Histone acetyltransferase TaHAG1 interacts with TaPLATZ5 to activate TaPAD4 expression and positively contributes to powdery mildew resistance in wheat

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Summary

- Plants have evolved a two-branched innate immune system to detect and cope with pathogen attacks, which are initiated by cell-surface and intracellular immune receptors leading to pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively. A core transducer including PAD4-EDS1 node is proposed as the convergence point for a two-tiered immune system in conferring pathogen immunity. However, the transcriptional regulatory mechanisms controlling expression of these key transducers remain largely unknown.
- Here, we identified histone acetyltransferase TaHAG1 as a positive regulator of powdery mildew resistance in wheat. TaHAG1 regulates expression of key transducer gene TaPAD4 and promotes SA and reactive oxygen species accumulation to accomplish resistance to Bgt infection.
- Moreover, overexpression and CRISPR-mediated knockout of TaPAD4 validate its role in wheat powdery mildew resistance. Furthermore, TaHAG1 physically interacts with TaPLATZ5, a plant-specific zinc-binding protein. TaPLATZ5 directly binds to promoter of TaPAD4 and together with TaHAG1 to potentiate the expression of TaPAD4 by increasing the levels of H3 acetylation.
- Our study revealed a key transcription regulatory node in which TaHAG1 acts as an epigenetic modulator and interacts with TaPLATZ5 that confers powdery mildew resistance in wheat through activating a convergence point gene between PTI and ETI, which could be effective for genetic improvement of disease resistance in wheat and other crops.

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide and provides 20% of the daily protein and food calories globally (Shewry & Hey, 2015; FAOSTAT, 2020). Powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*) is a major devastating disease of wheat that leads to serious yield losses every year (Singh et al., 2016). In comparison to chemical control, the use of cultivars with disease resistance genes is a more comprehensive, economical and environmentally friendly approach (Randhawa et al., 2019). Understanding the molecular mechanisms and identifying genes involved in basal defense will be useful to exploit this new approach for disease resistance breeding.

Plants resist attacks by pathogens via innate immune responses, which are initiated by cell-surface immune receptors and intracellular immune receptors leading to pattern-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively (Jones & Dangl, 2006; Zhou & Zhang, 2020). Both PTI and ETI are associated with the induction of defense responses, including accumulation of defense hormone SA and production of reactive oxygen species (ROS). Reactive oxygen species have been proposed to act as signaling molecules that further activate immune responses (Levine et al., 1994; Wang et al., 2011; Qi et al., 2017; Alhoraibi et al., 2019; Ding & Ding, 2020; Ngou et al., 2021; Yuan et al., 2021). Induction of PTI and ETI also involves large-scale transcriptional reprogramming and this process is synergistically linked with signal transducers (Wang et al., 2006; Dempsey & Klessig, 2012; Li et al., 2019). A battery of key players, such as Phytoalexin Deficient 4 (PAD4) and Enhanced Disease Susceptibility 1 (EDS1), have been identified as the major signal transducer during these processes (Feyes et al., 2005; Zhang et al., 2010; Zhu et al., 2011). Both EDS1 and PAD4 encode lipase-like protein and function in *R* gene-

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mediated and basal disease resistance (Jirage et al., 1999). PAD4 physically interacts with EDS1 to transcriptionally mobilize antimicrobial defense pathways and the complex also is required for the accumulation of SA and hydrogen peroxide (H$_2$O$_2$) during systemic acquired acclimation and resistance (Mühlenbock et al., 2008; Rietz et al., 2011; Cui et al., 2017). Recent study further revealed that EDS1–PAD4 dimers act as convergence point for defence signalling cascades and play a broader role promoting basal immune responses that can be initiated by both PTI and ETI (Pruitt et al., 2021; Tian et al., 2021). Previous study revealed that EDS1 acts as a positive regulator against powdery mildew in wheat (G. Chen et al., 2018). However, little is known about the involvement of PAD4 homologs in wheat upon pathogen attacks, despite its key role in signal molecule-triggered immunity and integrated multiple signaling hub in stress response (Zhou et al., 1998; Wituszynska et al., 2013; Chen et al., 2015; Bernacki et al., 2019). Moreover, the underlying transcriptional mechanisms of PAD4 and EDS1 genes remain largely unknown.

As a major epigenetic regulatory mechanism, histone acetylation plays an important role in gene regulation involved in plant development and response to environmental changes (Servet et al., 2010; Shen et al., 2015; Kim et al., 2018; Y. Chen et al., 2018; Zhao et al., 2019). Histone acetylation homeostasis is regulated by two types of inverse enzymatic reactions mediated by histone acetyltransferase (HAT) and histone deacetylase (HDAC), respectively. An increasing body of work has suggested histone acetylation also as a new layer of regulation for transcriptional reprogramming during the activation of the pathogen defense systems (Zhou et al., 2005; Kim et al., 2008, 2020; Choi et al., 2012; Song & Walley, 2016; Wang et al., 2017; Liu et al., 2019). Intriguingly, the systemic acquired resistance can even be passed on to progeny through histone acetylation (Jaskiewicz et al., 2011; Luna et al., 2012; Fu & Dong, 2013). Although indicative of an involvement of histone acetylation in regulating pathogen defense responses, direct proof disclosing the function of HATs in establishing defense and whether acetylation modifiers induce specific regulatory pathways with other interaction factors to confer pathogen resistance has remained elusive.

In this study, we show that the histone acetyltransferase TaHAG1 plays a pivotal role in resistance to powdery mildew via promoting SA and ROS accumulation in wheat. We demonstrate that TaHAG1 interacts directly with a plant-specific zinc-dependent protein TaPLATZ5 to potentiate the expression of TaPAD4 by increasing the levels of histone acetylation. Our study reveals that a key transcription regulatory node in wheat confers powdery mildew resistance through activating pathogen-responsive genes, which could be effective for genetic improvement of disease resistance in wheat and other crops.

Materials and Methods

Plant materials and growth conditions

Wheat plants were cultivated in an experimental field in Beijing (lat. 39°57′N, long. 116°17′E) under natural growing seasons. For powdery mildew resistance phenotyping, plants were grown in the glasshouse under a 12 h : 12 h, light : dark photoperiod (24°C : 18°C, 70% relative humidity (RH)). Nicotiana benthamiana plants, used as host plants, were grown in a glasshouse at 24–25°C under a 16 h : 8 h, light : dark cycle for 4–6 wk before agroinfection.

