# Identification of proteins induced or upregulated by Fusarium head blight infection in the spikes of hexaploid wheat (*Triticum aestivum*)

**Wenchun Zhou, Frederic L. Kolb, and Dean E. Riechers**

**Abstract:** Fusarium head blight (FHB) caused by *Fusarium graminearum* is a destructive disease of wheat and barley. It causes economic losses due to reduction in both yield and quality. Although FHB resistance has been well documented and resistant cultivars have been developed to reduce incidence and severity of FHB, there is a limited understanding of the molecular mechanisms involved in plant resistance against the infection and spread of *F. graminearum*. In the current study, 2-dimensional displays of proteins extracted from wheat spikelets infected with *F. graminearum* were compared with those from spikelets inoculated with sterile $H_2O$. Fifteen protein spots were detected that were either induced (qualitatively different) or upregulated (quantitatively increased) following *F. graminearum* infection of spikelets of 'Ning7840', a resistant cultivar. These proteins were identified by LC-MS/MS analysis. Proteins with an antioxidant function such as superoxide dismutase, dehydroascorbate reductase, and glutathione *S*-transferases (GSTs) were upregulated or induced 5 d after inoculation with *F. graminearum,* indicating an oxidative burst of $H_2O_2$ inside the tissues infected by FHB. An ascorbate-glutathione cycle is likely involved in reduction of $H_2O_2$. Expression of proteins with highest similarity to dehydroascorbate reductase and TaGSTF5 (a glutathione *S*-transferase) differed following FHB infection in susceptible and resistant cultivars. A 14-3-3 protein homolog was also upregulated in FHB-infected spikelets. In addition, a PR-2 protein (β-1, 3 glucanase) was upregulated in FHB-infected spikes, which is in accord with a previous study that analyzed transcript accumulation.

*Key words:* Fusarium head blight, scab, 2-dimensional electrophoresis, LC-MS/MS, protein, wheat, *Triticum aestivum*.

**Résumé :** La fusariose de l'épi (FHB), causée par le *Fusarium graminearum*, est une maladie dévastatrice chez le blé et l'orge. Elle cause des pertes économiques en raison de réductions du rendement et de la qualité. Bien que la résistance à la fusariose soit bien documentée et que des cultivars résistants aient été développés pour réduire l'incidence et la sévérité de la fusariose, les mécanismes moléculaires impliqués dans la résistance à l'infection et à la propagation du *F. graminearum* demeurent peu connus. Dans le présent travail, des gels bidimensionnels ont été employés pour comparer les profils protéiques d'épillets de blé infectés par le *F. graminearum* ou inoculés avec de l'eau stérile. Quinze protéines montrant une induction (différence qualitative) ou surexpression (augmentation quantitative) ont été détectées suite à l'infection des épillets de 'Ning7840', un cultivar résistant. Ces protéines ont été identifiées par analyse LC-MS/MS. Des protéines antioxydantes telles qu'une superoxyde dismutase, une déshydroascorbate réductase et des glutathion S-transférases (GST) étaient surexprimées ou induites 5 jours après inoculation avec le *F. graminearum*, ce qui suggère un burst oxydatif de $H_2O_2$ au sein des tissus infectés par le FHB. Un cycle ascorbate-glutathion est vraisemblablement impliqué dans la réduction de la quantité de $H_2O_2$. L'expression de protéines montrant la plus forte similarité avec la déshydroascorbate réductase et la TaGSTF5 (une glutathion *S*-transférase) différait suite à l'infection chez les cultivars résistants et sensibles. Un homologue de protéine 14-3-3 était également surexprimé chez les épillets infectés. De plus, une protéine PR-2 (β-1,3 glucanase) était surexprimée chez les épillets infectés, une observation qui concorde avec une étude antérieure qui portait sur l'abondance des transcrits.

*Mots clés :* fusariose de l'épi, électrophorèse bidimensionnelle, LC-MS/MS, protéine, blé, *Triticum aestivum*.

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## Introduction

Fusarium head blight (FHB) or scab, caused mainly by *Fusarium graminearum* Schwabe and sometimes by other *Fusarium* species such as *Fusarium culmorum* and *Fusarium avenaceum*, is a severe disease of *Triticum* spp. and *Hordeum vulgare* (Stack 2000). Fusarium head blight causes significantly lower grain yield, lower test weight, reduced grain quality, and reduced milling yield. The fungus also produces trichothecene mycotoxins (such as deoxynivenol) that are detrimental to both humans and livestock. Shriveled grains contaminated with mycotoxins are commonly observed in susceptible cultivars infected by FHB (Bai and Shaner 1994). This results in significant loss in value for both the producer and the wheat and barley milling industry (McMullen et al. 1997).

Although application of fungicides at flowering may reduce damage due to scab, the best means of control for this disease is the development of resistant cultivars. Introduction of scab-resistant wheat cultivars would contribute to improved food safety and reduce losses suffered by wheat growers and industry. As a result, the development of scab-resistant cultivars is a high priority breeding objective for many wheat breeding programs.

Sources that are FHB-resistant have been identified in wheat and used to breed FHB-resistant cultivars adapted to local environments. Since evaluation of FHB resistance of breeding lines is laborious, time-consuming, and costly, PCR-based molecular markers, such as simple sequence repeats (SSR) linked to FHB resistance quantitative trait loci (QTL) from resistant cultivars such as 'Sumai 3' and its derived lines, have been developed and applied in marker-assisted selection for FHB resistance (Anderson et al. 2001; Bai et al. 1999; Buerstmayr et al. 2002, 2003; Zhou et al. 2000, 2002, and 2003). However, there is a limited understanding of the molecular mechanisms involved in plant resistance against the infection and spread of *F. graminearum*.

