


# *Thinopyrum intermedium* TiAP1 interacts with a chitin deacetylase from *Blumeria graminis* f. sp. *tritici* and increases the resistance to *Bgt* in wheat

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## Summary

The biotrophic fungal pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*) is a crucial factor causing reduction in global wheat production. Wild wheat relatives, for example *Thinopyrum intermedium*, is one of the wild-used parents in wheat disease-resistant breeding. From *T. intermedium* line, we identified the aspartic protease gene, *TiAP1*, which is involved in resistance against *Bgt*. TiAP1 is a secreted protein that accumulates in large amounts at the infection sites of *Bgt* and extends to the intercellular space. Yeast two-hybrid, luciferase complementation imaging and bimolecular fluorescent complimentary analysis showed that TiAP1 interacted with the chitin deacetylase (BgtCDA1) of *Bgt*. The yeast expression, purification and *in vitro* test confirmed the chitin deacetylase activity of BgtCDA1. The bombardment and VIGS-mediated host-induced gene silencing showed that *BgtCDA1* promotes the invasion of *Bgt*. Transcriptome analysis showed the cell wall xylan metabolism, lignin biosynthesis-related and defence genes involved in the signal transduction were up-regulated in the transgenic *TiAP1* wheat induced by *Bgt*. The TiAP1 in wheat may inactivate the deacetylation function of BgtCDA1, cause chitin oligomers expose to wheat chitin receptor, then trigger the wheat immune response to inhibit the growth and penetration of *Bgt*, and thereby enhance the resistance of wheat to pathogens.

## Introduction

Wheat (*Triticum aestivum* L.) is one of the main crops in the world, therefore the production of wheat is closely related to food security. In recent years, pathogenic infections have seriously affected the yield of wheat. Wheat powdery mildew caused by the obligate biotrophic fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*) can result in severe reductions in grain yield (Singh *et al.*, 2016). When powdery mildew invades the plant, it releases proteinaceous exudates that contain the mechanical and molecular features required for the full virulence of the pathogen (Zhang *et al.*, 2005). Fungal exudates function in the attachment of conidia and in the adherence of the penetrating appressoria (App) to invade plants (Deising and Werner, 2000). At the same time, the plant responds immediately to the pathogen invasion, and a number of secreted proteins, especially protease, from plant cells accumulate in the apoplastic space to carry an apoplastic battle between hosts and pathogen (Geziel, 2015; Wang and Wang, 2020).

In wheat, more than 66 powdery mildew resistance loci (*Pm1*–*Pm66*) located on different chromosomes of the common wheat have been named (Li *et al.*, 2020a). Most of them originated from wild relative species of wheat, such as *Pm21* from *Haynaldia villosa* has been introduced into the common wheat using the translocation line T6VS-6AL (Chen *et al.*, 2013). Li *et al.* (2020b) cloned *Pm41*, which was a powdery mildew resistance gene

deriving from the wild emmer wheat. *Thinopyrum intermedium* had been hybridized extensively with wheat and proven a useful source of resistance to various diseases of hexaploid wheat, and several powdery mildew resistance genes, such as *Pm40*, *Pm43* and *PmL962* (He *et al.*, 2009; Luo *et al.*, 2009; Shen *et al.*, 2015), have been identified and genetic mapped. The mining and utilization of these new resistance genes are vital means to improve current wheat varieties and increase their resistance to powdery mildew.

Aspartic proteases consist of four main proteolytic enzymes that are widely present in animals, plants, yeast, microorganisms and viruses (Rawlings and Barrett, 1995). Xia *et al.* (2004) reported that the peptide signal system of CDR1 was involved in the activation of the resistance mechanism, while Prasad *et al.* (2009, 2010) found that *OsCDR1/OsAP5* could enhance the disease resistance of rice and *Arabidopsis thaliana*. The aspartic protease gene, *AP13*, from grape could promote the salicylic acid-dependent signal transduction pathway (Guo *et al.*, 2016). Moreover, Alam *et al.* (2014) found that the rice *OsAP77* gene accumulated in the extracellular space and sieve tubes to defend against the pathogens. Hence, these studies have shown aspartic proteases participate in the biotic stresses response of plants to enhance the resistance.

To resist pathogens, plants have developed a complex immune system including the plasma membrane receptors that recognize pathogen-related molecular patterns, such as chitin from the

fungal cell walls that can trigger defence responses. Kamakura *et al.* (2002) found a new germination tube-specific gene, *CBP1*, from the rice blast fungus *Magnaporthe grisea*, which encoded a chitin-binding protein (CBP) having two similar chitin-binding domains at both sides of the central domain, might play a vital role in the hydrophobic surface sensing of *M. grisea* during App differentiation. Kuroki *et al.* (2017) confirmed *CBP1* encoded a chitin deacetylase and participated in differentiation stage of App in *M. oryzae*. These results proved the chitin deacetylase activity of CBP1 is necessary for the formation of App. In addition, Yang *et al.* (2019) identified the chitinase gene *MoChia1* from *M. oryzae*. This gene can trigger a plant's defence response to *M. oryzae* in rice under an inducible promoter. *MoChia1* was also a functional chitinase that is required for the growth and development of *M. oryzae*. The *MoChia1* binding to free chitin could inhibit the plant immune response, while another protein, *OsTPR1*, competitively binding with *MoChia1*, would re-establish the immune response (Yang *et al.*, 2019). Han *et al.* (2019) also found the mechanism of *MoChia1* that targeted the host lectin to inhibit rice immunity and promote colonization. Furthermore, they also found rice protein *OsMBL1* could interact with *MoChia1* to enhance the resistance against *M. oryzae*. Overexpressing of *OsMBL1* can lead to the activation of defence response genes of rice and the burst of chitin-induced reactive oxygen species. Moreover, Gao *et al.* (2019) identified a secreted polysaccharide deacetylase (PDA1) from soil-borne *Verticillium dahliae*. PDA1 can promote the deacetylation of chitin oligomers, whose *N*-acetyl group contributes to the host lysine motif (LysM)-containing receptor that perceives ligand-triggered immunity and facilitates virulence. Thus, the silencing of PDA1 allows the *N*-acetyl group of the chitin-triggered host immunity to occur.

In a previous study, we obtained an aspartic protease gene, *TiAP1*, from trititrigia SN6306 through a comparative transcriptome analysis. Further sequence analysis showed *TiAP1* originated from *T. intermedium* (Tian *et al.*, 2017). Through gene expression and Barley Stripe Mosaic Virus (BSMV) virus-induced gene silencing (VIGS) analyses, we found the *TiAP1* gene might be involved in the resistance to wheat powdery mildew. In addition, subcellular localization analysis showed that *TiAP1* is a secretory protein. During the *Bgt* invasion, *TiAP1* would assemble in large numbers at the infect site. The yeast two-hybrid (Y2H) verification, luciferase (LUC) complementation imaging (LCI) and bimolecular fluorescent complimentary (BiFC) analysis show that *TiAP1* interacts with chitin deacetylase (*BgtCDA1*) of *Bgt*, while *BgtCDA1* could promote the invasion of *Bgt*. Therefore, we propose that *TiAP1* induces the plant to resist the invasion of pathogens by interacting with *BgtCDA1*, inactivating the deacetylation function of *BgtCDA1*, then triggering the wheat immune response to inhibit the growth and penetration of *Bgt*.

## Results

### The expression of *TiAP1* mediates the resistance in trititrigia SN6306 to *Bgt* invasion

Since Li *et al.* (2016) analysed the transcriptome of SN6306 and YN15, and obtained a gene that was up-regulated by *Bgt* induction, which encoded an aspartic protease, while Tian *et al.* (2017) cloned the *TiAP1* gene and found that it came from *T. intermedium*. Here, we found that the *TiAP1* gene was expressed in SN6306 not in wheat YN15, and was up-regulated by the induction at the early stages of the *Bgt* invasion (Figure 1a).

To investigate the effect of endogenous *TiAP1* on SN6306 resistance to *Bgt*, we reduced the endogenous *TiAP1* gene levels by suppressing *TiAP1* expression using BSMV-VIGS. The *TiAP1* expression levels were significantly suppressed in the leaves of BSMV:*TiAP1*as SN6306 relative to the BSMV:00 negative control (Figure 1b). When the BSMV:*TiAP1*as SN6306 was inoculated with *Bgt* at the three-leaf stage, the infection in BSMV:*TiAP1*as SN6306 was much more severe than in the BSMV:00 control at 10 days post-inoculation (dpi) (Figure 1c,d). This proved that the *TiAP1* gene was resistant to *Bgt* invasion and could play a role in the resistance.

