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Comparative Proteomic Analysis of Wheat Developing Grains between Chinese Spring and 1S^l/1B Substitution Line

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A comparative proteomic analysis of grain proteins during five grain developmental stages of wheat cultivar Chinese Spring (CS) and its 1S^l/1B substitution line CS-1S^l(1B) was carried out in the current study. A total of 78 differentially expressed protein (DEP) spots with at least 2-fold expression difference were detected by two-dimensional electrophoresis (2-DE). Among these, 73 protein spots representing 55 differentially expressed proteins (DEPs) were successfully identified by matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS). Differential protein spots between the two genotypes were analyzed by cluster software, which revealed significant proteome differences. There were 39 common spots (including 33 DEPs) that showed significant difference between the two lines across five grain developmental stages, of which 14 DEP spots (including 11 DEPs) were mainly involved in carbohydrate metabolism that were encoded by the genes on 1B chromosome while 25 DEP spots (including 12 DEPs) were mainly related to stress response and gluten quality that were encoded by 1S^l chromosome. These results indicated that the S^l genome harbors more stress and quality related genes that are potential valuable for improving wheat stress resistance and product quality.

Keywords: wheat, differentially expressed proteins, 1S^l/1B substitution, comparative proteome, expression patterns

Introduction

Wheat is the most important grain crops in the world and its yield and quality have been the main research objectives in the past several decades. Wheat grain protein compositions have been extensively studied, including endosperm proteins at grain developmental stage (Skylas et al. 2000), key enzymatic functional proteins (Badea et al. 2008), etc. The proteins in wheat grains can be divided into two major categories according to their

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functions: storage proteins including gliadins and glutenins, and metabolism proteins consisting of water-soluble albumins and salt-soluble globulins (Bietz and Wall 1972). Gliadins and glutenins account for about 85% of the total grain's proteins (Salt et al. 2005), while albumins and globulins only account for about 15% (Vensel et al. 2007). Gliadin proteins were mainly related to dough extensibility. Glutenins consist of high and low molecular weight glutenin subunits (HMW-GS, LMW-GS) (Payne et al. 1988) and are closely linked to dough elasticity, stickiness, and strength. Albumins and globulins possess multiple significant functions during wheat growth and development.

Bread wheat is an allohexaploid species with three related diploid genomes denoted by A, B and D and is composed of seven pairs of chromosomes in each genome (Marcussen et al. 2014). The wheat genome has large amount of repetitive sequences (>80%) and its size is up to 17 Gb, which is five times larger than the human genome (Paux et al. 2008). Although wheat genome project is not completed yet, considerable wheat proteome as well as phosphoproteome studies have been carried out (Gao et al. 2009; Ge et al. 2011; Guo et al. 2012; Zhang et al. 2014). In particular, some quality-related albumins and globulins were identified (Gao et al. 2009; Dong et al. 2012). The recent release of the whole-genome shotgun sequencing data for bread wheat 3B genome, common wheat and its progenitor A^u and D^t genomes (Brenchley et al. 2012; Jia et al. 2013; Ling et al. 2013; IWGSC, 2014) will greatly accelerate wheat proteome studies due to the enhanced protein peptides identification depending on the homology-based matching.

It is widely accepted that the S genome is the progenitor of the B genome in bread wheat. The section *Sitopsis* of *Aegilops* genus includes *Ae. speltoides* (S), *Ae. longissima* (S^l), *Ae. bicornis* (S^b), *Ae. searsii* (S^s) and *Ae. sharonensis* (S^{sh}), which are closely related based on cytogenetic and molecular genetic investigations (Sasanuma et al. 2004). *Ae. longissima* was found to have extraordinary features such as eyespot and pre-harvest sprouting resistance (Sheng et al. 2012). The recent report has shown that the $1S^l/1B$ substitution in Chinese Spring leads to a significant improvement for dough and bread-making quality due to the introgression of two novel HMW-GS $1S^l$x2.3* and $1S^l$y16* (Wang et al. 2013). This indicated that the $1S^l$ genome contains desirable genes for wheat cultivar improvement.

