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# Proteomic analysis of leaves of different wheat genotypes subjected to PEG 6000 stress and rewatering

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**Abstract**

Drought is an abiotic stress that strongly influences plant growth, development and productivity. To understand the drought tolerance mechanism at the protein level in wheat, a differential proteomics study was carried out on young spring wheat leaves of different genotypes in PEG-stressed and rewatered, using two-dimensional polyacrylamide gel electrophoresis (2-DE). A 2-DE pattern with high resolution and good reproducibility was obtained after staining with Coomassie brilliant blue G-250. Using PDQuest software, 600 protein spots were clearly identified from the treatment and control groups with isoelectric points ranging from 4.0 to 7.0. Thirty-eight differentially expressed protein spots were MALDI-TOF/TOF-MS fingerprinted using 2-DE gel and 35 spots were identified by search through the NCBInr database using Mascot software. Of 35 proteins, twenty-one proteins changed in abundance after PEG stress, with 15 proteins up-regulated, whereas 6 proteins down-regulated. Twenty four hour after rewatering, there were 5 proteins up-regulated and 9 proteins down-regulated compared to the well-watered control. Twenty-two differentially expressed proteins were detected in Qingchun 38 and 13 proteins in Abbondanza. They were involved in photosynthesis, protein biosynthesis, energy pathway, carbon metabolism, cell defense, oxidation reduction, transportation and signal transduction. Our proteomics results suggested that drought stress significantly affects wheat photosynthesis.

**Keywords:** Spring wheat; PEG6000 stress; rewatering; differential proteomics; MALDI-TOF-TOF-MS.

## Introduction

Currently, about 43% of the world's arable fields are in either arid or semi-arid regions. Like many other countries, China faces great challenges of water deficiency, which causes considerable decreases in crop production every year (Belder et al., 2005; Fulda et al., 2011). For example, the average rainfall of Qinghai province in Qinghai-Tibet Plateau, northwestern China, is only 300 mm in 2011 and about 66% of the crop fields in the area are seriously affected by drought (Qinghai Bureau of Statistics, 2012). Every year the province experiences severe drought from April to May, when the spring wheat is right in the tillering stage. Severe dry condition leads to a dramatic drop in wheat yield and quality. Therefore, study and understanding the drought tolerance mechanisms of wheat during the seedling stage are important in cultivating new varieties of drought-resistant wheat.

Understanding the drought tolerance mechanisms of wheat involves isolation and characterization of drought stress-related proteins and genes (Salekdeh et al., 2002). Many genes related to drought tolerance have been identified. However, findings from these genes are generally limited to the mRNA level and the mRNA characteristics of the genes cannot be completely accounted for the actual processes that occur in the drought-stressed wheat. Complete elucidation of these processes requires a study of the proteins expressed by the genes because proteins are more physiologically and biochemically responsive to stress and better correlate with plant characteristics (Pandey and Mann, 2000; Bazargani et al., 2011; Caruso et al., 2009). Therefore, a proteomic research concerning the structure and function of stress-induced proteins will provide a better understanding of the drought tolerance mechanism of plants (Peng et al., 2009). Proteomics studies on the drought resistance mechanisms of some crops, such as rice, have been carried out yet (Ali and Komastu, 2006; Salekdeh et al., 2002; Yan et al., 2005). However, research on the proteomics profiles of wheat is still limited (Bazargani et al., 2011; Caruso et al., 2009; Peng et al., 2009). Several studies reported that the drought resistance mechanism involved biochemical and genomic changes (Xu et al., 2009; Xu et al., 2008). To the best of our knowledge, no study has been reported on the drought-resistance mechanism of spring wheat at the protein level under PEG6000 stress and re-watering during the seedling stage. Elucidation of the proteomic changes in drought-stressed and rewatered spring wheat leaves will be of major significance in understanding the drought resistant mechanisms of wheat.

## Results

### *Establishment and analysis of 2DE maps for PEG-stressed and rewatered wheat leaves*

The wheat leaves of Qingchun 38 and Abbondanza were stressed by PEG 6000 for 72 h and then rewatered for 24 h. The total leaf proteins from each wheat variety were extracted with TCA and separated by IEF/SDS-PAGE. Proteins were stained with CBB G-250. An equal amount (900 μg) of total proteins

was loaded onto each gel strip. The representative gel profiles of the total proteins from the control and the treatment group (PEG6000 stressed and rewatered) are shown in Fig 1. The profiles showed high resolution and good repeatability (3 replicates). About 600 clear and reproducible protein spots were recognized on each gel profiles using the PDQuest software. The representative gels allowed the reproducible detection of more than 500 common spots, corresponding to about 90% of the total number of spots between the two samples (control and treatment groups).

***Mass spectrometry (MS) analysis and database search of differentially expressed proteins***

As shown in Fig 1 and Tables 1 and 2, proteins from the treatment and control groups were differentially expressed with variations in both quantity and quality. Proteins larger than two-fold difference in protein expression were considered differentially expressed, while those with lower than two-fold difference in expression were considered as a not differentially expressed due to systematic variation. In the master gel, 72 differentially expressed protein spots (as shown by the arrows in Fig 1) were obtained through automatic matching and manual adjustment. Of these spots, 16 spots were located the same with another corresponding variety or treatment (as shown by the dashed arrows in Fig 1). To improve the MS identification, 38 spots of the 56 differentially expressed protein spots were analyzed by MALDI-TOF-TOF-MS (Supplementary data). The relative abundance ratios of the 38 proteins are shown in Fig 2 (Supplementary Table S1). Sixteen spots were successfully identified by PMF (Table 1); 19 spots were identified through MS/MS analysis (Table 2) and 3 spots were not recognized.