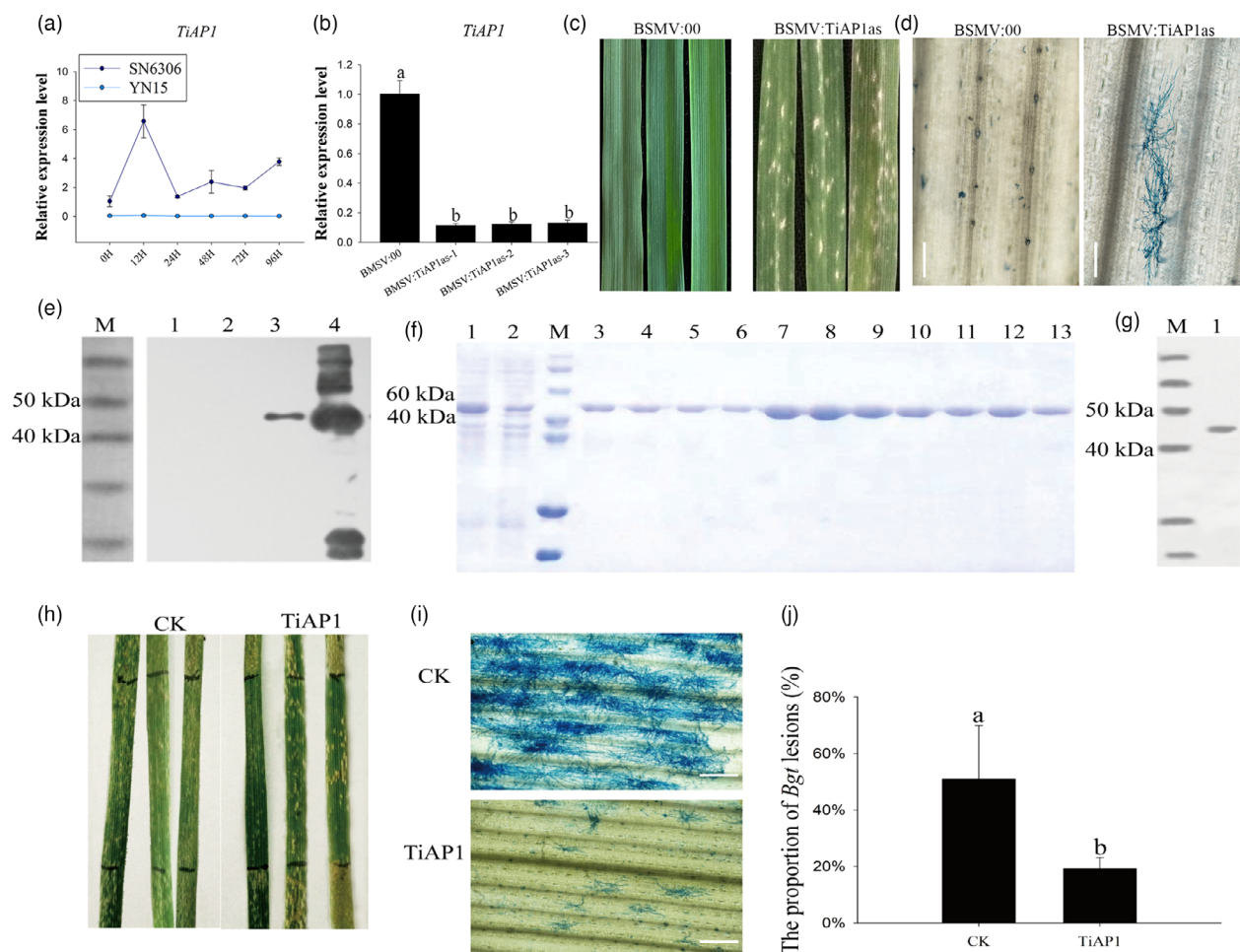
### The *TiAP1* protein enhances resistance to *Bgt* in vitro

Western blot analysis showed that *TiAP1* was induced in the precipitation through prokaryotic expression (Figure 1e,f, Figure S1). After renaturation, the protein concentration was determined to be 0.437 mg/mL by the Bradford method (Figure 1g; Bradford, 1976). Moreover, regarding the *TiAP1* protein effect on the *Bgt* invasion, the number of *Bgt* conidia on YN15 leaf after smearing *TiAP1* for 5 days was significantly lower than that of YN15 with CK (pET28a). In addition, there was a smaller number of hyphae in the leaves smeared with *TiAP1*, less hyphal length and the diseased area than those of the leaves smeared with CK (Figure 1h,i). Lesion areas of the leaves treated with *TiAP1* were also significantly less than that of the control (Figure 1j), suggesting that *TiAP1* can resist the *Bgt* invasion in vitro.

### *TiAP1* gene transfer can elevate the resistance to *Bgt* of the wheat recipients

To investigate the function of *TiAP1*, the pMUBi-*TiAP1* vector and the screening marker *Bar* gene vector, were co-transformed into the susceptible hexaploid wheat cultivar, Bobwhite. The obtained transgenic Bobwhite with the *TiAP1* gene was subjected to PCR identification of the *Bar* and *TiAP1* genes (Figure S2A,B) to obtain the positive transgenic plants. The qRT-PCR analysis showed that the expression level of the *TiAP1* gene was 20 and 110 times higher in OE1 and OE2 than that of SN6306 when infected by *Bgt* 2 days respectively (Figure 2a). After multiple comparisons, we found that the expression level of *TiAP1* when infected by *Bgt* in OE2-2 was significantly different from that of OE2-0 ( $P < 0.05$ ) (Figure 2a), indicating that the expression of *TiAP1* could be involved in the response to *Bgt*. In addition, we found that the overexpression of *TiAP1* gene in Bobwhite (OE1 or OE2) showed moderate resistance, while the leaf surface was still entirely green and had less conidia at 5 dpi than those of the control wheat Bobwhite (Figure 2b). A large number of *Bgt* in the OE1-2, OE2-2 and SN6306 were retained at App of 41.2%, 43.4% and 83.7%, and of 16.2%, 14.5% and 0.34% retained at hyphae stage, respectively, while in the control Bobwhite, 28.9% of the *Bgt* were present in the form of App, and 25.6% in the form of hyphae (Figure 2c,d). The HI in Bob-2 was 68.6%, while in OE1-2, OE2-2 and SN6306 it was 53.5%, 48.8% and 6.7% respectively (Figure 2e). In addition, Figure 2d,e suggested that the transgenic *TiAP1* gene could affect the formation of *Bgt* Hau, thereby resisting the invasion of *Bgt*.

Meanwhile, we crossbred between the homozygous transgenic *TiAP1* Bobwhite and YN15 to obtain the hybrid F<sub>1</sub>. Gene amplification and phenotype investigation confirmed the inheritance of the *TiAP1* gene and its function in resisting *Bgt* in the hybrid F<sub>1</sub>. A chi-square test showed that the proportion of resistant-susceptible separation in the F<sub>2</sub> generation was 3:1. The

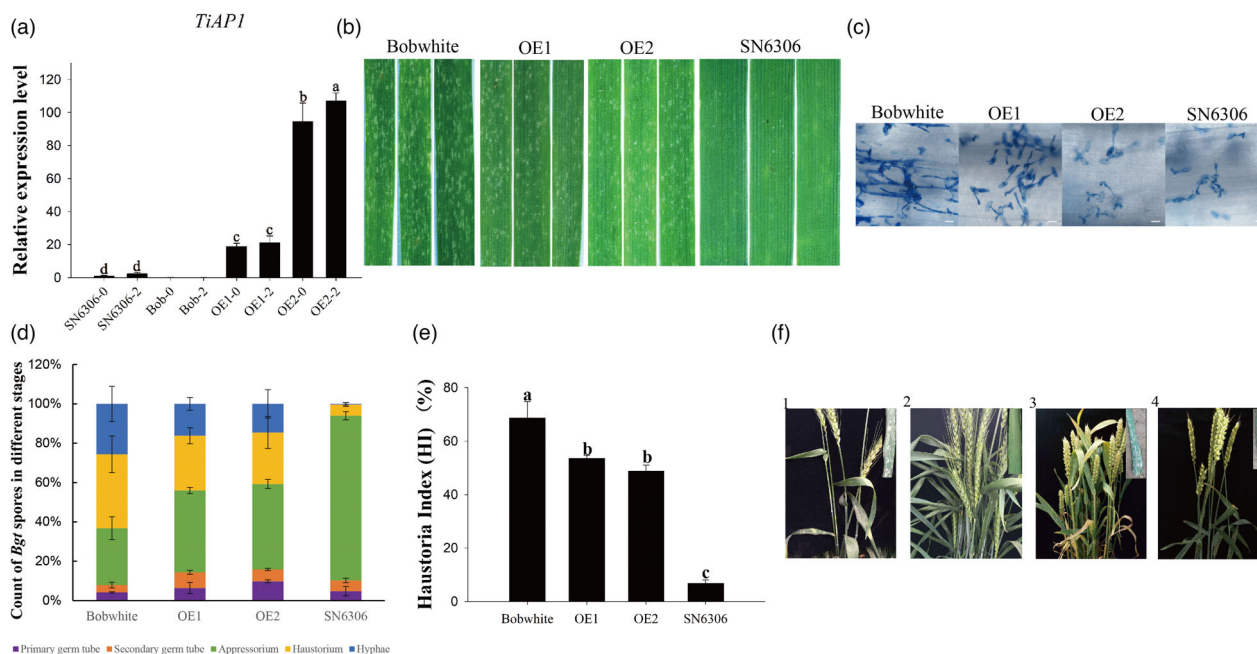


**Figure 1** qRT-PCR and VIGS analysis of the *TiAP1* effect on the *Bgt* resistance, the *TiAP1* prokaryotic expression and purification and the reduction in the powdery mildew severity (lesions area) on the wheat seedlings by a recombination protein treatment. (a) Expression levels of *TiAP1* in YN15 and SN6306 that were induced by *Bgt*. Total RNA from the leaves of YN15 and SN6306 that were induced by *Bgt* at 0, 12, 24, 48, 72 and 96 h were extracted as the template to be detected by qRT-PCR. Data represent mean  $\pm$  SD of  $n = 3$  technical replicates. (b) Relative quantification of expression levels of *TiAP1*. Total RNA from the leaves of barley stripe mosaic virus (BSMV):00 and BSMV: *TiAP1as* inoculated with *E09* for 10 days were extracted as the template for the detection by qRT-PCR. (c) Disease phenotype of the BSMV:00 and BSMV: *TiAP1as* inoculated with *E09*. Photographs were taken at 10 dpi. (d) Microscopic observation of the disease symptoms of BSMV:00 and BSMV: *TiAP1as* at 10 dpi. Bar = 100  $\mu$ m. (e) The *TiAP1* protein expression detection by Western blotting before and after induction. M: Pre-stained protein ladder; Lane 1: Supernatant of non-induced; Lane 2: Precipitation of non-induced; Lane 3: Supernatant of induced; Lane 4: Precipitation of induced. (f) SDS-PAGE analysis of the inclusion body purification. M: Low-molecular weight protein ladder; Lane 1: Inclusions after dissolving; Lane 2: The effluent absorbed by nickel iminodiacetic acid (Ni-IDA); Lane 3–6: The eluent by 50 mM imidazole elution; Lane 7–11: The eluent by 100 mM imidazole elution; Lane 12–13: The eluent by 300 mM imidazole elution; (g) *TiAP1* quality assurance after renaturation. M: Pre-stained protein ladder; Lane 1: *TiAP1* protein (1.5  $\mu$ g). (h) *Bgt* phenotype on YN15 leaves smeared with *TiAP1* and the tag protein from pET28a (CK) and inoculated with *E09* for 5 days. (i) Microscopic (Nikon Ni-U, Tokyo, Japan) analysis of the *Bgt* in YN15 leaves smeared with *TiAP1* and CK and inoculated with *E09* for 5 days. Bar = 100  $\mu$ m. (j) The proportion of lesions area on the leaf of YN15 that was pre-treated with the recombinant *TiAP1* and inoculated by *Bgt* *E09* for 5 days. Control: the purified recombinant tag protein of pET28a construct. Different letters above the bars indicate statistically significant differences ( $P < 0.05$ ) as obtained by one-way analysis of variance with least significance difference and Duncan's multiple range test method.

subsequent  $F_3$  generation at the seedling stage (Figures S2C and S3; Table S1) also double confirmed *TiAP1* endue the resistance to *Bgt* for wheat.