Pathogen inoculation

Bgt isolate E09 was used for powdery mildew evaluations. Wheat seedlings were inoculated with Bgt E09 as described previously (Qiu et al., 2021). Leaf samples were collected for examination from different genotypes after inoculation at indicated time points.

Staining and microscopy

Hyphae and leaf cell death were observed at 8 or 9 d post-inoculation (dpi) by Trypan blue staining as described previously (Zou et al., 2018). To visualize H$_2$O$_2$ accumulation, the first leaves were cut from plants of transgenic lines and wild-type (WT) at 2 dpi and were incubated in a 3,3′-diaminobenzidine (DAB) solution (1 mg ml$^{-1}$) for 12 h; staining and microscopy screen were performed as described previously (Lu et al., 2020).

RNA sequencing and statistical analysis

Ten-day-old seedlings of the WT Fielder and TaHAG1-OE lines were inoculated with Bgt isolate E09, and collected 0, 24, 48 and 72 h post-inoculation (hpi) for RNA extraction. Three biological replicates were performed for each time point. Two micrograms total RNA of each replication were used to construct RNA-seq libraries using the NovaSeq platform. The RNA-seq reads were aligned to the Chinese Spring reference genome using STAR with default parameters. The read counts were normalized to fragments per kilobase of exon per million mapped fragments (FPKM) values to show the relative gene expression levels and detailed analysis of RNA-seq data was conducted as previously described (Zheng et al., 2021). Analyses of enriched functional categories were performed using the Triticeae Genetribe source option (Chen et al., 2020).

Plasmid construction and plant transformation

In order to make the candidate gene knockout constructs, the sgRNA target sequences were designed according to conserved region within the first or second exon using the E-Crispr Design Website. Then reverse complementary sgRNA sequences were synthesized, digested and inserted into the terminal vector pBUE411. The open reading frame (ORF) of TaHAG1-D and TaPAD4-B were amplified and inserted into pMWB110 using BamHI sites to achieve the Ubi:TaHAG1 and Ubi:TaPAD4 construct. All binary vectors harboring the desired constructs were transferred into strain EHA105 and transformed into wheat cultivar Fielder by Agrobacterium-mediated transformation.
Subcellular localization

The full-length TaPAD4 or TaPLATZ5 cDNA was cloned into pCAMBIA1300-GFP vector to generate a fusion construct (GFP, green fluorescent protein). The construct was co-infiltrated into wheat protoplasts or leaves of N. benthamiana. 35S: GFP was transferred as a control of GFP signal. The fluorescent signals were observed on a confocal microscope (LSM880; Carl Zeiss).

SA quantification

The samples were homogenized into powder in liquid nitrogen and 50 mg samples put into 2-ml centrifuge tubes. Then 50 μl of internal standard solution and 500 μl of extraction solution (IPA : H2O : HCl = 1 : 1 : 0.002) were added and tubes shaken at 900 rpm for 30 min at 4°C, then 1 ml CHCl3 extract was added and mixed, and the tubes shaken again. After phase separation, the extract was transferred to a new centrifuge tube and dried. Samples were dissolved in 0.1 ml MeOH and divided equally for total and free SA analysis. The content of SA was quantified using UPLC I-Class.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChiP) assays were performed as described elsewhere (Hu et al., 2015). Four grams of 10-d-old wheat seedling materials were collected and subjected to vacuum infiltration in 1% (v/v) formaldehyde for 15 min at 25°C. After isolation and lysis of nuclei, chromatin was sonicated and incubated with anti-TaHAG1 antibody and anti-H3K9ac and H3K14ac antibody (Millipore). The immunoprecipitated DNA fragments then were purified and the enriched DNA fragments were analyzed by quantitative reverse transcription (qRT)-PCR. Amplified DNA from the chromatin fractions before antibody incubation were used as the controls. The enrichments were normalized to the input sample. Relevant primer sequences are listed in Supporting Information Table S1.

Yeast one-hybrid (Y1H) assay

The R1 and R2 sequences were amplified and inserted into the pAbAi vector (BGI, Shenzhen, China). The construct was linearized by BstBI digestion and transformed into Y1HGold cells to generate bait strain. TaPLATZ5 cDNA was inserted into pGADT7 and transformed into Y1H bait strain. The TaHAG1-GFP and TaPLATZ5-MYC fusion constructs were co-transformed into yeast strain AH109. Colonies were cultured on SD/-Leu/-Trp liquid media. The cultures then were serially diluted and plated on synthetic dropout media (SD-L-W-H-A) and kept at 30°C for 2 d before taking photos.

Luciferase complementation assays (LCI)

The LCI assay of TaHAG1 and TaPLATZ5 was carried out as described previously (Zou et al., 2018). The pCAMBIA1300 n-LUC or pCAMBIA1300 c-LUC vectors were used as negative controls.

Co-immunoprecipitation (Co-IP) assay

The TaHAG1-GFP and TaPLATZ5-MYC fusion constructs were co-transformed into N. benthamiana leaves. Total proteins were isolated and incubated with anti-GFP beads at 4°C for 4–6 h with gently shaking. Input and eluted proteins were electrophoretically separated and specific proteins detected by immunoblotting with anti-GFP (HT801-01; TransGen Biotech, Beijing, China) or anti-MYC (HT101-01) antibodies.

Bimolecular fluorescence complementation (BiFC) assay

The fused proteins, or one of them combined with its corresponding empty GFPN/GFPC vector, were co-transformed into N. benthamiana leaves. After 48 hpi, the GFP fluorescence signals were imaged with a confocal microscope (LSM880; Carl Zeiss).

Virus-induced gene silencing (VIGS)

For silencing of TaPLATZ5, a 211-bp fragment of the TaPLATZ5 was inserted in reverse orientation into the Barley stripe mosaic virus RNAγ to form the recombinant vector BSMV: TaPLATZ5. The experiment was performed as described previously (Wan et al., 2022).

Previously (Zheng et al., 2021). Each sample had five independent transfections and primers are listed in Table S1.

