Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2018) 19(6), 1343–1352 DOI: 10.1111/mpp.12617

Silencing of copine genes confers common wheat enhanced resistance to powdery mildew

BAOHONG ZOU[1,†], YUAN DING[1,†], HE LIU[1] AND JIAN HUA[1,2,*]

[1]State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, Nanjing 210095, China
[2]Plant Biology Section, School of Integrated Plant Science, Cornell University, Ithaca NY 14853, USA

SUMMARY

Powdery mildew, caused by the biotrophic fungal pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*), is a major threat to the production of wheat (*Triticum aestivum*). It is of great importance to identify new resistance genes for the generation of *Bgt*-resistant or *Bgt*-tolerant wheat varieties. Here, we show that the wheat copine genes *TaBON1* and *TaBON3* negatively regulate wheat disease resistance to *Bgt*. Two copies of *TaBON1* and three copies of *TaBON3*, located on chromosomes 6AS, 6BL, 1AL, 1BL and 1DL, respectively, were identified from the current common wheat genome sequences. The expression of *TaBON1* and *TaBON3* is responsive to both pathogen infection and temperature changes. Knocking down of *TaBON1* or *TaBON3* by virus-induced gene silencing (VIGS) induces the up-regulation of defence responses in wheat. These *TaBON1*- or *TaBON3*-silenced plants exhibit enhanced wheat disease resistance to *Bgt*, accompanied by greater accumulation of hydrogen peroxide and heightened cell death. In addition, high temperature has little effect on the up-regulation of defence response genes conferred by the silencing of *TaBON1* or *TaBON3*. Our study shows a conserved function of plant copine genes in plant immunity and provides new genetic resources for the improvement of resistance to powdery mildew in wheat.

Keywords: copine, disease resistance, powdery mildew, temperature, *Triticum aestivum*.

INTRODUCTION

Hexaploid wheat (*Triticum aestivum* L. AABBDD) is a major food crop that provides dietary carbohydrates for more than one-third of the world's population. Its production is critical for global food security, but its grain yield and quality are negatively affected by a variety of pathogenic viruses, bacteria, fungi, oomycetes and nematodes. Amongst these, *Blumeria graminis* f. sp. *tritici* (*Bgt*) causes wheat powdery mildew which is one of the most destructive wheat diseases worldwide (Yao *et al.*, 2007). The utilization of resistant wheat cultivars is the most effective and economic strategy for the control of wheat powdery mildew. It is therefore important to identify new powdery mildew resistance regulators from the wheat genome for traditional and molecular breeding.

As sessile organisms, plants cannot hide or escape when attacked by various pathogens. They have evolved at least two layers of innate immunity for defence against these would-be invaders (Jones and Dangl, 2006). The first layer, known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), is achieved by a set of plant plasma membrane-located pattern recognition receptors (PRRs), which confer basal defence by the perception of conserved PAMPs, such as flagellin, EF-Tu and chitin (Bernoux *et al.*, 2011; Jones and Dangl, 2006; Segonzac and Zipfel, 2011; Zhang and Zhou, 2010). The induced PTI defence responses, including reactive oxygen species accumulation, callose deposition and the activation of defence-related genes, inhibit or prevent the colonization of plants by pathogens. Some pathogens can overcome PTI by producing effectors to suppress the defence response and therefore to enhance disease development. Plants have evolved a second layer of innate immunity, namely effector-triggered immunity (ETI), which is activated on direct or indirect recognition of effectors by disease resistance (*R*) genes, mostly coding intracellular receptors, namely NLRs (Nod-Like Receptors) (Jones and Dangl, 2006). The detection of pathogen effectors by plant NLR proteins often culminates in a hypersensitive response (HR) associated with locally induced cell death that blocks the spread of the pathogen (Hofius *et al.*, 2007; Morel and Dangl, 1997). More than 58 *R* genes for powdery mildew have been designated in wheat (McIntosh *et al.*, 2017). Most are race-specific resistance genes based on a gene-for-gene system. This type of resistance is usually short lived because of the high evolutionary potential of the pathogen (McDonald and Linde, 2002).

Copines are calcium-dependent phospholipid-binding proteins, consisting of highly conserved N-terminal C2 domains (named C2A and C2B) and a C-terminal von Willebrand factor A (vWA) domain (Creutz *et al.*, 1998). In Arabidopsis, the C2 domains and the vWA domain of the copine protein BON1 have been shown to play critical functions through their calcium-binding and protein–protein interacting activities, respectively (Bennypaul *et al.*, 2012). Copines are present in diverse species of plants, and can be grouped into type I and type III copines in each of the 16 species analysed (Zou *et al.*, 2016). In the dicot plant Arabidopsis, all

*Correspondence: Email: jh299@njau.edu.cn
†These authors contributed equally to this work.

three copine genes, *AtBON1* and *AtBON2* in type I, and *AtBON3* in type III, are involved in disease resistance regulation. *AtBON1* in Arabidopsis negatively regulates the immune receptor NLR gene *SNC1* (*Suppressor of npr1-1, constitutive 1*), and the *bon1* loss-of-function mutant has an enhanced disease resistance and, consequently, a temperature-dependent growth defect (Yang and Hua, 2004). The Arabidopsis *BON1* is also a positive regulator of the abscisic acid- and bacterial pathogen-triggered stomatal closure response, indicating an opposing role of *BON1* at pre-invasion and post-invasion defence responses (Gou *et al*., 2015). In addition, *AtBON1* has overlapping functions with *AtBON2* and *AtBON3* in repressing programmed cell death and defence responses through multiple NLR-like genes (Li *et al*., 2009), and *bon1bon2bon3* triple loss-of-function mutants die from heightened defence responses (Yang *et al*., 2006). Although the functions of copine genes have been studied in Arabidopsis, the roles of these genes in monocots or crop plants, such as common wheat, remain unknown.

Virus-induced gene silencing (VIGS) is a useful strategy for the study of gene function in cereals. It uses a *Barley stripe mosaic virus* (BSMV)-based silencing system in monocot plants (Bennypaul *et al*., 2012; Holzberg *et al*., 2002), and has been successfully utilized in wheat gene function studies (Ahmed *et al*., 2017; Kage *et al*., 2017; Liu *et al*., 2016).

In this study, we investigated the function of copine genes in common wheat: *TaBON1* and *TaBON3*. We found that the expression of *TaBON1* and *TaBON3* is altered by pathogen infection and temperature changes. Most importantly, the silencing of copine genes *TaBON1* or *TaBON3* up-regulates defence response genes in the absence of pathogen invasion and enhances resistance against powdery mildew in wheat. Our results thus reveal a conserved function of copine genes in the regulation of disease resistance in dicots and monocots.