In this study, we conducted a comparative proteomic analysis of wheat developing grains using Chinese Spring (CS-1B) and its substitution line CS-$1S^l$(1B). Numerous differentially expressed proteins (DEPs) associated with the S^l and B genomes were identified, which were involved in various metabolisms and play important roles in grain development. Our results provide new insights into the expression and functions of grain proteins from different wheat genomes.

Materials and Methods

Plant material

Common wheat (*Triticum aestivum* L.) Chinese Spring (CS) and its substitution lines CS-$1S^l$(1B) developed in the Institute for Plant Breeding, Technical University of Munich

(Wang et al. 2013) were used in this study. The same grain sizes of the two lines were selected and treated for 30 minutes with 20% sodium hypochlorite, then washed 4 times with sterile water. After the seeds germinate in petridish, seedlings were selected and planted in greenhouse of the Chinese Academy of Agricultural Science (CAAS). Both lines were planted in three biological replicates and each replicate had 200 plants. Samples from five grain developmental stages (5, 10, 15, 20 and 30 days post-anthesis, DPA) used for two-dimensional electrophoresis (2-DE) analysis were collected and all samples were stored at –80 °C prior to analysis.

Protein preparation

Samples of 500 mg from three biological replicates were used to extract proteins following the method outlined by Gao et al. (2009). Grains from five stages of the two lines were used as samples. Finally, lysis buffer of 300 µl (7 M urea, 2 M thiourea, 4% CHAPS, 40 M Tris-base) was added to the pellet. After 4 h, insoluble material was removed by centrifuging 15 min at 13,000 rpm. Finally, protein concentrations were determined with a 2-D Quant Kit (Amersham Bioscience, USA).

2-DE and image analysis

2-DE was performed according to Ge et al. (2011), and 600 µg of proteins was loaded into analytical and preparative gels (pH 3–10, 18 cm, GE Healthcare). The 2-DE experiments of each sample were repeated 2–3 times. After SDS-PAGE, gels were scanned, and protein spots were detected, quantified and analyzed. The background was subtracted with ImageMaster 2D Platinum Software Version 7.0 (GE Healthcare). For protein spot profile analysis, the 2-DE gels of seed samples during five developmental stages in CS were selected as the reference gels. All analyzed gels from CS-1S^{l}(1B) substitution line were matched to the corresponding reference gel. Then, the protein spots that existed in all three independent sample sets were selected and all the matched spots were checked manually. The abundance of each spot was estimated by the percentage volume (% Vol). At last, differentially accumulated protein spots were determined according to statistically significant changes between samples by Student's *t*-test (abundance variation at least 2-fold, $p < 0.05$). And then they were analyzed to find differentially expressed protein (DEP) spots (Gao et al. 2009).

Protein identification through tandem mass spectrometry

The selected DEP spots were excised from 2-D gels, and pellets were washed with 100 µl bleaching solution containing 50% 25 mM NH_4HCO_3 and 50% acetonitrile. This step was repeated for twice to three times until the pellets were colorless. Then 100 µl acetonitriles were added to the micro-centrifuge tubes, and the peptides were extracted three times with 0.1% trifluoroacetic acid in 60% acetonitrile and further analyzed using a 4800 Plus MALDI TOF/TOF™ Analyzer (Applied Biosystems, USA). Proteins were identified by

searching against the NCBI non-redundant (NCBInr) databases using softwares GPS Explorer (Applied Biosystems) with the following parameter settings: two missed cleavages allowed, mass tolerance set to ± 0.2 Da, fragment tolerance set to ± 0.3 Da. Protein Score C.I.% and Total Ion Score C.I.% all above 95% were identified as the creditable results.