Electrophoresis mobility shift assay (EMSA)

The ORF of TaPLATZ5 was cloned into pGEX6P-1 vector to produce a GST fusion protein. GST protein expressed from the empty vector was purified and used as a negative control. For EMSA, biotinylated probe derived from a 47-bp TaPAD4-B promoter fragment containing the A/T-rich motif was synthesized as DNA probe (Table S1). The unlabelled probes with WT or mutated A/T-rich motif were used as competitors. The reaction products were separated by 6% polyacrylamide gel, transferred to a nylon membrane and detection of biotin-labeled DNA were performed according to the manufacturer’s instructions.
Results

Knock-out of TaHAG1 by CRISPR/Cas9 causes strong susceptibility of wheat to powdery mildew

In order to examine whether histone acetylation is required for powdery mildew resistance in wheat, a high-throughput genome editing program of wheat HAT/HDAC family genes were performed. Generally, a highly conserved 19-nt sequences within the first exon of homoeologous genes were chosen as target site for Cas9 cleavage. Agrobacterium-mediated transformation of the susceptible bread wheat cultivar Fielder immature embryos successfully generated T₀ editing events of 10 target genes, including three TaHATs and seven TaHDs (Fig. S1). The target regions of multiple putative transgenic lines were sequenced after PCR amplification. Based on the genotyping combined with phenotype screening, the knockout line of wheat histone acetyltransferase TaHAG1 was noted in the initial screen for its severe susceptibility to powdery mildew infection (Fig. S2). TaHAG1 is an ortholog of Arabidopsis HAG1/GCN5, which belongs to the GNAT subfamily of HATs and functions as a transcriptional coactivator involved in various physiological programs through the regulation of histone modifications (Yin et al., 2019; Kim et al., 2020). For more careful examination of the role of TaHAG1 in powdery mildew resistance, 87 progenies of TaHAG1-KO T₂ plants were further sequenced for homozygous mutant screen. However, the line with all three TaHAG1 homoeologs simultaneously knocked out was not obtained, suggesting that the mutation in three homoeologs of TaHAG1 may be lethal for wheat, consistent with a recent study (Zheng et al., 2021).

Thus, the T₃ lines with simultaneous mutations at the two TaHAG1 homoeologs (1 bp insertion in the first exon of TaHAG1-A and 25 bp deletion in TaHAG1-B, respectively) were selected for further study (Fig. S3). When inoculated with Bgt isolate E09 at the seedling stage, the TaHAG1-KO lines was highly susceptible, with a large number of visible conidiophores produced compared to WT Fielder plants (Fig. 1a). At 9 dpi, a larger number of spores with more branches were observed in TaHAG1-KO lines than in WT plants after Trypan blue staining (Fig. 1b). Moreover, Trypan blue-DAB staining showed smaller conidiophores and less accumulation of H₂O₂ in RNAi lines than WT plants (Fig. 1c). In addition, compared with the MI of 20% in Fielder controls, a significantly higher colonization with an MI of 29% was found in TaHAG1-RNAi lines (Fig. S4c,d). These data indicate that suppressing the expression of TaHAG1 in wheat results in faster and higher susceptibility to powdery mildew.

We used the TaHAG1 overexpression lines with significantly enhanced transcription to further validate its function in powdery mildew resistance (Fig. S5b; Zheng et al., 2021). As expected, the TaHAG1-OE lines showed enhanced resistance to Bgt E09. The pathogen produced fewer spores on leaves of TaHAG1-OE lines than on the Fielder controls (Fig. 1a). The formation of mature Bgt mycelium was significantly inhibited in the leaves of TaHAG1-OE lines compared with the Fielder controls (Fig. 1b). Moreover, Trypan blue and DAB staining also showed robust accumulation of H₂O₂ and substantially restrained development of hyphae in TaHAG1-OE lines, compared to WT plants (Fig. 1c). In addition, TaHAG1-OE lines had significantly fewer conidia with an MI of only 7% compared with MI of > 18% in WT controls (Fig. S4e,f). Together, these results of the overexpression, CRISPR-mediated knockout and RNAi experiments strongly suggest that the TaHAG1 functions as a positive regulator in powdery mildew resistance in wheat.

Transcriptomic analysis of TaHAG1-OE lines under powdery mildew infection

In order to explore the underlying molecular basis of the TaHAG1 in regulation of powdery mildew resistance in wheat, we performed RNA sequencing experiments at 0, 24, 48 and 72 hpi with Bgt E09 in 10-d-old seedlings of TaHAG1-OE and WT Fielder plants. At 24 hpi, the WT and TaHAG1-OE lines showed similar transcriptional response, 5212 and 4518 genes were upregulated (fold-change ≥ 2, FDR ≤ 0.05), whereas 3746 and 3745 genes were downregulated (fold-change ≤ –2, FDR ≤ 0.05) in WT and TaHAG1-OE lines, respectively (Figs 2a, S6; Table S2). Bgt E09 infection caused a more remarkable transcriptomic change in TaHAG1-OE plants relative to the WT plants at 48 and 72 hpi. We totally identified 8559 upregulated and 7365 downregulated genes in the WT at 48 and 72 h detection points, whereas 9693 genes were upregulated and 11138 downregulated in TaHAG1-OE plants at 48 and 72 h detection points (Figs 2a, S6; Table S2).

In view of histone acetylation generally associated with activation of gene transcription (Gan et al., 2021), we mainly focused on the upregulated genes that affected by TaHAG1 in response to powdery mildew infection. Our genome-wide survey provided
a list of 1548 genes whose expression was upregulated in TaHAG1-OE lines compared with WT plants at all time points, designated TaHAG1-OE > WT, and 8113 genes were upregulated in the TaHAG1-OE lines after inoculation at all time points, designated TaHAG1-OE hpi > Control (Fig. 2b; Table S3). Among them, 449 genes were upregulated simultaneously in the TaHAG1-OE lines under Bgt E09 infection (Fig. 2b; Tables S4, S5). We reasoned that TaHAG1-regulated genes involved in powdery mildew resistance would be enriched in the gene clusters where their expression was upregulated in TaHAG1-OE lines compared with WT plants and induced by Bgt E09 infection. Therefore, these 449 genes were considered as TaHAG1-regulated genes in response to Bgt E09 infection. The most significantly enriched functional classes of these genes were those responses to oxidation–reduction process, H2O2 metabolism, cell wall and chitin catabolic processes, defense response to biotic stimulus and systemic acquired resistance (Fig. 2c). A large number of genes reported to be involved in the regulation of defense pathways were detected in these 449 genes, such as disease resistance protein gene TaPik-1, TaEDR2, pathogenesis-related gene TaPR2, TaPRBl, transcription factors TaWRKY53, TaWRKY62, TaERF012, TaIBL1, systemic acquired resistance regulator TaMESI, and receptor-like kinases TaLRR1, TaFER, TaWAK1 (Fig. 2d; Table S5). Notably, a series of typical genes such as PAD4 and EDS1 that are involved in SA and ROS biosynthesis and signallng transducers of systemic acquired resistance were upregulated significantly in TaHAG1-OE lines than WT plants under Bgt E09 infection (Fig. 2d; Table S5). These results are consistent with our above observation that TaHAG1 enhanced powdery mildew resistance in wheat by promoting oxidative burst in pathogen defense response. Moreover, this suggested that elevated SA and/or ROS content may be part of the resistance mechanism mediated by TaHAG1 overexpression.

