

Research Article

Proteomic and Physiological Response of Spring Barley Leaves to Cold Stress

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Abstract

Cold is one of the most significant abiotic stresses that restrict crop growth and productivity worldwide. In order to investigate how spring barley (*Athys cultivar*) adapts to short-term cold stress, the present study attempted to explore proteomic, physiological, and biochemical changes that occur in leaves. Barley seedlings were exposed to low temperature (4°C) for 48 hours, and third leaves were harvested and compared with plants grown in normal conditions (25°C). In cold-stressed plants, results indicated a significant increase in hydrogen peroxide content, associated with a highly significant rise of lipid peroxidation (as demonstrated by MDA measurements) and catalase activity; the latter represents one of the oxidative stress resistance strategies adopted to promote cell survival. Cold stress also caused a significant reduction in chlorophyll b (chl_b) content with a parallel increase in the chl_a/chl_b ratio, which is probably related to defense mechanisms against cold-induced production of reactive oxygen species. On the contrary, other physiological and biochemical traits [namely, the membrane stability index (MSI), peroxidase activity, electron leakage, carotenoid and chl_a content] showed no statistically significant differences. The proteomic analysis revealed fifteen statistically significant cold-modulated spots, seven of which were successfully identified by LC-MS/MS. These responsive proteins are related to the Calvin cycle, photosynthetic electron transport, light reactions, and signal transduction. An increase in abundance of proteins involved in the regulation of the chloroplast system probably reflects plant acclimation to cold, thus confirming that cold stress severely affects photosynthesis in spring barley.

INTRODUCTION

Barley is akin to other plants from the tribe Triticeae and exists in spring and winter varieties. In order to avoid heat and drought stress at the end of the season, spring cultivars must be planted earlier [1]. Frost in the late spring during seedling development is a major factor that affects spring barley production, as it can result in mortality, poor crop establishment, and subsequent yield reduction [2]. Spring cold stress can damage wheat and barley, causing yield penalties as high as 30-50% [3]. The risk of spring freeze is likely to be overlooked in a preponderance of breeding programs due to the significance of early maturity. As a result, presently spring freeze is considered much less than cold acclimatization and freeze hardiness [4]. Cold stress leads to a broad range of responses in plants, including physiobiochemical

responses, which take place together with gene expression fluctuations [5]. These changes make plants more tolerant to cold conditions. Many studies have been conducted over the last years that explore plant response to cold stress in wheat and barley [6,7].

The cell membrane is considered to be the first possible target of cold. Cold disfigures the membrane and disturbs its activity [8]. It causes membrane electrolyte leakage for tissues in the sensitive plants that are incapable of increasing the fluidity of the living membrane by increasing unsaturated fatty acids [9]. Freeze and cold stress create disorder in plant metabolism and intensify the production of various active oxygen molecules. These free radicals damage cell macromolecules through the oxidation of biotic molecules and impose oxidative stress on the plant [10]. One of

these active oxygen molecules is hydrogen peroxide (H_2O_2), which, through the peroxidation of unsaturated fatty acids in membrane lipids, affects their selective permeability, thereby damaging membranes [11]. The destruction of membrane unsaturated fatty acids leads to the production of malondialdehyde (MDA), which is measured as a suitable marker for identifying the rate and intensity of oxidative damage to biotic membranes [12]. To resist active oxygen radicals and diminish their detrimental effects, the cell produces or activates antioxidant enzymes, among which are catalase [13]. Proteins perform a major role in plant stress response because they participate in both structural and metabolic changes directly. They also participate in energy metabolism, photosynthesis, and reactive oxygen species (ROS) scavenging, storage, protection from stress, regulation of the cell cycle, and plant development during freezing conditions and cold stress [14]. Studies on proteomics have substantially contributed to the identification and characterization of numerous new proteins that are involved in the plant cold response. There is a preponderance of works regarding proteomics that have been published on cold response in the wheat leaf and crown [15-21], whereas only limited information is available regarding the proteomic response of barley.

This study was carried out in order to explore physiological, biochemical, and proteome changes of barley leaves exposed to low temperature (4 °C) for 48 hours by two-dimensional gel electrophoresis (2D-GE) coupled with peptide mass fingerprinting for protein identification using LC-MS/MS.

MATERIAL AND METHODS

Plant growth conditions and cold stress treatments

The plant material (barley line Aths) was a sensitive genotype according to freezing measurements reported in [22]. The seeds were obtained from the Seed and Plant Improvement Institute (SPII) in Karaj, Iran. After initial germination on filter paper, the seeds were planted in pots that were 15 cm in diameter and 50 cm in length, and contained fine sand and perlite (10:1). Plants were grown in an experimental greenhouse under 70% humidity, in 16-hour daylight at 25 °C, and with light intensity of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$. They were regularly irrigated up to the three-leaf stage. Afterwards, a number of randomly chosen pots were subjected to 4 °C acclimation for 48 hours (cold treatment) in a freezing machine under the same light regime of control plants. Plant leaves were harvested and compared with those grown at a normal temperature to measure physiological and biochemical traits and perform proteome analysis.

Membrane stability index (MSI)

This index was determined by measuring the electrical conductivity of the substances leaked from the leaf samples into double-distilled water at 40 and 100 °C [23]. The MSI was determined in percentage terms according to the following formula: $\text{MSI} = [1 - (C_1/C_2)] \times 100$.

H_2O_2 content

The H_2O_2 content was measured according to the method provided by Gong et al. [24]. In 0.1% (v/w) trichloroacetic acid and liquid nitrogen, 0.2 g of the leaf sample was homogenized.

The homogenized solution was centrifuged at 12000 g for 15 minutes. Then 0.5 ml of a 10 mmol potassium phosphate buffer (pH = 7.5) and 1 ml of 1 mol potassium iodide were added to 0.5 ml of the supernatant. The absorbance was read at 390 nm. The H_2O_2 content was determined by using the standard curve for pure H_2O_2 .

Lipid peroxidation rate

The level of lipid peroxidation was measured with regard to the thiobarbituric acid reactive content. In 10 ml of 0.1% (v/w) trichloroacetic acid, 0.5 g of the leaf sample was homogenized. The homogenized solution was centrifuged at 15000 g for 5 minutes. Then 4 ml of 0.5% (v/w) thiobarbituric acid in 20% (v/w) trichloroacetic acid was added to 2 ml of the supernatant. The mixture was heated at 95 °C for 30 minutes and then was cooled in an ice bar. After being centrifuged at 10000 g for 10 minutes, supernatant absorbance was read at 532 nm. Nonspecific absorbance at 600 nm was deducted from this content. The total content of thiobarbituric acid was measured with the extinction coefficient of 155 $\text{mm}^{-1}\text{cm}^{-1}$ [25].

