Proteomics of Bulked Rachides Combined with Documented QTL Uncovers Genotype Nonspecific Players of the *Fusarium* Head Blight Responses in Wheat

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ABSTRACT

Fusarium head blight (FHB) is a destructive disease of wheat that reduces yield and grain quality. High-throughput proteomic techniques have been used to identify a wide range of candidate proteins involved in host resistance. The majority of the published works on the proteomics of the wheat response to *Fusarium graminearum* infection are case specific. In the current study, a high-throughput quantitative label-free strategy was employed on bulked rachides of *F. graminearum*-infected wheat collected from multiple genotypes. Differentially accumulated proteins among the following four pools were identified: mock-inoculated FHB-resistant accessions (RM), mock-inoculated FHB-susceptible accessions (SM), *F. graminearum*-inoculated FHB-resistant accessions (RFg), and

F. graminearum-inoculated FHB-susceptible accessions (SFg). Four pairs of comparisons were made: RFg versus RM, SFg versus SM, RM versus SM, and RFg versus SFg. Proteins were projected onto the consensus intervals of previously reported quantitative trait loci in the FHB-resistant pool by blasting against the Chinese Spring reference sequences. In addition to proteins previously reported in the host response to *Fusarium* spp., new candidates have emerged in association with resistance or susceptibility, including a group 3 late embryogenesis abundant as a resistance-related protein and a purple acid phosphatase as a susceptibility protein. The protein atlas presented here provides new perspectives on the interaction between *F. graminearum* and wheat.

Fusarium head blight (FHB), mainly caused by *Fusarium graminearum* and related species, is a devastating disease of wheat worldwide. The fungus produces trichothecene mycotoxins such as deoxynivalenol (DON), which are harmful to humans and animals (Pestka 2010). Resistance to FHB is quantitative (Buerstmayr et al. 2009); as such, no wheat germplasm with immunity to this disease has been identified. The two best-described mechanisms of resistance are resistance to initial infection of the spike (type 1) and resistance to disease spread from spikelet to spikelet (type 2) (Schroeder and Christensen 1963). More than 100 quantitative trait loci (QTL) have been reported for FHB resistance (Buerstmayr et al. 2009; Liu et al. 2009). Perhaps the best-described source of resistance is the Sumai 3 *Fhb1* locus, recently described as a chimeric lectin (Rawat et al. 2016), which has been widely used in breeding programs around the world due to the strong contributions of this QTL, especially in type 2 resistance (Anderson et al. 2001; Cuthbert et al. 2006).

Proteomics strategies have led to the identification of various defense-related gene products involved in the host response. A reduction in carbon metabolism and photosynthesis gene products occurs prior to the upregulation of stress-related proteins (Wang

et al. 2005). Oxidative burst and pathogenesis-related (PR) proteins have been linked both with susceptible and resistant responses of wheat and barley (Geddes et al. 2008; Zhou et al. 2005, 2006). Previous studies have all employed a two-dimensional polyacrylamide gel electrophoresis (2DE) approach and, although 2DE still offers some advantages over the gel-free methods (Rogowska-Wrzesinska et al. 2013), the throughput of 2DE does not match that of the latter.

Many of the “omics” studies in the FHB–host interaction are carried out in spikelets, which may limit the information provided on type 2 resistance. Type 2 resistance is important for delaying pathogen progression in the rachis; therefore, the rachis architecture likely plays a crucial role in shaping this form of resistance (Jansen et al. 2005; Kang and Buchenauer 2000). A bulked-sample analysis for omics studies can remove genotype-dependent variability from the data set (Zou et al. 2016). This work was carried out on bulked rachides from a panel of well-documented FHB-resistant (carrying type 2 resistance QTL) and FHB-susceptible wheat genotypes, in order to focus on the responses at the rachis node. Additionally, proteins were mapped to the genome-wide QTL, providing a panel of candidate genes for further investigations. Taking advantage of high-throughput proteomics techniques, this is the first study addressing the host response to *F. graminearum* at the rachis node by employing a bulked-sample analysis approach toward identification of candidate proteins involved in type 2 resistance in wheat.

MATERIALS AND METHODS

Plant material, inoculations, and sampling. Ten FHB-resistant accessions with known resistance QTL were used, including *Fhb1*-NIL-R (Bernardo et al. 2012), Sumai 3 (Anderson et al. 2001), Wangshuibai (Lin et al. 2004; Yu et al. 2008b; Zhou et al.

