Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 5, No. 5, p. 291-298, 2014
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Comparative analyses of wheat leaf proteome under drought stress using 2D-PAGE

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Article published on November 19, 2014

Key words: Wheat /Leaf proteome /Drought stress.

Abstract

Drought is one of the most important abiotic stresses throughout the world. Wheat as a major crop is mostly cultivated in area that encountered with drought stress at least in a period of year. Proteomics is one of the approaches to identify proteins involved in plant tolerance to drought stress. To study the effects of drought stress on wheat leaf proteome pattern in susceptible (*Bahar*) and tolerant (*Kavir*) cultivars of spring wheat; comparisons between drought stressed and control samples of both varieties was performed in terms of morpho-physiological traits in addition to proteome changes. Leaf proteins were extracted using TCA/aceton method and protein expression pattern was obtained using two-dimensional electrophoresis. Proteins involved in drought stress were identified by comparison of expression profile between drought stressed and control samples of both varieties. The results showed that there are significant differences between the treatments for almost all of the traits. The leaf proteome pattern analysis identified 13 protein spots in each of the comparisons (a total of 26 spots) representing a reproducible significant expression changes. The protein spots classified into functional groups include: photosynthesis, metabolic pathways, stress defense/response, photorespiration, protein synthesis/assembly and proteins with unknown functions. The reasonable effects and roles of identified proteins in drought stress were discussed. These results would help for better understanding of drought response molecular basis in plants to improve drought resistance in wheat.

Introduction

Drought has a great impact on wheat production because, although wheat is relatively tolerant to abiotic stresses, it is frequently grown in environments in which water deficit is a common occurrence (Caruso *et al*., 2009). With increasing pressure on water supply, a major shift is now underway to improve its level of abiotic tolerance (Peng *et al*., 2009). Therefore, researches for understanding wheat drought response mechanisms for producing water-stress tolerant cultivars is necessary (Caruso *et al*., 2009). Usually plants, in order to survive under unfavorable growth conditions, preserve homeostasis, carry out detoxification of harmful elements and recovery of growth by developing several responses at physiological and molecular levels (Xiong and Zhu, 2002). Plant responses to drought stress in terms of physiological changes include decrease of photosynthesis through stomatal closure or metabolic impairment, increase of oxidative stress, alteration of cell wall elasticity, and generation of toxic metabolites causing plant death (Caruso *et al*., 2009; Reddy *et al*., 2004). Distortion in mitochondrial and photosynthetic electron transport due to water deficit lead to the generation of highly toxic reactive oxygen species (ROS) such as superoxide and peroxides, that cause chemical damage to DNA and proteins leading to serious effects on cellular metabolism (Mittler, 2006). Abiotic stresses usually cause protein dysfunction. General responses to environmental stress conditions include establishing a set of stress proteins that protects the organism from cellular damage (Kamal *et al*., 2010). The correlation between transcriptional and translational patterns of differentially expressed proteins (DEPs) was poor because many gene products are subject to post-translational modification, which cannot be detected by transcriptomics analyses (Peng *et al.,* 2009). Since proteins are the main effectors of most cellular functions proteomics has been particularly useful in comparative analyses of protein abundance between untreated and stress-treated, and/or tolerant and intolerant crops (Caruso *et al*., 2009; Nanjo *et al*., 2011).The aim of the present work was to perform a comparative study by measuring some morpho-physiological traits and by proteomics approach, based on two-dimensional poly-acrylamide gel electrophoresis (2D-PAGE) in order to identify drought-related proteins in wheat (*Triticum aestivum*) varieties named Kavir and Bahar that differ in tolerance to the drought stress.

Materials and methods

Plant growth and stress treatment

Plant material used in this study was two wheat (*Tristicum aestivum* L.) cultivars; "Kavir" and "Bahar" known as tolerant and susceptible to drought stress, respectively. These seeds were taken from "Seed and Plant Improvement Institute", Karaj, IRAN. Plants were grown under controlled conditions in the growth chamber in Proteomics Laboratory, University of Tabriz, IRAN. Experiment was conducted in completely randomized design with four replications. Treatments were combination of wheat, cultivars of wheat ("Kavir" and "Bahar") and two irrigation levels including normal and drought stress. Stress was imposed by holding irrigation for stress samples at the stage of two leaves and continued for four days. Then the leaves and roots were harvested for protein extraction and morpho-physiological traits measurements including: relative water content of leaf (RWC), shoot fresh and dry weight, root length and fresh weight.