Pigment content

For the measurement of the chlorophyll and carotenoid content, the leaf sample (0.05 g) was incubated in 5 ml of dimethyl sulfoxide (DMSO) at 65 °C for 4 hours. Absorbance was recorded at 645, 665, and 470 nm, and the quantities of chlorophyll a (chl a), chlorophyll b (chl b), and chl a/chl b were calculated [26].

Rate of catalase activity

The rate of catalase activity was measured according to the method provided by Aebi [27]. Next, 0.5 g of the leaf sample was homogenized in a 0.1 M cold potassium phosphate buffer (pH = 7.5) with 0.5 M of EDTA. The homogenized sample was centrifuged at 15000 g for 15 minutes at 4 °C. Then, 0.05 ml of the supernatant was added to 1.5 ml of a 0.1 mM phosphate buffer (pH = 7) and 1.45 ml of double-distilled water. The reaction began by adding 0.5 ml of 75 mM H_2O_2 . The reduction in absorbance was recorded at 240 nm for 1 minute.

Rate of peroxidase activity

The activity of this enzyme was assessed according to the method described by Macadam et al. [28]. Next, 0.5 g of the leaf sample was homogenized in a 0.1 M cold potassium phosphate buffer (pH = 7.5) with 0.5 M of EDTA. The homogenized sample was centrifuged at 15000 g at 4 °C for 15 minutes. Then, 20 μl of the supernatant was added to 0.81 ml of a 50 M potassium phosphate buffer (pH = 6.6). Next, 90 μl of 1% guaiacol as an electron donor was added to the resultant mixture. The reaction mixture was added to the cuvette. Moreover, before measuring the rate of the reaction, 90 μl of 3% H_2O_2 was added to the reaction mixture as an electron acceptor. Also, absorbance was measured at 470 nm for 60 seconds at 25 °C.

Data analysis for physiological and biochemical traits

A t-test was used to test the statistical difference between cold stress and control pots after clearing homogeneity of within-group variances. Data analysis was performed using SPSS software.

Extraction of proteins

Total protein extracts were isolated from approximately 0.5 g of a frozen root per biological replicate ($n = 3$) to obtain a fine powder, which was then suspended in cold acetone containing 10% TCA and 0.07% 2-Mercapthoethanol. The resultant powder was dissolved in the lysis buffer containing 7 M Urea, 2 M thiourea, 2% CHAPS, 60 mM DDT, and 1% ampholyte (pH = 3-10). Protein concentration was determined by the Bradford method [29].

2DE

To remove lipids, 600 μ g of protein was precipitated from a desired volume of each sample with a cold mix of tri-*n*-butyl phosphate/acetone/methanol (1:12:1). After incubation at 4 °C for 90 min, the precipitate was pelleted by centrifugation at 13500 rpm for 20 min at 4 °C. The pellet was air-dried and then solubilized in a buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 40 mM Tris-HCL. The sample was subsequently reduced (5 mM tributylphosphine, 1 h) and alkylated (7.7 mM IAA, 1 h). To prevent over-alkylation, iodoacetamide (IAA) excess was neutralized by adding 10 mM DTE. The sample was included in the rehydration solution (7M Urea, 2 M Thiourea, 4% CHAPS and 0.5% w/v pH 3-10 carrier ampholyte) (Bio-Rad, CA,USA) and was taken up into the 17 cm IPG strips pH 3-10 (Bio-Rad, CA, USA) passively during rehydration over night. IEF was run on a Protean IEF Cell (Bio-Rad, CA, USA) at a 20 °C constant temperature and the total product time \times voltage applied was 80,000 V-h. After IEF, the IPG gel strips were incubated at room temperature for 30 min in 6 M Urea, 30% w/v glycerol, 2% w/v SDS, 50 mM Tris-HCL, and pH 8.8. The strips were sealed at the top of a 1.5 mm vertical second dimensional gel home-made with 0.5% agarose in 25 mM Tris, 192 mM glycine, 0.1% SDS, and pH 8.3. SDS-PAGE was carried out on homogeneous running gels 12% T, 2.6% C. Run conditions were 40mA/gel until the bromophenol blue reached the bottom of the gel. Protein spots were stained by sensitive Coomassie brilliant blue G-250 stain.

Image analysis

Three biological replicates and two technical replicates were carried out, making a total of 12 gels per growth conditions. 2DE maps were digitalized using a ChemiDoc™ XRS+ Systems with the Image Lab™ Software (Bio-Rad Hercules, CA). The scanned images were processed and statistically evaluated with the Progenesis SameSpots software (Nonlinear Dynamics, UK). All spots were pre-filtered and manually checked before applying the statistical criteria (ANOVA $p < 0.05$ and fold ≥ 1.5).

In-gel digestion

Gel bands were carefully excised from the gel and subjected to in-gel trypsin digestion according to Shevchenko et al. [30], with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/ml trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, and WI) in an ice bath. After 30 min, the supernatant was removed and discarded; then, 20 ml of 50 mM NH_4HCO_3 was added to the gel pieces, and digestion was allowed to proceed overnight at 37 °C. The supernatant containing tryptic peptides was dried by vacuum centrifugation.

LC-MS/MS analysis

Peptide extracts were analyzed by using a split-free nano-flow liquid chromatography system (EASY-nLC II, Proxeon, Odense, Denmark) coupled with a 3D-ion trap (model AmaZon ETD, Bruker Daltonik, Germany) equipped with an online ESI nanospray (the spray capillary was a fused silica capillary, 0.090 mm OD, 0.020 mm ID) in the positive-ion mode. For all experiments, a sample volume of 15 μ l was loaded by the autosampler onto a homemade 2-cm fused silica precolumn (100 μ m I.D.; 375 μ m O.D.; Reprosil C18-AQ, 5 μ m, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Sequential elution of peptides was accomplished by using a flow rate of 300 nl/min and a linear gradient from Solution A (2% acetonitrile; 0.1% formic acid) to 50% of Solution B (98% acetonitrile; 0.1% formic acid) in 40 min over the precolumn on-line with a homemade 15-cm resolving column (75 μ m ID; 375 μ m OD; Reprosil C18-AQ, 3 μ m, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). The acquisition parameters for the mass spectrometer were as follows: dry gas temperature, 220 °C; dry gas, 4.0 l/min; nebulizer gas, 10 psi; electrospray voltage, 4000 v; high-voltage end-plate offset, -200 v; capillary exit, 140 v; trap drive, 63.2; funnel 1 in 100 v out of 35 v and funnel 2 in 12 v out of 10 v; ICC target, 200,000; and maximum accumulation time, 50 ms. The sample was measured with the Enhanced Resolution Mode at 8100 m/z per second (which allows monoisotopic resolution up to four charge stages), scan range from m/z 300 to 1500, 5 spectra averaged, and rolling average of 1. The "Smart Decomposition" was set to "auto." Automatic MS/MS fragmentation was performed on the three most intense precursor ions of each spectrum. Precursor ions were excluded from MS/MS fragmentation after appearance in 2 spectra and released after 1 minute. Acquired MS/MS spectra were processed in Data Analysis 4.0 and submitted to the Mascot search program (in-house version 2.2, Matrix Science, London, UK). The following parameters were adopted for database searches: NCBI nr database; taxonomy = Viridiplantae; peptide and fragment mass tolerance = ± 0.3 Da; enzyme specificity trypsin with 1 missed cleavages considered; fixed modifications: carbamidomethyl (C); variable modifications: oxidation (M).