Wheat responds to *F. graminearum* infection by inducing various defenses. Molecular characterization of cDNA clones and expressed sequence tags from FHB-infected spikes revealed that transcript levels of many pathogenesis-related (PR) genes increased upon FHB infection (Li et al. 2001; Pritsch et al. 2000, 2001). Different classes of pathogenesis-related proteins (PR-proteins) including PR-1, PR-2 (β-1,3 glucanases), PR-3 (chitinases), PR-5 (thaumatin-like protein), and PR-9 (peroxidases) were induced within 6–12 h of inoculation. Transcript levels reached peak levels within 36–48 h after inoculation (Li et al. 2001; Pritsch et al. 2000, 2001). Through measurement of guaiacol-peroxidase and polyphenol oxidase activities in susceptible and resistant wheat spikes at flowering, milk, dough, and ripening stages after inoculation with *F. graminearum* conidia at anthesis, a 3-fold increase in the polyphenol oxidase activity was detected in resistant wheat cultivars when compared with the uninoculated controls (Mohammadi and Kazemi 2002).

Proteomic techniques provide a novel approach to studying the mechanism of plant resistance against biotic and abiotic stresses. Using 2-dimensional electrophoresis (2-DE) and proteomic techniques, specific proteins have been shown to be differentially expressed in salt-stressed wheat (Majoul et al. 2000; Ouerghi et al. 2000), heat-stressed wheat (Majoul et al. 2003), herbicide safener-treated wheat (Zhang and Riechers 2004), ozone-stressed rice (Agrawal et al. 2002), wounded rice (Shen et al. 2003*a*), and gibberellin-treated rice (Shen et al. 2003*b*). One of the major advantages of the 2-DE technique is that differentially expressed proteins can clearly and reproducibly be detected between sensitive versus tolerant lines, or between infected (stressed) versus uninfected (nonstressed) conditions. Proteins that are qualitatively or quantitatively different in their expression levels among treatments have a high likelihood of playing an important role in the response of the plant to a given stress. Further identification of these differentially expressed proteins by LC-MS/MS can provide powerful insight into the molecular mechanism of resistance and underlying functions of these proteins in determining resistance or tolerance in plants. The objective in the current study was to compare and identify differentially expressed proteins in FHB-susceptible and -resistant wheat lines under infected and uninfected conditions.

## Materials and methods

### Preparation of conidia inoculum

Cultures of *F. graminearum* were isolated from infected hexaploid wheat seed. About 30 *F. graminearum* isolates were isolated from infected kernels of 20 wheat genotypes including both susceptible and resistant genotypes. The media used for conidiospore production contained 1.5% carboxymethylcellulose (CMC), 0.1% $NH_4NO_3$, 0.1% $KH_2PO_4$ monobasic, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.1% yeast extract. Media were prepared by mixing the boiling solution vigorously with a magnetic stirrer. The flask was covered to control evaporation during heating. Stirring continued until all CMC was dissolved. About 70 mL of liquid CMC was poured into each 250 mL flask. A stopper was placed in the top of each flask, and the flasks were autoclaved at 250 °C and 18 psi for 20 min. Each flask was inoculated in a laminar flow hood with an agar plug from a clean *F. graminearum* isolate. The flasks were placed on a shaker table and swirled gently at 150 rpm for 4–5 d at room temperature (about 22 °C). The number of conidiospores per mL was determined by counting spores using a hemacytometer. To include approximately the same number of conidiospores from each isolate in the mixture of isolates used for inoculation the amount of inoculum of each isolate was adjusted based on the conidiospore counts.

### Plant culture and sample preparation

Fusarium head blight-resistant cultivar 'Ning7840' was used for this study, and a susceptible cultivar, 'Clark', was also examined for comparison purposes. 'Ning7840' and 'Clark' were previously used to develop a mapping population for detecting resistance QTL in 'Ning7840' (Bai et al. 1999, Zhou et al. 2002).

Seeds were germinated in plastic trays filled with vermiculite. After seedling emergence, plants were transferred to a vernalization chamber for 8 weeks at 4 °C with a 16-h photoperiod. Vernalized plants were transplanted into 15-cm pots and grown in a greenhouse. About 30 pots of each

cultivar were placed randomly on a bench in a greenhouse maintained at 24 °C with a 16-h photoperiod. Identical water and fertilizer management procedures were used for all plants during the entire growing period.

Wheat spikes at midanthesis were inoculated with conidiospores or deionized water using separate syringes. About 1000 conidiospores in a volume of 10 µL were injected into 2 flowering florets of a spikelet. As a control, the same volume of deionized water was injected into flowering spikelets on a different plant of the same cultivar. The inoculated spikelets were marked; and the time and date of inoculation were recorded. Inoculated plants were placed in a mist chamber immediately after inoculation. Four nozzles on a pipe near the top of the chamber provided a fine spray mist with a volume of 18 mL of water every 10 min to maintain a high relative humidity inside the chamber. The mist chamber was in the same greenhouse room where the plants were grown. The plants remained in the mist chamber for 72 h and were then moved back to the original bench. Five days postinoculation, spikes were harvested by cutting with scissors. Immediately, the spikes were placed on ice and then transferred into a –80 °C freezer for storage until protein extraction. Spikelets from 15 plants inoculated with either $H_2O$ or *F. graminearum* were mixed together as an independent sample for each cultivar (1 spikelet from 15 different plants). This sampling process was repeated 3 times for each cultivar.

