Yeast two-hybrid screening for proteins that interact with PFT in wheat

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Fusarium head blight (FHB) is a devastating disease of wheat worldwide. Fhb1 is the most consistently reported quantitative trait locus (QTL) for FHB resistance breeding. A pore-forming toxin-like (PFT) gene at Fhb1 was first cloned by map-based cloning and found to confer FHB resistance in wheat. Proteins often interact with each other to execute their functions. Characterization of the proteins interacting with PFT might therefore provide information on the molecular mechanisms of PFT functions. In this study, a high-quality yeast two-hybrid (Y2H) library using RNA extracted from Fusarium graminearum (Fg)-infected wheat spikes of Sumai 3 was constructed. The agglutinin domains of PFT exhibited no self-activation and toxicity to yeast cells and were used as bait to screen the Y2H library. Twenty-three proteins that interact with PFT were obtained, which were mainly involved in the ubiquitination process, clathrin coat assembly, the oxidation-reduction process, and protein phosphorylation. The expression pattern of these interacting genes was analyzed by quantitative real-time PCR. This study clarifies the protein interactions of PFT and raises a regulatory network for PFT regarding FHB resistance in wheat.

Wheat is one of the world's most important food crops, and demand for wheat is increasing due to the growth of the human population. However, wheat production is facing continuous threat from different biotic and abiotic stress factors. Globally, Fusarium head blight (FHB) is one of the most destructive fungal diseases of wheat. The disease not only results in heavy yield losses but also reduces grain quality by producing harmful deoxynivalenol and other trichothecene toxins posing a risk to global food security1,2.

Development of FHB-resistant cultivars is an effective approach to reduce damage from FHB. However, FHB resistance in wheat is an overly complex quantitative trait controlled by multiple quantitative trait loci (QTLs) with significant genotype–environment interactions3–5. Over 100 QTLs associated with FHB resistance have been reported on all 21 chromosomes of wheat5. Among these, Fhb1, identified in Sumai 3 and other Chinese cultivars, is the strongest and best validated resistance QTL6,7. Laborious and continuous studies have been carried out to identify candidate genes in Fhb1 by various approaches, including transcriptome-based analysis and genomic contig sequencing8,9. Functional validation of candidate genes in wheat remained elusive until pore-forming toxin-like (PFT) in the Fhb1 region was reported to confer FHB resistance by mutation analysis, gene silencing, and transgenic overexpression in wheat10. PFT is predicted to encode a chimeric lectin with two agglutinin domains and an ETX/MTX2 toxin domain; further studies are needed to understand the mechanism underlying PFT action.

Protein–protein interaction networks are important sources of information related to biological processes and complex metabolic functions in living cells. The yeast two-hybrid (Y2H) system, originally developed by Field and Song, is one of the most practical tools to identify the interacting partners of proteins in regulatory complexes11. The Y2H system exploits eukaryotic transcriptional activators containing separable functional domains for DNA-binding and transactivation, and it represents a powerful and fast approach to identify new protein interacting partners for a protein of interest that has been widely used in various organisms, including plants, animals, and fungi. Several wheat proteins in the regulatory network have been revealed using the Y2H screen system. The key vernalization gene TaVRN-A1 was used as bait to elucidate interacting proteins in wheat12. A cytosolic glutamine synthetase GS1b has been found to interact with the mitogen-activated protein kinase tMEK2 in plant defence against pathogen attacks, by Y2H screening13. The proteins encoded by the
photosynthesis-related genes PSK-I and PsbS1 have been confirmed to interact with the truncated haemoglobin protein trHb in photosynthesis14. These proteins are good entry points to further elucidate their regulatory network in the development process.

In this study, to screen proteins that interact with PFT, a high-quality Y2H library using *Fusarium graminearum* (*Fg*)-infected wheat spikes of the FHB-resistant cultivar Sumai 3 was constructed. Using the Y2H screening system, we screened some proteins that interact with PFT and provide information on the proteins interacting with PFT that are putatively involved in FHB resistance in wheat.

Results

Evaluation of the effect of yeast growth. PFT contains a chimeric lectin with two agglutinin domains and an ETX/MTX2 toxin domain. The ETX/MTX2 proteins are potent toxins posing potential threats to living cells10. To test whether PFT is toxic to *Saccharomyces cerevisiae* (yeast), the full-length PFT and its two domains were separately transformed into the *S. cerevisiae* strain Y2HGold. The transformants were plated on synthetically defined (SD) medium lacking tryptophan (SD/-Trp), and the ETX/MTX2 domain had a toxic effect on yeast growth.