Network analysis

The sequences of all proteins were used for BLAST analysis through the databases of the National Center for Biotechnology Information (NCBI) to obtain the accession numbers of those proteins and then obtain the eukaryotic orthologous group (KOG) numbers in a database (http://eggnog.embl.de/version 3.0). A data set containing all the KOG numbers was then used for protein–protein interaction (PPI) analysis by using the Search Tool for Retrieval of Interacting Genes/Proteins (STRING) database (http://string-db.org/newstring_cgi/ show_input_page.pl). The confidence level (score) was set to a high value (0.700). Then, the PPI network was extracted from the whole interaction network and reconstructed by using the Cytoscape software (version 3.02).

Results

Protein expression profiling during grain development of CS and CS-1S^{l}/1B

The comparative 2-DE mapping of grain protein dynamics at 5, 10, 15, 20 and 30 DPA during grain development in CS and the substitution line CS-1S^{l}(1B) (Fig. S1*) revealed that, at the early periods of grain development, grain proteins were mostly distributed in acidic area, while the basic proteins appeared after 15 DPA. The proteome expression differences between CS and CS-1S^{l}(1B) increased gradually in the later stages. In total, 78 DEP spots with at least 2-fold expression differences were detected at five different developmental stages of the two genotypes, including 39 spots common for both genotypes, 14 and 25 DEP spots expressed specifically in CS and CS-1S^{1}(1B), respectively (Fig. S2A). Through matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS) analysis, 73 DEP spots representing 55 unique proteins were identified. All identified proteins are listed in Table S1, and their detailed peptide information of tandem mass spectrometry analysis and volume data changes of differentially expressed protein spots by 2-DE and their statistical analyses were shown in Tables S2 and S3, respectively.

The identified DEPs included various enzymes with different physiological functions, which were mainly classified into 9 groups as shown in Fig. S2B: carbon metabolism (28.77%), storage proteins (27.40%), stress-defense (16.44%), protein synthesis and decomposition (6.85%), nitrogen metabolism (4.11%), cell metabolism (2.74%), photosynthesis (2.74%), signal transduction (1.37%), and unknown (9.59%). Some protein spots with different *pI* and molecular mass were identified as the same protein such as spot B1

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

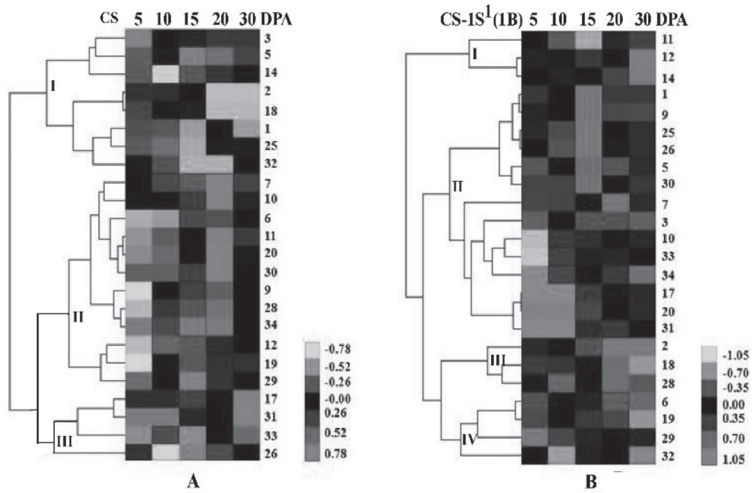

Figure 1. The expression patterns of spots with expression differences of at least 2-fold between CS and CS-1S^{l}(1B) during grain development (the numbers on the right sides of the figures stand for common DEP spots in two lines, which are presented in Table S1)

and B2, S16 and S17. The results from functional catalogue (Fig. S2B) clearly showed that more than 70% identified DEPs during grain development of CS and CS-1S^{l}(1B) were involved in substance and energy metabolism, storage proteins and stress-defense.