In the adult stage, through infection by *Bgt* *E09* in the controlled glasshouse, the overexpression of *TiAP1* gene in Bobwhite showed high resistance to *Bgt* (Figure 2f2, Figure S4; Table S2), whereas Bobwhite (Figure 2f1) and YN15 (Figure 2f3)

showed high susceptibility to *Bgt* on the leaves with many lesions of more than 1 mm in diameter and more hyphae. At the same time, the hybrid  $F_1$  between the overexpression of *TiAP1* gene in Bobwhite and YN15, showed high resistance to *Bgt*, where the diameter of the lesions was  $<1$  mm, the amount of the lesions was little and the whole leaf surface was still green (Figure 2f4).



**Figure 2** Identifying the *Bgt* resistance of Bobwhite. SN6306, two transgenic *TiAP1* wheat and the offspring of the hybrid. (a) Expression levels of *TiAP1* in SN6306, Bobwhite and the transgenic Bobwhite with *TiAP1* when inoculated with *E09* for 2 days. The symbols '-0' and '-2' means treated 0 and 2 days respectively. (b) Phenotype of Bobwhite, SN6306, and two transgenic Bobwhite with *TiAP1* (OE1 and OE2) when inoculated with *E09* for 5 days. (c) Microscopic (Nikon Ni-U, Tokyo, Japan) observation of the disease symptoms of Bobwhite, SN6306, and the transgenic Bobwhite with *TiAP1* when inoculated with *E09* for 2 days. Bar = 100  $\mu$ m. (d) Count of *Bgt* spores at different stages inoculated for 2 days in Bobwhite, SN6306, and the transgenic Bobwhite with *TiAP1*. (e) The HI statistics of *Bgt* *E09* inoculated for 2 days in Bobwhite, SN6306, and the transgenic Bobwhite with *TiAP1*. Different letters above the bars indicate statistically significant differences ( $P < 0.05$ ) as obtained by one-way analysis of variance with least significance difference and Duncan's multiple range test method. (f) Identifying the resistance of different wheat at the adult stage infected by *Bgt* *E09* in the controlled glasshouse. (1) Bobwhite; (2) The transgenic Bobwhite with *TiAP1* (OE1); (3) YN15; (4) F<sub>1</sub> obtained from cross breeding between YN15 and homozygous transgenic *TiAP1* wheat OE1.

### TiAP1 is a secretory protein that accumulates at the infection site

To determine the localization of TiAP1 in plant cells, we utilized the TiAP1-GFP,  $\Delta$ sp-TiAP1-GFP and free GFP transient transformation in *Nicotiana benthamiana*. Through plasmolysis methods, we found that there was much fluorescent signal of TiAP1-GFP outside the cell membrane, while that of  $\Delta$ sp-TiAP1-GFP (without signal peptide) was in the cytoplasm (Figure 3a,b,c, Figure S5). To further confirm whether TiAP1 is a secretory protein, the mCherry-TaSYP51, which is a member of the syntaxin superfamily on plasma membrane, was co-bombarded with TiAP1-mYFP into the barley leaf epidermal cells. We found a large numbers of TiAP1 and TaSYP51 proteins accumulated at the infection site of the primary and App germination tube of *Bgt*, and TiAP1 extended to the barley intercellular space (Figure 3d; Video S1). Integrating with our observations of the *Bgt* on the leaves of *TiAP1* gene transferred wheat, we speculated that TiAP1 restrains the powdery mildew from penetration, assisting the plants in resisting the invasion of *Bgt*.

### The BgCDA1 interact with TiAP1

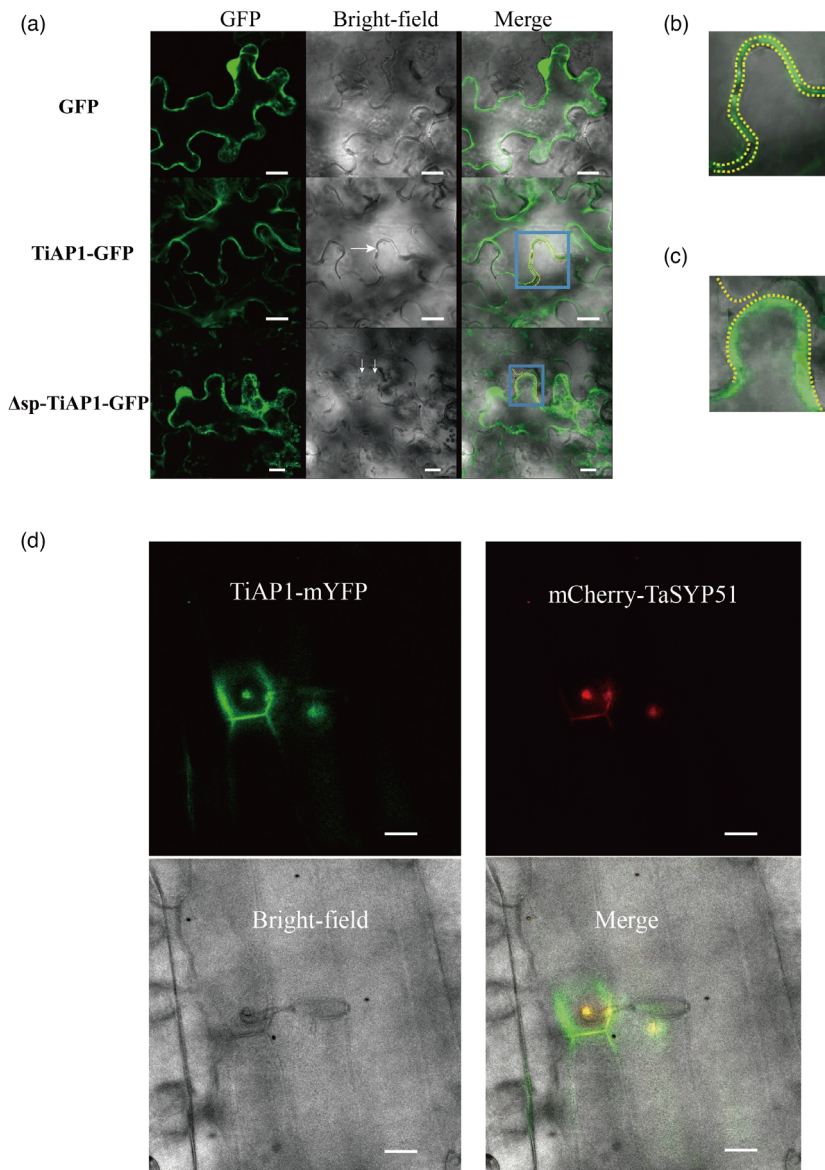
To clarify how TiAP1 can regulate wheat resistance to *Bgt*, CDS of TiAP1 without signal peptide was fused with the GAL4 DNA-binding domain sequence to generate the bait vector. We performed a Y2H screen assay using *Bgt* cDNAs as a prey library. Interestingly, one of the obtained interactors was highly similar

(95.06%) to the chitin deacetylase (EPQ66796.1) of *Bgt*. Furthermore, the analysis of the cDNA revealed that the related protein contains a polysaccharide deacetylation domain. Therefore, we named this gene chitin deacetylase (*BgtCDA1*) of *Bgt* (Figure S6A, B). The *BgtCDA1* has an N-terminal 19-aminoacid signal peptide (<http://www.cbs.dtu.dk/services/SignalP>) and five conserved motifs that are required for hydrolysing the acetyl groups of the substrates (Figure S6C,D). Moreover, the point-to-point hybridization analysis verified the *BgtCDA1* interacted with TiAP1 (Figure 4a).

Subsequently, we further confirmed the interaction through LCI, and BiFC. As expected, the LUC activity in the *N. benthamiana* leaves was high when co-expressed using the  $\Delta$ spTiAP1-nLuc and  $\Delta$ spBgCDA1-cLuc, while the other three combinations were used as negative controls (Figure 4b). The BiFC analysis also confirmed that the co-expression of nGFP- $\Delta$ spBgCDA1 and cGFP- $\Delta$ spTiAP1 resulted in a clear GFP signal in the cytoplasm. In contrast, no visible signal was detected in any of the corresponding negative controls (Figure 4c). Therefore, these observations indicate that there is an *in vivo* interaction between TiAP1 and *BgtCDA1*.