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2004), Nyubai (Ban et al. 2008; Cuthbert et al. 2006), Ning7840 (Bai et al. 1999), Huangfangzhu (Li et al. 2012), Baishanyuehuang (Zhang et al. 2012), Huangcandou (Cai and Bai 2014), Taiwan wheat (Li et al. 2016a), and Tokai-66 (Yen et al. 2010). Each of these lines has consistently shown a high level of FHB resistance, with reported disease severities of less than 20%. All of the resistant lines carry *Fhb1* in addition to other resistance QTL. Six FHB-susceptible accessions, including *Fhb1*-NIL-S (Bernardo et al. 2012), Wheaton, Bobwhite, Jagger, Chinese Spring, and Clark, were previously shown to be highly susceptible to FHB, with disease severities greater than 60% (Li et al. 2016a,b; Yu et al. 2006, 2008a). Wheat plants were grown in a greenhouse in Yangzhou, China, maintained at $22 \pm 5^\circ\text{C}$ (day) and $17 \pm 2^\circ\text{C}$ (night) with a 12-h photoperiod.

Plants were inoculated with macroconidial spores of an *F. graminearum* strain, isolate Fg65, collected in the province of Jiangsu. Macroconidia were produced in mung bean broth according to the protocol described by Bai et al. (1999). For each of the 16 accessions, 10 spikes were inoculated at early anthesis by injecting 10 μl of the spore suspension (100 conidia μl^{-1}) into a floret of the central spikelet in a spike. Mock-inoculated control plants were treated the same way, where 10 μl of mung bean broth was used instead of spores. Inoculated spikes were individually covered with a thin plastic sandwich bag to provide the humidity required to initiate fungal infection. The rachides below the pathogen- and mock-inoculated spikelets were harvested 3 days postinoculation (dpi) and stored at -80°C prior to protein and RNA extraction. Three independent biological replicates were prepared for each extraction.

Protein extractions. The rachides collected from each accession and treatment were ground to a powder in liquid nitrogen with a mortar and pestle. For each treatment, equal amounts of ground rachides from the 10 resistant accessions or the 6 susceptible accessions were combined for the extraction. Thus, for each of three replicates, four pools of ground tissue were generated: an FHB-resistant pool (consisting of 10 resistant genotypes) following mock inoculation (RM) or inoculation with *F. graminearum* (RFg), and an FHB-susceptible pool (consisting of 6 susceptible genotypes) following mock inoculation (SM) or inoculation with *F. graminearum* (SFg). Protein extraction was carried out by Shanghai Applied Protein Technology Co., Ltd., where a trichloroacetic acid (TCA)-acetone precipitation method was employed. The ground rachis tissues were mixed with 5 $\times$ volume of TCA-acetone (1:9) and proteins were precipitated at -20°C for 4 h. The precipitate was pelleted by centrifugation (6,000 $\times$ g, 40 min, 4°C) and the pellets were washed three times in cold acetone, air dried, and, finally, resuspended in 30 $\times$ volume of STD buffer (4% sodium dodecyl sulfate, 100 mM Tris-HCl, and 1 mM dithiothreitol, pH7.6). Samples in STD buffer were boiled for 5 min, sonicated, and then boiled again for 15 min. In order to remove debris, samples were then spun at 14,000 $\times$ g for 40 min and the supernatant passed through 0.22- μm filters. Protein concentration was determined using a BCA Protein Assay Kit (Bio-Rad) and samples were stored at -80°C prior to protein digestion and mass spectrometry (MS).

Sample preparation and MS. Protein digestion and MS were carried out at Shanghai Applied Protein Technology Co., Ltd. Filter-aided sample preparation digestion was performed according to Wiśniewski et al. (2009). Briefly, for each protein extraction, 200 μg of protein in 30 μl of STD buffer was transferred to an ultrafiltration unit with a 10-kDa molecular mass cutoff. STD buffer was removed by centrifugation-assisted washes carried out in UA buffer (8 M urea and 150 mM Tris-HCl, pH 8.0). Reduced cysteine residues were then alkylated using 100 mM iodoacetamide in 100 μl of UA buffer. Following a 20-min incubation, the filter was washed with UA buffer, then with 25 mM NH_4HCO_3 . Finally, the protein was suspended in 40 μl of 25 mM NH_4HCO_3 buffer and an on-filter digestion was carried out with 4 μg of trypsin (Promega Corp.). Following an overnight incubation at 37°C , peptides were

eluted from the column and the filtrate was desalted on a C18 Cartridge (Empore SPE Cartridges C18 [standard density]), bed inner diameter = 7 mm, and volume = 3 ml; Sigma). Desalted samples were concentrated by vacuum centrifugation and reconstituted in 40 μl of 0.1% formic acid. The peptide concentration was estimated by near-UV absorption at 280 nm, using an extinction coefficient of 1.1 of 0.1% (gram per liter) solution.

