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The bHLH transcription factor GmPIB1 facilitates resistance to *Phytophthora sojae* in *Glycine max*

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Received 13 September 2017; Editorial decision 05 March 2018; Accepted 16 March 2018

Editor: Katherine Denby, York University, UK

Abstract

*Phytophthora sojae* Kaufmann and Gerdemann causes Phytophthora root rot, a destructive soybean disease worldwide. A basic helix–loop–helix (bHLH) transcription factor is thought to be involved in the response to *P. sojae* infection in soybean, as revealed by RNA sequencing (RNA-seq). However, the molecular mechanism underlying this response is currently unclear. Here, we explored the function and underlying mechanisms of a bHLH transcription factor in soybean, designated GmPIB1 (*P. sojae*-inducible bHLH transcription factor), during host responses to *P. sojae*. GmPIB1 was significantly induced by *P. sojae* in the resistant soybean cultivar ‘L77-1863’. Analysis of transgenic soybean hairy roots with elevated or reduced expression of GmPIB1 demonstrated that GmPIB1 enhances resistance to *P. sojae* and reduces reactive oxygen species (ROS) accumulation. Quantitative reverse transcription PCR and chromatin immunoprecipitation–quantitative PCR assays revealed that GmPIB1 binds directly to the promoter of GmSPOD1 and represses its expression; this gene encodes a key enzyme in ROS production. Moreover, transgenic soybean hairy roots with GmSPOD1 silencing through RNA interference exhibited improved resistance to *P. sojae* and reduced ROS generation. These findings suggest that GmPIB1 enhances resistance to *P. sojae* by repressing the expression of GmSPOD1.

Keywords: bHLH transcription factor, *Glycine max*, *Phytophthora sojae*, root, ROS.

Introduction

Phytophthora root and stem rot caused by *Phytophthora sojae* is one of the most destructive soybean diseases worldwide, resulting in annual losses of $1–2 billion globally (Tyler, 2007). The most economical and effective way to protect soybeans against *P. sojae* infection is by breeding for dominant resistance to *P. sojae* (*Rps*) genes (Sugimoto *et al.*, 2012). However, the continuous utilization of a single *Rps* gene can result in selective pressure that promotes the evolution of more pathogenic races of *P. sojae*. Thus, a particular *Rps* gene is effective for only 8–15 years (Walker and Schmitthenner, 1984; Tooley and Grau, 1984; Sugimoto *et al.*, 2012). Moreover, some genes encode proteins that most likely function in direct protection, such as...
key enzymes for osmolyte biosynthesis, antioxidant and reactive oxygen species (ROS) scavengers, and enzymes involved in many metabolic processes (Yan et al., 2014; Cheng et al., 2015; Wang et al., 2015a; Yan et al., 2016). The products of regulatory genes, including membrane-localized receptors, calcium sensors, kinases, and transcription factors (TFs), participate in further signal transduction and the regulation of gene expression (Wang et al., 2015a). Several TF families play important roles in plant stress tolerance, such as basic helix–loop–helix (bHLH), DREB, ERF, WRKY, MYB, bZIP, and NAC TFs (Tran et al., 2004; Hu et al., 2006; Kim and Kim, 2006; Liao et al., 2008a,b; Zhou et al., 2008; Seo et al., 2010; Hao et al., 2011; Niu et al., 2012; Liu et al., 2014; Dong et al., 2015). These TFs separately or cooperatively affect the expression of various downstream genes and constitute gene networks for stress adaptation (Wang et al., 2015a).

Members of the bHLH family, which are distinguished by the bHLH domain, are universally found in eukaryotes (Doeck and Fankhauser, 2005; Liu et al., 2014). The bHLH domain consists of 50–60 amino acids with two functionally distinct regions: the basic region (containing 13–17 primarily basic amino acids for DNA binding) and the HLH region (which enables the formation of homodimers or heterodimers with one or several different partners) (Toledo-Ortiz et al., 2003; Feller et al., 2011). The bHLH TFs are involved in essential plant physiological and developmental processes by binding to E-box (CANNTG)/G-box (CACGTG) sequences in the promoters of stress-response genes (Kim and Kim, 2006; Liu et al., 2013; Liu et al., 2014). For instance, CIB1 is a bHLH TF that binds to the G-box DNA motif in vitro but heterodimerizes with other CIB1-related proteins that in turn bind to E-box sequences to regulate transcription in vivo (Liu et al., 2013). bHLH122 binds directly to the G-box/E-box cis-elements in the CYP707A3 promoter and represses its expression, and bHLH122 is strongly induced by drought, NaCl, and osmotic stress in Arabidopsis (Liu et al., 2014). Increasing evidence indicates that bHLHs regulate plant responses to biotic and abiotic stresses (Zhang et al., 2011; Liu et al., 2014; Wang et al., 2015b; Turnbull et al., 2017). For example, phytochrome-interacting factor 4 (PIF4), a nucleus-localized bHLH protein, interacts directly with brassinazole-resistant 1 (BZR1) and forms a module that integrates steroid and environmental signaling (Oh et al., 2012). Abscisic acid (ABA)-inducible bHLH TF/jasmonic acid (JA)-associated MYC2-like 1 (JAM1), a repressor of JA signaling, plays a pivotal role in the fine-tuning of JA-mediated stress responses and plant growth (Nakata et al., 2013). ABA-inducible gene (AtAIG1), encoding a bHLH-type TF in Arabidopsis, is up-regulated after exposure to ABA but not to cold or NaCl, suggesting that AtAIG1 might be involved in ABA-mediated responses (Kim and Kim, 2006). ICE1, which is constitutively expressed in Arabidopsis, encodes a bHLH TF that regulates the expression of CBF genes in response to cold stress (Chinnusamy et al., 2003; Lee et al., 2005). Overexpressing OrbHLH001 improves freezing and salt tolerance in Arabidopsis. Moreover, the Arabidopsis bHLH TF HBI1 is a negative regulator of the basal defense response. Loss-of-function of HBI1 increases resistance to bacterial infection, and constitutive overexpression of HBI1 reduces pathogen-associated molecular pattern (PAMP)-induced immune responses (Fan et al., 2014). The transient overexpression of SbCHL1 significantly increases leaf colonization of Nicotiana benthamiana by P. infestans, which is consistent with the finding that its homologs, HB11 and CIB1, are negative regulators of immunity responses (Turnbull et al., 2017). However, the potential functions of most bHLH family members in soybean are still unclear.