RESULTS

Identification of copines in common wheat

Given that the copines play key roles in disease resistance in Arabidopsis, we were interested in elucidating the roles of copines in common wheat, especially in disease resistance. In Arabidopsis, the loss of *AtBON1* function leads to enhanced disease resistance to the virulent hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the biotrophic oomycete pathogen *Hyaloperonospora parasitica* (Yang and Hua, 2004). Therefore, we hypothesized that its homologues in common wheat might also regulate resistance to obligate biotrophic fungi, such as powdery mildew. To identify copine genes in wheat, we used the cDNA sequences of the Arabidopsis *AtBON1* gene to blast search the genome sequences of wheat at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Two cDNAs (Sequence IDs: AK334076.1 and AK330585.1) were identified and their corresponding genes were named as *TaBON1* and *TaBON3* to indicate the type I and type III copines to which they belong (Zou *et al*., 2016). The deduced TaBON1 and TaBON3 protein sequences have 66%–68% sequence identity to AtBON1. Because common wheat is hexaploid and has A, B, D sets of genomes, we expected that there would be three copies of each of the *TaBON1* and *TaBON3* genes. We determined the chromosomal locations of these *TaBON* genes using their cDNA sequences to blast the chromosome-based draft sequence of hexaploid wheat (https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=BLAST&method=Org_Taestivum_er). Two genes corresponding to the *TaBON1* cDNA sequences were found on chromosomes 6AS and 6BL, and were therefore designated as homeologous to *TaBON-A1* and *TaBON-B1*. Three genes corresponding to *TaBON3* cDNA were located on chromosomes 1AL, 1BL and 1DL, and were therefore designated as homeologous to *TaBON-A3*, *TaBON-B3* and *TaBON-D3*. Phylogenetic analysis of the copine proteins from Arabidopsis, rice and wheat revealed that TaBON1 is closely related to the rice OsBON1 (Os02g0521300) protein, with a sequence identity of 86%. TaBON3 is closely related to the OsBON3 protein (Os05g0373300) with a sequence identity of 82%–85% (Fig. 1A). Based on the previous classification of plant copine proteins (Zou *et al*., 2016), TaBON1 is a member of type I and TaBON3 is a member of type III copines. We further compared homeologous copies of the *TaBON1* and *TaBON3* genes based on the annotation of the draft genome sequences. All predicted TaBON proteins contain features of copine, including two C2 domains (C2A and C2B) at the N-terminus and a vWA domain at the C-terminus (Fig. 1B). These domains are almost identical in amino acid sequences amongst homeologous copies of TaBON1 or TaBON3 (Figs S1 and S2, see Supporting Information). All copies of *TaBON1* and *TaBON3* genes have 13–16 exons based on annotation (Fig. 1C). The exon/intron junction sites are conserved not only amongst homeologues, but also amongst all *TaBON* genes. The lengths of the corresponding introns are almost identical amongst homeologues, but less conserved between *TaBON1* and *TaBON3* genes. The major differences amongst homeologues lie in the 5′ and/or 3′ end of the gene. *TaBON1-B* lacks two exons at the 5′ and one exon at the 3′ end compared with *TaBON-A1*, whereas *TaBON-D3* lacks one exon at the 5′ end compared with *TaBON-A3* or *TaBON-B3*. Whether or not these differences are a result of mis-annotation has yet to be determined experimentally. Nevertheless, we conclude that common wheat has at least two homeologous *TaBON1* genes and three homeologous *TaBON3* genes.

We subsequently used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay to assess the expression patterns of these genes in different tissues, including root, stem, young leaf, senescent leaf and spike. Specific primers were

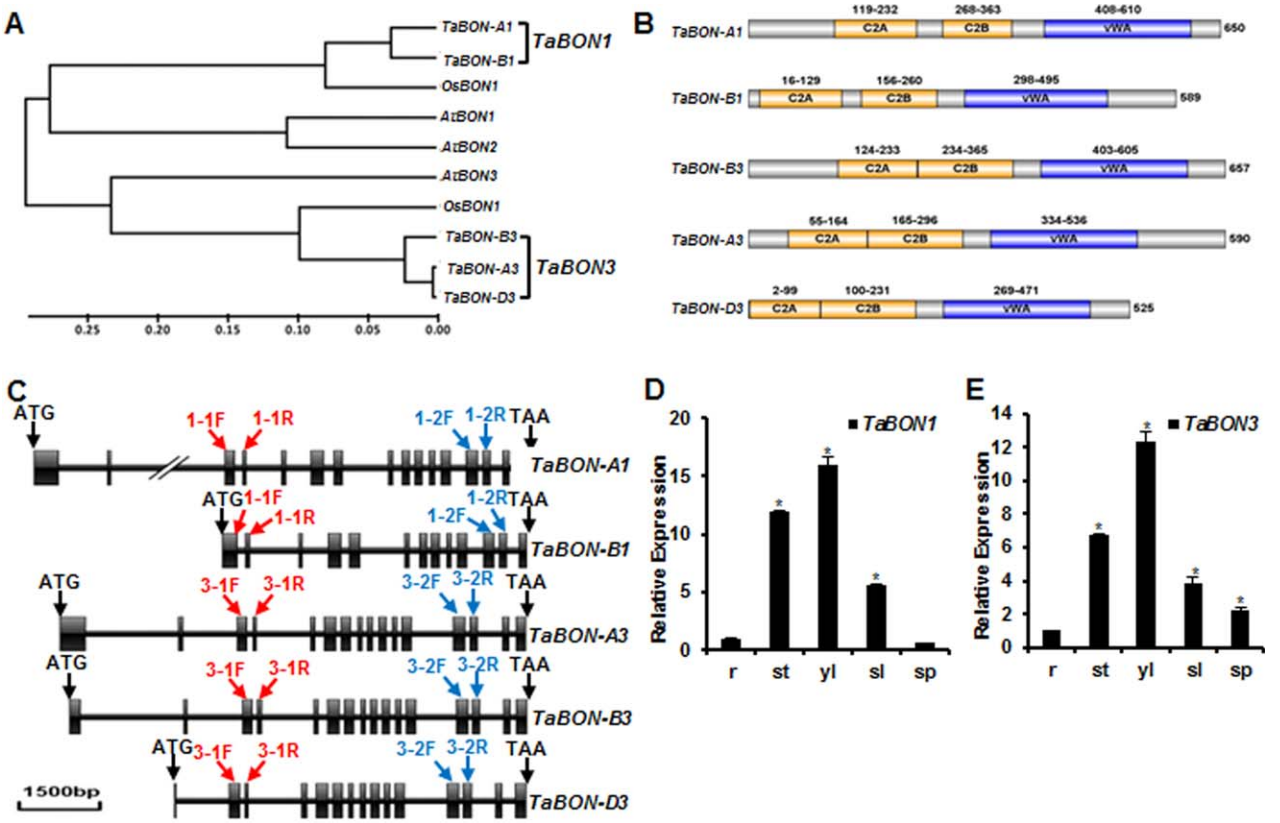

Fig. 1 Phylogenetic tree, domain organization and tissue expression pattern of *TaBON1* and *TaBON3*. (A) Neighbour-joining tree of copine family members from *Arabidopsis thaliana* (*At*), *Oryza sativa* (*Os*) and *Triticum aestivum* (*Ta*) generated by MEGA5.02 software. The inferred phylogeny was tested by 1000 bootstrap replicates. The scale bar indicates the branch length. (B) Conserved domains identified in SMART and displayed on the IBS1.0.2 program (http://ibs.biocuckoo.org/download.php). Numbers represent the amino acid residues in the domain or the whole protein. (C) Diagram of gene structure of *TaBON1* and *TaBON3*. Boxes and lines represent exons and introns, respectively. The red arrows indicate the primer sites for the amplification of fragments used in virus-induced gene silencing (VIGS) constructs of *TaBON1* and *TaBON3*. The blue arrows indicate the primer sites for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to check the expression of *TaBON1* or *TaBON3*. 1-1F stands for *TaBON1*-1F, 3-1F for *TaBON3*-1F, and so on. (D, E) qRT-PCR analysis of *TaBON1* (D) and *TaBON3* (E) expression levels in various tissues, including the root (r), stem (st), young leaf (yl) (McCouch *et al.*, 2013), senescent leaf (sl) and spike (sp). The expression is normalized by the expression level in the root. *TaActin* was used as the internal control. Similar results were seen in all three biological replicates and one biological experiment with three technical repeats is shown. Asterisks indicate statistically significant differences in comparison with the root at $P \leq 0.01$ (Student's *t*-test).

designed to differentiate between *TaBON1* and *TaBON3*, but they were not able to differentiate homeologous copies. Expression of *TaBON1* and *TaBON3* was detectable in all tested wheat tissues and the tissue expression pattern was similar between *TaBON1* and *TaBON3*. Both genes had the highest expression in young leaves and the lowest expression in roots (Fig. 1D,E).

