

# Identification of changes in *Triticum aestivum* L. leaf proteome in response to drought stress by 2D-PAGE and MALDI-TOF/TOF mass spectrometry

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**Abstract** Drought is an abiotic stress that strongly influences plant growth, development and productivity. To gain a better understanding of the drought-stress responses at physiological and molecular level in wheat plants (*Triticum aestivum* cv. KTC86211), we performed a comparative physiological and proteomics analysis. Eight-day-old wheat seedlings were treated with polyethylene glycol-simulated drought stress for 0, 24, 48 and 72 h. Drought treatment resulted in alterations of morphology, increased relative electrolyte leakage and reduced length and weight on leaf and root. Stress-induced proteome changes were analyzed by two-dimensional gel electrophoresis in conjunction with MALDI-TOF/TOF. Twenty-three spots differed significantly between control and treated plants following 48 h of drought stress, with 19 upregulated, and 4 downregulated, in leaf tissues. All of the differentially expressed protein spots were identified, revealing that the majority of proteins altered by drought treatment were involved in reactive oxygen species scavenging enzymes and photosynthesis. Other proteins identified were involved in protein metabolism, cytoskeleton structure, defense response, acid metabolism and signal transduction. All proteins might contribute cooperatively to reestablish

cellular homeostasis under drought stress. The present study not only provides new insights into the mechanisms of acclimation and tolerance to drought stress in wheat plants, but also provides clues for improving wheat's drought tolerance through breeding or genetic engineering.

**Keywords** Drought stress · Proteomics · Two-dimensional electrophoresis · Matrix-assisted laser desorption/ionization time of flight · Wheat

## Introduction

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world and provides about 20 % of the calories consumed by humans (Brenchley et al. 2012). Furthermore, wheat is an important source of protein, vitamins and minerals. During the growing season, wheat plants are often susceptible to various environmental stresses. Drought stress is a major environmental stress factor limiting plant growth and development and causes yields loss in cultivated crops worldwide (Waraich et al. 2011). The mechanisms underlying drought tolerance and acclimation have been intensively studied in recent years (Chaves et al. 2009; McDowell 2011).

To defend against the drought stress, plants undergo a process of stress acclimation. This process may require changes in gene (Harb et al. 2010) and protein expression profiles (Plomion et al. 2006; Aranjuelo et al. 2011). The transcriptome analysis was frequently employed to clarify the global gene expression profile, revealing that some genes are induced or repressed upon drought stress (Bogeat-Triboulot et al. 2007). These data contributed greatly to our understanding of drought responses mechanisms. However, the changes in gene expression at

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transcript level do not always correlate to the changes at protein expression level, probably owing to large differences in protein turnover and post-translational modifications (Pradet-Balade et al. 2001). As proteins, unlike transcripts, are direct effectors of plant stress response, investigation of changes in plant proteome can be playing an increasingly important role in addressing plant response to environmental changes (Kosová et al. 2011).

With the determination of the genome sequence of wheat, it is now possible to attempt a comprehensive understanding of complex biological processes in this species. Many recent proteomics studies have been performed on various species under different abiotic stresses, such as drought (Jorge et al. 2006; Vincent et al. 2005), osmotic stress (Toorchi et al. 2009), high salinity (Manaa et al. 2011), low temperature (Cheng et al. 2010), heat (Lee et al. 2007), waterlogging (Alam et al. 2010). However, changes in protein profiles also differ depending on species, plant organs and duration (Ashraf and Harrisb 2004). There are limited reports concerning systematic proteomics analyses of protein abundances in wheat under drought stress. Under severe drought conditions, Caruso et al. (2009) found that certain drought-responsive proteins that have evolved in glycolysis and gluconeogenesis, Calvin cycle, reactive oxygen species (ROS) scavenging, amino acid biosynthesis, defense mechanisms and post-transcriptional regulation. Hajheidari et al. (2007) revealed that two-thirds of identified proteins were thioredoxin (Trx) targets, in three wheat genotypes differing in genetic background. Compared with previous studies, we obtained some similar results. For instance, the responsive proteins also included ROS scavenging, photosynthesis and carbon fixation, amino acid biosynthesis, defense mechanism. Although the exact proportion is different among various groups, ROS scavenging enzymes and photosynthesis constitute the major two groups in protein classification. Meanwhile, we observed other groups changed from past studies include protein metabolism, signal transduction, cytoskeleton and membrane lipid modification. In this study, wheat protein profiles from PEG-treated plants were performed by using a proteomics approach in order to elucidate the mechanisms underlying drought stress.

## Materials and methods

### Plant materials and stress treatment

Wheat (*T. aestivum* L.) cv. KTC86211, an ABA-insensitive somaclone, was used as experimental material. KTC86211 was more tolerant to drought than other genotypes by comparison of some physiological parameters (Lu et al.

1989). Seeds of uniform size were surfaced sterilized with 75 % ethyl alcohol followed by rinsing several times with distilled water. The sterilized seeds were soaked in distilled water in darkness (25 °C, 24 h). After seeds germinated, wheat seedlings were grown in plastic pots (15 cm deep and 15 cm in diameter) filled with washed sand under white fluorescent light (150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 16 h light/8 h dark photoperiod) at 25/19 °C (light/dark) and 75 % relative humidity in a growth chamber. During the growth period, plants were watered daily until water drained from the bottom of the pots and fertilized twice a week with full-strength Hoagland's nutrient solution (Hoagland and Arnon 1938).

When the second leaf was fully expanded (about 8 days), seedlings were subjected to drought stress. The plants were irrigated daily with water (control) or with polyethylene glycol (PEG) 6,000 solution to induce drought stress for 0, 24, 48 and 72 h. Drought stress was obtained by adding PEG solution with osmotic potential of  $-0.50$  MPa twice a day for everyday. Each treatment for each sampling point was replicated in four pots. At each time of watering, sufficient water or PEG solution was applied until the sand medium was fully saturated. For morphological analysis of wheat seedlings under drought stress, the length of leaf and root and fresh weight was measured every day. For proteome analysis, 8-day-old seedlings were treated for 48 h and then used. All physiological and proteomics analysis was performed on the second leaf, which was fully expanded at the beginning of the treatment.

The experiments were based on a completely randomized design and three biologically independent replicates were performed at separate times with 20 plants.