A bHLH TF gene was shown to be up-regulated in all 10 near-isogenic lines (NILs) examined, each with a unique Rps gene/allele, based on sequencing and comparative transcriptome analysis of the NILs and the susceptible parent ‘Williams’ pre- and post-inoculation with P. sojae (Lin et al., 2014). Therefore, in the current study, we isolated this bHLH TF gene from P. sojae-resistant soybean cultivar ‘L77–1863’, which we designated GmPIB1 (P. sojae-inducible bHLH transcription factor; Glyma.01g129700). Overexpressing GmPIB1 in transgenic soybean hairy roots increased resistance to P. sojae, whereas RNA interference (RNAi) of this gene in transgenic soybean hairy roots increased susceptibility to this pathogen. GmPIB1 bound directly to the promoter of GmSPOD1 and inhibited its expression, leading to improve resistance to P. sojae. Taken together, these results indicate that GmPIB1 facilitates the resistance response of soybean to P. sojae infection by repressing the expression of GmSPOD1.

Materials and methods

Plant material, treatments, and primers

The P. sojae-susceptible soybean cultivar ‘Williams’ (rps1b) and the resistant cultivar ‘L77–1863’ (Rps1b) (Shan et al., 2004) were used in this study. The seeds were sown in pots in a growth chamber maintained at 25 °C and 70% relative humidity with a 16 h light/8 h dark cycle. Fourteen days after planting, seedlings at the first-node stage (V1; Fehr et al., 1971) were subjected to various treatments.

For abiotic treatments, ‘L77–1863’ plants were exposed to one of three different hormones, namely, methyl jasmonate (MeJA), ethylene (ET), or salicylic acid (SA). SA (2 mM) and MeJA (100 µM) were dissolved in 0.01% Tween 20 and sprayed onto young leaves for 0, 1, 3, 6, 9, 12, or 24 h. Ethylene treatment was performed by injecting gaseous ethylene at a concentration of 200 µl l⁻¹ into a sealed Plexiglas chamber for 0, 1, 3, 6, 9, 12, or 24 h. The control leaves were sprayed with an equal volume of 0.01% (v/v) Tween 20.

For P. sojae treatment, plants of the susceptible cultivar ‘Williams’ and the resistant cultivar ‘L77–1863’ were inoculated with P. sojae race 1 (Zhang et al., 2010) zoospores as described by Ward et al. (1979). Unifoliate leaves were treated for 0, 6, 9, 12, 24, 36, 48, or 72 h. The susceptible soybean cultivar ‘Williams’ and resistant cultivar ‘L77–1863’ were obtained from the Key Laboratory of Soybean Biology at the Chinese Ministry of Education, Harbin, and used for the gene transformation experiments. All primers used for vector construction, PCR, and quantitative reverse transcription (qRT)-PCR assays for all target genes are listed in Supplementary Table S1 at JXB online.

