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Proteomic analysis reveals the fungal resistance of soft wheat during storage

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ABSTRACT

The prevention of fungal growth is of great importance for the safe storage of wheat. Previous studies have shown that soft wheat is more resistant to the xerophilic fungus *Aspergillus glaucus* than hard wheat during storage under the same conditions. We conducted a comparative proteomic analysis of embryos of soft wheat cv. Yangmai 15 and of hard wheat cv. Yangmai 16 to identify specific differentially expressed proteins. Dot-blot analysis of embryo lysates revealed 65 and 15 specific differential protein spots from Yangmai 15 and Yangmai 16, respectively. Four specific differential protein spots were revealed from Yangmai matched antifungal proteins, including a peroxidase, endogenous alpha-amylase/subtilisin inhibitor, xylanase inhibitor protein 1, and puroindoline B, while spots from Yangmai 16 did not match any antifungal proteins. This proteomic study provided novel insights to investigate the discrepancy of storage tolerances among different wheat varieties.

1. Introduction

Wheat (*Triticum aestivum*) is an important staple food worldwide used for the manufacture of flour-derived foodstuffs such as bread and noodles (Lim, 2013). Harvested wheat is commonly stored long-term to guarantee grain supply. Fungal spoilage of stored grain, which is significantly affected by storage temperature and moisture content, deteriorates the edibility and processing quality and can even be associated with the production of mycotoxins (Jian and Jayas, 2012; Zhang et al., 2014). When the moisture content of stored wheat is elevated through water adsorption or moisture transfer owing to the heat balance of the grain bulk, xerophilic fungi, such as *Aspergillus glaucus* group, can appear at the beginning of storage (Cai et al., 2012). Moreover, the moisture content and temperature in the grain bulk can further increase along with the growth of the xerophilic fungi, which could lead to the growth of other storage fungi including toxigenic species, resulting in serious grain spoilage (Cai et al., 2012). Therefore, preventing the growth of xerophilic fungi is critical for the safe storage of wheat.

With the aim to reduce the possibility of fungal spoilage, abiotic factors affecting wheat storage, such as moisture content, temperature, and warehouse type, have been extensively studied (Sharma and Bhandari, 2013). However, the role of wheat intrinsic characteristics in fungal spoilage has been seldom reported. We recently reported that the rate of fungal growth significantly varied between hard and soft wheat varieties under the same storage condition and soft wheat were found more resistant to the xerophilic *A. glaucus* (Cai et al., 2013a). Microscopic observations have revealed that fungal mycelia are mainly located in the embryo within the wheat kernel (Li et al., 2016). Hence, identifying the inhibitory substance in the soft wheat embryo would be interesting and helpful to elucidate the resistance of soft wheat to *A. glaucus.*

Biochemical composition varies among wheat of different hardness (Pasha et al., 2010). In the last two decades, several pathogenesis-related proteins have been characterized, and their antifungal properties were confirmed in wheat kernels (Golshani et al., 2015). Thus, we reasoned that soft wheat kernels may contain more (powerful) antifungal proteins than hard wheat kernels. To reveal the antifungal properties of soft wheat kernels, a comparative proteomic analysis of soft and hard wheat embryos was performed. The results showed that the soft wheat embryo contains more antifungal proteins than the hard wheat

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embryo. It was suggested that the difference in antifungal proteins in the hard and soft wheat embryos might be responsible for the distinct resistance to *A. glaucus* growth during wheat storage.

2. Materials and methods

2.1. Plant material

The soft red hexaploid wheat cv. Yangmai 15 and the hard red wheat hexaploid cv. Yangmai 16 were purchased from the Jiangsu Lixiahe Institute of Agricultural Science in the year of 2014. The two cultivars are widely grown in the Yangtze-Huaihe region of China. Healthy and homogeneous grain kernels of the two cultivars were used for proteomic analysis.

2.2. Protein extraction

Wheat embryos of each cultivar were ground in liquid nitrogen. One gram of sample was added to 1.0 ml of extraction buffer solution (7 M urea, 2 M thiourea, 4% CHAPS, 18 mM Tris-HCl [pH 8], 53 μ/ml DNase I, 4.9 μ/ml RNase A and 1 mM PMSF) to solubilize crude protein. After a 30-min incubation at 4 °C, dithiothreitol was added at a final concentration of 15 mM and the samples were centrifuged for 10 min at 12,000 × *g* at 4 °C. The protein concentration of the supernatant was measured using the BCA Protein Assay Kit (Beyotime, Shanghai, China) with bovine serum albumin as a standard (Beyotime, Shanghai, China).