Confirmation of bait autoactivation. The expression of the full-length PFT had a negligible toxic effect on yeast growth; therefore, we chose the partial PFT containing only the agglutinin domains (PFTa) to further analyze protein–protein interactions. The vectors pGBK7, pCL1, and pGBK7-PFTa were transformed into the Y190 yeast strain, and the resulting transformants were plated on SD medium lacking tryptophan but containing a chromogenic substrate for yeast galactosidase (SD/-Trp/X-Gal) for the autoactivation test. The yeast transfected with plasmids pGBK7 (A) and pGBK7-PFTa (C) did not turn blue in the β-galactosidase assay, indicating that pGBK7-PFTa does not autonomously activate the reporter genes in yeast cells without a prey protein.

**Figure 1.** Effect of PFT and its two domains on the growth of *S. cerevisiae*. (A) Schematic presentation of the PFT domains and deletion constructs used to transform the yeast cells. (B) The full-length PFT, its two domains, and pGBK7 used as the control, were separately transformed into the *S. cerevisiae* strain Y2HGold. The transformants were plated on synthetically defined (SD) medium lacking tryptophan (SD/-Trp), and the ETX/MTX2 domain had a toxic effect on yeast growth.

**Figure 2.** β-galactosidase assay indicating no self-activation of PFTa. The Y190 yeast cells that were transformed with negative control pGBK7 vectors (A), positive control pCL1 (which encodes full-length wild-type GAL4) (B), and pGBK7-PFTa (which contains only the agglutinin domains) (C), were plated on synthetically defined (SD) medium lacking tryptophan (SD/-Trp), and the ETX/MTX2 domain had a toxic effect on yeast growth.

**Figure 2.** β-galactosidase assay indicating no self-activation of PFTa. The Y190 yeast cells that were transformed with negative control pGBK7 vectors (A), positive control pCL1 (which encodes full-length wild-type GAL4) (B), and pGBK7-PFTa (which contains only the agglutinin domains) (C), were plated on synthetically defined (SD) medium lacking tryptophan (SD/-Trp), and the ETX/MTX2 domain had a toxic effect on yeast growth.
Construction of the Y2H library. Total RNA from the spike of Sumai 3 after Fg inoculation was used for the construction of the Y2H library. The integrity of total RNA after DNase I digestion was identified by electrophoresis on a 1% agarose gel. Several bands corresponding to ribosomal 28S and 18S were observed (Fig. 3A), indicating that the total RNA was complete and did not degrade. To obtain a high-quality cDNA for the subsequent library construction, the cDNA was synthesized using the SMART technology (Clontech). The synthesized cDNA was then normalized and analyzed by electrophoresis on a 1% agarose gel. The bands of cDNA fragments ranged from 0.5 to 2.25 kb in size (Fig. 3B). Small cDNA fragments were eliminated after purification using a Chroma Spin-1000 column (Fig. 3C), and then transferred to the pGADT7 vector to construct a Y2H library. The transformation reaction mixture was diluted from 1:10 to 1:1000, and then spread on LB plates to calculate transformation efficiency. The Y2H library had ~2 × 10^6 total primary clones and the final library titre was 1.0 × 10^9 cfu/mL, which far exceeded the minimum cell density, 2 × 10^7 cells/mL, required for Y2H screening. To determine the length of the inserts of the cDNA library, 16 positive clones were randomly selected and identified by polymerase chain reaction (PCR). The results showed that the inserted fragments ranged from 0.5 to 2.25 kb in size (Fig. 3D). These findings indicated that the Y2H library could be used for further research.

Screening of PFT-interacting proteins. The transformants expressing interacting pairs of pGBKT7-PFTa and AD library proteins in Y190 yeast cells were incubated on SD medium lacking Trp, Leu, and histidine but containing 30 mM 3-amino-1,2,4-triazole (SD/-Trp/-Leu/-His + 30 mM 3-AT) for the first screening. A total of 212 clones grew out. Plasmids from all of them were isolated and sequenced and 57 different genes were identified. As there were false positive clones, we transformed the plasmid pGBKT7-PFTa and the 57 plasmids representing different genes into Y2HGold yeast cells individually. The Y2HGold strain is unable to synthesize His and adenine (Ade) and it is therefore unable to grow on media that lack either of the two essential amino acids. When bait and prey proteins interact, Gal4-responsive His3 and Ade2 expression allow the cell to biosynthesize both of these amino acids, and grow on –His–Ade minimal medium. The cotransformants were then grown on SD/-Trp/-Leu/-His/X-α-Gal and SD/-Trp/-Leu/-Ade/X-α-Gal media. Finally, 23 positive clones representing different genes were identified through screening (Fig. 4).