RT-PCR and qRT-PCR analysis

Total RNA was isolated from ‘Williams’ and ‘L77–1863’ soybean leaves using Trizol reagent (Invitrogen, Shanghai, China). cDNA synthesis was conducted using an M-MLV reverse transcriptase kit (Takara, Dalian, China) according to the manufacturer’s instructions. RT-PCR was performed to analyse GmPIB1 transcript levels in ‘Williams’ and ‘L77–1863’ plants according to Zhang et al. (2012). The soybean housekeeping gene GmEF1β (GenBank accession no. NM_001248778) was used as the internal control. qRT-PCR analysis was performed to measure GmPIB1.
The coding sequence of GmPIB1 was cloned into the plant expression vector pCAMBIA3301 with a C-terminal 4×Myc fusion coding sequence of p35S:GmPIB1-Myc. Hairy roots were generated via Agrobacterium rhizogenes-mediated transformation as described by Graham et al. (2007) and Kereszt et al. (2007) with some modifications. The cotyledons were cut into rough triangles and immediately placed in Petri dishes containing 0.6% agar medium to keep them moist. The cut surface was treated with 20 µl A. rhizogenes suspension. The dishes were sealed with Parafilm and placed in an incubator at 25 °C. Transformed hairy roots were abundant along a callus ridge on the inoculated cotyledons after approximately 3 weeks. Overexpression of the target gene in transgenic hairy roots was tested via quantitative PCR (qPCR) and immunoblotting, and RNAi transgenic hairy roots were verified by qPCR and Southern blot analysis.

Promoter–GUS analysis

The 1494 bp promoter sequence of GmPIB1 was amplified using gene-specific primers GmPIB1P1 and GmPIB1P2 and cloned into the pBI121 expression vector. The GmPIB1 promoter–GUS construct was transformed into the hairy roots of L77–1863 soybean plants by A. rhizogenes-mediated transformation. When the hairy roots generated at the infection site were approximately 8 cm long, the original main roots were treated with P. sojae zoospores for 48 h, or MeJA, ET, or SA for 6 h. Soybean hairy roots transformed with empty vector (EV) were used as controls. Histochemical GUS staining was performed 3 h after treatment using GUS buffer (1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide solution in 100 mM sodium phosphate pH 7.0, 0.1 mM EDTA, 0.5 mM ferrocyanide, 0.5 mM ferricyanide, and 0.1% Triton X-100) at 37 °C overnight. GUS activity was measured as described by Jefferson et al. (1987).

Pathogen response assays of transgenic soybean hairy roots

To investigate whether GmPIB1-transformed hairy roots were resistant to pathogen infection, artificial inoculation procedures were performed as described by Ward et al. (1979). When the hairy roots generated at the infection site were approximately 8 cm long, the original main roots were incubated with P. sojae zoospores in a mist chamber at 25 °C with 100% relative humidity for 2 d. EV soybean hairy roots were used as controls. Disease symptoms on each root were observed after inoculation and photographed with a Nikon B7000 camera.

In situ ROS detection

To investigate whether GmPIB1-transformed soybean hairy roots would respond to oxidative stress, GmPIB1 transgenic and EV (control) hairy roots were treated with P. sojae zoospores for 48 h as described by Ward et al. (1979). In situ H2O2 and O2 detection were performed using diaminobenzidine (DAB) or Nitro blue tetrazolium (NBT) as described by Lu et al. (2011). Total ROS levels were measured according to the instructions supplied with the Reactive Oxygen Species Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Fluorescence was detected at 485 nm for excitation and 530 nm for emission with a fluorescence microplate reader (Bio-TEK, USA; Qian et al., 2009). Relative ROS levels, i.e. the ratio of total ROS levels in hairy roots under P. sojae zoospore versus water treatment (mock) at the same time point were measured.

Yeast two-hybrid assays

For interaction studies, full-length GmPIB1 was amplified using gene-specific primers GmPIB1YF and GmPIB1YR and cloned in the pGBK7T7 vector and pGAD7T7 vector. Fusion plasmids pGAD7T7-GmPIB1 and pGBK7T7-GmPIB1 were transformed into yeast strain Y2HGold (Clontech). After selection on SD (–Trp, –Leu) medium, the transformants were transferred to SD (–Trp, –His, –Trp, +Ade) medium to identify protein–protein interactions.
Bimolecular fluorescence complementation assays

The coding sequence of GmPIB1 was cloned into serial pSAT6 vectors encoding either N- and C-terminal-enhanced yellow fluorescent protein fragments. The resulting constructs were used for transient assays via PEG transfection of Arabidopsis protoplasts as described by Yoo et al. (2007). Transfected cells were imaged using a TCS SP2 confocal spectral microscope imaging system (Leica).

Chromatin immunoprecipitation–qPCR assays

For chromatin immunoprecipitation (ChIP)–qPCR assays, EV and p35S:GmPIB1-Myc transgenic lines were subjected to chromatin extraction and immunoprecipitation as described by Saleh et al. (2008). Briefly, soybean hairy roots were harvested for fixation. Chromatin was isolated and sonicated to generate DNA fragments with an average size of 500 bp. The soluble chromatin fragments were isolated and pre-absorbed with 30 µl Protein G Plus/Protein A Agarose Suspension (Mercck Millipore Biotechnology) to eliminate non-specific binding and immunoprecipitated by 30 µl Protein G Plus/Protein A Agarose Suspension with anti-Myc (Santa Cruz Biotechnology). The precipitated DNA was recovered and analysed by qRT-PCR with SYBR Premix ExTaq Mix (Takara Bio). The precipitated and input DNA samples were analysed by qPCR with the gene-specific primers. The data were normalized to input transcript levels and represent the means from three biological replicates.