### Physiological analyses

When wheat plants were treated by PEG at different sampling points (0, 24, 48 and 72 h), control and treatment groups were harvested and separated into leaves, stems, and roots. The leaves and roots tissue were used to measure the growth indicators including leaf length, root length, leaf fresh weight and root fresh weight. For the relative electrolyte leakage (REL) assay, 150 mg of fresh seedlings were rinsed with ddH<sub>2</sub>O, placed in test tubes containing 10 mL of ddH<sub>2</sub>O, and incubated at room temperature for 2 h, with the initial electrical conductivity of the medium ( $C_1$ ) measured using a conductivity meter (Orion 115Aplus; Thermo Electron). Then, the tubes were boiled for 15 min to release all electrolytes, cooled to room temperature, and the final electrical conductivity ( $C_2$ ) was measured again. The REL was calculated by the formula  $C_1/C_2 \times 100$  % (Cao et al. 2007).

## Protein extraction

Protein extraction was performed following a protocol using acetone/trichloroacetic acid (TCA) precipitation method developed by Damerval et al. (1986), applying some modifications. At 48 h of treatment, leaves were harvested and immediately frozen in liquid nitrogen, and then stored at  $-80^{\circ}\text{C}$  prior to analysis. Three independent samples were harvested from each treatment. Leaf tissue (about 1 g) was homogenized and incubated with 10 mL of precipitation solution (10 % TCA and 0.07 % 2-mercaptoethanol in acetone) for 2 h at  $-20^{\circ}\text{C}$ . The precipitated proteins were pelleted and washed with 10 % TCA/acetone twice and cold acetone twice to remove pigments and other non-protein compounds. The supernatant was removed and the pellet was vacuum-dried, resuspended in the isoelectric focusing (IEF) extraction solution consisting of 7 M urea, 2 M thiourea, 4 % CHAPS, 20 mM DTT, and 0.2 % pH 3–10 pharmalytes. In order to obtain a complete protein solubilization, the sample was incubated for 30 min at room temperature. Insoluble tissue was removed by centrifugation at 15,000g for 40 min and the supernatant was subjected to IEF. Protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard.

## Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis of proteins was performed in accordance with the method of O'Farrell (1975), with some modifications. Briefly, 350  $\mu\text{L}$  of solution containing 800  $\mu\text{g}$  of proteins were applied into an immobilized pH gradient (IPG) strip (17 cm, pH 4–7 linear, BioRad) and covered with paraffin oil. IEF was performed with a PROTEAN IEF system (BioRad, USA) applying the following conditions. For the rehydration step the voltage was maintained for 16 h at 50 V, then the proteins were focused for 1 h at 250 V, 1 h at 500 V, 1 h at 1,000 V, 5 h at 8,000 V, and 10 h at 8,000 V. The temperature was maintained at  $20^{\circ}\text{C}$  and the current was 50  $\mu\text{A}$  per strip. Prior to the second dimension separation, the strips were equilibrated in DTT followed by iodoacetamide as described by Chivasa et al. (2002). The second dimension separation of proteins was performed on a 12 % SDS-PAGE gel using a PROTEAN II XL two electrophoresis unit (BioRad, USA). The electrophoresis was carried out at  $25^{\circ}\text{C}$  and 1 W per gel for 30 min and then 8 W per gel for 5 h and 40 min until the bromophenol blue dye front arrived at the bottom of the gels. Following SDS-PAGE, gels were washed in  $\text{ddH}_2\text{O}$  three times for 15 min and proteins were detected by a modified colloidal Coomassie brilliant blue staining-blue silver protocol (Candiano et al. 2004). A total of six gels were analyzed, three gels for untreated plants and three gels for PEG-treated plants.

## Image and statistical analysis

The 2-DE image and statistical analysis were performed by using the GS-800 scanner (BioRad) and the PDQuest ver. 8.0 software (BioRad, USA). Image analysis included image filtration, spot detection and measurement, background subtraction and spot matching. All gels were compared with one of the selected gels as a reference gel and spots were automatically matched followed by adding unmatched spots to the gels, manually. The amount of a protein spot was expressed as the volume of that spot, which was defined as the sum of the intensities of all the pixels that make up that spot. To accurately compare spot quantities between gels, the spot volumes were normalized as a percentage of the total volume of all of the spots in the gel. The quantitative comparison of protein spots was based on their volumes. Only statistically significant spots ( $p < 0.05$ ) were accepted, and they had to be consistently present in all replications.

## Spot excision and protein in-gel digestion

Those differently expressed spots were manually excised from the gel, and minced into pieces. Tryptic in-gel digestion was based, with some modifications, on the protocol of Parker et al. (2006). Briefly, the gel piece was destained with 30 % acetonitrile (ACN) in 100 mM ammonium bicarbonate until colorless, incubated at room temperature for 15 min with ammonium bicarbonate, and then the gel pieces were dehydrated for 10 min with acetonitrile. The enzymatic digestion was performed at  $37^{\circ}\text{C}$  with trypsin ( $10\text{ ng } \mu\text{L}^{-1}$ ) for 20 h. The gel pieces were immersed in 60 % ACN/0.1 % trifluoroacetic acid (TFA) solution and were ultrasonic treated for 15 min. The supernatant was combined and vacuum-dried.

## MS analysis and database searching

The peptides were resuspended in 2  $\mu\text{L}$  of 0.1 % TFA, 50 % ACN, and then immediately spotted onto a MALDI target plate with CHCA as matrix. The MS was analyzed using a 4800 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (AB SCIEX, USA). Protein identification was performed using protein sequences databases downloaded from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) using the MASCOT program (in-house version 2.2, Matrix Science, UK). The search parameters were as follows: complete carbamidomethylation of cysteines and partial oxidation of methionines, peptide mass tolerance  $\pm 100$  ppm, fragment mass tolerance  $\pm 0.4$  Da, missed cleavages 1. The criteria for protein identification were as follows: the protein score had to be more than the significance threshold level

( $p < 0.05$ ) and higher than 50, and at least two MS/MS sequences matched.

### Statistical analyses

The SPSS v. 17 was employed to perform the entire statistical analysis. For protein abundance analysis, electrophoresis was run in three extraction samples from each replicate. The relative expression of the identified proteins is represented as the mean of three replicates. Physiological and protein abundance data were compared using one-way analysis of variance (ANOVA). The significance of differences among means was carried out using Duncan's multiple range test (DMRT) at  $p = 0.05$ . The results were expressed as the mean  $\pm$  SE of three repeated experiments.