**Protein extraction and quantification**

Frozen spikelet samples were ground using a mortar and pestle prechilled with liquid nitrogen. Protein extractions from ground tissue were prepared by the TCA-acetone precipitation method (Damerval et al. 1986). Briefly, 0.3 g of finely ground plant tissue was collected into 2-mL microcentrifuge tubes, and 1 mL 10% (*w/v*) trichloroacetic acid and 0.05% (*v/v*) 2-mercaptoethanol in cold (–20 °C) acetone was added. The samples were incubated for 2 h at –20 °C to precipitate proteins and then centrifuged for 20 min at 14 000*g*. The pellet of precipitated proteins was washed with 1 mL cold acetone containing 0.05% *v/v* 2-mercaptoethanol several times until the pellet was colorless. A 10-min centrifugation at 14 000*g* was used to precipitate the pellet in each wash. The pellet was dried under vacuum for 10 min, and the proteins were resuspended in 1 mL rehydration buffer (8 mol urea/L, 2% 3-[3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 20 mmol DTT/L, and 0.5% carrier ampholytes pH 3–10). After centrifugation at 14 000*g* for 10 min, the supernatant was collected, and a 10 µL sample was removed for protein assay. The remaining supernatant was stored at –80 °C until 2-DE. Protein concentration of 2-D samples was determined using Bradford's method with bovine serum albumin as standards (Bradford 1976).

**Isoelectric focusing (IEF) and SDS–PAGE**

A calculated volume containing 120 µg of protein sample was added and mixed with rehydration buffer from Bio-Rad (Hercules, California, USA) to a total volume of 350 µL. The protein solution was passively rehydrated for 16 h and absorbed into 17-cm pH 4–7 and pH 5–8 Bio-Rad Ready Gel Strips (Hercules, California, USA) as described by the manufacturer's manual. The IEF steps were 250 V for 1 h, 500 V for 1 h, 500–10 000 V for 5 h with a linear gradient, 10 000 V for 6 h, and a 500-V hold. After IEF, the strips were either kept at –20˚ C or directly used in SDS–PAGE. The strips were equilibrated in equilibration buffer I (6 mol urea/L, 2% (*w/v*) SDS, 0.05 mol Tris–HCl/L, (pH 8.8), 20% (*v/v*) glycerol, 2% (*w/v*) dithiothreitol) at ambient temperature for 15 min, and then in equilibration buffer II (6 mol urea/L, 2% (*w/v*) SDS, 0.05 mol Tris–HCl/L, (pH 8.8), 20% (*v/v*) glycerol, 2.5% (*w/v*) iodoacetamide) for another 15 min. After equilibration, the strips were positioned on top of the 2nd-dimension gel and sealed with 1% (*w/v*) agarose. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis was performed on 15% (*w/v*) polyacrylamide gels. The gels were run for 30 min at 30 mA followed by 60 mA for 5 h.

**Staining of 2-DE gels and protein imaging**

Analytical gels were fixed in solution I (40% (*v/v*) ethanol, 10% (*v/v*) acetic acid) for 1 h and then in solution II (5% (*v/v*) ethanol, 5% (*v/v*) acetic acid) overnight. Gels were washed with distilled water twice, 20 min per wash. The gels were further fixed with 5% (*v/v*) glutaraldehyde for 1 h and then washed 4 times with distilled water, each time for at least 30 min. The gels were stained in silver solution (0.02 N NaOH, 0.6% (*w/v*) $NH_4OH$, 0.8% (*w/v*) $AgNO_3$) for 30 min. Gels were quickly washed 3 times with distilled water and then developed in buffer containing 0.005% (*w/v*) citric acid and 0.02% (*v/v*) formaldehyde until the spots on the gels were visible. The reaction was stopped with 5% acetic acid. For preparative purposes (samples for submission for LC-MS/MS), the Silver Stain Plus kit (Bio-Rad, Hercules, California, USA) was used according to the manufacturer's instructions. Silver-stained gels were scanned with a flatbed scanner. The image analysis in this study was performed visually, and the changes observed were both qualitative and quantitative in nature. To confirm that the induced changes in proteins were reproducible, triplicate gels were run for each sample. Those proteins that were visually identifiable as not present in controls, or increased over controls, were selected for further analysis by LC-MS/MS protein identification. The induced (qualitatively different) and upregulated (quantitatively increased) protein spots were excised from gels and dried for LC-MS/MS analysis. The protein image annotation was analyzed with Melanie 4 Viewer (http://us.expasy.org/melanie/).

**LC-MS/MS**

Silver-stained protein spots were manually excised individually from the 2D-PAGE gels and dried by vacuum centrifugation. Identification of protein spots was conducted by the Stanford University Mass Spectrometry facility, using µ-ESI-LC-MS/MS. The spots were destained and reduced with dithiothreitol, alkylated with acrylamide and digested with trypsin (Promega, Madison, Wisconsin, USA). The resulting peptide solution was analyzed on a Micromass CapLC and Q-Tof API US (Manchester, UK) LC-MS system. A peptide CapTrap (Michrom Bioresources, Auburn, California, USA) was used for online desalting, followed by back flushing onto a 0.075 × 100 mm PepMap™ $C_{18}$ column (LC Packings, Amsterdam, Netherlands). Peptides were eluted from the column with a 30-min linear gradient of 3%–45% solvent B (solvent A: $H_2O$, 2% ACN, 0.1% formic acid; solvent B: ACN, 2% $H_2O$, 0.1% formic acid) at a flow

rate of ~300 nL/min. Solvent A and solvent B were used to equilibrate the HPLC column and resolve and elute peptides, respectively. The standard Micromass nanospray source with blunt-tip 90 μm OD, 20 μm ID fused silica emitter was held at 80 °C, capillary voltage +3.4 kV, cone voltage 32 V. Data acquisition was performed in data dependent mode, with up to 3 precursors for MS/MS selected from each MS survey scan. The.pkl files generated by Micromass ProteinLynx (Waters Corporation, Milford, Mass.) software were searched against the NCBI NR protein database using the Mascot MS/MS Ion Search (http://www.matrixscience.com).