TaHAG1 affects SA and H2O2 accumulation under powdery mildew infection

In order to test this possibility, we first examined the total SA accumulation in TaHAG1 transgenic lines and WT Fielder plants under Bgt E09 infection conditions. The SA accumulation of the TaHAG1-KO, TaHAG1-RNAi and TaHAG1-OE lines was comparable to that of the WT in control conditions (Fig. 3a). After 48 hpi with Bgt E09 inoculation, both the WT plants and transgenic lines displayed significantly greater SA accumulation. However, the TaHAG1-OE lines produced much higher amounts of SA compared to the WT, whereas TaHAG1-KO and TaHAG1-RNAi lines accumulated less SA than WT plants.
Moreover, we further compared the ROS content of TaHAG1-OE, TaHAG1-RNAi lines and WT plants by histochemical stain. Both NBT and DAB staining reveals that the TaHAG1-KO and TaHAG1-RNAi lines produce lower amounts of H2O2 in the inoculated leaves than in WT Fielder. By contrast, the TaHAG1-OE lines accumulated much greater H2O2 concentrations than WT plants under Bgt infection, resulting in darker staining (Figs 3b, c, S7a). In addition, the H2O2 content also was monitored by H2DCFDA labeling assay and the results are consistent with NBT and DAB staining (Fig. S7b).

Together, these results suggest TaHAG1 contributes to powdery mildew resistance in wheat by elevating the SA and H2O2 accumulation under infection.

TaHAG1 is physically associated with TaPAD4 and facilitates its expression by modulating histone acetylation

For further investigation of the molecular mechanism by which TaHAG1 enhances powdery mildew resistance, we focused on TaPAD4 and TaEDS1 for the following considerations: (1) mutation of TaHAG1 reduced the SA and H2O2 accumulation under powdery mildew infection (Figs 3, S7); (2) both TaPAD4 and TaEDS1 homologs encode positive regulator protein that contributed to basal disease resistance via regulating the SA biosynthesis and H2O2 accumulation (Rietz et al., 2011) and (3) TaPAD4 and TaEDS1 are upregulated in the TaHAG1-OE lines compared with WT plants after Bgt E09 infection (Fig. 2d). To

|        | 24 hpi | 48 hpi | 72 hpi |
|--------|--------|--------|--------|
| WT     |        |        |        |
| OE     | 5212   | 4518   | 3624   |
| OE     | 3866   | 4935   | 5827   |

Fig. 2 Transcriptome analyses of histone acetyltransferase TaHAG1-OE and wild-type (WT) Fielder seedlings under powdery mildew infection by RNA-seq. (a) Numbers of significantly differentially expressed genes (DEGs) at 24, 48 or 72 h post-inoculation (hpi) with Bgt isolate E09 (fold-change ≥2 or ≤−2, FDR ≤0.05). (b) Venn diagrams showing an overlap between genes upregulated in TaHAG1-OE plants by E09 inoculation and upregulated genes in the TaHAG1-OE lines compared with WT plants. (c) Gene ontology (GO) enrichment analyses of the 449 genes whose expression was upregulated significantly upon E09 inoculation in TaHAG1-OE lines than WT Fielder plants. (d) Heatmap showing the expression patterns of the typical genes involved in the regulation of pathogen defense pathways.
this end, we first conducted qRT-PCR to examine the transcript levels of TaPAD4 and TaEDS1 genes in seedlings of TaHAG1 transgenic lines and WT plants following Bgt E09 inoculation. The expression of TaPAD4 and TaEDS1 was downregulated in TaHAG1-KO line but upregulated in TaHAG1-OE line, compared with the WT post-inoculation with Bgt E09 (Fig. 4a,b). The result further confirmed that TaHAG1 positively regulates TaPAD4 and TaEDS1 expression in response to powdery mildew penetration.

In order to explore the possible association of TaHAG1 binding to TaPAD4 or TaEDS1 in response to powdery mildew resistance, the seedlings of wheat plants 2 dpi with Bgt E09 were collected for ChIP assay using antibodies against specific to TaHAG1 protein. The precipitated chromatin DNA was analyzed by qPCR to examine enrichment levels relative to those of nonprecipitated (input) genomic DNA. The occupancy of TaHAG1 to the genomic regions near the transcriptional start sites (TSSs) and coding regions of TaPAD4 or TaEDS1 was analyzed (Fig. 4c). As shown in Fig. 4(d), relatively high enrichment of the fragments near the TSSs of TaPAD4 was detected after E09 inoculation compared with mock-treated plants. By contrast, the enrichment was significantly reduced in the coding regions of this gene before and after E09 inoculation (Fig. 4d). These data suggest that TaHAG1 may specifically bind to the regions near the TSSs of TaPAD4 under powdery mildew infection. In addition, no significant enrichment was detected both at the TSSs and coding region of TaEDS1 gene before and after E09 inoculation, suggesting that it is not the direct target of TaHAG1 (Fig. 4d).