The MS experiment was carried out with an EASY-nLC (Proxeon Biosystems) instrument, equipped with a C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, and 3 μm resin), and coupled to a Q Exactive Mass Spectrometer (Thermo Fisher Scientific). Samples (5 μg) were loaded onto the column in solvent A (0.1% formic acid) and eluted with an increasing concentration of solvent B (0.1% formic acid and 46.5% acetonitrile) as follows: linear gradient of 0 to 55% solvent B over 110 min and then 55 to 100% solvent B over 5 min, followed by a washout at 100% solvent B for 5 min (flow rate of 250 nl min^{-1}). Precursor ions were generated by electrospray ionization in positive ion mode. A data-dependent top-10 method was employed during the survey scans (300 to 1,800 m/z , with a resolution of 70,000 at m/z 200) to select for the most abundant precursor ions for high-energy collision-induced dissociation (HCD). The dynamic exclusion duration was set at 40 s. A collision energy of 30 eV was employed, and automatic gain control was set at 3×10^6 with a maximum injection time of 10 s. The HCD resolution was 17,500 at m/z 200. The underfill ratio was set at 0.1%.

Sequence database searching and data analysis. The MS data were analyzed by Shanghai Applied Protein Technology Co., Ltd. with MaxQuant software (version 1.3.0.5; Max Plank Institute of Biochemistry) (Cox and Mann 2008) and searched against Uniprot (137,970 total entries) (<https://www.uniprot.org/>). The following search parameters were employed: Main search parts per million = 6; missed cleavage = 2; MS/MS tolerance ppm = 20; deisotopic = TRUE; enzyme = trypsin; database = uniprot_Triticum_137970_20150724.fasta; fixed modification = carbamidomethyl (C); variable modification = oxidation (M), acetyl (protein N-term); decoy database pattern = reverse; iBAQ = TRUE; iBAQ min. ratio count = 1; match between runs = 2 min; peptide false discovery rate (FDR) = 0.01; and protein FDR = 0.01.

Label-free quantification was carried out using the MaxQuant software as previously described (Luber et al. 2010). Protein abundance was calculated based on the normalized label-free quantitation spectral intensity. Missing values were assigned in Perseus (Tyanova et al. 2016) (software version 1.3.0.4) by replacing missing values from a normal distribution to simulate low-abundance values in a typical MS performance. Differentially accumulated proteins were defined as those with at least a twofold difference for the mean ratio ($P < 0.05$). The sequences of the detected differentially accumulated proteins were searched locally against the SwissProt database using the National Center for Biotechnology Information BLAST+ client software (ncbi-blast-2.2.28+-win32.exe) to find homologous sequences for functional annotation. The top 10 blast hits (E values $< 1e-3$) for each query sequence were selected for Blast2GO2 (version 2.7.2) analysis and subsequent GO3 mapping and annotation.

Consensus QTL intervals and database construction. The intervals of previously reported QTL from each resistant wheat accession in the panel were compared and consensus QTL intervals were established (Buerstmayr et al. 2009; Liu et al. 2009). Peptides were first projected to the cDNA library of Chinese Spring (International Wheat Genome Sequencing Consortium 2014); then, the cDNA were blasted to the Chinese Spring reference sequences (<https://wheat-urgi.versailles.inra.fr>).

Primers from GrainGenes (<https://wheat.pw.usda.gov>) were selected for electronic polymerase chain reaction (e-PCR). The e-PCR program was used to provide a reliable graphical view of the primer positions, and parameters were set as follows, -w 9, -f 1, -m 100, -t 3, D = 80 to 500, n = 2, G = 2, T = 3, and others were set

as default. The source code for the e-PCR package is freely available (<ftp://ncbi.nlm.nih.gov/pub/schuler/e-PCR/>). The proteins that fell into the target QTL intervals are listed in Supplementary Table S1.