Silencing of *TaBON1* or *TaBON3* induces defence response genes in wheat

To assess the function of *TaBON1* and *TaBON3* in wheat, we silenced these two genes via a BSMV-based VIGS approach (Zhou *et al.*, 2007). This approach has been shown to be effective for reverse genetics in wheat (Scofield *et al.*, 2005). Because homeologous genes probably have redundant functions, we silenced all *TaBON1* (*TaBON-A1* and *TaBON-B1*) and *TaBON3* (*TaBON-A3*, *TaBON-B3* and *TaBON-D3*) genes using regions of the cDNA sequences specific to *TaBON1* or *TaBON3* gene VIGS, respectively. The chlorosis phenotype was observed by 8 days post-inoculation (dpi), indicating successful virus infection (Fig. 2A). The efficiency of VIGS was monitored using the wheat phytoene desaturase gene (*PDS*) as a control for VIGS, and leaf bleaching was observed by 10 dpi at 22 °C, indicating an efficient silencing of the *PDS* gene (Fig. S3A, see Supporting Information). The efficiency of VIGS of *TaBON* was analysed through qRT-PCR using the fourth leaves of seedlings. The endogenous *TaBON1* and *TaBON3* transcripts were 80%–90% reduced in leaves inoculated with the BSMV virus containing the respective *TaBON1* and

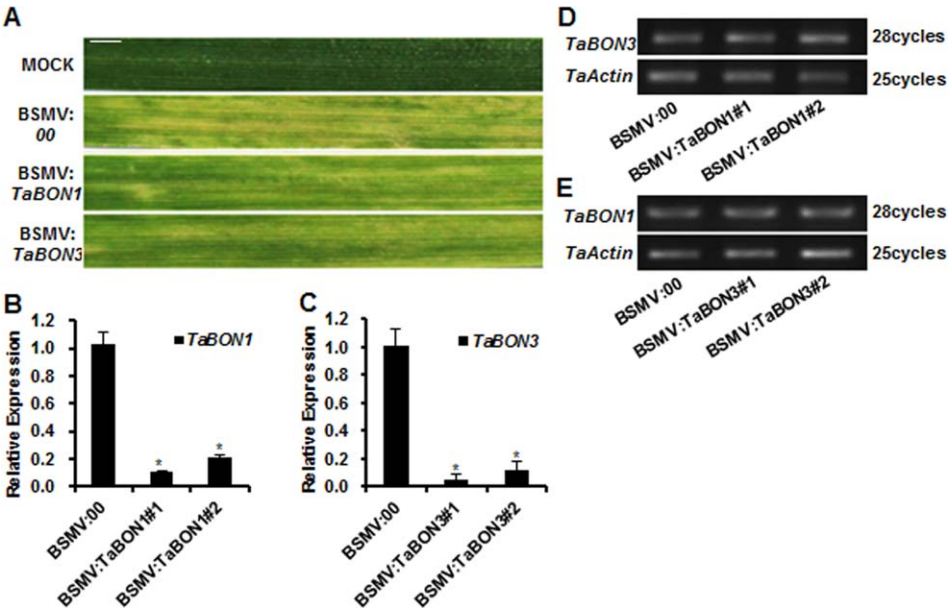

Fig. 2 Knocking down of *TaBON1* and *TaBON3* expression by *Barley stripe mosaic virus* (BSMV)-mediated gene silencing. (A) The leaf phenotype of successful virus infection. Photographs were captured at 8 days after virus inoculation. MOCK: wheat leaves treated with 2 × GKP buffer. Scale bars, 1 cm. (B, C) Relative transcript levels of *TaBON1* (B) and *TaBON3* (C) in plants at 22 °C inoculated with the BSMV-based silencing system and assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Expression was normalized by the expression level in the control (BSMV:00). *TaActin* was used as the internal control. Similar results were seen in all three biological replicates and one biological set with three technical repeats is shown. Asterisks indicate statistically significant differences in comparison with the control (BSMV:00) at $P \leq 0.01$ (Student's *t*-test). (D, E) RNA expression levels of *TaBON3* in *TaBON1*-silenced (D) and *TaBON1* in *TaBON3*-silenced (E) plants before *Blumeria graminis* f. sp. *tritici* (*Bgt*) infection determined by semi-quantitative RT-PCR analysis.

TaBON3 fragments compared with control plants inoculated with the BSMV virus only (Fig. 2B,C). The specificity of silencing was also shown by RT-PCR: the expression of *TaBON3* was not altered in *TaBON1*-silenced lines and the expression of *TaBON1* was not altered in *TaBON3*-silenced lines (Fig. 2D,E).

Because knocking out of the Arabidopsis *BON1* gene induces autoimmunity with the up-regulation of defence genes (Yang and Hua, 2004), we tested whether or not the silencing of *TaBON* genes would also affect the transcript levels of defence response genes in the absence of pathogens. We chose *TaPR2* and *TaPR10* as marker genes for defence responses because they are highly induced during plant defence (Caruso *et al.*, 1999; Mohammadi *et al.*, 2012). By qRT-PCR analysis, the transcripts of *TaPR2* and *TaPR10* were found to accumulate to higher levels in the *TaBON1*- and *TaBON3*-silenced lines than in BSMV:00 control plants (Fig. 3A,B). We further analysed H_2O_2 accumulation and cell death in silenced plants, because such events occurred in the Arabidopsis *bon1* mutant. Using 3,3′-diaminobenzidine (DAB) and trypan blue staining to assess H_2O_2 and cell death, respectively, we did not find a difference between silenced plants and control plants (Fig. 3C,D). These results suggest that the knockdown of *TaBON1* and *TaBON3* causes the up-regulation of defence response genes, but not significant cell death, in the absence of the pathogen in wheat.

TaBON1 and *TaBON3* negatively regulate wheat resistance against powdery mildew

To assess the roles of *TaBON* genes in resistance against the powdery mildew pathogen *Bgt*, we analysed their expression during *Bgt* infection. Plants of the common wheat variety Sumai 3 were infected with *Bgt* at the 2-week-old stage, and gene expression was analysed on infected leaves collected at different time points by qRT-PCR (Fig. 4A,B). The expression of *TaBON1* was increased by two- to nine-fold at 1, 2 and 4 h post-inoculation (hpi), whereas the expression of *TaBON3* was up-regulated by two- to 11-fold at 1, 2 and 4 hpi. The induction of both genes reached a maximum at 4 hpi and decreased slightly at 8 and 12 hpi. At 24, 36 and 48 hpi, the expression of these two genes decreased to one- to two-fold of the pre-infection level. The induction of wheat *PR* genes *TaPR2* and *TaPR10* by *Bgt* in common wheat occurred at 2 hpi and reached a maximum at 12 hpi (Fig. S4, see Supporting Information). Therefore, *TaBON1* and *TaBON3* appear to show an early induction by *Bgt*, and maximum induction occurs earlier than that of the *PR* gene, suggesting a role of these genes in the early defence response to *Bgt*.

