The Crop Journal xxx (xxxx) xxx

Contents lists available at ScienceDirect

The Crop Journal

journal homepage: www.keaipublishing.com/en/journals/the-crop-journal/

*Hv*WRKY2 acts as an immunity suppressor and targets *HvCEBiP* to regulate powdery mildew resistance in barley

Deshui Yu [a,b,c,*], Renchun Fan [b], Ling Zhang [b], Pengya Xue [b,c], Libing Liao [a], Meizhen Hu [b], Yanjun Cheng [b], Jine Li [b,c], Ting Qi [b], Shaojuan Jing [b], Qiuyun Wang [b], Arvind Bhatt [a], Qian-Hua Shen [b,c,*]

[a] *Lushan Botanical Garden, Chinese Academy of Science, Jiujiang 332900, Jiangxi, China*
[b] *State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Innovation Academy for Seed Design, Beijing 100101, China*
[c] *CAS Center for Excellence in Biotic Interactions, University of Chinese Academy of Sciences, Beijing 100049, China*

ARTICLE INFO

Article history:
Received 29 January 2022
Revised 17 April 2022
Accepted 17 June 2022
Available online xxxx

Keywords:
*Hv*WRKY2
Basal immunity
Chromatin immunoprecipitation (ChIP)
HvCEBiP

ABSTRACT

Plants use a sophisticated immune system to perceive pathogen infection and activate immune responses in a tightly controlled manner. In barley, *Hv*WRKY2 acts as a repressor in barley disease resistance to the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*). However, the molecular features of *Hv*WRKY2 in its DNA-binding and repressor functions, as well as its target genes, are uncharacterized. We show that the W-box binding of *Hv*WRKY2 requires an intact WRKY domain and an upstream sequence of ~75 amino acids, and the *Hv*WRKY2 W-box binding activity is linked to its repressor function in disease resistance. Chromatin immunoprecipitation (ChIP)-seq analysis identified *HvCEBiP*, a putative chitin receptor gene, as a target gene of *Hv*WRKY2 in overexpressing transgenic barley plants. ChIP-qPCR and Electrophoretic Mobility Shift Assay (EMSA) verified the direct binding of *Hv*WRKY2 to a W-box-containing sequence in the *HvCEBiP* promoter. *Hv*CEBiP positively regulates resistance against *Bgh* in barley. Our findings suggest that *Hv*WRKY2 represses barley basal immunity by directly targeting pathogen-associated molecular pattern (PAMP) recognition receptor genes, suggesting that *Hv*CEBiP and likely chitin signaling function in barley PAMP-triggered immune responses to *Bgh* infection.

1. Introduction

Plants rely on a two-tiered innate immune system to protect them against pathogens [1–3]. In this immune system, pathogen or microbe-associated molecular patterns (PAMPs or MAMPs) and damage-associated molecular patterns (DAMPs) are recognized via cell surface-localized pattern-recognition receptors (PRRs) and trigger PAMP-triggered immunity (PTI) [4–7]. PRRs belong mainly to two types: receptor-like proteins (RLP) and receptor-like kinases (RLK) [2,4,6]. Successful pathogens secrete various effectors that, when delivered into host plant cells or the apoplast, disturb or suppress host immunity, thereby promoting pathogen virulence [8]. Plants have evolved numerous intracellular nucleotide-binding site/leucine-rich repeat (NLR) receptors to directly or indirectly recognize pathogen effectors and launch effector-triggered immunity (ETI), which is more potent and usually accompanied by localized host-cell death (hypersensitive response, HR) [3,9,10]. PTI and ETI can converge in signaling pathways and lead to dynamically differing but similar and interdependent immune responses [11–18].

Chitin is a major component of the fungal cell wall, and chitin elicitors serve as PAMPs to trigger immune responses in both plants and animals [19]. In plants, chitin oligomers are perceived by a plasma membrane-localized receptor complex composed of multiple lysine motif (LysM)-containing receptor-like kinases (LysM-RLKs) or receptor-like proteins (LysM-RLPs) [20]. In rice, the LysM-RLP *Os*CEBiP is the major high-affinity chitin receptor [21]. Chitin elicitation induces homodimerization of *Os*CEBiP itself and heterodimerization between *Os*CEBiP and *Os*CERK1, and *Os*CERK1 as a LysM receptor-like kinase is indispensable for intracellular chitin signaling in rice [22–25]. Barley *Hv*CEBiP, a homolog of *Os*CEBiP, is involved in barley basal defense against appressorium-mediated infection by *Magnaporthe oryzae*, and this basal defense is likely triggered by chitin oligosaccharides derived from *M. oryzae* [26].

WRKY transcription factors (TFs) constitute one of the largest TF families in plants and function in diverse developmental and

* Corresponding authors.
E-mail addresses: qhshen@genetics.ac.cn (Q.-H. Shen), ds-yu@163.com (D. Yu).

https://doi.org/10.1016/j.cj.2022.05.010

physiological processes [27–29]. WRKY TFs comprise either one or two WRKY domains of about 60 conserved amino acids that serve as the DNA binding domain, which contains a highly conserved WRKYGQK motif followed by a zinc-finger motif of C2H2 or C2HC type [30,31]. WRKY TFs are classified into groups I, II, and III and each group is further divided into subgroups according to the numbers of WRKY domains and the zinc-finger structure [31]. Previous studies have shown that WRKY TFs bind specifically to the W-box (TTGAC-C/T) DNA sequence in the promoters of target genes [30–36].

Plant WRKY transcription factors play important roles in regulating defense transcription in response to pathogen infection [27,37]. The WRKY-IIa subgroup members from *Arabidopsis*, barley, and rice act mainly as repressors of plant immunity to diverse pathogens [38–45]. The *Arabidopsis Atwrky18wrky40* double mutant showed increased resistance to *Golovinomyces orontii* and *Pseudomonas syringae* [41,42]. Resistance in *Atwrky18wrky40* plants is accompanied by massive transcriptional reprogramming and imbalance in salicylic acid (SA) and jasmonic acid (JA) signaling, altered *Enhanced Disease Susceptibility1* (*EDS1*) expression, and accumulation of the phytoalexin camalexin [40]. *At*WRKY40 binds directly to the promoter region of *EDS1*, *JAZ8*, and *RRTF1* (an AP2/ERF-type transcription factor), as demonstrated by chromatin immunoprecipitation (ChIP) [40]. Genome-wide binding analysis revealed that *At*WRKY18 and *At*WRKY40 target more than 1000 gene loci and act as negative regulators of flg22-induced PTI responses [38]. In barley, we have shown that *Hv*WRKY1/2/3, homologs of *At*WRKY18/40/60, are all repressors of barley basal immunity to powdery mildew fungus [41,46]. The *Mla* locus in barley encodes ~30 allelic CC-NB-LRR-type MLA receptors that each confer isolate-specific disease resistance against the *Bgh* fungal pathogen [41,47,48]. Upon activation, MLA receptors interact with *Hv*WRKY1/2 in the nucleus, derepressing and potentiating PAMP-triggered immunity [41]. MLA receptors also interact with *Hv*MYB6, a positive regulator of barley immunity, to stimulate the DNA-binding activity of *Hv*MYB6 and increase barley immunity to the *Bgh* fungus [49]. We have recently identified a conserved sucrose non-fermenting-related kinase 1 (SnRK1) in barley that specifically targets *Hv*WRKY3 for phosphorylation and promotes its proteasomal degradation to also derepress barley immunity against *Bgh* [46]. It is not yet fully understood how different types of TFs, including negative and positive regulators, dynamically associate with one another to regulate defense gene expressions, and whether they target the same or different genes for immune responses.

In this study, we show that the activity of *Hv*WRKY2 W-box binding requires an intact WRKY domain and the upstream sequence of ~75 amino acids, which is associated with its immune repressor function in barley resistance against the *Bgh* fungus. ChIP-seq analysis using barley transgenic plants overexpressing *Hv*WRKY2 identified a putative chitin receptor gene *HvCEBiP* as a potential target gene. ChIP-qPCR and EMSA further showed that *Hv*WRKY2 binds directly to the W-box fragment in the promoter of *HvCEBiP*. Functional analyses showed that *Hv*CEBiP positively regulates resistance against *Bgh* in barley. Our findings suggest that *Hv*WRKY2 represses barley basal immunity by directly targeting PAMP-recognition receptor genes, indicating that *Hv*CEBiP is part of PTI signaling in barley immunity to the *Bgh* fungus.

