Proteomic analysis of cold acclimation in winter wheat under field conditions

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ABSTRACT

A proteomics analysis was used to investigate how a winter wheat (*Triticum aestivum* L. cv Pishgam) adapted to LT during the autumn season and then initiated reproductive growth under field conditions. Cross-comparisons of leaf proteome were performed for consecutive stages including T_1 (before the beginning of cold acclimation; zero-tolerance), T_2 (initiation of the cold acclimation; LT tolerance=~-6 °C), T_3 (vernalization fulfillment stage; LT tolerance=~-15 °C) and T_4 (first weeks of reproductive stage; LT tolerance=~-10 °C). Approximately 400 protein spots were reproducibly detected on each gel. However, only 56 differentially abundant protein spots showed any considerable change ($p \leq 0.05$) between experimental stages. Proteomic analysis indicated a substantial decrease in the abundance of several proteins such as oxygen-evolving enhancer protein, NADH dehydrogenase and dehydroascorbate reductase during the initiation of cold acclimation. However, the abundance of some metabolic regulator, ion transporter, redox and photosynthetic proteins increased by achieving maximum LT tolerance (T_3). By initiation of the reproductive phase (T_4) the abundance of some proteins that mainly participate in photosynthesis and carbon metabolism significantly increased.

Keywords: Cold stress, comparative proteomic, two-dimensional electrophoresis, vernalization

YFIRLIT

Greining á próteinsamsetningu kuldaaðlagaðs vetrarhveitis í útiræktun.

Heildar próteingreining á vetrarhveiti (*Triticum aestivum* L. cv Pishgam) var gerð í þeim tilgangi að skoða þær breytingar sem verða í próteinsamsetningu plantnanna á mismunandi stigum kuldaaðlögunar og herslu. Sýni til greiningar og samanburðar voru tekin á eftirfarandi stigum þroska og kuldaaðlögunar: T_1 (áður en hersluferill gegn lækkuðum útihita hefst; án kuldaþols), T_2 (í upphafi hersluferils; kuldaþol LT=~-6 °C), T_3 (við fulla herslu; kuldaþol LT=~-15 °C) ogT_4 (á fyrstu viku kynvaxtar; kuldaþol LT =~-10 °C). Aðgreindir voru 400 próteinblettir með rafdrætti en af þeim sýndu aðeins 56 blettir marktækan mun ($p \leq 0.05$) á milli ofangreindra herslu- /þroskastiga. Við upphaf hersluferils minnkaði framboð nokkurrra próteina þar á meðal ljóstíllífunarpróteinsins oxygen-evolving enhancer, NADH dehydrogenasa og dehydroascorbate afoxunarensímsins. Við fulla herslu jókst magn nokkurra próteina sem tengjast stýrihlutverkum í efnaskiptum, jónaflutningi yfir himnur, afoxun og ljóstillífun. Við upphaf kynvaxtar kom fram marktæk aukning í nokkrum próteinum sem tengjast ljóstillífun og sykruefnaskiptum.

INTRODUCTION

Environmental stresses adversely affect growth and productivity and cause a series of morphological, physiological, biochemical and molecular modifications in plants (Xin & Browse 2001). Low temperature (LT) is a major environmental restriction on plant performance, especially in cold climates at high latitudes or altitudes (Mahfoozi et al. 2006). In cold regions, the problem of coping with LT is exacerbated through the requirement to lengthen the growing season beyond the short summer.

Plants differ in their cold response and LT-tolerant species may develop efficient strategies to adapt to a chilling environment. Many plants can increase their freezing tolerance when experiencing low but non-freezing temperature, which is known as cold acclimation (Chinnusamy et al. 2007). Therefore, even hardy plants are not freezing-tolerant during all growth stages. It has been documented that in winter cereals, cold acclimation, developmental stage and genotype can influence the expression of LT tolerance (Kosová et al. 2013). Some hardy plants delay transition from vegetative growth to the reproductive stage until they have been exposed to a period of low but non-freezing temperatures, a process called vernalization that allows plants to over-winter as seedlings (Limin &Fowler 2006). LT tolerance is generally regulated through expression of different inducible genes, which are fully expressed after cold acclimation during the vegetative stage; in contrast, this control appears to be switched off by the start of the reproductive stage when the plant has limited capability to acclimate to LT (Janmohammadi 2012). Consequently, after the completion of vernalization and at the end of the vegetative phase, the LT tolerances of winter plants gradually decrease (Limin & Fowler 2006). The vernalization process operates as a fine mechanism that maintains plants in the vegetative stage during the cold season and enters into the more sensitive reproductive phase when both a long day photoperiod and mild climate occur (Mahfoozi et al. 2001). Spring cereals do not have a vernalization requirement, normally developing rapidly into their reproductive phase when the risk of frost is resolved (Kosová et al. 2013). Acclimation to LT stress is mediated via profound changes in gene expression which result in changes in composition of the plant transcriptome, proteome and metabolome (Chinnusamy et al. 2007).