The dynamic expression patterns of DEP spots that were detected in all five grain developmental stages in CS and CS-1S^{l}(1B) were analyzed by cluster software and the results are shown in Fig. 1. From an overall perspective, although protein expression patterns in both lines were divided into two main categories, clear expression differences between the two lines were present. Further analysis revealed that the protein expression patterns of different spots over five grain developmental stages in Chinese Spring could be categorized into three subclasses: Group I containing 8 DEP spots (No. 3, 5, 14, 2, 18, 1, 25 and 32) with down-regulation, Group II including 12 DEP spots (No. 7, 10, 6, 11, 20, 30, 9, 28, 34, 12, 19 and 29) with up-down regulation, and Group III having four DEP spots (No. 17, 31, 33 and 26) with up-regulation. The DEPs in these Groups were mainly involved in carbohydrate metabolism. In the 1S^{l}(1B) substitution line, the expression patterns of different spots over five grain developmental stages were divided into four groups: Group I with three DEP spots (No. 11, 12 and 14) showing up-regulation expression, Group II with 14 DEP spots (No. 1, 9, 25, 26, 5, 30, 7, 3, 10, 33, 34, 17, 20 and 31) displaying up-down regulation expression, Group III with three DEP spots (No. 2, 18, and 28) indicating down-regulation expression and Group IV with four DEP spots (No. 6, 19, 29 and 32) exhibiting down-up-down regulation expression. The DEPs in Groups I and II were mainly involved in glycolysis and stress response and those in Groups III and IV were mainly related to starch synthesis and nitrogen metabolism.

Comparative proteome analysis of developing grains between CS and CS-1S^{l}(1B)

Comparative proteome analysis revealed that the 1S^{1}/1B chromosome substitution resulted in significant changes of proteome compositions and expression patterns during grain development. However, the proteome expression profiles between the two lines were almost similar from 5 to 10 DPA, and no specifically expressed protein spots were found. The numbers of DEP spots gradually increased from 10 to 30 DPA (Fig. S1). During the five grain developmental stages, 39 common protein spots including 33 DEPs were identified in both lines, of which 8 protein spots (2, 7, 9, 10, 18, 20, 30 and 34) showed similar expression patterns in both lines (Fig. S2), while the others displayed different expression levels between CS and CS-1S^{l}(1B). As shown in Fig. S3A, the abundance of spot 2 (succinyl-CoA ligase) and spot 18 (glutamine synthetase isoform GSr1) was higher in CS-1S^{l}(1B) and decreased gradually during grain development in both lines. The protein spots 7, 9, 10, 20, 30 and 34 showed an up-down expression trend in both lines, of which three spots (10, 20 and 34) had a higher expression level in CS (Fig. S3B–D). These proteins are mainly related to basic carbohydrate metabolism such as glycolysis, tricarboxylicacid cycle and starch synthesis. Particularly, the protein spot 3 (chaperone protein ClpC1) showed a down-regulated trend in CS, but an up-down expression pattern during grain development in CS-1S^{l}(1B), which is related to protein synthesis, assembly and degradation. The protein spot 11 identified as dimeric alpha-amylase inhibitor decreased gradually in CS, but increased then decreased during grain development in CS-1S^{l}(1B) as shown in Fig. S3E.

According to the results listed in Table S1, 11 DEPs were encoded specifically by the genes on the 1B chromosome (including 3 HMW-GS), which were mainly involved in carbohydrate and protein metabolisms. Among these proteins, alpha-amylase/trypsin inhibitor CM2 (spot B7) is related to stress response and oxygen-evolving complex protein (spot B5) is involved in photosynthesis. Both proteins are important for the basic physiological activities of plant growth and development.

A total of 12 DEP spots representing 9 unique proteins were found to be encoded specifically by the genes of the S^{1} genome, including 3 HMW-GS proteins; they were mainly involved in carbohydrate and protein metabolisms and stress response. Particularly, some important stress resistance related proteins from 1S^{1} chromosome were identified, such as serine proteinase inhibitors (spots S15, S16, S17 and S22), heat shock protein (spot S14), triticin precursor (spot S8), low-temperature-induced protein (spots S10) and LEA1 protein (spot S25). As shown in Fig. S3F–H, spots 8, 14, and 25 showed a higher expression level during grain development in CS-1S^{l}(1B), of which triticin precursor was up-down regulated, LEA1 protein exhibited a much higher expression at 20–30 DPA and heat shock protein had two expression peaks at 10 and 30 DPA.