We subsequently assessed VIGS lines of *TaBON1* or *TaBON3* for their resistance to the virulent *Bgt* field-isolated strain. Wheat plants were pre-inoculated with buffer alone (MOCK), virus with

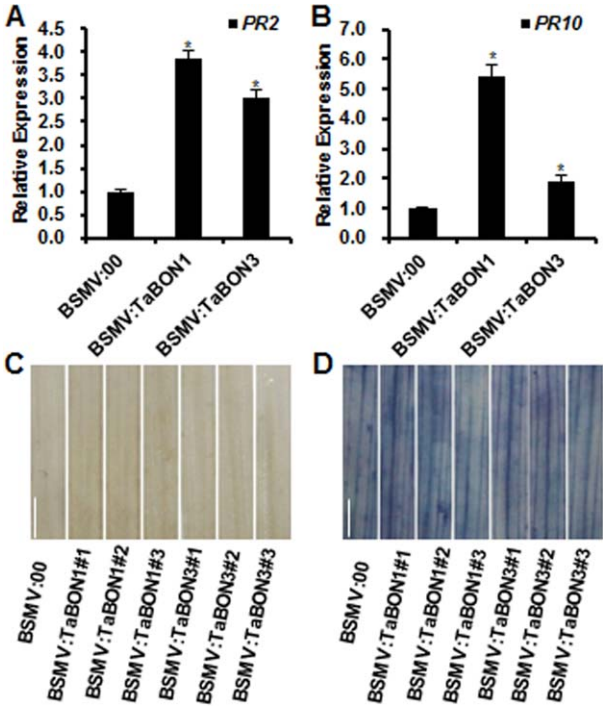

Fig. 3 Up-regulation of defence-related features in silenced wheat plants without *Blumeria graminis* f. sp. *tritici* (*Bgt*) infection at 22 °C. (A, B) RNA expression of pathogenesis-related genes *TaPR2* (A) and *TaPR10* (B) analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in BSMV:00-, BSMV:*TaBON1*- and BSMV:*TaBON3*-inoculated plants at 22 °C. RNA samples were isolated from the fourth leaves of wheat seedlings. Expression is normalized by the expression level in the control (BSMV:00). *TaActin* was used as the internal control. Similar results were seen in all three biological replicates and one biological replicate with three technical repeats is shown. Asterisks indicate statistically significant differences in comparison with the control (BSMV:00) at $P \leq 0.01$ (Student's *t*-test). (C, D) The fourth leaves at 14 days before challenge with *Bgt* were stained with 3,3′-diaminobenzidine (DAB) (C) and trypan blue (D). Scale bars, 1 cm. Three plants for *TaBON1* and *TaBON3* silencing were analysed.

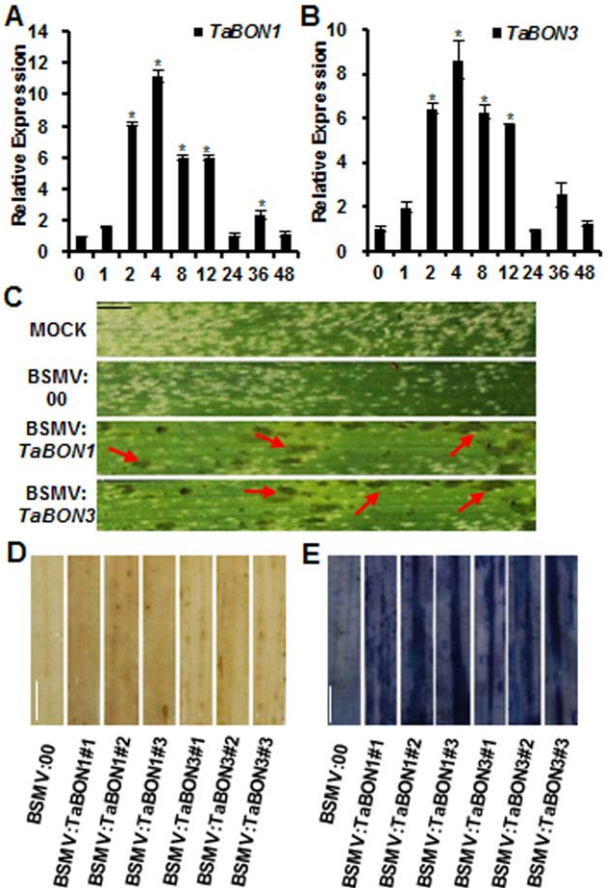

Fig. 4 Silencing of *TaBON1* or *TaBON3* enhanced resistance to *Blumeria graminis* f. sp. *tritici* (*Bgt*). (A, B) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of the relative expression levels of *TaBON1* (A) and *TaBON3* (B) in *Bgt*-infected common wheat leaves. RNA samples were isolated from the leaves of *Bgt*-infected common wheat Sumai 3 at 0, 1, 2, 4, 8, 12, 24, 36 and 48 h post-inoculation (hpi). Expression was normalized by the expression level at 0 hpi. *TaActin* was used as the internal control. Similar results were seen in all three biological replicates and one biological replicate with three technical repeats is shown. Asterisks indicate statistically significant differences in comparison with 0 hpi at $P \leq 0.01$ (Student's *t*-test). (C) The fourth leaves at 14 days after infection with spores of *Bgt* isolated from the field. Representative leaves were photographed at 5 days post-inoculation (dpi). The red arrows indicate the location of the lesions. (D) The fourth leaves at 14 days were challenged with *Bgt* and sampled at 3 dpi. Three silenced plants for *TaBON1* and *TaBON3* were analysed. H_2O_2 accumulation at infection sites was stained using 3,3′-diaminobenzidine (DAB). (E) The fourth leaves at 14 days were challenged with *Bgt* and sampled at 24 hpi. Three silenced plants for each of *TaBON1* and *TaBON3* were analysed. Scale bars, 1 cm. Cell death accumulation at infection sites was stained with trypan blue.

the silencing vector BSMV:00 or *TaBON1*- and *TaBON3*-silenced constructs on the second leaf. At 14 days after virus inoculation, the fourth leaf was detached and infected with fresh spores of virulent *Bgt*. Disease symptoms were analysed at 5 days after *Bgt* inoculation. Whereas leaves from plants pre-inoculated with MOCK or BSMV:00 vector developed extensive powdery growth on the surface, leaves from both *TaBON1*- and *TaBON3*-silenced lines showed significantly less powdery growth, indicating that the knockdown of *TaBON1* and *TaBON3* enhanced resistance to powdery mildew. In addition, we assessed resistance to *Bgt* using un-detached leaves, and reduced powdery growth was also observed in *TaBON1*- and *TaBON3*-silenced lines (Fig. S3D). Interestingly, extensive lesions developed on *Bgt*-infected leaves in *TaBON1*- and *TaBON3*-silenced lines, but not the control lines (Fig. 4C). DAB staining revealed that H_2O_2 accumulation was enhanced in *TaBON1*- and *TaBON3*-silenced plants compared with BSMV:00-infected control plants at 3 dpi of *Bgt* (Fig. 4D). Trypan blue staining showed that cell death was also significantly increased in *TaBON1*- and *TaBON3*-silenced plants compared with BSMV:00-infected control plants at 24 hpi (Fig. 4E). Therefore,