Because TaHAG1 is mainly involved in vivo in histone H3 lysine 9 and 14 acetylation in wheat (Zheng et al., 2021), we further investigated whether TaHAG1 affect the expression of TaPAD4 gene by H3K9 and/or H3K14 acetylation. The ChIP assays were performed with antibodies against H3K9ac and H3K14ac. The levels of H3K9ac and H3K14ac on TaPAD4 were low in mock-treated plants but significantly increased after E09 inoculation. Moreover, the levels of H3K9ac and H3K14ac in TaPAD4 were decreased in TaHAG1-KO plants but were significantly increased in TaHAG1-OE plants compared to the WT, especially upon E09 inoculation (Fig. 4e,f). These changes indicated that the enrichment of TaHAG1 triggers increased H3K9ac, H3K14ac and transcriptional upregulation of this loci. The effect of TaHAG1 regulating TaPAD4 loci also was examined using a transient expression assay (Fig. 4g). Co-expression of the TaHAG1 with the reporter construct resulted in significantly increased LUC expression compared with the control (Fig. 4h), indicating that TaHAG1 could activate the expression of TaPAD4. Together, based on these findings, we propose that TaHAG1 directly binds to the promoter of TaPAD4 gene to trigger the epigenetic changes and, in turn, facilitates their expression in response to Bgt E09 infection.
CRISPR-mediated knockout and overexpression of TaPAD4 in wheat validate its role in powdery mildew resistance

TaPAD4 belongs to the abhydrolase super family and contains a typical hydrolases domain (Fig. S8). Consistent with the AtPAD4 in Arabidopsis, subcellular localization assay indicated that the TaPAD4 protein localizes both in the cytoplasm and nucleus (Fig. S9). To examine the role of TaPAD4 in powdery mildew resistance in wheat, we generated TaPAD4 mutant plants. The guide RNA was designed to target a highly conserved region in TaPAD4 (Fig. 4a, b). TaPAD4 and TaEDS1 expression in wild-type (WT) Fielder, TaHAG1-OE and TaHAG1-KO plants at 0, 24, 48 and 72 h post-inoculation (hpi) with Bgt E09, measured by quantitative reverse transcription (qRT)-PCR (EDS1, Enhanced Disease Susceptibility 1). The expression of β-Actin was used to normalize mRNA levels. Error bars represent ±SD of values obtained from three independent biological samples. (c) Schematic representation of gene structures indicating regions examined by the chromatin immunoprecipitation (ChIP) assay. The positions of the primer sets (dumbbell) used in the ChIP assay relative to the transcriptional start sites (TSSs) and ATG codon are shown. (d) Enrichment of TaHAG1 binding determined on the TaPAD4 and TaEDS1 loci by ChIP assays. Signals are given as percentages of the input chromatin value. P1 and P2 indicate TSSs and coding regions, respectively. The contig423638 (retrotransposon) is negative control site for TaHAG1 binding and histone acetylation enrichments (Zheng et al., 2021). (e, f) Enrichment of H3K9ac and H3K14ac on promoter region of TaPAD4 gene in mock and 48 hpi with Bgt E09 conditions. Asterisks indicate significant differences between TaHAG1 transgenic lines and WT plants under the same treatment. (g, h) Dual luciferase transcriptional activity assays to assess the ability of TaHAG1 to transactivate target gene expression. Schematic diagrams of the effector and reporter constructs (g). LUC : REN indicates the ratio of the signal detected for firefly luciferase (LUC) to renilla luciferase (REN) activity (h). Error bars represent SD for five replicates (**, P < 0.01, by Student’s t-test).
the second exon of three TaPAD4 homoeologs (Fig. 5a). Homozygous mutants with simultaneous knockout of all three TaPAD4 homeologs were identified. Two independent knockout lines, KO#1 and KO#2, harboring in-frame shift mutations in the second exon of TaPAD4-A, B, D, were generated by the CRISPR-Cas9 system (Fig. 5a). KO#1 confers a 38-bp deletion

![Diagram of TaPAD4 sequence comparison between wild-type (WT) Fielder and mutants. The targeted sequence is underlined and deleted nucleotides are represented by ‘-’, and inserted nucleotides are highlighted in red. The numbers on the right represent the number of nucleotides involved in the indel-created events with ‘+’ or ‘/’.](image)

**Fig. 5** Transduction gene TaPAD4 is required for the powdery mildew resistance in wheat (PAD4, Phytoalexin Deficient 4). (a) Comparison of CRISPR-Cas9 targeted TaPAD4 sequences between wild-type (WT) Fielder and mutants. The targeted sequence is underlined and deleted nucleotides are represented by ‘-’, and inserted nucleotides are highlighted in red. The numbers on the right represent the number of nucleotides involved in the indel-created events with ‘+’ or ‘/’. (b) Ten-day-old TaPAD4-KO and WT Fielder plants were inoculated with Bgt E09. (c) Transcript levels of TaPAD4 in Fielder and TaPAD4 overexpression plants. Values are means ± SD of three biological repeats. (d) Ten-day-old TaPAD4-OE and WT plants were inoculated with Bgt E09. In (b) and (d), representative leaves were removed and photographed at 8 d post-inoculation (dpi). Bar, 2 mm. Trypan blue staining of the leaves infected with Bgt E09 at 8 dpi to visualize fungal structures and plant cell death. Bar, 25 μm. DAB staining of leaves infected with Bgt E09 at 2 dpi. Brown staining shows the accumulation of hydrogen peroxide (H2O2). Bar, 25 μm. (e, f) Measurements of the SA (e) and H2O2 contents (f) in the seedling of the WT Fielder and different TaPAD4 transgenic lines under E09 infection conditions. Values are means ± SD (n = 3 biological repeats). The data in (c), (e) and (f) were analyzed by ANOVA one-way comparison followed by LSD test. Different letters above the bars indicate a significant difference at P < 0.05.
in TaPAD4-A, a 5-bp deletion in TaPAD4-B and a 1-bp insertion in TaPAD4-D; and line #2, confers a 5-bp deletion in TaPAD4-A, a 4-bp deletion in TaPAD4-B and a 1-bp insertion in TaPAD4-D, respectively. The resistance of TaPAD4-KO plants was tested by evaluating the conidiophores formation following infection with Bgt E09. As shown in Fig. 5(b), leaves of TaPAD4-KO plants had significantly more mildew microcolonies and enhanced susceptibility to Bgt E09 infection compared with Fielder plants. At 8 dpi, a larger number of mycelia with more branches were observed in TaPAD4-KO lines than in WT plants after Trypan blue staining. Moreover, Trypan blue-DAB staining showed visible conidiophores and lower accumulation of H2O2 in TaPAD4-KO lines than in WT plants (Figs 5b, S10). These data indicate that knockout of TaPAD4 in wheat results in increased susceptibility to powdery mildew.