Network analysis of 1B and 1S^{l} encoded key DEPs

To understand the interaction network among the key DEPs of 1B and 1S^{1} chromosomes, PPI analysis was conducted by using STRING. All of the 23 DEP spots that had KOG numbers in the database and represented 20 unique proteins (Table S1) were used to con-

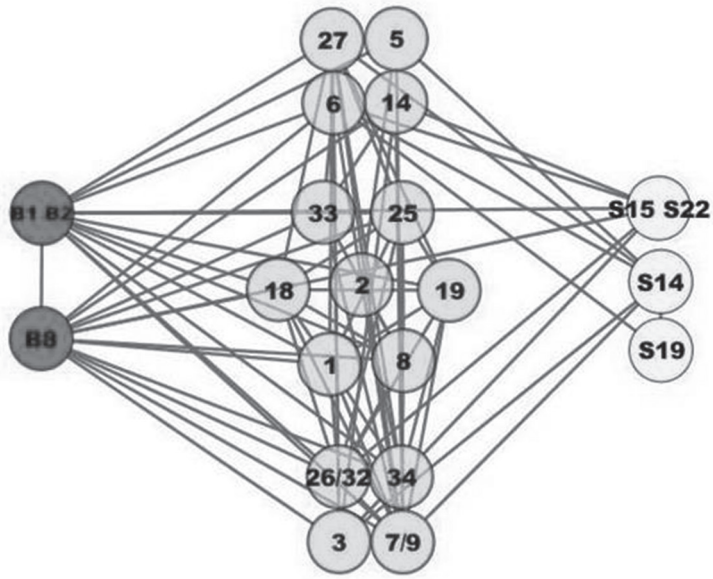

Figure 2. Protein–protein interaction (PPI) network of some important DEPs encoded by the B and S1 genomes constructed by STRING. The numbers in the cycle were same as those in Table S1

struct the interaction network, of which spots B1, B2 and B8 were associated with 1B chromosome and spots S14, S15, S22 and S29 with $1S^1$ chromosome. As shown in Fig. 2, two DEPs (glyceraldehyde-3-phosphate dehydrogenase and aldose reductase) expressed in CS and three (serpin-Z2A, putative heat shock 70 KD protein and translational initiation factor eIF-4A) in CS-$1S^l$(1B) interacted with the proteins closely related to substance and energy metabolism. Particularly, the DEPs in CS-$1S^l$(1B) were specifically interacted with those involved in stress response. This reflects that more stress-related proteins present in the S^1 genome of the substitution line.

Discussion

In this study, we found that the grain proteome in Chinese Spring and the $1S^1$(1B) substitution line had significant expression differences and most of the expressed proteins were acidic at the early stage of the grain development. At the later stages, many basic proteins appeared and DEP spots between them surged, resulting in more than 30 DEP spots present in the last period (Fig. S1). And we can found that some protein spots with different *pI* and molecular mass were identified as the same protein such as spot B1 and B2, S16 and S17. This could be resulted from various post-translational modifications, such as protein phosphorylation and glycosylation, and isomerides encoded by highly relevant gene sequences. Our recent research revealed that the starch synthases SSI could be phosphorylated at different locations of the gel (Cao et al. 2015). Other reasons may be from

the activity of proteases, minor breakage due to sonication and deamidation. Among the DEPs, 33 common proteins were identified between the two lines during five grain developmental stages, which were mainly involved in substance metabolism, protein synthesis/assembly/degradation and stress response (Table S1).

Some DEPs were found to be specifically encoded by the genes on the 1B chromosome of CS, which were mainly involved in carbohydrate/protein metabolism and photosynthesis, the basic metabolism activities necessary for plant growth and development.