## Results and discussion

### Identification of upregulated or induced proteins in *F. graminearum*-infected spikelets

Comparison of 2-DE images indicated there were both qualitative and quantitative differences between protein profiles from FHB-infected and control spikes in 'Ning7840' as shown in Fig. 1 (proteins separated from pH 4 to 7) and Fig. 2 (proteins separated from pH 5 to 8). In total, 2 proteins (spots 5 and 6) were detected and categorized as qualitatively different (new proteins not detected in the control), and 11 proteins were categorized as quantitatively increased (upregulated) after infection with *F. graminearum* from spikelets harvested 5 d after inoculation. Two additional proteins (spots 14 and 15) were identified as fungal proteins (Fig. 2) both in 'Ning7840' and 'Clark' spikes inoculated with *F. graminearum*. These 15 proteins were further analyzed by LC-MS/MS for protein identification (Table 1), and were characterized according to their potential functional roles through bioinformatic analyses.

### PR-2 proteins

Protein spot 2 in Fig. 1 was found to be upregulated after FHB infection in the spikelets of 'Ning7840' and was identified as β-1, 3 glucanase based on LC-MS/MS results. This confirmed a previous report examining mRNA levels in which β-1, 3 glucanases accumulated in response to FHB infection (Li et al. 2001). β-1, 3 glucanase transcripts were observed to accumulate as early as 6 to 12 h after infection with *F. graminearum* both in a resistant wheat cultivar, 'Sumai 3', and in a susceptible wheat cultivar, 'Wheaton' (Pritsch et al. 2000). β-1, 3 glucanases are important components of plant defense responses to pathogen infection, and they are classified as PR-2 proteins (Bowles 1990; Collinge et al. 1993). β-1, 3 glucanases inhibit the growth of many fungi in vitro by hydrolyzing β-glucan of fungal cell walls. Furthermore, oligomeric products of digested β-glucan can act as signal molecules to stimulate further defense responses (Kim and Hwang 1997).

### Proteins with antioxidant functions

Protein spot 11 (Fig. 1) was upregulated in 'Ning7840' spikelets inoculated with *F. graminearum* and showed the highest identity with superoxide dismutase (Mn) in wheat. Spot 5 was only induced in FHB-infected 'Ning7840' but not in 'Clark' spikelets and was identified as dehydroascorbate reductase (DHAR) of wheat (Fig. 1A). Spot 10 (Fig. 1) was found to be upregulated after FHB infection and had the highest identity with a glutathione-dependent DHAR of rice.

The plasma membrane of plant cells produces $H_2O_2$ in response to both biotic and abiotic stimuli (Doke et al. 1994). Reactive oxygen species such as $H_2O_2$ and $O_2^-$ are suggested to play an important role in plant–pathogen interactions (Levine et al. 1994). Superoxide dismutase, ascorbate peroxidases, DHAR, and glutathione reductase are key enzymes involved in reducing $H_2O_2$ in the ascorbate–glutathione cycle in plant cells (Vanacker et al. 1998). Superoxide dismutase and DHAR were found to be either induced or upregulated in the FHB-infected 'Ning7840' spikelets compared with spikelets inoculated with $H_2O$.

Identification of these proteins provides the first direct evidence for induced expression of superoxide dismutase and DHAR in wheat spikes in response to FHB infection. This also suggests, indirectly, that there are $H_2O_2$ bursts in the wheat spikes in response to FHB infection and spread. Pathogen-induced $H_2O_2$ production has several effects. (*i*) It hinders penetration of the pathogen by stimulating peroxidase activity and by cross-linking cell walls at the site of contact; (*ii*) it poses a stress on the pathogen as well as the host cell generating the oxidative burst; and (*iii*) it acts as a diffusible signal that leads to systemic acquired resistance (Noctor and Foyer 1998). High intracellular levels of $H_2O_2$ cause the activation of plant cell death and defense mechanisms during pathogen invasion (Takahashi et al. 1997). Superoxide dismutase and DHAR are critical $H_2O_2$ scavenging enzymes found in the cytosol, chloroplast, and mitochondria of higher plants (Asada 1992; Karpinski et al. 1997; Mittler and Lam 1995; Patterson and Poulos 1995). These proteins are induced in response to many environmental stresses and result in the detoxification of reactive oxygen species (Jiménez et al. 1997). Typically, 3 d after inoculation with *F. graminearum*, wheat spikelets start to show lesions due to plant cell death. Induced DHAR resulting from *F. graminearum* invasion might lower $H_2O_2$ levels and limit cell death in neighboring spikelets. 'Ning7840' is highly resistant to FHB spread within spikes after infection; the observed symptoms are usually restricted to inoculated spikelets, while symptoms start spreading to neighboring spikelets within susceptible wheat spikes.