2D-PAGE

Protein extraction was performed in TCA/Aceton method according to Toorchi, *et al.* (2009) with some modifications. A volume of 100µL, corresponding to 450 µg of proteins, was loaded onto tubes containing hand casting 8% urea gels (11 cm, pH 5–8 linear gradient). IEF was carried out in three steps: 30 min at 200 V, then 16h at 400 V and finally one hour at 600 V. After IEF the tubular gels were soak in SDS for 15 min, two times. The second dimension was performed on a 15% SDS-PAGE at 25°C applying 35mA constant current (for each gel) for about three hours (until the marker bromophenol blue's line

reach end of the gels). Protein spots were visualized by Coomassie Brilliant Blue. Three gels for each sample extract were run, and four extracts obtained from each plant set (control and stressed) were separated by 2D-PAGE, therefore a total of 12 gels were analyzed. In order to evaluate if the observed qualitative or quantitative changes arise from analytical variability or from biological conditions; three biologically independent samples were prepared for each group of plants, stressed plants and control plants.

Statistical analysis and spot identifying

Gels were scanned using Bio Rad GS-800 scanner. Image analyses was performed in PDQuest™ by designating three gel images for each set, and then applying spot automatic detection and measurement, background subtraction, and then spot matching. Beside spot automatic detection, manual validation and addition/removal of spots were performed, in order to include spots that were missed by automatic identification. To obtain protein expression pattern, the volume percentage (%V) of each spot in the four gel sets were compared. A sample replicate set was consisting of three gels of each sample. Data analysis was carried out in two comparisons: between the tolerant cultivar, "Kavir" gel sets of stressed and control, and the susceptible cultivar, "Bahar" gel sets of stressed and control. Protein spots with significant changes and up to two-fold induction factor in each comparison was selected and their point of isoelectric (based on position of spot on gel within pH range of 5-8) and molecular weight (using protein marker) were determined. Selected spots were attributed to corresponding proteins by searching and reconciling their point of isoelectric (pI) and molecular weight (MW) within bioinformatics' databases especially "SWISS-2DPAGE" option in ExPASy.

Results and discussion

Summary of morpho-physiological measurements

A statistical comparison was performed between treatment combinations including "Bahar" cultivar in well watered and under drought stress conditions (BC and BS respectively) and "Kavir" cultivar in well watered and under drought stress conditions (KC and KS respectively). The results were based on three replicates of RWC and four replicates for the rest of the traits and the data were subjected to ANOVA at probability level of $p < 0.05$. Under well watered condition, the two cultivars did not show significant difference except for shoot dry weight in which the susceptible cultivar had lower mean compared to the tolerant cultivar. But under drought stress condition the two cultivars indicated decrease in all traits except shoot dry weight and root length so that the tolerant cultivar did not show decrease. Also under drought stress condition decrease in RWC in susceptible cultivar is significantly more than decrease in tolerant cultivar (Fig. 1).

Generally the experiments showed that the drought tolerant cultivar, Kavir has better morpho-physiological performance. Drought may affect homeostasis, and provoke several toxic effects on plants by means of complex mechanisms that actually have not been fully characterized (Munns, 2002).

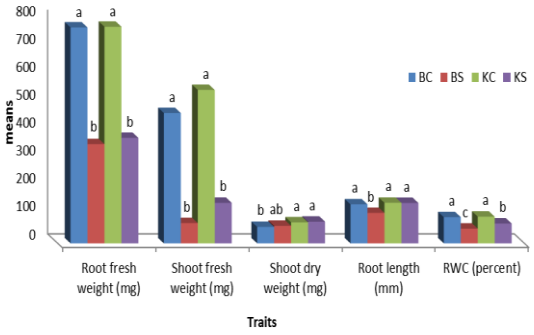

Fig. 1. Traits compare mean between treat combinations include: the tolerant cultivar, kavir well watered (KC), and under drought stress (KS), the susceptible cultivar Bahar under drought stress (BS) and Well watered (BC). The characters show significant differences according to Duncan test.