***Protein changes in PEG-stressed and rewatered wheat leaves***

Compared with the control group, the protein expression profiles of the treatment group stressed by PEG for 72 h exhibited responsive changes: some proteins were up-regulated, down-regulated, or specifically induced (Supplementary Table S2). All the observed changes were considered the consequence of adaptation to the changes in the stress (as shown with arrows in Fig 1).

After 24 h of rewatering, the differences of protein expression between the control and the treatment groups of the two wheat varieties decreased relatively (Fig 1). Compared to the control group, the number of specifically expressed or up-regulated proteins in the treatment group was less than that in down-regulated proteins. The physiological implication of this observation requires further investigation.

Some differentially expressed proteins (shown with dashed arrows in Fig. 1A) from the same variety remain constant in both PEG stress and rewatering conditions. In addition, several differentially expressed proteins were also similar for different varieties (shown with dashed arrows in Fig 1).

***Differences of protein profiles between wheat varieties***

As shown in Fig 1, there is no significant difference between the 2-DE protein profiles for the two wheat varieties. Most abundant proteins and some less-abundant ones exhibited very similar patterns. Either PEG-stressed for 72 h or subsequently rewatered for 24 h, the difference of differentially expressed proteins between the control and treatment groups was more significant for Qingchun 38 than Abbondanza. This indicates that Abbondanza is less responsive to drought than Qingchun 38.

**Discussion**

Discrepancy between experimental and theoretical Mr/pI values was observed similar to previously reports (Caruso et al., 2009; Dani et al., 2005). Such discrepancy may be attributed to post-translational modifications, such as glycosylation and phosphorylation, which can change the molecular weight and/or protein charge. Another reason could be the fact that the theoretical Mr/PI comes and is calculated from other species rather than wheat proteins. Some proteins were found in more than one spot of the same gel, such as ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) B, transketolase and chloroplast–like proteins. This phenomenon is probably due to post-translational modification, a biological mechanism that plays a key role in signal transduction.

The 35 identified proteins (Tables 1 and 2) were divided into nine categories based on their metabolic functions in leaf development such as photosynthesis, protein synthesis, energy metabolism, carbon metabolism, cell defense, oxidation-reduction, transportation, signal transduction, and unclassified and unknown proteins. As shown in Fig 3, most of the identified proteins were involved in photosynthesis.

***Photosynthesis related proteins***

Water deficiency leads to stomatal closure in leaves; thereby, decreasing the carbon dioxide flow into leaves and inducing the increased hydrolysis of starches and accumulation of sugars as well as the decreased output of photosynthetic products. All of these changes result in decreased photosynthesis (Lawlor and Cornic, 2002). The Calvin cycle, which consists of carboxylation, reduction and renewal phases, is the primary pathway of photosynthesis in plants.

Protein spots 12 and 18 were identified as ribulose-1,5-bisphosphate carboxylase; Spots 2, 5, 6, 7, 8, 9, 21, 23, 32, 37 were identified as ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit; Spot 17 was identified as ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit; Spot 27 was identified as ribulose-1,5-bisphosphate carboxylase/oxygenase activase small isoform; Spots 11 and 26 were identified as GAPDH B and GAPDH, respectively; Spot 35 was identified as ructose-1,6-bisphosphatase; and spots 1 and 15 were identified as ransketolase and chloroplast-like proteins, respectively. All of these proteins are involved in the Calvin cycle.

Rubisco, a key enzyme in carbon dioxide fixation in photosynthesis, is composed of several catalytic large subunits and regulative small subunits (Spreitzer and Salvucci, 2002). GAPDH plays a key role in reducing glycerate 3-phosphate into glyceraldehyde-3-phosphate. The latter is not only a photosynthetic product but also the substrate of ribulose 5-phosphate. GAPDH B was previously identified by Yang et al. (2008) as a new protein responsible for light reaction, the activities of which can affect the efficiency of the Calvin cycle, the accumulation of photosynthesis products, and the products of crops (Pillai et al., 2002). Fructose-1,6 -bisphosphatase, which catalyzes fructose-1,6-diphosphate to fructose-6-phosphate, plays an important regulatory role in the Calvin cycle and the transportation of photosynthetic intermediates (Kiddle et al., 1999; Jaleh et al., 1993). In the Calvin cycle, transketolase catalyzes glycerate 3-phosphate and fructose-6-phosphate into xylose-5-phosphate and erythrose-4