### HIGS analysis showed that the silencing of *BgtCDA1* inhibits the penetration and Hau formation of *Bgt*

To confirm the role of *BgtCDA1* in the growth and development of *Bgt*, we used a transient transformation system based on the particle bombardment of RNAi constructs (Douchkov *et al.*, 2005). The HI of the empty vector control was set to 100%, then



**Figure 3** Subcellular localization of the fusion protein through transient expression of agroinfiltrated GFP, TiAP-GFP and  $\Delta$ sp-TiAP-GFP in the epidermal cells of *N. benthamiana* after plasmolysis, and co-transient expression of TiAP1-mYFP and mCherry-TaSYP51 in the epidermal cells of barley through bombardment. (a) Confocal laser scanning microscopy (Leica SP5-X, Wetzlar, Germany) observation of the leaf epidermal cells revealed that  $\Delta$ sp-TiAP-GFP appears to be localized in the cytoplasm, while TiAP1-GFP is predominantly localized in the extracellular region, where white arrows indicate plasmolysis and the yellow dotted line shows the location of the cell membrane of the two adjacent cells. A GFP signal was used as the control. Bar = 20  $\mu$ m. (b) The enlargement of the blue box in TiAP1-GFP. (c) The enlargement of the blue box in  $\Delta$ sp-TiAP1-GFP. (d) *B. graminis* f. sp. *hordei* induced the accumulation of TiAP1-mYFP and mCherry-TaSYP51 at the infection site. This figure is part of Video S1. Bar = 20  $\mu$ m.

the HI of the positive control, Mlo-RNAi, was 47.44%, while that of *BgtCDA1* RNAi was 55.36% (Figure 5a,b), *Bgt* haustorial formation rate was significantly decreased during silencing of *BgtCDA1*. And through BSMV-VIGS, we also found that the lesions area in the leaves of BSMV: *BgtCDA1* as wheat infected with powdery mildew for 5 days and the hyphal density score of *Bgt* infection for 3 days were significantly lower than those of BSMV:00 wheat (Figure 5c–f). This indicated that *BgtCDA1* gene silencing suppressed the growth of *Bgt*, and that is the *BgtCDA1* gene promoted the invasion of *Bgt*.

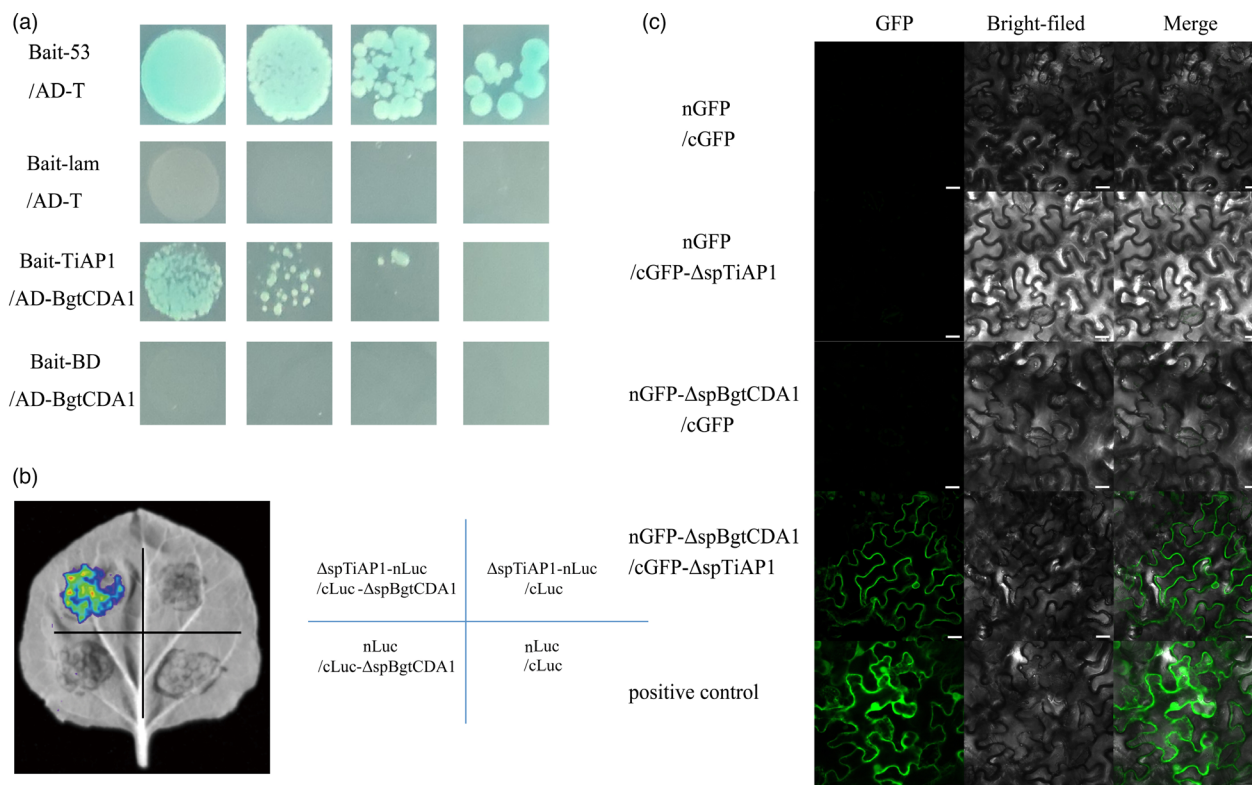
#### The chitin deacetylase activity of *BgtCDA1*

We examined the chitin deacetylase activity of *BgtCDA1*. After *Pichi* yeast expression, the purified *BgtCDA1*-His (2  $\mu$ m) was incubated with 1 mM Chitooctose with six GlcNAc moieties (A6) at 37 °C in 50 mM Tris-HCl buffer (pH8.0), followed by detection of the enzymatic reaction products using MALDI-TOF-MS. As shown in Figure 5g, the deacetylated intermediate products A5D1 (where A is N-acetyl-D-glucosamine and D is D-

glucosamine) were detected after 5- or 30-min incubation. It showed that *BgtCDA1* has the deacetylase activity.

#### TiAP1 and *BgtCDA1* co-regulate the expression of wheat pathogen-responsive genes

To evaluate the impact of TiAP1 and *BgtCDA1* on the pathogen-responsive gene expression on a genome-wide scale, we performed RNA-Seq experiments using Bobwhite and OE2 seedlings inoculated with *E09* for 2 days (Figure S7A). Genes with more than twofold change in expression ( $P < 0.01$ ) were considered to be differentially expressed. Total 274 differentially expressed genes were detected in the OE2-vs-Bobwhite at 2 dpi, of which 116 genes were up-regulated in the OE2-2 (Figure S7B, C; Table S3). Gene ontology (GO) analysis indicated that these differentially expressed genes were enriched in the chitin catabolic process, cell wall macromolecule catabolic process, defence response to bacterium and fungus, chitinase activity and chitin-binding (Figure S7D; Table S4). Defence-related genes were also up-regulated in OE2-2 relative to Bob-2 (Figure 6a;



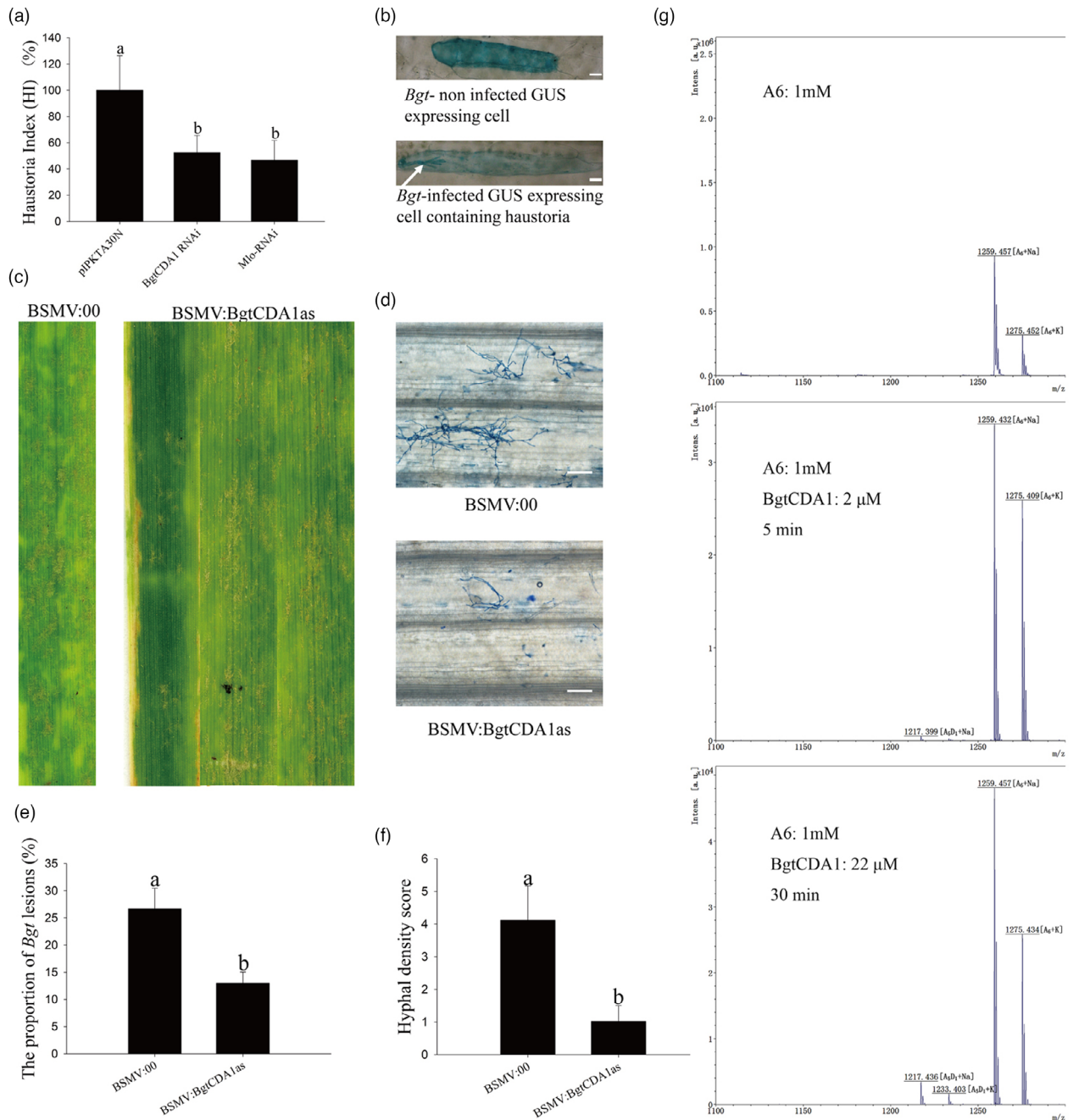
**Figure 4** Interaction of TiAP1 with BgtCDA1. (a) Yeast two-hybrid analysis of the interaction between TiAP1 and BgtCDA1. Yeasts expressing the interacting combinations of bait and prey were spotted on the SD-HAWL/X- $\alpha$ -Gal/ABA. Bait-53 and AD-T (prey) were the positive controls, while Bait-Lam and AD-T as well as Bait-BD and AD-BgtCDA1 were used as the negative controls. (b) Luciferase complementation imaging assay showing that TiAP1 interacted with and BgtCDA1. The  $\Delta\text{spTiAP1-nLuc}$  and cLuc- $\Delta\text{spBgtCDA1}$  were transiently co-expressed in *N. benthamiana*. Co-infiltration of cLuc/nLuc-tagged  $\Delta\text{spTiAP1}$  protein with the nLuc/cLuc-tagged  $\Delta\text{spBgtCDA1}$  or the empty vectors were used as negative controls, where the images were observed using chemiluminescence imaging 2 days later. (c) Bimolecular fluorescent complimentary assay showing TiAP1 interaction with BgtCDA1. The cGFP- $\Delta\text{spTiAP1}$  and nGFP- $\Delta\text{spBgtCDA1}$  were transiently co-expressed in *N. benthamiana*. Co-infiltrations of nGFP/cGFP- $\Delta\text{spTiAP1}$ , cGFP/nGFP- $\Delta\text{spBgtCDA1}$  or the vectors control nGFP/cGFP were used as negative controls. The positive controls were nYFP-14-3-3/14-3-3-cCFP. The green fluorescent protein (GFP) signal was visualized using confocal microscopy (Leica SP5-X). Bar = 20  $\mu\text{m}$ .