Identification of proteins interacting with PFT. The 23 positive clones were further analyzed using the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) and UniProt databases (Table 1). Among them, only five proteins were characterized, namely, an acetolactate synthase (A0A3B6QKH3), a proteasome subunit beta (W5GEL1), a superoxide dismutase (Q96185), a mitogen-activated protein kinase (A0A3B5ZVC6), and a serine/threonine-protein phosphatase (A0A3B6MZU1). Five other proteins A0A3B6EIL3, A0A3B6NP03, A0A3B6AYL3, A0A3B6GUJ7, and A0A3B5YVG0, presented the highest screening frequency with 118 appearances, covering 55.6% of the 212 clones. To obtain more information on these proteins, we analyzed their homology function in the wheat species Aegilops tauschii subsp. tauschii (Table 1). Proteins related to DNA/RNA binding were the most frequent, including A0A3B6GUJ7, A0A3B6TJ86, A0A3B5XY63, A0A3B6DMV5, and A0A3B6INR9. Proteins related to the ubiquitination pathway were also frequent, including A0A3B6EIL3, A0A3B5YVG0, and W5GEL1. A protein with superoxide dismutase activity
named Q96185 was also found. The function of these interacting proteins with PFT was further subject to gene ontology (GO) analysis (Table 2). These proteins participated in the regulation of transcription, proteolysis, the oxidation-reduction process, protein phosphorylation, and clathrin coat assembly. Protein domain architectures provide useful information to determine protein functions. We further analyzed domains of the 23 interacting proteins using the SMART and Pfam databases. Consistent with the above homology function and GO analysis, diverse domains were found in these proteins including zinc finger (Znf), RING domain, ENTH domain, DDT domain, PP2A, serine/threonine protein kinase (S_TKc) domain, and RRM domain (Supplementary Fig. S1).

Expression profiles of the interacting genes. The transcript levels of PFT were low in the root, stem, and flag leaf, and high in the early developing spikes. PFT was also induced after Fg inoculation in Sumai 315. When two proteins interact, they are more likely to be co-expressed16. To observe the expression profiles of the interacting genes in Sumai 3, we analyzed the relative expression of these genes in the root, stem, leaf, and spike. The graphical representation of the expression profiles of 23 interacting genes in the four tissues is shown in Fig. 5. Most of the interacting genes were highly expressed in the leaf. TraesCS6A02G154100 and TraesCS6B02G181800 were specially expressed in the spike. To identify whether the interacting genes were induced after Fg treatment, the expression levels of these genes after 12, 24, and 48 h of Fg inoculation were investigated. Eight genes, including TraesCS3A02G288900, TraesCS2A02G295900, TraesCS4D02G021400, TraesCS6A02G154100, TraesCS6D02G245800, TraesCS2A02G191000, TraesCS6B02G181800, and TraesCS2D02G529100 were up-regulated by at least 1.2-fold 12 h after Fg inoculation, while eight genes, including TraesCS6D02G268700, TraesCS2A02G295900, TraesCS3D02G245800, TraesCS2D02G538300, TraesCS1D02G192200, TraesCS1A02G150600, TraesCS3A02G250100 and TraesCS6B02G455800 were up-regulated by at least 1.2-fold 48 h after Fg inoculation (Fig. 6).

Discussion
Proteins often work together with other partner proteins to accomplish their essential functions in living organisms. Thus, the determination of protein–protein interactions is helpful for elucidating the molecular functions of relevant protein molecules involved in plant development and responses to stresses. The PFT gene at Fhb1 has been reported to confer FHB resistance in wheat, but the role of PFT in FHB resistance is controversial10,17,18. Although PFT was up-regulated after Fg inoculation, its expression pattern has been reported to differ among some resistant and susceptible varieties15. It is possible that the expression and function of PFT is influenced by other genes. Therefore, it is necessary to identify proteins that interact with PFT to understand its resistance mechanism. In this study, we constructed a high-quality Y2H library using RNA extracted from Fg-infected wheat spikes of the FHB-resistant cultivar Sumai 3, and performed Y2H screening to identify putative proteins that interact with PFT.
A high-quality Y2H library can provide molecular resources to analyze protein functions and interactions based on a known protein to facilitate the construction of protein networks\(^\text{24}\). These proteins provide baseline information for further elucidating their regulatory network in the development process. Library titre, recombination rate, and the size of inserted fragments indicated the quality of the cDNA library\(^\text{20}\). The library titre should be not less than 1.7 × 10\(^5\) cfu. Our primary library storage capacity was over 2.0 × 10\(^5\) cfu, the recombination rate was approximately 100%, and the final library titre was over 1.0 × 10\(^5\) cfu/mL. Clones were randomly selected and showed that the inserted fragments in our library were ranged from 0.5 to 2.25 kb, indicating that the Y2H library was of high quality and could therefore be used for further research.