**Table 1.** Differentially expressed proteins identified by peptide mass fingerprinting.

| Spot No.[a)] | Accession No. [b)] | Theoretical Mr(kD)/pI | Experimental Mr(kD)/pI | Sequence coverage [c)] | Score [d)] | Protein name | Species |
|---|---|---|---|---|---|---|---|
| 1 | gi\|357110873 | 80.1/5.93 | 95.0/5.80 | 17% | 230 | PREDICTED: transketolase, chloroplastic-like | *Brachypodium distachyon* |
| 2 | gi\|2500666 | 50.7/6.58 | 36.4/6.07 | 41% | 97 | Ribulose bisphosphate carboxylase large chain | *Galium lucidum* |
| 4 | gi\|326506328 | 19.8/10.17 | 17.2/5.10 | 29% | 85 | predicted protein | *Hordeum vulgare subsp. vulgare* |
| 11 | gi\|357114230 | 47.7/6.03 | 45.8/6.02 | 23% | 250 | PREDICTED: glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic-like | *Brachypodium distachyon* |
| 12 | gi\|552516 | 50.2/6.41 | 37.2/6.41 | 28% | 74 | Ribulose 1,5-bisphosphate carboxylase | *Cyperus alternifolius* |
| 13 | gi\|242037499 | 65.5/6.09 | 32.2/4.82 | 15% | 77 | Hypothetical protein SORBIDRAFT_01g002140 | *Sorghum bicolor* |
| 14 | gi\|340842127 | 34.3/6.00 | 23.4/5.70 | 25% | 153 | 26s proteasome non-ATPase regulatory subunit | *Triticum aestivum* |
| 18 | gi\|74179280 | 51.9/6.13 | 31.9/5.58 | 21% | 102 | Ribulose-1,5-bisphosphate carboxylase/oxygenase | *Thottea borneensis* |
| 19 | gi\|52548246 | 32.4/9.22 | 32.6/5.98 | 33% | 74 | Chloroplast inositol phosphatase-like protein | *Triticum aestivum* |
| 20 | gi\|326506340 | 22.2/5.79 | 18.6/4.84 | 29% | 104 | Predicted protein | *Hordeum vulgare subsp. vulgare* |
| 22 | gi\|90025017 | 68.8/5.23 | 64.9//5.833 | 43% | 252 | Vacuolar proton-ATPase subunit A | *Triticum aestivum* |
| 24 | gi\|20302473 | 40.5/6.92 | 39.5/5.84 | 45% | 119 | Ferredoxin-NADP(H) oxidoreductase | *Triticum aestivum* |
| 27 | gi\|313574196 | 47.3/7.59 | 47.5/6.41 | 32% | 118 | Ribulose-1,5-bisphosphate carboxylase/oxygenase activase small isoform | *Hordeum vulgare subsp. vulgare* |
| 28 | gi\|50897038 | 84.8/ 5.68 | 95.6/6.33 | 31% | 157 | Methionine synthase | *Hordeum vulgare subsp. vulgare* |
| 33 | gi\|326533372 | 74.0/5.45 | | 38% | 103 | Predicted protein | *Hordeum vulgare subsp. vulgare* |
| 34 | gi\|229610841 | 56.7/6.40 | | 55% | 179 | Small subunit of ADP-glucose pyrophosphorylase | *Hordeum vulgare subsp. vulgare* |

(a) Spot No.: the numbers of proteins on gels.
(b) Accession No.: the number of the predicted protein in NCBInr.
(c) Coverage: percentage of predicated protein sequence covered by matched sequences.
(d) Score: statistical probability of true positive identification of the predicted protein calculated by MASCOT with 0.3 peptide tolerance and one allowed missed cleavage (score ≧ 73 against NCBInr).

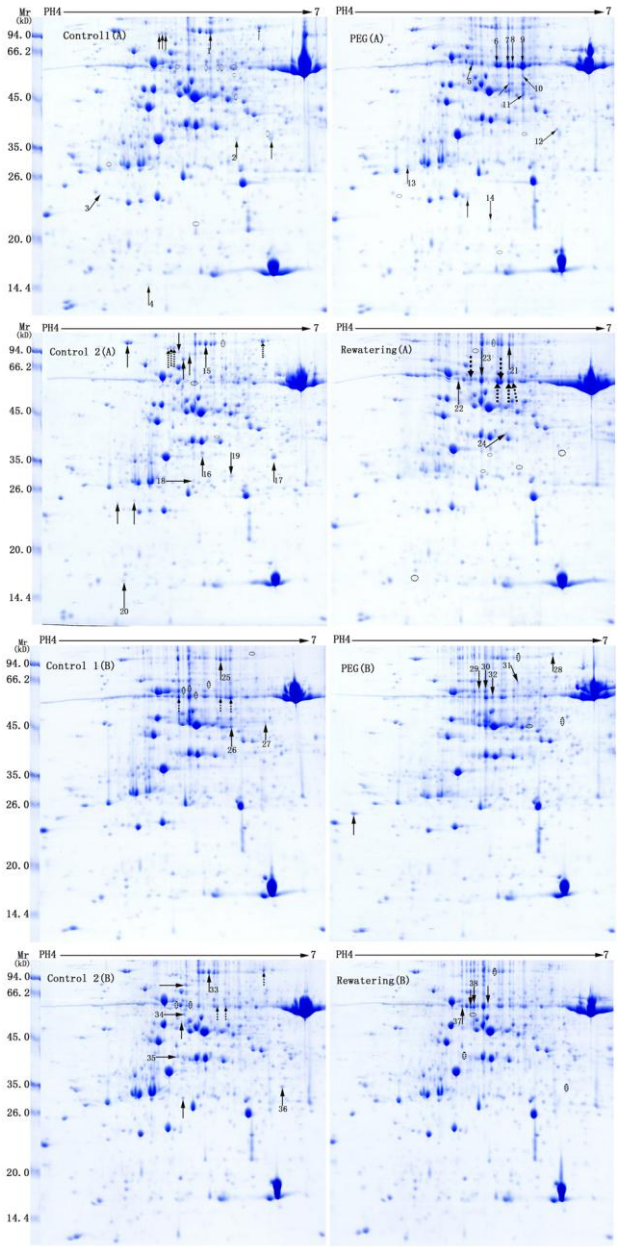


**Fig 1.** The 2-DE image analysis of proteins extracted from wheat leaves of Qingchun 38 and Abbondanza under PEG stress and rewatering. Control 1(A), well-watered from Qingchun 38; PEG (A), stressed by PEG 6000 from Qingchun 38; Control 2(A), well-watered from Qingchun 38; Rewatering (A), rewatered for 24 h after PEG 6000 stress for 72 h from Qingchun 38; Control 1(B), well-watered from Abbondanza; PEG (B), stressed by PEG 6000 from Abbondanza; Control 2(B), well-watered from Abbondanza; Rewatering (B), rewatered for 24 h after PEG 6000 stress for 72 h from Abbondanza.