Table S5), such as *traesCS1A02G410500*, *traesCS2B02G187500*, *traesCS2A02G161500*, *traesCS3B02G379200* and *traesCS1B02G440300* that encode proteins containing WRKY domains; *traesCS5A02G049600* encodes phytochrome-interacting bHLH; *traesCS7A02G201100* encodes TIFY family gene; *traesCS5D02G188600* encodes protein phosphatase 2C (PP2C); *traesCS6D02G217800* and *traesCS3A02G404400* encode ERF transcription factors; *traesCS6B02G018700* encodes LigB domain-containing protein, and may involve in betaine metabolism; *traesCS7D02G201600* and *traesCS6D02G237900* are ubiquitin E3 ligase RING-type related genes and *traesCS6A02G266100* that encodes the xyloglucan glycosyltransferase/hydrolase and may be involved in cell wall xylan metabolism. By qRT-PCR, we further analysed 12 genes which were differential expression induced by *Bgt* for 2 days. Among these 12 genes, the expression levels of *TraesCS1B02G440300*, *TraesCS2A02G161500*, *TraesCS2A02G199300*, *TraesCS3B02G379200*, *TraesCS5D02G188600* and *TraesCS5A02G049600* which related to plant hormone signal transduction were all up-regulated in OE2-2 relative to Bob-2, beside *TraesCS5D02G188600.0* which was no difference in OE2-2 relative to Bob-2 (Figure 6b). The *TraesCS3B02G259500*, *TraesCS2B02G224300*, *TraesCS6A02G266100* and *TraesCS2D02G099900* involved in cell wall anabolism, in which *TraesCS3B02G259500* and *TraesCS6A02G266100* were consistent with

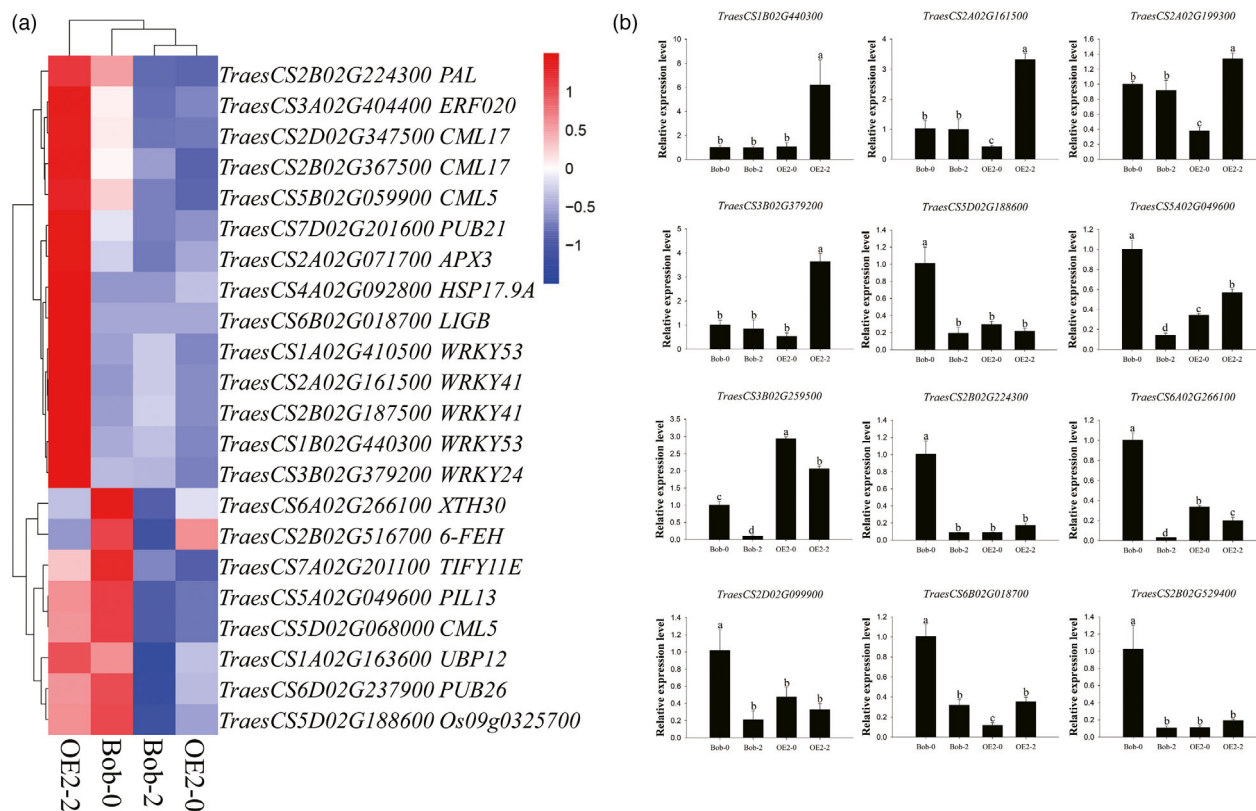
transcriptome were up-regulated in OE2-2 relative to Bob-2, *TraesCS2B02G224300* and *TraesCS2D02G099900* were no difference in OE2-2 relative to Bob-2, but the expression in OE2-2 was higher than Bob-2, and *TraesCS6B02G018700* and *TraesCS2B02G529400* genes expression pattern were similar with *TraesCS2B02G224300* and *TraesCS2D02G099900* (Figure 6b). These quantitative results were slightly different from those at the transcriptome, possibly because of the differences between individuals. In summary, the up-regulated expression of these genes provided favourable evidence to understand the disease-resistant mechanism of the *TiAP1* gene.

## Discussion

Aspartic proteases play a vital regulatory role in plant growth and may be involved in the senescence process (Chen *et al.*, 2015), plant reproduction (Niu *et al.*, 2013), programmed cell death (Chen and Foolad, 1997), resistance to pathogens (Prasad *et al.*, 2010), response to stress (Guo *et al.*, 2013) and endoproteases in plants (Rawlings and Barrett, 2016). If the endoproteases encoding genes were deleted or inhibited, the plant's sensitivity to pathogens increases (Díaz *et al.*, 2018; Jashni *et al.*, 2015). Through multiple sequence comparisons, we found that the *TiAP1* gene is 98% similar to scaffold 208 assembled from the



**Figure 5** The silencing of *BgtCDA1* reduced *Bgt* infection and the BgtCDA1-mediated deacetylation of chitoheptaose (A6). (a) The haustorial index of wheat YN15 leaves, bombarded different RNAi together with GUS reporter constructs, and infected with *Bgt*. The haustorial index was calculated as the ratio of haustoria-containing transformed cells (GUS expressing cells) divided by the total number of transformed cells. Data shown are mean values,  $n = 4$ , and a minimum of 150 transformed cells was counted in each repeat. The relative haustorial index was computed relative to the empty vector pPKTA30N control of each experiment, which was set to 100%. The Mlo-RNAi was another control. Different letters above the bars indicate statistically significant differences ( $P < 0.05$ ) as obtained by one-way analysis of variance with least significance difference and Duncan's multiple range test method. (b) Wheat YN15 leaves epidermal cells, bombarded with RNAi and GUS reporter constructs, and infected with *Bgt*. Arrow indicates haustoria. Bar = 20  $\mu\text{m}$ . (c) Disease phenotype of the BSMV:00 and BSMV: BgtCDA1as inoculated with *E09*. Photographs were taken at 5 dpi. (d) Microscopic observation of the disease symptoms of BSMV:00 and BSMV: BgtCDA1as 3 dpi using *E09*. Bar = 100  $\mu\text{m}$ . (e) The proportion of lesions area on the leaves of BSMV:00 and BSMV: BgtCDA1as inoculated with *E09* for 5 days. (f) The hyphal density score of BSMV:00 and BSMV: BgtCDA1as 3 dpi using *E09*. Different letters above the bars indicate statistically significant differences ( $P < 0.05$ ) as obtained by one-way analysis of variance with least significance difference and Duncan's multiple range test method. (g) The deacetylation function of purified BgtCDA1. The partially deacetylated product A5D1 was detected after incubated for 5, or 30 min. A, N-acetyl-D-glucosamine; D, D-glucosamine.



