**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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**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 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, OstP1, 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 OsMLB1 could interact with MoChia1 to enhance the resistance against M. oryzae. Overexpressing of OsMLB1 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 tritigriga 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 florescent complementary (BiFC) analysis show that TiAP1 interacts with chitin deacetylase (BgtCDCA1) of Bgt, while BgtCDCA1 could promote the invasion of Bgt. Therefore, we propose that TiAP1 induces the plant to resist the invasion of pathogens by interacting with BgtCDCA1, inactivating the deacetylation function of BgtCDCA1, then triggering the wheat immune response to inhibit the growth and penetration of Bgt.

Results

The expression of TiAP1 mediates the resistance in tritigriga 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 BMSV:TiAP1as SN6306 relative to the BMSV:00 negative control (Figure 1b). When the BMSV:TiAP1as SN6306 was inoculated with Bgt at the three-leaf stage, the infection in BMSV:TiAP1as SN6306 was much more severe than in the BMSV: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<0.055) (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 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 Bob2 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 F1. Gene amplification and phenotype investigation confirmed the inheritance of the TiAP1 gene and its function in resisting Bgt in the hybrid F1. A chi-square test showed that the proportion of resistant-susceptible separation in the F2 generation was 3:1.
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).
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, Dsp-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 Dsp-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 Bgh, 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 BgtCDA1 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 DspTiAP1-nLuc and DspBgtCDA1-cLuc, while the other three combinations were used as negative controls (Figure 4b). The BiFC analysis also confirmed that the co-expression of nGFP-DspBgtCDA1 and cGFP-DspTiAP1 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
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: BgtCDA1as 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 μM) was incubated with 1 mM Chitooctaose with six GlcNAc moieties (A6) at 37 °C in 50 nM 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 ASD1 (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 3 Subcellular localization of the fusion protein through transient expression of agroinfiltrated GFP, TIAP-GFP and Δsp-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 SPS-X, Wetzlar, Germany) observation of the leaf epidermal cells revealed that Δsp-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 μm. (b) The enlargement of the blue box in TIAP1-GFP. (c) The enlargement of the blue box in Δsp-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 μm.
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; `traesCS6D02G188600` 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 `traesCS3D02G18860.0` which was no difference in OE2-2 relative to Bob-2 (Figure 6b). The 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 chitoheaxose (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 pIpT30N 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, bombard with RNAi and GUS reporter constructs, and infected with Bgt. Arrow indicates haustoria. Bar = 20 μm. (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 μm. (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 ASD1 was detected after incubated for 5, or 30 min. A, N-acetyl-D-glucosamine; D, D-glucosamine.
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 expressed under 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 (Bracket, 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 F1 generation showed high resistance to powdery mildew in the adult stage (Figure 2f2, f4, 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 condia 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 super-family 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 (Upadhyya 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; Hurlbut 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 secret an apoplastic aspartic protease, GmAP5, to bind and degrade a pathogen-secreted apoplastic endoglucanase PcsXEG1 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 active 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 signal transduction in plant cells to trigger an immune response. 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 oligos 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 BgtCDA1 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 *Tridritigia 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) at a ratio 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/m²/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* of *E09* (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.
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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:TiAP1as and BSMV:BgtCDA1as 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 Norriss 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 β-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 (Nicon Ni-U). The empty vectors pIPK- TA30N, and the Mlo-RNAi (pIPKTA36) 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 β-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 (KJS13672) 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 SPS-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% Phytagel™ Petri dishes containing 40 mg/mL of benzimidazole (Sigma-Aldrich, St Louis MO, USA) 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 SPS-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 BgCDA1 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 BgCDA1 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 SP5X, 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 production and purification in yeast, BgtCDA1 coding sequences with a 6×His 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-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The standard reaction mixture (200 μl) containing 2 μM BgtCDA1, 50 mM Tris-HCl (pH 8.0) and 1 μM 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.

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-urugi.versailles.inra.fr/SeqRepository/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 EQ066796 between KE374986.1:123143 and KE374986.1:123187 and 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: Saccharomycetes cerevisiae CDA1 (NP_013410) and Saccharomycetes cerevisiae CDA2 (NP_013411). TaSYF51 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., P.F., J.L., W.X., N.L., Z.N., Q.L., J.S., Q.T. and Y.B. 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 F2, 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 F2 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/mtCherry-TaSY5P1 on barley leaf epidermal cell under the infection of Bgy.