PFT contains one ETX/MTX2 domain, which are potent bacterial toxins that form channels in the cell membrane of the host leading to cell death\(^\text{25}\). We then checked the effects of PFT on yeast growth using the full-length protein and its two domains separately. Although the effect was negligible at a high concentration (OD600 = 1) in all the transformed yeast strains, growth inhibition of the full-length PFT and its ETX/MTX2 domain-transformed yeast strains was observed at low concentrations (OD600 = 0.01 to 0.001), but not in agglutinin domain-transformed yeast strains. Agglutinin interacts with other proteins in various biological processes. The interactions of garlic (Allium sativum) leaf agglutinin with a chaperonin group of unique receptor protein isolated from a bacterial endosymbiont of mustard aphid have been identified in a symbionin-mediated virus transmission study\(^\text{21}\). Therefore, we chose the integrant PFT sequences containing only agglutinin domains for further analysis of protein–protein interaction, and this partial PFT was used as bait to screen the Y2H library in this study as it could not autonomously activate the reporter genes without prey protein.

In the present study, we identified 23 proteins that putatively interacted with PFT. Among them, five proteins presented the highest screening frequency with 118 appearances, covering 55.6% of the 212 clones. These five proteins were A0A3B6EIL3 (46), A0A3B6NP03 (31), A0A3B6AYL3 (16), A0A3B6GUJ7 (15), and A0A3B5YVG0 (10). Both A0A3B6EIL3 and A0A3B6NP03 are proteins with DNA-binding domains. They are supposed to involve in the initiation of clathrin-coated pit formation in the plasma membrane during endocytosis process. An Arabidopsis clathrin assembly protein with a predicted role in plant defence was recently identified\(^\text{24}\). Proteins with DNA-binding domains are involved in the transcriptional regulation of key developmental processes, and proteins with RNA-binding domains also play vital roles in plant gene expression and regulation. Loss of function of these proteins can have a detrimental effect on some critical processes such as photosynthesis and respiration, sometimes leading to lethality\(^\text{21}\). Several such proteins have been found to interact with the PFT protein in this study. Among the interacting proteins, Q96185 is a superoxide dismutase.

### Table 1. The information of the genes interacting with PFT.

| No. | Gene Name | UniProtKB Name | Protein Name | Appearance | Homology Function in Aegilops tauschii subsp. tauschii |
|-----|-----------|----------------|--------------|------------|---------------------------------------------------------|
| 7   | TraeCS3A02G288900 | A0A3B6EIL3 | Uncharacterized protein | 46 | E3 ubiquitin-protein ligase MIEL1-like |
| 10  | TraeCS1B02G159900 | A0A3B5YVG0 | Uncharacterized protein | 10 | E3 ubiquitin-protein ligase MIEL1-like |
| 14  | TraeCS6D02G268700 | A0A3B6QKH3 | Acetolactate synthase | 1 | Acetolactate synthase 1 |
| 16  | TraeCS2A02G295900 | A0A3B6AYL3 | Uncharacterized protein | 16 | OTU domain-containing protein 5-B |
| 32  | TraeCS6B02G174200 | W5GEL1 | Proteasome subunit beta | 1 | Proteasome subunit beta type-3-like |
| 39  | TraeCS4D02G021400 | A0A3B6DMV5 | Uncharacterized protein | 6 | Phosphomethylpyrimidine synthase |
| 40  | TraeCS6A02G154100 | A0A3B6NP03 | Uncharacterized protein | 31 | Clathrin assembly protein |
| 61  | TraeCS3D02G245800 | A0A3B6GUJ7 | Uncharacterized protein | 15 | Homeobox DD7 domain protein RLT2-like |
| 79  | TraeCS2D02G538300 | Q96185 | Superoxide dismutase | 1 | Superoxide dismutase |
| 83  | TraeCS2D02G267600 | A0A2X0S5V9 | Uncharacterized protein | 1 | Uncharacterized protein |
| 125 | TraeCS2A02G191000 | A0A3B6AV80 | Uncharacterized protein | 1 | MO25-like protein |
| 127 | TraeCS2D02G192200 | A0A3B5YVC6 | Mitogen-activated protein kinase | 1 | Mitogen-activated protein kinase 6 |
| 185 | TraeCS7D02G70800 | A0A3B6R16 | Uncharacterized protein | 1 | Nuclear speckle RNA-binding protein A-like |
| 191 | TraeCS1B02G244100 | A0A3B5YZ23 | Uncharacterized protein | 1 | Chaperone protein dnal 49 |
| 199 | TraeCS1A02G150600 | A0A3B5X6Y3 | Uncharacterized protein | 1 | Heterogeneous nuclear ribonucleoprotein 1-like |
| 206 | TraeCS6B02G181800 | A0A3B6PLA2 | Uncharacterized protein | 1 | Clathrin assembly protein |
| 207 | TraeCS2D02G529100 | A0A3B6DMV5 | Uncharacterized protein | 1 | UBP1-associated protein 2B-like |
| 231 | TraeCS5A02G250100 | A0A3B6R95 | Uncharacterized protein | 1 | Uncharacterized protein |
| 244 | TraeCS6D02G402000 | A0A3B6FL97 | Uncharacterized protein | 2 | Tankyrase 1-like |
| 266 | TraeCSS02G465400 | A0A3B6M2121 | Serine/threonine-protein phosphatase | 1 | Serine/threonine-protein phosphatase PP2A-2 catalytic subunit |
| 282 | TraeCS4B02G153900 | A0A3B6NR9 | Uncharacterized protein | 2 | Polypyrimidine tract-binding protein homolog 1-like |
| 286 | TraeCS2A02G306800 | A0A3B6A2H5 | Uncharacterized protein | 1 | NAC domain-containing protein 104 |
| 316 | TraeCSS5B02G455800 | A0A3B6LV94 | Uncharacterized protein | 1 | Uncharacterized protein |
Rapid production of reactive oxygen species (ROS) after a pathogen attack has been proposed to orchestrate the establishment of different defensive barriers against infection. However, scavenging ROS to an appropriate level in plants is helpful to promote plant development and induce resistance against environmental stresses26.

