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Title: The wheat LLM-domain-containing transcription factor TaGATA1 positively modulates host immune response to *Rhizoctonia cerealis*

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Highlight:

A wheat LLM-domain-containing B-GATA transcription factor positively regulates host immune response to the important pathogen *Rhizoctonia cerealis*.

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Abstract

Wheat (*Triticum aestivum*) is essential for global food security. *Rhizoctonia cerealis* is the causal pathogen of sharp eyespot, an important disease of wheat. GATA proteins in model plants have been implicated in growth and development; however, little is known about their roles in immunity. Here, we reported a defence role of a wheat LLM-domain-containing B-GATA transcription factor, TaGATA1, against *R. cerealis* infection and explored the underlying mechanism. Through transcriptomic analysis, *TaGATA1* was identified to be more highly expressed in resistant wheat genotypes than in susceptible wheat genotypes. *TaGATA1* was located on chromosome 3B and had two homoeologous genes on chromosomes 3A and 3D. TaGATA1 was demonstrated to localize in the nucleus, possess transcriptional-activation activity, and bind to GATA-core *cis*-elements. *TaGATA1* overexpression significantly enhanced resistance of transgenic wheat to *R. cerealis*, whereas silencing of *TaGATA1* suppressed the resistance. RT-qPCR and chromatin immunoprecipitation-qPCR results indicated that TaGATA1 directly bound to and activated certain defence genes in host immune response to *R. cerealis*. Collectively, TaGATA1 positively regulates immune responses to *R. cerealis* through activating expression of defence genes in wheat. This study reveals a new function of plant GATAs in immunity and provides a candidate gene for improving crop resistance to *R. cerealis*.

Key words: Bread wheat (*Triticum aestivum*), B-GATA transcription factor, defence gene, immune response, *Rhizoctonia cerealis*, transcriptional activation

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Introduction

GATA transcription factors (TFs) are a group of transcriptional regulatory proteins containing a type IV zinc finger $CX_2CX_{17–20}CX_2C$ (C, cysteine; X, any residue) DNA-binding domain that can recognize GATA-core *cis*-elements in promoters(Reyes *et al.*, 2004). GATA TFs are divided into A-, B-, C-, and D-GATA classes based on conservation of amino-acid sequences and the exon-intron gene structure of the respective gene (Behringer *et al.*, 2014; Ranftl *et al.*, 2016; Reyes *et al.*, 2004). In *Arabidopsis*, B-GATA proteins can be further subdivided into two subfamilies based on the presence of additional conserved domains apart from the GATA domain: GATAs with a conserved LLM (leucine-leucine-methionine) domain at their C terminus, and GATAs with a conserved HAN (HANABA TARANU) domain in their N terminus. Based on the amino-acid sequence length between the N-terminus to the GATA domain, LLM domain-containing GATA proteins were further divided into short B-GATAs with an LLM domain and long B-GATAs with an LLM-domain. *Arabidopsis GATA23* is a long B-GATA member with a degenerate LLM-domain, while *GATA29* contains a degenerate HAN-domain (Behringer *et al.*, 2014; Behringer and Schwechheimer, 2015). LLM- and HAN-domain containing B-GATA proteins have been identified in all the sequenced monocot and dicot species (Behringer *et al.*, 2014; Chen *et al.*, 2017; Ranftl *et al.*, 2016; Reyes *et al.*, 2004). For instance, genome-wide surveys of GATA domain-containing sequences identified 30 GATA members in *Arabidopsis*, and 29 in rice (*Oryza sativa*) (Bi *et al.*, 2005; Chen *et al.*, 2017; Reyes *et al.*, 2004).

In model plants, B-GATA factors have been shown to participate in various plant growth and developmental processes (Behringer and Schwechheimer, 2015; Houston *et al.*, 2012; Hudson *et al.*,2013; Klermund *et al.*, 2016; Lu *et al.*, 2017; Ranftl *et al.*,

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2016; Wang *et al.*, 2009). For instance, GNC (GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED) and its paralog CGA1/GNL (CYTOKININ-INDUCED GATA1/GNC-LIKE), two representative long LLM domain-containing B-GATA factors, promote greening and chloroplast biogenesis, hypocotyl elongation, stomata development, leaf development, and plant architecture, but suppress germination in *Arabidopsis* (Richter *et al.*, 2010, 2013a; Chiang *et al.*, 2012; Bihringer *et al.*, 2014; Klermund *et al.*, 2016). *GNC* and *GNL* also delay flowering time and participate in the response to cold stress (Richter *et al.*, 2013b). In *Arabidopsis*, loss-of-function mutants of *GATA15*, *GATA16*, *GATA17*, and *GATA17L*, short LLM-domain B-GATA members, displayed early flowering and similar phenotypes to *gnc* and *gnl* mutants (Ranftl *et al.*, 2016). Additionally, *GATA15*, *GATA16*, *GATA17*, and *GATA17L* function in phyllotactic patterning, floral organ initiation, and accessory meristem formation (Ranftl *et al.*, 2016). These reports suggest that LLM-domain B-GATA factors share redundant biological roles in development and greening and chloroplast biogenesis. In *Arabidopsis*, HAN factor, a HAN-domain containing B-GATA member, has been shown to regulate embryo development and floral development, and to act as a repressor of cell proliferation (Zhao *et al.* 2004; Nawy *et al.*, 2010). Three HAN paralogous factors, NL1 (NECK LEAF1) in rice, TSH1 (TASSEL SHEATH1) in maize, and TRD (THIRD OUTER GLUME) in barley, were found to repress growth and cell cycle activities in the shoot meristem (Wang *et al.*, 2009; Whipple *et al.*, 2010). However, no genetic evidence for the roles of GATAs in immune responses has been reported in plant species.

Bread wheat (*Triticum aestivum*) is one of the most important staple crops in the world. Numerous diseases, caused by various pathogens, reduce yield and grain quality of wheat. Sharp eyespot, caused mainly by the necrotrophic fungus

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Rhizoctonia cerealis, is a devastating disease of wheat worldwide (Chen *et al.*, 2013; Chen *et al.*, 2008; Hamada *et al.*, 2011). Since 2005, more than 6.67 million hectares of wheat plants each year have been harmed by sharp eyespot in China, which has become the largest epidemic region in the world (Chen *et al.*, 2013; Zhu *et al.*, 2015). Infection of *R. cerealis* causes not only sharp eyespot of other cereal crops including barley, oats, and rye but also other diseases in important economic crops and bioenergy plants, such as root rot in sugar beet, cotton, potato, and several legumes (Toda *et al.*, 1999; Tomaso-Peterson and Trevathan, 2007). Breeding crop varieties with resistance is an environmentally friendly and effective method to control the diseases caused by *R. cerealis*. It is necessary to isolate and characterize the key elements in wheat responses to infection with *R. cerealis*, and to unravel their underlying mechanisms.

In this study, we identified a short LLM-domain B-GATA TF-encoding gene, *TaGATA1*, in the wheat response to *R. cerealis* infection through comparative transcriptomic analysis. Molecular biology assays revealed that TaGATA1 was a GATA domain-binding transcription activator localized in the nucleus. Genetic functional assays demonstrated that *TaGATA1* positively regulated the immune response to *R. cerealis* infection in wheat. Both RT-qPCR and chromatin immunoprecipitation (ChIP)–qPCR analyses showed that TaGATA1 could bind to and activate the expression of certain defence genes. This study reveals a novel functional role of plant GATA TFs in innate immunity.

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Materials and Methods

Plant and fungal materials, primers, and treatments

Six wheat cultivars (cvs.), including CI12633, Shanhongmai, Niavt14, Shannong0431, Yangmai 16, and Wenmai 6, showing different extents of resistance to sharp eyespot caused by *R. cerealis* (Zhu *et al.*, 2015), were used to investigate the expression profile or function of *TaGATA1*. The wheat cv. Chinese spring nulli-tetrasomic (NT) lines, including N3A/T3B, N3A/T3D, N3B/T3A, N3B/T3D, N3D/T3A, and N3D/T3B, were used for chromosomal localization of *TaGATA1*.

The fungal pathogen *R. cerealis* isolate R0301, which is dominant in Jiangsu and Anhui provinces of China, and the strain WK207, which is dominant in North China, were used in this study. The wheat growth conditions, *R. cerealis* inoculation, and sampling were conducted following (Zhu *et al.*, 2015). The sequences of all primers are listed in Table S1.