RESULTS AND DISCUSSION

Cold effects on physiological and biochemical traits

After verifying the normality of the data, the t-test was performed, and the results showed that the difference between the control condition (25 °C) and the cold stress condition (4 °C) in five physiological and biochemical traits, namely H_2O_2 , catalase activity, MDA, chlorophyll-b content, and chl-a/chl-b ratio, was significant (Figure 1, panels A-C, H, I); however, the peroxidase activity, MSI, carotenoids, and chl-a content had no statistically significant differences (Figure 1, panels D-G).

After being subjected to cold stress at 4 °C, the H_2O_2 content increased significantly ($p < 0.001$) (Figure 1, panel A). This increase may be due to the following reasons: (1) upon the water shortage through plant stress, oxygenation reaction of RuBisCO in the chloroplast increases, thus causing a rise in the glycolate content. Glycolate in peroxisomes is converted into glyoxylate by the glycolate oxidase enzyme, which is a reaction leading to the generation of H_2O_2 [31]; (2) under stresses,

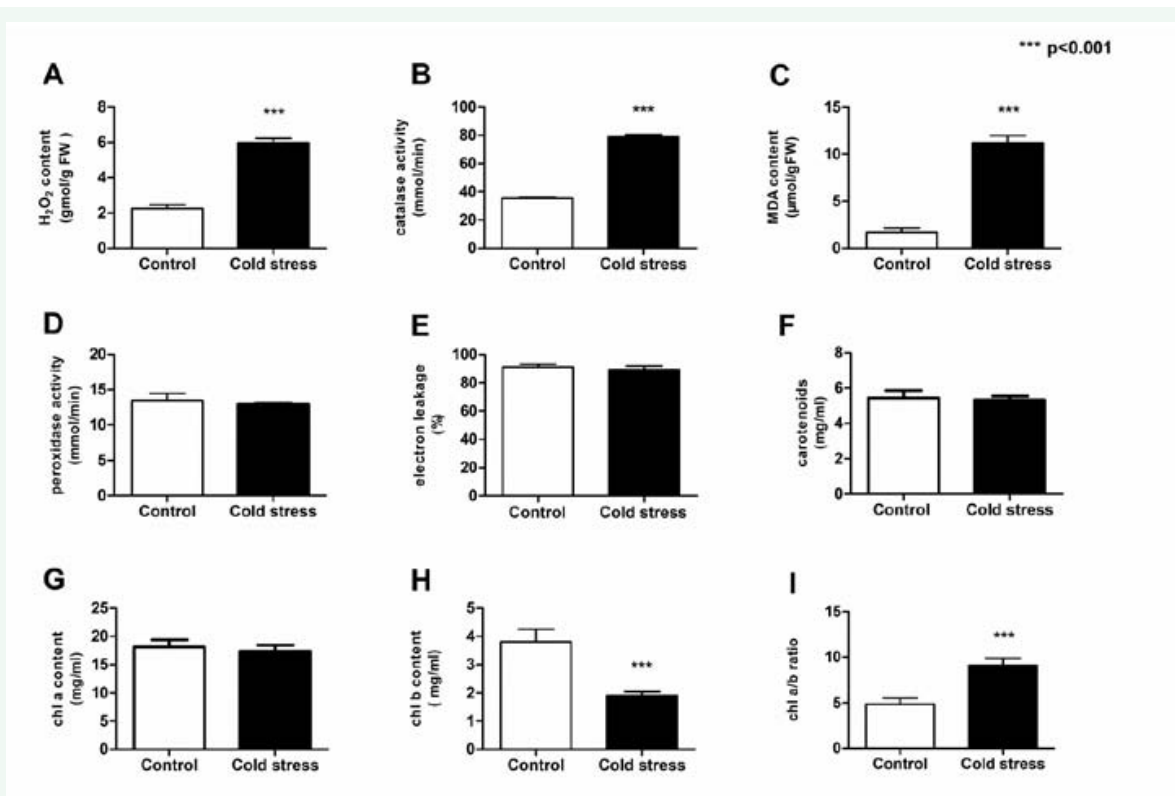


Figure 1 Changes on physiological and biochemical traits at control conditions and cold stress.

oxygen in peroxisomes acquires the hydrogens that result from the dehydrogenation of the fatty acids produced from the beta-oxidation process and ultimately changes to H₂O₂ [32]; (3) the formation of H₂O₂ by means of amine oxidase activity in the apoplast is also induced by stress [33]. The increase in the H₂O₂ level through cold stress has been previously reported, although in different plants [7]. In parallel with the H₂O₂ accumulation we also found a cold-induced increase in the catalase activity (Figure 1, panel B), confirming previous reports that show the plant need of augmenting this antioxidant defense [33]. High levels of H₂O₂ directly mediate lipid peroxidation [11], and this is in agreement with our evidence of a statistically significant increase in the MDA content after cold stress ((Figure 1), panel C), as also reported on rice and on rape seeds by other authors [34,35]. As low-temperature (LT) stress is known to affect plant photosynthesis and reduce the need for the utilization of light [36], we measured chl-a and chl-b content in control and 48h-LT-treated plants. The results showed a significant decrease ($p < 0.001$) of the chlb content after cold stress ((Figure 1), panel H). During cold stress, the photosynthetic electron transport chain is disturbed, and the electron is transported to the oxygen molecule [37]. At this moment, the high amount of chlorophyll elevates only the level of ROS. One of the methods for reducing the production of ROS is to diminish the leaf chlorophyll content, particularly chlb, which takes place in plants upon the increase in chlorophyllase activity [38]. This enzyme detaches the linear chain of chlorophyll from the cyclic part. This chain is the phytol, which acts as a precursor of alpha-tocopherol. Moreover, during the breakdown of chlorophyll, chl b converts to chl a [39]. A greater reduction in chl b than in chl a and, subsequently, an increase in the chl a/

chl b ratio indicate the conversion of chl b to chl a for maintaining the content of this flavonoid at a high level during exposure to cold stress. Considering the effect of cold stress on rice [37], oat [40], and rapeseed [35] plants, it has been stated that cold stress reduces the chlorophyll content.