Quantitative PCR. Reverse-transcription quantitative PCR (RT-qPCR) was carried out for five genes of interest to investigate the consistency between transcript levels and corresponding protein accumulation of predicted gene products. RT-qPCR was performed using bulked RNA from exactly the same bulked samples used for proteomics to keep consistency. Total RNA was isolated and purified from each pool using TRIzol reagent (Invitrogen). cDNA synthesis was carried out with PrimeScript RT Reagent Kit with gDNA Eraser (Takara) according to the manufacturer's instructions. Primers were designed in Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>) to amplify genes of interest (Supplementary Table S2), and actin (5'-GACAATGGAACCGGAATGGTC-3' and 5'-GTGTGATGCCAGATTTTCTCCAT-3') was used as a housekeeping gene. RT-qPCR was carried out using SYBR premix Ex Taq (Takara) on an Applied Biosystems ViiA 7 Real-Time PCR system. The samples were incubated at 50°C for 2 min, then 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 45 s. A melt curve was employed at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The experiment was carried out on three biological replicates for each pool, and the three technical replicates were included in the RT-qPCR. Relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen 2001). A multiple comparison analysis of relative expression data was performed with the Matlab software (R2014b) to determine significant differences, and a *P*-value cutoff of 0.05 was employed.

RESULTS

Protein identification. Label-free quantification resulted in the identification of 19,706 unique peptides and 4,627 proteins. Among the 4,627 proteins, the majority of the peptide sequence

coverage was less than 30% (Fig. 1A). Putative protein identifications were assigned based on at least one peptide match, where 20% of the proteins had five or more peptide hits, 1,825 proteins had two to four hits, and 1,865 proteins had only one peptide match (Fig. 1B). Putative protein names or functional categories were only assigned to those with multiple (two or more) peptide hits. Among all of the detected proteins, 2,766 proteins have predicted molecular mass of 20 to 60 kDa, 774 of 60 to 100 kDa, 760 of 1 to 20 kDa, and 327 of >100 kDa (Fig. 1C). Detected peptides were distributed across the wheat genome (Fig. 2): 6,488 peptides were mapped on the A genome, 5,943 peptides on the B genome, 4,468 peptides on the D genome, and the location of 2,807 were undetermined. In the seven homologous groups, group 2 had the largest number of peptides and group 7 had the lowest. In total, 1,508 peptides were mapped on chromosome 3B, which made up the largest proportion of peptides on the B genome.

Comparative analysis of wheat proteome. Differences in the abundance of proteins represented among the four pairs of the pools (RFg versus RM, SFg versus SM, RM versus SM, and RFg versus SFg) were analyzed (Fig. 3; Supplementary Fig. S1; Supplementary Table S3). In total, 237 proteins were differentially accumulated between the RFg and the RM pools, 238 between the SFg and the SM pools, 234 between the RM and the SM pools, and 206 between the RFg and the SFg pools. Thirty-eight common proteins were identified between the two pairs of the pools RFg versus RM and SFg versus SM. Thirty-nine proteins were in common between RM versus SM and RFg versus SFg. Gene ontology (GO) for differentially expressed proteins is presented in Figure 4 and Supplementary Table S4.

***Fusarium*-induced changes in protein accumulation in resistant or susceptible wheat.** In total, 237 proteins differed significantly in abundance between the RFg and RM pools, among which 165 proteins were upregulated in the RFg pool. Among the proteins with two or more peptide hits, 126 were annotated based on GO terms, revealing that 36 differentially regulated proteins are involved in catalytic activity, 25 proteins are plastid components,

Fig. 1. Proteomic data. **A**, Peptide sequence coverage among the identified proteins. **B**, Distribution of unique peptides identified. **C**, Molecular mass distribution of the identified proteins.

and another 25 proteins are predicted to take part in cellular processes.

In the susceptible genotypes, 238 proteins were differentially accumulated between the SFg and SM pools. Among these, 150 putative proteins significantly increased in abundance in the SFg pool. β -1,3-Glucanase (PR-2) showed seven times higher accumulation in the SFg pool. Analysis of GO terms suggests that differentially accumulated proteins in the SFg versus SM pool are mainly involved in hydrolase activity and biosynthetic process.

Fusarium-induced changes in protein accumulation in common among resistant and susceptible wheat. Some commonalities were observed in the resistant versus susceptible response to *Fusarium* challenge. Peptides from 27 proteins were upregulated in both the RFg and SFg pools when compared with their respective mock-inoculated pools, and 6 proteins in common were downregulated in both RFg and SFg. PR-1, PR-2, peroxidase (POD) 12, and peptidyl-prolyl *cis-trans* isomerase (PPIase) were upregulated in both the RFg pool and SFg pool. A nonspecific lipid-transfer protein (nsLTP) was downregulated in RFg and SFg.