Transient expression assay

A transient dual-luciferase assay was performed as previously described (Shang et al., 2010; Song et al., 2013). Briefly, the 1.761 kb promoter sequence of pGmSPOD1 was cloned using gene-specific primers GmSPOD1P-F/R, and inserted into the SacI and XbaI sites of the pBI121 vector (Clontech, CA, USA) after its GUS gene had been replaced with the firefly luciferase gene. The reporter construct pGmSPOD1:GUS and the effector construct p35S::GmPIB1-Myc were transformed into A. rhizogenes strain K599 and transfected into soybean hairy roots by A. rhizogenes-mediated transformation. When the hairy roots generated at the infection site were approximately 8 cm long, the original main roots were stained for GUS. The reporter construct pGmSPOD1:LUC and the effector construct p35S::GmPIB1-Myc were transformed into Agrobacterium tumefaciens strain GV3101 and transfected into healthy 21-day-old N. benthamiana tobacco leaves by agroinfiltration as described previously (Liu et al., 2012, Meng et al., 2013). The plants were incubated 3 d after infiltration, sprayed with luciferin (1 mM), and photographed with a CCD camera (Berthold Technologies) at 72 h after infiltration.

Protein extraction, immunoblotting, and Southern blotting

To analyse protein expression in transgenic plants, total proteins were extracted with protein extraction buffer (50 mM Tris–HCl at pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, and protease inhibitor cocktail (Roche)). Total proteins (200 mg) were separated by SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed using anti-Myc antibodies (Santa Cruz Biotechnology).

Southern blotting was conducted according to the modified protocol of Zhang et al. (2012), in which 20 µg of genomic DNA digested with the restriction enzyme HindIII was hybridized to a probe derived from the bar-specific fragment (354 bp).

Results

GmPIB1 expression is induced upon P. sojae infection

To evaluate whether GmPIB1 is involved in the response of soybean to P. sojae infection, we performed RT-PCR and qRT-PCR to examine the transcript levels of this gene in the susceptible soybean cultivar ‘Williams’ and the resistant cultivar ‘L77-1863’. As shown in Fig. 1A, B, the expression level of GmPIB1 was much higher in the resistant cultivar ‘L77-1863’ than in the susceptible cultivar ‘Williams’. qRT-PCR assays showed that GmPIB1 transcript levels were significantly elevated and reached a maximum level at 36 h after P. sojae treatment in ‘L77-1863’ (Fig. 1D). However, in ‘Williams’, GmPIB1 transcript levels did not increase under P. sojae treatment (Fig. 1E).

We used the 1494 bp promoter region of GmPIB1 to drive the expression of the GUS reporter gene in the pBI121 expression vector, which we transformed into ‘L77-1863’ soybean hairy roots via high-efficiency A. rhizogenes-mediated transformation as described by Graham et al. (2007) and Kereszt et al. (2007). When the hairy roots generated at the infection site were approximately 8 cm long, we subjected the original main roots to gene expression analysis and P. sojae treatment. Compared with control roots (treated with water), GmPIB1 promoter activity was highly induced in roots subjected to P. sojae treatment (Fig. 1C, D). Together, these results suggest that GmPIB1 is involved in the defense response of soybean to P. sojae.

Cloning full-length GmPIB1 cDNA

We then examined whether GmPIB1 gene and promoter sequences differ between ‘Williams’ and ‘L77-1863’. We cloned and sequenced the cDNA and promoter of GmPIB1 in ‘Williams’ and ‘L77-1863’ and found no difference in sequence between the two cultivars (data not shown). GmPIB1 encodes a deduced 151 amino acid polypeptide with a bHLH domain at amino acid positions 9–63 (see Supplementary Fig. S1A). The predicted three-dimensional model of GmPIB1 consists of two α-helices (Supplementary Fig. S1C). To further explore the evolutionary relationship among plant bHLH proteins, we constructed a phylogenetic tree using MEGA4.0 (Tamura et al., 2007) based on amino acid sequences. Sequence alignment and phylogenetic tree analysis revealed that GmPIB1 shares 65.5–95.2% identity in overall amino acid sequence with bHLH TFs from Glycine max (XP_003551597), Arachis ipaensis (XP_016186634), Theobroma cacao (XP_017974773), Vigna radiata var. radiata (XP_014491943), Vitis vinifera (XP_002268100), Gossypium arboresum (XP_017609785), and Cicer arietinum bHLH (XP_004492536) (Supplementary Fig. S1B, D).