Proteomics

Drought stress related proteins were investigated by 2D-PAGE and bioinformatics' databases in two *Triticum aestivum* cultivars: Kavir (drought tolerant)

and Bahar (drought susceptible). Two comparison was conducted: first, Kavir under well watered and stress and second: between Bahar under well watered and stress condition that lead to reproducible detection of 177 spots in first comparison and 121 spots in second comparison. Using 2D-PAGE a total of 26 spots exhibited significant changes ($p < 0.10$) in the expression level due to stress conditions (Fig. 3) Out of 13 differentially expressed protein spots in Kavir, nine spots showed up-regulation, two spots, absence and two spots, presence under drought stress, and among 13 differentially expressed protein spots in Bahar, five spots indicate up-regulation, two spots, down-regulation, three spots, absence and three spots presence in drought stress condition (Fig. 2).

Differentially expressed spots (DEPs) under drought stress and corresponding proteins in both cultivars have been listed in **Error! Reference source not found.**. There were some differences between theoretical and experimental pI and MW values. These are probably due to post translational modifications (Gobom *et al.*, 2002). Stress condition and particularly drought exposure (Caruso *et al.*, 2009) can significantly create changes in protein structure and isoform composition via PTMs such as phosphorilation. Drought stress responsible proteins identified in this study were not common between the two cultivars, which could represent different mechanisms of two cultivars to deal with stress. However the selected proteins could be classified into similar functional groups. These groups include: photosynthesis, metabolic pathways, stress defense/response, photorespiration, protein synthesis/assembly and proteins with unknown function (Fig. 4).

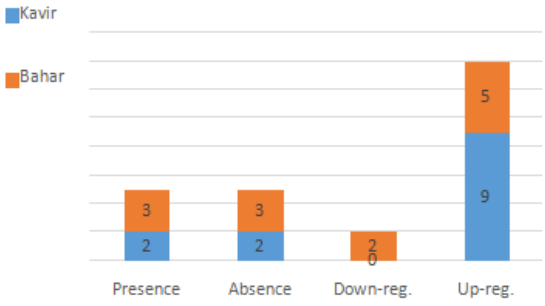

Fig. 2. The numbers of differentially expresssed spots in tolerant (Kavir) and susceptible (Bahar) cultivars.

Table 1. Drought responsive proteins identified in Bahar (up) and Kavir (down) cultivars.

Spot no [a]	Identified protein	pI/MW experimental	pI/MW theoritical	Express. Level [b]	Accession no	Species
4204	ATP synthase beta subunite	6.2/37.6	5.4/39.9	↓	114574	*Triticum aestivum*
7103	Peroxiredoxin-5 like	6.7/21.8	6.2/23	↓	BAD15391	*Oryza sativa*
7102	Hypothetical protein	7.4/26	7.7/26	↑	Q5NKR1	*monococcum*
4903	Glycine dehydrogenase	6.3/108.2	6.32/112	↑	356514615	*Glycine max*
5101	Cu/Zn superoxide dismutase	6.4/18.4	5.35/20	↑	1654387	*Triticum aestivum*
3101	Glyceraldehyde 3-phosphate dehydrogenase	6.1/18.5	5.4/18	↑	BAD93961	*Arabidopsis thaliana*
6105	RAS-related proteim RAB-7	6.6/20.3	5.5/23.4	↑	226494367	*Zea mays*
1704	S-adenosylmethionine synthetase 1	5.5/63	6.2/64	Ab.	TA62080_4565	*Hordeum vulgare*
2208	Putative thioredoxin-like protein CDSP32	5.9/33.9	8.1/33	Ab.	CAA71103	*Solanum tuberosum*
0801	Phosphoglycerate mutase, (fragment)	5.5/78	5.58/78	Ab.	Q7XYD2	*Triticum aestivum*
3103	Triose phosphate isomerase	6.1/31.1	6.0/32	Pr.	136063	*Triticum aestivum*
5106	Photosystem II oxygen evolving complex protein 1	6.5/32.5	5.89/35	Pr.	T02066	*Triticum aestivum*
7002	Cold regulated protein	6.8/19.8	6.3/19	Pr.	CD914580	*Triticum aestivum*
4617	HSP70	6.2/63.3	5.76/67.1	↑	476003	*Hordeom vulgare*
5503	Cell division protease ftsH homolog 2	6.7/51.7	5.5/73	↑	Q655S1	*Oryza sativa*
5204	50S ribosomal protein L4, chloroplast precursor	6.4/34.5	6.1/35	↑	ABF95133	*Oryza sativa*
5611	ATP synthase cf1 alpha chain	6.6/57	6.11/55.3	↑	17371040	*Triticum aestivum*
3205	GrpE protein homolog	5.8/33	9.5/39	↑	TA68734_4565	*Oryza sativa*
4201	Alternative oxidase	6.0/33	6.3/33.1	↑	19912725	*Triticum aestivum*
5603	glucose 6 phosphate dehydrogenase	6.4/61	5.92/59	↑	3023817	*Nicotiana tabacum*
4505	polyphenol oxidase (catechol oxidase)	6.2/55.5	5.8/55.6	↑	343489333	*Triticum aestivum*
4107	50S ribosomal protein L12-1, chloroplast precursor	6.1/23	5.5/23	↑	CD862473	*Secale cereale*
3605	Calreticulin-1, precursor	5.3/61.5	4.41/55	Ab.	O04151	*Arabidopsis thaliana*
4404	Plastid glutamine synthase isoforme GS2c	6.0/47.9	5.75/47	Ab.	71362640	*Triticum aestivum*
6404	Rubisco activatase a	6.4/46.5	6.4/45	Pr.	109940135	*Oryza sativa*
5205	Caffeoyl-CoA O-methyltransferase	6.1/32.5	5.9/28	Pr.	Q41720	*Zinnia violacea*