2.3. Two-dimensional electrophoresis (2DE) and image analysis

For each cultivar, 150 μg of crude protein was used for passive rehydration overnight at room temperature on 24-cm immobiline pH 3–10 gel strips (GE Healthcare, Uppsala Sweden) for the first dimension of 2-DE. Isoelectric focusing electrophoresis was carried out on an Ettan IPGphor 2 unit using the following voltage gradient: 12 h at 50 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 0 V, and 11 h at 10,000 V. Prior to second dimension, each strip was equilibrated after reduction of the proteins for 15 min in 10 ml of an equilibration solution (6 M urea, 50 mM Tris-HCl [pH 8.8], 30% glycerol, 2% SDS, 2% DTT, and a trace of bromophenol blue), followed by an alkylation step in 10 ml of equilibration solution containing 25 mg/ml iodoacetamide for 15 min. Then, the strips were placed on top of 24 × 24 cm 12.5% polyacrylamide resolving gels, and proteins were separated by electrophoresis using the Ettan Dalt Twelve System (Amersham Biosciences). The voltage gradient for SDS-PAGE was: 45 min at 100 V and 4 h at 200 V. For each protein sample, three experimental replicates were performed. Protein was fixed and stained with Coomassie brilliant blue G-250. The 2-DE gel was scanned with an ImageScanner (GE Healthcare, Uppsala, Sweden) at 300 dpi and 16-bit grey scale level. Gel images were analyzed using PDquest 8.0 software (Bio-Rad Laboratories, Hercules, CA, USA) with normalized spot volume as quantitative parameter. Power analysis using the SameSpots software and based on principal component analysis of all 2D gel images indicated that any possible difference in normalized spot volumes between genotypes could be revealed for 98% of the spots with only three replicates. For each comparison, spot data were statistically analyzed using ANOVA and $P < 0.05$ was considered significant. Qualitative differences between gels were analyzed by defining a threshold value for normalized spot volume; spots with a normalized spot volume >0.045 were considered as present.

2.4. Trypsin digestion, characterization, and identification of proteins by mass spectrometry

Specific differential protein spots were excised from the replicate gels and pooled. Four hundred microliters of ammonium bicarbonate-acetonitrile buffer (100 mmol/l) was added to destain the samples and then, 5 μl 10 ng/μl trypsin solution (Promega, Madison WI, USA) was added to digest the protein at 37 °C for 20 min. The peptides were identified using a mass spectrometer (Voyager-DE Pro MALDI-TOF Applied Biosystems, Framingham, MA, USA). The monoisotopic peptide masses were used for interrogation of proteindatabase4 yaping (September 18, 2015, 94,854 sequences) using the Mascot software (Matrix Science, London, UK, http://www.matrixscience.com). Proteins were considered identified by MALDI-TOF if at least four non-redundant peptides matched a single reference in one of the databases.

3. Results

3.1. 2-DE analysis of embryos of wheat cvs. Yangmai 15 and Yangmai 16

To reveal the biochemical basis of the storage tolerance of wheat cv. Yangmai 15, the protein profile of Yangmai 15 embryos was investigated in comparison to that of Yangmai 16 embryos through 2-DE analysis. The Coomassie brilliant blue-stained 2D gels are shown in Fig. 1A and Fig. 1B. In total, 65 and 15 differential, specific spots were distinguished in Yangmai 15 and Yangmai 16 blots, respectively.

3.2. Identification of proteins showing differential expression in Yangmai 15

The majority of the specific protein spots could not be characterized based on peptide mapping owing to the limited data available in our local database. However, four proteins that existed only in Yangmai 15 were identified as potential antifungal proteins, while no antifungal proteins were identified in Yangmai 16 (Table 1). The protein at spot 7408 was identified as a peroxidase (UniProt accession no.: W5DLG4), with 11% sequence coverage; spot 5107 matched an endogenous alpha-amylase/subtilisin inhibitor (accession no.: P16347) with a sequence coverage of 46%; spot 6216 matched xylanase inhibitor protein 1 (accession no.: Q8L5C6) with 13% sequence coverage; spot 8005 matched a puroindoline B (accession no.: Q10464) with 12% sequence coverage.

4. Discussion

Wheat kernels are dormant seeds that are generally stored long term. During this post-harvest storage, molds can readily infect and damage the stored grains if storage conditions are unfavorable (Cai et al., 2013b). Thus, the grain moisture content and temperature must be strictly controlled to prevent fungal growth during storage. However, due to water adsorption, the moisture content of dried wheat can increase under humid climatic conditions (Li et al., 2011). Hence, cultivars with high storage tolerance are favored for long-term storage. Soft wheat has been found to be more resistant to *A. glaucus* than hard wheat under the same storage conditions (Cai et al., 2013a; Li et al., 2016), and thus, offers valuable material to investigate storage tolerance in wheat. However, the biochemical basis of the resistance of soft wheat to *A. glaucus* remained unknown.

In recent years, proteomic studies to investigate wheat kernel development and mature kernels have been reported (Irar et al.,