Fusarium-induced changes in protein accumulation—Comparison of resistant and susceptible wheat. In total, 206 protein profiles differed between the RFg and the SFg pools, where 75 proteins were higher in the RFg pool. Of the 206 proteins, 26 are predicted to partake in biosynthetic processes and 11 are involved in

the stress response. Two proteins accumulated in RFg at three times the abundance of SFg: a photosystem II reaction center protein and a PR protein.

Comparison of *Fusarium*-induced host response proteins reported in the literature. To analyze agreements and differences with previously published studies, our results were compared with the proteins differentially enriched in response to *Fusarium*

Fig. 3. Venn diagram depicting the number of differentially accumulated proteins. **A**, Effect of *Fusarium* spp. in resistant or susceptible accessions. **B**, Effect of inoculation with and without *Fusarium* spp. Pools: mock-inoculated *Fusarium* head blight (FHB)-resistant accessions (RM), mock-inoculated FHB-susceptible accessions (SM), *Fusarium graminearum*-inoculated FHB-resistant accessions (RFg), and *F. graminearum*-inoculated FHB-susceptible accessions (SFg).

Fig. 2. Peptide distribution on the seven wheat chromosomes.

spp. in resistant genotypes (Gunnaiah et al. 2012; Zhou et al. 2005), susceptible genotypes (Chetouhi et al. 2015; Zhou et al. 2006), or both (Ding et al. 2011). These studies identified 15 to 101 differentially accumulated proteins. From the gel-free approach presented herein, a nonredundant list of differentially accumulated proteins identified in this study was compiled: in total, 632 proteins were found in at least one of the four comparisons: RFg versus RM, SFg versus SM, RM versus SM, and RFg versus SFg. When comparing the protein lists from each of the five studies with the nonredundant list generated here, there was a 15 to 37% overlap in the proteins identified (Supplementary Table S5). Meanwhile, when comparing each of the five studies one on one, less than 10% (and in some cases even less than 5%) overlap was observed. Notably, PR proteins, POD, malate dehydrogenase, alcohol dehydrogenase, GAPDH, and PPIase are frequently identified as *Fusarium*-responsive proteins.

Proteins within the consensus QTL intervals. We carried out an exhaustive review of 17 QTL intervals previously reported in the genotypes of the resistant pool to construct a consensus map underlying these QTL, and these QTL were distributed on all of the wheat chromosomes except 1D, 4A, 4D, 6A, 6D, and 7B. Proteins were projected to the target QTL regions, and the proteins localized within these QTL intervals were counted (Table 1). In total, 404 proteins were mapped onto the target QTL intervals—and, among these, 38 proteins were differentially accumulated among the four different comparisons. Those with only one peptide hit were not considered, including three proteins localized within the QTL on 2BL. In all, 81 proteins hit the QTL on 3BSc, 4 of which were differentially accumulated; and 13, 16, and 9 proteins were projected onto the QTL on 2DS, 5AS, and 6BS chromosomes, respectively. None of the proteins are aligned with the fine-mapped *Fhb1* interval on 3BS, and the proteins falling into the QTL on 3DL, 5BL, 6BS, and 7AL did not show significant differences in abundance among the four pairs. Seven differentially accumulated proteins are located within the QTL on 7DL, flanked by GWM44 and WMC702.

RT-qPCR validation of proteomics data. The putative genes encoding five of the differentially accumulated proteins were selected for RT-qPCR validation (Fig. 5). The differential gene expression patterns generally correspond with the observed protein accumulation. Transcripts of *PR-1* and *FLAVONE O-METHYLTRANSFERASE 1 (TaCOMT1)* were upregulated in both RFg and SFg but showed no significance between the two pools. No difference in protein accumulation or gene expression of PR-1 was observed between RFg and SFg. Although TaCOMT1 differed in protein accumulation between the two inoculated pools, no difference in gene expression was observed for these two comparisons. An ATP-binding cassette (*ABC*) *SUBFAMILY C TRANSPORTER (ABCC)* was upregulated in SFg when compared with either SM or RFg, and protein accumulation patterns reflected this trend. The opposite trend was observed for *GROUP 3 LATE EMBRYOGENESIS ABUNDANT PROTEIN (G3LEA)* and *PURPLE ACID PHOSPHATASE (PAP)*, both of which were downregulated in SFg compared with either SM or RFg. The putative PAP protein showed an accumulation pattern similar to that of the *PAP* transcript, whereas no peptide matching G3LEA was detected in SM and SFg. In addition, PAP displayed no significance between RM and RFg at either the transcript level or protein accumulation, whereas G3LEA was upregulated in RFg as compared with RM.