[a] spot numbers given by PDQuest software/ [b] The component increased (↑), decreased (↓), absence (Ab.) and presence (Pr.) in drought treated plants

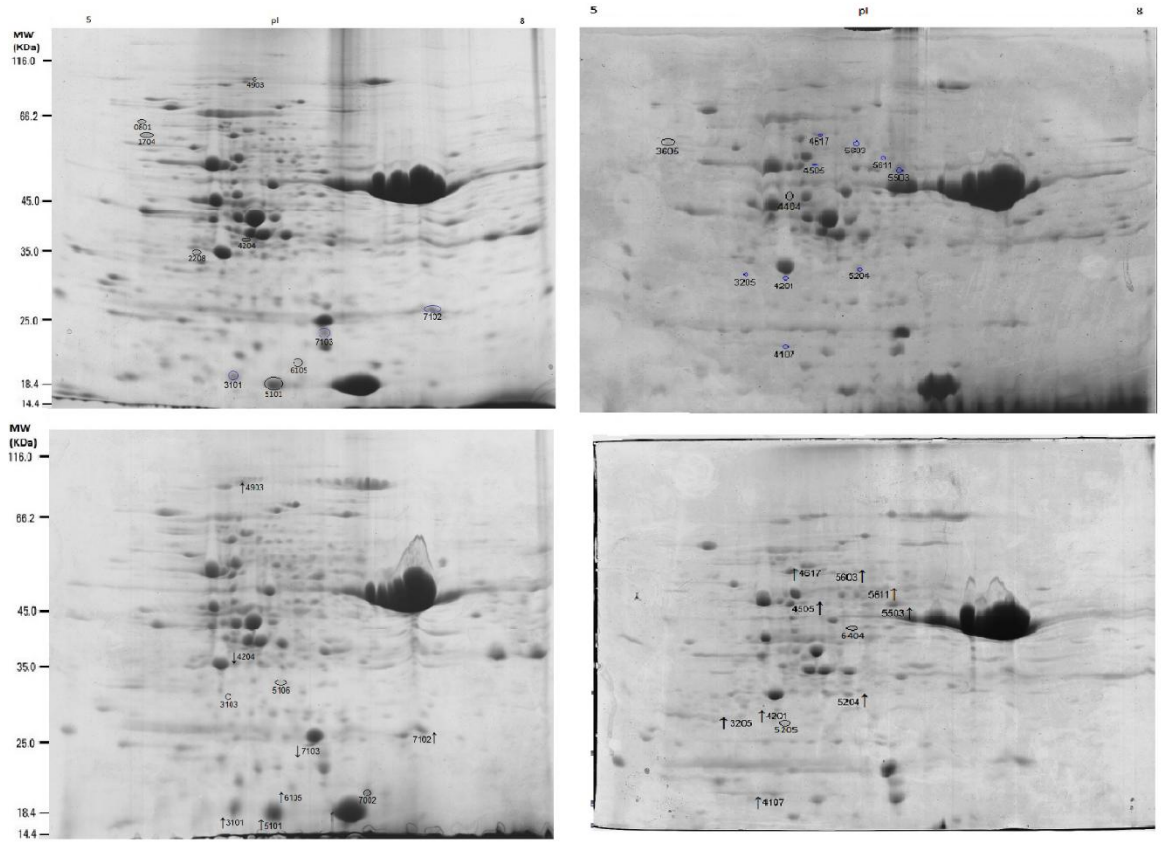

Fig. 3. The position of differentially expressed spots on 2-DE gels in susceptible cultivar, Bahar (Left) and tolerant cultivar, Kavir (Right) under well watered (Up) and stress (Down) condition.

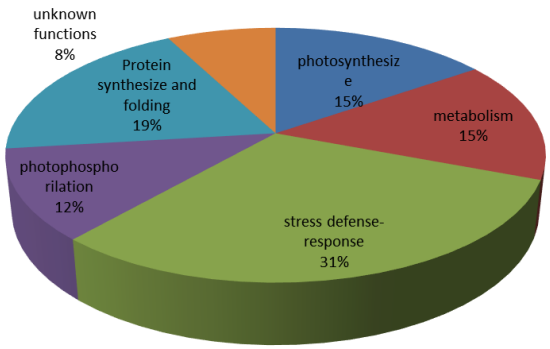

Fig. 4. Identified protein's classifying to biological functional groups.

The susceptible cultivar, Bahar, had no proteins in protein synthesis/assembly group and it had more proteins in metabolic pathway group. It seems this cultivar in contrast to Kavir, used plant storages to deal with drought stress instead of trying to preservation producing systems of cell and conservation of cellular structures.

Photosynthesis

In drought stress condition, both cultivar try to keep the photosynthesis active via expression of oxygen evolving complex (spot 5106 in Bahar) and rubisco activase a (spot 6404 in Kavir), but decrease and increase in expression of ATP synthase subunits respectively in Bahar (spot 4204) and Kavir (spot 5611) can show the success of the tolerant cultivar. Up and down regulation of ATP synthase subunits under drought stress have been observed (Kosová *et al.*, 2011; Sobhanian *et al.*, 2010). Increment in expression of oxygen evolving complex (OEC) in wheat (Caruso *et al*; 2009) and rice (Ali and Komatsu, 2006) under drought stress was reported. Expression of rubisco activase and other rubisco related proteins in susceptible (Bazargani *et al.*, 2011) and tolerant (Demirevska *et al.*, 2009) cultivars of wheat