Previously, it has been proven that the alterations in gene expression at transcript level do not constantly match with the alterations at protein level (Bogeat-Triboulot et al. 2007). Consequently, examination of alterations in plant proteome is very important since proteins, unlike transcripts, are direct effectors of plant stress response. Proteomics is a powerful tool for investigating the molecular mechanisms of the plant response to stresses, and it provides a path toward increasing the efficiency of indirect selection for inherited traits. This approach has been applied to investigate the temporal changes of total proteins in different crop plants after LT stress. To provide a few examples, Rinalducci et al. (2011a) analysed the response of spring wheat seedlings (*T. aestivum* cv Kohdasht) to prolonged LT stress (42 days). In this study, the authors managed to identify 71 differentially expressed proteins; future protein classification revealed that the largest functional category of protein influenced by LT was devoted to photosynthesis and the Krebs cycle. Sarhadi et al. (2010) investigated the interrelationship between vernalization fulfillment and the expression of LT-induced proteins in winter wheat cultivars under controlled conditions. Cold acclimation resulted in significant changes in expression of some proteins which were categorized into cold-regulated proteins, antifreezing proteins, oxidative stress defense, photosynthesis, chloroplast post-transcriptional regulation, metabolisms, and protein synthesis. In this study, after vernalization fulfillment a considerable decline was observed in the protein abundance. Rinalducci et al. (2011b) used proteomic techniques to isolate cold-responsive proteins in leaf tissues of winter wheat (*Triticum aestivum* L. cv Cheyenne) after prolonged cold acclima-

tion. They identified a decrease in abundance of several photosynthesis-related proteins and a concomitant increase in abundance of some Calvin cycle enzymes. Similar results were also reported by Kosová et al. (2013). Although these studies provide initial insight into plant response to LT stress under controlled conditions, proteome analysis of LT tolerance and the interrelationship between the switch from vegetative to reproductive growth and the expression pattern of LT-associated proteins has not been studied in wheat cultivars under field conditions and in natural environments. To fill this gap, we looked for LT-induced protein changes by cross-comparison of leaf proteomes for consecutive developmental stages of winter wheat plants grown under field conditions in the Zanjan region, Iran.

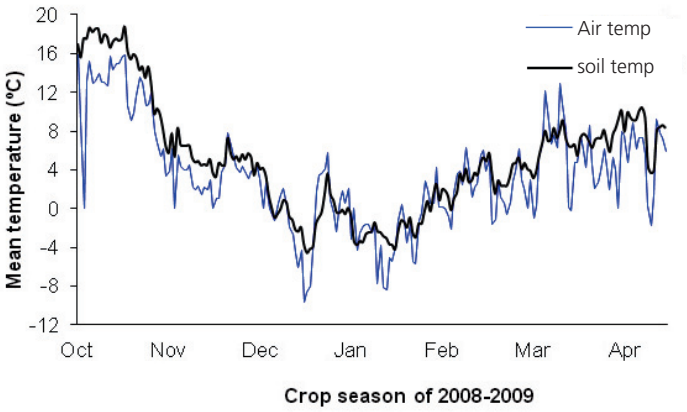

Figure 1. Average daily air and soil temperature (°C) at 5 cm depth as recorded at the Zanjan Agricultural Research Station, Iran in 2008–2009.

MATERIALS AND METHODS

Sample Preparation.

A winter wheat cultivar (*Triticum aestivum* L. cv Pishgam) with a relatively medium vernalization requirement was planted at the agricultural research stations of Kheyrabad in Zanjan province (36° 31' N, 48° 47' E; 1770 m) as a cold region with long cold winters in northwest Iran. Mean air temperatures (at 5 cm above ground) and soil temperatures at 5 cm depth were recorded from October 1, 2008, to March 31, 2009 (Figure 1) using an on-location weather station. Plants were sampled from November 4 to March 16 in 2008–2009 to determine their LT tolerance and stage of phenological development. Determination of LT tolerance was carried out during different developmental stages. The procedure outlined by Limin & Fowler (1988) was used to determine the LT_{50} (the mean lethal temperature) at each LT acclimation period. The stage of phenological development was determined through dissection of the plant crowns with the assessment of the changes in the morphology of the shoot apex expressed in decimal code (Natrova & Jokes 1993).

In order to perform protein extraction and proteome analysis, fully expanded upper leaves of wheat plants in each sampling date were harvested and then stored at -80 °C. After the completion of sampling and revealing the results of LT tolerance, proteome analysis was carried out for four sampling dates including T_1 (4 Nov: before the beginning of cold acclimation), T_2 (23 Nov: initiation of cold acclimation; LT tolerance=~-6 °C), T_3 (26 Dec: vernalization fulfillment; LT tolerance=~-15 °C) and T_4 (21 Feb: early reproductive growth stage LT tolerance=~-10 °C). Changes induced in leaf proteins were studied by two-dimensional gel electrophoresis and quantitatively analysed using image analysis software.

Protein extraction

One gram of harvested leaves was finely ground in liquid nitrogen using mortar and pestle, then the proteins were extracted following the procedure described by Damerval et al. (1986) with some modifications. Briefly, 1 g of powder was suspended in an ice-cold solution of 10% w/v trichloroacetic acid (TCA) in acetone with 0.07% w/v DTT for at least 1 h at -20 °C, and centrifuged for 20 min at 35 000× g.