**Figure 6** The transcriptome and qRT-PCR analysis of the transgenic wheat with *TiAP1*. (a) Heat map of selected pathogen-responsive genes in Bobwhite without and with induction of *Bgt* for 2 days (Bob-0 and Bob-2 respectively), and overexpression line OE2 as the same treatment (OE2-0 and OE2-2 respectively). (b) qRT-PCR analysis of 12 selected genes induced by *Bgt* for 2 days.

*T. intermedium* genome (<https://jgi.doe.gov/>), and Yang and Feng (2020) analysed the aspartic protease gene family and their response under powdery mildew stress in wheat. Among those family genes, the AP gene which was most similar to *TiAP1*, TRIAE\_CS42\_5DS\_TGACv1\_456657\_AA1475640.1, was located on the chromosome 5DS with 70% identity. The qRT-PCR analysis showed that the *TiAP1* gene was rapidly expressed under the induction of *Bgt* in SN6306 with no expression in YN15 (Figure 1a). The silencing of *TiAP1* significantly destroyed the resistance to *Bgt* in the leaves of BMSV:TiAP1as SN6306 (Figure 1b–d). The lesion area on the leaves that smeared with the TiAP1 protein was remarkably decreased when compared with the control (Figure 1h–j). These results indicated that *TiAP1* might resist the invasion of powdery mildew.

Furthermore, the identification of the *Bgt* resistance phenotype at the seedling stage showed that the overexpression of *TiAP1* gene in wheat was moderately resistant to powdery mildew (Figure 2b), while the life cycle of *Bgt* in the 2-day-infected leaves of the overexpression of *TiAP1* gene in Bobwhite was primarily retained at the App stage. In contrast, the *Bgt* on the Bobwhite leaves were mainly at Hau stage. Hau was wrapped in the extrahaustorial membrane (EHM), which was usually used for signal exchange and nutrient absorption (Panstruga, 2013), where the development of the Hau reflected the growth and metabolism of the entire pathogen to a certain extent. It was a vital trait for evaluating host cell resistance (Brackett, 1968).

The HI of *Bgt* in Bobwhite was significantly higher than that of the transgenic *TiAP1* gene lines, indicating that overexpression of

*TiAP1* gene in Bobwhite was resistant to the *Bgt* invasion (Figure 2e). This resistance may be because the transgenic wheat inhibited the penetration of the *Bgt*, thereby affecting their infection rate. Subsequently, we found that the transgenic *TiAP1* Bobwhite and the F<sub>1</sub> generation showed high resistance to powdery mildew in the adult stage (Figure 2f, 2g, Figure S4). Many studies refer to this trait, which showed less significant resistance to the disease at the seedling stage than that of the adult stage (Hautea *et al.*, 1987; Griffey and Das 1994), or of a chronic disease-resistant type (Shaner, 1973). Moreover, Shaner (1973) found conidia of *Konx*, a typical slow-powdery mildew variety, could not successfully invade. Therefore, we speculated that the transgenic wheat line with the *TiAP1* gene might be used as a chronic powdery mildew-resistant variety to prevent the invasion of *Bgt*. Syntaxins were members of the SNARE superfamily of proteins that mediated membrane fusion events (Collins *et al.*, 2003). Since TiAP1 was a secreted protein (Figure 3a–c) which expressed in large quantities at intercellular space of the infection site (Figure 3D, Video S1), we speculate that syntaxin TaSYP51 may help to transport the TiAP1 protein to the outside of the cell, where the detailed mechanisms need to be further elucidated.

Through the Y2H, LCI and BiFC *in vivo* verification, we determined TiAP1 interacts with *BgtCDA1* (Figure 4). Subsequently, we found that when the *BgtCDA1* gene was silenced, the HI, lesions area and hyphal density score were significantly reduced (Figure 5). Zhao and Park (2010) found that chitin deacetylase was secreted when it interacted with other



substances. The chitin deacetylase of *C. lindemuthianum* was secreted during the process of fungal hyphae invading plants to modify the chitin that can be recognized by the plant's resistance system (Tsigos and Bouriotis, 1995). Geoghegan and Gurr (2017) also found that CDA1 and CDA4 were necessary for chitin deacetylation in the cell wall of the mycelium in *M. oryzae*. During infection, the ability of the fungal cells to convert chitin to chitosan was critical for masking the cell wall chitin from being recognized by the host chitinases, thereby promoting the fungal attack (Upadhyaya et al., 2018). Chitin oligomers can also act as ligands that were recognized by LysM receptors on the cell surface and triggered pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Gao et al., 2019; Hurlburt et al., 2018). We found the silencing of *BgtCDA1* can inhibit the invasion of *Bgt* and speculated that the interaction between *BgtCDA1* and *TiAP1* made *BgtCDA1* be degraded by *TiAP1* which inhibits the deacetylation function of *BgtCDA1*. The chitinase of the wheat was secreted to release the chitin oligomers, which were recognized by the wheat chitin receptor, such as LysM. This led to PTI and caused the transgenic wheat with the *TiAP1* gene to be resistant to powdery mildew (Figure 7). When the pathogen invades the host, an extremely complex and precise arms race of 'attack and defence' occurs in the apoplastic space. Xia et al. (2020) found that soybean could secrete an apoplastic aspartic protease, GmAP5, to bind and degrade a pathogen-secreted apoplastic endoglucanase PsXEG1 to destroy its virulence in *Phytophthora sojae* invasion, and proposed a 'multi-layered immune model' of the plants against pathogens in the extracellular region. Therefore, this study revealed the complexity of the interaction between pathogens and hosts as well as provided a theoretical basis to further study the role of *TiAP1* in resistance mechanisms.

When chitin receptors recognize the chitin, the plant can activate defence genes and release some metabolites that initiate the signal transduction in plant cells to trigger an immune response. Many defence-related genes were up-regulated in OE2-2 relative to Bob-2 (Figure 6A; Table S5). WRKY TFs are key nodes in abscisic acid (ABA)-responsive signalling networks (Rushton et al., 2012) and Zhang et al. (2015) found the OsWRKY24, -53 and -70 are involved in gibberellic acid. Therefore, we speculate that these genes may be involved in the ABA or gibberellic acid (GA) signalling pathways. Phytochrome-interacting bHLH, which are known to play important roles in red light-mediated (phyA and/or phyB-dependent) signal transduction pathways at immediate positions downstream of the photoreceptor in *A. thaliana* (Nakamura et al., 2007). In *A. thaliana*, the TIFY family may be involved in jasmonic acid (JA) pathway (Chung and Howe, 2009). PP2Cs are vitally involved in ABA signalling (Park et al., 2009). The ERF transcription factor family is involved in ethylene-activated signalling pathway (Xu et al., 2008). The metabolites include ABA, ethylene, JA, GA and ubiquitination that caused the plants to respond to the invasion of pathogens (Figure 7; Table S3). Moreover, RNA-Seq and qRT-PCR analysis revealed that there was a cell wall xylan metabolism gene, *traesCS6A02G266100*, whose expression in Bobwhite was sharply reduced after being induced by powdery mildew, while the gene in OE2-2 was significantly up-regulated relative to that of Bob-2 (Figure 6).

Since xylan was a type of hemicellulose that was closely related to functional proteins in the cell wall, it had a great influence on the ductility, pore elasticity, flow and rheological properties of plant cells during growth (Zhou et al., 2017). Therefore, we infer that the penetration during the powdery mildew infection of the

transgenic wheat with the *TiAP1* gene was blocked. We also found that the *TraesCS2B02G224300* gene encoded a phenylalanine ammonia-lyase (PAL), which was up-regulated and significant difference in OE2-2 relative to Bob-2 in transcriptome, (Figure 6a; Table S5) and in qRT-PCR the expression of *TraesCS2B02G224300* in OE2-2 was twice higher than Bob-2 (Figure 6b) and the PAL was pivotal for the enzymes related to the biosynthesis of lignin, salicylic acid and other phenylalanine-derived metabolites (You et al., 2020). As a main component of the plant cell wall, lignin contributes to the formation of the physical and chemical barriers in plant immunity. Zhou et al. (2018) also found that *OsPAL1*-mediated resistance to rice blast might be due to the accumulation of lignin, while the broad-spectrum blast-resistant rice varieties *Pigm*- and *PIBP1*-mediated resistance might be related to lignin content (Zhai et al., 2019). Similarly, the overexpression of *OsPAL8* in rice can lead to increased lignin content and enhanced brown planthopper resistance (He et al., 2020). Thus, these results demonstrate that the transgenic wheat with the *TiAP1* gene could enhance resistance to *Bgt*.