2-DE Analysis

Two-dimensional electrophoresis (2-DE) analysis (Figure 2) led to the detection of 72 reproducible protein spots, 15 of which showed statistically significant differences in abundance between control and cold stress condition (Table 1). Out of the 15 LT-modulated protein spots, 10 showed over-expression and 5 showed a decrease in abundance through cold stress at 4 °C for 48 hours. On the whole, 7 protein spots were successfully identified by using LC-MS/MS (Table 1). The identified proteins were assigned to four functional categories (Figure 3).

Proteins involved in the light reactions of photosynthesis

The protein spots 2115 and 3770 were placed in this group. The spot 2115 is a chloroplast stem-loop binding protein of 41b (CSP41b), which demonstrated up-regulation. Chloroplast gene expression is indispensable for the accumulation of photosynthetic complexes. The processes of transcription and translation significantly rely on chloroplast proteins, which are regulated, for the most part, by nuclear encoding factors. Certain groups of regulators are comprised of proteins that either cleave RNA or are bound to RNA (generally in correspondence to a stem-loop structure present in the 3'-untranslated region) in order to

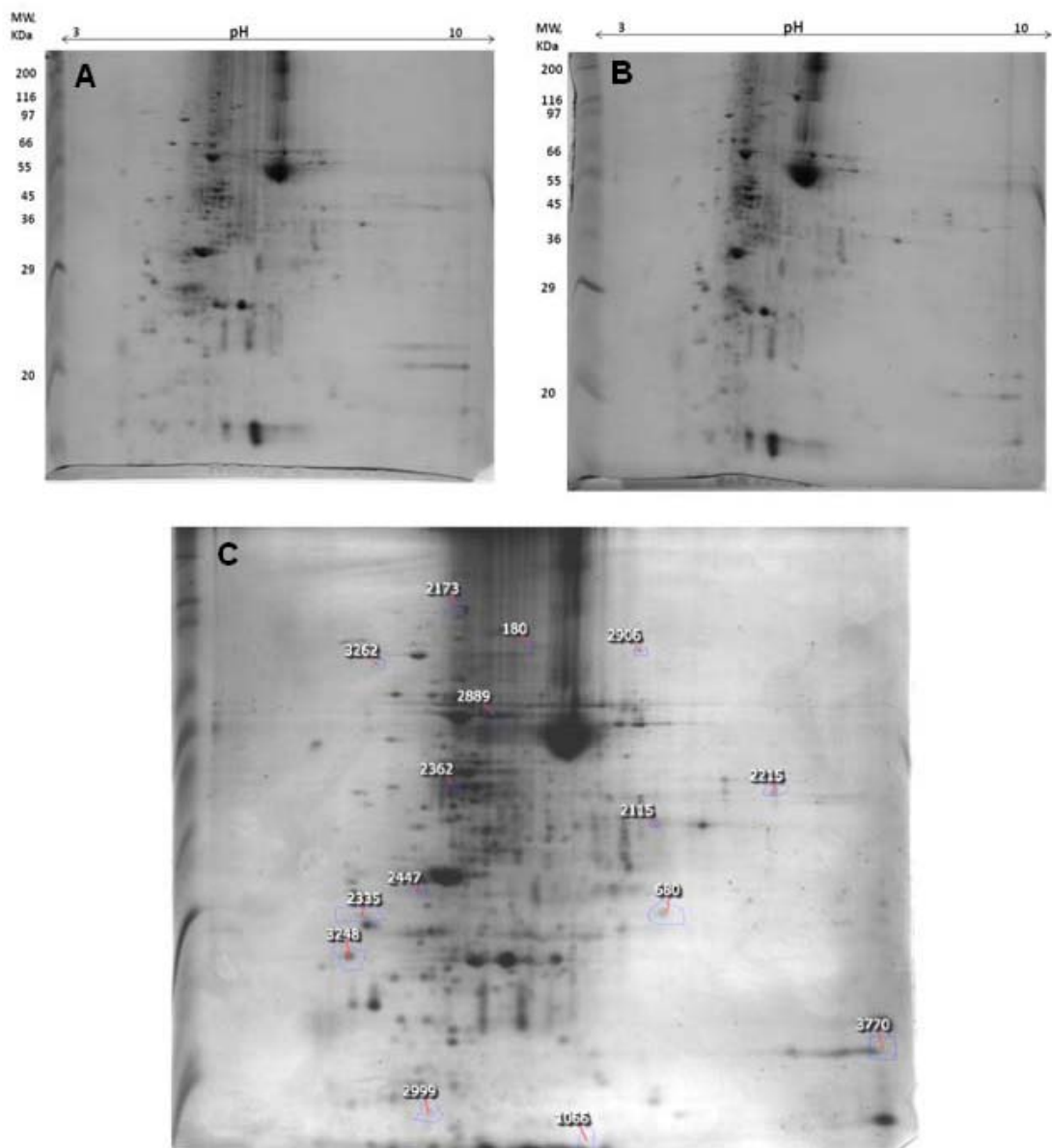


Figure 2 2-DE proteomic profiling of Aths leaves under control condition (A) and cold stress at 4 °C (B). Panel C shows the reference map derived from computerized image analysis performed by using Progenesis SameSpots software. Numbers indicate the variable protein spots.

mediate RNA splicing, editing stability, and translation. Among chloroplast proteins that are bound to RNA and have definite biochemical properties, one could refer to CSP41, which has a and b homologs [41]. In the spinach chloroplast as well, CSP41a has been demonstrated both as ribo nuclease and as a RNA-binding protein for its activity in mRNA30s [42]. CSP41b was also purified as a homolog of CSP41a from *Nicotiana tabacum* [43] and *Arabidopsis* seedlings, where it was shown that RNA target for this protein is the PetD RNA [44]. The chloroplast *petD* gene encodes the subunit of the cytochrome b6 complex, which mediates the electron transport between PSII and PSI. Moreover, the expression of this protein is affected by growth regulators, environmental factors, senescence, and water stress [44].