We also generated TaPAD4 overexpression lines (TaPAD4-ox) to further validate the function of TaPAD4 in wheat powdery mildew resistance. Two independent overexpression lines, ox1 and ox2 with significantly elevated transcript abundance of TaPAD4 were selected for further study (Fig. 5c). The overexpression lines showed enhanced resistance to Bgt E09 (Fig. 5d). The transgenic lines produced fewer spores on leaves of TaPAD4-ox lines than on the Fielder controls. In agreement with these findings, Trypan blue stain indicated the formation of mature Bgt mycelium was significantly inhibited in the leaves of TaPAD4-ox lines compared with the Fielder controls (Fig. 5d). Moreover, at 2 dpi, Trypan blue-DAB staining also showed robust accumulation of H2O2 and with no visible conidia produced in TaPAD4-ox lines, compared to WT plants (Fig. 5d). These results strongly suggest that the TaPAD4 indeed plays a crucial role in powdery mildew resistance in bread wheat.

We further examined the total SA accumulation in the TaPAD4 transgenic lines and WT Fielder plants under Bgt E09 infection conditions. After Bgt E09 inoculation, the TaPAD4-KO lines accumulate less SA compared to WT plants, whereas TaPAD4-ox lines produce significantly higher amounts of SA than WT plants (Fig. 5e). Moreover, both NBT and DAB staining revealed that TaPAD4-KO lines produce lower amounts of H2O2 in the inoculated leaves than WT Fielder (Figs 5f, S10). By contrast, the TaPAD4-ox lines accumulated much higher H2O2 than WT plants under Bgt E09 infection, resulting in darker staining (Figs 5f, S10). Together, these results suggest that TaPAD4 contributes to powdery mildew resistance in wheat by promoting accumulation of SA and H2O2 under infection.

TaHAG1 interacts with zinc-dependent DNA-binding protein TaPLATZ5

In order to further explore the regulatory mechanisms of TaHAG1, we performed Y2H screening and 24 candidate interactors of TaHAG1 were obtained (Table S6). A putative plant AT-rich zinc-binding protein (PLATZ) encoded by TraesCS2D02G447400 was confirmed to interact with TaHAG1 by Y2H assays (Fig. 6a). Sequence alignment indicated that TraesCS2D02G447400 contained a PLATZ domain and shared the highest sequence identity with LOC_Os04g50120 in rice and ZmPLATZ5 in maize, respectively; thus, it was named TaPLATZ5 (Fig. S11). TaPLATZ5 is localized in the nucleus (Fig. S12). We then corroborated this TaHAG1-TaPLATZ5 interaction using LCI assays, where the LUC activity signal was produced after nLUC-TaHAG1 and cLUC-TaPLATZ5 had been co-infiltrated into N. benthamiana leaves (Fig. 6b). Furthermore, we performed pull-down assays and observed that TaPLATZ5-GST can interact directly with TaHAG1-MBP (Fig. 6c). We also created transgenic tobacco plants expressing TaPLATZ5-MYC and crossed them with TaHAG1-GFP plants to perform Co-IP, which showed that TaPLATZ5-MYC can interact with TaHAG1-GFP in planta (Fig. 6d). Moreover, to investigate the subcellular localizations of TaHAG1-TaPLATZ5 interaction inside plant cells, we conducted BiFC assays. Coexpression of TaHAG1-nGFP and TaPLATZ5-cGFP in the pavement cells of N. benthamiana yielded strong green fluorescence in the nucleus; however, no fluorescence signal was detected in the pavement cells transfected with control constructs (Fig. 6e). Collectively, these results demonstrate that TaHAG1 physically interacts with TaPLATZ5.

Transcription of TaPLATZ5 was significantly enhanced in wheat after E09 inoculation, and its expression was comparable between TaHAG1-OE, TaHAG1-KO lines and WT, indicating that TaPLATZ5 expression responds to E09 infection but could not be affected by TaHAG1 (Fig. S13). Using the yeast GAL4 system, we verified that TaPLATZ5 has transcriptional activation ability, although weaker than positive control P53 (Fig. S14). To test the function of TaPLATZ5 in powdery mildew resistance, we knocked down its expression in wheat by VIGS using BSMV. The BSMV-TaPLATZ5 plants showed reduced TaPLATZ5 expression level and increased susceptibility to Bgt E09 (Fig. 6f, g). At 10 dpi, the pathogen produced more sporophores on leaves of BSMV-TaPLATZ5 lines than on the control plants (BSMV:γ). Moreover, Trypan blue-DAB staining showed more conidiophores and lower accumulation of H2O2 in BSMV-TaPLATZ5 lines than in BSMV:γ plants (Fig. 6g).

TaPLATZ5 directly binds to the promoter of TaPAD4

The above findings that TaHAG1 targets and facilitates TaPAD4 expression by modulating histone acetylation, together with the physical interaction of TaHAG1 and TaPLATZ5, promoted us to investigate whether TaPAD4 is a direct target of TaPLATZ5. The PLATZ preferentially targets sequence that is rich in A/T bases (Q. Li et al., 2017). To address this possibility, we first analyzed the promoter sequence (2001 bp upstream from the ATG start codon) of TaPAD4 (TraesCS4B02G100100) in wheat. There are two A/T base-rich motif regions in the promoter of TaPAD4 (Fig. 7a). We found that TaPLATZ5 directly binds to TaPAD4 promoter region R1 containing an A/T-rich motif from −955 to −167 (Fig. 7a). Consistent with this finding, a transient transactivation assay showed that TaPLATZ5 was able to mildly but distinctly activate the expression of TaPAD4 promoter-driven LUC reporter (Fig. 7b, c). When the A/T base-rich motif in region R1 is mutated, transactivation of the promoter by TaPLATZ5 was drastically reduced (Fig. 7b, c). We also
conducted an EMSA to confirm whether TaPLATZ5 directly binds to TaPAD4 regulatory regions using a recombinant protein TaPLATZ5-GST. It was shown that TaPLATZ5 physically bound to the biotin-labeled TaPAD4 promoter in an A/T-rich motif-dependent manner and the TaPLATZ5-TaPAD4 promoter binding was competed against unlabelled WT probes but not mutated probes (Fig. 7d). Moreover, TaPAD4 transcript abundance and H3K9, H3K14 acetylation statuses of its
promoter were significantly reduced in BSMV-TaPLATZ5 plants compared with the control plants (Fig. 6h–j). Notably, the identified that the TaPLATZ5 binding region in promoter of TaPAD4 overlaps substantially with the aforementioned enrichment region of TaHAG1 (Fig. 4c,d), suggesting the co-regulation of TaPLATZ5 and TaHAG1 on the target gene TaPAD4.

