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Analysis and identification of cold responsive proteins in Kohdasht spring wheat (Triticum aestivum)

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Abstract

Spring wheat is cultivated early in the spring in moderate climatic areas or cultivated late in the winter when freezing period is over. To understand plant's response to the cold stress, former studies have led researchers to the identification of several proteins either in wheat or in other plants. In order to achieve further information regarding with the mechanism of spring wheat response to the cold stress at protein expression level, proteomics was applied for an Iranian local spring wheat cv. Kohdasht (LT50 = - 4 to - 6°C). Seedlings were first grown in a controlled growth room at 20 °C for 14 days and subsequently were transferred to 4 °C (experimental day 0) for 42 days (cold treatment), or they were maintained at 20 °C (control treatment). Sampling was done on days 0, 21 and 42 of the cold exposure. Protein extraction was carried out and two dimensional analysis were done. Proteins that showed at least two levels of expression changes were identified and mass spectrometry was carried out for their identification. The study led us to the identification of 92 proteins several of which seem to play important role in plant's response to the cold stress such as cold regulated protein and ferredoxin-NADP(H) oxidoreductase as well as heat shock protein 70. Here we report proteomics analysis of cold responsive proteins and discuss their role in Kohdasht spring wheat cultivar.

Key words: Cold stress, Spring wheat, Kohdasht, Proteomics, COR protein, heat shock protein (HSP)

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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important crops with wide adaptation to different environmental conditions. The success of wheat for adapting into such diverse environmental conditions could be attributed to it's wide evolutionary range of development and differentiation into different genotypes including spring, intermediate (facultative), and winter wheat with different behavior and environmental requirement [1]**.** Among such environmental conditions and outstandingly is their response to the temperature and day-length. Temperature changes that trigger cold acclimation may be analyzed by studying the photosynthetic apparatus [2]. A change in temperature modifies the balance of the absorbed energy during light phase of photosynthesis and the energy metabolized in dark processes. These changes influence the relative state of photosystem II (PSII) oxidation. Such changes can modify the balance between energy utilization in the processes of growth. In order to achieve further information regarding with the mechanism of spring wheat response to the early season cold stress at protein expression level, proteomics was applied for an Iranian local Kohdasht spring wheat cultivar (Triticum aestivum L., LT50 = - 5 °C).

2. Protein extraction and 2-DE

Total soluble proteins were extracted from leaves of four independent replications from each treatment according to the methods of Damerval et al. (1986) with some modifications.Four riplicate tissue samples of each stage were ground in liquid nitrogen and suspended in 10% w/v TCA in acetone with 0.07% w/v DTT at -20°C for 1 h, followed by centrifugation for 15 min at 4°C with 14000rpm. The pellets were washed with icecold acetone containing 0.07% DTT, incubated at -20°C for 1 h and centrifuged again at 4°C nad . This step was repeated three times and then pellets were lyophilized. The sample powder was then solubilized in lysis buffer (9 M urea, 2% w/v CHAPS, 0.8% w/v Pharmalyte, pH 3–10, 1% w/v DTT), and the protein concentration was determined by the Bradford assay with BSA as the standard. IEF and SDS PAGE were carried out according to Gorg et al with minor modification. For analytical and preparative gels, the 17 cm IPG strips (pH 4–7) were rehydrated overnight with 300 µl of rehydration buffer (8 M urea, 0.5% CHAPS, 20 mM DTT, 0.5% v/v IPG buffers) in a reswelling tray at room temperature. For analytical and preparative gels, 750µg of protein were loaded. IEF was conducted at 20°C with a Biorad multiphore II and Dry Strip Kit (Bio-Rad- Protean-IEF-Cell-System).

Separation in the second dimension was performed by SDSPAGE in a vertical slab of acrylamide (12% total monomer, with 2.6% crosslinker) using a PROTEAN II Multi Cell. About 750 µg of proteins were loaded for analytical and preparative gels, respectively. Analytical gels were stained with were stained with comassi brilliant blue.

3. Staining and image analysis E-surveys are done via two methods (personal computers and network systems) defined in the following:

The stained gels were scanned at a resolution of 300 dots per inch on a Epson Perfection V700 densitometer. The scanned gels were saved as TIF images for subsequent analysis. Spot quantitation was carried out using the progenesis samespots V2.02.

4. Statistical analyses and protein classification Considering the above information, E-questionnaires are of the following kinds:

First dimension gel images were digitized using a ImageScanner II (GE Healthcare, Uppsala, Sweden) with a resolution of 300 dpi and 16-bit greyscale pixel depth. Image analysis was carried out with TotalLab software vers. 2.01 (Bio-Rad), which allows bands detection, quantification, background subtraction. In an effort to reduce the effects of biological variation, each stage of leaves development was analyzed at least as biological triplicates (three different leaves from three different plants) pooled in one sample for each day of measurement. Protein bands were selected for profile analysis only if they were found and positively assigned in all the analyzed samples. To

compensate for subtle difference in sample loading, the volume of each band was normalized as relative volume. This normalization method divides each spot volume value by the sum of total band volume values to obtain individual relative band volumes. Total band volume refers to the sum volume of all bands chosen for analysis. The normalised volume of each bands was plotted using the software SigmaPlot vers. 9.0 (Systat, Erkrath, Germany).