In addition to the above mentioned duties, there are other functions that have been attributed to this protein, such as regulation of the chloroplast system both in the light and the dark, integrity of chloroplast, accumulation of ribosomal units, and regulation of hetero polysaccharides [45,46]. Therefore, the increase in the abundance of this protein in stress conditions can help to resist the effects of cold stress, which brings about the degradation of chloroplast and reduction in transcription. In addition, it can probably increase the electron transport between PSII and PSI indirectly through an increase in the stability of the PetD protein, which might be the attempt by chloroplast to reach a recovery state in the electron transport from PSII and the production of energy.

Table 1: List of differentially abundant proteins.

spot No.	Trend (ctr vs stress)	Fold of variation	MW (kDa) theor/exp	pI theor/exp	NCBI GI No.	Mascot Score	Protein Name	Functional category
180	Up	2.1	53.478/87.2	6.13/6.24	gi 294117	79	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	Calvin cycle
680	Down	1.6	29.0	7.5	-	-	Not identified	-
1066	Up	1.6	16.0	7.1	-	-	Not identified	-
2115	Up	1.7	41.636/38.3	8.31/7.4	gi 573963758	83	chloroplast stem-loop binding protein of 41 kDa b, chloroplastic-like	Light reactions of photosynthesis
2173	Up	1.7	53.899/114.1	5.11/5.6	gi 11583	167	ATPase, beta subunit chloroplast	Electron transport
2215	Up	1.5	43.0	9.0	-	-	Not identified	-
2335	Up	1.6	29.0	4.6	-	-	Not identified	-
2362	Up	1.5	47.34/43.2	8.62/5.48	gi 167096	54	Ribulose 1,5 biphosphate carboxylase activase isoform 1	Calvin cycle
2447	Down	1.5	29.36/31.1	4.83/5.17	gi 22607	308	14-3-3 protein homologue	Signal transduction
2889	Down	1.6	60.0	5.8	-	-	Not identified	-
2906	Up	1.5	87.0	7.2	-	-	Not identified	-
2999	Down	4	20.0	5.3	-	-	Not identified	-
3248	Up	1.5	20.341/27.2	9.41/4.4	gi 329750617	56	Mitochondrial thioredoxin	Electron transport
3262	Up	1.5	70.0	4.8	-	-	Not identified	-
3770	Down	1.5	15.447/22.1	9.82/9.70	gi 131176	53	Photosystem I reaction center subunit IV	Light reactions of photosynthesis

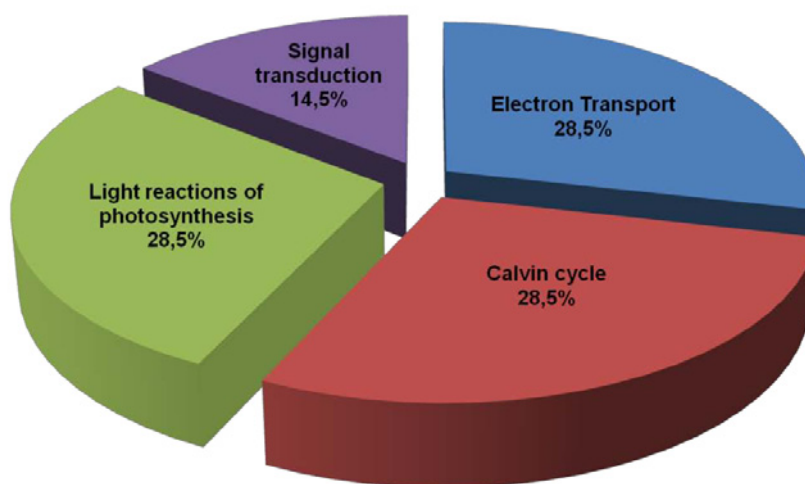


Figure 3 Gene Ontology-based functional enrichment analysis of cold-modulated proteins.

The spot 3770 is the photosystem I reaction IV center subunit protein, which decreased under cold stress conditions. This protein is abbreviated as PsaE; different functions have been reported for PsaE, including assistance in binding ferredoxin to PSI and interaction with Ferredoxin-NADP⁺ oxidoreductase (FNR) [47]. Numerous pieces of evidence demonstrate that PSI can be the target of photoinhibition, particularly under cold

and freezing conditions, where the electron transport chain is unbalanced [48]. Moreover, the decrease in the activity or sensitivity of PSI to cold conditions has been attributed to the decrease in the capacity of antioxidant enzymes to detoxify ROS [49]. In other words, the increase in ROS leads to a decrease in the activity of PSI. As a result, the PsaE protein abundance reduction in the sensitive cultivar Aths could be due to an increase in the

production of H_2O_2 and hydroxyl radicals in the Mahler cycle. This decrease in expression may lead to a decrease in the binding of ferredoxin to PSI and may create impairment in the electron transport chain, thereby reducing the production of NADPH. There are several reports on the reduction in the activity of PSI and its components in various type of stresses, such as drought, salinity, and metal [50,52].

Electron transport

The up-regulated spot 2173 was identified as the beta subunit of the chloroplast ATP synthase. The beta subunit plays a major role in the energy metabolism by converting ADP to ATP when there is the intramembrane proton gradient. By proteomic analysis, Wu et al. [54] demonstrated an over-expression of the ATPase beta subunit in birch leaves under cold stress. An increase in abundance of the same protein has also been observed in Arabidopsis as a part of defensive processes against cold stress [55]. Accordingly, the 48-h-cold-induced up-regulation of this enzyme found in our study is probably due to the fact that the cells require a large quantity of energy to increase the tolerance of plants to cold stress. A similar increase has been reported in rice under cold stress for 24 hours [56]. However, there are also several reports that demonstrate a decrease in the expression of this protein under cold stress [17,19,57,58]. The reason for this is said to be the effect of cold on the degradation of chloroplast or the strategy of the plant for acclimatizing to photosynthesis. Another protein involved in this pathway is the spot 3248, which contained the mitochondrial thioredoxin protein and displayed up-modulation under LT stress. Thioredoxins (Trxs) are ubiquitous and evolutionarily conserved enzymes involved in regulating the redox activation/inactivation of several metabolic and antioxidant proteins [59]. Barranco-Medina et al. [60] declared the existence of a heterocomplex between plant mitochondrial Trx-o and peroxiredoxin-IIIF, which ensures a high and efficient detoxification of H_2O_2 . Furthermore, Trx has a negative impact on the activity of ascorbate peroxidase [61]. Considering physiological findings reported in the current research (i.e., accumulation of H_2O_2), the rise in Trx content after cold treatment of *Aths* leaves could be ascribed to a possible defense mechanism for detoxifying free radicals in cold stress and developing resistance in this cultivar. The increase in chloroplast Trx under cold and freezing conditions has previously been reported in wheat leaves as well [62]. Upon exploring cold stress effects on a resistant and semi-resistant wheat cultivar, Sarhadi and coworkers [16] also demonstrated that the M-type Trx content was higher in semi-hardy plants.