-phosphate, and glycerate 3-phosphate and sedoheptulose 7-phosphate into xylose-5-phosphate and erythrose-4-phosphate, respectively.

After PEG stress for 72 h, ribulose-1,5-bisphosphate carboxylase/oxygenase exhibited up-regulated expression, while GAPDH was specifically expressed. These changes may enable the wheat to survive during drought stress by increasing carbon dioxide utilization and assimilation efficiency in the Calvin cycle. Wan et al. (2008) observed the same phenomenon in ribulose -1,5 -bisphosphate carboxylase/oxygenase in hydrogen peroxide-stressed young rice leaves. Wang et al. (2008) reported the up-regulated expression of GAPDH in wheat roots stressed with NaCl. However, after stress and rewatering, transketolase and fructose-1,6-bisphosphatase B exhibited down-regulated expressions. These down-regulations significantly decrease the conversion rate of glycerate 3-phosphate and may lead to the specific expression of GAPDH B. A decrease in transketolase expression could reduce the efficiency of both the Calvin cycle and the entire photosynthetic pathway.

***Carbon metabolism related protein***

Spot 34 was identified to be a small subunit of ADP-glucose pyrophosphorylase (AGPase). AGPase, a rate-limiting enzyme in biosynthesis, consists of two large subunits (l-AGPase) and two small subunits (s-AGPase). The small subunits have catalytic function and play a key role in starch synthesis (Kim et al., 2002). Under PEG stress and rewatering, AGPase expression decreased, indicating that the effect of drought stress could linger in wheat even after rewatering and that normal physiological functions have yet to be restored.

***Cell defense related protein***

Spot 14 was identified to be the 26S proteasome non-ATPase regulatory subunit. 26S proteasome is composed of a 20S core subunit and two 19S regulatory subunits (Hanna and Finley, 2007). The 19S regulatory subunit, which consists of 17 or 18 other subunits, is composed of a base and a lid. The base is made up of nine subunits that can be divided into two classes: ATPase-active and non-ATPase-active (Fu et al., 2001). In the present study, spot 14 was specifically expressed under drought stress, suggesting that wheat survives drought by increasing protein hydrolysis and recycling. Zang and Komatsu, (2007) reported a similar result in rice seedlings stressed by osmosis.

***Oxidation-reduction related protein***

Spot 24 was identified as ferredoxin-NADP (H) oxidoreductase, a key enzyme in energy transfer pathway that catalyzes electron transfer between NADPH and ferredoxin. During rewatering, the enzyme was specifically expressed, which is believed to be a possible compensatory mechanism for adapting to restored normal living conditions.

***Transportation related protein***

Spot 22 was identified to be vacuolar proton-ATPase subunit A. Vacuolar proton-ATPase subunit A consumes ATP to transfer protons into vacuole. Through that, the proton transmembrane gradient is produced, which provides the power to transport various ions and metabolites (Ratajczak, 2000). Previous studies has shown that stress conditions, such as drought and salt stresses, induce V-ATPase to exhibit high flexibility and plasticity, which are essential for plant survival (Kluge et al., 2003). In the present study, spot 22 exhibited up-regulated expression during rewatering, which may increase the efficiency of water utilization.

***Signal transduction related protein***

Various extracellular stimuli are considered to be primary messengers in transmembrane signal transduction pathway,