| Gene Name          | Molecular function                                                                 | Biological process                                                                 | Cellular component                   |
|--------------------|------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------|
| TraesCS3A02G288900 | GO:0008270 zinc ion binding                                                         | GO:1902456 regulation of stomatal opening                                            |                                      |
| TraesCS1B02G159900 | GO:0008270 zinc ion binding                                                         | GO:0008652 cellular amino acid biosynthetic process                                 | GO:0009507 chloroplast               |
|                    | GO:0003984 acetyl-CoA synthase activity                                              | GO:0009082 branched-chain amino acid biosynthetic process                            | GO:0009570 chloroplast stroma         |
|                    | GO:0016740 transferase activity                                                     | GO:0009097 isoleucine biosynthetic process                                          |                                      |
|                    | GO:0016829 lyase activity                                                          | GO:0009099 valine biosynthetic process                                              |                                      |
| TraesCS2A02G295900 | GO:0004843 thiol-dependent ubiquitin-specific protease activity                     | GO:0016579 protein deubiquitination                                                  |                                      |
|                    | GO:003682 chromatin binding                                                        | GO:0016578 histone deubiquitination                                                  |                                      |
|                    | GO:0031491 nucleosome binding                                                       | GO:0045892 negative regulation of transcription, DNA-templated                      |                                      |
|                    | GO:0042393 histone binding                                                         | GO:0045892 negative regulation of transcription, DNA-templated                      |                                      |
| TraesCS6B02G174200 | GO:0001475 endopeptidase activity                                                   | GO:000502 proteasome complex                                                        |                                      |
|                    | GO:004298 threonine-type endopeptidase activity                                     | GO:0043161 proteasome-mediated ubiquitin-dependent protein catabolic process         | GO:0005634 nucleus                   |
|                    | GO:0008233 peptidase activity                                                      | GO:0016491 oxidoreductase activity                                                  | GO:0005737 cytoplasm                 |
|                    | GO:0016787 hydrolase activity                                                      | GO:0005839 proteasome core complex                                                  |                                      |
| TraesCS4D02G021400 | GO:0016830 carbon–carbon lyase activity                                             | GO:0009228 thiamine biosynthetic process                                            | GO:0009536 plastid                  |
|                    | GO:0019904 protein domain-specific binding                                          | GO:0009570 chloroplast                                                               |                                      |
| TraesCS6A02G154100 | GO:0005543 phospholipid binding                                                     | GO:0048268 clathrin coat assembly                                                   | GO:0030136 clathrin-coated vesicle    |
|                    | GO:0030276 clathrin binding                                                        | GO:0048268 clathrin coat assembly                                                   | GO:0030136 clathrin-coated vesicle    |
| TraesCS3D02G245800 | GO:0003677 DNA binding                                                               | GO:006355 regulation of transcription, DNA-templated                                |                                      |
| TraesCS2D02G538300 | GO:0004784 superoxide dismutase activity                                            | GO:0019430 removal of superoxide radicals                                            |                                      |
|                    | GO:0016491 oxidoreductase activity                                                  | GO:005114 oxidation-reduction process                                               |                                      |
| TraesCS1D02G192200 | GO:0004672 protein kinase activity                                                  | GO:000165 MAPK cascade                                                              | GO:0005622 intracellular              |
|                    | GO:000166 nucleotide binding                                                       | GO:0006468 protein phosphorylation                                                  |                                      |
|                    | GO:0004707 MAP kinase activity                                                     | GO:0016310 phosphorylation                                                         |                                      |
|                    | GO:0016301 kinase activity                                                         | GO:0071339 MLL1 complex                                                             |                                      |
| TraesCS7D02G270800 | GO:0003723 RNA binding                                                              | GO:0030136 clathrin-coated vesicle                                                  |                                      |
| TraesCS1A02G150600 | GO:0003723 RNA binding                                                              | GO:0030136 clathrin-coated vesicle                                                  |                                      |
|                    | GO:0003676 nucleic acid binding                                                     | GO:0030136 clathrin-coated vesicle                                                  |                                      |
| TraesCS6B02G181800 | GO:0005543 phospholipid binding                                                     | GO:0048268 clathrin coat assembly                                                   | GO:0030136 clathrin-coated vesicle    |
|                    | GO:0030276 clathrin binding                                                        | GO:0048268 clathrin coat assembly                                                   | GO:0030136 clathrin-coated vesicle    |
| TraesCS2D02G529100 | GO:0003723 RNA binding                                                              | GO:0030136 clathrin-coated vesicle                                                  |                                      |
|                    | GO:0003676 nucleic acid binding                                                     | GO:0030136 clathrin-coated vesicle                                                  |                                      |
| TraesCS5A02G250100 | GO:0003677 DNA binding                                                               | GO:0030136 clathrin-coated vesicle                                                  |                                      |
|                    | GO:0003676 nucleic acid binding                                                     | GO:0030136 clathrin-coated vesicle                                                  |                                      |
| TraesCSSD02G465400 | GO:0016787 hydrolase activity                                                       | GO:0006470 protein dephosphorylation                                                |                                      |
|                    | GO:0004721 phosphoprotein phosphatase activity                                     | GO:0006470 protein dephosphorylation                                                |                                      |
| TraesCS4B02G153900 | GO:0003723 RNA binding                                                              | GO:0006355 regulation of transcription, DNA-templated                              |                                      |
|                    | GO:0003676 nucleic acid binding                                                     | GO:0006355 regulation of transcription, DNA-templated                              |                                      |
| TraesCS2A02G306800 | GO:0003677 DNA binding                                                               | GO:0006355 regulation of transcription, DNA-templated                              |                                      |
| TraesCS5B02G455800 | GO:0002151 G-quadruplex RNA binding                                                | GO:0031011 Ino80 complex                                                            |                                      |
|                    | GO:0005515 protein binding                                                          | GO:0031011 Ino80 complex                                                            |                                      |