The signal transduction pathway

The spot 2447 was identified as the 14-3-3 protein, which displayed a decrease in abundance under cold stress. 14-3-3 proteins constitute a family of conserved regulatory proteins that are uniquely present in eukaryotes and involved in protein-protein interactions mediating signal transduction pathways [63]. In Arabidopsis, acclimatization to cold is done by a complex network of signal transduction pathways, including those mediated by the expression of C-Repeated/DRE Binding Factors (CBFs) or by the Abscisic acid (ABA) and ethylene hormones [64]. It has been reported that the expression of 14-3-

3 proteins in Arabidopsis increases the production of ethylene by regulating the stability of the 1-aminocyclopropane-1-carboxylic acid synthase (ACS), which catalyzes the rate-limiting step of ethylene biosynthesis [65]. Specifically, if on one hand a decrease in expression of the 14-3-3 protein has been correlated to the accumulation of ABA and thus to a decrease in cold tolerance, on the other hand its down-regulation negatively influences ethylene production, thereby causing sensitivity to cold stress.

Hence, one of the factors responsible for the sensitivity of the cultivar *Aths* to cold stress is likely to be a decrease in abundance of this protein. A down-regulation of the 14-3-3 protein has been previously declared in wheat lines under cold stress [15,18]. However, Han et al. [62], demonstrated an up-modulation of this protein in common wheat under spring cold conditions, supporting that it plays a major role in signal transduction pathways for the regulation of the gene expression under cold stress.

The Calvin cycle

The spot 180 was identified as the RuBisCO large subunit (LSU). RuBisCO represents 12-35% of total leaf proteins and 50% of soluble proteins, particularly in C3 plants [66]. Compared with the control, this spot demonstrates a 2-fold increase in abundance under cold conditions. Cold-induced fragmentation of the RuBisCO LSU was shown by Yan et al. [67], whereas the up-regulation of the intact protein has been reported on the growth stage of wheat at the end of autumn [57] but also in a resistant wheat cultivar under freeze stress at -8 °C [19] and after cold stress at 4 °C for 7 days in Alfalfa leaves [68]. The large subunit of RuBisCO has a documented role in various processes such as cell death, protein folding, and response to cold and acquired resistance [57]. Additionally, this increase could be due to the low ratio of carbon dioxide (CO_2) to oxygen that results from a reduction in stomatal conductance during stress, where this enzyme begins its oxygenase activity for photorespiration [69]. The spot 2362 contained the RuBisCO activase isoform I, which displayed overexpression. As chaperonin molecules, this enzyme could provide assistance in accumulating or isolating protein fragments. Upon hydrolyzing ATP, RuBisCO activase promotes the dissociation of sugar-phosphate inhibitors (e.g., Ribulose-1,5-bisphosphate) from the active site of RuBisCO, so that CO_2 keeps the enzyme RuBisCO active though carbamylation [70,71]. In the present study, both RuBisCO and RuBisCO activase showed up-regulation. Therefore, it could be stated that the up-regulation of RuBisCO is unlikely to be due to an increase in photorespiration, but rather it may be due to the increase in the fixation of CO_2 for resisting cold stress or removing any remaining CO_2 , which prevents energy loss by the oxygenase reaction of photorespiration. The overexpression of RuBisCO activase in the strawberry [72] and wheat [17] under cold stress has been reported, and it has been attributed to the role of this enzyme in stabilizing the active form of RuBisCO. On the other hand, PSI activity decreased under cold stress in the sensitive cultivar *Aths* and photosynthetic light reactions were inhibited; therefore, it is possible that RuBisCO entered the photo respiratory cycle and displayed over-expression. As a consequence, respiratory products close the active site of RuBisCO [73], and the RuBisCO activase over-expression keeps this site free [71], so that the

remaining CO₂ is fixed and the plant displays resistance against cold stress.

CONCLUSIONS

In conclusion, our data demonstrate that cold stress (48 h of exposure at 4 °C) induces physiological and proteome-level responses in the spring barley *Aths* cultivar. To counteract cold effects, plants performed the following: (i) strengthened antioxidant defenses by acting on free radical detoxification through an increase of catalase activity and a reduction of chl b content; (ii) up-modulated chloroplast proteins involved in the light/dark system and in its integrity; (iii) increased transcription through the accumulation of ribosomal units; and (iv) responded to the necessity of more energy by the up-regulation of ATPase. The identification of cold tolerance mechanisms that are adapted to each given plant is necessary to improve breeding programs. To accomplish this goal, intensive *in vitro* and *in vivo* analyses of plant responses to cold conditions are needed.