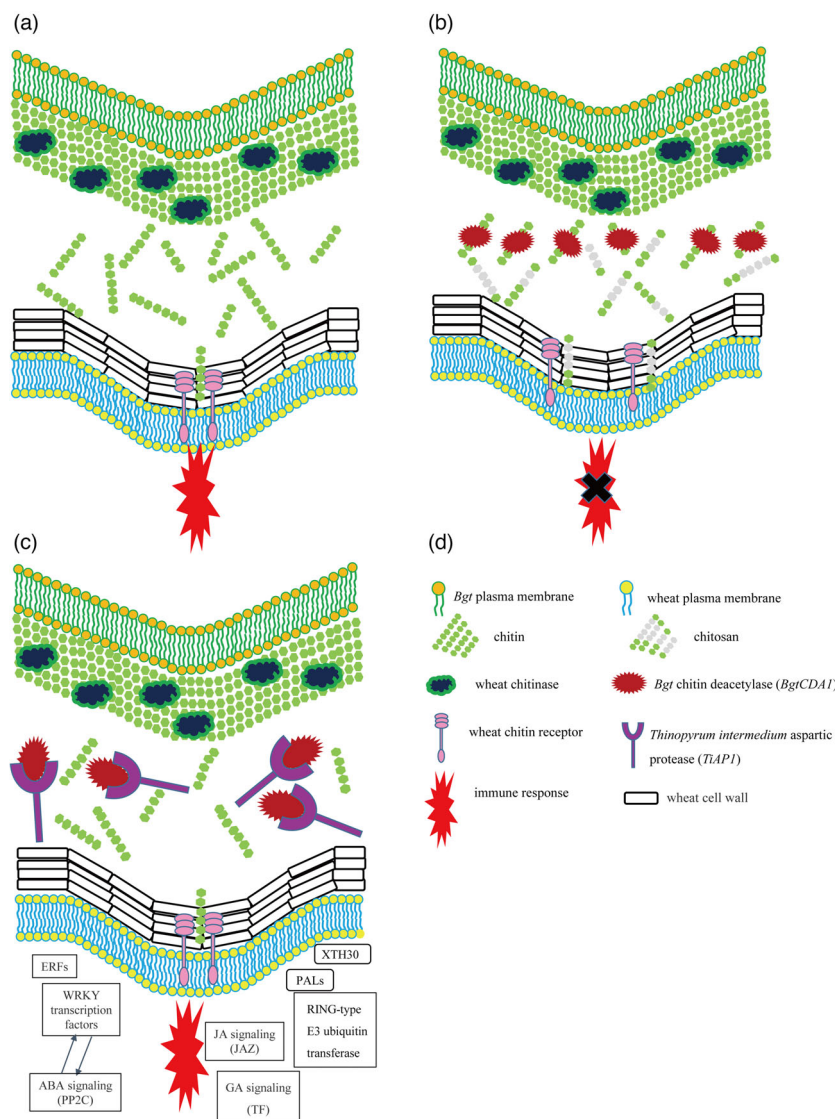
In summary, we speculate the fungal chitin deacetylase *BgtCDA1* can block the PAMP processes that the fungal-derived chitin oligomers are recognized by wheat chitin receptor protein and then trigger an immune response, while *TiAP1* competitively interacting with *BgtCDA1* is the causal reason to re-establish resistance to fungal pathogen *Bgt*. Moreover, the overexpression of *TiAP1* up-regulates the cell wall-related genes in transgenic *TiAP1* wheat thereby blocks the penetration of powdery mildew and enhances the resistance to pathogens. Furthermore, we conclude many other plants may share a similar mechanism and this mechanism can be used for future genetic improvement of crops. However, the mechanism of *BgtCDA1* degraded by *TiAP1*, and the resulting detailed disease resistance pathway remains to be studied.

## Materials and methods

### Plant material and pathogen infection

The tritrigia SN6306 was highly resistant to the powdery mildew and obtained by hybridization between the highly susceptible to powdery mildew wheat (*Triticum aestivum* L.) cultivar Yannong 15 (YN15) and *T. intermedium* (Li et al., 2016). Wheat Bobwhite was highly susceptible to the powdery mildew. The transgenic overexpression *TiAP1* in Bobwhite line 1 and 2 were named as OE1 and OE2 respectively. The untreated with *Bgt* wheat lines Bobwhite, OE1 and OE2 were named as Bob-0, OE1-0 and OE2-0 respectively. The Bobwhite, OE1 and OE2 wheat inoculated with *E09* for 2 days were named as Bob-2, OE1-2 and OE2-2 respectively. The seeds of these materials above and *N. benthamiana* were sown in a mixed soil (loess, matrix and vermiculite of 1 : 1 : 1, v/v/v) at 25°C, 70% humidity and a long-term photoperiod of a 16 h/8 h light/dark cycle in a glasshouse with a light intensity of 600 mmol/m<sup>2</sup>/s. The virulent isolate *E09* of the wheat powdery mildew fungus (*Bgt*) was propagated on the wheat variety YN15 via a weekly inoculum transfer. The barley (*Hordeum vulgare* L.) line Ingrid *ror2* (Collins et al., 2003) was highly susceptible to *Blumeria graminis* f. sp. *hordei* (*Bgh*) and used for the localization and expression profiling of *TiAP1*. The *Bgh* isolate B103 was propagated on barley P-10 via a weekly inoculum transfer, while the *Escherichia coli* (*E. coli*) strain BL21 (DE3) was used for the prokaryotic expression of *TiAP1*. The prevailing *Bgt* isolate *E09* was kindly provided by Dr. Hongjie Li,

**Figure 7** A proposed model of the TiAP1 interaction with chitin deacetylase to increase the resistance to *Bgt* in wheat. (a) The *Bgt* cell walls were targeted by wheat-secreted chitinases to release the chitin fragments that can further activate the host immune system. (b) *Bgt* would secrete BgtCDA1 to convert the cell wall chitin into chitosan to protect the hyphae of the pathogenic fungi from being hydrolysed by the extracellular plant chitinases, thereby protecting the integrity of the fungal cell wall and inhibiting the resistance response mechanism of the plant. (c) Interaction between TiAP1 and BgtCDA1 causing BgtCDA1 to inactivate its deacetylation function, therefore the chitin in fungal cell walls would be targeted by the wheat-secreted chitinases that liberate chitin fragments that can further activate the host immune system. When the transgenic *TiAP1* wheat was infected by *Bgt*, the hormone signals related gene expression *in vivo* would interact to activate plant disease resistance and up-regulate the expression of phenylalanine ammonia lyases in the lignin biosynthesis and XTH30 in the xylan metabolism of the transgenic *TiAP1* wheat. Hence, it enhances the ability of the plant cell walls to resist the penetration by pathogens.



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### VIGS, BSMV and host-induced gene silencing (HIGS)

Virus-induced gene silencing analysis was conducted according to Yuan *et al.* (2011) to insert the conserved sequence of the target *TiAP1* or *BgtCDA1* gene (316 or 300 bp respectively) into the BSMV vector to create BSMV:*TiAP1*as and BSMV:*BgtCDA1*as and transform the *Agrobacterium*. The detailed method was described in Methods S1. Ten days after inoculation with BSMV, six leaves were harvested to test the expression level of *TiAP1* or *BgtCDA1* gene, while the other six leaves were inoculated with *Bgt* race *E09* and were observed and used to evaluate the resistance to the powdery mildew using a biological microscope (Nikon Ni-U, Tokyo, Japan) after the Coomassie Brilliant Blue staining and the statistics of hyphal density score refers to the method of Norris and Frost-Smith (2007) and three biological replicates.

*BgtCDA1* gene silencing in *Bgt* was performed using the HIGS method as described by Nowara *et al.* (2010). The RNA interference (RNAi) construct *BgtCDA1* RNAi and a  $\beta$ -glucuronidase (GUS) reporter gene construct were co-transformed into the leaf

epidermal cells of the wheat YN15 that grew for 7 days using particle bombardment. The fungal haustorial formation was examined for the transformed (GUS expressing) blue cells using a biological microscope (Nikon Ni-U). The empty vectors pPK-TA30N, and the Mlo-RNAi (pPKTA36) constructs, were used as negative and positive controls, respectively, and four biological replicates were conducted per analysis with three technical replicates. The detailed method was described in Methods S1 according to Ahmed *et al.* (2015).

### Constructing the *TiAP1* prokaryotic expression plasmid and the protein expression

To improve the expression of *TiAP1*, the gene sequence was optimized according to *E. coli* bias, and constructed into the pET28a vector (containing 6xHis tags). Recombinant plasmids were transformed into BL21 (DE3) chemically competent cells where a single-colony transformant was cultured and inoculated at 15 °C overnight induced expression with 0.25 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). The cells were harvested and subjected to a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to detect the protein expression.

Then, the recombinant TiAP1 was purified according to the method described in Methods S1.

### Determination of the antifungal activity of TiAP1

To confirm the effect of TiAP1 protein on *Bgt*, we used the renatured TiAP1 and pET28a tag protein to add 0.025% Tween-20 to smear the leaves of YN15 and inoculate with *E09*. After 5 days, the infectivity of *Bgt* was observed and the leaves inoculated for 2 days were stained with the Coomassie Brilliant Blue stain, as described previously (Göllner *et al.*, 2008) and three biological replicates were conducted per analysis with three technical replicates. Lesion areas on the leaf surface were calculated according to Goodwin and Hsiang (2010) to analyse the pixels of the *Bgt* infection area relative to the normal leaf area.

### Constructing the particle bombardment genetic transformation vector to transform wheat and identify the disease resistance phenotype

The coding sequence (CDS) of *TiAP1* (KJ513672) was cloned into the *Ubi-gene-Tnos* vector with a ubiquitin (Ubi) promoter, yielding a recombinant pMUBi-*TiAP1* vector. The resulting plasmid pMUBi-*TiAP1* and the vector harbouring the *Bar* gene were mixed and co-bombarded with a particle bombardment of 1100 psi, 1 µg per gun and 60 µg gold powder into the callus that was induced from the mature embryos of the Bobwhite wheat, which was highly susceptible to *Bgt*. During the induction and regeneration, the transformed wheat was screened using the herbicide Basta, while the presence of the transgenes was determined by amplifying the target gene using the pUbi-ASPF/R primers (Table S6).

Next, the disease responses to powdery mildew of the transgenic plants were tested. When the Bobwhite and the transgenic Bobwhite with the *TiAP1* gene grew to the three-leaf stage, the first leaves of them were spread out on an acrylic plate with the conidium of the *Bgt* race *E09* sprayed evenly. The leaves were harvested at 48 h post-inoculation with *E09* and stained with Coomassie Brilliant Blue as described previously (Göllner *et al.*, 2008) and three biological replicates were conducted per stains with three technical replicates. Furthermore, to evaluate the resistance to *Bgt*, of the Bobwhite, SN6306, the transgenic Bobwhite with the *TiAP1* gene, and the F1 obtained from cross breeding between YN15 and homozygous transgenic *TiAP1* Bobwhite, they were evaluated under controlled greenhouse conditions. The infection type (IT) was recorded 15 days after inoculation and reconfirmed after 20 days with a scale of 0–4, according to Wang *et al.* (2015). The values 0–2 were classified as resistant, and 3–4 were as susceptible.