GmPIB1 enhances resistance to P. sojae in transgenic soybean hairy roots

To examine the effect of the loss and overexpression of GmPIB1 on resistance to P. sojae, we generated GmPIB1-overexpressing (GmPIB1-OE) and GmPIB1-RNAi transgenic soybean hairy roots by high-efficiency A. rhizogenes-mediated transformation (Graham et al., 2007; Kereszt et al., 2007) in susceptible cultivar ‘Williams’ and resistant cultivar ‘L77-1863’. We examined the GmPIB1-OE transgenic hairy roots by immunoblotting (see Supplementary Fig. S2A) and qRT-PCR (Fig. 1F) and the GmPIB1-RNAi transgenic hairy roots by Southern blot
GmPIB1 enhances disease resistance

As shown in Fig. 1H, ~75% of EV (vector control) transgenic hairy roots of the susceptible cultivar ‘Williams’ inoculated with P. sojae were completely dead at 5 d of treatment, whereas only ~18% of inoculated GmPIB1-OE transgenic hairy roots were completely dead. However, ~35% of inoculated EV transgenic hairy roots of resistant cultivar ‘L77-1863’ and ~95% of inoculated GmPIB1-RNAi transgenic hairy roots were completely dead at 5 d of inoculation with P. sojae (Fig. 1I). After 2 d of incubation with P. sojae zoospores, the three GmPIB1-OE lines displayed almost no visible lesions compared with EV control roots in susceptible cultivar ‘Williams’ (Fig. 1J). By contrast, the three GmPIB1-RNAi transgenic hairy root lines exhibited enhanced wilting symptoms and chlorosis compared with EV hairy roots in resistant cultivar ‘L77-1863’ (Fig. 1K).

We also analysed the relative biomass of P. sojae in infected soybean hairy roots after 2 d of incubation with P. sojae zoospores. The biomass of P. sojae (based on the transcript level of Phytophthora sojae TEF1 (EU079791) transcript levels in infected soybean hairy roots (2 d) were plotted relative to soybean GmEF1β (NM_001248778) expression levels, as determined by qRT-PCR. The amplification of soybean GmEF1β was used as an internal control to normalize all data. The experiment was performed using three biological replicates, each with three technical replicates, and differences were statistically analysed using Student’s t-test (*P<0.05, **P<0.01). Bars indicate standard error of the mean. (This figure is available in color at JXB online.)
lines than in EV hairy roots (Fig. 1M). These results indicate that overexpressing GmPIB1 in soybean hairy roots improves resistance to P. sojae and that silencing this gene increases susceptibility to P. sojae.

GmPIB1 transcript levels under different hormone treatments

To investigate the expression pattern of GmPIB1 in response to phytohormone treatment, we performed qRT-PCR to examine GmPIB1 transcript levels in ‘L77-1863’ soybean plants. GmPIB1 expression was responsive to MeJA, ET, and SA treatment. GmPIB1 mRNA levels rapidly increased under these treatments, reaching a maximum level at 6 h after treatment, followed by a rapid decline (Fig. 2A–C). In ‘L77-1863’ plants, GmPIB1 was constitutively and highly expressed in stems, followed by roots and leaves (Fig. 2D). To elucidate the regulatory mechanism of GmPIB1 under MeJA, ET, and SA treatment, we measured GmPIB1 promoter activity in hairy roots at 6 h after treatment. GUS activity driven by the GmPIB1 promoter (pGmPIB1) was weak under control (water) conditions, but it increased approximately 8- and 2.5-fold compared with the control under MeJA and SA treatment, respectively (Fig. 2E, F). These results suggest that GmPIB1 is primarily involved in the response to MeJA treatment.

GmPIB1 is a transcriptional repressor that binds to the E-box sequence

To investigate the subcellular localization of GmPIB1, we expressed a gene construct encoding GmPIB1–humanized GFP (hGFP) fusion protein under the control of the 35S promoter in Arabidopsis protoplasts. Confocal immunofluorescence and immunoblot analysis showed that hGFP alone was uniformly distributed throughout the cell, whereas transfected cells carrying GmPIB1–hGFP fusion protein localized to the cytoplasm and nuclei (Fig. 3A, B).

To express GmPIB1 in Transetta (DE3) E. coli cells, we cloned the coding sequence of GmPIB1 into pET-29b, an expression vector with a His-tag. Upon induction by IPTG, GmPIB1 was expressed as a major soluble protein product at 1, 2, and 4 h (Supplementary Fig. S3, lanes 2, 3, and 4). The molecular mass of the purified protein was approximately 21 kDa, as revealed by SDS-PAGE (Supplementary Fig. S3, lane 5), which is consistent with its calculated molecular mass (21.33 kDa). Immunoblotting of purified recombinant GmPIB1 protein confirmed its specific immune reactivity to anti-His antibodies (Supplementary Fig. S3, lane 6).

To determine whether GmPIB1 binds to the cis-acting element of the E-box in its target promoters in vitro, we subjected purified His-tagged GmPIB1 to an EMSA with a digoxigenin–ddUTP-labeled double-stranded oligonucleotide E-box probe. The sequences of the E-box and mutared E-box (mE-box) are shown in Fig. 3C. When the E-box was used as a probe, GmPIB1 caused a mobility shift in labeled E-box probe (Fig. 3D, lane 1), which migrated more slowly than the free probe (Fig. 3D, lane 5). Furthermore, when mE-box was used in the assay, this mobility shift was not observed (Fig. 3D, lane 2). We conducted competition experiments to examine the specificity of the mobility shift. When the ratio of unlabeled-to-labeled E-box probe was 100:1, almost no labeled probe was bound (Fig. 3D, lane 4), and when 100-fold unlabeled mE-box probe was used as the competitor, no binding competition was observed (Fig. 3D, lane 3).