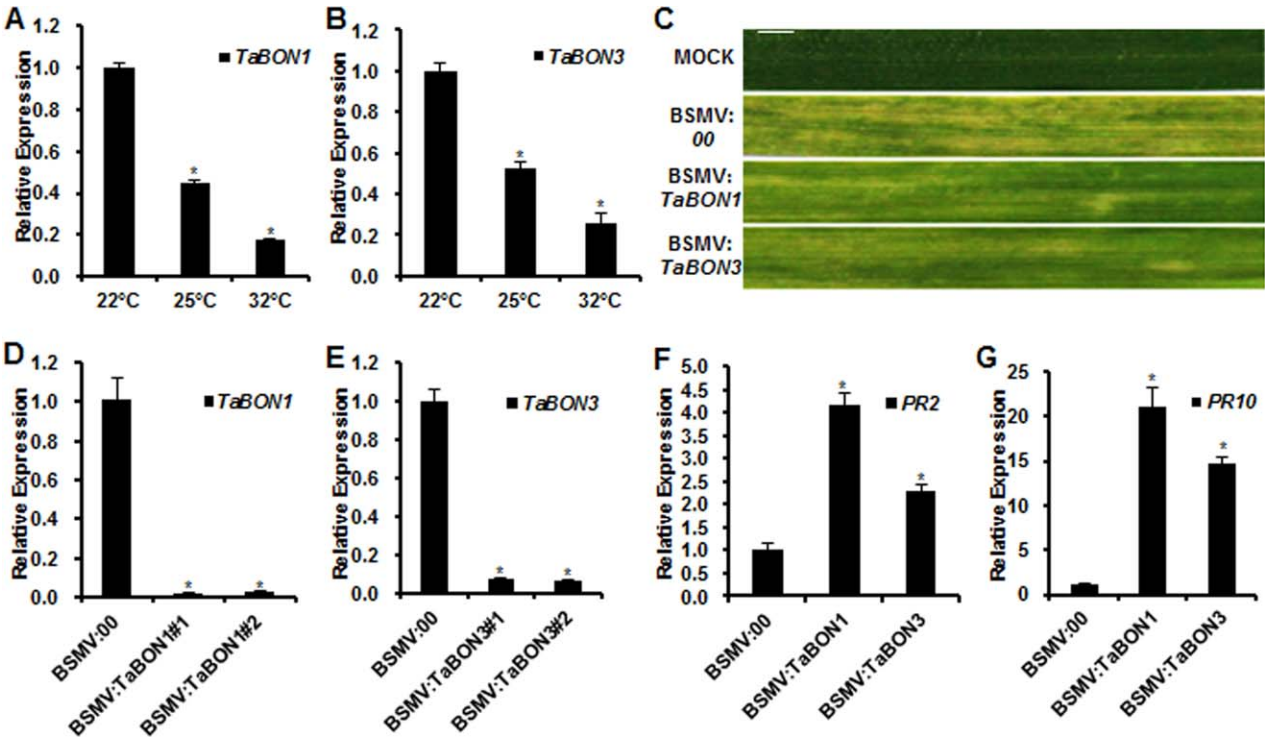

Fig. 5 Temperature effects on the expression of *TaBON1* and *TaBON3*, as well as defence induced by the silencing of *TaBON1* and *TaBON3*. (A, B) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *TaBON1* (A) and *TaBON3* (B) relative expression levels at different temperatures in common wheat leaves. RNA samples were isolated from the leaves of high-temperature-treated common wheat Sumai 3 at 22, 25 and 32 °C. Expression was normalized by the expression level at 22 °C. (C) The leaf phenotype of successful virus infection. Photographs were captured at 8 days after virus inoculation. MOCK: wheat leaves treated with 2 × GKP buffer. Scale bars, 1 cm. (D, E) Relative transcript levels of *TaBON1* (D) and *TaBON3* (E) in plants at 28 °C inoculated with a *Barley stripe mosaic virus* (BSMV)-based silencing system and assayed by qRT-PCR. (F, G) RNA expression of pathogenesis-related genes *TaPR2* (F) and *TaPR10* (G) analysed by qRT-PCR in BSMV:00-, BSMV:*TaBON1*- and BSMV:*TaBON3*-inoculated plants at 28 °C. Expression was normalized by the expression level in the control (BSMV:00). *TaActin* was used as the internal control. Similar results were seen in all three biological replicates, and one biological replicate with three technical repeats is shown. Asterisks indicate statistically significant differences in comparison with the control at $P \leq 0.01$ (Student's *t*-test).

TaBON1 and *TaBON3* negatively regulate wheat disease resistance to *Bgt* and its associated H_2O_2 accumulation and cell death.

Silencing of *TaBON1* or *TaBON3* induces the up-regulation of defence response genes in wheat at 28°C

Because the Arabidopsis *BON1* gene shows a higher expression at lower temperature (Hua *et al.*, 2001; Yang and Hua, 2004), we analysed the expression of the two wheat copine genes in leaves at three different temperatures using qRT-PCR. The expression of *TaBON1* and *TaBON3* was significantly lower at higher temperatures of 25 and 32 °C than at the normal growth temperature of 22 °C, with the lowest expression at 32 °C (Fig. 5A,B).

Moderately high temperature was found to suppress the enhanced disease resistance in Arabidopsis *BON1* knockout plants resulting from NLR up-regulation. We therefore analysed the disease resistance phenotypes of the *TaBON*-silenced plants at 28 °C, a moderately higher growth temperature for wheat. The VIGS system was efficient to knock down the *TaBON1* and *TaBON3* genes at 28 °C. Chlorosis was observed by 8 days after virus inoculation on the third leaves at 28 °C, indicating successful virus infection (Fig. 5C). Analysis by qRT-PCR of the fourth leaves revealed a substantial reduction in endogenous *TaBON1* and *TaBON3* expression by VIGS (Fig. 5D,E). Because *Bgt* does not grow at 28 °C and therefore cannot be used to test disease resistance, we analysed the expression of defence genes by qRT-PCR in *TaBON1* and *TaBON3* knockdown plants without *Bgt* infection. Marker genes *TaPR2* and *TaPR10* were both significantly up-regulated in the *TaBON1* and *TaBON3* knockdown plants compared with the control plants (Fig. 5F,G), and the extent of up-regulation was comparable between 28 and 22 °C (Fig. 3).

DISCUSSION

The wheat powdery mildew fungus *Bgt* is an obligate biotrophic fungus and a causal agent of one of the most economically important diseases of wheat. As a result of its rapid evolution, novel

virulent *Bgt* races often appear to circumvent race-specific resistance genes in wheat. Thus, it is of great importance to identify new resistance genes from the wheat genome for the generation of *Bgt*-tolerant wheat. In Arabidopsis, the evolutionarily conserved copine proteins have been shown to be negative regulators of plant disease resistance to both bacterial and fungal pathogens (Yang *et al.*, 2006). In this study, we found a role of the wheat copine *TaBON1* and *TaBON3* genes in disease resistance to powdery mildew in wheat, which provides new gene resources for resistance to powdery mildew in wheat.