## Results and discussion

### Plant growth response to drought stress in wheat

The present results showed that drought-stress treatment significantly decreased the whole plant growth of the wheat plant. With stress duration, the suppression of growth became more serious. These seedlings showed obviously retarded growth morphology compared with untreated samples, such as shorter plant stature, rolled leaves and lighter shoot pigmentation (Fig. 1a). Analysis of morphological characteristics showed that leaf and root growth was changed dramatically, compared with the untreated plants. The impact of stress on leaf and root mainly included extension and weight. The changes in leaf length, root length, leaf fresh weight and root fresh weight were evaluated at each time point (Fig. 1b–e). As expected, the measured value of treated plants was smaller than that in untreated at the same sampling point. By stressing time increasing, the leaf length of wheat seedlings in treated samples reduced gradually, but increased in untreated samples (Fig. 1b). Similar law was observed in leaf fresh weight (Fig. 1d) and root fresh weight (Fig. 1e), but their altered scope was larger than the former. However, the root length of either the treated samples or the untreated increased with stress proceeding (Fig. 1c), which showed a different change trend. This suggested that stress suppressed the growth of wheat seedlings, but the slight elongation of the root under drought conditions may reflect the resistance to negative surroundings.

Relative electrolyte leakage is known as an indicator of membrane damage caused by drought stress. As shown in Fig. 1f, the REL gradually increased with the stress treatment prolonged. The value of REL in treated seedlings at 0 h was 18 %. After 24 h treatments, it increased to 29 % and further increased to 36 and 44 % after PEG was

maintained for 48 and 72 h, respectively. When compared with the untreated samples, the REL sharply increased 1.41, 1.85 and 1.88 times higher at 24, 48 and 72 h, respectively. This suggested that stress-triggered cellular damage accumulated throughout the duration of the treatment. It is worthy to note that most of the physiological indices changed remarkably in treated plants at 48 h of treatment and the difference was significant, but the difference was not significant compared with the values elevated in treated plants at 72 h treatment (Fig. 1b–e). Therefore, 48 h of treatment time was selected in the following proteomics analysis.

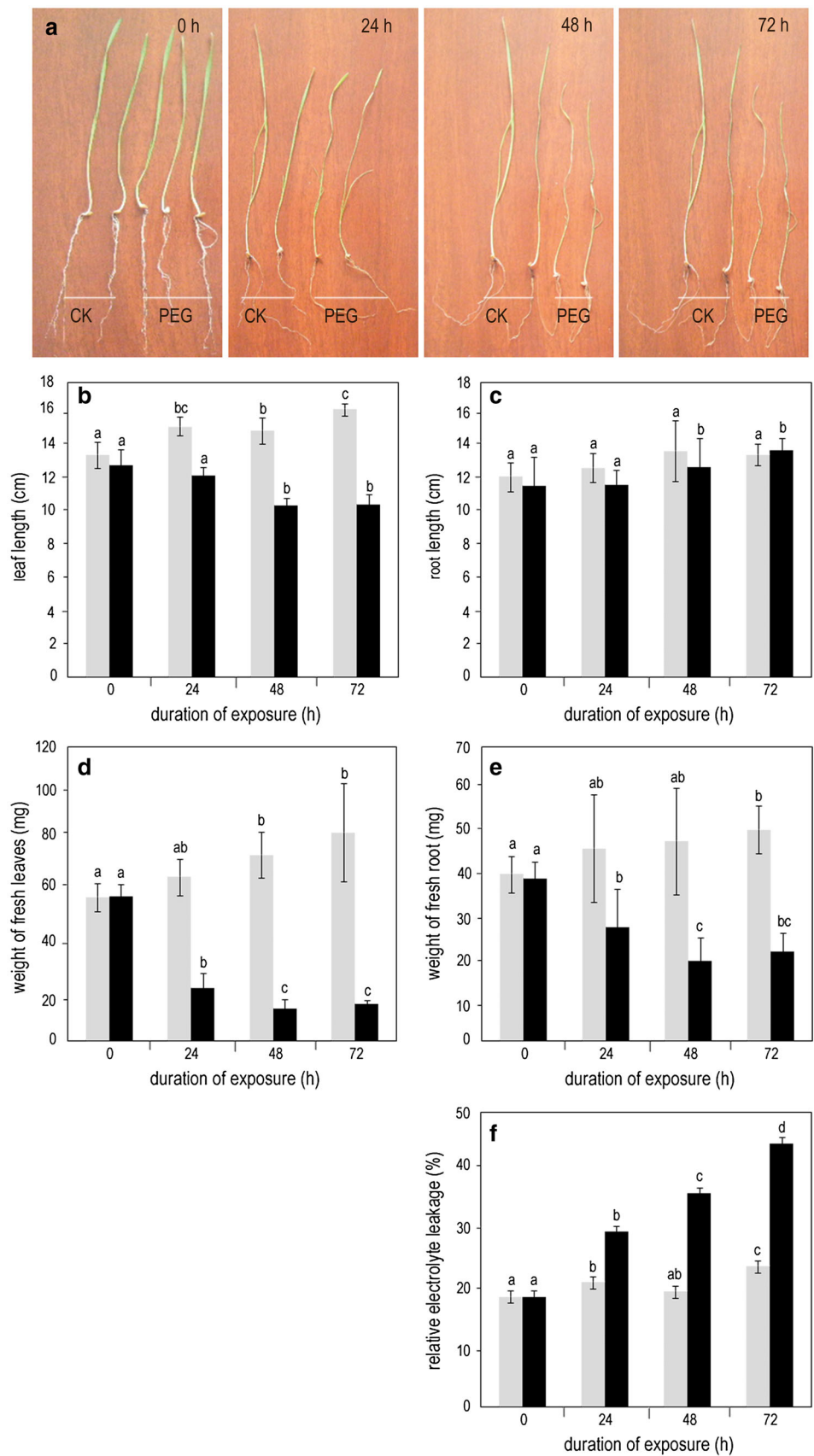
### 2-DE analysis of drought stress-responsive proteins in wheat leaves

To investigate the changes of protein profiles in response to drought stress, 2-DE analysis of the total proteins in *T. aestivum* leaves from three biologically independent replicate experiments was carried out. Figure 2 shows the reference 2-DE electrophoretic maps obtained from the control (Fig. 2a) and the drought-treated samples (Fig. 2b). A broad distribution of the proteins was displayed in the pI range from 4.0 to 7.0 and the mass range from 10 to 100 kDa. Approximately 530 protein spots were detected on the colloidal Coomassie brilliant blue stained gels and about 350 protein spots were matched between three control gels and three treatment gels. To find the drought-responsive proteins, changes in spot volume between untreated and treated samples were quantified by software analysis (see "Materials and methods"). Protein spots were selected only when they were reproducibly detected in three independent experiments. Of the differently expressed protein spots, 19 spots were upregulated and 4 were downregulated when compared with the control gels. Figure 3 shows examples of protein spots with a significant difference ( $p < 0.05$ ) in abundance between control and drought-treated plants.