**Table 2**. Differentially expressed proteins identified by MS/MS.

| SpotNo.[a] | Accession No.[b] | Theoretical Mr(kD)/pI | Experimental Mr(kD)/pI | Sequence Coverage[c] | Score[d] | Annotation | Species | Peptide fragment | Start[e] | End[f] |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 | gi\|1141727 | 48.8.4/6.42 | 64.2/5.45 | 7% | 106 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | *Prostanthera rotundifolia* | K.TFQGPPHGIQVER.D | 113 | 125 |
| 6 | gi\|14585745 | 49.1/6.80 | 63.4/5.75 | 2% | 101 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | *Veronica arguta* | K.TFQGPPHGIKVER.X | 138 | 150 |
| 7 | gi\|18652329 | 48.7/6.72 | 62.6/5.88 | 2% | 109 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | *Adenophorus abietinus* | K.TFQGPPHGIQVER.D | 134 | 146 |
| 8 | gi\|14585745 | 49.1/6.80 | 62.4/5.90 | 2% | 102 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | *Veronica arguta* | K.TFQGPPHGIKVER.X | 138 | 150 |
| 9 | gi\|13548898 | 49.3/6.30 | 62.1/6.02 | 5% | 100 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | *Sundacarpus amarus* | K.TFQGPPHGIQVER.D | 134 | 146 |
| 10 | gi\|357149925 | 50.6/5.88 | 51.5/6.02 | 5% | 95 | PREDICTED: elongation factor Tu, chloroplastic-like | *Brachypodium distachyon* | K.KYDEIDAAPEER.A | 108 | 119 |
| 15 | gi\|357110873 | 80.1/5.93 | 98.3/5.72 | 3% | 196 | PREDICTED: transketolase, chloroplastic-like | *Brachypodium distachyon* | K.SIITGELPAGWADALPQYTTESPADATR.N | 408 | 435 |
| 17 | gi\|207080698 | 78.9/9.42 | 36.1/6.42 | 18% | 100 | Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit | *Secale cereale* | R.EHNASPGYYDGR.Y | 46 | 57 |
| 21 | gi\|131899 | 52.2/6.13 | 98.0/5.87 | 2% | 67 | Ribulose bisphosphate carboxylase large chain | *Adoxa moschatellina* | K.TFQGPPHGIQVER.D | 138 | 150 |
| 23 | gi\|13548898 | 49.3/6.30 | 64.8/5.33 | 5% | 102 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit | *Sundacarpus amarus* | K.TFQGPPHGIQVER.D | 134 | 146 |
| 25 | gi\|326533372 | 74.0/5.45 | 95.0/5.93 | 2% | 30 | Predicted protein | *Hordeum vulgare subsp. vulgare* | K.FAQYEQKYPEDAATLK.S | 330 | 345 |
| 26 | gi\|18076106 | 34.2/7.15 | 45.6/6.05 | 5% | 62 | Glyceraldehyde-3-phosphate dehydrogenase | *Sphagnum cuspidatum* | R.KDSPLEIVVINDTGGLK.Q | 22 | 38 |
| 29 | gi\|14017579 | 53.9/5.06 | 68.2/5.56 | 13% | 176 | ATP synthase CF1 beta subunit | *Triticum aestivum* | R.IFNVLGEPVDNLGPVDSSATFPIHR.S | 110 | 134 |
| 30 | gi\|110915710 | 53.2/5.17 | 68.4/5.63 | 17% | 128 | ATP synthase beta subunit | *Vulpia microstachys* | R.IFNVLGEPVDNLGPVDSSATFPIHR.S | 102 | 126 |
| 31 | gi\|195627844 | 63.3/6.18 | 73.1/5.98 | 4% | 88 | Ketol-acid reductoisomerase | *Zea mays* | K.VSLAGHEEYIVR.G | 81 | 92 |
| 32 | gi\|343013 | 52.1/6.34 | 63.6/5.71 | 2% | 109 | Ribulose-1,5-bisphosphate carboxylase | *Pandanus tectorius* | K.TFKGPPHGIQVER.D | 136 | 148 |
| 35 | gi\|300681469 | 37.9/5.38 | 39.1/5.43 | 3% | 79 | Fructose-1,6-bisphosphatase, cytosolic,putative,expressed | *Triticum aestivum* | R.SLDLIPTDIHER.S | 305 | 316 |
| 36 | gi\|7384808 | 34.5/5.69 | 34.3/6.56 | 3% | 74 | Cysteine synthasel | *Allium tuberosum* | K.LIVVVFPSFGER.Y | 293 | 304 |
| 37 | gi\|33317784 | 49.8/6.75 | 64.2/5.41 | 5% | 105 | Ribulose-1,5-bisphosphate carboxylase large subunit | *Podocarpus chinensis* | K.TFQGPPHGIQVER.D | 136 | 148 |

(a) Spot No.: the numbers of proteins on gels. (b) Accession No.: the number of the predicted protein in NCBInr (c) Sequence coverage: by the assigned tryptic peptides in percentage. (d) Score: statistical probability of true positive identification of the predicted protein calculated by MASCOT with ±100 ppm peptide tolerance, ±0.6 Da MS/MS tolerance and one allowed missed cleavage (score≧47 against NCBInr). e) Start: the start position of the identified peptide fragment in the protein sequence. (f) End: the end position of the identified peptide fragment in the protein sequence.

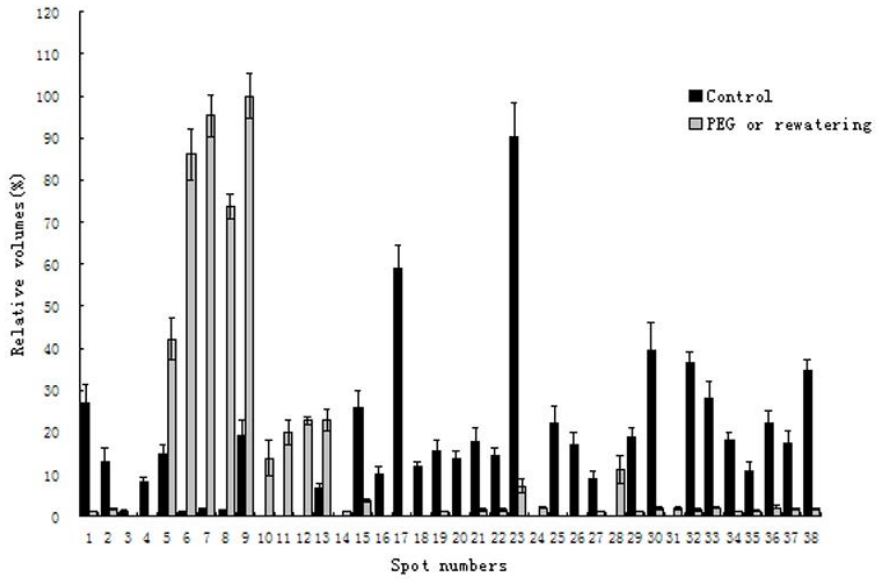


**Fig 2.** Relative abundance ratio of both up- and down-regulated proteins. The X axis denotes the spot number and the Y axis denotes the relative levels of protein expression, such as relative volume values (%V). The bars in the graphs represent the average volume ± SD. The black bar represents the controls and the gray bar represents the treated samples.