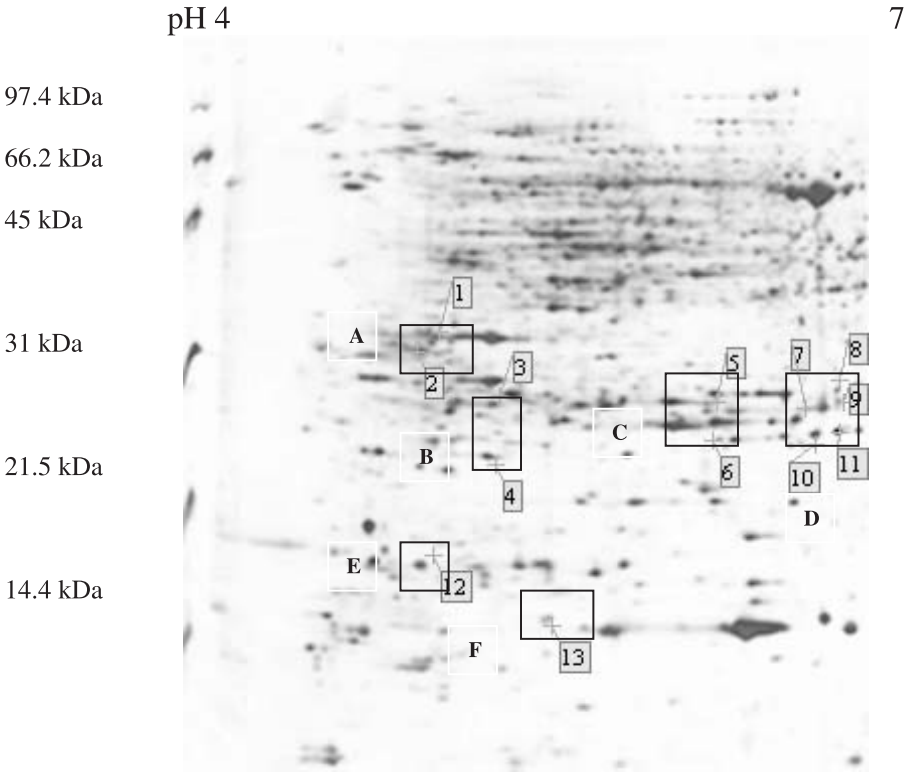
### Peroxiredoxins

Protein spot 12 was most similar to a peroxiredoxin-like protein in *Arabidopsis*. It was upregulated in the FHB-infected spikelets in 'Ning7840' (Fig. 1). Peroxiredoxins can reduce $H_2O_2$, alkyl hydroperoxides and hydroxyl radicals, and they have been shown to have antioxidant activity in vitro through protection of lipids, enzymes, and DNA against radical attack (Baier and Dietz 1997; Lim et al. 1993; Netto and Stadtman 1996). Detection of this protein in FHB-infected spikelets is consistent with the oxidative burst in infected cells as mentioned previously.

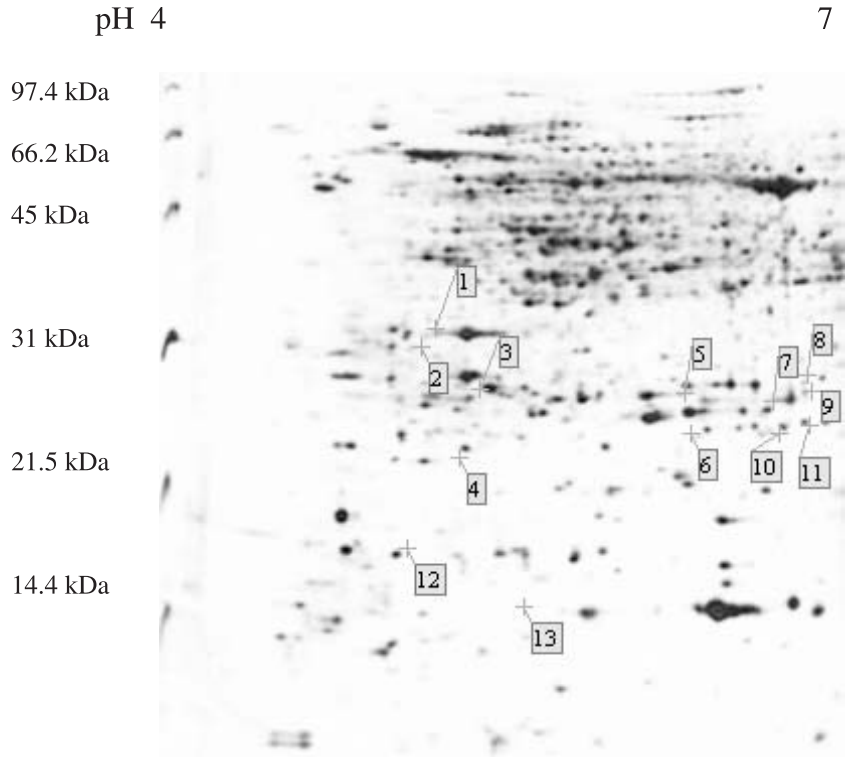
### Glutathione *S*-transferases

Six proteins showed high similarity to glutathione *S*-transferases (GSTs). One GST (spot 6 in Fig. 1) was qualitatively different, and 5 other GST proteins (spots 3, 4, 7, 8, and 9 in Fig. 1) were upregulated in *F. graminearum*-infected spikelets of 'Ning7840'.

**Fig. 1.** Silver-stained patterns of protein samples in the pH range of 4 to 7 extracted from 'Ning7840' spikelets 5 d after inoculation with *Fusarium graminearum* (A) or after inoculation with $H_2O$ (B). Spots 1–13 were analyzed with LC-MS/MS identification in Table 1. Boxed regions (A, B, C, D, E, and F) are enlarged in Fig. 3.