DISCUSSION

This is the first article on the *F. graminearum*–wheat interaction that focuses on the proteomics of rachis tissues in wheat. A high-throughput bulked proteomics approach was employed in order to remove genotype-specific responses, thereby enabling a holistic comparison of resistance versus susceptibility. Multiple genotypes were pooled from either FHB-resistant or -susceptible sources for differential protein analysis. Differentially accumulated proteins were associated with known QTL for FHB resistance, in hopes of facilitating the identification of resistant genes linked to these QTL.

Fig. 4. Top 20 gene ontology terms for differentially accumulated proteins: mock-inoculated *Fusarium* head blight (FHB)-resistant accessions (RM), mock-inoculated FHB-susceptible accessions (SM), *Fusarium graminearum*-inoculated FHB-resistant accessions (RFg), and *F. graminearum*-inoculated FHB-susceptible accessions (SFg). **A**, RFg versus SFg and RM versus SM and **B**, RFg versus RM and SFg versus SM.

It is anticipated that this information will benefit breeding programs through the development of diagnostic markers.

PR genes are involved in the plant defense response against different pathogens. In the host response to FHB, differential transcript (Golkari et al. 2009; Xiao et al. 2013) and protein (Ding et al. 2011; Gunnaiah et al. 2012; Zhou et al. 2005, 2006) accumulation has been reported for numerous *PR* genes. In the work presented herein, seven *PR* proteins—*PR*-1, *PR*-2, *PR*-3 (chitinase), *PR*-4 (Wheatwin-1), and *PR*-5 (thaumatin-like protein), *PR*-10 (ribonuclease-like protein), and *PR*-14 (nsLTP)—were differentially accumulated in response to *F. graminearum*. However, in the case of *PR*-4 and *PR*-10, only single peptide matches were observed, thereby reducing confidence in the identity assigned for these two gene products. Gene expression analysis validated the *F. graminearum*-induced upregulation of *PR*-1 in both resistant and susceptible pools (Fig. 5), suggesting that *PR*-1 accumulation might not be exclusive to resistant or susceptible genotypes. Similarly, other groups have shown that expression of *PR*-1 through -5 is

upregulated by *F. graminearum* in both resistant and susceptible wheat (Pritsch et al. 2000). However, an earlier or stronger response of *PR* expression may contribute to a resistance response (Bernardo et al. 2007; Pritsch et al. 2000).

Members of the *PR*-14 family, nsLTP, were more pronounced in the *F. graminearum*-treated susceptible pool (SFg) than that in the resistant one (RFg). Interestingly, constitutive or transgenic over-expression, as opposed to induced expression, of various LTP have been associated with resistant phenotypes (Foroud et al. 2012; Schweiger et al. 2013; Zhu et al. 2012). It should also be noted that a subset of wheat nsLTP were previously found to have antifungal activity toward *F. graminearum* in axenic cultures (Sun et al. 2008).

PR gene expression is often regulated by plant signaling hormones. For example, jasmonic acid (JA) and ethylene (ET) coregulate *PR*-3, *PR*-4, and *PR*-12 (also called *PDF1.2*) genes. Both the JA and ET pathways have been implicated in the FHB resistance response (Gottwald et al. 2012; Li and Yen 2008; Makandar et al. 2010; Qi et al. 2016). In this work, accumulation of ET and JA

TABLE 1. Consensus quantitative trait loci region known for Fusarium head blight resistance flanked by two markers

Chromosome	Intervals	Proteins (<i>n</i>)		Wheat variety
		Mapped ^a	Accumulated ^b	
1AS	WMC24–WMC120	55	3	Huangfangzhu
1AL	WMC59–BARC158	8	1	Wangshuibai
1BS	BARC128–GWM18	29	2	Wangshuibai, Huangfangzhu
2AS	BARC212–GWM515	55	8	Ning 7840, Wangshuibai
2BL	GWM120–BARC101	0	0	Tokai-66, Ning 7840
2DS	GWM296–GWM484	13	3	Wangshuibai, Sumai 3, Nyubai
3AS	GWM2–GWM674	33	2	Tokai-66, Baishanyuehuang, Wangshuibai, Huangcandou
3BS	BARC133–SNP7	0	0	Baishanyuehuang, Huangcandou, Huangfangzhu, Ning 7840, Sumai3, Wangshuibai, Tokai-66, Nyubai, Taiwan Wheat
3BSc	GWM566–GWM131	81	4	Nyubai, Wangshuibai, Huangcandou
3DL	GWM664–GWM645	4	0	Wangshuibai
4BL	WMC511–WMC349	43	4	Wangshuibai
5AS	GWM205–GWM129	16	3	Nyubai, Baishanyuehuang, Huangfangzhu, Wangshuibai
5BL	GWM335–GWM371	9	0	Wangshuibai
5DL	CFD57–GWM212	4	1	Wangshuibai
6BS	GWM518–GWM644	9	0	Sumai3, Wangshuibai
7AL	GWM276–GWM282	4	0	Sumai3, Wangshuibai, Huangfangzhu
7DL	GWM44–WMC702	41	7	Wangshuibai

^a Number of mapped proteins.