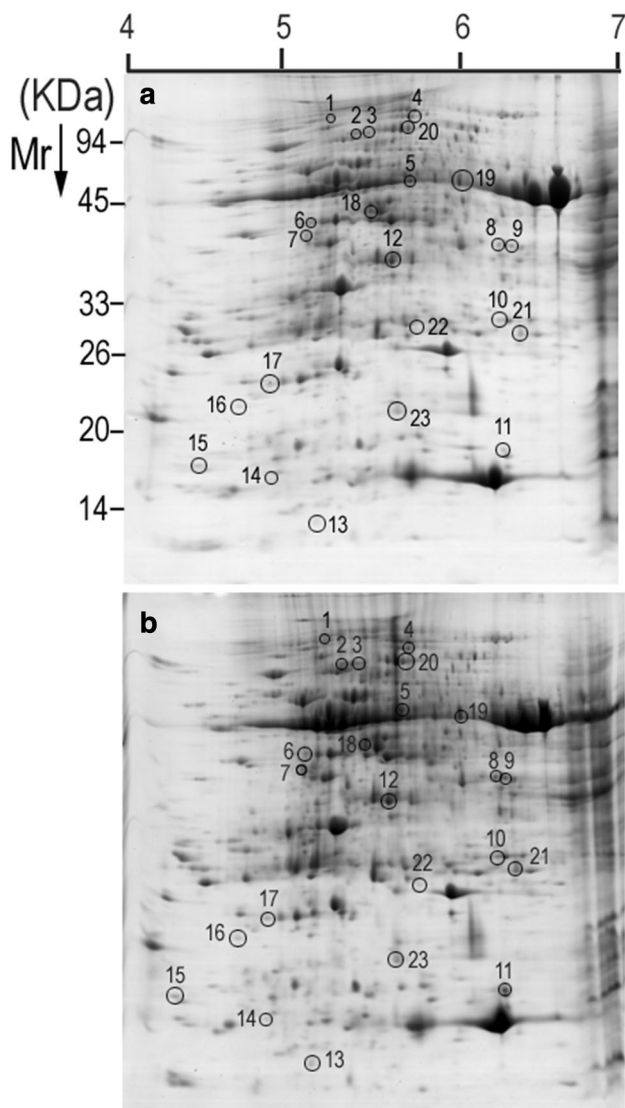
### MALDI-TOF/TOF identification and classification of drought-responsive proteins

The spots of differentially expressed proteins were excised from the gels, in-gel digested by trypsin, and analyzed by mass spectrometer. In total, 23 protein spots were successfully identified by MS/MS analysis representing 22 individual proteins. These identified protein spots whose expression level was observed to change in response to drought stress are shown and listed in Table 1 and Fig. 2. Some drought-responsive protein spots were not excised owing to their low abundance. Indeed, these protein spots were below the threshold of colloidal Coomassie brilliant blue staining and were not well visible for picking up.

**Fig. 1** The physiological responses induced by PEG-simulated drought stress in wheat. Eight-day-old seedlings were subjected to drought stress for 0, 24, 48 and 72 h and sampled for further analysis. Morphological changes of the plants under drought stress are shown in **a**. Leaf length (**b**), root length (**c**), weight of fresh leaves (**d**), weight of fresh root (**e**) and REL (**f**) were evaluated. *Gray columns* and *black columns* represent the untreated drought-treated samples, respectively. *Bars* represent the mean  $\pm$  SE. *Bars* denoted by the *same letter* within response variables are not significantly different ( $p = 0.05$ ) using DMRT







**Fig. 2** Two-dimensional gel electrophoresis of proteins extracted from wheat control sample (a) and treated sample (b). The leaves were sampled at 48 h. The proteins were separated on a 17 cm immobilized pH gradient strip with a linear gradient of pH 4–7, followed by a 12 % (v/v) sodium dodecyl sulfate polyacrylamide gel. Proteins were visualized by Coomassie R250. Numbered circles indicate spots showing an expression level change between control and stressed samples. Proteins are listed in Table 1

Table 1 provided the information on the identified proteins, 19 proteins were upregulated and 4 of them were downregulated. It was found that one protein, iron–sulfur subunit of cytochrome b6-f complex, was identified in two spots (spots 2 and 11), although they were excised from the same gel (Table 1; Fig. 2). Proteins present in more than one spot could be isoforms, perhaps due to post-translational modification, or degradation. Post-translational modifications such as glycosylation, phosphorylation, etc., can alter the molecular weight and/or the pI of proteins (Caruso et al. 2008). Degradation was another important cause of

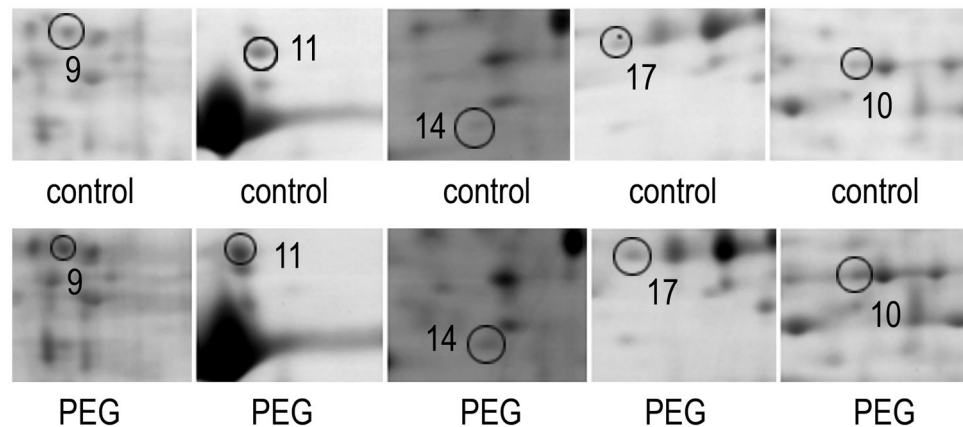
this phenomenon. Proteomics studies have also demonstrated that some proteins may be degraded under environmental stress conditions. For example, the RuBisCo large subunit was identified as three separate fragments plus the intact protein in drought-treated rice leaves (Taylor et al. 2005). It is possible that ROS may also contribute to the degradation of proteins under stress conditions (Kingston-Smith and Foyer 2000).