To investigate whether GmPIB1 is a transcriptional repressor, we performed a transactivation assay in Arabidopsis protoplasts using a reporter gene with four tandem copies of the E-box and effector plasmids with GmPIB1 (Fig. 3E). As shown in Fig. 3F, GmPIB1 appeared to repress reporter gene expression, since GUS expression was reduced to 71% of control levels in the presence of this protein. Overall, these results suggest that GmPIB1 is an E-box-specific DNA binding protein that acts as a transcriptional repressor in plant cells.

GmPIB1 can form homodimers

The bHLH TFs form homodimers or heterodimers, which is a prerequisite for DNA binding, and each partner binds to half of the DNA recognition sequence (Ma et al., 1994; Shimizu et al., 1997; Feller et al., 2011). To determine whether GmPIB1 forms homodimers in yeast cells, we fused full-length GmPIB1 to the DNA-binding domain of GAL4 (BD) (Clontech, Palo Alto, CA, USA) and subjected it to a transcriptional activation activity by growing the yeast cells on SD/-Leu/-Trp (DDO) and SD/-Ade/-His/-Leu/-Trp (QDO) media. Together with the GAL4 activation domain (AD), yeast cells carrying full-length GmPIB1 fused to the GAL4 DNA binding domain grew on DDO, but not on QDO medium (Fig. 4A). Further analysis suggested that in yeast cells carrying BD-GmPIB1 and AD-GmPIB1, the transcription of downstream reporter genes was activated, and the cells grew on QDO medium (Fig. 4A).

To further confirm the occurrence of these interactions in planta, we performed a bimolecular fluorescence complementation (BiFC) assay involving transient expression in Arabidopsis protoplasts. Co-expression of both N-terminal yellow fluorescent protein (YFP<sup>N</sup>)-tagged GmPIB1 and C-terminal YFP (YFP<sup>C</sup>)-tagged GmPIB1 resulted in significant fluorescence in the chloroplasts of Arabidopsis protoplasts (Fig. 4B). However, no fluorescence was detected in Arabidopsis protoplasts co-transformed with YFP<sup>N</sup>–GmPIB1 and YFP<sup>C</sup> or YFP<sup>C</sup>–GmPIB1 and YFP<sup>N</sup>. These results suggest that GmPIB1 interacts with itself in planta.

Expression of GmPIB1 in soybean hairy root affects ROS levels

ROS are key signaling molecules that are produced in response to biotic and abiotic stress and trigger a variety of plant defense responses (Hückelhoven and Kogel, 2003; Soosaar et al., 2005; Takabatake et al., 2007; Shetty et al., 2008; Perez and Brown, 2014). H<sub>2</sub>O<sub>2</sub> and superoxide (O<sub>2</sub><sup>-</sup>) are the primary ROS components (Mittler et al., 2004; Foyer and Shigeoka, 2011). We therefore compared ROS production in EV, GmPIB1–OE, and GmPIB1–RNAi hairy roots after P. sojae zoospore inoculation by in situ NBT staining of superoxide anions and DAB staining of H<sub>2</sub>O<sub>2</sub>. Upon infection with P. sojae zoospores, we
observed a dramatic increase in superoxide anion and H₂O₂ contents in EV hairy roots at 48 h after inoculation (Fig. 5A, B). Compared with EV hairy roots, lower levels of superoxide anion and H₂O₂ were detected in GmPIB1-OE roots, whereas higher levels were detected in GmPIB1-RNAi roots (Fig. 5A, B). We also measured relative ROS levels in EV, GmPIB1-OE, and GmPIB1-RNAi transgenic hairy roots at 0, 3, 6, 12, 24, and 48 h after incubation with P. sojae. The relative ROS levels gradually increased in EV, GmPIB1-OE, and GmPIB1-RNAi with increasing incubation time (Fig. 5C) and were significantly lower in the GmPIB1-OE lines and significantly higher in the GmPIB1-RNAi lines compared with EV hairy roots at the same time point (Fig. 5C). These results suggest that overexpressing GmPIB1 efficiently reduces ROS accumulation in soybean.

GmPIB1 represses the expression of GmSPOD1 in transgenic soybean hairy roots

To address how GmPIB1 affects ROS generation, we performed qRT-PCR in EV, GmPIB1-OE, and GmPIB1-RNAi hairy roots to measure the relative expression of genes that are known to take part in ROS production, such as the peroxidase gene GmSPOD1 (NM_001252802); the ascorbate peroxidase gene GmAPX (L10292.1); the catalase gene GmCAT (AK286272.1); the superoxide dismutase gene GmSOD (XM_003526765.3); the glutathione peroxidase gene GmGPX (XM_006600055.2); the TF genes GmNAC29 (XM_003556741), GmWRKY27 (DQ322695), and GmMYB174 (DQ822939); and the isoflavone reductase gene GmIFR (NM_001254100). SPOD1 was significantly down-regulated in GmPIB1-OE hairy roots but markedly up-regulated in the GmPIB1-RNAi lines compared with EV hairy roots at the same time point (Fig. 5C). These results suggest that overexpressing GmPIB1 efficiently reduces ROS accumulation in soybean.