^b Number of differentially accumulated proteins.

Fig. 5. Changes in relative gene expression (black bars) and protein accumulation (white bars) of five wheat genes. Pools: mock-inoculated Fusarium head blight (FHB)-resistant accessions (RM), mock-inoculated FHB-susceptible accessions (SM), *Fusarium graminearum*-inoculated FHB-resistant accessions (RFg), and *F. graminearum*-inoculated FHB-susceptible accessions (SFg). Reverse-transcription quantitative polymerase chain reaction data were normalized to the reference gene *TaActin*, where the relative expression of this gene was set to 1 in RM. Label-free quantitation intensity data (protein) from each experiment was log₁₀ transformed. *PR*-1 = pathogenesis-related protein 1, *TaCOMT1* = flavone O-methyltransferase 1, *ABCC* = ATP-binding cassette subfamily C transporter, *G3LEA* = group 3 late embryogenesis abundant protein, and *PAP* = purple-acid phosphatase.

biosynthesis enzymes 1-aminocyclopropane-1-carboxylate oxidase and 12-oxo-phytodienoic acid reductase 2 (OPR2), respectively, did not differ significantly among the four pools. The *OPR2* gene is located within the QTL on chromosome 7DL. However, *OPR1* was upregulated in RFg compared with RM, suggesting that JA biosynthesis is activated at 3 dpi, if not sooner. Similar results were reported in a study on *Fusarium* crown rot disease of wheat; in their work, Powell et al. (2017) also measured JA but did not observe any changes in JA accumulation despite upregulation of *OPR* transcripts.

Lignin biosynthesis can be positively regulated by JA signaling (Xue et al. 2008), and the activities of COMT and phenylalanine ammonia lyase (PAL) are involved in this process (https://www.genome.jp/kegg-bin/show_pathway?ko00940+C00482). *TaCOMT1* was markedly upregulated in the RFg pool compared with the SFg pool, whereas the level of *TaCOMT1* transcript was not affected (Fig. 5). PAL catalyzes the first step of the phenylpropanoid pathway (Dixon et al. 2002). Unexpectedly, PAL was observed at a higher abundance in SFg when compared with either SM or RFg. This differs from previous reports of *Fusarium*-induced PAL upregulation at the transcript level in Sumai 3 and CM82036 (Golkari et al. 2009; Steiner et al. 2009). The expression of this enzyme in a resistance response may have spatial or temporal order during wheat infection. Alternatively, PAL may not be specific to resistant genotypes.

In an effort to provide new insights into potential core factors behind QTL-mediated defense against FHB, 404 QTL-oriented proteins were distributed on 17 intervals, and the number of proteins falling into each QTL varied with the QTL and QTL interval size.

The *Fhb1* QTL from Sumai 3 was reported to encode a *PORE-FORMING TOXIN-LIKE (PFT)* gene conferring FHB resistance (Rawat et al. 2016). In the present study, all 10 resistant accessions in the bulk carry the *PFT* allele (Rawat et al. 2016), which is absent in all 6 susceptible accessions. Because the Chinese Spring reference genome does not carry *Fhb1*, the peptide sequences identified in the current study were screened for matches directly against the encoded PFT gene product (accession number AOZ21512). Two peptide hits were observed (data not shown) in the resistant accessions but only in the mock-inoculated pool (RM). PFT was absent in the susceptible pools (SM or SFg) but was also not detected as part of the *Fusarium* response (i.e., RFg pool). The proteomic analysis presented here captured one time point after inoculation (3 dpi) in one tissue type (rachis node). It may be that *Fusarium*-induced PFT accumulation occurs in different tissues or at different times during the host–pathogen interaction. Recently, He et al. (2018) observed differences in the *PFT* expression profile patterns among 12 wheat varieties carrying the Sumai 3-derived *Fhb1* QTL. The reference genome derived from Chinese Spring, a susceptible accession, may be a masking factor for identifying proteins located within the *Fhb1* region, as was the case for PFT.