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REFERENCES

1. Gusta LV, Fowler DB. Effects of temperature on dehardening and rehardening of winter cereals. *Cana J Plant Sci.* 1976; 56: 673-678.
2. Capo-chichi L, Nyachiro J, Juskiw P, Beattie A, Slaski J. *Botany 2015: Science and plants for people.* The Shaw Conference Centre. 2015; 25-29.
3. Zhong X, Mei X, Li Y, Yoshida H, Zhao P, Wang X, et al. Changes in frost resistance of wheat young ears with development during jointing stage. *J Agron Crop Sci.* 2008; 194: 343-349.
4. Galiba G, Vágújfalvi A, Li C, Soltész A, Dubcovsky J. Regulatory genes involved in the determination of frost tolerance in temperate cereals. *Plant Sci.* 2009; 176: 12-19.
5. Heidarvand L, Amiri RM. What happens in plant molecular responses to cold stress? *Acta Physiol Plant.* 2010; 32: 419-431.
6. Janda T, Szalai G, Rios-Gonzalez K, Veisz O, Páldi E. Comparative study of frost tolerance and antioxidant activity in cereals. *Plant Sci.* 2003; 164: 301-306.
7. Apostolova P, Yordanova R, Popova L. Response of antioxidant defence system to low temperature stress in two wheat cultivars. *Gen. Appl. Plant Physiol.* 2008; 34: 281-294.
8. Blum A. *Plant breeding for stress environments.* Boca Raton, Florida: CRC Press. 1988; 223.
9. Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK. Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *Plant J.* 2006; 45: 523-539.
10. Desican R, Hancock JT, Neill S. *Oxidative stress signaling. plant response to abiotic stress.* Springer. 2004; 120-150.
11. Yamazaki JY, Ohashi A, Hashimoto Y, Negishi E, Kumagai S, Kubo T, et al. Effects of high light and low temperature during harsh winter on needle photodamage of *Abies mariesii* growing at the forest limit on Mt. Norikura in Central Japan. *Plant Sci.* 2003; 165: 257-264.
12. Kim SJ, Tai TH. Evaluation of seedling cold tolerance in rice cultivars: a comparison of visual ratings and quantitative indicators of physiological changes. *Euphytica.* 2011; 178: 437-447.
13. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Ann Bot.* 2003; 91: 179-194.
14. Kosová K, Vítámvás P, Prášil IT. Proteomics of stress responses in wheat and barley-search for potential protein markers of stress tolerance. *Front Plant Sci.* 2014; 5: 10-33.
15. Vítámvás P, Saalbach G, Prášil IT, Čapková V, Opatrná J, Ahmed J. WCS120 protein family and proteins soluble upon boiling in cold-acclimated winter wheat. *J Plant Physiol.* 2007; 164: 1197-1207.
16. Sarhadi E, Mahfoozi S, Hosseini SA, Salekdeh GH. Cold acclimation proteome analysis reveals close link between the up-regulation of low-temperature associated proteins and vernalization fulfillment. *J Proteome Res.* 2010; 9: 5658-5667.
17. Rinalducci S, Egidi MG, Karimzadeh G, Jazii FR, Zolla L. Proteomic analysis of a spring wheat cultivar in response to prolonged cold stress. *Electrophoresis.* 2011; 32: 1807-1818.
18. Vítámvás P, Prášil IT, Kosova K, Planchon S, Renaut J. Analysis of proteome and frost tolerance in chromosome 5A and 5B reciprocal substitution lines between two winter wheats during long-term cold acclimation. *J Proteomics.* 2012; 12: 68-85.
19. Xu J, Li Y, Sun J, Du L, Zhang Y, Yu Q, et al. Comparative physiological and proteomic response to abrupt low temperature stress between two winter wheat cultivars differing in low temperature tolerance. *Plant Biol.* 2013; 15: 292-303.
20. Kosová K, Vítámvás P, Planchon S, Renaut J, Vanková R, Prášil IT. Proteome analysis of cold response in spring and winter wheat (*Triticum aestivum*) crowns reveals similarities in stress adaptation and differences in regulatory processes between the growth habits. *J Proteome Res.* 2013; 12: 4830-4845.
21. Gharechahi J, Alizadeh H, Naghavi MR, Sharifi G. A proteomic analysis to identify cold acclimation associated proteins in wild wheat (*Triticum urartu* L.). *Mol Biol Rep.* 2014; 41: 3897-3905.
22. Valizadeh Kamran R, Toochri M, Mogaddam M, Mohammadi H. Genetic evaluation of crown freezing tolerance and some physiological traits in barley (*Hordeum vulgare* L.) lines. *Vegetos.* 2016; 29: 2-9.
23. Sairam RK, Srivastava GC. Changes in antioxidant activity in sub-cellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. *Plant Sci.* 2002; 162: 897-904.
24. Gong H, Zhu X, Chen K, Wang S, Zhang C. Silicon alleviates oxidative damage of wheat plants in pots under drought. *Plant Sci.* 2005; 169: 313-321.
25. Stepien P, Klobus G. Antioxidant defense in the leaves of C3 and C4 plants under salinity stress. *Physiol. Plant.* 2005; 125: 31-40.
26. Prochazkova D, Sairam RK, Srivastava GC, Singh DV. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. *Plant Sci.* 2001; 161: 765-771.
27. Aebi H. Catalase in vitro. *Methods Enzymol.* 1984; 105: 121-126.
28. MacAdam JW, Nelson CJ, Sharp RE. Peroxidase activity in the leaf elongation zone of tall fescue I. Spatial distribution of ionically bound peroxidase activity in genotypes differing in length of the elongation zone. *Plant Physiol.* 1992; 99: 872-878.
29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72: 248-254.
30. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal.*