Cloning and sequence analysis of *TaGATA1*

RNA extraction was performed following Zhang *et al.* ((Zhang *et al.*, 2007). The primers for 3'-RACE and 5'-RACE were designed based on the sequence of the microarray probe TC415152, and synthesized. Through two rounds of PCR reactions using 3'RACE primers and 3-RACE kit v.2.0 (TaKaRa, Japan), the 3'-UTR sequence of *TaGATA1* was amplified from cDNA of CI12633 stems inoculated with *R. cerealis* R0301 for 4 d. The 5'-UTR sequence of *TaGATA1* was amplified through three rounds of PCR reactions using 5'RACE primers from the CI12633 cDNA. Based on the assembled sequences of the gene, two pairs of *TaGATA1*-specific primers were

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designed and used for nested PCR to amplify the full length cDNA and DNA sequences of *TaGATA1* from cDNA and genomic DNA of CI12633 stems.

TaGATA1 promoter sequence was cloned from CI12633 genomic DNA using the primers *TaGATA1*-pro-all-F/R. *Cis*-elements in the *TaGATA1* promoter were analyzed using https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?sid=&lang=en&pj=640&action= page&page=newplace. A phylogenetic tree was constructed using a neighbor-joining method implemented in MEGA V 5.0 with 1000 bootstrap replications.

Subcellular localization of TaGATA1

The *TaGATA1* coding sequence without a stop codon was sub-cloned to fuse with the 5'-end of GFP (green fluorescent protein) coding sequence in a p*35S:GFP* vector. The *TaGATA1-GFP* transcript was controlled using a *CaMV35S* promoter. The plasmid DNA of the resulting fusion construct and p*35S:GFP* control were separately introduced into wheat protoplasts by PEG4000 or white onion epidermal cells using biolistic bombardment as described previously (Zhang *et al.*, 2007). After incubation at 25 °C for 20 h, GFP signals were observed and photographed (Qi *et al.*, 2017) using a confocal laser scanning microscope (Zeiss LSM 700, Germany) with a Fluor 10X/0.50 M27 objective lens and SP640 filter.

Electrophoretic mobility shift assay (EMSA)

TaGATA1 was fused into a His-Trigger Factor (His-TF) tag of a p*Cold-TF* vector. The resulting p*His-TF-TaGATA1* recombinant construct was transformed into competent cells of *Escherichia coli* Transetta (DE3) (Transgen, China). Subsequently, the His-TF-TaGATA1 recombinant protein was expressed after induction with 0.5 mM IPTG

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(isopropyl β-D-1-thiogalactopyranoside) at 16 °C, and purified using Ni-NTA Resin (Transgen, China). The biotin-labeled DNA oligonucleotides listed in Table S1 were synthesized and used as probes, and unlabeled DNA fragments of the same sequences were used as the competitors. Double-stranded DNA was obtained by heating oligonucleotides at 95 °C for 15 min and annealing at room temperature. EMSA was performed using the LightShift Chemiluminescent EMSA kit (Thermo, USA) according to the manufacturer's instructions. The His-TF protein alone was used as the negative control.

Yeast hybrid assay on transcriptional-activation activity of TaGATA1

The yeast strain (AH109) containing the reporter genes *HIS3* and *ADE2*, was used in the transcription-activation assay of TaGATA1. The *TaGATA1* coding sequence was fused with the GAL4 DNA-binding domain (BD) in the p*GBKT7* vector to generate p*BD-TaGATA1* expressing BD-TaGATA1 fused protein. The resulting vector DNA was transformed into competent cells of yeast AH109 via a PEG-mediated method. The empty p*GBKT7* (p*BD*) vector was used as a negative control. Transcription-activation activity of TaGATA1 was evaluated according to the growth on SD/Trp (Yeast Synthetic Drop-out Medium Supplement without tryptophan) (Clontech, USA) and SD/Trp/His/Ade (Yeast Synthetic Drop-out medium supplement without tryptophan, histidine, or adenine) (Clontech, USA) .

Virus-induced gene silencing for the defence function of *TaGATA1* in wheat

The defensive role of *TaGATA1* was investigated using a barley stripe mosaic virus (BSMV)-based virus induced gene silencing (VIGS) method (Holzberg *et al.* 2002; Scofield *et al.*, 2005). A 200-bp fragment of *TaGATA1* (no. 756 to 955 nt in *TaGATA1* cDNA sequence) was sub-cloned in an antisense orientation into the *Nhe* I

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restriction site of RNAγ of BSMV, resulting in a *BSMV:TaGATA1* recombinant construct (Fig. S1) (Donald and Jackson, 1996). At the three-leaf stage, at least 20 plants of resistant wheat line CI12633 were inoculated with *BSMV:TaGATA1* or *BSMV:GFP* (as a control) following (Zhu *et al.*, 2015). At 20 d after virus infection, the fourth leaves were sampled to examine BSMV infection and the transcriptional level of *TaGATA1*. At 22-25 d after BSMV inoculation, these CI12633 plants were further inoculated with *R. cerealis* WK207. Following(Chen *et al.*, 2008), their infection types (ITs) and disease indexes were scored at 40 dpi with *R. cerealis* WK207.

Generation of *TaGATA1*-overexpressing transgenic wheat

The full ORF sequence of *TaGATA1* was sub-cloned into a modified p*AHC25* vector (Christensen and Quail, 1996) with a c-myc epitope tag (Christensen & Quail, 1996; Zhu *et al.*, 2015). In the resulting transformation vector p*Ubi:myc-TaGATA1* (Fig. S2), *c-myc-TaGATA1* was driven by the maize *ubiquitin* (*Ubi*) promoter and terminated by the 3′ non-transcribed region of *Agrobacterium tumefaciens* nopaline synthase gene (*Tnos*). p*UBI:myc-TaGATA1* plasmid DNA was introduced into immature embryos of the wheat cv. Yangmai16 by biolistic bombardment (von Arnim, 2007).

PCR and western blotting analyses on *TaGATA1* transgenic wheat

The presence of the introduced *TaGATA1* transgene was monitored by PCR using the transgene-specific primer pair (TaGATA1-TF & TaGATA1-TR) that spans the intron of *TaGATA1* genomic sequence. PCR was performed in a 20 μl volume containing 1 μl genomic DNA (200 ng/μl), 10 μl 2×PCR Mixture (Transgen, China), 0.5 μl each primer (10 μM), and 8 μl ddH_2O.

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Total proteins were extracted from 1 g of stems from each transgenic wheat line using a tissue protein extraction kit (CWBIO, China). Western blotting was deployed to investigate c-myc-TaGATA1 fusion protein with 100-fold diluted anti-c-myc antibody. The c-myc-TaGATA1 protein hybridized with 1000-fold diluted secondary antibody conjugated to horseradish peroxidase was visualized using the Pro-light HRP Chemiluminescent Kit (Transgen, China).

RT-PCR and RT-qPCR

The transcriptional levels of *TaGATA1*, *BSMV-CP*, and defence-associated genes in wheat were analyzed by RT-PCR or RT-qPCR. RT-qPCR was done on an ABI 7500 real time PCR system (Applied Biosystems, USA) following ((Dong *et al.*, 2010). The relative expression of the tested genes was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), where the wheat *Actin* gene *TaActin* was used as the internal reference. Three independent replications were performed.

Assessment of response in transgenic wheat plants to *R. cerealis*

R. cerealis isolate R0301 was used to inoculate T_1 and T_2 plants grown in a greenhouse, and *R. cerealis* isolate WK207 was used to inoculate T_3 plants grown in the field. At the tillering stage, the wheat plants were inoculated on each stem base with 8-10 wheat grains harboring *R. cerealis* mycelia. To enhance *R. cerealis* infection, the plants were sprinkled with water twice a day during the first seven days, then with a frequency depending on rainfall and soil moisture until final disease was recorded. Ten to 30 plants of each line were assessed for disease severity. ITs and disease indexes of wheat plants/lines were scored at the harvest stage following (Chen *et al.*, 2008).

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ChIP-qPCR

A rapid and efficient ChIP analysis was performed following a modified method (Lee *et al.*, 2017; Nelson *et al.*, 2006). Briefly, wheat protoplasts were isolated from two-leaf-stage seedlings. p*35S:GFP* and p*35S:TaGATA1-GFP* vectors were introduced into protoplasts. Total protein was extracted from 100 μl protoplast solution using a tissue protein extraction kit (CWBIO, China). Transient expression of GFP and TaGATA1-GFP was confirmed by western blot using 400-fold diluted monoclonal anti-GFP antibody. The remaining protoplasts expressing GFP or TaGATA1-GFP were crosslinked with 1% formaldehyde for 15 min. The nuclei were isolated, then chromatins were sonicated and sheared to 100-500 bp using a Bioruptor UCD-200.