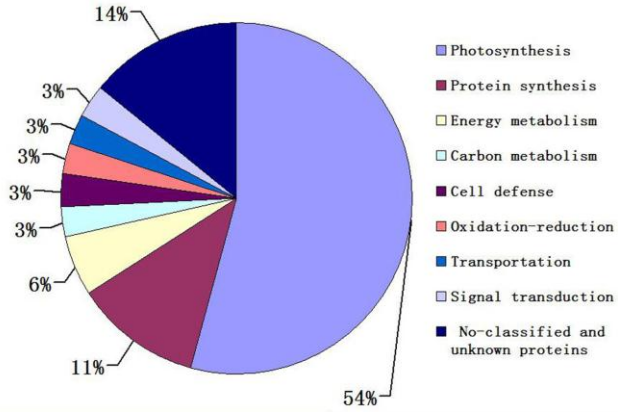


**Fig 3.** Functional categorization and distribution of 35 identified proteins. Unknown proteins include those whose functions have not been described but may be deduced based on sequence homology analysis as listed in Tables 1 and 2.

which stimulate various intracellular cytokines (considered as secondary messengers). Spot 19 was identified to be chloroplast inositol phosphatase-like protein, a secondary messenger in the phosphoinositide cycle. During rewatering, the enzyme exhibited down-regulated expression, which may result from the partial closure of the phosphoinositide signal pathway by drought stress.

***Protein synthesis related proteins***

Spots 10, 28 and 31 were identified to be elongation factor Tu, methionine synthase and ketol acid reductoisomerase, respectively. Elongation factor Tu is an aminoacyl-transfer-tRNA functional protein required during protein synthesis for peptide chain elongation. Methionine synthase is a key enzyme in methionine synthesis. Ketol acid reductoisomerase is a key enzyme in branched-chain amino acid synthesis. These enzymes were all specifically expressed in PEG–stressed leaves, especially in the drought-resistant variety. The changes observed may improve drought adaptation by directly increasing the synthesis of soluble proteins. Spot 36 was identified to be L-cysteine synthase, an isomer of cysteine. Cysteine synthase, together with serine acetyltransferase, participates in cysteine biosynthesis as the rate-limiting enzyme, whose catalytic reaction is the last step in sulfur absorption as well as the start in synthesis of other sulfur metabolites such as methionine, GSH and sulfur secondary metabolites (Harada et al., 2001). When Abbondanza was rewatered after 24 h, L-cysteine synthase exhibited down-regulated expression. This finding indicates that a large amount of sulfur metabolites accumulate when plants are rewatered after PEG stress, leading to a decrease in sulfur absorption and cysteine requirement. Eventually these decreases result in the down-regulated expression of L-cysteine synthase.

***Energy metabolism related proteins***

ATP synthase is composed of CF1 and CF0. CF1, an enzyme complex, is made up of five subunits. Of those, the β-subunit, composed of a catalytic and ADP-binding unit, plays an

important role in energy metabolism by converting ADP into ATP in the presence of a transmembrane proton gradient. Previous research has reported that the expression of ATP synthase decreases under drought stress (Tezara et al., 1999). In the present study, under PEG stress conditions, the expression of the β-subunit of ATP synthase (spots 29 and 30) was significantly reduced, inevitably leading to a decrease in ATP production and further affecting the Calvin cycle. These changes may account for the observed decrease in photosynthesis rate.

***Unclassified and unknown proteins***

Spot 13 was identified to be the hypothetical protein SORBIDRAFT_01g002140 and spots 4, 20, 25, and 33 are also identified to be hypothetical proteins. These proteins may be involved in drought stress but what functions they actually have require further investigation. Spots 3, 16 and 38 were not meaningfully identified partly due to inherent imperfections in the recently available database and the identification methods employed. To obtain better insights into the drought tolerance mechanism of wheat, the functions of these proteins must be investigated upon the improvement of the database and pertinent identification methods.

**Materials and methods**

***Plant materials instruments and reagents***

Two major spring wheat cultivars are available in Qinghai: Qingchun 38, which is sensitive to drought, and Abbondanza, an exotic variety resistant to drought.

The isoelectric focusing electrophoresis system PROTEAN IEF cell (Bio-Rad, USA), the vertical electrophoresis system PROTEAN II xi Cell (Bio-Rad, USA) and the gel image processing software PDQuest 8.o.1 (Bio-Rad, USA) were used in this study. The following materials were also used: scanning densitometer UMAX PowerLook 2100XL (Taiwan Power Company), BRUKER Ultraflex TOF/TOF (Germany), linear IPG strips, amphielectrolytes (Bio-Rad), phenylmethylsulfonyl fluoride (PMSF), 2-mercaptoethanol, CHAPS, acrylamide, bis-acrylamide, TEMED, ammonium persulfate, iodoacetamide and urea (Sigma). All other materials used were analytically pure and were obtained from China.

***Seedling culture and PEG stress***

Wheat seeds of the same plumpness were sterilized with 70% alcohol for 30 sec, rinsed several times with distilled water, and then placed in Petri dishes for culturing. Seedling culture was carried out in a plant growth chamber at 25 °C in a 12-h light-dark cycle and 60%~70% relative humidity. After sprouting one and a half leaves, seedlings that grew best and with similar growth rate were transplanted into conical flasks (5 plants per flask) and cultivated in Hoagland nutrient solution (Supplementary Table S3). Plastic film was used to fix the plants and prevent water from vaporizing. Silver paper was used to wrap the flasks to provide the roots darkness and allow them to grow. When two and a half leaves had emerged on the seedlings, half of the seedlings were continuously cultivated as the control group in Hoagland nutrient solution. The rest of the seedlings were stressed as the treatment group in PEG 6000 (–1.0 MPa) solution for 72 h and then rewatered for 24 h. A factorial design with three replicates was used.