**Table 2.** Gene ontology analysis on proteins interacting with PFT.

Rapid production of reactive oxygen species (ROS) after a pathogen attack has been proposed to orchestrate the establishment of different defensive barriers against infection. However, scavenging ROS to an appropriate level in plants is helpful to promote plant development and induce resistance against environmental stresses26.
Interacting proteins are more likely to be involved in similar biological functions and processes, and thus are likely to be co-expressed\(^1\). Thus, quantitative real-time PCR (qRT-PCR) analysis was performed for several tissues, and also under \(F_g\) stress, to evaluate the expression of the interacting genes. Unlike the PFT expression pattern, most of the interacting genes were highly expressed in the leaf. It should also be noted that all the interacting genes were expressed in the spikes regardless of the expression level. Two genes encoding clathrin assembly proteins, TraeCS6A02G154100 and TraeCS6B02G181800, were particularly expressed in the spikes. Eight genes, including the above two genes encoding clathrin-related proteins, were up-regulated by at least 1.2-fold 12 h after \(F_g\) inoculation, and eight genes were up-regulated 48 h after \(F_g\) inoculation. These \(F_g\)-induced genes seem to be involved in the ubiquitination process, clathrin coat assembly, the oxidation-reduction process, and protein phosphorylation, and might contribute for PFT function.