Interestingly, 11 DEPs were specifically encoded by the genes on the $1S^1$ chromosome of CS-$1S^l$/1B, which are mainly involved in stress resistance and storage protein metabolism (Table S1). A specific triticin precursor (spot S8) was identified in the $1S^1$/1B substitution line. Wheat triticin polypeptides are synthesized specifically during seed development 8 to 21 days after anthesis, and are deposited in the endosperm protein bodies. It may play an important role in determining the functionality of wheat flour dough by participating in thioldisulphide inter-change reactions (Singh et al. 1993). Our results from 2-DE also indicated that this protein accumulated at the early stages and reduced gradually at the later stages (Fig. S1). Heat shock 70 KD protein (spot S14) is another protein that relates to stress response. It was a member of HSP, which are a group of proteins induced by heat shock. It was mainly involved in the folding and unfolding of other proteins and its expression is increased when cells are exposed to elevated temperatures or other stress (De Mao 1999). Taken example of molecular chaperones, the HSP family is involved in regulating normal plant growth processes. Previous studies in *Arabidopsis* revealed that HSP genes were overexpressed in response to environmental stressors, including drought, heat, and cold (Sung et al. 2001). Cooperation among different classes of HSPs under abiotic stress is essential to cellular protection mechanisms such as maintaining protein conformation, preventing aggregation of non-native proteins, refolding of denatured proteins, and removing harmful polypeptides (Wang et al. 2004). Furthermore, Hsp70 chaperones can interact and cooperate with Hsp40 to perform its chaperone activities and act as essential substrate-targeting factors for ClpB/Hsp104 (Winkler et al. 2012).

Our recent study identified two novel HMW-GS from the $1S^l$ genome of *Aegilops longissima*, $1S^l$x2.3*+$1S^l$y16*, which resulted in a significant improvement of breadmaking quality (Wang et al. 2013). Both subunits possessed long repetitive domain and had a higher level of mRNA expressions during grain development, which contribute to superior dough quality. In this study, five DEP spots (S2, S3, S4, S5 and S6) were identified as HMW-GS $1S^1$y16* in CS-$1S^l$(1B). Both subunits began to accumulate at 15 DPA and then displayed up-regulation expression until grain maturity (Fig. S1).

To date, various chromosome additions, alien substitutions and fragment translocations have been used as effective ways to transfer valuable genes from *Aegilops* genus into bread wheat to improve agronomic performances (Wang and Zeller 1992), such as biotic and abiotic resistance, protein content, gluten quality, and yield (Harper et al. 2011). Our research in this work provides useful information for the proteome characterization of the S^1 genome from *Aegilops longissima*, which are potential gene resources for bread wheat improvement, particularly in the aspects of stress resistance and gluten quality.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Differentially expressed proteins (DEPs) at five grain developmental stages in CS and CS-$1S^l$(1B) identified by MALDI-TOF/TOF-MS

Electronic Supplementary *Table S2*. Peptide sequences of the identified spots in Table 1 by MALDI-TOF/TOF-MS

Electronic Supplementary *Table S3*. The volume data of differentially expressed protein spots by 2-DE and statistical analyses

Electronic Supplementary *Figure S1*. 2D maps of grain proteins from developing grain 5, 10, 15, 20 and 30 DPA of CS and CS-1S^{l}(1B). The 39 differentially expressed protein spots found in both lines are numbered (1–39). The 14 DEP spots specific to the genome of CS are labeled in blue (B1–B14) and the 29 DEP spots specific to the S^{l} genome are labeled in red (S1–S29)

Electronic Supplementary *Figure S2*. The DEP spots identified in developing grains in CS and CS-1S^{l}(1B) and their functional catalogues. (A) Venn diagram illustrating the comparison of DEP spots in developing grains between CS and CS-1S^{l}(1B). The numbers indicated the common and specific DEP spots expressed in two lines. (B) Functional distribution of the identified differentially expressed proteins during grain development of CS and CS-1S^{l}(1B)

Electronic Supplementary *Figure S3*. (A–E) Expression pattern comparison of some key DEPs in CS and CS-1S^{l}(1B). The red and blue lines represented protein expression in CS-1B and CS-1S^{l}(1B), respectively. (F–H) The expression patterns of protein spot S8, S25 and S14 which expressed specifically in CS-1S^{l}(1B). The numbers on the right side of figures were the same as the spot numbers in Table S1