A previous study has found that copines in higher plants can be grouped into types I and III, where *AtBON1* and *AtBON2* belong to type I and *AtBON3* belongs to type III (Zou *et al.*, 2016). In monocot species, there is usually one gene of the type I group and one gene of the type III group. Common wheat is a hexaploid with an A, B and D genome, and we found A and B copies of the *TaBON1* gene in the type I group, as well as A, B and D copies of *TaBON3* in the type III group. Apparently, a D copy of *TaBON1* is absent from the wheat genome. This could be a result of missing information from the current draft genome sequence, or a real loss of the copy from the D genome. Amongst the different copies of the *TaBON1* or *TaBON3* genes, the sequence identity is 97%–99% at the amino acid sequence level. The variations amongst gene copies often reside in the N-terminal region which is important for protein localization. These variations might therefore confer functional diversity to different copies of the *TaBON* genes (Bennypaul *et al.*, 2012). It will be interesting to investigate whether or not differences are indeed true (not a result of misannotation) and, if so, the biological consequence of the differences.

In Arabidopsis, *AtBON1* plays an important role in plant immunity and stomatal closure control (Gou *et al.*, 2015; Hua *et al.*, 2001; Yang and Hua, 2004), whereas *AtBON2* and *AtBON3* play a minor role in immunity as their single knockout mutants do not show a defect, but have a synergistic effect with the *AtBON1* mutant in plant immunity (Yang *et al.*, 2006). Unlike the Arabidopsis copines, silencing of *TaBON1* or *TaBON3* leads to enhanced disease resistance to *Bgt*. It appears that *TaBON* genes of the two types have an equal and non-redundant function in plant immunity. It is likely that the two types of copine genes have distinct functions in plants, which enables the maintenance of copine genes in two groups in both monocots and dicots. The distinct function is not yet known from this study, but the results strongly indicate that copine genes have a conserved role in plant disease resistance from monocots to dicots.

In Arabidopsis, high temperature suppresses *bon1* autoimmunity (Yang and Hua, 2004), Here, we found that high temperature has no drastic effect on the induction of defence response genes conferred by silencing of *TaBON1* or *TaBON3*. This resembles the *bon1bon3* and *bon1bon2* double mutants, where high temperature does not suppress autoimmunity (Yang *et al.*, 2006). It is possible that the temperature-sensitive *SNC1* is the major *NLR* gene activated in *bon1*, and that multiple *NLR* and *NLR*-like genes are activated in *bon1bon3* and *bon1bon2* double mutants, and these *NLR*-like genes are not all temperature sensitive. The involvement of *NLR* genes in the enhanced resistance of *TaBON*-silenced wheat plants has not been studied, but it is possible that *R* genes could be up-regulated in *TaBON*-silenced plants and not all of them are temperature sensitive.

The production of ROS is one of the earliest events in plant cells after infection by pathogens including *Bgt* (Fig. S5, see Supporting Information). ROS accumulation in plant cells may induce callose deposition, defence gene activation and hypersensitive cell death, which is usually beneficial for plants for resisting biotrophic and obligate hemibiotrophic pathogens (Kim *et al.*, 2012; Lehmann *et al.*, 2015; Luna *et al.*, 2011; Torres *et al.*, 2006). Unlike the Arabidopsis *BON1* knockout mutant, *TaBON1* or *TaBON3* knockdown plants do not exhibit over-accumulation of ROS or spontaneous cell death in the absence of pathogen infection, Whether or not this difference between Arabidopsis and wheat copines is caused by residual function of *TaBON1* and *TaBON3* in knockdown plants is yet to be experimentally determined. Nevertheless, these plants produced more ROS and showed more extensive cell death after *Bgt* infection than control plants. This heightened response probably leads to the enhanced resistance phenotype of *TaBON1* or *TaBON3* knockdown plants. In summary, the wheat copine genes *TaBON1* and *TaBON3* negatively modulate disease resistance against wheat powdery mildew. This is probably through the modulation of H_2O_2 and cell death accumulation during the interaction of wheat and *Bgt*. This finding indicates a conserved function of plant copines in plant immunity and provides new genetic resources for the improvement of resistance to powdery mildew in wheat.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Common wheat variety Sumai 3, a highly susceptible variety to powdery mildew, was used throughout these experiments. Wheat plants were grown in a light climate box under long-day conditions (16 h : 8 h, light : dark) and 60% relative humidity. For temperature treatments, plants were grown to 2 weeks at 22 °C and then transferred to 25 and 32 °C, respectively, for 5 days.

Sequence analysis, domain prediction and phylogenetic tree construction

Full-length copine protein sequences were collected from NCBI (http://www.ncbi.nlm.nih.gov/), TAIR (http://www.arabidopsis.org/), Gramene (http://www.gramene.org/) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Conserved domains were predicted using tools at

Table 1 Information for the primers used in this study.

Name of the primer	Primer sequence (5′–3′)	Note
TaBON1-VIGS-1F	CCTTAATTAAGCAATCCCATAGTTGTCGTG	VIGS vector construction
TaBON1-VIGS-1R	CCGCGGCCGCATGAAACTGCGGATCAATAT	
TaBON1–2F	GGTCCTCTACTTAGCACC	qRT-PCR primers for *TaBON1*
TaBON1–2R	TTTCCTTGAAGTCAGCCC	
TaBON3-VIGS-1F	CCTTAATTAAAGTGATCCTATGTTGGTGGT	VIGS vector construction
TaBON3-VIGS-1R	CCGCGGCCGCTTCAGTGGTGTGTTATGGTA	
TaBON3-2F	AGTTGGAGTTGAAGGCAT	qRT-PCR primers for *TaBON3*
TaBON3-2R	TCTTTTGTTTCTTGCTGG	
PR2-F	GCGTGAAGGTGGTGATTT	qRT-PCR primers for *TaPR2* (DQ090946)
PR2-R	GTGCCCGTTACACTTGGAT	
PR10-F	ACGGAGCGGATGTGGAAG	qRT-PCR primers for *TaPR10* (CV778999)
PR10-R	GCCACCTGCGACTTGAGC	
Actin -F	CCTTCGTTTGGACCTTGCTG	qRT-PCR primers for *TaActin*
Actin-R	AGCTGCTCCTAGCCGTTTCC	

SMART (http://smart.embl-heidelberg.de/). Multiple sequence alignment of full-length copine proteins was carried out using the MEGA5.02 program (http://www.megasoftware.net/history.php) with default parameters, and then displayed on GeneDoc (http://www.softpedia.com/get/Science-CAD/GeneDoc.shtml). MEGA5.02 software was also used to create the phylogenetic trees of the copine family members.

Bgt isolates and inoculation

The naturally occurring *Bgt* population was collected from the field in Nanjing, Jiangsu Province, China (latitude 31°14″N to 32°37″N, longitude 118°22″E to 119°14″E) and was propagated for disease resistance tests. Sumai 3 plants were used as inoculum to breed fungi under spore-proof glasshouse conditions. The infected Sumai 3 plants were grown in a 16 h/8 h, 20 °C/18 °C, day/night cycle, 80% relative humidity, and used to inoculate the surface of the tested leaves by gently shaking conidia (Chen *et al.*, 2007).