The pellets were rinsed twice with acetone containing 0.07% w/v DTT for 1 h at -20 °C and then lyophilized. The resulting pellet was solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 35 mM TRIS-HCl, 1% w/v DTT, and 1% v/v Ampholyte pH 4-7) for 1 h at room temperature and then centrifuged at 12 000× g for 15 min. The supernatant was carefully collected and aliquoted and stored at -80 °C until 2-DE. The protein concentrations were quantified according to the Bradford method using BSA as standard (Bradford 1976).

Two-Dimensional Gel Electrophoresis

150 µg of protein were loaded by rehydration to 13 cm immobilized pH gradient (IPG) strips pH 4-7 and separated on IPGphor unit (Amersham Biosciences). The running conditions were as follows: 1 h gradient 250 V, 1 h gradient 500 V, 1 h gradient from 500 to 4000 V, and 5 h 4000 V with a total of about 24 kVh. Focused strips were equilibrated for 15 min in 8 ml equilibration solution (50 mM Tris-HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v sodium dodecyl sulfate (SDS), 20 mM DTT, 0.01% w/v bromphenol blue), and mounted on top of a 11.25% SDS–polyacrylamide gel with stacking gel (Laemmli, 1970) in a Hoefer S600 apparatus (Amersham Biosciences). After washing three times with dH_2O, gels were stained with colloidal Coomassie Brilliant Blue (CBB) G-250 solution (GelCode Blue Stain Reagent, Pierce) for 1 h, washed with dH_2O for 5 min, and stored in dH_2O.

Gel imaging and data analysis

After SDS–PAGE, CBB stained 2-DE gels were digitized using a flatbed scanner (UMAX Power Look III) with Power Scan 3.0 software (Nonlinear Dynamics). Image analysis was carried out with Progenesis SameSpots software package Phoretix 2D Evolution v2005 (Nonlinear Dynamics, Newcastle, UK), which allows spot detection, background subtraction and protein spot OD intensity quantification (spot quantity definition). After image analysis including the Student's t-test, spots with a significant difference ($p \leq 0.10$) in expression intensity in pairwise comparisons were picked from the respective 2-DE gel and subjected to identification by mass spectrometry. Detailed expression profiling for each selected protein spot throughout all experimental conditions was performed using PDQuest Advanced 8.0 software (BioRad).

Protein Identification and Database Search

Three biological and three technical replicates per condition were performed and cross-compared by the image software analysis. Protein spots of interest were manually cut out from the 2DE gels and washed for 30 minutes at room temperature with 400 ml buffer consisting of 100% acetonitrile/50 mM ammonium bicarbonate (NH_4CHO_3) (50:50 v/v). After removing the supernatant, the gel pieces were dried. Proteins were digested using a trypsin solution containing 12 ng/μL (10 µL) trypsin in 50 mM ammonium bicarbonate solution. After incubation for 5 h at 37°C, the reaction was stopped by adding 2 µl 1% trifluoroacetic acid (TFA) (Schlesier & Mock, 2006).

Protein identification was performed by MALDI-TOF mass spectrometry (MS) following the procedure described by Amme et al. (2006). For this purpose, 1 µl of the protein spot digest was mixed with 2 µl of the matrix solution (5 mg α-cyano-4-hydroxycinnamic acid (CHCA) in 80% v/v ACN and 0.1% w/v TFA) and 1 µl of this mixture was deposited onto the MALDI target. Tryptic peptides were analysed with a REFLEX III MALDI-TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany). Spectra were calibrated using trypsin autolysis products as internal standards under application of the XMASS software Version 5.1.5 (Bruker Daltonics, Bremen, Germany). Protein identification was performed by searching for Viridiplantae in the non-redundant NCBI, HarvestHv, TIGRPoa and the SwissProt database using the MASCOT search engine (Matrix Science, London, UK) with the following parameters: monoisotopic mass accuracy, 100 ppm; missed cleavages, 1; allowed variable modifications, oxidation (Met) and propionamide (Cys). Protein

MASCOT scores higher than 70 for MS were considered to be statistically significant ($p \leq 0.05$).

RESULTS

Analysis of variance indicated that differences in LT_{50} due to the acclimation period were highly significant. Plants grown at cool temperatures in autumn started to acclimate at a rapid rate. The rate of change in LT tolerance then gradually slowed until LT tolerance began to be lost (Figure 3). Plants reached their maximum LT tolerance (LT_{50}) -15 °C) between 60 and 75 d, which is about the same time as vernalization saturation occurred under field conditions in the Zanjan region (Figure 2). Shoot apex dissection, which is commonly recognized as a good indicator of wheat developmental stage, was also performed (Figure 2) and expressed by decimal code (DC). At the T_1 stage, both genotypes were in an early vegetative stage, i.e., their shoot apices were short and produced only leaf primordia. Based on trend of LT tolerance (Fig. 3) and phenological development we decided to perform a proteomic study on samples harvested during a vegetative phase (T_1 and T_2 ; DC of about 11 and 16), point of vernalization fulfillment and formation of double ridge DR-1 stage (T_3; DC= 20) and reproductive phase (T4; DC=26). During the experiment, plants were in the 3–5 leaf stage. At the T_4 stage, plants started to develop the first tiller. We found 397 spots that were common to all the gels (Figure 4); among these 185 spots were changed ($p \leq 0.10$) due to the cold acclimation process and phenological development (Fig. 6). However, only a total of 56 spots showed considerably different abundance levels ($p \leq 0.05$, absolute variation).