The identified, differentially expressed proteins were classified into several groups according to their functions (Fig. 4). The first group is involved in the ROS scavenging, including ascorbate peroxidase (APX, spot 10), glutathione-S-transferase (GST, spot 21), thiol-specific antioxidant protein (spot 17), ferritin-1 (spot 22) and thioredoxin which includes Trx h (spots 13 and 23) and Trx m type (spot 14). The second group of proteins is related to photosynthesis and carbon fixation, including RuBisCO activase (spot 8), iron–sulfur subunit of cytochrome b6-f complex (spots 2 and 11), transketolase (spot 20), ATP synthase F0 subunit 1 (spot 19), 2-phosphoglycerate dehydratase (spot 5) and fructose 1, 6-bisphosphate aldolase (spot 12). Meanwhile, the third group of proteins is associated with protein metabolism, such as 40S ribosomal protein SA-like (spot 7), 60S acidic ribosomal protein P1-like (spot 15), Heat shock protein70 (HSP70, spot 3) and presequence protease1 (PreP, spot 1). 80 % of the identified proteins were implicated in photosynthesis and carbon fixation, ROS scavenging and protein metabolism (e.g., translation, processing, and degradation). The proportion of other groups of protein, such as membrane lipid modification, signal transduction, defense mechanisms, cytoskeleton and amino acid biosynthesis, identified by proteomics experiment, was small and similar in the present study. With the exception of two proteins including PreP1 and TIR-NBS-LRR-TIR type disease resistance protein, most of that identified in our experiments have already been reported in the context of drought-stress responses. Some of the identified proteins have been well characterized in terms of their response to drought and other abiotic stress. However, the role of other proteins including the two novel drought-responsive proteins remains unclear yet in drought stress. The selected stress-responsive proteins are discussed below.

#### ROS scavenging and detoxifying enzymes

Drought stress often causes accumulation of ROS such as singlet oxygen, superoxide radical and hydroxyl radical, and the enhanced amount of ROS under stress can be viewed as a threat for the cell, and they can also act as secondary messengers involved in the stress signal transduction pathway (Foyer et al. 1994). Plants can regulate the ROS level through sophisticated mechanisms of several enzymes whose functions are implicated in cell

**Fig. 3** Examples of protein spots showing a significant difference ( $p < 0.05$ ) in abundance between control and PEG-treated plants; the numbers refer to those in Fig. 2



detoxification. Until now, several unique factors have been characterized in the leaves of wheat, which potentially functions as scavengers of ROS produced under drought stress (Peng et al. 2009; Hajheidari et al. 2007; Caruso et al. 2009). In our study, several proteins involved in these mechanisms were found to be upregulated during wheat acclimation to drought stress, including one APX (spot 10), one glutathione-*S*-transferase (spot 21), three thioredoxins (spots 13, 23 and 14), one thiol-specific antioxidant protein (spot 17) and one ferritin-1A (spot 22).

Ascorbate peroxidase is one of the major antioxidant enzymes and free radical scavengers in plants, which can directly detoxify  $H_2O_2$  by oxidizing specific substrates such as ascorbate (Hernández et al. 1995). Previous reports indicated that cytosolic isozymes of APX showed an increased expression in pea shoots (Zhang and Kirkham 1994), in two poplar species (Yang et al. 2010), but the activity of APX remained unchanged in leaves of *Ramonda serbica* during drought stress (Veljovic-Jovanovic et al. 2006). Jiang et al. (2012) proposed that the upregulation or downregulation in abundance of APX often reflected the resistance of different varieties to drought, i.e., the upregulation or unchanged state occurred in the variety of resistance of drought. In the present work, the accumulation of APX was induced by water deficit maybe is in accordance with the characteristic of the tested variety, which is verified by Lu et al. (1989).

The enzymes glutathione-*S*-transferases (GSTs) have been associated with both normal cellular metabolism as well as in the detoxification of xenobiotics, limiting oxidative damage and other stress responses in plants. The endogenous products of oxidation including membrane lipid peroxides and products of oxidative DNA degradation are highly cytotoxic. GSTs detoxify such endogenously produced electrophiles by their conjugation with GSH. Previous studies revealed that GSTs are responsive to various environmental stresses, including cold, heat, and drought stress in different plant species (Kosmala et al.

2009; Lee et al. 2007; Gazanchian et al. 2007). In most proteomics experiments related to drought stress, GSTs were upregulated in different plant species (Gazanchian et al. 2007; Yoshimura et al. 2008). In our study, GST was markedly upregulated after 48 h of drought-stress treatment, indicating its involvement in drought-stress tolerance.

Thioredoxin is involved in cellular redox regulation by two approaches. Trx could donate electrons to redox enzymes like ribonucleotide reductase, methionine sulf-oxide reductase and peroxiredoxin. Meanwhile, Trx could contribute by reducing disulfide bonds of oxidized proteins. In plants, three main types of Trx have been identified, each consisting of a multigene family. Trx h is located in the cytosol, while Trx m and f are in chloroplast (Jacquot et al. 1997). Different types of thioredoxins have been reported in response to drought stress in *Solanum tuberosum*, in wheat (Rey et al. 1998; Hajheidari et al. 2007). In our study, both Trx h and m exhibited an increase in abundance under drought conditions, which is in accordance with previous study (Bazargani et al. 2011).

Thiol-specific antioxidant protein was the first perox-iredoxin to be identified. The peroxiredoxins are a family of multiple isozymes that catalyze the reduction of  $H_2O_2$  and protect the cells against oxidative damage in plants. In previous literatures, we have observed the upregulation in wheat leaves by salt stress and drought stress (Caruso et al. 2008, 2009). Here, a similar result was obtained with that from Caruso, indicating that this antioxidant protein might play a principal role in ROS scavenging under drought stress.