Based on these findings, a regulatory network of wheat resistance against \(F_g\) invasion can be constructed using PFT and its interacting proteins. Once \(F_g\) invasion is signalled, the expression of \(PFT\) is up-regulated to improve the host’s antifungal ability. Simultaneously, PFT arouses many pathways to act together to confer \(F_g\) invasion. For example, it regulates the ubiquitination process by interacting with A0A3B6EIL3, A0A3B5YVG0, and W5GEL1, and the possible endocytosis process involved in plant defence by interacting with A0A3B6NPO3 and A0A3B6PLA2. It is also involved in the transcriptional regulation of downstream gene expression by interacting with proteins with DNA/RNA-binding domains, and scavenging the ROS increased level due to the \(F_g\) invasion by interacting with Q96185. However, it should be acknowledged that all methods have their limitations, and validation of these interacting proteins using different methods, such as immunoprecipitation or bimolecular fluorescence complementation in planta is recommended to obtain reliable results. Another aspect to be noted is that, despite the controversial role of PFT in FHB resistance, the proteins identified here are good indicators to understand the mechanism of PFT action.

**Methods**

**Plant materials.** The highly FHB-resistant wheat cultivar Sumai 3 was used to construct the Y2H library. Plants were grown in a greenhouse under 14/10 h light/dark and 24/15 °C in Nanjing, China. Spikelets at early anthesis were chosen for further \(F_g\) inoculation as described previously\(^7\). After 12, 24, and 48 h of inoculation, samples were harvested and frozen immediately in liquid nitrogen, and then stored at −80 °C until further use.

**Construction of the Y2H library.** Total RNA was harvested from the spikes of Sumai 3 8 h after \(F_g\) inoculation and then treated with DNasel (Takara, Japan) to eliminate contaminated genomic DNA. The cDNA was synthesized with CDS 4 M adapter (Supplemental Table 1) using the SMART\(^\text{TM}\) cDNA Library Construction Kit (Clontech, USA), and then normalized using the TRIMMER-DIRECT cDNA Normalization Kit (Evrogen, USA) according to the manufacturer's instructions. To eliminate low-molecular-weight cDNA fragments and small DNA contaminants, the cDNA after SfiI digestion (> 200 bp) was excised from a 1% agarose gel and purified using the CHROMA SPIN-1000 column (Clontech, USA). The purified cDNA was merged into the pGADT7-SfiI vector (library of prey proteins with the Gal4 activation domain; Clontech, USA) by directional cloning at SfiI A (5′-GGCCATTACGGCC-3′) and SfiI B (3′-CCGCGGAGCCGG-5′) sites. Dilutions of the transformed mixture (from 1:10 to 1:1000) were spread on LB media (Amp\(^+\)) and incubated at 37 °C until colonies appeared, to calculate the transformation efficiency and the number of primary colonies. Sixteen colonies were randomly selected and amplified by PCR using primer pGADT7-F/R (Supplemental Table 1) to check insert sizes and the recombinant ratio of the Y2H library using 1% agarose gel electrophoresis. The primary library was retransformed into competent *Escherichia coli*, and the plasmids were then harvested using the Plasmid Maxi Preparation Kit (Qiagen, Germany). The amplified library was stored at −80 °C by adding dimethyl sulfoxide to a final concentration of 7%

**Cloning and testing bait for toxicity and autoactivation.** The sequences of full-length PFT and its two domains (agglutinin and ETX/MTX2) were obtained by PCR using the primers listed in Supplemental Table 1. The fragments were inserted into pGBK7 vectors individually. To evaluate the effect on yeast growth, pGBK7-PFT, pGBK7-PFTA (PFTI-agglutinin)), pGBK7-ETX/MTX2, and pGBK7 vectors were separately transformed into *S. cerevisiae* strain Y2HG0ld (Clontech, USA), using Yeastmaker Yeast Transformation System 2 (Clontech, USA). The transformed cells were grown in SD-Trp medium at 30 °C for 3–5 days, and then diluted at different concentrations (OD 600 = 1, diluted from 1 to 1:1000). One microliter of these dilutions was spotted separately on SD-Trp medium and grown at 30 °C for 3 days. To test the bait PFTA for autoactivation, pGBK7-PFTA, the negative control pGBK7, and the positive control pGBK7-PCL1, which encodes full-length PFTa, were transformed into the prey Y2H AD library with the Gal4 activation domain, was co-transformed into the Y190 strain (Clontech, USA), using Yeastmaker Yeast Transformation System 2 (Clontech, USA). The transformed cells were grown in SD-Trp medium at 30 °C for 3–5 days, and then diluted at different concentrations (OD 600 = 1, diluted from 1 to 1:1000). One microliter of these dilutions was spotted separately on SD-Trp medium and grown at 30 °C for 3 days. To test the bait PFTA for autoactivation, pGBK7-PFTA, the negative control pGBK7, and the positive control pGBK7-PCL1, which encodes full-length wild-type GAL4 (able to constitutively activate transcription) were transformed into Y190 yeast cells separately. After culturing at 30 °C for 2 days, colonies were copied to a filter paper and transferred to liquid nitrogen for at least 30 s. The papers were placed in a fresh Z buffer/X-gal solution as described in the Yeast Protocols Handbook (PT3024-1) in a clean 150-mm plate and incubated at 30 °C until blue colonies appeared, which indicated self-activation of the plasmid.