Table 1 shows the list of proteins which their abundance significantly changed in comparative image analysis. The most increase in protein abundance during early weeks of LT hardening was recorded for heat-shock protein/chaperone that is responsible for protein folding, assembly, translocation and degradation in many normal cellular processes (De Maio 1999). In addition, abundance of key enzymes of the glycolysis pathway (spot 1357), a transcription factor from MADS-box family (spot 2432), proteins involved in protein transport (spot 641) and ribulose bisphosphate carboxylase/oxygenase large sub-unit (RuBisCO LSU) significantly increased with LT acclimation (Table 1).

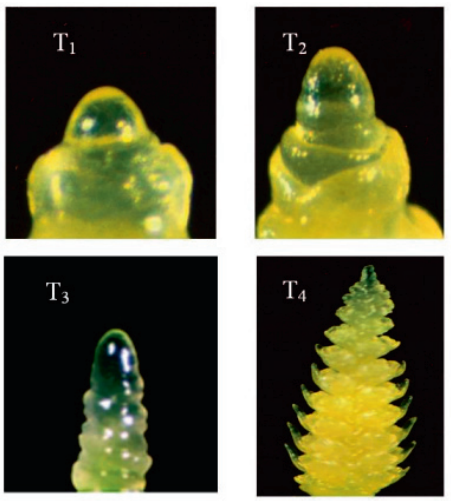

Figure 2. Phenological growth stages of Pishgam winter wheat cultivar as estimated from shoot apex developmental morphology.

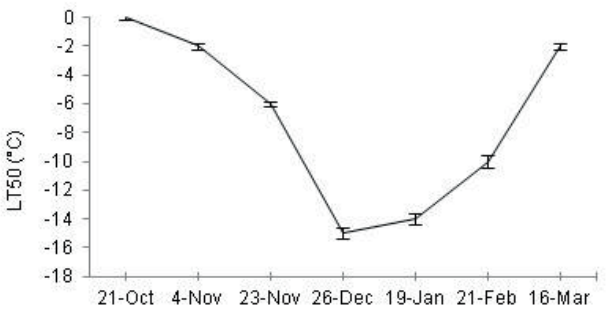

Figure 3. Low-temperature tolerance of Pishgam winter wheat acclimated under field conditions at the Zanjan Agricultural Research Station, Iran in 2008–2009.

Table 1. Differentially expressed proteins during different developmental stages in winter wheat identified by MALDI-TOF MS.