TaHAG1 and TaPLATZ5 additively activate TaPAD4 expression

The in vitro and in vivo protein interaction assays, ChIP-qPCR and EMSA suggest that TaHAG1 and TaPLATZ5 may form protein complexes for binding to promoter of TaPAD4 for its elevated expression. Therefore, we tested whether co-expression of TaHAG1 is necessary to enhance TaPLATZ5-mediated transcriptional regulation of TaPAD4 using dual LUC transcriptional activity assay. The LUC signal detection was used to determine their transactivation ability and the co-expression effects on the promoter of TaPAD4 gene (Fig. 8a). Consistent with the aforementioned finding, both TaHAG1 and TaPLATZ5 can activate TaPAD4 promoter, but the LUC expression was only slightly increased when TaPLATZ5 was expressed alone, suggesting its lower activation efficiency (Fig. 8b). However, co-expression of TaHAG1 with TaPLATZ5 led to a significant increase in TaPAD4 promoter activation compared with the expression of each single effector constructs. By contrast, when the A/T base-rich motif is mutated in TaPAD4 promoter, transactivation of the promoter by TaPLATZ5 alone or co-expression with TaHAG1 was drastically reduced and resulted in comparable LUC expression compared with the control, suggesting the binding ability of TaPLATZ5 to the target motif is important for gene expression (Fig. 8b). Taken together, these results indicate that TaHAG1 functions collaboratively with TaPLATZ5 and has additive effects with TaPLATZ5 in driving TaPAD4 gene expression.

Discussion

Exploring the role of regulatory factors and underlying molecular mechanisms in plant disease resistance will be crucial for developing a strategy to defend against plant diseases and improve crop productivity. In the past few decades, several important genetic loci and key factors that have critical roles in powdery mildew resistance have been identified and cloned in crops (Hurni et al., 2013; Sánchez-Martín et al., 2016; He et al., 2018; Xing et al., 2018; Zou et al., 2018; Lu et al., 2020). However, the underlying mechanisms and regulatory pathways in defending against pathogens remain obscure. This is particularly challenging for common wheat, which carries a large and polyploid genome and has only limited functional genomic platforms. In this study, we demonstrate that the histone acetyltransferase TaHAG1 is involved in oxidative burst upon pathogen infection and essential for powdery mildew resistance in wheat. Knock-out and knock-down of TaHAG1 in wheat causes decreased SA and H2O2 accumulation and strong susceptibility to powdery mildew, whereas overexpression of TaHAG1 conferred elevated SA content and ROS burst and enhanced resistance against powdery mildew. We further reveal a key transcription regulatory node in which TaHAG1 acts as an epigenetic modulator interacting with TaPLATZ5 that renders wheat to confer powdery mildew resistance through potentiating the expression of TaPAD4.

In Arabidopsis, PAD4 encodes a lipase-like protein that is important for the SA signaling pathway and is required for multiple defense responses (Feys et al., 2001). Mutation of PAD4 leads to impaired SA and ROS accumulation and displays enhanced susceptibility to mutiple pathogens (Rietz et al., 2011). Moreover, the upregulated expression of PAD4 in F1 hybrids contributes to its heterosis for biotrophic pathogen resistance in Arabidopsis (Yang et al., 2015). Interestingly, another study revealed that PAD4/EDS1 complexes bolster pathogen defense though interference with MYC2, a master regulator of SA-antagonizing JA hormone pathways in Arabidopsis (Cui et al., 2018). Recent study further revealed the EDS1-PAD4-ADR1 node is a convergence point for defence signaling cascades in conferring pathogen immunity (Pruitt et al., 2021). These corroborating results pinpoint that PAD4 plays a pivotal role in basal defense against biotrophic pathogens. However, few upstream regulators of PAD4 in response to pathogen defense have been isolated, except that Giri et al. (2017) found that the GBF1
Fig. 7 Zinc-binding protein TaPLATZ5 directly binds the promoter of transduction gene TaPAD4 (PAD4, Phytoalexin Deficient 4). (a) TaPLATZ5 could bind to the core motif of TaPAD4 promoter via yeast one-hybrid (Y1H) assay. Schematic representation of the TaPAD4 promoter showing putative TaPLATZ5 binding sites (orange triangles). Yeast cells were co-transformed with the bait vector, containing the R1 or R2 sequence fused to the AbAi reporter gene and with the prey vector, encoding TaPLATZ5 protein fused to the GAL4 activation domain. The strains were grown on the SD/-Leu/-Ura with or without 200 ng ml\(^{-1}\) ABA for 3 d. Numbers at the top represent the dilution times of an optical density at 600 nm. (b, c) Dual luciferase transcriptional activity assays showing ability of TaPLATZ5 to transactivate wild-type (WT) and mutated TaPAD4 promoter expression. (b) Schematic diagrams of the effector and reporter plasmids. (c) The transactivation ability of TaPLATZ5 is indicated by the ratio of firefly luciferase (LUC) to renilla luciferase (REN). The empty vector (EV) was used as control. Error bars represent SD for five replicates. Different letters above the bars indicate a significant difference by one-way ANOVA and Tukey’s multiple comparison test at \(P < 0.05\). (d) EMSA analysis of TaPLATZ5 binding to TaPAD4 promoter. Unlabelled probe containing WT or mutated AT-rich motif from R1 fragment was used as competitor. ‘+’ and ‘−’ indicate the presence and absence, respectively, of the corresponding probes and protein.
positively influences the defense by regulating \( \text{AtPAD4} \) transcription in Arabidopsis. In this study, we demonstrate \( \text{TaPAD4} \) is a direct downstream target of \( \text{TaHAG1} \) and plays a critical role in powdery mildew resistance of wheat. \( \text{TaHAG1} \) associates directly with the promoter of \( \text{TaPAD4} \) and triggers increased H3 acetylation in response to powdery mildew infection, which provides further epigenetic regulatory evidence that it contributes to powdery mildew resistance. These results are reminiscent of the findings reported in the aforementioned observation that increased H3 acetylation of key defense genes correlates with their upregulation in infected organisms (Yang et al., 2015). Accordingly, we speculate that the expression of \( \text{TaHAG1} \) upon pathogen infection might induce a primed state characterized by enhanced histone acetylation in the promoter of these defense-associated transcriptional reprogramming factors such as \( \text{TaPAD4} \), which then enables a more rapid and stronger activation of defense-related genes on pathogen invasion. Furthermore, overexpression and knockout of \( \text{TaPAD4} \) validated its role in powdery mildew resistance in wheat, supporting the notion that \( \text{TaHAG1} \)-activated \( \text{TaPAD4} \) expression is essential for powdery mildew resistance. Moreover, we also found several genes that are involved in cellulose biosynthetic process and systemic acquired resistance pathways, including glucan synthase, WRKY and receptor-like kinase, were significantly upregulated in \( \text{TaHAG1} \) OE plants compared to the WT under powdery mildew infection (Table S5), implying that these genes and involved pathways also might participate in the \( \text{TaHAG1} \)-mediated powdery mildew resistance. In addition, besides disease resistance pathways,  