Immunoprecipitation was performed with 400-fold diluted monoclonal anti-GFP antibody (Transgen, China). Immune complexes were collected by Dynabeads protein G (Invitrogen, USA) and then DNA fragments were recovered using the phenol-chloroform method. Subsequently, qPCR was used to investigate the degree of enrichment of the GATA-containing DNA fragment bound by GFP-TaGATA1. ChIP-qPCR comparisons were made between lines carrying the TaGATA1-GFP-expressing protoplasts versus GFP-expressing protoplasts.

Results

Identification and transcriptional profiles of *TaGATA1* in wheat response to *R. cerealis*

To mine resistance response-related genes of wheat to *R. cerealis*, we compared the microarray data (GEO accession number GSE69245) of *R. cerealis*-resistant wheat cvs. CI12633/Shanghongmai and the susceptible cv. Wenmai 6 at 4, 7, and 21 d post inoculation (dpi) with *R. cerealis* isolate R0301. One important regulatory gene

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occupied by the probe (ID: A_99_P340746 in Agilent Wheat GeneChip) was identified and corresponded to 3' sequence of a wheat EST sequence with TIGR number TC415152. Hereafter, this gene sequence was cloned from resistant wheat CI12633 and designated *TaGATA1* since it encodes an LLM-domain B-GATA protein. The microarray data showed that at 4, 7, or 21 dpi with *R. cerealis* R0301, the gene transcriptional levels were higher in both resistant wheat cvs CI12633 and Shanhongmai than in susceptible wheat cv. Wenmai 6 (Fig. 1A).

Further RT-qPCR results showed that at 1, 4, 10, and 21 dpi with *R. cerealis* R0301, the transcriptional levels of *TaGATA1* were higher in resistant wheat cv. CI12633 than in susceptible cv. Wenmai 6, consistent with the trend in microarray data (Fig. 1B). The transcription of *TaGATA1* in resistant wheat cv. CI12633 was obviously increased after pathogen infection, and reached a peak at 10 dpi (Fig. 1B). After inoculation with *R. cerealis* R0301, *TaGATA1* transcription was significantly higher in the partially resistant wheat cvs (Shanhongmai, CI12633, Niavt14, and Shannong0431) than in susceptible cv. Wenmai 6 (Fig. 1C). Transcriptional analyses in different organs at the heading stage showed that at 3 dpi with *R. cerealis* R0301, *TaGATA1* transcription was the highest in stems where sharp eyespot disease primarily occurs (Fig. 1D). These results suggested that *TaGATA1* might participate in the wheat immune response to *R. cerealis*.

Sequence characteristics and chromosomal localization of *TaGATA1*

The full-length cDNA of *TaGATA1* (1096 bp) was cloned from resistant wheat CI12633 stem cDNA and has been deposited in GenBank (accession number MG461317). It contains an open reading frame (ORF) that is 546 bp in length, with a 5'-UTR of 249 bp, and a 3'-UTR of 301 bp. The cDNA sequence of *TaGATA1* shares

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92.04% identity with the matched sequence TC415152. Genomic sequence 1251 bp in length was also cloned from CI12633. Comparison of the cDNA and genomic sequences showed that *TaGATA1* genomic sequence comprises 1 intron (155 bp) and 2 exons (Fig. 2A). Furthermore, the promoter sequence (1793 bp) upstream of the start codon of *TaGATA1* was cloned from CI12633 genomic DNA, and includes several biotic stress-responsive *cis*- elements, phytohormone responsive *cis*-elements, and light responsive elements (Table S2). The deduced protein TaGATA1 consisted of 181 amino acid residues with a molecular weight of 19.817 kD and a theoretical pI of 9.313. TaGATA1 contains an acidic region (amino acids 2-32) that possibly acts as a transcription activation domain, a conserved GATA-motif binding domain (amino acids 29-79), two putative nuclear localization signals (NLS1 and NLS2, located in amino acids 58-79 and 145-157, respectively), and an LLM-domain (amino acids 167-174) (Fig. 2B).

TaGATA1 and some GATA proteins from wheat, *Arabidopsis*, rice, barley, and *Brachypodium distachyon* were subjected to phylogenetic analysis. The phylogenetic analysis revealed that these GATA proteins belong to short LLM domain B-GATA subfamily, GATA23 with degenerate LLM-domain, long LLM domain B-GATA subfamily, long B-GATAs with an HAN-domain subfamily, and GATA29 with degenerate HAN-domain. TaGATA1 fell into the short LLM-domain B-GATA subfamily, while HvGATA6 (TRD1, Houston *et al.*, 2012), its paralog in wheat named TaGATA1AL, TaGATA18, AtGATA18-20, OsGATA8-9 and OsGATA15 as well BdGATA7-8 fell into the long B-GATAs HAN-domain subfamily (Fig. S3). TaGATA1 is closer to TaGATA16 with 76.50% identity then to HvGATA2 (70.62% identity), whose functions have not been reported yet. The whole amino acid sequence of TaGATA1 shared 64.44%, 47.51%, 28.72%, 32.80%, 29.74%, and 30.35%

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identities with those of function-unknown *Brachypodium distachyon* BdGATA4 and function-known OsGATA12, AtGATA15, AtGATA16, AtGATA17, and AtGATA17LIKE, respectively. These data showed that TaGATA1 is a short LLM-domain B-GATA protein in wheat.

A BLAST analysis against the hexaploid wheat genome sequence showed that the *TaGATA1* genomic sequence shared 98% identity to the sequence TRIAE_CS42_3B_TGACv1_221429_AA0740590 on wheat chromosome 3B, suggesting that *TaGATA1* should be located on wheat chromosome 3B. Using *TaGATA1*-specific primers and the templates from genomic DNAs of nulli-tetrasomic (NT) lines of the wheat cultivar Chinese Spring, PCR results proved that *TaGATA1* was located on wheat chromosome 3B (Fig. 2C). Additionally, two homoeologous genes from chromosomes 3A and 3D, *TaGATA1-3A* and *TaGATA1-3D*, were cloned from CI12633, respectively. At the ORF region, *TaGATA1* on chromosome 3B (*TaGATA1-3B*), displayed 83.90% and 87.90% sequence identities with *TaGATA1-3A* and *TaGATA1-3D*, respectively (Fig. S4). The pairwise comparison indicated that the protein sequence of TaGATA1 on chromosome 3B shares 88.20% and 86.30% identities with the homoeologous proteins TaGATA1-3A and TaGATA1-3D, respectively, and all the three homoeologous proteins contain the conserved GATA-binding domain (Fig. S5).

TaGATA1 is localized in the nucleus

To investigate the subcellular localization, the full coding sequence of *TaGATA1* was fused to the N-terminus of GFP (Fig. 3A). DNAs of the resulting p*35S:TaGATA1-GFP* and p*35S:GFP* control constructs were individually introduced into wheat mesophyll protoplasts or onion epidermal cells. These fluorescent proteins were

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transiently expressed and observed via a confocal microscope. The fluorescent images in wheat mesophyll protoplasts showed that TaGATA1-GFP accumulated in the nucleus but the control GFP was distributed throughout the cell (Fig. 3A). Accordingly, in onion epidermal cells, the fluorescent images of TaGATA1-GFP and the control GFP displayed the same patterns as those in the wheat protoplasts (Fig. 3A). These results showed that the TaGATA1 TF localized in the nucleus.

TaGATA1 is a GATA-binding transcription activator

The transcription activation assay was performed in yeast. As shown in Fig. 3B, although all the yeast cells were able to grow well on the SD/Trp medium, only yeast cells expressing BD-TaGATA1 could grow on selective medium (SD/Trp/His/Ade). These results indicated that TaGATA1 could activate the transcription of reporter genes *Ade2* and *His3* in the yeast genome, and suggested that TaGATA1 might possess transcriptional-activation activity.

To examine the binding ability of TaGATA1 to GATA *cis*-elements, the recombinant protein His-TF-TaGATA1 was constructed and expressed in *E. coli*. The purified His-TF-TaGATA1 protein was mixed with the probe containing a GATA-core *cis*- element (the recognition sequence of GATA TFs) in binding reaction buffer. EMSA results showed that the gel mobility shift was present in the combination of His-TF-TaGATA1 protein with the probe containing the GATA-core *cis*-element (lanes 1-4 in Fig. 3C) but absent in the combination of His-TF protein with the probe (lane 5 in Fig. 3C). Furthermore, the binding was reduced with addition of unlabeled competitors with the same sequences (lanes 2-4 in Fig. 3C). These results proved that TaGATA1 could bind to the GATA-core *cis*- element.