Fe is an important element in photosynthesis apparatus and free Fe participates in the production of excess hydroxyl radicals ( $OH\cdot$ ) via the Fenton reaction (Ravet et al. 2009). The control of free iron in plant cells is important because  $Fe^{3+}$  resulting from the Fenton reaction can be reduced back to  $Fe^{2+}$  by  $O_2^{\cdot-}$  radicals or by ascorbate, thus sustaining the reaction. Ferritin can

**Table 1** Identification of drought-responsive proteins using MALDI-TOF/TOF analysis

Spot ID	Drought stress <sup>a</sup>	Theoretical pI/Mr	Experimental pI/Mr	Protein identification and species	Accession no.	MOWSE score	PM <sup>b</sup>	C% <sup>c</sup>	p value <sup>d</sup>
1	↑	5.42/121.82	5.4/128	Presequence protease 1 <i>Brachypodium distachyon</i>	gil357137982	255	18	18	0.048
2	↑	8.47/24.11	5.5/110	Iron–sulfur subunit of cytochrome b6-f complex, chloroplastic <i>Triticum aestivum</i>	gil68566191	582	08	55	0.035
3	↑	5.76/67.14	5.6/111	HSP70 <i>Hordeum vulgare</i> subsp. <i>vulgare</i>	gil476003	646	13	30	0.049
4	↑	5.61/97.90	5.8/127	Lipoxygenase 6 <i>Zea mays</i>	gil162463394	152	11	17	0.011
5	↑	5.41/48.28	5.8/81	2-Phosphoglycerate dehydratase <i>Oryza sativa Japonica Group</i>	gil90110845	397	12	39	0.047
6	↑	5.75/47.02	5.3/66	plastid glutamine synthetase 2 <i>Triticum aestivum</i>	gil71362640	120	08	33	0.045
7	↑	5.02/32.68	5.3/58	40S Ribosomal protein SA-like <i>Brachypodium distachyon</i>	gil357111906	338	09	37	0.043
8	↑	6.52/40.26	6.3/54	Chloroplast ribulose-1,5-bisphosphate carboxylase activase <i>Triticum aestivum</i>	gil115392208	126	07	26	0.010
9	↑	5.94/14.73	6.4/54	TIR-NBS-LRR-TIR type disease resistance protein <i>Populus trichocarpa</i>	gil105922786	062	17	16	0.021
10	↑	5.54/26.77	6.3/34	Ascorbate peroxidase <i>Triticum aestivum</i>	gil226897533	247	07	42	0.017
11	↑	8.47/24.11	6.3/15	Iron–sulfur subunit of cytochrome b6-f complex, chloroplastic <i>Triticum aestivum</i>	gil68566191	623	10	67	0.038
12	↓	6.26/42.18	5.7/50	Fructose-bisphosphate aldolase, chloroplastic-like <i>Brachypodium distachyon</i>	gil357157399	508	13	38	0.024
13	↑	5.12/13.62	5.3/10	Thioredoxin H-type <i>Triticum aestivum</i>	gil11135126	357	07	57	0.015
14	↑	8.67/19.68	5.1/13	Thioredoxin M-type, chloroplastic <i>Triticum aestivum</i>	gil11135474	140	04	30	0.040
15	↑	4.52/11.29	4.6/14	60S acidic ribosomal protein P1-like <i>Brachypodium distachyon</i>	gil357144469	475	05	65	0.012
16	↑	4.55/18.82	5.0/20	Translationally controlled tumor protein <i>Triticum aestivum</i>	gil146285306	194	04	27	0.015
17	↑	5.71/23.42	5.1/23	Thiol-specific antioxidant protein (precursor) <i>Triticum aestivum</i>	gil2829687	153	02	20	0.023
18	↓	5.24/31.87	5.6/71	Actin <i>Elymus nutans</i>	gil345647450	596	14	65	0.017
19	↓	5.85/55.62	6.1/81	ATP synthase F0 subunit 1 <i>Oryza sativa Japonica Group</i>	gil194033257	404	17	39	0.015
20	↓	5.47/73.34	5.8/114	Transketolase, chloroplastic <i>Zea mays</i>	gil75140229	195	07	10	0.020
21	↑	5.79/23.60	6.4/32	Glutathione-S-transferase <i>Triticum aestivum</i>	gil5923877	582	08	45	0.013



**Table 1** continued

Spot ID	Drought stress <sup>a</sup>	Theoretical pI/Mr	Experimental pI/Mr	Protein identification and species	Accession no.	MOWSE score	PM <sup>b</sup>	C% <sup>c</sup>	p value <sup>d</sup>
22	↑	5.21/22.72	5.8/32	Ferritin1A <i>Triticum aestivum</i>	gil210061129	433	13	48	0.041
23	↑	5.29/12.85	5.7/19	Thioredoxin H <i>Triticum aestivum</i>	gil27461140	053	06	64	0.018

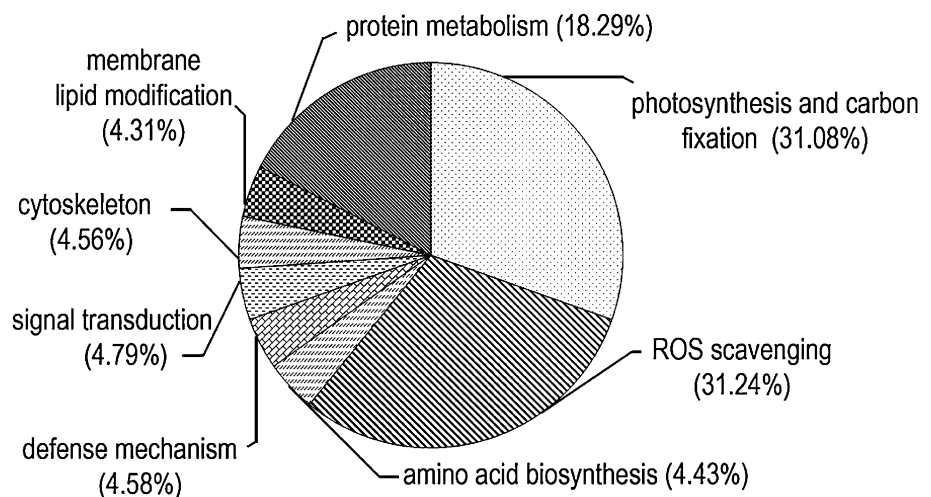
<sup>a</sup> Drought effect; (↑) indicates the upregulation of protein, (↓) indicates the downregulation of protein

<sup>b</sup> Number of polypeptide matched

<sup>c</sup> Sequence coverage percentage

<sup>d</sup> p value, indicates the significance of up- or downregulation of spots according to ANOVA ( $p < 0.05$ )

**Fig. 4** Distribution representation of the identified proteins based on their function in biological processes. Function of the protein was obtained via the MASCOT software from the NCBI database



sequester intracellular iron and thereby can limit the generation of hydroxyl radicals (Briat et al. 2010). Protein abundance of ferritin was also reported increasing in response to water stress in soybean root (Yamaguchi et al. 2010), and in leaves of chickpea (Bhushan et al. 2007). In the present study, the increased abundance of one ferritin subunit, ferritin-1A, under water stress indicates that regulation of free iron levels is an important component of adaptation to drought stress in the wheat leaves.