Spot No.[a]	Protein name	Accession no.	Organism	Database	Fold of variation[b]	Proteome comparisons
Increased						
771	70 kDa heat shock protein	gi|290131414	*Triticum aestivum*	NCBI	2.13	T_2/T_1
1357	Putative fructose-bisphosphate aldolase	35_1820	*Oryza sativa*	HarvestHv	2.08	T_2/T_1
2432	MADS-box protein	35_27943	*Oryza sativa*	HarvestHv	2.05	T_2/T_1
1042	RuBisCO large subunit	gi|2493650	*Triticum aestivum*	NCBI	1.95	T_2/T_1
641	Os03g0108400	gi|255674149	*Oryza sativa*	NCBI	1.88	T_2/T_1
1292	actin	gi|281485191	*Persea americana*	NCBI	1.86	T_2/T_1
1133	NADH dehydrogenase subunit 1	gi|18378414	*Cucurbita argyrosperma*	NCBI	1.76	T_3/T_2
1169	Thioredoxin-like protein	35_50073	*Oryza sativa*	HarvestHv	1.68	T_3/T_2
2199	Oxygen-evolving enhancer protein 2	gi|131394	*Triticum aestivum*	NCBI	1.44	T_3/T_2
2234	Manganese superoxide dismutase	gi|125663927	*Triticum aestivum*	NCBI	1.40	T_3/T_2
2169	NADH dehydrogenase subunit 1	gi|18378406	*Cucurbita ecuadorensis*	NCBI	1.36	T_3/T_2
2351	metal ion transmembrane transporter	gi|240256271	*Arabidopsis thaliana*	NCBI	1.34	T_3/T_2
2190	Oxygen-evolving enhancer protein 2,	35_1423	*Oryza sativa*	HarvestHv	1.96	T_4/T_3
1510	ribosomal protein S1	gi|159161283	*Cuscuta exaltata*	NCBI	1.92	T_4/T_3
1108	UDP-glucose pyrophosphorylase	gi|88866516	*Oryza sativa*	NCBI	1.84	T_4/T_3
1359	Fructose-bisphosphate aldolase	Ta_TC235339	*Arabidopsis thaliana*	TIGERPoa	1.79	T_4/T_3
2648	ribulose 1,5-bisphosphate carboxylase, large subunit	Ta_TC263613	*Arabidopsis thaliana*	TIGERPoa	1.78	T_4/T_3
1007	RuBisCO large subunit-binding protein subunit alpha,	gi|134102	*Triticum aestivum*	NCBI	1.70	T_4/T_3
Decreased						
2199	Oxygen-evolving enhancer protein 2, chloroplastic	gi|131394	*Triticum aestivum*	NCBI	0.68	T_2/T_1
2351	metal ion transmembrane transporter	gi|240256271	*Arabidopsis thaliana*	NCBI	0.54	T_2/T_1
2169	NADH dehydrogenase subunit 1	gi|18378406	*Cucurbita ecuadorensis*	NCBI	0.52	T_2/T_1
1821	oxygen-evolving complex protein 1	gi|739292	*Triticum aestivum*	NCBI	0.50	T_2/T_1
2265	undecaprenyl diphosphate synthase, putative	gi|255582903	*Ricinus communis*	NCBI	0.50	T_2/T_1
2191	Oxygen-evolving enhancer protein 2	gi|131394	*Triticum aestivum*	NCBI	0.48	T_2/T_1
2207	dehydroascorbate reductase	gi|259017810	*Triticum aestivum*	NCBI	0.48	T_2/T_1
1062	Os05g0291700 protein	gi|3176645	*Oryza sativa*	HarvestHv	0.58	T_3/T_2
1237	Transcription factor, putative	gi|255574095	*Ricinus communis*	NCBI	0.48	T_3/T_2
1042	RuBisCO large subunit-binding protein subunit beta, chloroplastic	gi|2493650	*Triticum aestivum*	NCBI	0.48	T_3/T_2
1244	Translational elongation factor Tu	35_976	*Oryza sativa*	HarvestHv	0.46	T_3/T_2
1069	ATP synthase CF1 beta subunit	gi|14017579	*Triticum aestivum*	NCBI	0.46	T_3/T_2
1366	ribulose 1,5-bisphosphate carboxylase activase isoform 1	gi|167096	*Hordeum vulgare*	NCBI	0.44	T_3/T_2
1357	Putative fructose-bisphosphate aldolase	35_1820	*Oryza sativa*	HarvestHv	0.43	T_3/T_2
948	protein P0668H12.12	35_24827	*Oryza sativa*	HarvestHv	0.49	T_4/T_3
1502	Putative aldehyde oxidase-like protein	35_3416	*Oryza sativa*	HarvestHv	0.44	T_4/T_3
689	Cytochrome P450-like	35_12050	*Oryza sativa*	NCBI	0.40	T_4/T_3
525	Electron transporter, putative	gi|255576550	*Ricinus communis*	NCBI	0.38	T_4/T_3
2690	cell-autonomous heat shock cognate protein 70	gi|26985223	*Cucurbita maxima*	NCBI	0.38	T_4/T_3
1335	Photosystem II 44 kDa reaction center protein (P6 protein)	Og_TC291300	*Oryza sativa*	TIGERPoa	0.36	T_4/T_3

[a] Spot number represents the number on the master gel (see Figure 4).

[b] Fold of protein variation is calculated by standardizing the mean of the normalized spot volumes of samples at different harvesting times (T_1, T2, T_3 and T_4) with the mean of the normalized spot.

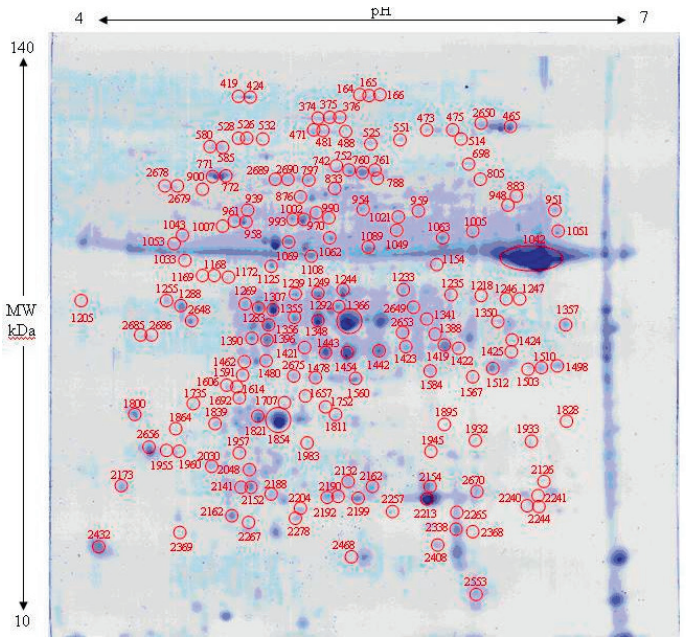

Figure 4. 2-DE gel analysis of proteins extracted from leaves of Pishgam winter wheat harvested at different developmental stages. Panel shows the reference map derived from computerized image analysis performed by using Progenesis SameSpots software. Numbers indicate the variable protein spots.