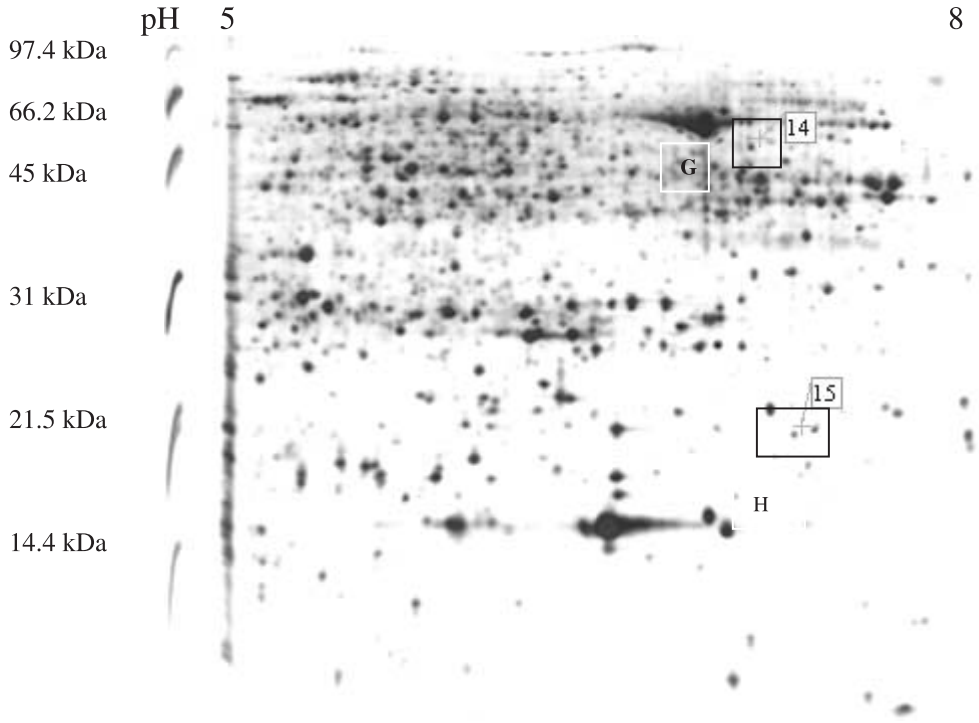



A

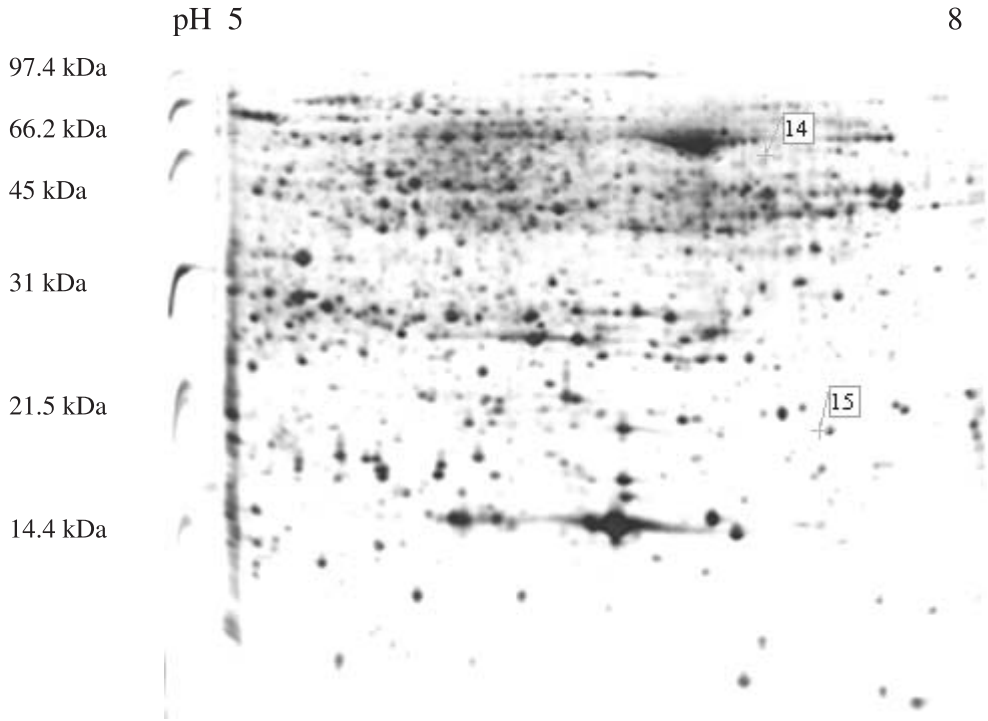



B

**Fig. 2.** Silver-stained patterns of protein samples in the pH range of 5 to 8 extracted from 'Ning7840' spikelets 5 d after inoculation with *Fusarium graminearum* (A) or after inoculation with $H_2O$ (B). Spots 14 and 15 were analyzed with LC-MS/MS identification in Table 1. Boxed regions (G and H) are enlarged in Fig. 3.




A




B

**Table 1.** Identification of proteins from spikes of 'Ning7840' responsive to *Fusarium graminearum* infection by LC-MS/MS.