**Screening of the Y2H library.** Bait vector pGBK7-PFTA with the Gal4 DNA-binding domain, together with the prey Y2H AD library with the Gal4 activation domain, was co-transformed into the Y190 strain (Clontech, USA) according to the Yeast Protocols Handbook (PT3024-1) and plated on SD-Trp/-Leu/-His/+30 mM 3-AT medium at 30 °C for 10 days. Colonies with diameter > 2 mm were selected as primary interacting proteins for further analysis and then retransformed into SD-Trp/-Leu liquid medium cultivating for 2 days for isolating vectors using the Yeast Plasmid Extraction Kit (Solarbio, China). The vectors were identified by PCR using the specific primers 5′AD and 3′AD, and then sequenced and analyzed using BLAST (https://blast.ncbi.nlm.nih.gov).
Confirmation and analysis of positive interactions. The primary interacting proteins and the bait pGBKT7-PPi vectors were co-transformed into Y2HGold yeast cells individually to confirm their interactions. The transformants were grown in SD/-Trp/-Leu, SD/-Trp/-Leu/-His/X-α-Gal, and SD/-Trp/-Leu/-His/-Ade/X-α-Gal media at 30 °C for 3–5 days. Three replicates were carried out. The sequences from the positive interactions were analyzed using the wheat genome database (TGACv1) at Ensemble Plants (http://plants.ensembl.org/Triticum_aestivum/) to identify gene names and GO terms, and Uniprot (https://www.uniprot.org/uniprot/) to identify protein names and function. Homology function in A. tauschii subsp. tauschii was determined using BLAST. The identified protein sequences were further analyzed using the PFAM (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) databases to search their function domains.

Figure 5. Different expression levels of the interacting genes in the root, stem, flag leaf, and spike of Sumai 3. The transcript levels of the 23 genes were analyzed by quantitative real-time PCR. The wheat tubulin gene was used as the endogenous control gene. The gene expression levels were expressed as relative values compared to the value in root, and error bars indicate mean ± standard error (SE) from the results of three replicates.

Figure 6. Different expression levels of the interacting genes in wheat spikes after Fg inoculation. Spikes of Sumai 3 at early anthesis were injected with 10 μl Fg suspension (1 × 10⁶ conidia/mL). The inoculated spikes were covered with plastic bags to maintain the moisture for fungal infection. Samples were collected at 0, 12, 24 and 48 h after Fg inoculation. The wheat tubulin gene was used as the endogenous control gene. The gene expression levels were expressed as relative values compared to the value in non-Fg inoculated spikes, and were analyzed by two-way ANOVA with Tukey’s significant difference test. The values are means ± standard error (SE) from the results of three replicates, and significant differences are indicated by different lowercase letters (p < 0.05).
Extraction of RNA and qRT-PCR analysis. Sumai 3 root, stem, leaf, and spikes, as well as spikes treated with Fg were used to extract RNA using the Promega RNA Isolation System (Promega, USA). Quantitative real-time PCR analyses were then performed on a Roche (Switzerland) thermal cycler 96 using SYBR Green (Takara, Dalian, China) to detect gene expression. The endogenous control gene, and the primers used for the qRT-PCR are listed in Supplemental Table 1. The qRT-PCR conditions were as follows: 45 cycles at 95 °C for 30 s, 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 10 s, followed by 95 °C for 10 s, 65 °C for 10 s, and 95 °C for 5 s. The relative abundance of each genes was determined by the comparative Ct method28. All reactions were performed in triplicate.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions
H.M. and Y.H. designed the experiments. Y.H. carried out most of the experiments and analyzed the data. L.W., X.L., X.Z. and P.J. assisted in yeast two-hybrid assay and analyzing the data. Y.H. and H.M. wrote the article.

Competing interests
The authors declare no competing interests.

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