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Silencing of *TaGATA1* suppresses wheat resistance to *R. cerealis*

The cDNA fragment specific to *TaGATA1* on chromosome 3B, with 67.60% and 79.50% sequence identities to the target regions of homoeologous genes on 3A and 3D, respectively, was used to construct the BSMV-based VIGS vector for specifically silencing *TaGATA1* in the resistant wheat cv. CI12633. At 20 d after transfection of BSMV-derived RNAs into leaves of CI12633, symptoms of BSMV infection appeared on these leaves and the transcript of BSMV coat protein (*cp*) was detected (Fig. 4A), indicating that BSMV infected these wheat plants. The transcriptional levels of *TaGATA1* were significantly decreased in *BSMV:TaGATA1*-infected CI12633 plants compared to *BSMV:GFP*-infected CI12633 plants (Fig. 4B), suggesting that *TaGATA1* was successfully silenced in *BSMV:TaGATA1*-infected (*TaGATA1*-silenced) plants.

Subsequently, *TaGATA1*-silenced and *BSMV:GFP*-infected plants were further inoculated with *R. cerealis* isolate WK207. At 21 dpi with *R. cerealis*, the stems of *TaGATA1*-silenced CI12633 plants displayed more serious necrosis of sharp eyespot than did *BSMV:GFP*-infected (control) plants (Fig. 4C). Based on two batches of VIGS and disease scoring at 40 dpi with *R. cerealis*, the average ITs and disease indexes of *TaGATA1*-silenced CI12633 plants were 2.33/3.78 and 46.6/75.60 but those of *BSMV:GFP*-infected CI12633 (control) plants were 1.43/2.53 and 28.60/50.60, respectively (Fig. 4D). These results indicated that silencing of *TaGATA1* suppressed resistance of wheat CI12633 to *R. cerealis*.

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TaGATA1 overexpression increases resistance of transgenic wheat to *R. cerealis*

To generate *TaGATA1*-overexpressing transgenic wheat plants and further explore the defence role of *TaGATA1*, the transformation vector p*Ubi:myc-TaGATA1* (Fig. S2) was constructed and bombarded into immature embryos of susceptible wheat cultivar Yangmai 16. The presence of alien *TaGATA1* transgene was detected by the PCR product using transgene-specific primers (Fig. S6). Based on results of PCR detections in four successive generations (T_0-T_3), five stable transgenic wheat lines (GO1-GO5) were screened. RT-qPCR analyses showed that transcriptional levels of *TaGATA1* in these five transgenic wheat lines were significantly elevated compared to non-transformed (wild type, WT) wheat Yangmai 16 (Fig. 5A). Western blotting analysis indicated that the introduced *myc-TaGATA1* was translated into myc-TaGATA1 protein in these five overexpressing transgenic lines, but not in WT Yangmai 16 (Fig. 5B).

After *R. cerealis* infection, all five *TaGATA1*-overexpressing lines in three successive (T_1-T_3) generations displayed significantly enhanced resistance to sharp eyespot compared with WT Yangmai 16 (Fig. 5C-5D, Table S3). For example, average infection types of these 5 *TaGATA1*-overexpressing lines in the T_2 generation were 1.00, 1.37, 1.50, 1.50, and 1.00, whereas an average infection type of WT Yangmai 16 was 3.06. The disease index of WT Yangmai 16 was 63.48, whereas those of these *TaGATA1*-overexpressing lines in the T_2 generation were 20-30 (Fig. 5D, Table S3), showing that the degree of resistance was significantly increased in all transgenic lines. These results suggest that *TaGATA1* positively regulates the resistance response to *R. cerealis* infection.

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TaGATA1 activates the expression of certain defence genes

The above trans-activation assay revealed that TaGATA1 is a transcriptional activator. To uncover if defence genes were activated by TaGATA1, RT-qPCR was deployed to analyze the transcriptional patterns of several defence-marker genes in wheat, including *pathogenesis-related* (*PR*) genes *PR10* and *PR17c*, and *Chitinase3*, in *TaGATA1*-overexpressing and *TaGATA1*-silenced wheat plants as well their control plants. RT-qPCR results showed that 7 dpi with *R. cerealis* WK207, transcriptional levels of *PR10, PR17C*, and *Chitinase3* were significantly elevated in *TaGATA1*-overexpressing lines compared to WT Yangmai 16, whereas they were significantly decreased in *TaGATA1*-silenced plants compared to BSMV:GFP-infected control plants (Fig. 6). These results suggest that TaGATA1 can activate the expression of certain defence genes in the wheat immune response to *R. cerealis.*

ChIP indicates binding of TaGATA1 to *PR10, PR17C,* and *Chitinase3* in wheat

The promoter sequences of the wheat defence-marker genes, *PR10*, *PR17C,* and *Chitinase3*, encompass 13, 10, and 5 GATA-core *cis*-elements, respectively (Table S4). A rapid ChIP protocol was deployed to examine whether TaGATA1 directly binds to GATA-core *cis*-element containing regions of the above defence-marker genes' promoters. The transient expression of GFP and TaGATA1-GFP in wheat mesophyll protoplasts were confirmed by western blot with monoclonal GFP antibody (Fig. 7A). Subsequently, ChIP-qPCR was used to amplify GATA-containing regions in promoters of *PR10*, *PR17C*, and *Chitinase3*. The ChIP-qPCR results showed that the GATA-core *cis*-element fragments in the promoters of *PR10*, *Chitinase3*, and *PR17C* were enriched 5.6-, 3.7-, and 2.4-fold more by TaGATA1-GFP than by GFP (Fig. 7B, C, D). These data showed that TaGATA1 could directly bind *in planta* to

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these GATA-core *cis*-element containing sites present in *PR10*, *PR17C*, and *Chitinase3* promoters and directly activate expression of these target genes in wheat.

TaGATA1 **and its activated defence genes are regulated by cytokinin and jasmonate**

Arabidopsis B-GATA genes and rice *CGA1* are responsive to the phytohormone cytokinin (Ranftl *et al.,* 2016; Hudson *et al.,* 2013). Jasmonate (JA) is a primary phytohormone in regulation of plant defence responses to necrotrophic pathogens (Pieterse *et al.*, 2009; Thomma *et al.*, 1998). *TaGATA1* promoter contains both cytokinin and JA responsive *cis*-elements (Table S2). To investigate how *TaGATA1* responds to application of external cytokinin and JA, we analyzed transcriptional profiles of *TaGATA1* in wheat cultivar Yangmai16 leaves after external cytokinin (6-benzylaminopurine, 6-BA) and methyl jasmonate (MeJA, JA analog) as well as mock treatments for 0.5, 1, 3, 6, and 12 h. After 6-BA treatment, *TaGATA1* transcription was significantly elevated, e.g. ~2.86-fold at 1 h and ~2.47-fold at 6 h compared to the mock (Fig. 8A). In response to MeJA stimulus, *TaGATA1* transcription was elevated at 1 h, reached a peak at 3 h and maintained a high level at 6 h relative to the mock (Fig. 8B). Furthermore, all the promoter sequences of *PR10*, *PR17C*, and *Chitinase3* contained both cytokinin and JA responsive *cis*-elements (Table S4). Thus, we investigated transcriptional profiles of *PR10*, *PR17C*, and *Chitinase3* in wheat cv. Yangmai16 leaves treated with 6-BA (for 1 and 6 h), MeJA (for 3 and 6 h), or mock. As shown in Fig. 8C, *PR10* transcription significantly increased after exogenous 6-BA treatment, whereas *PR17C* and *Chitinase3* were down-regulated by 6-BA treatment. Following MeJA treatment, transcription levels of *PR10*, *PR17C*, and *Chitinase3* were significantly increased compared with mock treatment (Fig. 8D).

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These results suggested that *TaGATA1* and its activated defence genes (*PR10*, *PR17C*, and *Chitinase3*) were responsive to cytokinin and/or JA stimuli.

Discussion

In *Arabidopsis*, rice, and barley, some B-GATA TFs play regulatory roles in many aspects of growth and development (Behringer and Schwechheimer, 2015; Houston *et al.*, 2012; Klermund *et al.*, 2016; Lu *et al.*, 2017; Ranftl *et al.*, 2016). However, GATA TFs in wheat have not been reported. In this study, through comparative transcriptome analyses, the wheat LLM-domain B-GATA gene *TaGATA1* was identified in the host immune response to *R. cerealis*. *TaGATA1* transcription was higher in resistant wheat cvs. Shanghongmai and CI12633 than in susceptible wheat cv. Wenmai 6, and significantly elevated after *R. cerealis* infection. *TaGATA1* is expressed at the highest level in stems where sharp eyespot disease primarily occurs. Accordingly, the *TaGATA1* promoter contains biotic stress responsive *cis*-elements.