- Biochem. 1996; 68: 850-858.
31. Gupta PK. Cell and molecular biology. Rastogi Publications. 2002.
32. Corpas FJ, Barroso JB, del Río LA. Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. Trends Plant Sci. 2001; 6: 145-150.
33. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 2002; 7: 405-410.
34. Fahimirad S, Karimzadeh G, Ghanati F. Cold-induced changes of antioxidant enzymes activity and lipid peroxidation in two canola (*Brassica napus* L.) cultivars. J Plant Physiol. Breed. 2013; 3: 1-11.
35. Moieni-Korbekandi Z, Karimzadeh G, Sharifi M. Cold-induced changes of proline, malondialdehyde and chlorophyll in spring canola cultivars. J Plant Physiol Breed. 2014; 4: 1-11.
36. Wu J, Lightner J, Warwick N, Browse J. Low-temperature damage and subsequent recovery of fab1 mutant arabidopsis exposed to 2°C. Plant Physiol. 1997; 113: 347-356.
37. Hassibi P, Moradi F, Nabipour M. Screening of rice genotypes for low temperature stress-using chlorophyll fluorescence. Iranian J. Crop Sci. 2007; 9: 14-31.
38. Guo Z, Tan H, Zhu Z, Lu S, Zhou B. Effect of intermediates on ascorbic acid and oxalate biosynthesis of rice and in relation to its stress resistance. Plant Physiol. Biochem. 2005; 43: 955-962.
39. Santos CV. Regulation of chlorophyll biosynthesis and degradation by salt stress in sunflower leaves. Sci Hort. 2004; 103: 93-99.
40. Liu W, Yu K, He T, Li F, Zhang D, Liu J. The low temperature induced physiological responses of *Avenanuda* L., a cold-tolerant plant species. Scientific World J. 2013; 658793.
41. Bollenbach TJ, Sharwood RE, Gutierrez R, Lerbs-Mache S, Stern DB. The RNA-binding proteins CSP41a and CSP41b may regulate transcription and translation of chloroplast-encoded RNAs in Arabidopsis. Plant Mol. Biol. 2009; 69: 541-552.
42. Yang J, Schuster G, Stern DB. CSP41, a sequence-specific chloroplast mRNA binding protein, is an endoribonuclease. Plant Cell. 1996; 8: 1409-1420.
43. Pfannschmidt T, Ogrzewalla K, Baginsky S, Sickmann A, Meyer HE, Link G. The multisubunit chloroplast RNA polymerase A from mustard (*Sinapis alba* L.). Eur J Biochem. 2000; 267: 253-261.
44. Raab S, Toth Z, de Groot C, Stamminger T, Hoth S. ABA-responsive RNA-binding proteins are involved in chloroplast and stromule function in Arabidopsis seedlings. Planta. 2006; 224: 900-914.
45. Fettke J, Nunes-Nesi A, Fernie AR, Steup M. Identification of a novel heteroglycan-interacting protein, HIP 1.3, from Arabidopsis thaliana. J. Plant Physiol. 2011; 168: 1415-1425.
46. Beligni MV, Mayfield SP. Arabidopsis thaliana mutants reveal a role for CSP41a and CSP41b, two ribosome-associated endonucleases, in chloroplast ribosomal RNA metabolism. Plant Mol Biol. 2008; 67: 389-401.
47. Andersen B, Scheller HV, Møller BL. The PSI-E subunit of photosystem I binds ferredoxin: NADP+ oxidoreductase. FEBS Lett. 1992; 311: 169-173.
48. Sonoike K, Terashima I. Mechanism of photosystem-I photoinhibition in leaves of *Cucumis sativus* L. Planta, 1994; 194: 287-293.
49. Ivanov AG, Morgan RM, Gray GR, Velitchkova MY, Huner NPA. Temperature/light dependent development of selective resistance to photoinhibition of photosystem I. FEBS Lett. 1998; 430: 288-292.
50. Ford KL, Cassin A, Bacic A. Quantitative proteomic analysis of wheat cultivars with differing drought stress tolerance. Front Plant Sci. 2011; 2: 44-51.
51. Rasoulnia A, Bihanta MR, Peyghambari SA, Alizadeh H, Rahnama A. Proteomic response of barley leaves to salinity. Mol. Biol. Rep. 2011; 38: 5055-5063.
52. Li G, Peng X, Xuan H, Wei L, Yang Y, Guo T, et al. Proteomic analysis of leaves and roots of common wheat (*Triticum aestivum* L.) under copper-stress conditions. J Proteome Res. 2013; 12: 4846-4861.
53. Ye J, Wang S, Zhang F, Xie D, Yao Y. Proteomic analysis of leaves of different wheat genotypes subjected to PEG 6000 stress and rewatering. Plant Omics. 2013; 6: 286-296.
54. Wu FZ, Wang BC, Yang CP. Proteomic analysis of the cold stress response in the leaves of birch (*Betula platyphylla* Suk). Plant Omics J. 2014; 7: 195-204.
55. Goulas E, Schubert M, Kieselbach T, Kleczkowski LA, Gardeström P, Schröder W, et al. The chloroplast lumen and stromal proteomes of Arabidopsis thaliana show differential sensitivity to short-and long-term exposure to low temperature. Plant J. 2006; 47: 720-734.
56. Cui S, Huang F, Wang J, Ma X, Cheng Y, Liu J. A proteomic analysis of cold stress responses in rice seedlings. Proteomics. 2005; 5: 3162-3172.
57. Janmohammadi M, Matros A, Mock HP. Proteomic analysis of cold acclimation in winter wheat under field condition. Icel Agric Sci. 2014; 27: 3-15.
58. Gao F, Zhou Y, Zhu W, Li X, Fan L, Zhang G. Proteomic analysis of cold stress-responsive proteins in *Thellungiella* rosette leaves. Planta. 2009; 230: 1033-1046.
59. Laloi C, Mestres-Ortega D, Marco Y, Meyer Y, Reichheld JP. The Arabidopsis cytosolic thioredoxin h5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. Plant Physiol. 2004; 134: 1006-1016.
60. Barranco-Medina S, Krell T, Bernier-Villamor L, Sevilla F, Lázaro JJ, Dietz KJ. Hexameric oligomerization of mitochondrial peroxiredoxin PrxIIIF and formation of an ultrahigh affinity complex with its electron donor thioredoxin Trx-o. J Exp Bot. 2008; 59: 3259-3269.
61. Gelhaye E, Navrot N, Macdonald IK, Rouhier N, Raven EL, Jacquot JP. Ascorbate peroxidase-thioredoxin interaction. Photosynth Res. 2006; 89: 193-200.
62. Han Q, Kang G, Guo T. Proteomic analysis of spring freeze-stress responsive proteins in leaves of bread wheat (*Triticum aestivum* L.). Plant Physiol Biochem. 2013; 63: 236-244.
63. Gökirmak T, Paul AL, Ferl RJ. Plant phosphopeptide-binding proteins as signaling mediators. Cur Opin Plant Biol. 2010; 13: 527-532.
64. Medina J, Catalá R, Salinas J. The CBFs: three Arabidopsis transcription factors to cold acclimate. Plant Sci. 2011; 180: 3-11.
65. Yoon GM, Kieber JJ. 14-3-3 regulates 1-aminocyclopropane-1-carboxylate synthase protein turnover in Arabidopsis. Plant Cell. 2013; 25: 1016-1028.
66. Zhang M, Lv D, Ge P, Bian Y, Chen G, Zhu G, et al. Phosphoproteome analysis reveals new drought response and defense mechanisms of seedling leaves in bread wheat (*Triticum aestivum* L.). J Proteomics. 2014; 109: 290-308.
67. Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN. Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol. Cell. Proteomics. 2006; 5: 484-496.
68. Chen J, Han G, Shang C, Li J, Zhang H, Liu F, et al. Proteomic analyses reveal differences in cold acclimation mechanisms in freezing-tolerant

- and freezing-sensitive cultivars of alfalfa. *Front Plant Sci.* 2015; 6: 1-14.
69. Wingler A, Lea PJ, Quick WP, Leegood RC. Photorespiration: metabolic pathway and their role in stress protection. *Philos Trans R Soc Lond B Biol Sci.* 2000; 335: 1517-1529.
70. Portis Jr AR. Rubisco activase–Rubisco’s catalytic chaperone. *Photosynth. Res.* 2003; 75: 11-27.
71. Portis Jr AR, Parry MA. Discoveries in Rubisco (Ribulose 1, 5-bisphosphate carboxylase/oxygenase): a historical perspective. *Photosynth Res.* 2007; 94: 121-143.
72. Gu X, Gao Z, Zhuang W, Qiao Y, Wang X, Mi L, et al. Comparative proteomic analysis of rd29A: RdreB1BI transgenic and non-transgenic strawberries exposed to low temperature. *J Plant Physiol.* 2013; 170: 696-706.
73. Pearce FG, Andrews TJ. The relationship between side reactions and slow inhibition of ribulose-bisphosphate carboxylase revealed by a loop 6 mutants of the tobacco enzyme. *J Biol Chem.* 2003; 278: 32526-32536.

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