During the early weeks of LT acclimation the abundance of some proteins such as oxygen-evolving enhancer protein 2 (spot 2199), metal ion transmembrane transporter (spot 2351), NADH dehydrogenase subunit 1 (spot 2169), oxygen-evolving complex protein 1 (spot 1821), a putative undecaprenyl diphosphate synthase (spot 2265), and a dehydroascorbate reductase (spot 2191) decreased. The most decrease in protein abundance was related to some proteins involved in light reactions of photosynthesis (spot 2199) and ion transport (spot 2351).

The comparative image analysis in samples from T_2 and T_3 revealed that abundance of proteins involved in photosynthesis (spot 2199), reduction/oxidation (redox) (spot 1133, 1169, and 2169), reactive oxygen species (ROS) scavenging (spot 2234) and ion transport were increased. Also at vernalization fulfillment stage (T3) the abundance of some proteins that were involved in metabolic processes (spot 1062), protein folding, response to LT (spot 1042), transcription (spot 1237), protein biosynthesis (spot 1244), ATP synthesis (spot 1069), photosynthesis (spot 1366) and glycolysis (spot 1357) were decreased.

During the transition from the vernalization fulfillment point (T3) to the early reproductive stage (T4) some abundance of some proteins such as OEE2, ribosomal protein S1, UDP glucose pyrophosphorylase (UGPase), fructose-bisphosphate aldolase and both α and β subunits of RuBisCO as well as Rubisco Activaseincreased significantly. At the occurrence of the vegetative/reproductive transition the abundance of some defense proteins such as cytochrome P450-like (spot 689) and heat shock 70 (spot 2690) were decreased.

Investigation of the protein abundance trends between different stages revealed that there were 14 proteins with similar abundance trends (Figure 5). In this cluster proteins showed a relatively constant trend during the first two stages (T_1 and T_2) and then their abundance considerably decreased with vernalization fulfillment (T_3), and finally, upon entering the reproductive phase, their abundance significantly increased. Some of the identified spots from the abovementioned cluster included the RuBisCO large subunit-binding protein subunit beta (spot 933), UDP-glucose pyrophosphorylase (spot 1108), fructose-bisphosphate aldolase (spot 1359), ribosomal protein S11 (spot 1510), two-component response regulator-like PRR73 (spot 1684), putative dehydrogenase (spot 2653) and cp31BHv (spot 2656). These proteins are involved in photosynthesis (993), metabolic processes (1108), glycolysis (1359), translation (1510), biological rhythms

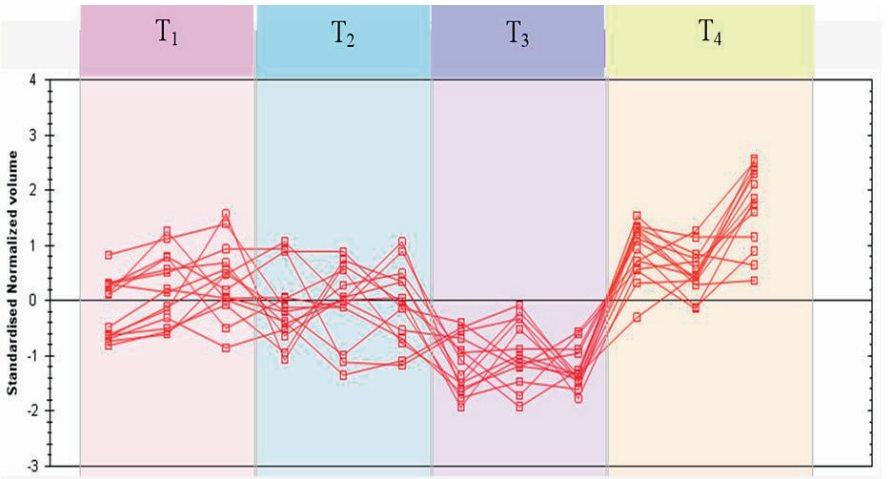

Figure 5. Phenological development and cold-induced changes in proteins of Pishgam winter wheat grown under field conditions at the Zanjan Agricultural Research Station, Iran in 2008–2009. Values are shown as log-normalized volumes. Three single dots in each stage indicate the normalized volume in gel replications.

and transcription (1684), threonine and methionine biosynthesis as well as in cellular redox balance (2653).

Among the all reproducible spots nearly 185 proteins were changed ($p \leq 0.10$) due to the cold acclimation process and vernalization fulfillment. These proteins were classified into seven functional categories including metabolism, protein synthesis, stress defense, signaling, redox regulation, transport and energy production (Figure 6).

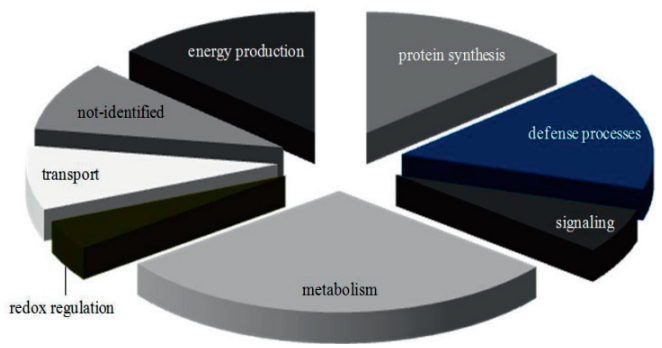

Figure 6. Functional classification of protein detected in the comparative proteome analysis.