Previous studies reported that plant TF genes were induced to a higher extent in resistant lines after infection of pathogens and were demonstrated to regulate resistance responses (McGrath *et al.*, 2005; Zhang *et al.*, 2012; Zhang *et al.*, 2007). Here, *TaGATA1*-silenced wheat plants and *TaGATA1*-overexpression transgenic wheat lines were generated and their resistance responses after pathogen inoculation were assessed. The genetic functional assays revealed that overexpression of *TaGATA1* significantly increased resistance of the transgenic wheat to *R. cerealis* and silencing of *TaGATA1* significantly impaired host resistance to the pathogen. These results show that *TaGATA1* acts as a positive regulator and is required for the wheat immune responses to *R. cerealis* infection. This is the first report uncovering a defence role of plant GATAs in response to pathogens. This study broadens our

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understanding of the biological function of GATA in plant species. Additionally, reports of plant responses to necrotrophic pathogens have been limited. This study extends the current knowledge of plant immune responses against necrotrophic pathogens.

In this report, the phylogenetic analysis showed that TaGATA1 belongs to the short LLM-domain-containing B-GATA class. In this class, TaGATA1 shares quite low identity with function-known GATA factors, such as OsGATA12, AtGATA15, AtGATA16, AtGATA17, and AtGATA17LIKE. OsGATA12, AtGATA15, AtGATA16, AtGATA17, and AtGATA17LIKE have been shown to regulate greening, tillering, senescence, flowering time, hypocotyl elongation, and stomata formation in hypocotyls (Klermund *et al.*, 2016; Lu *et al.*, 2017; Ranftl *et al.*, 2016). Herein, the defensive role of *TaGATA1* provides a novel function of plant B-GATAs, which may be due to *TaGATA1*-specific sequence during wheat evolution. It supports reports of the neofunctionalization of monocot-specific B-GATAs during plant evolution to expand their functional repertoire (Behringer *et al.*, 2014; Behringer and Schwechheimer, 2015; Reyes *et al.*, 2004).

GATA TFs all should include a conserved GATA-motif binding domain and at least one NLS domain (Lu *et al.*, 2017). Some GATA members showed transcriptional-activation activity due to possessing a transcription activation domain (Shaikhali *et al.*, 2012; Shikata M *et al.*, 2003; Sugimoto *et al.*, 2003). Here, the protein sequence analysis indicated that TaGATA1 contained a conserved GATA-motif binding domain, two conserved nuclear localization signal motifs, and a transcription activation domain. Thus, TaGATA1 was speculated to be an activator-type LLM-domain B-GATA TF. Our subcellular localization results confirmed that TaGATA1 is a nucleus-expressing protein. EMSA, transcription-activation and ChIP-

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qPCR results indicated that TaGATA1 is a transcription activator and can bind to GATA-core *cis*-elements. These molecular biology results prove that TaGATA1 is a GATA-binding transcription activator localizing in the nucleus, which is consistent with TaGATA1 sequence analysis and LLM-domain B-GATA protein characteristics.

Previous studied showed that several defence genes, such as chitinases, *PR10*, and *PR17C*, contributed to resistance of transgenic wheat to fungal pathogens (Anand *et al.*, 2003; Chen *et al.*, 2008; Li *et al.*, 2011; Zhang *et al.*, 2012; Zhu *et al.*, 2014). Here, the results showed that after *R. cerealis* inoculation, transcriptional levels of *PR10*, *PR17C*, and *Chitinase3* were significantly elevated in resistant *TaGATA1*-overexpressing wheat plants compared to WT Yangmai 16, and were the lowest in susceptible *TaGATA1*-silenced wheat plants. These data reveal that TaGATA1 activates the expression of *PR10*, *PR17C*, and *Chitinase3*. Moreover, ChIP-qPCR results confirm that TaGATA1 directly binds in wheat to GATA-core *cis*-elements present in the promoters of the defence genes targeted by TaGATA1 and activates expression of these genes. Taken together, these results verify that TaGATA1, acting as an LLM B-GATA transcription activator, can activate expression of defence genes followed by interaction with GATA-core *cis*-elements in the promoters of these target genes in wheat, leading to enhanced resistance against *R. cerealis* infection.

In *Arabidopsis thaliana*, GNC and CGA1/GNL control different aspects of cytokinin-regulated development (Ranftl *et al.*, 2016), and modulate crosstalk between auxin and gibberellin signaling (Richter *et al.*, 2010; Richter *et al.*, 2013). JA is primarily associated with necrotrophic pathogen resistance responses and regulates the expression of certain defence genes (McGrath *et al.*, 2005; Pieterse *et al.*, 2009; Thomma *et al.*, 1998). However, little is known about the effect of JA on GATAs. This study indicated that both external cytokinin and JA stimuli up-regulated the

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expression of *TaGATA1*. TaGATA1-activated defence genes, including *PR10*, *PR17C*, and *Chitinase3,* were up-regulated by exogenous JA application, but responded differently to exogenous cytokinin treatment. Upon exogenous cytokinin stimulus, *PR10* was up-regulated but *PR17C* and *Chitinase3* were down-regulated. Thus, we speculate that JA signaling might play a major role in the *TaGATA1*-mediated immune response to *R. cerealis.* In order to further clarify the issue, it is very interesting to investigate expression of the above genes and the pathogen biomass in the *TaGATA1*-overexpressing and silenced wheat plants as well their controls treated with exogenous JA in the future.

Conclusions

TaGATA1 was identified through transcriptomic analysis and was verified as a positive regulator in the wheat immune response to *R. cerealis* infection. TaGATA1 was demonstrated to be an LLM-domain B-GATA transcription activator. It directly bound to the GATA-core *cis*-element containing sequences in promoters of certain defence genes and activated their expression. *TaGATA1* and its activated defence genes were up-regulated by JA stimulus. Thus, *TaGATA1* positively regulates the immune response to *R. cerealis* through activating the expression of certain defence genes. This is the first investigation to reveal a defence role of plant LLM-domain B-GATA transcription factors. *TaGATA1* is a promising gene that can be used to improve resistance of wheat, other cereal and economic crops against *R. cerealis*.

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Figure legends

Figure 1 Transcriptional analysis of *TaGATA1* in *Rhizoctonia cerealis*-inoculated wheat. **A** microarray data for transcriptional up-regulation fold of *TaGATA1* between resistant wheat cultivars CI12633/Shanhongmai and susceptible wheat cultivar Wenmai 6 at 4, 7, and 21 d post inoculation (dpi) with *R. cerealis*. **B** Transcription of *TaGATA1* in *R. cerealis*-resistant wheat line CI12633 and susceptible wheat cultivar Wenmai 6 at 1, 4, 10, and 21 dpi with *R. cerealis* R0301 and mock treatment. *TaGATA1* transcription in mock-treated Wenmai 6 was set to 1. **C** *TaGATA1* transcription in five wheat cultivars at 7 dpi with *R. cerealis* R0301. The expression level of *TaGATA1* in Wenmai 6 was set to 1. DI indicates disease index of sharp eyespot. **D** Transcription of *TaGATA1* in roots, stems, leaves, sheath, and spikes of wheat Yangmai 16 at 3 dpi with *R. cerealis* R0301. *TaGATA1* transcription in roots of Wenmai 6 was set to 1. Statistically significant differences are derived from the results of three independent replications (*t*-test: **, $P< 0.01$). Error bars indicate SE.

Figure 2 Gene structure, amino acid sequence and chromosomal location of *TaGATA1*. **A** Genomic structure of *TaGATA1*. Grid grey portions represent untranslated regions (UTR). Exons and intron are marked by grid and dotted line, respectively. **B** Amino acid sequence of TaGATA1 protein. The transcriptional-activation domain and GATA-binding domain are marked by yellow and underline, respectively. Two nuclear localization signals and a LLM-domain are marked by red and pink. **C** Chromosome localization of *TaGATA1* using nulli-tetrasomic and double ditelosomic lines derived from wheat cv. Chinese Spring (CS). Marker indicates DL2, 000 DNA marker; N3A/T3B, N3A/T3D, N3B/T3A, N3B/T3D, N3D/T3A, and N3D/T3B indicate six CS nulli-tetrasomic lines.