2. Materials and methods

2.1. Primers and bacterial strains

All primers used in this study were synthesized by Invitrogen, Life Technologies (Beijing, China) and are listed in Table S1. *Escherichia coli* strains Trans5a (for normal vectors) and TransDB3.1 (for constructs harboring the *ccdB* gene) competent cells were purchased from TransGen Biotech (Beijing, China).

2.2. HvWRKY2-3×HA overexpressing transgenic barley plant generation and analysis

The *HvWRKY2-3×HA* overexpression vector was generated using overhanging primers (Yu01:Yu03, Yu01:Yu04, Yu01:Yu05, and Yu01:Yu06) in four cycles of PCR, fusing the 3×HA epitope-tag codon sequence and *att*B recombination sites with *HvWRKY2*, and then cloning it into pWBvec8 + Ga-b (with the pUbi promoter) using Gateway technique (Invitrogen). *HvWRKY2-3×HA* overexpressing transgenic plants were generated by *Agrobacterium*-mediated barley transformation following a previously developed protocol [50], using immature embryos of Golden Promise (GP) barley.

Southern blotting was used to detect the insert gene copy number in genomic DNA from transgenic barley plants. About 5 g of barley leaves from each sample were harvested for isolation of genomic DNA, which was digested with *Hin*dIII or *Xho*I (NEB). The digested DNA was then separated by electrophoresis in 0.8% (w/v) agarose gel, transferred onto a positively charged nylon membrane, and kept in an oven at 80 °C for 2 h to crosslink DNA to the membrane. The membrane was then hybridized with probes. p32-dCTP labeled probes were prepared using a Prime-a-Gene Labeling System (Promega Catalog number: U1100) with a hygromycin resistance gene in plasmid pWBvec8 + Ga-b used as a template, and following the product manual. The hybridized probe DNA was exposed to a phosphor screen and the images were developed by Typhoon trio (GE Healthcare).

Western blotting was performed for *Hv*WRKY2-3×HA fusion protein accumulation analysis in barley transgenic plants. Leaf samples were collected 4 h post infection (hpi) with powdery mildew fungus *Bgh*A6, from one-week old plants or from mock-infected plants. Total protein extraction and Western blotting were performed as in our previous study [51].

Microcolony formation rate analyses of transgenic GP lines overexpressing *Hv*WRKY2-3×HA were performed as in previous studies [46,52].

2.3. Single-cell transient gene expression assay

Expression vectors used in single-cell transient gene expression assays were constructed as in previous studies [47,49], with the exception of the *HvWRKY2-3×HA* fusion gene expression vector. *HvWRKY2-3×HA* fusion gene transient expression used the same plasmid as that used in the *HvWRKY2-3×HA* overexpression stable transformation experiment. Single-cell transient gene expression assays were performed as previously described [46,47,49,53,54]. A plasmid containing a β-glucuronidase (GUS) reporter driven by the ubiquitin promoter was co-expressed with tested gene expression plasmids at a molar ratio of 1:1 and then delivered into barley leaf epidermal cells by particle bombardment (Bio-Rad, Model PDS-1000/He). The leaves were infected with the powdery mildew fungus *Bgh*A6 4 h after bombardment. Transformed cells were stained with GUS staining solution, and the fungal haustorium index was scored under a microscope 48 h after inoculation with fungal spores. Each assay was repeated three times. Transiently induced gene silencing (TIGS) assay performed as previously described [49].

2.4. Yeast one-hybrid assay

First, a 3×W-box or 3×mW-box was inserted into yeast vector pHisi-1 (using site-directed mutagenesis as in the previous study

[55], to construct pHisi-1–3×W-box (primers Yu13:Yu14) and pHisi-1–3×mW-box (primers Yu15:Yu16), followed by linearizing and recombinant into the yeast strain YM4271 genome, generating two yeast strains: YM4271: 3×W-box and YM4271: 3×mW-box. The full-length cDNA of *HvWRKY1* was subcloned into the yeast expression vector pGADT7 at *Eco*R I and *Hin*dIII cut sites, and *HvWRKY2* and the *HvWRKY2* fragments were subcloned into the yeast expression vector pGADT7 at *Eco*R I and *Xho* I sites. Yeast expression vectors pGADT7-*Hv*WRKY2^{Q193K} and pGADT7-*Hv*WRKY2$_{\Delta WRKY}$ were generated based on pGADT7-*Hv*WRKY2 using site-directed mutagenesis with primers Yu19:Yu20/Yu21: Yu22, and then transformed into the two yeast strains YM4271: 3×W-box and YM4271: 3×mW-box, respectively. The transformed yeast was grown on SD/LH$^-$ medium at 30 °C for 5 days. Binding activity was tested by growing the positive yeast transformants on SD/LH$^-$ medium containing 60 mmol L^{-1} 3-amino-1,2,4-triazole (3AT) as described previously [55].

2.5. ChIP assays

Chromatin immunoprecipitation assays were performed using *HvWRKY2-3×HA* overexpressing transgenic plants following the protocol described previously [56]. The precipitated DNA fragments were used to generate Illumina sequencing libraries following the manufacturer's instructions. Sequencing and bioinformatic analysis were performed by the DNA Sequencing and Bioinformatics Platform of Beijing Institute of Genomics, Chinese Academy of Sciences. ChIP-qPCR was performed according to a previously published article [57], using the ABI Step-One Real-Time PCR system, with the *Actin* promoter used as control.

2.6. Electrophoretic mobility shift assays

EMSAs were performed to detect the binding activity of *Hv*WRKY2 to the *CEBiP* promoter. The *GST-HvWRKY2* fusions genes were cloned into pGEX-4T-1 and the fusion protein expressed in *E. coli* (BL21) and then purified according to the manual (GST gene Fusion System Handbook, Cytiva/GE). A pair of oligonucleotides, the *CEBiP* promoter region harboring the W-box motif, and that mutated in the W-box, were synthesized and labeled with biotin at the 3′ end (or left without biotin label). Gel-shift assays were performed as previously described [58].

3. Results

3.1. W-box binding of HvWRKY2 requires an intact WRKY domain

Plant WRKY transcription factors regulate transcription by binding the W-box (TTGACC/T) *cis*-element in the promoter of the target genes [31,36,59]. To test the W-box binding activity of *Hv*WRKY1 and *Hv*WRKY2, we performed a yeast one-hybrid assay. cDNA of *HvWRKY1* and *HvWRKY2* was subcloned into the pGADT7 vector and fused in frame to an activation domain (AD), and the plasmids were transformed into yeast strain YM4271: 3×W-box or YM4271: 3×mW-box carrying a plasmid to express a *HIS3* gene driven by a promoter sequence containing the 3×W-box or 3×mW-box, respectively (Fig. 1A, top). As shown in Fig. 1A, when pGADT7-*HvWRKY1*(AD-WRKY1) or pGADT7-*HvWRKY2*(AD-WRKY2) was transformed into the yeast strain YM4271: 3×W-box, yeast cells grew well on medium lacking histidine but supplemented with 60 mmol L^{-1} of 3-AT (3-amino-1,2,4-triazole, a competitive inhibitor of *HIS3* gene expression) (Fig. 1A, upper half), whereas yeast strain YM4271: 3×mW-box carrying the mutated W-box in the *HIS3* gene promoter was unable to grow (Fig. 1A, lower half). Yeast cells transformed with the pGADT7 empty vector (EV) were unable to grow. This result confirmed that *Hv*WRKY1 and *Hv*WRKY2 bound specifically to the wild-type, but not the mutated, W-box *cis*-element.