DISCUSSION

As noted, the reproductive stage is the most cold-sensitive phase in cereals and this was in agreement with phenological and LT_{50} observations. Results demonstrated the transition from the vegetative to the re-productive stage occurred in January. Fulfillment of the vernalization requirement was associated with a decline in LT tolerance. It seems that vernalization fulfillment can considerably reduce the expression of LT tolerance genes (Chinnusamy et al., 2007).

Significant differences between proteome statue of T_1 and T_2 indicated that cold acclimation stimulated one or more response mechanisms that resulted in changes of protein abundance and composition (Table 1). During early weeks of LT hardening a considerable increase was recorded for heatshock proteins (HSPs). These proteins play a crucial role in protecting plants against stress by reestablishing normal protein conformation and thus cell-

ular homeostasis. Previously, Wang et al. (2004) found that under LT stress conditions an increase in abundance of HSPs and chaperonins, especially for the HSP90, HSP70 and small HSPs, was recognizable. A subset of genes within the heat shock response might be triggered by increased levels of Reactive Oxygen Species (Larkindale & Knight 2002, Timperio et al. 2008). H_2O_2 is clearly able to induce the small HSPs class.

MADS-box genes seem to be involved in developmental regulation. It has been indicated that they control the transition from vegetative to generative growth and determine inflorescence meristem identity (Saedler et al. 2001). It has been suggested that actin is a critical player in many cellular functions, ranging from cell motility and the maintenance of cell shape and polarity to the regulation of transcription (Dominguez & Holmes 2011). LT stress induces change in membrane fluidity; hence the plasma membrane has been suggested to be the primary site of cold perception (Chinnusamy et al. 2007). An increase in the expression of actin and some other actin-binding proteins might be involved in dynamic reorganization of the cytoskeleton during LT acclimation (Orvar et al., 2000). The increased abundance of RuBisCO LSU in this study was consistent with the finding of Hashimoto & Komatsu (2007) who reported that LT stress (5 °C for 48 h) considerably increased the abundance of RuBisCO LSU in the leaf blades of rice seedlings. This result may suggest that RuBisCO LSU might also act as a cold-responsive protein, in addition to its role in photosynthesis. Also, in a recent study Rinalducci et al. (2011a) reported that the expression of some of RuBisCO subunits may be regulated by LT stresses.

Change in expression of oxygen evolving enhancer protein 2 (OEE2) is a common response to abiotic stress (Xin & Browse 2001). Recently, a proteomic analysis of Arabidopsis thaliana indicated that OEE2 is involved in plant adaptation to thermal stress (Rocco et al. 2013). Additionally, analysis of winter wheat proteome after prolonged LT stress revealed both an accumulation of soluble carbohydrates and a marked decrease of other photosynthesis-related proteins like OEE1, OEE2 and RuBisCO (Rinalducci et al. 2011b).

The results revealed that an abundance of undecaprenyl diphosphate synthase and oxygen-evolving complex protein 1 decreased by LT acclimation or transition from T_1 to T_2. Undecaprenyl diphosphate synthase enzyme is essential for the formation and breakdown of the cell wall through peptidoglycan synthesis. The functions of the oxygen-evolving complex protein 1 can be summarized as assembly and stabilization of photosystem II, participation in defense response against bacteria and water oxidation. In general, most changes during this stage were related to chloroplast organelles and photosynthetic apparatus.

An increased abundance of manganese superoxide dismutase (spot 2234) in this study corroborated this earlier finding of Janmohammadi et al. (2012) and Vítámvás et al. (2012), who reported that an abundance of ROS scavenging enzymes was significantly increased by LT acclimation in winter wheat. Xu et al. (2013) investigated the proteomic response to abrupt low temperature stress between two winter wheat cultivars differing in low temperature tolerance. They found that an LT-sensitive cultivar exhibited high levels of reactive oxygen species (ROS) and leaf cell death. Significant increases in relative abundance of antioxidant-related proteins were found in LT-tolerant cultivar leaves. The up-regulation of thioredoxin (spot 1169) seems to be consistent with other research which found that thioredoxin could act as a cold-responsive protein in *Oryza sativa* (Yan et al. 2006). NADH dehydrogenase is the largest and most complicated enzyme of the electron transport chain. NADH dehydrogenase is a flavoprotein that contains iron-sulfur centres and is located in the inner mitochondrial membrane that catalyses the transfer of electrons from NADH to coenzyme Q. It is the "entry enzyme" of oxidative phosphorylation in the mitochondria. This enzyme helps to build the electrochemical potential

used to produce ATP (Hatefi et al. 1985). The need for energy to maintain the LT tolerance at this stage can be a reason for the increase in abundance of this protein.