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Figure 3 Biochemical characteristic assays of TaGATA1. **A** Subcellular localization of TaGATA1 in wheat protoplasts and onion epidermal cells. The control GFP and fused TaGATA1-GFP are transient expressed in mesophyll protoplasts and onion epidermal cells. Bars = 20 μm (wheat protoplasts). Bars = 100 μm (onion epidermal cells). **B** Transcriptional-activation assay of TaGATA1 in yeast. The yeast AH109 cells containing *BD-TaGATA1* or *BD* or *BD-TaGATA1* all grow on the SD/Trp, whereas only yeast AH109 cells containing *BD-TaGATA1* grow on selective medium (SD/Trp/His/Ade). **C** EMSA assay for TaGATA1 binding to the GATA-core *cis*-element. Each biotin-labeled probe was incubated with the His-TF-TaGATA1 protein. Competitive binding analysis was performed with increasing amount of the unlabeled probe. The arrow, triangle, and asterisk indicate the shifted bands, non-specific binding, and free probe, respectively.

Figure 4 BSMV-induced *TaGATA1* silencing impairs resistance of wheat CI12633 to *Rhizoctonia cerealis.* **A** RT-PCR analysis of transcription level of BSMV coat protein (*cp*) gene and mild chlorotic mosaic symptoms were detected on leaves of *CI12633* plants infected by *BSMV:GFP* or *BSMV:TaGATA1* (BSMV:TaGATA1-1, BSMV:TaGATA1-2, BSMV:TaGATA1-3) for 10 d. **B** RT-qPCR analysis of *TaGATA1* transcription in *BSMV:GFP*- and *BSMV:TaGATA1*-infected wheat plants. The relative transcript level of *TaGATA1* in *BSMV:TaGATA1*-infected wheat CI12633 plants, BSMV:TaGATA1-1, BSMV:TaGATA1-2, BSMV:TaGATA1-3, is relative to that in *BSMV:GFP*-infected plants (set to 1). Significant differences were analyzed based on three replications (*t*-test: *, $P< 0.05$; **, $P< 0.01$). Error bars indicate SE. **C** Sharp eyespot symptoms of *BSMV:GFP*-infected and *TaGATA1*-silenced CI12633 plants (BSMV:TaGATA1-1, BSMV:TaGATA1-2, BSMV:TaGATA1-3) at 40 dpi with *R. cerealis* WK207. IT indicates infection type. **D** Average infection types of

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CI12633 plants infected by *BSMV:GFP* or *BSMV:TaGATA1*. Two independent batches were performed in two years.

Figure 5 Molecular characterizations of *TaGATA1*-overexpressing wheat plants and their responses to *Rhizoctonia cerealis*. **A** RT-qPCR analysis of *TaGATA1* transcription in *TaGATA1* transgenic lines. The transcriptional level in WT Yangmai 16 is set to 1. Three biological replicates per line were averaged and statistically treated (*t*-test; ** $P< 0.01$). Error bars indicate SE. **B** Western blot pattern of these *TaGATA1*-overexpressing transgenic lines and WT Yangmai 16 using anti-c-myc antibody. Similar results were obtained from three independent replicates. Coomassie brilliant blue (CBB) staining was used as a loading control. **C** Typical symptom of sharp eyespot in these five *TaGATA1*-overexpressing transgenic and WT wheat Yangmai 16 plants. IT indicates infection type. **D** Average infection types of *TaGATA1* transgenic lines in 3 generations and WT wheat Yangmai 16 plants.

Figure 6 Transcriptional analyses of defence genes (*PR10*, *PR17c*, and *Chitinase3*) in wheat. **A** The transcript levels of the tested genes in *TaGATA1*-overexpressing (GO1 and GO2) wheat Yangmai 16 or *TaGATA1*-silenced (*BSMV:TaGATA1*) wheat CI12633 plants are relative to those in the wild-type (WT) Yangmai 16 or *BSMV:GFP*-infected CI12633 plants, respectively. Statistically significant differences were analyzed based on three replications (*t*-test; $*P < 0.05$, $**P < 0.01$). Error bars indicate SE.

Figure 7 ChIP-qPCR assay of TaGATA1 binding and activation activity in wheat. **A** Western blot assay of GFP and TaGATA1-GFP transient expressed in wheat protoplasts. **B** TaGATA1 binding and activating *PR10*, *PR17c*, and *Chitinase3* in

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wheat. Schemata of *PR10*, *PR17c*, and *Chitinase3* promoters, including the predicted promoter regions, used in ChIP-qPCR assays are shown.

Figure 8 Transcriptional analyses of *TaGATA1* and its activated defence genes in wheat after exogenous cytokinin and jasmonate treatments. **A** & **B** Transcriptional profiles of *TaGATA1* in leaves of wheat cv. Yangmai 16 after exogenous applications of 10 μM cytokinin 6-BA (A) or 0.1 mM MeJA (B). **C** & **D** Expression of defence genes including *PR10*, *PR17c*, and *Chitinase3* in leaves of wheat Yangmai 16 after exogenous applications of 10 μM 6-BA (C) or 0.1 mM MeJA (D). The transcription level of the tested gene in mock-treated wheat plants is set to 1. Statistically significant differences (*P < 0.05, **P < 0.01) are analyzed based on three replications using Student's t-test. Error bars indicate SE.