To further identify sequences in *Hv*WRKY2 that contribute to W-box binding or interaction, a set of constructs were generated with pGADT7 to express fusions of *Hv*WRKY2 full-length or fragments or mutants for yeast one-hybrid analysis (Fig. 1B, left). The plasmid expressing fusion of wildtype *Hv*WRKY2 (1–319) enabled yeast to grow on SD media, as expected. The C-terminus deleted fragment *Hv*WRKY2 (1–242), including the WRKY domain (182–242, 60 aa), still enabled yeast growth, although weaker growth than the full-length protein (Fig. 1B, second panel). However, both the N-terminal half 1–175 fragment (without a WRKY domain) and the C-terminal half 176–319 fragments (with WRKY domain) reduced yeast growth (Fig. 1B, third and fourth panels), suggesting that the WRKY domain and some N-terminal sequence upstream of the WRKY domain are essential to W-box binding. Indeed, *Hv*WRKY2 (107–319) fragment with more sequence up to the nuclear localization signals (NLS) restored yeast growth (Fig. 1B, fifth panel), in contrast to the C-terminal half 176–319 fragment, indicating that both WRKY domain and the immediate N-terminal sequence up to the NLS are essential for W-box binding. A mutated full-length *Hv*WRKY2, m*Hv*WRKY2 (Q193K), with Gln193 mutated to Lys in the typical WRKY motif WRKYGQK, did not support yeast growth, nor did the WRKY domain-deleted mutant, Δ*Hv*WRKY2 (Fig. 1B, bottom two panels).

These results indicated that barley *Hv*WRKY1 and *Hv*WRKY2 bind to the W-box *cis*-element, and that *Hv*WRKY2 W-box binding requires an intact WRKY domain and the immediate upstream 75 amino acids with an NLS.

3.2. W-box binding activity of HvWRKY2 is associated with its function in repressing barley immunity

It was demonstrated by use of a single-cell transient gene expression assay that overexpression of *Hv*WRKY2 represses barley immunity against powdery mildew fungus [41]. We conducted a similar assay in barley line P01 by overexpressing two *Hv*WRKY2 fragments and a *Hv*WRKY2 mutant. The fragments were the 1–242 and 107–319 fragments, which conferred respectively weaker or similar W-box binding, as well as the Q193K mutant variant, which eliminated W-box binding (Figs. 2A, 1B). The *Hv*WRKY2-expressing constructs were co-delivered with a GUS reporter into barley leaf epidermal cells by particle bombardment. Following fungal spore inoculation of a compatible isolate *Bgh*A6, haustorium formation rate was scored in transformed cells as haustorium index (HI%), which represents the susceptibility level [60]. Overexpression of *Hv*WRKY2 led to almost doubled HI%: 80%, as compared to ~40% for the EV control (Fig. 2A, 1–2 column). Overexpression of *Hv*WRKY2 (1–242) and *Hv*WRKY2 (107–319) also significantly increased HI%, to ~60% and ~70%, respectively (Fig. 2A, 3–4 column). It appeared that the W-box binding activity of the fragments was associated with the immune-suppressing activity. Overexpression of the *Hv*WRKY2^{Q193K} mutant with fully eliminated W-box binding activity had no effect on fungal HI%, similar to that of the empty vector (Fig. 2A, columns 1 and 5). These data indicate that W-box binding of *Hv*WRKY2 is associated with its function in repressing barley basal immunity to the *Bgh* fungus, and that an intact WRKY domain is essential for the immune suppression function.

3.3. HvWRKY2 suppresses barley immunity in stable transgenic plants

In order to identify *Hv*WRKY2 target genes in barley, we generated barley transgenic plants overexpressing *Hv*WRKY2-3×HA fusion under the control of the maize ubiquitin promoter by

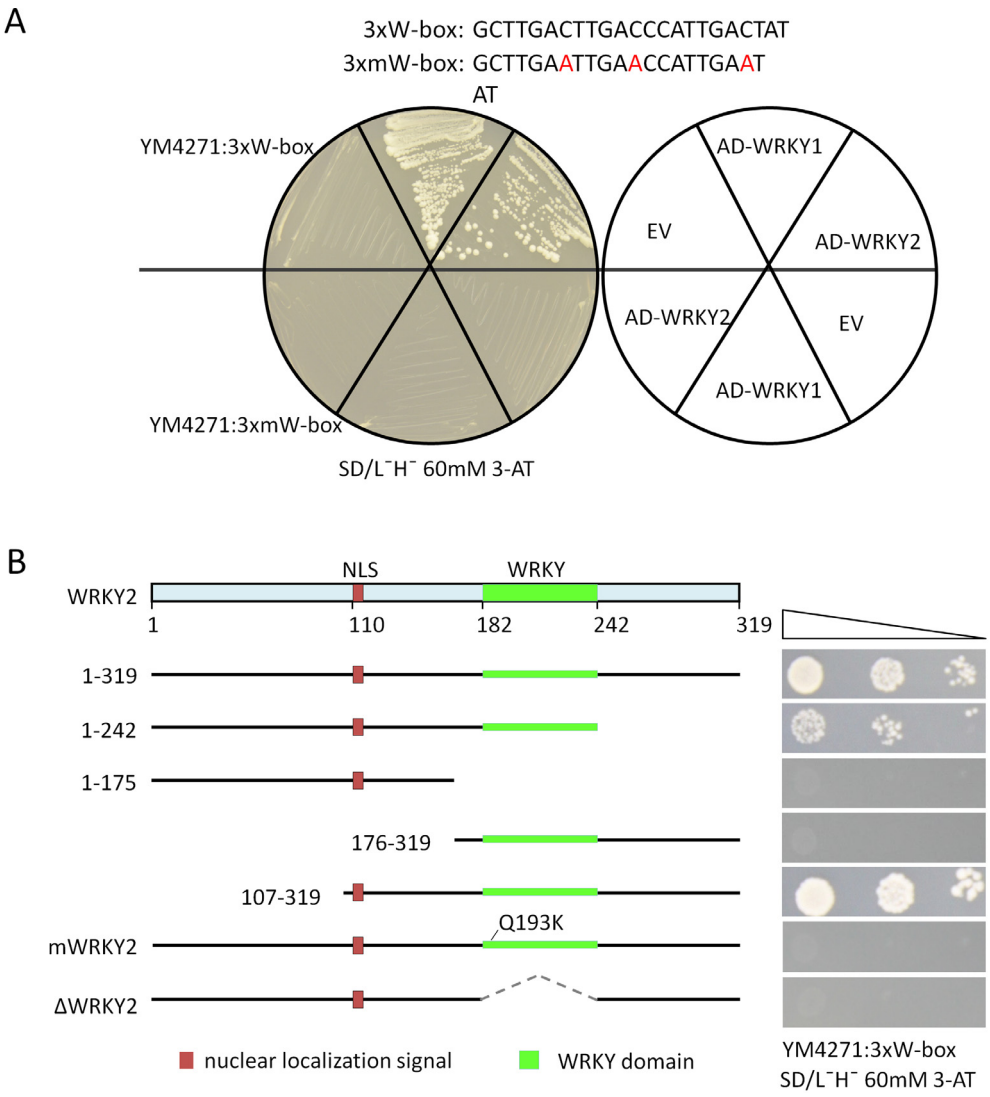

Fig. 1. *Hv*WRKY2 binds DNA with W-box element. (A) Yeast one-hybrid assay showing that barley *Hv*WRKY1 and *Hv*WRKY2 binds the 3xW-box DNA fragment. Three W-boxes and the mutated W-boxes are shown at top and mutated nucleotides are highlighted in red. Yeast strain of YM4271 containing 3×W-box was used and grown on SD media as indicated. (B) Yeast one-hybrid assay using *Hv*WRKY2 full-length, N- or C-terminal deleted fragments, and mutated *Hv*WRKY2 as effectors. pGADT7 construct expressing different *Hv*WRKY2 fragments was transformed into YM4271: 3×W-box yeast cells. A single transformant colony was inoculated in SD/L⁻H⁻ liquid culture for 24 h and then dropping transferred on SD/L⁻H⁻ plates supplemented with 60 mmol L^{-1} 3-AT after dilution to OD_{600} = 0.5 (1×), and further 10× and 100× dilution.