At the vernalization fulfillment stage the abundance of some proteins considerably decreased. Under LT stresses, a lot of proteins could be damaged, misfolded, become inefficient or unnecessary and need to be degraded. In winter cereals with the increase of LT acclimation, LT tolerance gradually increases and it seems that this is accompanied by alternations in expression of LT inducible genes. Our results point to the conclusion that LT inducible genes in plants grown under field conditions could be classified into different classes according to their function, including photosynthetic, respiratory, restoring redox balance and regulatory genes. However, Rinalducci et al. (2011b) reported that two proteins including a glycinerich RNA binding protein (GR-RBP) and the VER2 protein were connected with the vernalization-induced flowering process.

With initiation of reproductive growth the abundance of some of photosynthetic enzymes (2190, 2648, 1007, 1042 and 939) increased. The accumulation of photosynthetic enzymes could be due to the improvement in the thermal condition and the rapid growth of the plants with initiation of the reproductive phase. The modulation trend of RuBisCO activase protein appears to be related to its role in maintaining the active configuration of RuBisCO (Rinalducci et al. 2011b). Additionally investigation of the quantum efficiency of photosystem II showed a considerable increase during the periods noted (data not shown). UGPase represents an important activity in carbohydrate metabolism, catalysing a reversible production of Uridine diphosphate glucose (UDPG) and pyrophosphate (PPi) from Glucose -1-P and UTP (Kleczkowski et al. 2004). In young and mature leaves, UGPase is primarily involved in the sucrose biosynthesis pathway, providing UDPG for sucrose phosphate synthetase (SPS), whereas in other tissues, including immature apical leaves, which to some extent depend on imported carbon, UGPase may take part in sucrose breakdown, using the UDPG produced by sucrose synthase (Winter & Huber, 2000). Proteomics analysis in different LT tolerant winter wheat cultivars indicated that carbohydrate metabolism-related proteins were more abundant in LT-tolerant cultivars, correlating with observed accumulation of soluble sugars in their leaves. Amino acid analysis also revealed a strong response to LT stress in wheat leaves (Xu et al., 2013). It seems that the increase in the relative abundance of UGPase is due to an increase in photosynthetic production and plant rapid growth after the double ridge appearance on the apical meristem. Fructose-bisphosphate aldolase is a key enzyme in the glycolysis pathway that plays an important role in the production of intermediate components and energy.

Results revealed that the transition to the reproductive phase was associated with reduced abundance of cytochrome P450-like (spot 689) and heat shock 70 (spot 2690). The cytochrome P450 superfamily is a large and diverse group of enzymes that catalyses the oxidation of organic substances. In higher plants, cyt P450 is involved in the biosynthesis of various compounds such as lignins, UV protectants, pigments, defense compounds, fatty acids, phytohormones and signaling molecules (Schuler & Werck-Reichhart, 2003). It is logical that with increasing temperature and decreasing LT stress the abundance of numerous defense proteins dramatically decreases. The fulfillment of vernalization requirements coincides with the end of vegetative growth and initiation of the reproductive phase (that is detectable by formation of double ridges in the plant shoot apex) and this might be associated with substantial changes in gene expression and intercellular metabolic and defense processes. Proteomic study of the bread wheat mutant strain 5660M underlying spike development inhibition revealed a close relationship between transitions in the reproductive growth period and expression of LT-responsive protein (Zheng et al., 2013). Inhibition of spike development can result in oxidative

stress and causes an intensive response to cold stress.

Results showed that 14 proteins have similar abundance trends (Figure 5). Among them, Cp31BHv is a chloroplast RNA-binding protein and its expression is correlated with leaf development (Frances 2008). Furthermore, the results of Hou et al. (2009) suggest that the expression of these proteins is also influenced by low temperature under field conditions and this may suggest that it is a LT responsive protein. This approach can help the researchers to focus on the most affected biological processes, which would hopefully provide direct evidence of proteins positively or negatively related to LT acclimation and phase transition.

Classification of all changed proteins indicated that the largest functional category was related to metabolic regulators (29%), which were greatly affected during LT acclimation and also by the vegetative/reproductive phase transition (Figure 6). These results may refer to the very high importance of the regulation of metabolism during cold acclimation or phase transition.

CONCLUSION

In conclusion, it appears from this study that Iranian winter wheat (cv. Pishgam) plants can rapidly start LT-acclimation under field conditions. Furthermore, our study tried to shed light on the proteomic response during the cold winter months and in an advanced period of plant life, which is the reproductive stage. LT acclimation induces many modifications in the abundance of proteins, mainly in relation to primary metabolism, energy production and protein synthesis. A large number of all the selected proteins were part of the photosynthetic apparatus, confirming the key role of the chloroplast machinery during LT acclimation. Accordingly, proteome analysis of organelles such as the chloroplast and plasma membrane may be applied to widen our information about LT tolerance. The results highlight the ability of 2-DE based proteomic experiments to reveal significant, physiological differences in cellular systems as leads for new studies. In several regions of Iran some spring-habit cultivars like as Pishtaz are planted in the autumn. These cultivars are mostly disease resistant and have a relatively high grain yield and baking quality. Clearly, there is a need to examine and analyse LT stress responsive proteins in spring wheat plants under field condition.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Annegret Wolfan for her technical assistance. They thank the Ministry of Science, Research and Technology of Iran for financial support.

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Manuscript received 17 September 2013
Accepted 10 January 2014