***Leaf protein extraction and quantification***

Leaves were powdered in liquid nitrogen and the total protein was extracted with TCA/acetone following the methods of Kamo et al. (1995) and Wang et al. (2008) with minor modifications. The extraction solution was mixed with a –20°C pre-cooled solution of 10% TCA, 0.07% β-ME and 1 mM PMSF to precipitate the proteins. The mixture was stored at –20 °C overnight and centrifuged at 25 000×g for 25 min at 4 °C. The precipitate was rinsed with a pre-cooled acetone solution containing 0.07% β-ME and 1 mM PMSF, stored at –20 °C for 1 h, and then centrifuged (Eppendorf, Germany) at 25 000×g for 30 min. Rinse and centrifugation were performed three to four times until a bright white precipitate was obtained. This precipitate was lyophilized to a powder. The powder was dissolved in lysis solution containing 9 M urea, 4% (w/v) CHAPS, 65 mM DTT, 0.5% (v/v) IPG buffer (pH 4–7, Bio-Rad) and 0.001% (w/v) bromophenol blue, incubated at 32 °C for 30 min, and then cooled with liquid nitrogen (repeated twice). The lysis solution was centrifuged at 25 000×g for 20 min at 20 °C and the supernatant was collected for subsequent experiments. Protein contents were quantified according to the methods of Yao et al. (2006) using BSA as a standard.

***Two-dimensional gel electrophoresis (2D-PAGE)***

2D-PAGE was performed following the method of GÖrg (1999) and the instructions given in the manual for the IPGphor isoelectric focusing electrophoresis system. About 350 μL of the supernatant containing 900 μg of protein was loaded onto a commercially available precast IPG strip with a 17 cm linear pH 4–7 gradient and actively rehydrated at 50 V for 12 h at 20°C. Then, focusing was performed on the IPGphor apparatus (PROTEAN IEF Cell) under the following conditions: 250 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 4000 V for 1 h, 8000 V for 4h and 8000 V to achieve 80,000 V-h. Prior to SDS-PAGE, the strips were equilibrated for 15 min in 10 mL of reducing equilibration buffer (6 M urea, 2% (w/v) SDS, 0.375 M Tris-HCl at pH 8.8, 20% (v/v) glycerol, 2% (w/v) DTT) and then for another 15 min in alkylating equilibration buffer containing 2.5% (w/v) iodoacetamide instead of 2% DTT. The strips were placed on the top of vertical 11% SDS-polyacrylamide self-cast gels. Electrophoresis was carried out at 15 °C and 10 mA $gel^{-1}$ for 1 h and then at 20 mA $gel^{-1}$ until the dye front reached the bottom of the gel using the PROTEAN II xi Cell system. At least three replicates were performed for each sample.

The Coomassie blue staining was applied to the gel dyes as follows: watered with ultra-pure water twice for 10 min each; fixated in a fixative solution (40% methanol, 10% acetic acid, 50% ultra-pure water) for 2.5 h; rinsed quickly twice with ultra-pure water; immersed in staining solution (100 mg $L^{-1}$ $(NH_4)_2SO_4$, 1.2 g $L^{-1}$ Coomassie blue G-250, 116 mL $L^{-1}$ 85% $H_3PO_4$, 200 mL $L^{-1}$ methanol) for 12.5 h and then in destaining solution for 1 h; washed with ultra-pure water for 4 h with water changed every 30 min until the spots became clear.

***2-DE image analysis***

The 2-DE gels were scanned using a UMAX PowerLook 2100XL scanner (Taiwan) at a resolution of 600 dpi. Using the analytical software PDQuest 2DE 8.0.1 (Bio-Rad), the size and intensity of the protein spots were determined, the numbers were detected, and the molecular weight and isoelectric point were calculated.

Spots with two-fold or greater differences in protein expression were further analyzed using MALDI-TOF-TOF (Bruker).

***Analysis and identification of differentially expressed protein spots***

Protein spots showing significant changes in abundance during the treatments were selected and excised manually for protein identification. In-gel digestion of protein spots was performed according to the method described by Peng et al. (2009) and the peptide MS fingerprint of MALDI-TOF-TOF was analyzed using Ultraflex TOF/TOF (Wan and Liu, 2008). All protein spectrum were searched against NCBInr database using the online program Mascot (http://www.matrixscience.com). The search parameters were set as follows: ±100 ppm mass tolerance for peptides and ±0.6 Da mass tolerance for fragments; typsin as enzyme with one allowed miscleavage, carbamidomethyl (C) as a fixed modification, Gln->pyro-Glu (N-term Q) and Oxidation (M) as variable modifications. Two of the strongest peaks from the TOF spectra of each sample were chosen for MS/MS analysis.

**Conclusions**

Plant proteomes have been found to be influenced by environmental stresses. In this work, a proteomic approach was used for a large-scale, quantitative, and reproducible study on the effects of an abiotic stress. We described the results of a comparative proteomic analysis between Qingchun 38 (a drought-sensitive wheat variety) and Abbondanza (a drought-tolerant wheat variety) during the seedling stage under PEG 6000 stress and rewatering. A total of 72 differentially expressed protein spots were obtained, of which 35 were identified by MALDI-TOF and MALDI-TOF/TOF mass spectrometry. Those identified proteins were found to be involved in photosynthesis, protein biosynthesis, energy pathway, carbon metabolism, cell defense, oxidation–reduction, transportation and signal transduction. Drought stress was further shown to be strongly related to photosynthesis. While the wheat genome is very large and the physiology of drought adaptation is very complex, the functions of some drought stress-related wheat proteins remain unknown. Broadening research with advanced research techniques can help to enhance our knowledge of drought stress-related wheat proteins, improve our understanding of the molecular basis of drought responses and provide guidance for marker assisted gene selection. The findings of this work could help develop wheat varieties with improved drought tolerance or multi-tolerance.