#### Photosynthesis and carbon fixation

The reduction in photosynthetic activity under drought stress is due to several coordinated events, such as stomatal closure and the reduced activity of photosynthetic enzymes. However, the molecular-level mechanism is not yet fully understood. In this study, three proteins, identified as RuBisCo activase (spot 8), iron–sulfur subunit of cytochrome b6-f complex (spots 2 and 11) and ATP synthase F0 subunit 1 (spot 19), exhibited altered abundance playing a role in photosynthesis and electron transport. Among of these proteins, RuBisCo activase and iron–sulfur subunit of

cytochrome b6-f complex were upregulated under drought conditions, while ATP synthase F0 subunit 1 was downregulated (Table 1). RuBisCo activase is a key regulator of photosynthesis (Parry et al. 2003). The main role of RuBisCo activase is to remove inhibitory sugar phosphates from the active sites of uncarbamyated and carbamyated RuBisCo (Portis 2003). Photosynthesis is modulated up and down by RuBisCo activase with changing environmental conditions. As stomatal conductance decreases with water deficit, internal CO<sub>2</sub> concentrations in the leaf are predicted to be reduced, thus causing a slower rate of photosynthesis. Upregulation of RuBisCo activase was observed in drought-stressed rice and cold-stressed *Thellungie* (Salekdeh et al. 2002; Gao et al. 2009). The increases in RuBisCo activase could improve photosynthetic efficiency by increasing the amount of RuBisCo that is activated for CO<sub>2</sub> fixation, thus compensating for the reduced stomatal conductance (Cramer et al. 2007).

Cytochrome b6-f complex iron–sulfur protein, which mediates electron transfer between PSII and PSI, contributes to the photoprotection and repair of PS II during and after abiotic stress (Schroda et al. 1999). Since

photosynthetic CO<sub>2</sub> assimilation rate can be viewed as being limited either by the capacity of RuBisCo to consume ribulose 1, 5-bisP (RuBP) or by the capacity of the chloroplast electron transport to generate ATP and NADPH for RuBP regeneration (Yamori et al. 2011), the regulation of the Cyt b6/f complex and the ATP synthase are thus key components determining the rate of NADPH and ATP production for CO<sub>2</sub> fixation. Thus, it is reasonable that iron–sulfur subunit of cytochrome b6-f complex was upregulated in treated samples, in order to counteract decreasing of the photosynthetic activity via RuBP regeneration. Upregulation of iron–sulfur subunit of cytochrome b6-f complex has also been observed in wheat following drought stress (Kamal et al. 2013). With regard to ATP synthase F0 subunit 1, the downregulation of it suggested that ATP production was decreased through photophosphorylation and thus affected the Calvin cycle in photosynthesis. The decrease of ATP synthase could be associated with transiently decreased photosynthesis rates. Indeed, it might be possible to explain the deep deterioration of the photosynthetic machinery. The reduced expression of ATP synthase has been observed in drought-stressed poplar (Yang et al. 2010).

The primary metabolisms, such as metabolisms of carbon, needs to be modulated to establish a new homeostasis under abiotic stress (Thomashow 2001). As expected, PEG-induced water stress changed expression level of three proteins related to carbohydrate metabolism, including 2-phosphoglycerate dehydratase or enolase (spot 5), fructose 1, 6-bisphosphate aldolase (spot 12) and transketolase (spot 20). The prior two proteins, involved in glycolysis, were downregulated after drought treatment for 48 h and the latter, involved in pentose phosphate pathways, was upregulated under the same conditions. It is well known that carbohydrate metabolism and energy consumption is reduced in drought-treated wheat seedlings because of inhibition of photosynthesis under water stress conditions (Kosová et al. 2011). Thus, wheat seedlings can adjust their metabolic status in a manner of reducing related enzyme activity and reserve energy to cope with long-term submergence. The upregulation of enolase is probably due to a response as a consequence of the need for increased glycolysis to compensate for the lower ATP yield. Similar changes in the abundance of for these three proteins also presented in wheat following drought stress (Caruso et al. 2009; Chaves et al. 2009).

#### Protein metabolism

Regulation of gene expression is achieved at several levels, i.e., transcriptional, post-transcriptional, translational, and post-translational. 40S ribosomal protein SA (spot 7) and 60S acidic ribosomal protein P1 (spot 15), heat shock

protein70 (HSP70, spot 3) and presequence protease1 (PreP, spot 1) were proteins involved in protein translation, processing and degradation. All of these proteins exhibited upregulation under water deficit. Previous reports revealed that the regulation of ribosomal proteins have robust mechanisms under different abiotic stresses. For instance, some ribosomal proteins (ribosomal protein L10, ribosomal protein S3a and ribosomal protein S12) were decreased in abundance in *Arabidopsis* under NaCl stress (Jiang et al. 2007) and other ribosomal proteins (acidic ribosomal protein P3a, 60S ribosomal protein L16 and ribosomal S3 proteins) were upregulated in chilling-stressed rice and drought-stressed poplar (Yan et al. 2006; Xiao et al. 2009). The differential regulation of distinct components of the translation machinery suggests that there is a complicated mechanism controlling protein synthesis in response to stress. The induction of ribosomal proteins might enhance the translation process or help proper ribosome functioning under stress conditions (Kim et al. 2004).

Abiotic stresses usually cause protein dysfunction. HSPs are responsible for protein folding, assembly, translocation and degradation, playing a pivotal role in protecting plants against various abiotic stresses and in the reestablishment of cellular homeostasis (Wang et al. 2004). Xiao et al. (2009) observed in a proteomics analysis on drought-stressed *Populus cathayana* leaves that HSP70 was significantly upregulated. Hajheidari et al. (2007) observed upregulations of several HSPs among wheat genotypes with different tolerances in response to drought stress. Our results also indicate that HSPs play crucial roles in wheat acclimation to drought stress.