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Fig. 1

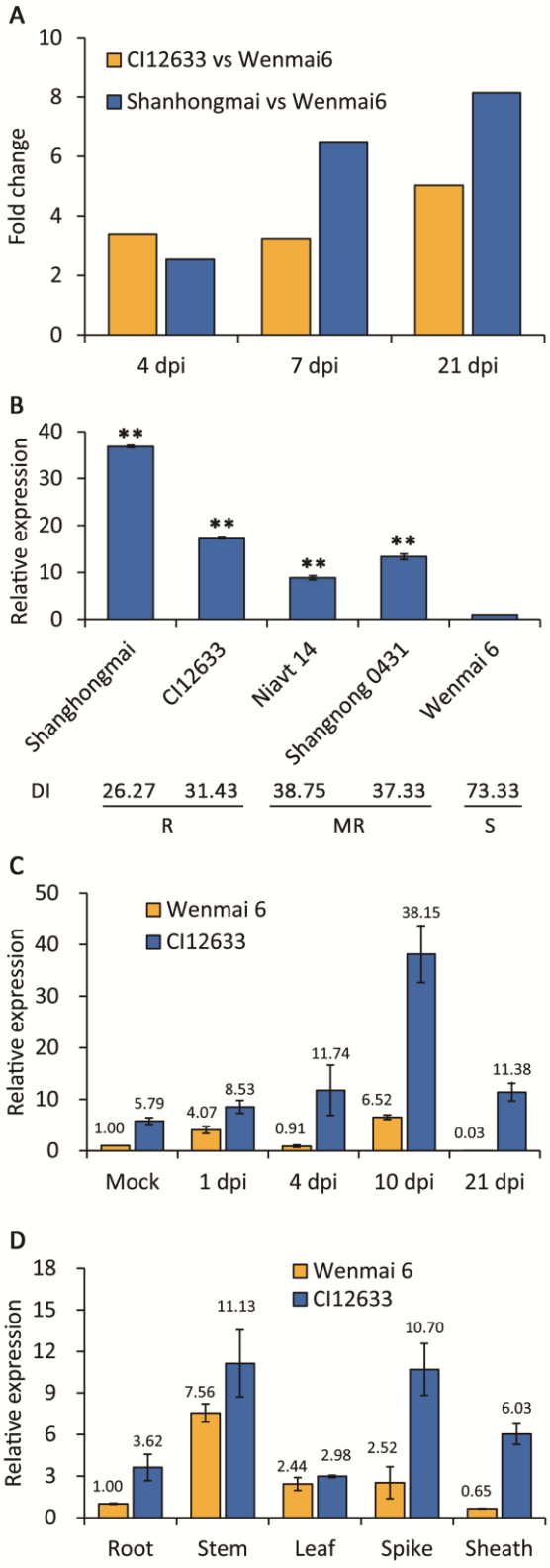

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Fig. 2

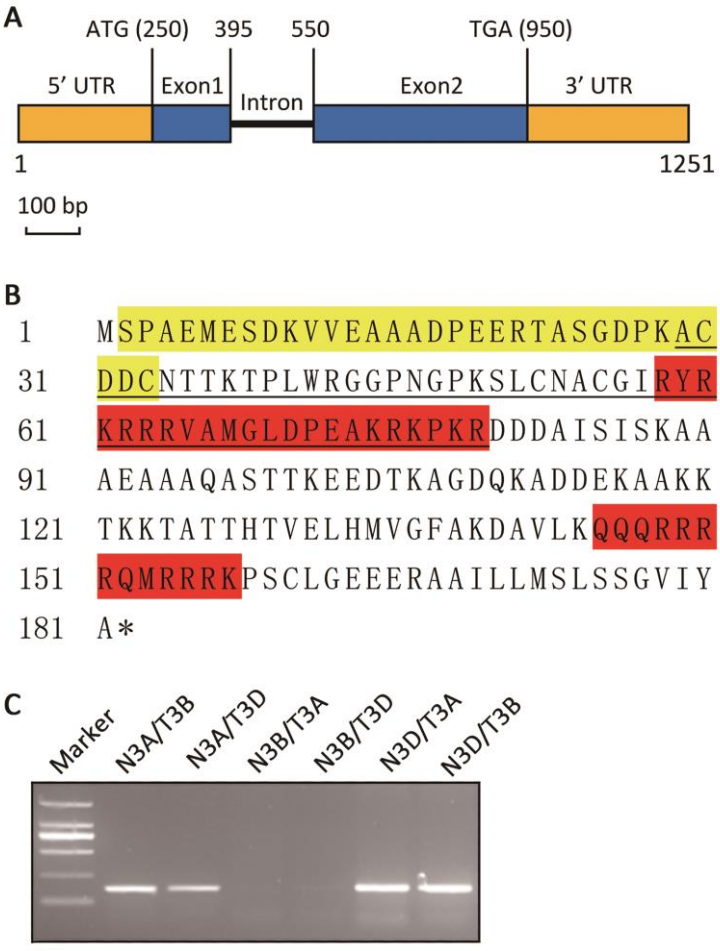

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Fig. 3

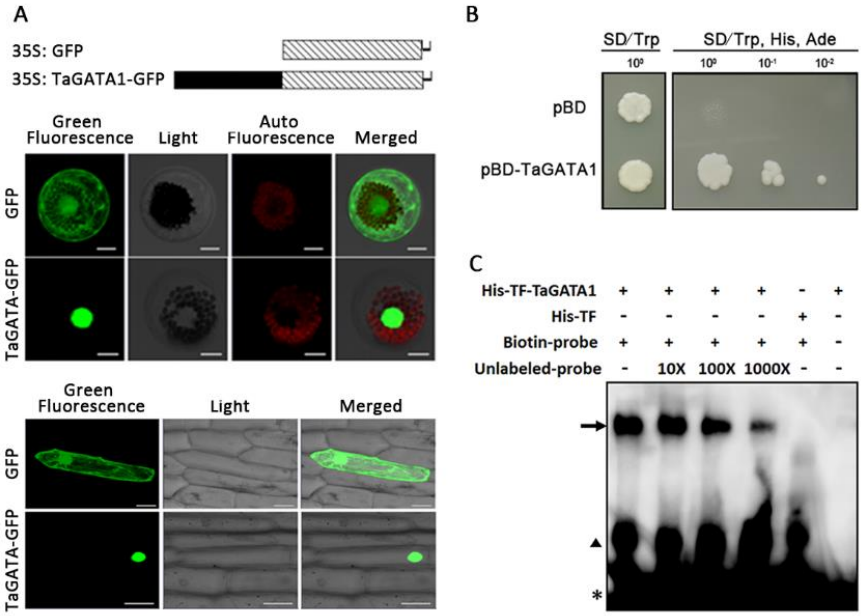

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Fig. 4

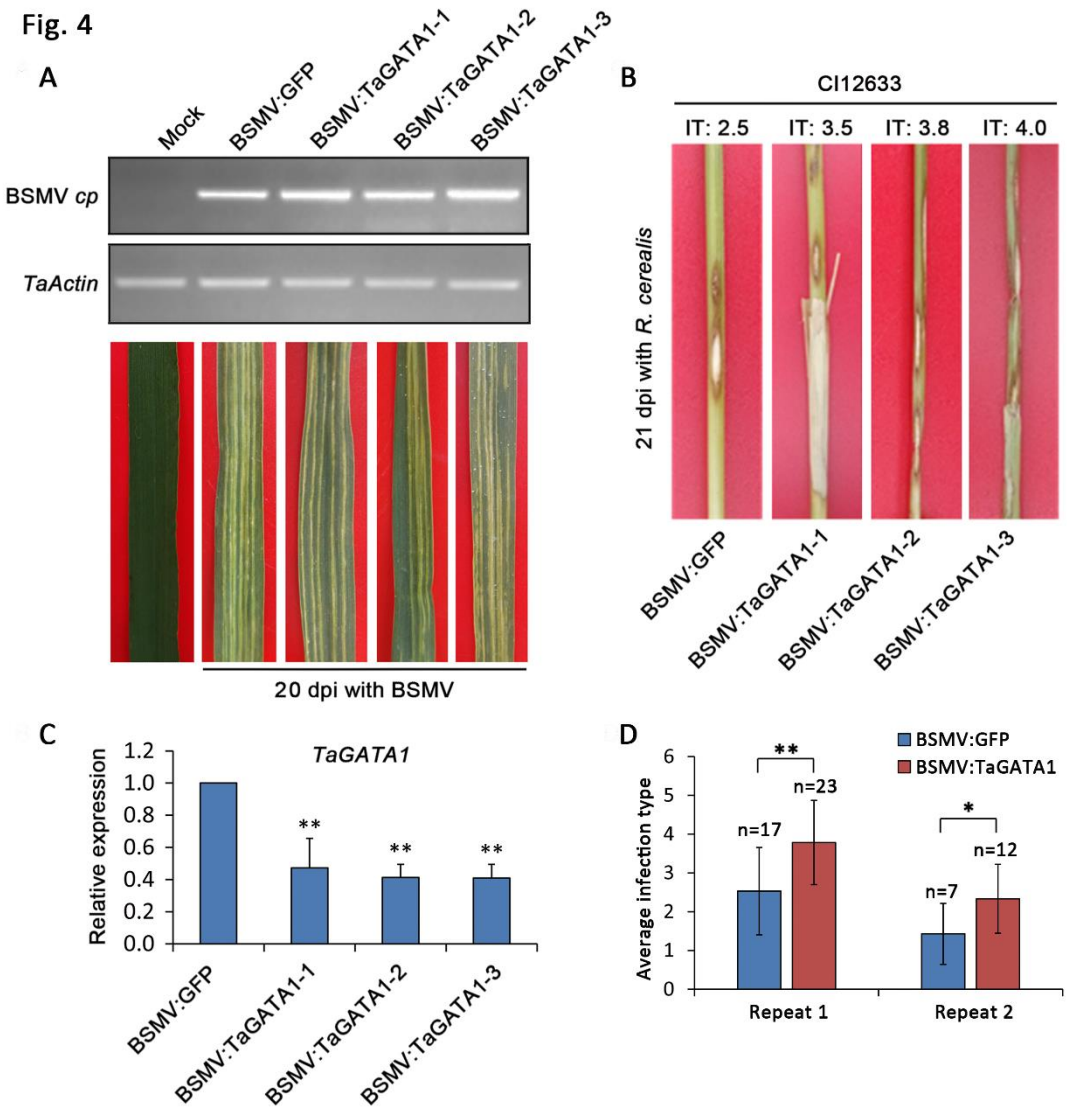