decreased and increased under drought stress respectively.

Metabolic pathways

Expression change of glycolysis pathway in susceptible cultivar, Bahar indicate increased activity of this pathway via overexpression of Glyceraldehyde 3-phosphate dehydrogenase a subunit (spot 3101). Stress derived reactive oxygen species cause degradation of proteins that can lead to different regulation of a particular protein subunits or fragments. Caruso *et al*., 2009 reported degradation of RuBisCO large subunit, (fragment) as well as one fragment of phosphoglycerate mutase. So absence of Phosphoglycerate mutase, (fragment) (spot 0801 in Bahar) can indicate increase in glycolysis and increased ROS scavenging activity that is in accordance with the changes in oxidative stress related proteins of this study. S-adenosylmethionine synthetase 1 (spot 1704) which showed absence in Bahar and Caffeoyl-CoA O-methyltransferase that showed presence in Kavir (spot 5205), both are methyl transferase and involved in biological pathways such as methylations and ethylene biosynthesis (Espartero *et al*., 1994). Methylation are well known as a mechanism in protein expression and of course ethylene as stress hormone in plants. It seems that the tolerant cultivar, Kavir by conserving this protein could succeed in establishing new homeostasis to deal with drought stress. Bazarghani *et al*., (2011) reported that overexpression of S-adenosylmethionine synthetase 1 lead to increased ethylene synthesis and accelerated plant senescence to avoid drought stress.

