Journal of Proteomics 130 (2016) 108–119

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Quantitative proteomics reveals the central changes of wheat in response to powdery mildew

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ARTICLE INFO

Article history:
Received 15 July 2015
Received in revised form 2 September 2015
Accepted 4 September 2015
Available online 14 September 2015

Keywords:
Wheat
iTRAQ
Quantitative proteomics
Powdery mildew

ABSTRACT

Powdery mildew (Pm), caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), is one of the most important crop diseases, causing severe economic losses to wheat production worldwide. However, there are few reports about the proteomic response to *Bgt* infection in resistant wheat. Hence, quantitative proteomic analysis of N9134, a resistant wheat line, was performed to explore the molecular mechanism of wheat in defense against *Bgt*. Comparing the leaf proteins of *Bgt*-inoculated N9134 with that of mock-inoculated controls, a total of 2182 protein-species were quantified by iTRAQ at 24, 48 and 72 h postinoculation (hpi) with *Bgt*, of which 394 showed differential accumulation. These differentially accumulated protein-species (DAPs) mainly included pathogenesis-related (PR) polypeptides, oxidative stress responsive proteins and components involved in primary metabolic pathways. KEGG enrichment analysis showed that phenylpropanoid biosynthesis, phenylalanine metabolism and photosynthesis-antenna proteins were the key pathways in response to *Bgt* infection. InterProScan 5 and the Gibbs Motif Sampler cluster 394 DAPs into eight conserved motifs, which shared leucine repeats and histidine sites in the sequence motifs. Moreover, eight separate protein–protein interaction (PPI) networks were predicted from STRING database. This study provides a powerful platform for further exploration of the molecular mechanism underlying resistant wheat responding to *Bgt*.

Significance: Powdery mildew, caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), is a destructive pathogenic disease in wheat-producing regions worldwide, resulting in severe yield reductions. Although many resistant wheat varieties have been cultivated, there are few reports about the proteomic response to *Bgt* infection in resistant wheat. Therefore, an iTRAQ-based quantitative proteomic analysis of a resistant wheat line (N9134) in response to *Bgt* infection has been performed. This paper provides new insights into the underlying molecular mechanism of wheat in response to *Bgt*. The proteomic analysis can significantly narrow the field of potential defense-related protein-species, and is conducive to recognize the critical or effector protein under *Bgt* infection more precisely. Taken together, large amounts of high-throughput data provide a powerful platform for further exploration of the molecular mechanism on wheat–*Bgt* interactions.

1. Introduction

Powdery mildew (Pm), caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), is a destructive wheat foliar disease, which occurs in regions with maritime and semi-continental climates and causes severe yield losses. Breeding and utilization of Pm-resistant varieties is the safest, most cost effective and environmentally friendly means for the control of wheat powdery mildew. Most of powdery mildew resistance genes have been transferred into wheat (*Triticum aestivum* L.) through traditional intergeneric and interspecific crosses [1,2]. Several sources of resistance genes have come from the related genera of wheat, such as *Aegilops*, *Elytrigia*, *Secale*, *Haynaldia*, and related species of *Triticum*, for instance, *T. boeoticum*, *T. dicoccoides*, *T. carthlicum* and *T. timopheevii* [3]. However, empirical selection for resistance variety is time-consuming. Additionally, the specific resistance is frequently overcome by pathogens because of the genetic diversity and rapid variation of the powdery mildew pathogen, which is besetting crop breeders. Genetic engineering has been successfully utilized to improve wheat resistance and is considered to have a remarkable feat, and showcases the power for creating crops with valuable traits [4,5]. Genome editing is another promising method for accurately operating target gene. Furthermore, transcriptomics and proteomics can provide comprehensive supports and gene targets candidates for genetic engineering and genome editing. Transcript profiling can reveal the transcriptional expression pattern of genes involved in defense response pathways, which can help to elucidate the defense mechanism controlled by resistance (R) genes. For example, affymetrix wheat microarrays and RNA-seq analysis have provided valuable information about globally genes expression changes in response to powdery mildew in wheat [6,7].

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http://dx.doi.org/10.1016/j.jprot.2015.09.006
1874-3919/

However, the detection of transcriptional changes cannot ensure the same tendency of the corresponding proteins expression for the existence of posttranscriptional, translational and/or posttranslational regulatory mechanisms [8,9], alternative splicing and even protein degradation [10]. Proteomics is a key tool which contributes to explaining complex biological processes directly on protein level, including two dimensional gel electrophoresis (2-DE), differential in gel electrophoresis (DIGE), tag-based labeling of proteins (isotope-coded affinity tags (ICAT), stable isotope labeling with amino acids in cell culture (SILAC), isobaric tags for relative and absolute quantification (iTRAQ) technologies, protein–protein interaction (PPI) and protein modifications. Wang et al. evaluated changes in the leaf proteome of wheat in response to powdery mildew infection, and found that 26 differentially accumulated protein-species were related to signal transduction, defense, transcription and translation, energy and metabolism [11]. Recently, changes in the protein profiles of resistant wheat (line N0308) in response to *Bgt* infection, and found that most of the up-accumulated protein-species were identified as stress- and defense-related protein-species [12]. In fact, most proteomic research employed 2-DE and DIGE primarily identified abundant soluble proteins [13], which are suffering from detecting and quantifying tiny or membrane proteins. Therefore, iTRAQ as the alternative and powerful protein approach has been the first-line technique in plant proteomics study, which adopts isotope labeling, multidimensional liquid chromatography and tandem mass spectrometry (MS) to accurately detect and quantify proteins [13–15].