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Fig. 5

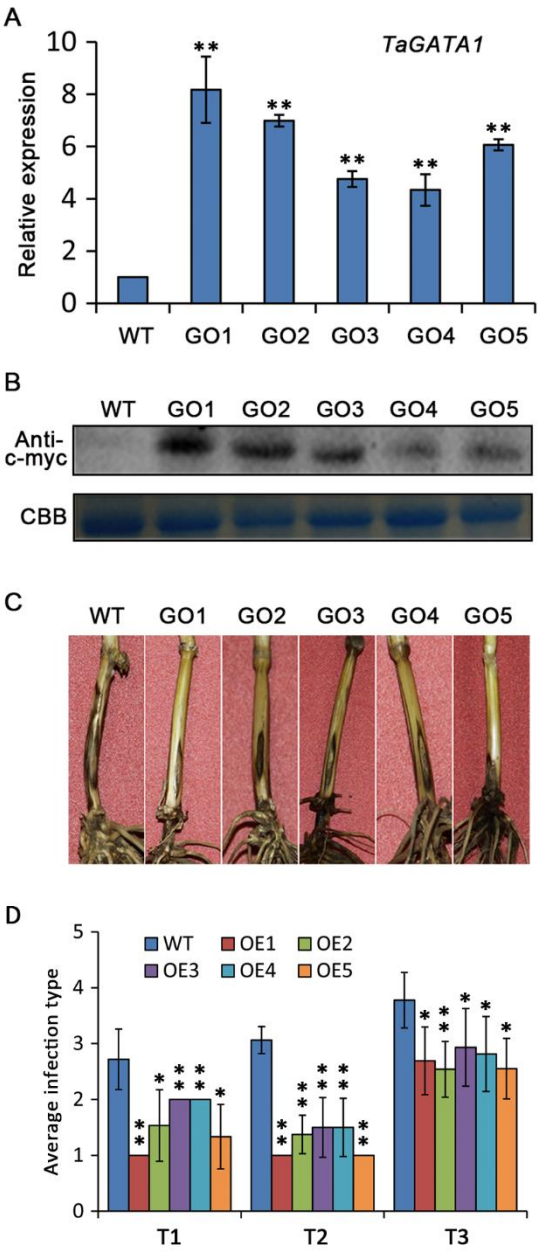

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Fig. 6

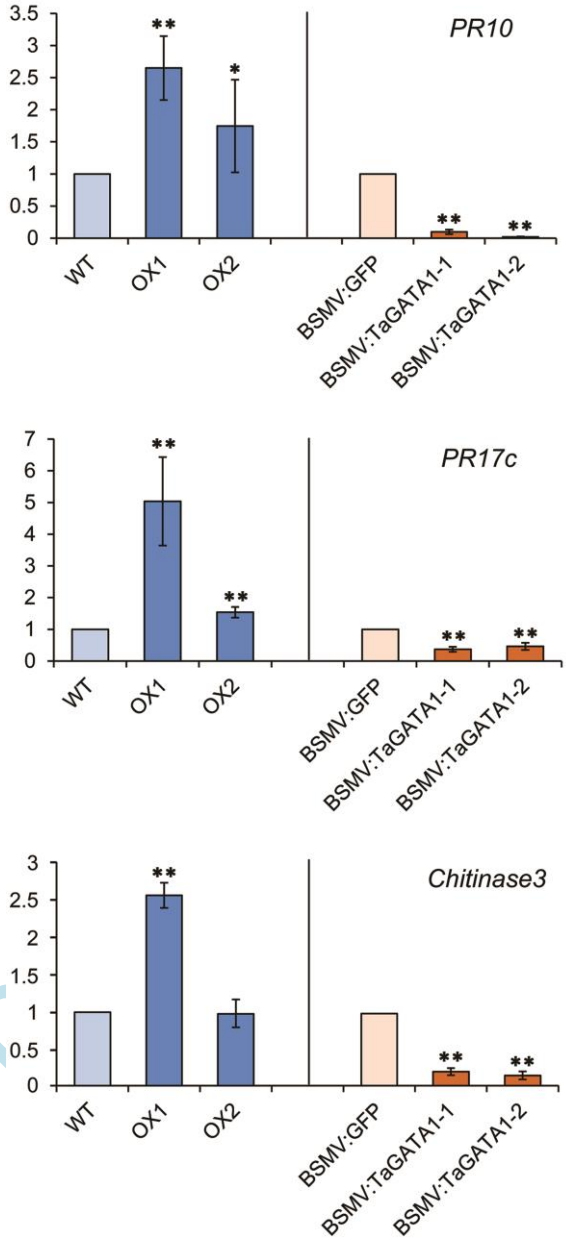

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Fig. 7

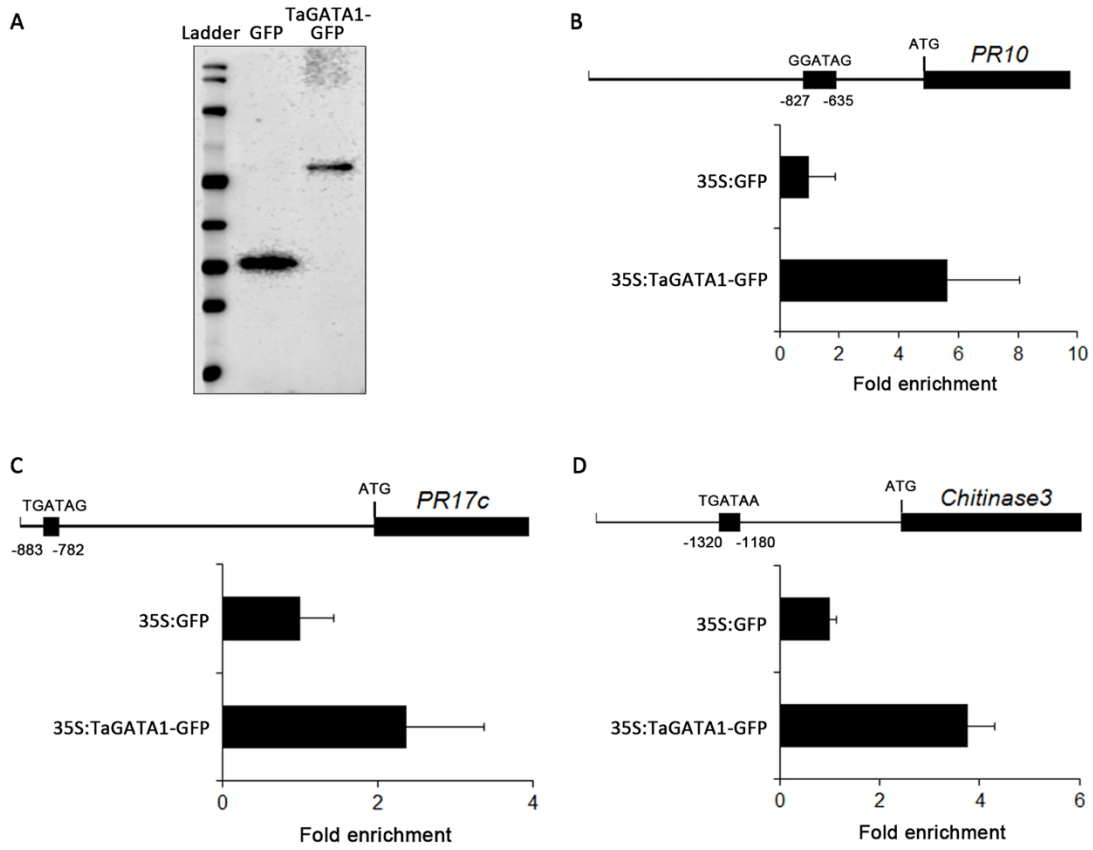

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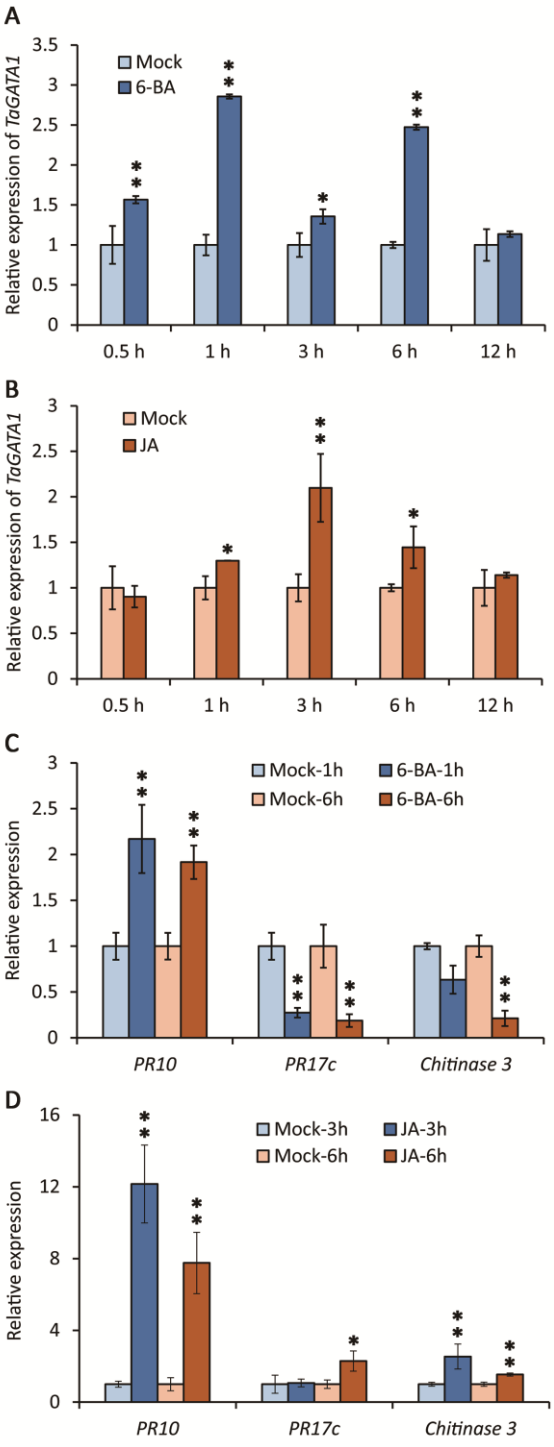
Fig. 8



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