It had also previously been proposed that the *Fhb1* locus encodes or regulates an enzyme with UDP-glycosyltransferase activity, which can detoxify DON by catalyzing the addition of a glucose at carbon-3, yielding DON-3-*O*-glucoside (Lemmens et al. 2005). In this study, two UDP-glycosyltransferases were identified in the proteome, neither of which were mapped to the *Fhb1* locus, and no significant changes were observed among the four pairs.

ABC transporters involved in trichothecene tolerance (Walter et al. 2015) have previously been detected with greater transcript abundance upon FHB challenge in resistant genotypes (Kosaka et al. 2015; Xiao et al. 2013). In the work presented herein, an ABCC positioned within the 3BSc QTL was higher in both transcript and protein abundance in the SFg than in the RFg pool. Wheat lines carrying *Fhb1*, on 3BS, were previously discriminated from the null lines in an expression profile study (Walter et al. 2008). Meanwhile, three ABC transporters have been offered as candidate resistance QTL on chromosomes 2DS (Handa et al. 2008), 2DL (Biselli et al. 2018), and 6BS (*Fhb2*) (Cuthbert et al. 2007).

Plants generate reactive oxygen species (ROS) by activating numerous oxidases in response to adverse environmental and biotic stresses (Apel and Hirt 2004). ROS scavenging enzymes such as catalase (CAT) and POD are commonly upregulated as part of the defense response (Mittler et al. 2004). Differential expression of genes encoding ROS scavengers and their gene products have been described in the host response to *Fusarium* spp. (Boddu et al. 2006; Golkari et al. 2007; Zhou et al. 2005, 2006). Transcript-based comparisons of resistant versus susceptible genotypes have suggested that the ROS pathway is either more active in susceptible genotypes (Xiao et al. 2013) or upregulated earlier in resistant accessions (Ding et al. 2011; Foroud et al. 2012). In this study, although two CAT and eight POD (some of which were mapped to QTL) were identified, no remarkable pattern of accumulation was observed for these two classes of ROS scavengers. However, two other candidate for ROS metabolism (Li et al. 2008; Liu et al. 2011; Mowla et al. 2006; Veljanovski et al. 2006) were differentially accumulated: a 55-kDa PAP protein (located on the 7DL QTL for FHB resistance) and the LEA protein G3LEA (mapped within the QTL on 4BL flanked by WMC511 and WMC349). Two additional LEA proteins are associated with the QTL localized on chromosome 1AL (flanked by WMC59 and BARC158); however, their accumulation patterns did not differ among the four pairs. The PAP protein was downregulated in the susceptible pool following inoculation with *F. graminearum* (SFg versus SM). Surprisingly, this protein was not detected in the resistant pools, whereas transcripts of the encoding gene were present in both RM and RFg (Fig. 5).

The G3LEA protein accumulated in the resistant pools but was undetected in the two susceptible pools. RT-qPCR shows that *G3LEA* is significantly increased in RFg as compared with its mock (RM) and also with SFg (Fig. 5). Of note, *G3LEA* was downregulated in the SFg when compared with the SM, whereas the encoded protein was not detected in either pool. Thus, G3LEA may play a positive role in regulating FHB resistance during fungal infection. Accumulation of LEA proteins and expression of genes involved in desiccation stress are predicted to protect cellular structures (Bray 1993; Toller et al. 2007; Welin et al. 1994). A barley *HVA1 (LEA3)* gene, a member of the G3LEA protein family, confers drought tolerance in transgenic wheat and rice (Sivamani et al. 2000; Xu et al. 1996). The LEA superfamily of proteins has been described with a vast array of functions involving the cytoskeleton, membrane stabilization, Ca²⁺ binding, and molecular chaperone activities (Hundertmark et al. 2011; Wise and Tunnacliffe 2004). Thus, in addition to or as part of their putative functions in protection against oxidative stress (Liu et al. 2011; Mowla et al. 2006), LEA proteins may have activities in cellular membrane channels and signal transduction.

By eliminating the genotype-specific responses through a bulked-proteomics approach, we have identified new candidates for FHB resistance (G3LEA) and susceptibility (PAP). In addition, numerous proteins previously reported in wheat or barley spikelets following *Fusarium* inoculation, including PR proteins, PAL, PPIase, and ABC transporters, were also found to be differentially accumulated at the rachis node. By focusing the analysis on QTL regions for FHB resistance, these results can be used to further develop markers for wheat breeding programs.

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