Agrobacterium-mediated transformation. We first investigated the function of the *Hv*WRKY2-3×HA fusion by single-cell transient gene expression assay. Transient overexpression of *Hv*WRKY2-3×HA led to significantly increased HI% in leaf epidermal cells of barley P01 as compared to EV control, and similar to the wild type *Hv*WRKY2 (Fig. 2B), indicating that the *Hv*WRKY2-3×HA fusion retained full function in repressing barley immunity. We further generated barley stable transgenic lines overexpressing the *Hv*WRKY2-3×HA fusion by *Agrobacterium*-mediated transformation, and obtained two independent transgenic lines: OE1 (*OE1-HvWRKY2-3×HA*) and OE2 (*OE2-HvWRKY2-3×HA*), each of them with a single-copy insertion, verified by Southern blotting (Fig. S1A). Highly increased accumulation of *HvWRKY2* transcripts was verified by qRT-PCR in two overexpressing transgenic lines as compared to the GP recipient (Fig. S1B). Fusion protein accumulation in healthy and *Bgh* inoculated transgenic lines was detected by Western blotting analysis (Fig. S1C). The two transgenic lines OE1 and OE2 were inoculated with the compatible isolate *Bgh*A6, and the frequency of fungal microcolonies (microcolony index, MI%) was scored at 48 h post infection. We observed significantly increased MI% by respectively ~20% and ~30% in the OE1 and OE2 lines, in comparison with the GP recipient (Fig. 2C), in agreement with the results from the transient gene expression analysis (Fig. 2B). The transgenic barley plants overexpressing *Hv*WRKY2-3×HA fusion also provide materials for further identification of potential *Hv*WRKY2 targets *in vivo*.

3.4. HvWRKY2 bound directly to the promoter of the barley HvCEBiP gene

We performed ChIP-seq to identify genome-wide binding sites of *Hv*WRKY2 in barley using OE1 transgenic plants. Leaf materials of OE1 *BghA6* infected plants were collected at 4 hpi for ChIP assay, a time point when the endogenous *HvWRKY2* gene was induced by *Bgh* infection and reached its peak point, determined by a time-course qRT-PCR analysis in compatible GP and *Bgh*A6 interaction (Fig. S2). Analysis of the ChIP-seq data indicated an enrichment of the promoter fragments of a barley chitin elicitor-binding protein (*CEBiP*) [26] (Fig. 3A, left, top panel), suggesting that *Hv*WRKY2 might bind to the promoter of the *HvCEBiP* gene. We amplified a

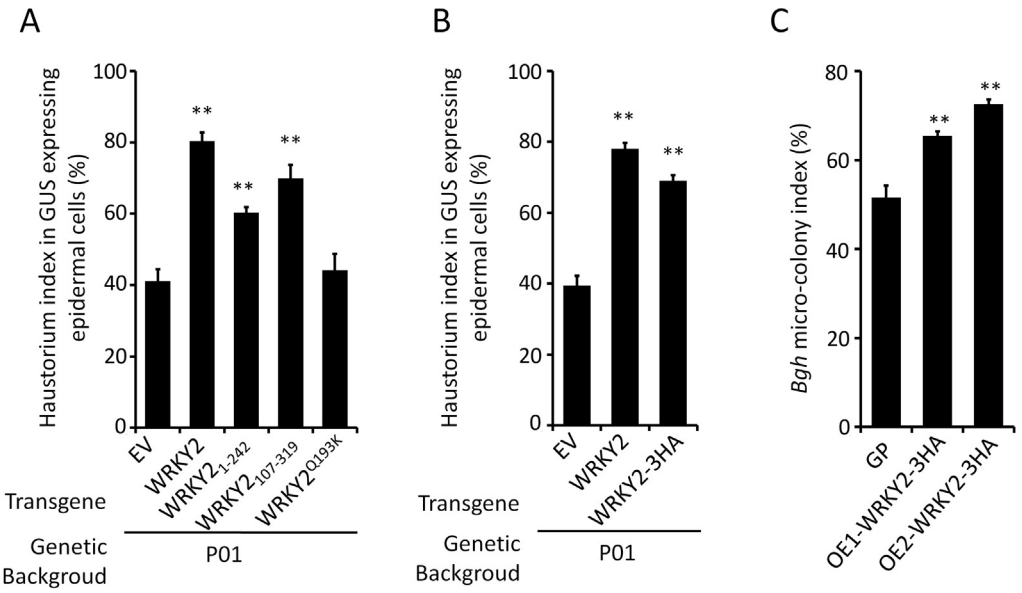

Fig. 2. *Hv*WRKY2 suppresses barley immunity to the *Bgh* fungus. (A) Single-cell transient gene expression analysis of *Hv*WRKY2 fragments and mutants. Barley leaves of P01 were bombarded with gold particles coated with DNA plasmids to express indicated *Hv*WRKY2 proteins. Relative susceptibility is shown by fungal haustorium index in leaf epidermal cells coexpressing a plasmid vector and a GUS reporter after inoculation with spores of the compatible *B. graminis* isolate *BghA6*. Fungal haustoria were microscopically scored at 48 h after inoculation. At least 50 GUS-expressing cells were examined in one experiment, and the values shown are means of three independent experiments. Student's *t*-tests were performed to calculate statistical significance (*, $P < 0.05$; **, $P < 0.01$). (B) Functional analysis of *Hv*WRKY2-3×HA fusion in transient expression assay. Experiments were performed as above (A). (C) Functional analysis of *Hv*WRKY2 fusion in stable transgenic barley plants. *Bgh* microcolony index (MI%) was compared between a Golden Promise (GP) recipient and two transgenic lines expressing *Hv*WRKY2-3×HA fusion (OE1 and OE2). Mean values of MI% were microscopically scored for at least 600 interaction sites after inoculation with *BghA6* at 48 h. Values shown are from three independent experiments. Student's *t* tests were performed to calculate statistical significance (**, $P < 0.01$).

2 kb upstream regulatory sequence of *HvCEBiP* from GP (1 kb upstream sequence of *HvCEBiP* is shown in Fig. S3), which contained two W-box/W-box-like elements at the sites of *Hv*WRKY2-3×HA enriched positions (Fig. 3A, left, bottom panel), suggesting the possibility that WRKY2 binds the promoter region of *HvCEBiP*. Indeed, ChIP-qPCR analysis resulted in a clear enrichment of a W-box-containing fragment in the *HvCEBiP* promoter by ~30-fold, as compared to the input (Fig. 3A, right).

We further performed EMSA to verify *Hv*WRKY2 binding to this fragment. Recombinant protein GST-*Hv*WRKY2 was obtained from *E. coli* and incubated with a biotin-labeled W-box-containing fragment derived from the *CEBiP* promoter (the probe). Indeed, GST-*Hv*WRKY2 formed a DNA–protein complex when the wildtype probe was used in the incubation (Fig. 3B, lane 1–2), and this signal of DNA–protein complex was eliminated by an unlabeled wild-type sequence as a competitor, but not by the mutated W-box sequences as a competitor (mCompetitor) (Fig. 3B, lane 3–4). These findings confirmed that *Hv*WRKY2 directly binds to the W-box containing region derived from the *HvCEBiP* promoter.

Together, these results suggested that *Hv*WRKY2 binds directly to the promoter region of the barley *HvCEBiP*.

3.5. HvCEBiP positively regulates barley immunity against the Bgh fungus

*Hv*CEBiP was shown to positively regulate basal immunity against *Magnaporthe oryzae* in barley [26]. We investigated the potential function of *Hv*CEBiP in barley immunity to powdery mildew using a single-cell transient gene expression assay. Overexpression of *Hv*CEBiP markedly reduced fungal HI% to ~30% in a compatible interaction, as compared to HI% of ~40% for the EV control (Fig. 4A). Moreover, we employed a transiently-induced gene silencing (TIGS) technique [61] to silence the *HvCEBiP* gene in barley leaf epidermal cells. Delivery of the *TIGS-HvCEBiP* vector to barley leaf cells led to a more than doubled fungal HI% upon inoculation with the compatible isolate *BghA6*, in comparison with the EV control (Fig. 4B).

Taken together, these findings indicated that *Hv*CEBiP positively regulates immunity against *Bgh* fungus in barley.