Second dimension gel images were digitized using a ImageScanner II with a resolution of 300 dpi and 16-bit gray scale pixel depth. Images were analyzed with the Progenesis SameSpots software vers. 2.0 (Nonlinear Dynamics, Newcastle, England). A match set was created from the protein patterns of five replicate gels for each independent chloroplast extract (at day = 0 and 10 of Cd treatment) which allows spot matching among multiple gels. Spot quantities of all gels were normalized to remove non expression–related variations in spot intensity. To normalize, the raw quantity of each spot in a gel was divided by the total quantity of all the spots in that gel that were considered in the analysis. The results were evaluated in terms of OD (optical density). Statistical analysis separated proteins that significantly increased or decreased after the treatments. A Student's t-test was performed in order to compare the two groups and identify sets of proteins that showed a statistically significant difference with a confidence level of 0.05. Image analysis allowed the identification of 18 proteins differently expressed by more than 50% ($p<0.05$).

5. Peptide analysis by MS and Protein Identification by MS/MS

Significantly modulated (2-fold or more) protein spots were carefully cut out from Sypro Ruby stained gels and subjected to in-gel trypsin digestion according to [3] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH4HCO3 and 12.5 ng/μL of trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 min, the supernatant was removed and discarded, 20 μL of 50 mM NH4HCO3 was added to the gel pieces, and digestion was allowed to proceed at 37 °C overnight. The supernatant containing tryptic peptides was dried by vacuum centrifugation. Prior to mass spectrometric analysis, the peptide mixtures were redissolved in 10 μL of 5% FA (Formic Acid).

Peptide mixtures were separated using a nanoflow-HPLC system (Ultimate; Switchos; Famos; LC Packings, Amsterdam, The Netherlands). A sample volume of 10 μL was loaded by the autosampler onto a homemade 2 cm fused silica precolumn (75 μm i.d.; 375 μm o.d.; Reprosil C18-AQ, 3 μm (Ammerbuch-Entringen, DE)) at a flow rate of 2 μL/min. Sequential elution of peptides was accomplished using a flow rate of 200 nL/min and a linear gradient from solution A (2% acetonitrile and 0.1% formic acid) to 50% of solution B (98% acetonitrile and 0.1% formic acid) in 40 min over the precolumn in-line with a homemade 10–15 cm resolving column (75 μm i.d.; 375 μm o.d.; Reprosil C18- AQ, 3 μm (Ammerbuch-Entringen, Germany). Peptides were eluted directly into a High Capacity ion Trap (model HCTplus, Bruker-Daltonik, Germany). Capillary voltage was 1.5–2 kV and a dry gas flow rate of 10 L/min was used with a temperature of 230 °C. The scan range used was from 300 to 1800 m/z. Protein identification was performed by searching in the National Center for Biotechnology Information nonredundant database (NCBInr) using the Mascot program (http://www.matrixscience.com). The following parameters were adopted for database searches: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide mass tolerance (1.2 Da, fragment mass tolerance (0.9

6. Conclusion

Image analysis has led us to the identification of 92 polypeptides whose expression was subjected to the change in response to cold stress. In response to the cold stress the number of proteins whose expression was subject to the change in expression was far less to the winter wheat demonstrated in our other presentation. A reason for this observation could be the low resistance of spring wheat as a result of lower genetic responses and thus lower protein expression level.

Ref The main protein that undergoes decreased expression was found to be 1-5 ribulose bisphosphate carboxylase / oxygenase small subunit. Changes in temperature modify the balance of energy absorbed during the light phase of photosynthesis and the energy metabolized in the dark processes. These changes influence the relative state of

photosystem II (PSII) oxidation. Such changes can modify the balance between energy utilization in the processes of growth [4]. Further confirmation on the proteomic result was investigated by using RT-PCR. The result of RT-PCR not only confirmed data obtained by proteomics but also shows that expression of 1-5 ribulose bisphosphate carboxylase / oxygenase is significantly affected by cold stress. This result verifies the reason for cold stress sensitivity of spring wheat.

Using proteomics we were able to identify 92 proteins of spring wheat in response to the cold stress. Among such proteins was 1-5 ribulose bisphosphate carboxylase / oxygenase whose expression was subject of change. Due to important role of this protein in plant metabolism, down regulation of which could affect plant survival and resistance to the stress [5], [6], [7] and [8]. There are other identified protein whose role in plant response to cold is known to some extent, but further scrutiny is required to unravel their exact role.

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