| Spot No. | GenBank protein identifier No. | Homologous protein (best matching proteins) | Mascot score* | Sequences/sites of peptides identified by LC-MS/MS |
|---|---|---|---|---|
| 1 | S18911 | 14-3-3 protein | 493 | 1. LAEQAERYEEMVEFMEK/17–33<br>2. TADVGELTVEER/37–48<br>3. IISSIEQKEESR/68–79<br>4. LLDSHLVPSATAAESK/109–124<br>5. KEAAENTLVAYKSAQDIALADLPTTHPIR/148–176<br>6. DNLTLWTSDNAEEGGDEIKEAASKPEGEGH/232–261 |
| 2 | AAD28734 | β-1,3-glucanase | 270 | 1. RACMFSVALALLGVLL†/5–20<br>2. AVQSIGVCY/26–34<br>3. YQSNGI/51–56<br>4. ALSGSNIDLIIDVANEDLAS† /71–90<br>5. PLLANVYPYFSYVDNQA†/191–207<br>6. HFGLFNPDKSPAYPISF/318–334 |
| 3 | CAC94005 | glutathione *S*-transferase | 512 | 1. AQMALAVKGLSYDYLPEDRWSTSDLLIASNPVYK†/24–57<br>2. FWAAYVNDK†/105–113<br>3. MEETLSG†/134–140<br>4. LAGLGPIIDPAR†/186–197<br>5. LAAWAERFSVAEPIK†/201–215<br>6. LEEYITTALYPK†/224–235 |
| 4 | CAC94005 | glutathione *S*-transferase | 90 | 1. KGLSYDYLPEDR†/32–42<br>2. FWAAYVNDK/105–113 |
| 5 | AAL71854 | dehydroascorbate reductase | 157 | 1. VLLTLEEK/25–32<br>2. ATKE NLIAGWAPK†/197–209 |
| 6 | CAD29478 | glutathione *S*-transferase F5 | 108 | 1. LNPFGQIPALQDGDEVVFESR/49–69<br>2. LLDVYEAHLAAGNK†/138–151 |
| 7 | CAC94001 | glutathione *S*-transferase | 555 | 1. GLVLLDFWVSPFGQR/6–20<br>2. GLPYEYAEEDLMAGK/29–43<br>3. IPVLLHDGRAVNESLIILQYLEEAFPDAPALLPSDPYAR†/57–95<br>4. FWADYVDK/100–107<br>5. LKGEPQAQARAEMLDILKTLDGALGDKPFFGGDK/119–152<br>6. YGEFSLPEVAPK/173–184<br>7. SLYSPDKVYDFIGLLK/201–216 |
| 8 | CAD29479 | glutathione *S*-transferase F6 | 297 | 1. VFGPAMSTNVAR/6–17<br>2. NPFGQIPAFQDGDLLLFESR/50–69<br>3. KNEVDLLR†/81–88<br>4. LAAQMVPK/209–218 |
| 9 | AAL73394 | glutathione *S*-transferase | 256 | 1. VFGPAMSTNVAR/6–17<br>2. NPFGQIPAFQDGDLLLFESR/50–69<br>3. GLPTNQTVVDESLEK/128–142<br>4. VLEVYEAR/146–153<br>5. LAAQMVPK/210–217 |
| 10 | BAA90672 | glutathione-dependent dehydroscorbate reductase | 172 | 1. VLLTLEEK/26–33<br>2. YPTPSLVTPPEYASVGSK/85–102<br>3. ALLTELQALEEHLK/123–136 |
| 11 | T06258 | superoxide dismutase (Mn) | 536 | 1. HATYVANYNKALEQLDAAVSKGDASAVVHLQSAIK/60–94<br>2. NLKPISEGGGEAPHGKLGWAIDEDFGSIEK/109–138<br>3. LSVETTPNQDPLVTK/167–181<br>4. NVRPDYLTNIWK/204–215<br>5. YAGEEYEK/221–228 |
| 12 | NP_190864 | peroxiredoxin-like protein | 69 | 1. YAILADDGVVK†/202–212 |
| 13 | BAC20708 | putative translational inhibitor protein | 75 | 1. APAALGPYSQAIK/65–77<br>2. STYQVAALPLNAR/159–171 |
| 14 | P32637 | glyceraldehyde 3-phosphate dehydrogenase | 220 | 1. YDSTHGVFK/48–56<br>2. FGIVEGLMTTVHSYTATQK/167–185<br>3. GAAQNIIPSSTGAAK/200–214<br>4. AGISLNDNFVKLVSWYDNEWGYSR/298–321 |
| 15 | JT0686 | peptidylprolyl isomerase | 112 | 1. IIPDFMLQGGDFTR /67–80<br>2. ALEATGSGSGAIK/154–166 |

**Note:** Spots 1–13 are shown in Fig. 1A. Spots 14 and 15 are shown in Fig. 2A.
*The Mascot score is defined as $S = -10 \times \log(P)$, where $P$ is the probability that the observed match is a random event.
†Unique sequence to the best matching protein.

| Sequence coverage (%) | Theoretical relative mass of matched protein (kDa/pI) | Species | Expression |
|---|---|---|---|
| 44 | 29.2/4.8 | *Hordeum vulgare* | upregulated |
| 25 | 34.6/4.4 | *Triticum aestivum* | upregulated |
| 37 | 26.4/5.0 | *Triticum aestivum* | upregulated |
| 8 | 26.4/5.0 | *Triticum aestivum* | upregulated |
| 9 | 23.3/5.9 | *Triticum aestivum* | induced |
| 16 | 23.4/5.8 | *Triticum aestivum* | induced |
| 63 | 24.9/6.4 | *Triticum aestivum* | upregulated |
| 22 | 24.8/5.9 | *Triticum aestivum* | upregulated |
| 28 | 24.9/5.8 | *Hordem vulgare* | upregulated |
| 18 | 23.5/5.7 | *Oryza sativa* | upregulated |
| 43 | 25.3/7.9 | *Triticum aestivum* | upregulated |
| 4 | 24.7/9.1 | *Arabidopsis thaliana* | upregulated |
| 14 | 18.8/9.4 | *Oryza sativa* | upregulated |
| 19 | 36.1/6.5 | *Podospora anserina* | — |
| 15 | 19.4/6.4 | *Fusarium sporotrichioides* | — |

Glutathione *S*-transferases play important roles in normal cellular metabolism as well as in the detoxification of diverse xenobiotic compounds, and they have been most intensively studied with regard to herbicide detoxification in plants (Marrs 1996; Riechers et al. 1997; Zhang and Riechers 2004). Many plant GSTs are induced by herbicides, herbicide safeners, heavy metals, pathogen attack, wounding, ethylene, and ozone. It has been suggested that GSTs are induced in response to oxidative stress to protect cellular components from damage when plants are challenged by biotic and abiotic stresses (Levine et al. 1994; Tenhaken et al. 1995; Ulmasov et al. 1994, 1995). Plant GST genes known to be induced by pathogen infection include the wheat *GstA1* mRNA (Dudler et al. 1991; Mauch and Dudler 1993), the potato *prp-1* mRNA (Taylor et al. 1990), and the *Arabidopsis AWI24* mRNA (Kim et al. 1994).