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**References**

Ali GM, Komatsu S (2006) Proteomic analysis of rice leaf sheath during drought stress. J Proteome Res 5:396–403

Bazargani MM, Sarhadi E, Bushehri AS, Matros A, Mock H, Naghavi M, Hajihoseini V, Mardi M, Hajirezaei M, Moradi F, Ehdaie B, Salekdeh GH (2011) A proteomics view on the role of drought-induced senescence and oxidative stress defense in enhanced stem reserves remobilization in wheat. J Proteomics 74:1959–1973

Belder P, Spiertz J, Bouman B, Lu G, Tuong T (2005) Nitrogen economy and water productivity of lowland rice under water-saving irrigation. Field Crop Res 93:169-185

Caruso G, Cavaliere C, Foglia P, Gubbiotti R, Samperi R, Laganà A (2009) Analysis of drought responsive proteins in wheat (*Triticum durum*) by 2D-PAGE and MALDI-TOF mass spectrometry. Plant Sci 177:570–576

Daie J (1993) Cytosolic fructose-1, 6-bisphosphatase: a key enzyme in the sucrose biosynthetic pathway. Photosynth Res 38:5-14

Dani V, Simon WJ, Duranti M, Croy RR (2005) Changes in the tobacco leaf apoplast proteome in response to salt stress. Proteomics 5:737–745

Fu H, Reis N, Lee Y, Glickman MH, Vierstra RD (2001) Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J. 20:7096–7107

Fulda S, Mikkat S, Stegmann H, Horn R (2011) Physiology and proteomics of drought stress acclimation in sunflower (*Helianthus annuus* L.). Plant Biol. 13:632–642

Görg A, Obermaier C, Boguth G, Weiss W (1999) Recent developments in two dimensional gel electrophoresis with immobilized pH gradients: Wide pH gradients up to pH 12, longer separation distances and simplified procedures. Electrophoresis 20:712–717

Hanna J, Finley D (2007) A proteasome for all occasions. FEBS Lett 581:2854–2861

Harada E, Choi Y-E, Tsuchisaka A, Obata H, Sano H (2001) Transgenic tobacco plants expressing a rice cysteine synthase gene are tolerant to toxic levels of cadmium. J Plant Physiol 158:655–661

Kamo M, Kawakami T, Miyatake N, Tsugita A (1995) Separation and characterization of Arabidopsis thaliana proteins by two- dimensional gel electrophoresis. Electrophoresis 16: 423-430

Kiddle G, Bennett R, Hick A, Wallsgrove R (1999) C-S lyase activities in leaves of crucifers and non-crucifers, and the characterization of three classes of C-S lyase activities from oilseed rape (*Brassica napus* L.). Plant Cell Environ 22:433–445

Kim SH, Mizuno K, Fujimura T (2002) Regulated expression of ADPglucose pyrophosphorylase and chalcone synthase during root development in sweet potato. Plant Growth Regul 38:173–179

Kluge C, Lamkemeyer P, Tavakoli N, Golldack D, Kandlbinder A, Dietz K-J (2003) cDNA cloning of 12 subunits of the V-type ATPase from *Mesembryanthemum crystallinum* and their expression under stress. Mol Membr Biol 20:171–183

Lawlor D, Cornic G (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ 25: 275-94

Pandey A, Mann M (2000) Proteomics to study genes and genomes. Nature 405:837–846

Peng Z, Wang M, Li F, Lv H, Li C, Xia G (2009) A proteomic study of the response to salinity and drought stress in an introgression strain of bread wheat. Mol Cell Proteomics 8:2676–2686

Pillai MA, Lihuang Z, Akiyama T (2002) Molecular cloning, characterization, expression and chromosomal location of OsGAPDH, a submergence responsive gene in rice (Oryza sativa L.). Theor Appl Genet 105:34–42

Qinghai Bureau of Statistics (2012) Qinghai statistical yearbook 2012. China statictics press, Beijing, China

Ratajczak R (2000) Structure, function and regulation of the plant vacuolar $H^+$-translocating ATPase. BBA-Biomembranes 1465:17–36

Salekdeh G, Siopongco J, Wade LJ, Ghareyazie B, Bennett J (2002) Proteomic analysis of rice leaves during drought stress and recovery. Proteomics 2:1131–1145

Spreitzer RJ, Salvucci ME (2002) Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. Annu Rev Plant Biol 53:449–475

Tezara W, Mitchell V, Driscoll S, Lawlor D (1999) Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature 401:914–917

Wan XY, Liu JY (2008) Comparative proteomics analysis reveals an intimate protein network provoked by hydrogen peroxide stress in rice seedling leaves. Mol Cell Proteomics 7:1469–1488

Wang MC, Peng ZY, Li CL, Li F, Liu C, Xia GM (2008) Proteomic analysis on a high salt tolerance introgression strain of *Triticum aestivum/Thinopyrum ponticum*. Proteomics 8:1470–1489

Xu ZS, Liu L, Ni ZY, Liu P, Chen M, Li LC, Chen YF, Ma YZ (2009) W55a encodes a novel protein kinase that is involved in multiple stress responses. J Integr Plant Biol 51:58–66

Xue G-P, Mcintyre CL, Glassop D, Shorter R (2008) Use of expression analysis to dissect alterations in carbohydrate metabolism in wheat leaves during drought stress. Plant Mol Biol 67:197–214

Yan S, Tang Z, Su W, Sun W (2005) Proteomic analysis of salt stress-responsive proteins in rice root. Proteomics 5:235–244

Yang YJ (2008) Proteome comparative analysis of Cryptochromes of *Arabidopsis*. Hunan University, Changsha, 2008

Yao Y, Yang YW, Liu JY (2006) An efficient protein preparation for proteomic analysis of developing cotton fibers by 2-DE. Electrophoresis 27:4559–4569

Zang X, Komatsu S (2007) A proteomics approach for identifying osmotic-stress-related proteins in rice. Phytochemisty 68:426–437