Using the PLACE program (Higo et al., 1999), we detected five E-box cis-elements in the 1.761-kb region upstream of the GmSPOD1 promoter (Fig. 6B). To further determine the binding capacity of GmPIB1 to the promoter of GmSPOD1,
we performed a ChIP-qPCR assay to compare the relative enrichment of specific GmSPOD1 sequences in GmPIB1-OE and EV hairy roots using anti-Myc antibodies. GmPIB1 protein was highly enriched in the GmSPOD1 promoter d site in the GmPIB1-OE lines, whereas it was present at extremely low levels in the EV control (Fig. 6C).

To further examine the regulatory effect of GmPIB1 on the expression of its target gene, we performed transient expression assays using 1.761 kb of the GmSPOD1 promoter fused to GUS or LUC as a reporter (pGmSPOD1:GUS or pGmSPOD1:LUC). The effector construct harbored GmPIB1 expressed under the control of the 35S promoter (p35S:GmPIB1-Myc). We transformed the reporter construct (pGmSPOD1:LUC) and the effector construct (p35S:GmPIB1-Myc) into healthy N. benthamiana leaves, finding that GmPIB1 significantly repressed the expression of GmSPOD1 (Fig. 6D). When we transformed the reporter construct (pGmSPOD1:GUS) and the effector construct (p35S:GmPIB1-Myc) into soybean hairy roots, we detected GUS activity driven by the GmSPOD1 promoter (Fig. 6Ea, F), but not by p35S:Myc (Fig. 6Eb, F) or p35S:GmPIB1-Myc (Fig. 6Ec, F). GmPIB1 significantly repressed the expression of GmSPOD1 (Fig. 6Ee, F), whereas there was no change in expression when pGmSPOD1:GUS and p35S:Myc were co-transformed into hairy roots (Fig. 6Ed, F).

Taken together, these findings strongly support the idea that GmPIB1 directly inhibits the expression of the downstream GmSPOD1 gene.
We also analysed the relative biomass of *P. sojae* in infected hairy roots after 2 d of incubation with *P. sojae* zoospores. The biomass of *P. sojae* (based on *P. sojae* TEF1 [GenBank accession no. EU079791] transcript levels) was significantly (*P*<0.01) higher in the roots of *GmSPOD1-OE* plants versus the EV control (Fig. 7G). The biomass of *P. sojae* was significantly (*P*<0.01) lower in the roots of *GmSPOD1-RNAi* plants compared with EV (Fig. 7H). Finally, we measured relative ROS levels in EV and *GmSPOD1-RNAi* transgenic hairy roots at 0 and 24 h after incubation with *P. sojae*. Relative ROS levels gradually increased with increasing incubation time in both EV and *GmSPOD1-RNAi* plants (Fig. 7I). However, the relative ROS levels were significantly lower in *GmSPOD1-RNAi* roots than in EV roots at the same time point (Fig. 7I). These results indicate that repressing *GmSPOD1* expression in soybean hairy roots improves resistance to *P. sojae*.

**Discussion**

A bHLH TF gene was previously found to be up-regulated in all 10 *Rps* NILs examined under *P. sojae* treatment, as revealed by RNA-seq (Lin *et al.*, 2014). In this study, we determined that the bHLH TF designated GmPIB1 plays a crucial role in the response of soybean to *P. sojae* infection. Consistent with this finding, we found that *GmPIB1* transcript levels were much higher in the *P. sojae*-resistant soybean cultivar ‘L77-1863’ than in the susceptible cultivar ‘Williams’ (Fig. 1A, B). Under *P. sojae* treatment, *GmPIB1* was significantly up-regulated in ‘L77-1863’ but not in ‘Williams’ (Fig. 1E). We also compared the gene and promoter sequences of *GmPIB1* between ‘Williams’ and ‘L77-1863’, finding no difference. Perhaps the difference in *GmPIB1* expression levels between the two cultivars is due to differences in *Rps*-mediated defense pathways. To date, a number of genes involved in *P. sojae* infection have been identified in soybean (Xu *et al.*, 2014; Cheng *et al.*, 2015; Dong *et al.*, 2015; Fan *et al.*, 2015, 2017; Jiang *et al.*, 2015; Yan *et al.*, 2016; Jing *et al.*, 2016; Zhao *et al.*, 2017). For example, in *GmERF5*-overexpressing soybean plants, *PR10*, *PR1-1*, and *PR10-1* are up-regulated and *P. sojae* resistance is significantly enhanced compared with wild type (Dong *et al.*, 2015). *GmiFR* encodes a NAD(P)H-dependent oxidoreductase and enhances resistance to *P. sojae* when overexpressed in soybean plants (Cheng *et al.*, 2015). Moreover, *Gmbips*, which are targets of the *P. sojae* RxLR effector, negatively regulate plant defense responses against *P. sojae* infection (Jing *et al.*, 2016). Although some genes were shown to be involved in *P. sojae* responses, little is known about the biological functions of bHLH family members in soybean. To explore the molecular function of GmPIB1 in the response to *P. sojae*, we overexpressed *GmPIB1* in transgenic soybean hairy roots. These hairy roots exhibited significantly increased resistance to *P. sojae*, whereas resistance to *P. sojae* was compromised in *GmPIB1-RNAi* transgenic hairy roots compared with the control (Fig. 1H–M). These results indicate that GmPIB1 plays an important role in defense responses to *P. sojae* in soybean.