Stress defense/response

Cold regulated protein (spot 7002) and putative thioredoxin-like protein CDSP32 (spot 2208), in susceptible cultivar, Bahar, showed presence and absence respectively; and HSP70 (spot 4617) was up-regulated in Kavir cultivar under drought condition. These proteins are known as stress responsible proteins. Oxidative stress induced by water stress causes impairment of photosynthetic electron transport in chloroplasts and mitochondria through the production of oxygen species, resulting destruction of cells and tissues (Navari-Izzo *et al*., 1997).

In general both cultivars of this study had increased activity in ROS scavenging and confronting with oxidative stress via increasing the expression levels of antioxidants including Cu/Zn superoxide dismutase (spot 5101) and RAS-related protein RAB-7 (spot 6105) in Bahar and alternative oxidase (spot 4201) and polyphenol oxidase (catechol oxidase, spot 4505) in Kavir. Different DEPs between the two cultivars suggest different mechanisms in dealing with oxidative stress. Unexpectedly an antioxidant protein (Peroxiredoxin-5 like, spot 7103) was down-regulated in susceptible cultivar. This, has been observed in wheat under drought stress (Bazargani *et al*., 2011); and probably due to damage caused by drought. Genotypes with lower lipid peroxidation, higher membrane stability, higher content of chlorophyll and carotenoid, indicated strong relation with antioxidant enzyme systems e.g. SOD, APO, GR and CAT (Sairam *et al*., 2002).

Photorespiration

Glycine dehydrogenase (spot 4903) and triose phosphate isomerase (spot 3103) from and glucose 6 phosphate dehydrogenase (spot 5603) from Kavir overexpressed and involved in photorespiration. Changes in expression of enzymes associated with photorespiration reflect higher activity of this cycle in both cultivars. Photorespiration occurs in CO_2 deficiency due to stomatal closure that occurs to decrease water loose from plant. Canvin *et al*. (1990) states that glycolate pathway of photorespiration has scavenger role. Every turn in glycolate cycle produces two phosphoglycolate molecules through oxygenation. One of these four carbon atoms is excreted in form of the carbon dioxide and the remaining three atoms will be returned to the chloroplast. Hence glycolate pathway returns 75 percent of the carbons into the chloroplast that otherwise would go to waste. It was shown that in the

absence of CO_2, if there be enough amounts of O_2 for photorespiration; the light did not damage the leaf. Apparently the CO_2 derived from photorespiration make electron transport system to continue working and through this prevents damage of photooxidation on leafs (Osmond and bjorkman, 1970). This mechanism can be ecologically beneficial in conditions with intense light and eliminated CO_2 amounts that occur in drought stress.

Protein synthesis/assembly

All of the proteins in this group were from Kavir, the tolerant cultivar; and except one protein (Calreticulin-1, precursor, spot 3605) that were absence in stress condition, the rest of the proteins was dramatically increased in expression. These proteins include: cell division protease ftsH homolog 2 (spot 5503), GrpE protein homolog (spot 3205), 50S ribosomal protein L4 (spot 5204) and L12-1 (spot 4107) chloroplast precursors. Increment of expression levels in ribosomal proteins chloroplast precursors and decline in expression levels of cell division protease ftsH homolog 2 and GrpE protein homolog in wheat under drought stress conditions have been observed (Bazargani *et al*., 2011).

Overexpression of these proteins may represent the tolerant cultivar's attempt to protect and sustain the correct folding of other proteins in addition to accelerated degradation of unfolded/incorrectly folded or stress damaged proteins. This manner could be considered the most important mechanism of Kavir cultivar's tolerance to drought stress.

The absence of Calreticulin-1, precursor indicate use of special proteins by plant for protein synthesis or assembly under drought stress condition.

Conclusion

Two wheat cultivars with different tolerance to response drought stress was compared in terms of morpho-physiological traits; and in terms of molecular responses using two dimensional polyacrilamid gel electrophoresis that allow monitoring of proteome changes under drought stress. This study, could identified some proteins that have key role in wheat response to drought stress. These proteins are involved in main metabolic pathways and investigating them could help to understand molecular basis of wheat response to drought stress. In tolerant cultivar the response was mainly related to protein synthesis and assembly. So these proteins could be choose as a putative key elements for understanding molecular mechanisms of responding to drought and particularly those involved in protein synthesis/assembly for improving drought resistant lines in wheat.

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