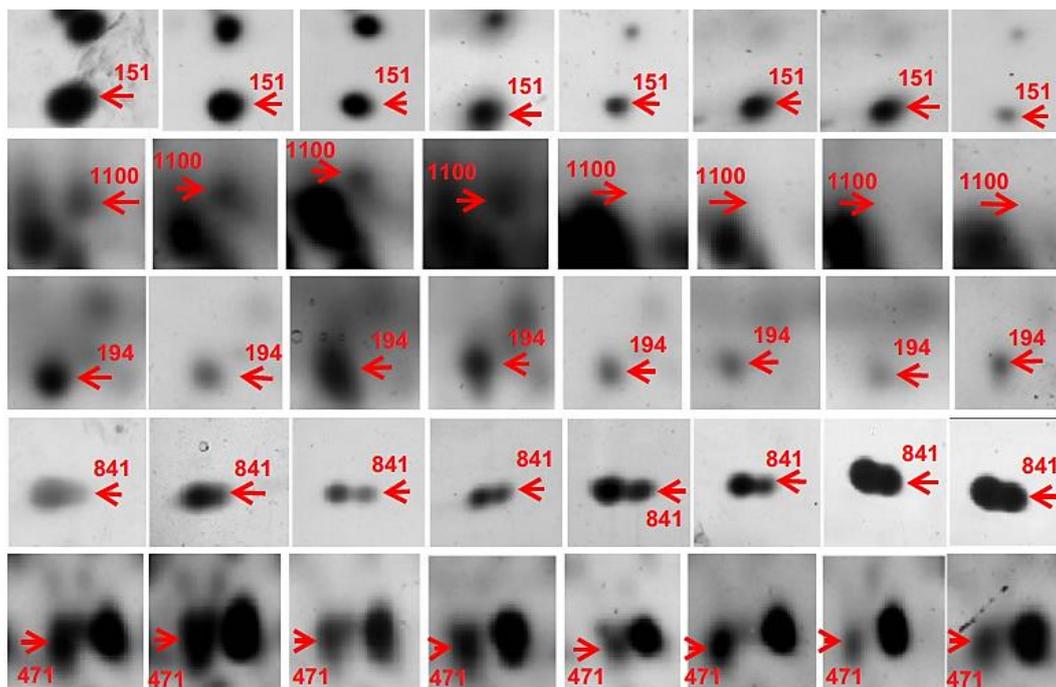


Fig 5. Five random spots with a significant difference in volume spots percentage. Each row represents one spot, from top to bottom, 151, 1100, 194, 841 and 471 respectively. Eight figures in each row represent Tolerant (Control) (12, 24 hours) – Tolerant (-12 bar) (12, 24 hours) – susceptible (control (12, 24 hours)) – susceptible (-12 bar) (12, 24 hours)) treatment from left to right respectively.

Proteins related to protein synthesis/degradation

Eukaryotic initiation factor 4A (spot 471) were identified in wheat embryo seed germination (Table 2). It was reported that this protein involved in elongation and initiation of a new peptides during protein synthesis (Wan and Liu, 2008). This protein is necessary for cap binding and activities of RNA helicase (von der Haar et al., 2004). In our study, it was indicated that major abundant of this protein was observed in tolerant cultivar in *sensu stricto* germination (12 and 24 HAI) and protein frequency decreased under stress, especially in susceptible cultivar (Figs 4, 5). In another study, it was shown that the level of this protein was constant during germination of *Arabidopsis thaliana* (Gallardo et al., 2001). Protein disulfide-isomerase, endoplasmic reticulum-located protein, is an enzyme that catalyzed redox (thiol) -disulfide reaction (oxidation, reduction, etc.) and is a vital role in redox signaling and found in all eukaryotic cells (Hatahet and Ruddock, 2009). Protein disulfide-isomerase was detected after sequencing 2-DE gel of wheat embryo (spot 738) (Table 2). It was observed that protein abundant was more during stress than control condition. Also with increasing in imbibition time, abundance of that increased, although abundance in tolerant was more than susceptible cultivar (Fig 4). This protein oxidized during *Arabidopsis* seed germination (Job et al., 2005). Protein abundant increased during alfalfa seed *sensu stricto* germination (Yacoubi et al., 2011). In *Arabidopsis* seed it was demonstrated that protein disulfide-isomerase was observed before program cell death in developing seed (Onda et al., 2011). Protein disulfide-isomerase up-regulated after protein analysis of rice as a model system for crop during oxidative stress (Kim et al., 2014).

Proteins related to signal transduction

Signaling proteins such as calmodulin were identified as the first class of sensors which binds Ca^{2+} (Ca^{2+} sensor) since

this protein have an importance role in regulation of activity of other proteins (Reddy and Reddy, 2004; Luan et al., 2002). This protein was demonstrated that was greatly conserved acidic protein in eukaryotes (Yang and Poovaiah, 2003). Calmodulin-3 (spot 151), one of the important signaling proteins, were identified in wheat embryo protein (Table 2). According to the proteome analysis, it was a major abundant protein in tolerant cultivar and control conditions and its abundance decreased during stress in both cultivars especially in susceptible cultivar. Thus, it was suggested that osmotic stress decreased abundance of this protein, also caused disturbance in signaling proteins (Figs 4, 5).