Proteases play fundamental roles in plants, maintaining strict protein quality control and degrading specific sets of proteins in response to diverse environmental and developmental stimuli (García-Lorenzo 2007). PreP, an ATP-independent protease involved in the removal of free targeting peptides, is a novel protease that degrades organellar targeting peptides as well as other unstructured peptides in both mitochondria and chloroplasts (Kmiec and Glaser 2012). Indeed, the replacement of misfolded and damaged proteins with newly formed ones is exacerbated in case of abiotic stresses (Smalle and Vierstra 2004). In our study, the upregulation of PreP maybe reflects this mechanism. To our knowledge, it is the first time to report the response of this protein to drought stress.

#### Signal transduction network

Another plant defense mechanism to drought may be represented by the variation of sensing and signaling pathways. In the current study, translationally controlled tumor protein (TCTP, spot 16) was induced by PEG treatment. In general, TCTP seems to be a cytoplasmic calcium binding

protein. TCTP have been found in a wide range of different organisms including human, mouse, rabbit, chicken, earthworm, yeast, and plants (Ermolayev et al. 2003). It has recently been reported that the expression of TCTP was enhanced by several abiotic stress, such as Al stress, water stress (Ermolayev et al. 2003; Yang et al. 2011). Suggestion of a possible function of the wheat TCTP is difficult. It is possible that this protein is involved in the maintenance of Ca homeostasis in stressed plant cells, because many extracellular signals such as biotic and abiotic stress can elicit changes in cellular  $\text{Ca}^{2+}$  concentration in plants (Luan et al. 2002).

#### Amino acid metabolism

An effect that may occur during abiotic stress condition exposure is the participation of amino acids metabolism. It is well known that the concentration of proline and certain other amino acids will increase under abiotic stress. These amino acids and amines act as an osmoprotector and as an osmoregulator to reduce harm caused by stress. In this work, one amino acid biosynthesis-related enzyme, glutamine synthase (GS, spot 6), was induced by PEG treatment. GS functions as the major assimilatory enzyme for ammonia, combining ammonia with glutamate to yield glutamine with expense of ATP. Recent data suggest that glutamine is the principal amino acid involved in proline synthesis. Plastidic isoforms of this enzyme have been detected in leaf tissues of wheat where the protein was found to be upregulated by drought stress (Caruso et al. 2009). In higher plants, GS has been known to occur as a number of distinct isozymes, plastidic and cytosolic, that are located in different organs and subcellular compartments (Pereira et al. 1996). In this work, a plastidic glutamine synthetase 2 was identified.

#### Cytoskeleton

The cytoskeleton is rapidly remodeled by various endogenous and external stimuli such as hormones, low temperature, aluminium, and NaCl (Abdrakhamanova et al. 2003). Here, a cytoskeleton-related protein, actin (spot 18), was observed to be downregulated after PEG treatment. Indeed actin plays diverse roles in the cytoskeleton of eukaryotic cells, and affects cell shape, cell division, motility, contraction, adhesion, phagocytosis, protein sorting and signal transduction (Uribe and Jay 2009). By downregulation of this cytoskeleton-related protein under drought stress, wheat plants can not only save the cell wall polysaccharides as carbohydrate sources, but also restrict cell elongation, which will consume much energy, thus making the wheat seedlings better prepared to undergo long periods of submergence. The restriction of cell

elongation is also consistent with the growth suppression of wheat seedlings by drought stress. Similar change of actin at the protein level has been described in several plant species following drought stress (Plomion et al. 2006; Vincent et al. 2007).

#### Defense-related proteins

Under drought conditions plants may active several cell defense mechanisms to cope with the negative impact. In our study, TIR-NBS-LRR-TIR type disease resistance protein (spot 9), one of defense-related proteins, was found to be induced by PEG treatment. TIR-NBS-LRR class disease resistance proteins often are described as responsive proteins related to biotic stress (Noutoshi et al. 2005). To our best knowledge, it is the first time this protein responsible for drought stress has been observed. However, the identification and upregulation in cold stress have already been reported in proteomics analysis in *Thellungiella halophila* (Gao et al. 2009). Since a cross-talk exists among different abiotic stresses and between abiotic and biotic stresses (Chinnusamy et al. 2004), it is reasonable to find this protein involved in drought response. To our knowledge, it is the first time the response of this protein to drought stress has been reported.

#### Membrane lipid modification-related proteins

Drought often results in reduction of membrane liquidity and initiation of photoinhibition. Here, one membrane lipid modification-related protein, lipoxygenase (spot 4), was increased in abundance by drought stress. Lipoxygenase catalyze the dioxygenation of polyunsaturated fatty acids and produce hydroperoxy fatty acids, which are highly toxic compounds to cells. In addition, drought conditions lead to a distinct decrease in leaf water potential paralleled by a substantial decrease in the net photosynthetic rate probably derived from lipid peroxidation. The high degree of lipid peroxidation could produce lipid derivatives acting as secondary messengers capable to activate some drought-stress-associated genes by means of specific transcription factors, in this manner activating the response of plant to desiccation. The upregulation of specific lipoxygenase isoenzymes was observed by Ye et al. (2000) in *Arabidopsis* flower tissues under drought conditions.

#### Conclusion

In this work, physiological and proteomics analysis was performed with wheat plants subjected to PEG treatment. Symptoms of stress, such as growth inhibition and electrolyte leakage were measured. With the aid of 2D-PAGE

combined with MALDI-TOF/TOF, 23 differently expressed proteins were identified. PEG-induced drought stress caused abundance reduction of some proteins involved in respiration, photosynthesis and cytoskeleton in wheat cultivars, which could be associated with the growth inhibition. Drought stress caused increased accumulation of ROS scavenging enzymes, which are probably of particular importance in the adaptation of the wheat leaves to drought-stress conditions. Drought stress also changed other proteins involved in other metabolisms, such as the defense, membrane lipid modification, etc. All results suggested that change induced by drought was related to diverse biochemical aspects in the wheat. It is worth noting that the functions of some of these differentially expressed proteins are not clearly understood, which warrants further investigation for revealing the underlying molecular and metabolic pathways.

**Author contribution** Dr. Hongmei Zhang designed and supervised the research project and wrote the paper. Dr. Dapeng Zhang, Dr. Weining Zhu, Hui Lv and Zhengyang Yu, contributed in lab experiment, data collection and analysis. Dr. Linsheng Zhang helped in manuscript discussion and editing. All authors have contributed their efforts to this work.

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