### 14-3-3 proteins

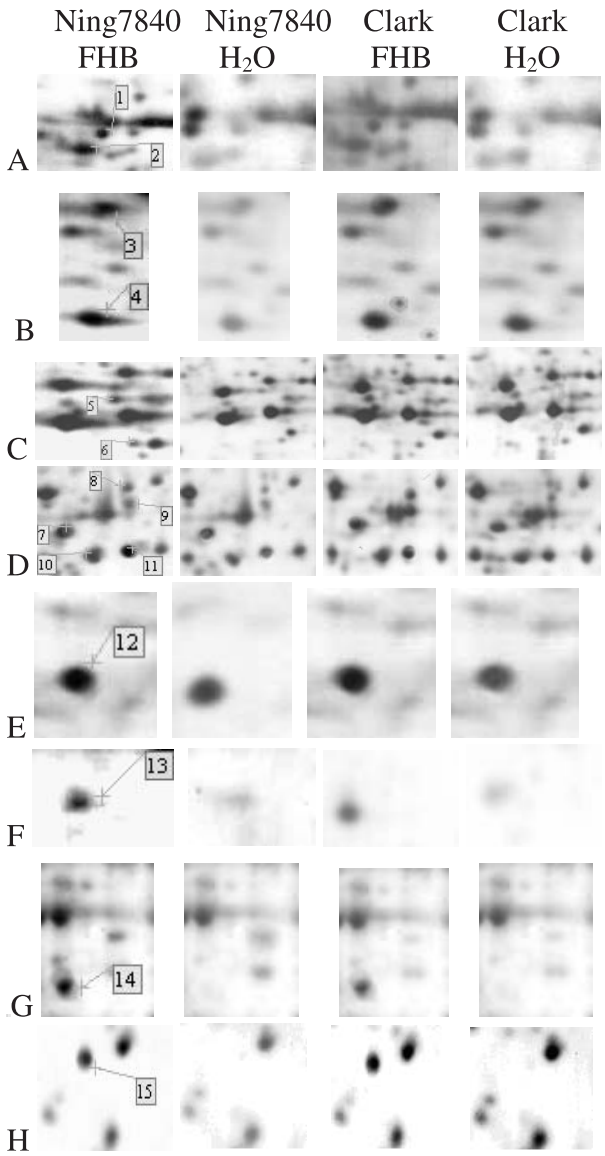
Protein spot 1 in Fig. 1A was upregulated in response to *F. graminearum* infection. LC-MS/MS analysis showed that this protein was most similar to barley 14-3-3 proteins. The 14-3-3 proteins comprise a family of highly conserved proteins with central roles in many eukaryotic signaling networks. In barley, 14-3-3 transcripts accumulated in the epidermis when leaves were inoculated with the fungus causing powdery mildew (*Erysiphe graminis* f. sp. *tritici)*; they bind to and activate plasma membrane $H^+$-ATPase, and they create a binding site for the phytotoxin fusicoccin (Brandt et al. 1992). Using a yeast 2-hybrid system, 14-3-3 proteins were shown to be involved in an epidermal-specific response to *E. graminis* f. sp. *tritici*, possibly through an activation of the plasma membrane $H^+$-ATPase (Finnie et al. 2002). Expression of $H^+$-ATPases was confirmed to be induced in barley infected with arbuscular mycorrhizal fungi (Murphy et al. 1997). *Fusarium graminearum* produces trichothecenes, which are acutely phytotoxic and act as virulence factors on sensitive host plants (Proctor et al. 1995). In this study, a 14-3-3 protein homolog induced in wheat spikes in response to *F. graminearum* infection suggests that the 14-3-3 proteins may be involved in the response to FHB and might be related to initial infection and mycotoxin accumulation in wheat kernels.

### Comparison of protein expression between susceptible and resistant cultivars

Differentially expressed proteins are indicated by the boxed regions marked in Figs. 1A and 2A. Closeup images comparing 2-D profiles from 4 protein samples, 'Ning7840' inoculated with *F. graminearum* and $H_2O$, and 'Clark' inoculated with *F. graminearum* and $H_2O$, are shown in Fig. 3.

Two proteins, DHAR and *Ta*GSTF5 (spots 5 and 6 in box C in Figs. 1A and 3), were induced by FHB infection in 'Ning7840' but were not detected in *F. graminearum*-infected spikelets of 'Clark' (box C in Fig. 3). This indicates that DHAR and *Ta*GSTF5 might play important roles in limiting FHB spread within spikes. The upregulated proteins (spots 1–4 and 7–13 in Fig. 1) in 'Ning7840' were also upregulated in FHB-infected spikelets of 'Clark' (Fig. 3). Two fungal proteins (spots 14 and 15 in Boxes G and H in Figs. 2A and 3) were found in both 'Ning7840' and 'Clark' spikes inoculated with *F. graminearum.* Since *F. graminearum* grows within the glumes after initial infection, it is not surprising that some fungal proteins were detected in FHB-infected spikelets.

**Fig. 3.** Enlarged comparisons of labeled spots in boxed regions (A, B, C, D, E, F, G, and H) marked in Figs. 1A and 2A among 4 protein samples. 1st column: 'Ning7840' 5 d after inoculation with *Fusarium graminearum*; 2nd column: 'Ning7840' 5 d after inoculation with $H_2O$; 3rd column: 'Clark' 5 d after inoculation with *F. graminearum;* 4th column: 'Clark' 5 d after inoculation with $H_2O$.




### Application and utility of proteomic techniques in studying wheat resistance against FHB

A proteomic strategy was used in this current study to successfully identify wheat proteins expressed in response to FHB infection in both susceptible and resistant cultivars. Thirteen wheat proteins from 'Ning7840' spikelets infected with FHB were determined to be upregulated or induced compared with controls, and they were identified by LC-MS/MS analysis. To the best of our knowledge, this is the first report of the application of proteomic techniques in

studying wheat resistance to FHB infection. This research opens a new gateway and sets the framework for future studies of the interaction between wheat and *F. graminearum*.

Our current study reports an initial experiment using proteomic techniques to assess the response of wheat spikes to FHB infection. Further studies on the effects of FHB infection on protein expression in wheat spikes during a time course may allow for the discovery of additional proteins produced in response to FHB initial infection, spread, and mycotoxin production by the fungus. Additionally, other protein detection methods such as fluorescent staining techniques can be used to measure protein expression levels more quantitatively; thus, the expression levels of those proteins that were differentially expressed and identified in our current study following FHB infection could be determined more precisely. The comparison of such protein expression-level changes between FHB-susceptible and -resistant cultivars (and the use of near-isogenic lines differing in FHB resistance), in conjunction with mRNA profiling methods, may help to determine whether these genes and their encoded proteins are associated with FHB resistance.

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