4. Discussion

Plants cope with various pathogen attacks using a complex immune system that is tightly controlled at transcriptional and posttranscriptional levels for transcriptional reprogramming [37,54,62–64]. WRKY TFs play a key role in plant immune regulation [27,37]. Previously, we have identified three WRKYs TFs, *Hv*WRKY1/2/3, and a R2R3 MYB transcription factor *Hv*MYB6 that play important roles in suppressing or increasing barley immunity against the powdery mildew fungus [41,46,49]. Our findings [41,49] also revealed that barley MLA immune receptors trigger ETI upon *Bgh* infection and that this involves MLA activation and interaction with *Hv*WRKY1/2 and *Hv*MYB6, but not *Hv*WRKY3, in the nucleus [41,49]. However, how these transcription factors regulate barley immunity and what the target genes are remain largely uncharacterized. In the present study, we conducted structure and functional analysis of *Hv*WRKY2, and attempted to identify potential target genes of *Hv*WRKY2. Our results reveal that the WRKY domain as well as some extra upstream sequence are essential for *Hv*WRKY2 W-box binding. We further identified *HvCEBiP*, a putative chitin receptor gene in barley, as a target gene of *Hv*WRKY2. *HvCEBiP* acts as a positive regulator in barley immunity against *Bgh* fungus. Our findings suggest that *Hv*WRKY2 binds the promoter region of a potential chitin-receptor to repress barley immunity, most likely to avoid unspecific immune gene expression and defense activation that is harmful for plant growth. The finding that *HvWRKY2* expression is induced at very early stages of *Bgh* infection is in accord with this notion (Fig. S2) [41].

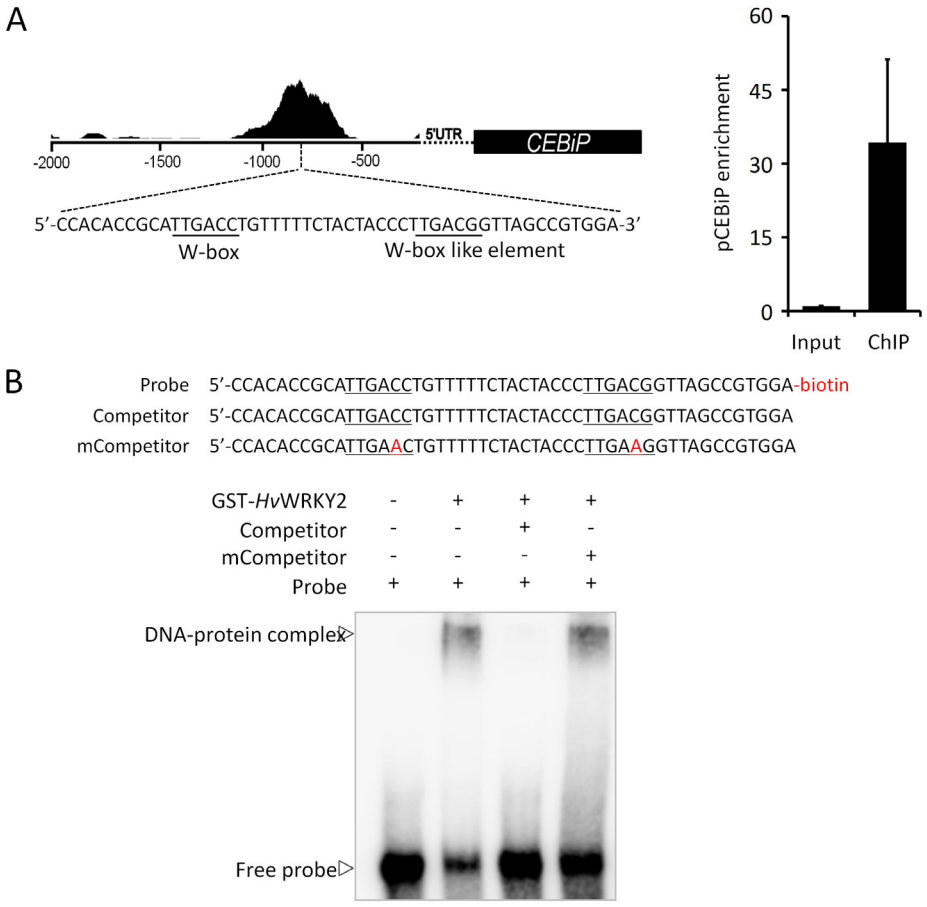

Fig. 3. *Hv*WRKY2 binds to the promoter region of *HvCEBiP*. (A) *Hv*WRKY2 binds the promoter of barley *CEBiP*. The promoter region of the barley *CEBiP* gene and the binding sites of *Hv*WRKY2 are shown schematically at top, with the specific sequence containing a W-box (TTGACC) and a W-box like element (TTGACG) at the indicated position highlighted below (left). Data were obtained and analyzed from ChIP-seq experiments showing that *Hv*WRKY2 binding sites are highly enriched around this position in the *HvCEBiP* promoter (right). For ChIP-qPCR, OE1 leaves were inoculated with *Bgh*A6 for 4 hpi. Input DNA before immunoprecipitation (Input) and coimmunoprecipitated DNA using an anti-HA (ChIP) were analyzed by qPCR employing gene-specific primer pairs and are expressed as fold enrichment relative to a promoter fragment of *HvActin*. (B) EMSA confirmed that *Hv*WRKY2 binds a W-box-containing promoter sequence of *HvCEBiP*. The probe and competitor probes containing W-box or mutated W-box are shown at top. GST-*Hv*WRKY2 fusion proteins were expressed and purified from *E. coli* and incubated with various DNA fragments. Lane 2 GST-*Hv*WRKY2 fusion proteins incubated with biotin-labeled oligo fragments of *HvCEBiP* promoter, Lanes 3 and 4 illustrate competition in the presence of unlabeled *HvCEBiP* promoter fragments and fragments mutated in W-box/W-box-like elements.

Most reported WRKY TFs regulate gene expression by binding to the W-box in the promoters of target genes. The WRKY domain, containing a highly conserved WRKYGQK core sequence motif, confers binding activity [30–32,35,36,59,65]. Alanine replacement of each of the amino acid residues in the WRKYGQK sequence reduces the W-box binding activity [32,65]. Three-dimensional structures of the carboxyl-terminal WRKY domains of several *Arabidopsis* WRKYs have strongly confirmed that the conserved WRKYGQK residues are directly involved in W-box binding [32,66–68]. However, previous studies have also shown that the N-terminal WRKY domain of group I WRKY TFs has no W-box binding activity [35,59,65,69], suggesting that the WRKY domain may not be sufficient for W-box binding. Our yeast-one hybrid results verified that both *Hv*WRKY1 and *Hv*WRKY2 bind to the W-box sequence in yeast (Fig. 1A). Further deletion and mutation analyses showed that an intact WRKY domain was indispensable for *Hv*WRKY2-W-box binding, given that either deletion of the WRKY domain (*Hv*WRKY2$_{\Delta WRKY}$ fragment) or one-amino-acid replacement in the WRKYGQK motif (m*Hv*WRKY2) resulted in loss of W-box binding (Fig. 1B). Although the *Hv*WRKY2$_{176\text{-}319}$ fragment showed no W-box binding activity, the *Hv*WRKY2$_{107\text{-}319}$ fragment restored W-box binding as well as suppression of barley immunity (Figs. 1B, 2A). Our results confirm that a conserved WRKY domain is essential but not sufficient for *in vivo* W-box binding of *Hv*WRKY2 and that the immediately upstream 75 amino acids are also essential for W-box binding and for immune suppression in barley.

Pathogen PAMP recognition by plant cell-surface localized PRRs triggers the first immune response to pathogens [1,6,70]. Chitin is one of the major components of fungal cell wall and can be hydrolyzed by plant chitinases to release chitin oligomers. Chitin oligomers are recognized as PAMP molecules by plant chitin receptors at the plasma membrane at early stages of fungal infection [20,70,71]. In rice, chitin-triggered plant immunity is conferred by two interacting proteins, *Os*CEBiP and *Os*CERK1, and intracellular downstream signaling [21,22,71–73]. *Hv*CEBiP, as a homolog of *Os*CEBiP, was reported to contribute to basal immunity against *Magnaporthe oryzae* [26]. Here we have shown that *Hv*CEBiP also positively regulates resistance against the *B. graminis* fungal pathogen. Given that chitin is a major component of the haustorial cell wall of powdery mildew fungi [74], it is reasonable to speculate that *Hv*CEBiP perceives chitin elicitors derived from the haustorial cell wall and induces defense signaling with the help of co-receptor (s) such as *Hv*CERK1. It is still unclear whether *Hv*CEBiP and *Hv*CERK1 cooperate in conferring disease resistance against fungal pathogen by forming a receptor complex. *Hv*CERK1 confers