In this study, an iTRAQ-based quantitative proteomic technique was used to analyze the proteome change in seedling leaves from *Bgt*-inoculated resistant wheat (line N9134) compared with that from mock-inoculated controls. N9134 is a bread wheat line carrying *PmAS846* and confers broad spectrum resistance to wheat powdery mildew. Our objective was to identify protein-species dysregulated by infection with *Bgt* in N9134, and to provide the ample and reliable proteome data which are conducive to further studies on the molecular mechanism of wheat in response to *Bgt*. This study has identified 394 protein-species that significantly changed in abundance at 24, 48 and 72 hpi with *Bgt* isolate E09, and systematically reveal the expressive characterization of the defense responsive protein-species. Moreover, the high-throughput proteome data will provide an understanding of the response mechanism of resistant wheat after *Bgt* infection.

2. Materials and methods

2.1. Plant materials and pathogen stress treatment

N9134 is a resistant bread wheat line carrying *PmAS846* introgressed from wild emmer accession AS846 (*T. dicoccoides*). In a previous work, this gene was physically located to wheat chromosome 5BL bin 0.75–0.76 within a gene-rich region [3]. It confers broad spectrum resistance to wheat powdery mildew.

The *Bgt* isolate E09 was maintained on susceptible wheat 'Shaanyou 225' by inoculating the fully expanded first leaves with conidia. Seeds of the control 'Shaanyou 225' and N9134 were planted in 8–10 cm pots in a growth chamber at 18 °C under a 16 h light/8 h dark photoperiod, and these pots were covered with some nonopaque and breathable hoods for prevention of other fungal contamination. Seedlings of N9134 were artificially inoculated by dusting *Bgt* E09 conidia from sporulating seedlings of 'Shaanyou 225' at the two to three leaf stages. The control 'Shaanyou 225' plants were simultaneously inoculated with E09 to check whether inoculation was successful or unsuccessful. *Bgt*-inoculated leaves were harvested at 24, 48 and 72 hpi, as well as mock-inoculated leaves at 0 hpi. The collected leaf samples were immediately frozen in liquid nitrogen and stored at −80 °C, and subjected to proteomics analysis. The experiment was carried out with three biological replications.

2.2. Microscopic examination of Bgt infection

Infected leaves were collected with three biological replications at 24, 48 and 72 hpi at the same condition. These leaf tissues were cut into small pieces (0.5–1 cm), fixed and decolorized in ethanol/acetic acid (3:1, *v*/*v*) containing 0.15% (*w*/*v*) trichloroacetic acid for 30 min at 70 °C, stained with 0.6% Coomassie Brillant Blue R-250 for 24 h and rinsed with ddH_2O. For microscopic observations, leaf segments were stored in acetic acid/glycerol (1:4, *v*/*v*) and examined under a microscope Olympus BX-43 (Olympus Corporation, Tokyo, Japan).

2.3. Protein extraction and iTRAQ labeling

Total proteins were extracted from the collected leaf tissue as previously described [16], and the extract procedure was optimized: Prior to trypsin digestion, the precipitated pellets were resuspended in a solution containing 7 Murea and 500 mM tetraethyl-ammonium bicarbonate (TEAB, pH 8.5) and then sonicated for 15 min. The method for protein resuspension can improve the labeling efficiency. Three biological replicates were carried out for each sample. The protein content was determined by Bradford's reagent (Supplementary Table 1) [17]. Total protein (100 μg) was taken out of each sample solution and then the protein was digested with Trypsin Gold with the ratio of protein: trypsin = 30:1 at 37 °C for 16 h. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M tetraethyl-ammonium bicarbonate (TEAB) and processed according to the manufacture's protocol for 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 μl isopropanol. Samples were labeled with the iTRAQ tags as follows (Supplementary Table 1). The peptides were labeled with the isobaric tags, incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

Next, the labeled peptide mixtures were reconstituted with 4 ml buffer A (25 mM NaH_2PO_4 in 25% *v*/*v* acetonitrile, pH 2.7) and were fractionated on a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) with an Ultremex SCX column (4.6 × 250 mm). The peptides were eluted at a flow rate of 1 ml/min with a gradient of buffer A for 10 min, 5–60% buffer B (25 mM NaH_2PO_4, 1 M KCl in 25% acetonitrile, pH 2.7) for 27 min, 60–100% buffer B for 1 min. The absorbance at 214 nm was monitored and 20 fractions were collected. The eluted peptides of each fraction were dried and desalted before LC-ESI MS/MS analysis.

2.4. LC-ESI-MS/MS analysis based on Q-EXACTIVE

The peptides of each fraction were resuspended in buffer A (2% acetonitrile, 0.1% formic acid) and centrifuged at 20,000 × *g* for 10 min; the final concentration of peptide was about 0.5 μg/μl on average. The supernatant (5 μl injection) was separated using a LC-20 AD Nano-HPLC by the auto-sampler with a flow rate of 300 nl/min. Peptides were eluted by application of a linear gradient from 2% buffer B (98% acetonitrile, 0.1% formic acid) to 35% buffer B over 40 min, followed by ramping up to 80% buffer B over 5 min, and maintained at 80% buffer B for 4 min, and finally return to 5% in 1 min, equilibrated in buffer A for 10 min. The fractions were subjected to nanoelectrospray ionization followed by tandem mass spectrometry (MS/MS) in a Q-EXACTIVE (Thermo Fisher Scientific, San Jose, CA) coupled online to the HPLC. Intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected for MS/MS using high-energy collision dissociation (HCD) operating mode with a normalized collision energy setting of 27.0; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 15 MS/MS scans was applied for the 15 most abundant precursor ions above a threshold ion count of 20,000 in the MS survey scan with a following Dynamic Exclusion duration of 15 s. The electrospray voltage applied was 1.6 kV. Automatic gain control (AGC) was used to optimize the spectra generated by the orbitrap.