### DNA and RNA extraction and qRT-PCR

Total DNA was extracted from the plant leaves using the cetyltrimethylammonium bromide method (Allen *et al.*, 2006). While total RNA (500 ng) was extracted using the EasyPure® Plant RNA kit (TransGen Biotech Co., Ltd, Beijing, China), reverse-transcribed (RT) using a RevertAid First Strand cDNA synthesis kit (TransGen Biotech Co. Ltd) and quantified on an ABI Quantitative PCR Q6 Detection System (Thermo Fisher Scientific, Waltham, USA) with the SYBR Premix Ex Taq kit (TransGene Biotech Co., Ltd). The wheat actin gene was used as the reference gene, in which three independent biological replicates were conducted per analysis with at least three technical replicates. The primers have been listed in Table S6.

### Subcellular localization

The CDS of the *TiAP1* fragments was fused green fluorescent protein (GFP) in the vector pCambia1300. Then the constructed TiAP1-GFP or Δsp-TiAP1-GFP (with or without signal peptide of TiAP1 respectively) were transferred into the *Agrobacterium tumefaciens* GV3101 and infiltrated into *N. benthamiana* leaves. Two days after transiently expression, they were observed using a confocal laser scanning microscope (Leica SP5-X, Germany), 10 *N. benthamiana* leaves were analysed in each of the three experiments and the detailed method was described in Methods S1.

### Transient expression of the TiAP1 protein induced by Bgh in barley leaves

The expression vector of the TiAP1 protein was constructed using the gateway system (Thermo Fisher Scientific, Waltham, USA). The two recombinant plasmids, TiAP1-mYFP and mCherry-TaSYP51, were co-bombarded onto the leaves of the 7-day-old barley by particle bombardment at a helium pressure of 1100 psi and placed on the 1% Phytigel™ Petri dishes containing 40 mg/mL of benzimidazole (Sigma-Aldrich, St Louis MO, USA) to culture for 24 h, and then inoculated with *Bgh* spores. After an additional 24 h of inoculation, the expression of TiAP1 and TaSYP51 proteins were observed and analysed using a confocal laser-scanning microscope (Leica SP5-X) according to the methodology of Smigielski *et al.* (2019). And three biological replicates were conducted per co-bombarded with six leaves.

### Protein interaction assay

We sampled the leaves of YN15 and SN6306 every day, which were infected with *Bgt* from 0 to 5 days, and then construct a primary cDNA library using the BP reaction of the CloneMiner II kit (Invitrogen, Thermo Fisher Scientific) by Qingdao Oebiotech Co. Ltd. (China). The quality of the cDNA library was identified. Next, we used the nuclear system Y2H mating standard operating procedure method as described in the manufacturer's instructions (Clontech Laboratories, Inc., Palo Alto, USA) to screen the protein that interacts with TiAP1 and further verified the obtained interaction protein, and three biological replicates were conducted per Y2H verify. The structure, amino acid sequence domain and the signal peptide of the obtained protein BgCDA1 were analysed using the Basic Local Alignment Search Tool (PBLAST; Altschul *et al.*, 1997), Pfam (Finn *et al.*, 2014) and SignalP 5.0 (Almagro Armenteros *et al.*, 2019) software respectively.

For LCI assay, by amplifying the sequence of TiAP1 and BgtCDA1 and applying homologous recombination with the nLuc and cLuc, as previously described (Chen *et al.*, 2008), the fusion protein and one of the corresponding co-injected empty vectors, nLuc/cLuc, was co-expressed in *N. benthamiana* as a negative control. At 48 h post-infiltration, the LUC activity of the leaves was observed using an *in vivo* imaging system (Berthold LB985 NightSHADE, Bad Wildbad, Germany). Ten *N. benthamiana* leaves were analysed in each of the three experiments.

For BiFC assay, the sequences of TiAP1 and BgtCDA1 were amplified and fused with the N and C terminals of the GFP, respectively, using the gateway system (Thermo Fisher Scientific, Waltham, USA). As mentioned previously (Bracha-Drori *et al.*, 2004), the fusion protein and one of the corresponding empty GFP-N/GFP-C vectors were co-expressed in *N. benthamiana* as a negative control. The GFP signal was observed 48 h after

infiltration using a confocal laser-scanning microscope (Leica SP5-X, Wetzlar, Germany). Ten *N. benthamiana* leaves were analysed in each of the three experiments.

### The yeast expression, purification and chitin deacetylase activity assay of BgtCDA1

For protein expression and purification in yeast, BgtCDA1 coding sequences with a 6xHis tag were amplified and ligated into pPIC9K-HIS vector (Invitrogen), and then transformed into *Pichia pastoris* strain GS115 (Invitrogen). The positive transformants were induced protein expression using methanol. The target proteins were purified using Ni-column affinity chromatography. Purified protein was used to detect the enzyme activity of BgtCDA1 by measuring the amount of released acetate using ion chromatography and qualitative analysis of deacetylated products by MALDI-TOF by Hoogen Biotech, Shanghai, China according to Gao *et al.* (2019). MALDI-TOF MS analysis was performed on an Ultraflex Extreme MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The standard reaction mixture (200  $\mu$ l) containing 2  $\mu$ M BgtCDA1, 50 mM Tris-HCl (pH8.0) and 1 mM chitin oligomers with 6 GlcNAc moieties (A6) as the substrate were incubated at 37 °C for 5, 30 min followed by heating at 100 °C for 10 min. The mixture without BgtCDA1 was the control. The yeast expression, purification and enzyme activity assay of BgtCDA1 were detailed performed described in Methods S1.

### RNA-Seq and data analysis

RNA-Seq analysis was performed using the three-leaf stage Bobwhite (Bob-0) and the transgenic Bobwhite with the *TiAP1* gene (OE2-0) as well as a Bobwhite inoculated with *E09* 2 days later (Bob-2) and the corresponding transgenic Bobwhite with the *TiAP1* gene (OE2-2). Three biological replicates were used for the RNA extraction, where total RNA was extracted and treated with DNase I, in which the quality test was performed, and then used to construct the library. The quality of the library was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, USA), while the libraries were sequenced on an Illumina HiSeq 2500 platform (Illumina, Inc., San Diego, USA). The RNA-Seq reads were aligned to the wheat genome of the International Wheat Genome Sequencing Consortium with the RefSeq v1.1 annotation (<https://wheat-urgi.versailles.inra.fr/Seq-Repository/Annotations>). Differentially expressed genes across the samples were identified using the DESeq2 package (Love and Huber, 2014) using the standard parameters with an adjusted false-discovery rate of  $P$ -value <0.01 and fold change >2.

### Statistical analysis

The statistical significance of the results was calculated using a one-way analysis of variance followed by least significant difference and Duncan's new multiple range test at a significant difference ( $P < 0.05$ ) using IBM SPSS software version 19.0 (Chicago, IL). All experiments reported include three to six biological replicates and a minimum of three technical replicates with similar results. All primers have been listed in Table S6.

### Accession numbers

The cDNA and coded protein sequence of *TiAP1* sequence in this research has been deposited in NCBI under the accession number KJ513672 and AJC64141.1; *Blumeria graminis* f. sp. *tritici* BgtCDA1 was coded\_by = " join (KE374986.1:122515..122759,

KE374986.1:122809..123142, KE374986.1:123188..123296, KE374986.1:123342..123381, KE374986.1:123432..123502, KE374986.1:123547..123801, KE374986.1:123919..123923, KE374986.1:123978..124142, KE374986.1:124319..124375), and had one and two additional alternative splicings compared with EPQ66796 between KE374986.1:123143 and KE374986.1:123187, KE374986.1:123802 and KE374986.1:123977 respectively; Other protein sequence data from this article can be found in the NCBI under the following accession numbers: *Saccharomyces cerevisiae* CDA1 (NP\_013410) and *Saccharomyces cerevisiae* CDA2 (NP\_013411). TaSYP51 was coded by sequence in chr5A of wheat between 67321475 and 67319313.

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### Conflict of interests

The authors have no competing interests to declare.

### Author contributions

D.F., H.W. and Y.Y. conceived and designed the experiments. Y.Y., P.F., J.L., W.X., N.L., Z.N., Q.L., J.S., Q.T. and Y.B. performed the experiments and analysed the data; Y.Y. and D.F. wrote the paper; D.F. and H.W. conceived, directed and coordinated the project.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figures S1.** The prokaryotic expression and purification of TiAP1.

**Figures S2.** Identification of the transgenic *TiAP1* gene Bobwhite and the subsequent hybrid generation.

**Figures S3.** Validation of *Bgt* resistance of the representative hybrid subsequent generation of F<sub>3</sub>, which showed resistance to *E09* at 7 dpi at the seedling stage.

**Figures S4.** Validation of the wheat powdery mildew resistance in seven transgenic wheat Bobwhite with the *TiAP1* gene at the adult stage, which showed high resistance to *Bgt*. Bobwhite was used as the susceptible control.

**Figures S5.** Subcellular localization of the fusion protein in the *N. benthamiana* leaf epidermal cells.

**Figures S6.** The *BgtCDA1* protein sequence analysis.

**Figures S7.** Transcriptome analysis of the *TiAP1* and *BgtCDA1* co-regulated genes.

**Tables S1.** Evaluation of the resistance to *E09* in YN15, wheat lines overexpressing *TiAP1*, and the hybrid subsequent generation of F<sub>3</sub> in the seedling stage.

**Tables S2.** Evaluation of the resistance to powdery mildew in Bobwhite and wheat lines overexpressing *TiAP1* at the adult stage.

**Tables S3.** Differential gene expression in the transgenic lines overexpression OE2 and Bobwhite when inoculated with *E09* for 2 days.

**Tables S4.** Gene ontology (GO) analysis of the differentially expressed genes in OE2-2 and Bob-2.

**Tables S5.** Expression of selected pathogen-responsive genes in Bob-0, Bob-2, OE2-0 and OE2-2 in the RNA-Seq experiments.

**Tables S6.** The primers used in this study.

Supplementary Material. Additional description of methods.

**Video S1.** Confocal microscopy (Leica SP5-X, Germany) z-scan image series of TiAP-mYFP/mCherry-TaSYP51 on barley leaf epidermal cell under the infection of *Bgh*.