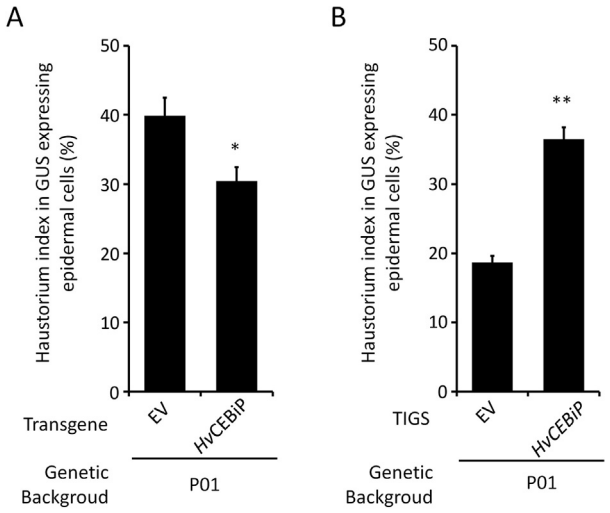

Fig. 4. *Hv*CEBiP positively regulates barley immunity against *Bgh*. (A) Single-cell transient gene expression showing that barley CEBiP positively regulates basal immunity to *Bgh* in barley. Transient *HvCEBiP* overexpression in barley leaves epidermal cells reduced haustoria formation compared with empty vector control (shown by HI% analysis). (B) Transiently induced gene silencing (TIGS) assay in barley epidermal cells showing that *HvCEBiP* is essential for basal immunity to *Bgh*. Transiently induced gene silencing of *HvCEBiP* was achieved by particle bombardment using a silencing construct harboring an antisense fragment of the *HvCEBiP* gene in barley leaf epidermal cells. Haustorial formation rate (HI%) was scored as in Fig. 2 and described in the Methods section. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

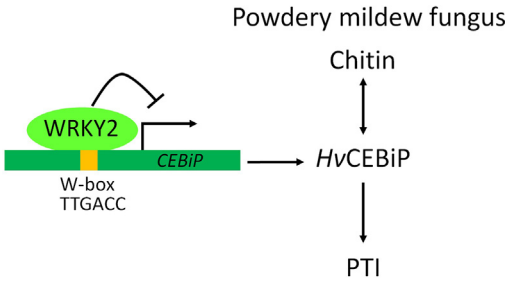

Fig. 5. Putative model of *Hv*WRKY2 suppressing barley immunity to powdery mildew fungus. When powdery mildew fungus infects barley, chitin as a PAMP molecule from the fungus is recognized by barley *Hv*CEBiP, activating PTI. *Hv*WRKY2 acts as a repressor of *Hv*CEBiP-triggered PTI by binding to the W-box element in the promoter region to suppress the transcription of *HvCEBiP*.

resistance against *Fusarium graminearum* in barley, most likely as a receptor for chitin elicitation and/or recognition [75]. This example and our findings strongly suggest that barley chitin receptor(s) function in plant perception of chitin elicitors derived from powdery mildew and other fungal pathogens. This suggestion is also supported by the identification of a lytic polysaccharide monooxygenase that is expressed in the haustorium of cucurbit powdery mildew and suppresses chitin-triggered immunity in cucurbits [76].

5. Conclusions

We have shown that the W-box binding of *Hv*WRKY2 requires an intact WRKY domain and the upstream sequence of ~75 amino acids. We identified *HvCEBiP* as a potential target gene of *Hv*WRKY2 in barley by ChIP-seq analysis, and confirmed binding of *Hv*WRKY2 to the W-box-containing sequence in the *HvCEBiP* promoter. *Hv*CEBiP positively regulates resistance against *Bgh* in barley. Based on these findings, we propose a model in which *Hv*WRKY2 negatively regulates barley basal immunity (PTI) by directly targeting PAMP-recognition receptor genes, such as *HvCEBiP*, and repressing their expression (Fig. 5).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Deshui Yu: Visualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition, Formal analysis. **Renchun Fan:** Conceptualization, Formal analysis. **Ling Zhang:** Visualization, Methodology. **Pengya Xue:** Visualization, Methodology. **Libing Liao:** Visualization, Methodology. **Meizhen Hu:** Visualization, Methodology. **Yanjun Cheng:** Visualization, Methodology. **Jine Li:** Visualization, Methodology. **Ting Qi:** Conceptualization, Formal analysis. **Shaojuan Jing:** Conceptualization, Formal analysis. **Qiuyun Wang:** Conceptualization, Formal analysis. **Arvind Bhatt:** Writing – original draft. **Qian-Hua Shen:** Conceptualization, Supervision, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Formal analysis.

Acknowledgments

This work was supported by National Key Research and Development Program of China (2018YFD1000703, 2018YFD1000700), Strategic Priority Research Program of the Chinese Academy of Sciences (XDB11020400), National Program on Research and Development of Transgenic Plants (2016ZX08009-003-001), Start-up Fund for Advanced Talents of Lushan Botanical Garden, Chinese Academy of Science (2020ZWZX03 and 2020ZWZX05), and the "Double Hundred and Double Thousand" Talent Project of Jiujiang City (jjsbsq2020026).

Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2022.05.010.

References

[1] W. Wang, B. Feng, J.M. Zhou, D. Tang, Plant immune signaling: advancing on two frontiers, J. Integr. Plant Biol. 62 (2020) 2–24.
[2] A.R. Bentham, J.C. de la Concepcion, N. Mukhi, R. Zdrzalek, M. Draeger, D. Gorenkin, R.K. Hughes, M.J. Banfield, A molecular roadmap to the plant immune system, J. Biol. Chem. 295 (2020) 14916–14935.
[3] J.D. Jones, J.L. Dangl, The plant immune system, Nature 444 (2006) 323–329.
[4] D. Tang, G. Wang, J.M. Zhou, Receptor kinases in plant-pathogen interactions: more than pattern recognition, Plant Cell 29 (2017) 618–637.
[5] C. Zipfel, Plant pattern-recognition receptors, Trends Immunol. 35 (2014) 345–351.
[6] Y. Wu, J. Zhou, Receptor-like kinases in plant innate immunity, J. Integr. Plant Biol. 55 (2013) 1271–1286.
[7] C. Zipfel, Early molecular events in PAMP-triggered immunity, Curr. Opin. Plant Biol. 12 (2009) 414–420.
[8] X.F. Xin, B. Kvitko, S.Y. He, Pseudomonas syringae: what it takes to be a pathogen, Nat. Rev. Microbiol. 16 (2018) 316–328.
[9] H. Cui, K. Tsuda, J.E. Parker, Effector-triggered immunity: from pathogen perception to robust defense, Annu. Rev. Plant Biol. 66 (2015) 487–511.
[10] S.T. Chisholm, G. Coaker, B. Day, B.J. Staskawicz, Host-microbe interactions: shaping the evolution of the plant immune response, Cell 124 (2006) 803–814.
[11] M. Yuan, B.P.M. Ngou, P. Ding, X.F. Xin, PTI-ETI crosstalk: an integrative view of plant immunity, Curr. Opin. Plant Biol. 62 (2021) 102030.
[12] D.E. Cook, C.H. Mesarich, B.P. Thomma, Understanding plant immunity as a surveillance system to detect invasion, Annu. Rev. Phytopathol. 53 (2015) 541–563.
[13] K. Tsuda, F. Katagiri, Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity, Curr. Opin. Plant Biol. 13 (2010) 459–465.