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The first strand of complementary DNA (cDNA) was synthesized using the Transcript 1st strand cDNA synthesis kit (Vazyme, Nanjing, China). qRT-PCR was performed in a CFX 96 Real Time PCR System (Bio-Rad Laboratories, USA) in 20 μL of reaction mixture containing 2 × SYBR Premix Ex Taq Kit (TaKaRa, http://www.takara-bio.com/), 0.4 μM of each primer and 1 μL of tenfold-diluted cDNA template. The PCR program used was as follows: 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C, 30 s at 59 °C and 30 s at 72 °C, and a final 10 min at 72 °C. Three technical replicates were run for each cDNA sample. The sequences of qRT-PCR primers are listed in Table 1.

BSMV-mediated *TaBON1* and *TaBON3* gene silencing

To generate the BSMV:*TaBON1* and BSMV:*TaBON3* constructs, the 194-bp and 224-bp sequences of *TaBON1* and *TaBON3* were amplified from Sumai 3. These two fragments were then each inserted into the BSMV:00 vector in reverse orientation to form the recombinant vectors BSMV:*TaBON1* and BSMV:*TaBON3*. The second fully expanded leaves of 2-week-old Sumai 3 seedlings were inoculated with virus containing BSMV:*TaBON1* or BSMV:*TaBON3* by gently sliding pinched fingers from the leaf base to the tip three times, with virus containing BSMV:*TaPDS* or BSMV:00 as the control. Three independent sets of plants were prepared for each treatment with 50 seedlings for each BSMV virus. Thirty seedlings were mock treated with 2 × GKP buffer (50 mM glycine, 30 mM K_2HPO_4, pH 9.2, 1% bentonite, 1% celite) as a negative control. Fourteen days after virus inoculation, the detached fourth leaves from 30 plants were placed on 6-Benzylaminopurine (6-BA) medium and inoculated with high densities of freshly collected *Bgt*. The phenotypes of inoculated leaves were observed and photographed at 5 dpi.

Observations of H_2O_2 and cell death accumulation

H_2O_2 production in plants was detected by an endogenous peroxidase-dependent *in situ* histochemical staining procedure using DAB (Thordal-Christensen *et al.*, 1997). The fourth leaves from silenced plants at 0 and 3 dpi after *Bgt* infection were stained with 1 mg/mL DAB dissolved in 1 M Tris-HCl (pH 7.5) for 6–8 h, discoloured in absolute ethyl alcohol and stored in 50% glycerol (Chen *et al.*, 2016). Trypan blue staining was performed to reveal dead plant cells (Thordal-Christensen *et al.*, 1997). Tissues were immersed in 0.4% trypan blue solution for 6 h, bleached with a solution of acetic acid–ethanol (3 : 1) at 37 °C and then stored in 50% glycerol.

DISCLOSURE

The authors have no conflicts of interest to declare.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Xiue Wang (Nanjing Agricultural University) for wheat materials and vectors. We thank Zongkuan Wang, Jia Zhao, Wentao Wan and Xiaobing Wang for technical assistance. This work was supported by grants from the Natural Science Foundation of Jiangsu Province (BK20150662), the Open Project Program of State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control (2010DS700124-KF1509) and Jiangsu Collaborative Innovation Center for Modern Crop Production.

REFERENCES

Ahmed, S.M., Liu, P., Xue, Q., Ji, C., Qi, T., Guo, J., Guo, J. and Kang, Z. (2017) TaDIR1–2, a wheat ortholog of lipid transfer protein AtDIR1 contributes to negative regulation of wheat resistance against *Puccinia striiformis* f. sp *tritici*. *Front. Plant Sci.* **8**, 521.

Bennypaul, H.S., Mutti, J.S., Rustgi, S., Kumar, N., Okubara, P.A. and Gill, K.S. (2012) Virus-induced gene silencing (VIGS) of genes expressed in root, leaf, and meiotic tissues of wheat. *Funct. Integr. Genomics*, **12**, 143–156.

Bernoux, M., Ellis, J.G. and Dodds, P.N. (2011) New insights in plant immunity signaling activation. *Curr. Opin. Plant Biol.* **14**, 512–518.

Caruso, C., Chilosi, G., Caporale, C., Leonardi, L., Bertini, L., Magro, P. and Buonocore, V. (1999) Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum*. *Plant Sci.* **140**, 87–97.

Chen, T., Xiao, J., Xu, J., Wan, W., Qin, B., Cao, A., Chen, W., Xing, L., Du, C., Gao, X., Zhang, S., Zhang, R., Shen, W., Wang, H. and Wang, X. (2016) Two members of TaRLK family confer powdery mildew resistance in common wheat. *BMC Plant Biol.* **16**, 1–17.

Chen, Y.-P., Xing, L.-P., Wu, G.-J., Wang, H.-Z., Wang, X.-E., Cao, A.-Z. and Chen, P.-D. (2007) Plastidial glutathione reductase from *Haynaldia villosa* is an enhancer of powdery mildew resistance in wheat (*Triticum aestivum*). *Plant Cell Physiol.* **48**, 1702–1712.

Creutz, C.E., Tomsig, J.L., Snyder, S.L., Gautier, M.-C., Skouri, F., Beisson, J. and Cohen, J. (1998) The copines, a novel class of C2 domain-containing, calcium-dependent, phospholipid-binding proteins conserved from *Paramecium* to humans. *J. Biol. Chem.* **273**, 1393–1402.

Gou, M.Y., Zhang, Z.M., Zhang, N., Huang, Q.S., Monaghan, J., Yang, H.J., Shi, Z., Zipfel, C. and Hua, J. (2015) Opposing effects on two phases of defense responses from concerted actions of HEAT SHOCK COGNATE70 and BONZAI1 in Arabidopsis. *Plant Physiol.* **169**, 2304–2323.

Hofius, D., Tsitsigiannis, D.I., Jones, J.D. and Mundy, J. (2007) Inducible cell death in plant immunity. *Semin. Cancer Biol.* **17**, 166–187.

Holzberg, S., Brosio, P., Gross, C. and Pogue, G.P. (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J.* **30**, 315–327.

Hua, J., Grisafi, P., Cheng, S.H. and Fink, G.R. (2001) Plant growth homeostasis is controlled by the Arabidopsis BON1 and BAP1 genes. *Gene Dev.* **15**, 2263–2272.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. *Nature*, **444**, 323–329.

Kage, U., Karre, S., Kushalappa, A.C. and McCartney, C. (2017) Identification and characterization of a fusarium head blight resistance gene TaACT in wheat QTL-2DL. *Plant Biotechnol. J.* **15**, 447–457.

Kim, C., Meskauskiene, R., Zhang, S.R., Lee, K.P., Ashok, M.L., Blajecka, K., Herrfurth, C., Feussner, I. and Apel, K. (2012) Chloroplasts of Arabidopsis are the source and a primary target of a plant-specific programmed cell death signaling pathway. *Plant Cell*, **24**, 3026–3039.

Lehmann, S., Serrano, M., L'Haridon, F., Tjamos, S.E. and Metraux, J.P. (2015) Reactive oxygen species and plant resistance to fungal pathogens. *Phytochemistry*, **112**, 54–62.