Materials and Methods

Plant materials

Susceptible and tolerant cultivar determination
Two wheat cultivars, namely, Sardari and Qods were treated under a simulated osmotic stress conditions using different solution of polyethylene glycol (PEG 6000) at 20° C to select susceptible and tolerant cultivars. Four replications of 50 seeds in each Petri dish with one filter paper and 16 osmotic stress levels (-1 to -16 bars) plus control level (0 bar) were used.

Critical stress level determination

After preliminary experiments, -12 bar potential was selected as the critical stress level because the germination percentage of susceptible cultivar was too low, but in tolerant cultivar was 50% under this osmotic potential (Fig 1). Seeds were exposed to this osmotic stress level and after 12 and 24 hours of imbibition, equivalent to the first and the second stages of germination, seeds were placed in liquid nitrogen and then embryos were isolated and stored at -80 °C until proteins extraction time.

Protein extraction

Protein extraction and 2-DE analysis were conducted according to the method of Sarhadi et al., (2010) with minor changes. Wheat embryos (200 mg) were powdered in a mortar with liquid nitrogen and 3 ml TCA extraction buffer (10% (w/v) TCA in acetone, 0/07 (w/v) DTT (dithiothreitol)) was added, then this mixture was suspended at -20 °C for 1 hour. After this time, the obtained suspension centrifuged at 21000 g for 20 min at 4 °C. The pellet with ice-cold acetone containing 0.07% (w/v) DTT was washed followed by incubation at -20 °C for 1 hour, then centrifuged at 12000g for 20 min at 4 °C. Pellet washing was done three times. Protein samples was solubilized in lysis buffer (4% (w/v) CHAPS, 9M urea, 1% (w/v) DTT, 1% (w/v) ampholyte, pH 3–10, 35 mM Tris–base). Bradford assay was used to determine protein concentration.

Two-dimensional polyacrylamide gel electrophoresis

Extracted proteins (120 µg and 2 mg) were loaded on to 350 µl of rehydration buffer (8M urea, 2% (w/v) CHAPS, 20 mM DTT, 0.5% (v/v) IPG buffer and a trace amount of bromophenol blue) for analytical and preparative gels, respectively. IPG strips (18 cm, pH 4–7 linear (Pharmacia)) were rehydrated in a re-swelling tray overnight (GE Healthcare), then IEF was conducted to Mutiphor II and a DryStrip kit (GE Healthcare) at 20°C (Finnie et al., 2004). For proteins separation, SDS-PAGE method was used by a vertical slab of acrylamide gels (12% total monomer, 2/5% crosslinker) and PROTEAN II Multi Cell (Bio-Rad) set. In order to visualize proteins in analytical and preparative gels, silver nitrate (AgNO₃) (Blum et al., 1987) and colloidal Coomassie Brilliant Blue G-250 (CBB) (Dyballa and Metzger, 2009) was used, respectively.

Image acquisition and data analysis

To scan analytical gels, GS-800 densitometer (Bio-Rad) was used at a resolution of 400 dots per square inch (dpi). Images obtained from scanning were saved as a TIF format. Protein spots were analyzed with Melanie 6 software (GeneBio, Geneva, Switzerland). Protein abundance was estimated according to the percentage volume (%vol), also in order to check relative differences between spots, Student's t-test was used. Only spots with significant changes (more than 1.5 fold change) were used for further analyses. The interesting spots (with significant changes) were excised from the preparative gels (CBB gels) using micro tips. Peptide masses were measured using a MALDI-TOF-TOF instrument in the University of Tehran, Iran.

Statistical analysis

Proteome analyses were conducted in a completely randomized design with three independent biological replications experiments. Germination tests were conducted in a completely randomized block design with four replications. For each replication before proteome analyses, a number of 500 wheat embryos from each treatment were used, then the results were analyzed using SAS software and the mean comparisons by Duncan's multiple range tests at $p < 0.05$ were performed.

Conclusion

Proteomics approach is a powerful technique to determinate different protein patterns, especially in stress studies. This

study was performed for the first time about the identification of proteins involved in *Triticum aestivum* embryo under osmotic stress, especially in *sensu stricto* germination, although these experiments on *Triticum durum* embryo have been conducted before (Irar et al., 2010). In our study, five protein categories (energy, stress/disease/defence, metabolism and protein synthesis/ degradation and signal transduction) were observed that had a vital role in seed germination (*Sensu stricto*). Among protein classes, metabolism-related protein (37%) was identified increasingly indicating that metabolic activity plays an important role in response mechanism to osmotic stress in seed germination, especially before radical protrusion (*sensu stricto*). However, we propose that genomics, metabolomics and transcriptomics approaches to be done to achieve a better understanding about the molecular occurrence in embryo axis (a miniature plant) of strategic plants such as wheat, especially under stress conditions.

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