[14] M. Chang, H. Chen, F. Liu, Z.Q. Fu, PTI and ETI: convergent pathways with diverse elicitors, Trends Plant Sci. 27 (2022) 113–115.
[15] B.P.M. Ngou, H.K. Ahn, P. Ding, J.D.G. Jones, Mutual potentiation of plant immunity by cell-surface and intracellular receptors, Nature 592 (2021) 110–115.
[16] R.N. Pruitt, F. Locci, F. Wanke, L. Zhang, S.C. Saile, A. Joe, D. Karelina, C. Hua, K. Frohlich, W.L. Wan, M. Hu, S. Rao, S.C. Stolze, A. Harzen, A.A. Gust, K. Harter, M. Joosten, B. Thomma, J.M. Zhou, J.L. Dangl, D. Weigel, H. Nakagami, C. Oecking, F.E. Kasmi, J.E. Parker, T. Nurnberger, The EDS1-PAD4-ADR1 node mediates *Arabidopsis* pattern-triggered immunity, Nature 598 (2021) 495–499.
[17] H. Tian, Z. Wu, S. Chen, K. Ao, W. Huang, H. Yaghmaiean, T. Sun, F. Xu, Y. Zhang, S. Wang, X. Li, Y. Zhang, Activation of TIR signalling boosts pattern-triggered immunity, Nature 598 (2021) 500–503.
[18] Z. Wu, L. Tian, X. Liu, Y. Zhang, X. Li, TIR signal promotes interactions between lipase-like proteins and ADR1-L1 receptor and ADR1-L1 oligomerization, Plant Physiol. 187 (2021) 681–686.
[19] N. Shibuya, E. Minami, Oligosaccharide signalling for defence responses in plant, Physiol. Mol. Plant P. 59 (2001) 223–233.
[20] B.Q. Gong, F.Z. Wang, J.F. Li, Hide-and-seek: chitin-triggered plant immunity and fungal counterstrategies, Trends Plant Sci. 25 (2020) 805–816.
[21] H. Kaku, Y. Nishizawa, N. Ishii-Minami, C. Akimoto-Tomiyama, N. Dohmae, K. Takio, E. Minami, N. Shibuya, Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 11086–11091.
[22] T. Shimizu, T. Nakano, D. Takamizawa, Y. Desaki, N. Ishii-Minami, Y. Nishizawa, E. Minami, K. Okada, H. Yamane, H. Kaku, N. Shibuya, Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice, Plant J. 64 (2010) 204–214.
[23] Y. Ao, Z. Li, D. Feng, F. Xiong, J. Liu, J.F. Li, M. Wang, J. Wang, B. Liu, H.B. Wang, OsCERK1 and OsRLCK176 play important roles in peptidoglycan and chitin signaling in rice innate immunity, Plant J. 80 (2014) 1072–1084.
[24] Y. Kouzai, S. Mochizuki, K. Nakajima, Y. Desaki, M. Hayafune, H. Miyazaki, N. Yokotani, K. Ozawa, E. Minami, H. Kaku, N. Shibuya, Y. Nishizawa, Targeted gene disruption of OsCERK1 reveals its indispensable role in chitin perception and involvement in the peptidoglycan response and immunity in rice, Mol. Plant-Microbe Interact. 27 (2014) 975–982.
[25] Y. Kouzai, K. Nakajima, M. Hayafune, K. Ozawa, H. Kaku, N. Shibuya, E. Minami, Y. Nishizawa, CEBiP is the major chitin oligomer-binding protein in rice and plays a main role in the perception of chitin oligomers, Plant Mol. Biol. 84 (2014) 519–528.
[26] S. Tanaka, A. Ichikawa, K. Yamada, G. Tsuji, T. Nishiuchi, M. Mori, H. Koga, Y. Nishizawa, R. O'Connell, Y. Kubo, HvCEBiP, a gene homologous to rice chitin receptor CEBiP, contributes to basal resistance of barley to *Magnaporthe oryzae*, BMC Plant Biol. 10 (2010) 288.
[27] S.P. Pandey, I.E. Somssich, The role of WRKY transcription factors in plant immunity, Plant Physiol. 150 (2009) 1648–1655.
[28] T. Eulgem, I.E. Somssich, Networks of WRKY transcription factors in defense signaling, Curr. Opin. Plant Biol. 10 (2007) 366–371.
[29] T. Eulgem, Dissecting the WRKY web of plant defense regulators, PLoS Pathog. 2 (2006) e126.
[30] B. Ulker, I.E. Somssich, WRKY transcription factors: from DNA binding towards biological function, Curr. Opin. Plant Biol. 7 (2004) 491–498.
[31] T. Eulgem, P.J. Rushton, S. Robatzek, I.E. Somssich, The WRKY superfamily of plant transcription factors, Trends Plant Sci. 5 (2000) 199–206.
[32] Y.P. Xu, H. Xu, B. Wang, X.D. Su, Crystal structures of N-terminal WRKY transcription factors and DNA complexes, Protein Cell 11 (2020) 208–213.
[33] P.J. Rushton, I.E. Somssich, P. Ringler, Q.J. Shen, WRKY transcription factors, Trends Plant Sci. 15 (2010) 247–258.
[34] K.L. Wu, Z.J. Guo, H.H. Wang, J. Li, The WRKY family of transcription factors in rice and *Arabidopsis* and their origins, DNA Res. 12 (2005) 9–26.
[35] T. Eulgem, P.J. Rushton, E. Schmelzer, K. Hahlbrock, I.E. Somssich, Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors, EMBO J. 18 (1999) 4689–4699.
[36] P.J. Rushton, J.T. Torres, M. Parniske, P. Wernert, K. Hahlbrock, I.E. Somssich, Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley *PR1* genes, EMBO J. 15 (1996) 5690–5700.
[37] K. Tsuda, I.E. Somssich, Transcriptional networks in plant immunity, New Phytol. 206 (2015) 932–947.
[38] R.P. Birkenbihl, B. Kracher, M. Roccaro, I.E. Somssich, Induced genome-wide binding of three *Arabidopsis* WRKY transcription factors during early MAMP-triggered immunity, Plant Cell 29 (2017) 20–38.
[39] M. Schon, A. Toller, C. Diezel, C. Roth, L. Westphal, M. Wiermer, I.E. Somssich, Analyses of *wrky18 wrky40* plants reveal critical roles of SA/EDS1 signaling and indole-glucosinolate biosynthesis for *Golovinomyces orontii* resistance and a loss-of resistance towards *Pseudomonas syringae* pv. *tomato* AvrRPS4, Mol. Plant-Microbe Interact. 26 (2013) 758–767.
[40] S.P. Pandey, M. Roccaro, M. Schon, E. Logemann, I.E. Somssich, Transcriptional reprogramming regulated by *WRKY18* and *WRKY40* facilitates powdery mildew infection of *Arabidopsis*, Plant J. 64 (2010) 912–923.
[41] Q.H. Shen, Y. Saijo, S. Mauch, C. Biskup, S. Bieri, B. Keller, H. Seki, B. Ulker, I.E. Somssich, P. Schulze-Lefert, Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses, Science 315 (2007) 1098–1103.
[42] X. Xu, C. Chen, B. Fan, Z. Chen, Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors, Plant Cell 18 (2006) 1310–1326.
[43] Y. Peng, L.E. Bartley, X. Chen, C. Dardick, M. Chern, R. Ruan, P.E. Canlas, P.C. Ronald, OsWRKY62 is a negative regulator of basal and Xa21-mediated defense against *Xanthomonas oryzae* pv. *oryzae* in rice, Mol. Plant 1 (2008) 446–458.
[44] J. Liu, X. Chen, X. Liang, X. Zhou, F. Yang, J. Liu, S.Y. He, Z. Guo, Alternative splicing of rice WRKY62 and WRKY76 transcription factor genes in pathogen defense, Plant Physiol. 171 (2016) 1427–1442.
[45] T. Chujo, K. Miyamoto, T. Shimogawa, T. Shimizu, Y. Otake, N. Yokotani, Y. Nishizawa, N. Shibuya, H. Nojiri, H. Yamane, E. Minami, K. Okada, OsWRKY28, a PAMP-responsive transrepressor, negatively regulates innate immune responses in rice against rice blast fungus, Plant Mol. Biol. 82 (2013) 23–37.
[46] X. Han, L. Zhang, L. Zhao, P. Xue, T. Qi, C. Zhang, H. Yuan, L. Zhou, D. Wang, J. Qiu, Q.H. Shen, SnRK1 phosphorylates and destabilizes WRKY3 to enhance barley immunity to powdery mildew, Plant Commun. 1 (2020) 100083.
[47] S. Bai, J. Liu, C. Chang, L. Zhang, T. Maekawa, Q. Wang, W. Xiao, Y. Liu, J. Chai, F. L. Takken, P. Schulze-Lefert, Q.H. Shen, Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance, PLoS Pathog. 8 (2012) e1002752.
[48] S. Seeholzer, T. Tsuchimatsu, T. Jordan, S. Bieri, S. Pajonk, W. Yang, A. Jahoor, K. K. Shimizu, B. Keller, P. Schulze-Lefert, Diversity at the Mla powdery mildew resistance locus from cultivated barley reveals sites of positive selection, Mol. Plant-Microbe Interact. 23 (2010) 497–509.
[49] C. Chang, D. Yu, J. Jiao, S. Jing, P. Schulze-Lefert, Q.H. Shen, Barley MLA immune receptors directly interfere with antagonistically acting transcription factors to initiate disease resistance signaling, Plant Cell 25 (2013) 1158–1173.
[50] J.G. Bartlett, S.C. Alves, M. Smedley, J.W. Snape, W.A. Harwood, High-throughput *Agrobacterium*-mediated barley transformation, Plant Methods 4 (2008) 22.
[51] D. Yu, L. Liao, Y. Zhang, K. Xu, J. Zhang, K. Liu, X. Li, G. Tan, J. Zheng, Y. He, C. Xu, J. Zhao, B. Fu, J. Xie, J. Mao, C. Li, Development of a Gateway-compatible pCAMBIA binary vector for RNAi-mediated gene knockdown in plants, Plasmid 98 (2018) 52–55.
[52] J. Zhang, D. Yu, Y. Zhang, K. Liu, K. Xu, F. Zhang, J. Wang, G. Tan, X. Nie, Q. Ji, L. Zhao, C. Li, Vacuum and co-cultivation *agroinfiltration* of (germinated) seeds results in *Tobacco rattle virus* (trv) mediated whole-plant virus-induced gene silencing (vigs) in wheat and maize, Front. Plant Sci. 8 (2017) 393.
[53] T. Wang, C. Chang, C. Gu, S. Tang, Q. Xie, Q.H. Shen, An E3 ligase affects the NLR receptor stability and immunity to powdery mildew, Plant Physiol. 172 (2016) 2504–2515.
[54] J. Liu, X. Cheng, D. Liu, W. Xu, R. Wise, Q.H. Shen, The miR9863 family regulates distinct Mla alleles in barley to attenuate NLR receptor-triggered disease resistance and cell-death signaling, PLoS Genet. 10 (2014) e1004755.
[55] D. Yu, L. Liao, J. Zhang, Y. Zhang, K. Xu, K. Liu, X. Li, G. Tan, R. Chen, Y. Wang, X. Liu, X. Zhang, X. Han, Z. Wei, C. Li, A novel, easy and rapid method for constructing yeast two-hybrid vectors using In-Fusion technology, BioTechniques 64 (2018) 219–224.
[56] M. Roccaro, I.E. Somssich, Chromatin immunoprecipitation to identify global targets of WRKY transcription factor family members involved in plant immunity, Methods Mol. Biol. 712 (2011) 45–58.
[57] S. Guo, Y. Xu, H. Liu, Z. Mao, C. Zhang, Y. Ma, Q. Zhang, Z. Meng, K. Chong, The interaction between OsMADS57 and OsTB1 modulates rice tillering via *DWARF14*, Nat. Commun. 4 (2013) 1566.
[58] W. Wei, J. Huang, Y.J. Hao, H.F. Zou, H.W. Wang, J.Y. Zhao, X.Y. Liu, W.K. Zhang, B. Ma, J.S. Zhang, S.Y. Chen, Soybean GmPHD-type transcription regulators improve stress tolerance in transgenic *Arabidopsis* plants, PLoS ONE 4 (2009) e7209.
[59] S. de Pater, V. Greco, K. Pham, J. Memelink, J. Kijne, Characterization of a zinc-dependent transcriptional activator from *Arabidopsis*, Nucleic Acids Res. 24 (1996) 4624–4631.
[60] Q.H. Shen, F. Zhou, S. Bieri, T. Haizel, K. Shirasu, P. Schulze-Lefert, Recognition specificity and RAR1/SGT1 dependence in barley Mla disease resistance genes to the powdery mildew fungus, Plant Cell 15 (2003) 732–744.
[61] D. Douchkov, D. Nowara, U. Zierold, P. Schweizer, A high-throughput gene-silencing system for the functional assessment of defense-related genes in barley epidermal cells, Mol. Plant-Microbe Interact. 18 (2005) 755–761.
[62] F. Delplace, C. Huard-Chauveau, R. Berthome, D. Roby, Network organization of the plant immune system: from pathogen perception to robust defense induction, Plant J. 109 (2022) 447–470.
[63] X. Yu, B. Li, G.J. Jang, S. Jiang, D. Jiang, J.C. Jang, S.H. Wu, L. Shan, P. He, Orchestration of processing body dynamics and mRNA decay in *Arabidopsis* immunity, Cell Rep. 28 (2019) 2194–2205.
[64] C. Chang, L. Zhang, Q.H. Shen, Partitioning, repressing and derepressing: dynamic regulations in MLA immune receptor triggered defense signaling, Front. Plant Sci. 4 (2013) 396.
[65] K. Maeo, S. Hayashi, H. Kojima-Suzuki, A. Morikami, K. Nakamura, Role of conserved residues of the WRKY domain in the DNA-binding of tobacco WRKY family proteins, Biosci. Biotechnol. Biochem. 65 (2001) 2428–2436.
[66] K. Yamasaki, T. Kigawa, S. Watanabe, M. Inoue, T. Yamasaki, M. Seki, K. Shinozaki, S. Yokoyama, Structural basis for sequence-specific DNA recognition by an *Arabidopsis* WRKY transcription factor, J. Biol. Chem. 287 (2012) 7683–7691.