Li, Y.Q., Pennington, B.O. and Hua, J. (2009) Multiple R-like genes are negatively regulated by BON1 and BON3 in Arabidopsis. *Mol. Plant–Microbe Interact.* **22**, 840–848.

Liu, J., Zhang, T.R., Jia, J.Z. and Sun, J.Q. (2016) The wheat mediator subunit TaMED25 interacts with the transcription factor TaEIL1 to negatively regulate disease resistance against powdery mildew. *Plant Physiol.* **170**, 1799–1816.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B. and Ton, J. (2011) Callose deposition: a multifaceted plant defense response. *Mol. Plant–Microbe Interact.* **24**, 183–193.

McCouch, S., Baute, G.J., Bradeen, J., Bramel, P., Bretting, P.K., Buckler, E., Burke, J.M., Charest, D., Cloutier, S., Cole, G., Dempewolf, H., Dingkuhn, M., Feuillet, C., Gepts, P., Grattapaglia, D., Guarino, L., Jackson, S., Knapp, S., Langridge, P., Lawton-Rauh, A., Lijua, Q., Lusty, C., Michael, T., Myles, S., Naito, K., Nelson, R.L., Pontarollo, R., Richards, C.M., Rieseberg, L., Ross-Ibarra, J., Rounsley, S., Hamilton, R.S., Schurr, U., Stein, N., Tomooka, N., van der Knaap, E., van Tassel, D., Toll, J., Valls, J., Varshney, R.K., Ward, J., Waugh, R., Wenzl, P. and Zamir, D. (2013) Agriculture: feeding the future. *Nature*, **499**, 23–24.

McDonald, B.A. and Linde, C. (2002) The population genetics of plant pathogens and breeding strategies for durable resistance. *Euphytica*, **124**, 163–180.

McIntosh, R.A., Dubcovsky, J., Rogers, W.J., Morris, C.F. and Xia, X.C. (2017) Catalogue of gene symbols for wheat: 2017 supplement. *Annu. Wheat News Lett.*

Mohammadi, M., Srivastava, S., Hall, J.C., Kav, N.N.V. and Deyholos, M.K. (2012) Two wheat (*Triticum aestivum*) pathogenesis-related 10 (PR-10) transcripts with distinct patterns of abundance in different organs. *Mol. Biotechnol.* **51**, 103–108.

Morel, J.B. and Dangl, J.L. (1997) The hypersensitive response and the induction of cell death in plants. *Cell Death Differ.* **4**, 671–683.

Scofield, S.R., Huang, L., Brandt, A.S. and Gill, B.S. (2005) Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. *Plant Physiol.* **138**, 2165–2173.

Segonzac, C. and Zipfel, C. (2011) Activation of plant pattern-recognition receptors by bacteria. *Curr. Opin. Microbiol.* **14**, 54–61.

Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D.B. (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *Plant J.* **11**, 1187–1194.

Torres, M.A., Jones, J.D.G. and Dangl, J.L. (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* **141**, 373–378.

Yang, S., Yang, H., Grisafi, P., Sanchatjate, S., Fink, G.R., Sun, Q. and Hua, J. (2006) The BON/CPN gene family represses cell death and promotes cell growth in Arabidopsis. *Plant J.* **45**, 166–179.

Yang, S.H. and Hua, J. (2004) A haplotype-specific resistance gene regulated by BONZAI1 mediates temperature-dependent growth control in Arabidopsis. *Plant Cell*, **16**, 1060–1071.

Yao, G., Zhang, J., Yang, L., Xu, H., Jiang, Y., Xiong, L., Zhang, C., Zhang, Z., Ma, Z. and Sorrells, M.E. (2007) Genetic mapping of two powdery mildew resistance genes in einkorn (*Triticum monococcum* L.) accessions. *Theor. Appl. Genet.* **114**, 351–358.

Zhang, J. and Zhou, J.M. (2010) Plant immunity triggered by microbial molecular signatures. *Mol. Plant*, **3**, 783–793.

Zhou, H., Li, S., Deng, Z., Wang, X., Chen, T., Zhang, J., Chen, S., Ling, H., Zhang, A., Wang, D. and Zhang, X. (2007) Molecular analysis of three new receptor-like kinase genes from hexaploid wheat and evidence for their participation in the wheat hypersensitive response to stripe rust fungus infection. *Plant J.* **52**, 420–434.

Zou, B., Hong, X., Ding, Y., Wang, X., Liu, H. and Hua, J. (2016) Identification and analysis of copine/BONZAI proteins among evolutionarily diverse plant species. *Genome*, **59**, 565–573.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Sequence alignment of three homeologues of TaBON1 in common wheat. Amino acid sequences of TaBON1. TaBON-A1 and TaBON-B1 represent TaBON1 proteins from the common wheat A and B genomes, respectively. The conserved C2A, C2B and von Willebrand factor A (vWA) domains are underlined separately in red, blue and orange, respectively.

Fig. S2 Sequence alignment of three homeologues of TaBON3 in common wheat. Amino acid sequences of TaBON3. TaBON-A3, TaBON-B3 and TaBON-D3 represent TaBON3 proteins from the common wheat A, B and D genomes, respectively. The conserved C2A, C2B and von Willebrand factor A (vWA) domains are underlined separately in red, blue and orange, respectively.

Fig. S3 Silencing of *TaBON1* or *TaBON3* enhanced resistance to *Blumeria graminis* f. sp. *tritici* (*Bgt*) in living wheat leaves. (A) The leaf phenotype of successful virus infection. Photographs were captured at 10 days after virus inoculation. MOCK: wheat leaves treated with 2 × GKP buffer. Scale bars, 1 cm. (B, C) The expression levels of TaBON1 (B) and TaBON3 (C) in plants at 22 °C inoculated with a *Barley stripe mosaic*

virus (BSMV)-based silencing system and assayed by reverse transcription-polymerase chain reaction (RT-PCR). TaActin was used as the internal control. Similar results were seen in all three biological replicates and one biological repeat is shown. (D) The disease phenotype of living leaves at 14 days after infection with spores of *Bgt* isolated from the field. Representative leaves were photographed at 5 dpi.

Fig. S4 *TaPR* genes are induced by *Blumeria graminis* f. sp. *tritici* (*Bgt*) infection. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis of the relative expression levels of *TaPR2* and *TaPR10* in *Bgt*-infected common wheat leaves. RNA samples were isolated from the leaves of *Bgt*-infected common wheat Sumai 3 at 0, 1, 2, 4, 8, 12, 24, 36 and 48 h post-inoculation (hpi). Expression is normalized by the expression level at 0 hpi. *TaActin* was used as the internal control. One biological replicate with three technical repeats is shown. Asterisks indicate statistically significant differences in comparison with 0 hpi at $P \leq 0.01$ (Student's *t*-test).

Fig. S5 H_2O_2 accumulation and cell death in response to *Blumeria graminis* f. sp. *tritici* (*Bgt*) infection in BSMV:00. The fourth leaves of BSMV:00-inoculated plants challenged with *Bgt* and stained with either 3,3′-diaminobenzidine (DAB) for H_2O_2 accumulation sampled at 0 and 3 days post-inoculation (dpi) (A) or with trypan blue for cell death at 0, 24, 48 and 72 hpi (B). Scale bars, 1 cm.