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**Fig. 8** Histone acetyltransferase \( \text{TaHAG1} \) and zinc-binding protein \( \text{TaPLATZ5} \) cooperate to activate transduction gene \( \text{TaPAD4} \) expression (PAD4, Phytoalexin Deficient 4). (a, b) Dual luciferase transcriptional activity assays showing ability of \( \text{TaHAG1} \) and \( \text{TaPLATZ5} \) to transactivate wild-type (WT) and mutated AT-rich motif from \( \text{TaPAD4} \) promoter expression. (a) Schematic diagrams of the effector and reporter plasmids. (b) The transactivation ability of \( \text{TaPLATZ5} \) and/or \( \text{TaHAG1} \) is indicated by the ratio of firefly luciferase (LUC) to renilla luciferase (REN). The empty vector (EV) was used as control. Error bars represent standard deviation for five replicates. Different letters above the bars indicate a significant difference by one-way ANOVA and Tukey’s multiple comparison test at \( P < 0.05 \). (c) A proposed working model of the \( \text{TaHAG1} \)–\( \text{TaPLATZ5} \) cooperation in activating \( \text{TaPAD4} \) expression for the powdery mildew resistance. \( \text{TaPLATZ5} \) alone through its binding to the A/T-rich motif establishes a basic transregulation system for activating the \( \text{TaPAD4} \) gene; the interaction of \( \text{TaHAG1} \) with \( \text{TaPLATZ5} \) is necessary for this enhanced transregulation system via increasing histone acetylation. Higher \( \text{TaPAD4} \) levels in turn result in accumulation of SA, hydrogen peroxide \((\text{H}_2\text{O}_2)\) and enhanced resistance to powdery mildew. Mutation of \( \text{TaHAG1} \) reduces the expression of \( \text{TaPAD4} \) by decreasing the levels of histone acetylation, reducing SA and \( \text{H}_2\text{O}_2 \) accumulation and leading to susceptibility.
TaHAG1 binds to the A/T-rich motif of TaPAD4 and is necessary for its promoter activation, indicating that TaHAG1 could serve as a target for both genetic engineering and selection for improvement of wheat powdery mildew resistance.

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Author contributions

NS, JCL and XBL performed the experiments with the help of ZHL, DBL, JC, YMC, SMC, QY and XYL; WLG, MMX, YYY, HRP and ZFN contributed to materials; ZRH, CJX and XBL contributed to materials and data processing; ZRH, CJX and XBL contributed to analysis of data. ZRH, CJX and XBL contributed to writing the paper with input from QXS and CJX; ZRH agrees to serve as the author responsible for contact and ensures communication. NS, JCL and XBL contributed equally to this work.

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Data availability

The data that support the findings of this study are available in the supplementary material of this article.

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Supporting Information

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

Fig. S1 Phylogenetic analysis of wheat histone acetyltransferase (TaHATS) and histone deacetylase (TaHDs) homologous copies.

Fig. S2 Ten-day-old TaHAG1-KO and wild-type Fielder plants were inoculated with Bgt isolate E09.

Fig. S3 Characterization of the TaHAG1 mutation in wheat.

Fig. S4 Validation the role of TaHAG1 in powdery mildew resistance of wheat.

Fig. S5 TaHAG1 transgenic lines confirmed by reverse transcription-PCR.

Fig. S6 Overlap genes upregulated and downregulated by inoculation with Bgt isolate E09.

Fig. S7 The reactive oxygen species production in leaves of wild-type Fielder and different TaHAG1 transgenic lines.
Fig. S8 Phylogenetic and amino acids analysis of PAD4 homoeologs in different species.

Fig. S9 Subcellular localization of TaPAD4 in wheat leaf protoplasts.

Fig. S10 NBT and DAB staining of Bgt E09 infection-induced reactive oxygen species production in leaves of wild-type Fielder and different TaPAD4 transgenic lines.

Fig. S11 Phylogenetic and amino acids analysis of TaPLATZ5 homoeologs in different species.

Fig. S12 Subcellular localization of TaPLATZ5.

Fig. S13 The TaPLATZ5 gene expression in wild-type Fielder, TaHAG1-OE and TaHAG1-KO plants after inoculation with Bgt E09.

Fig. S14 Yeast two-hybrid assay of the transcriptional activation ability by TaPLATZ5.

Fig. S15 TaHAG1 is physically associated with TaVrt2 but not TaHKT1;5-D, TaRht1 and TaVSR1.

Fig. S16 Phenotypes of different TaHAG1 transgenic lines and wild-type Fielder plants under a duration of Bgt E09 infection in vegetative and reproductive stages of wheat growth.

Table S1 Primer pairs used in this study.

Table S2 Genome-wide transcription profiles of TaHAG1-OE and wild-type Fielder post-inoculation (hpi) with Bgt isolate E09.

Table S3 Genes upregulated in the TaHAG1-OE lines compared with wild-type plants at each time point.

Table S4 Genes upregulated in the TaHAG1-OE lines post-inoculation with Bgt isolate E09 at all time points.

Table S5 Genes upregulated in the TaHAG1-OE lines compared with wild-type plants and induced by Bgt E09 infection.

Table S6 TaHAG1-interacting protein candidates identified using yeast-two-hybrid assay.

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