[67] M.R. Duan, J. Nan, Y.H. Liang, P. Mao, L. Lu, L. Li, C. Wei, L. Lai, Y. Li, X.D. Su, DNA binding mechanism revealed by high resolution crystal structure of *Arabidopsis thaliana* WRKY1 protein, Nucleic Acids Res. 35 (2007) 1145–1154.

[68] K. Yamasaki, T. Kigawa, M. Inoue, M. Tateno, T. Yamasaki, T. Yabuki, M. Aoki, E. Seki, T. Matsuda, Y. Tomo, N. Hayami, T. Terada, M. Shirouzu, A. Tanaka, M. Seki, K. Shinozaki, S. Yokoyama, Solution structure of an *Arabidopsis* WRKY DNA binding domain, Plant Cell 17 (2005) 944–956.

[69] S. Ishiguro, K. Nakamura, Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5' upstream regions of genes coding for sporamin and beta-amylase from sweet potato, Mol. Gen. Genet. 244 (1994) 563–571.

[70] Y. Kawano, K. Shimamoto, Early signaling network in rice PRR-mediated and R-mediated immunity, Curr. Opin. Plant Biol. 16 (2013) 496–504.

[71] A. Akamatsu, H.L. Wong, M. Fujiwara, J. Okuda, K. Nishide, K. Uno, K. Imai, K. Umemura, T. Kawasaki, Y. Kawano, K. Shimamoto, An OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 module is an essential early component of chitin-induced rice immunity, Cell Host Microbe 13 (2013) 465–476.

[72] K. Yamaguchi, K. Yamada, K. Ishikawa, S. Yoshimura, N. Hayashi, K. Uchihashi, N. Ishihama, M. Kishi-Kaboshi, A. Takahashi, S. Tsuge, A receptor-like cytoplasmic kinase targeted by a plant pathogen effector is directly phosphorylated by the chitin receptor and mediates rice immunity, Cell Host Microbe 13 (2013) 347–357.

[73] K. Kishimoto, Y. Kouzai, H. Kaku, N. Shibuya, E. Minami, Y. Nishizawa, Perception of the chitin oligosaccharides contributes to disease resistance to blast fungus *Magnaporthe oryzae* in rice, Plant J. 64 (2010) 343–354.

[74] C.O. Micali, U. Neumann, D. Grunewald, R. Panstruga, R. O'Connell, Biogenesis of a specialized plant-fungal interface during host cell internalization of *Golovinomyces orontii* haustoria, Cell Microbiol. 13 (2011) 210–226.

[75] S. Karre, A. Kumar, D. Dhokane, A.C. Kushalappa, Metabolo-transcriptome profiling of barley reveals induction of chitin elicitor receptor kinase gene (HvCERK1) conferring resistance against *Fusarium graminearum*, Plant Mol. Biol. 93 (2017) 247–267.

[76] A. Polonio, D. Fernandez-Ortuno, A. de Vicente, A. Perez-Garcia, A haustorial-expressed lytic polysaccharide monooxygenase from the cucurbit powdery mildew pathogen *Podosphaera xanthii* contributes to the suppression of chitin-triggered immunity, Mol. Plant Pathol. 22 (2021) 580–601.