Plants encounter many environmental stresses in their natural environments and have evolved a wide range of...
mechanisms to cope with these stresses (Dixon and Paiva, 1995; Zhang et al., 2008). When plants are overcome by certain pathogens, they recruit an inducible defense system to limit further pathogen ingression. The phytohormones SA, JA, and ET play central roles in biotic stress signaling following pathogen infection (Pieterse et al., 2009; Robert-Seilaniantz et al., 2011; Sugano et al., 2013). The transcriptional cofactor NPR1 plays a key role in the SA-signaling pathway in several plant species (Vlot et al., 2009). ERF1 plays a crucial role in ET-mediated disease resistance (Berrocal-Lobo et al., 2002). ERF1 also regulates other hormone responses, particularly the JA-mediated defense response (Lorenzo et al., 2003). ET and JA mediate defense responses against pathogen attack (partly) by inducing the expression of defense genes such as PLANT DEFENSIN1.2 (PDF1.2). In the current study, we analysed the expression of GmPIB1 following various hormone treatments (Fig. 2A–C) and determined that GmPIB1 might be primarily involved in responses to MeJA treatment.

The bHLH TFs play important roles in stress responses, which they mediate by binding to the E- and G-boxes present in the promoters of stress-related genes (Qian et al., 2007; Liu et al., 2014). AtbHLH122 specifically binds the E-box of the promoter regions of CYP707A3 and represses its expression, thereby increasing ABA content to positively regulate drought, salt, and osmotic stress signaling in Arabidopsis (Liu et al., 2014). PsGBF (a bHLH-type G-box binding factor) binds to the PsCHS1 promoter and activates its expression to regulate the phenylpropanoid biosynthesis pathway in pea (Qian et al., 2007). bHLH TFs also bind to the G- or E-box DNA motif to regulate plant development (Meng et al., 2013; Liu et al., 2013). For example, GmCIB1 (for cryptochrome-interacting bHLH1) interacts with the E-box-containing promoter sequence of WRKY53b to mediate light-induced regulation of leaf senescence in soybean (Meng et al., 2013).

In the current study, we demonstrated that GmPIB1 is localized to the nucleus and cytoplasm and specifically binds to the E-box in vitro (Fig. 3A–D). We also found that GmPIB1 suppressed the basal transcription levels of a reporter gene in Arabidopsis protoplasts (Fig. 3E, F). These findings suggest that GmPIB1 acts as an E-box-mediated transcriptional repressor.

ROS such as H₂O₂ and O₂⁻ act as signaling molecules to regulate plant responses to biotic stress (Mittler et al., 2004).
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...Therefore, we measured ROS levels in GmPIB1-OE and GmPIB1-RNAi hairy roots compared with EV, as revealed by qRT-PCR. Soybean GmEF1α was used as an internal control to normalize all data. (B, C) ChIP analysis of GmPIB1 binding to the GmSPOD1 promoter in GmPIB1-Myc transgenic soybean hairy roots and EV. Chromatin from GmPIB1-Myc transgenic and EV hairy roots was immunoprecipitated with anti-Myc antibody and treated without antibodies. The precipitated chromatin fragments were analysed by qPCR using four primer sets amplifying four regions upstream of GmSPOD1 (GmSPOD1a, GmSPOD1b, GmSPOD1c, GmSPOD1d), as indicated. One-tenth of the input (without antibody precipitation) of chromatin was analysed and used as a control. Three biological replicates, each with three technical replicates, were averaged and statistically analysed using Student’s t-test (*P<0.05, **P<0.01). Bars indicate standard error of the mean. (D) GmPIB1 represses GmSPOD1 promoter activity in N. benthamiana leaves. Agrobacterium tumefaciens GV3101 strains harboring pGmSPOD1:Luc and p35S: GmPIB1 were transfected into N. benthamiana leaves. Luciferase imaging was performed 72 h after infection. (E) GmPIB1 represses GmSPOD1 promoter activity in soybean hairy roots. Agrobacterium rhizogenes K599 strains harboring p35S: GmPIB1, and pGmSPOD1:GUS were transfected into soybean hairy roots. Line 1, pGmSPOD1:GUS; line 2, p35S:Myc; line 3, p35S: GmPIB1-Myc; line 4, p35S:Myc and pGmSPOD1:GUS; line 5, p35S: GmPIB1-Myc and pGmSPOD1:GUS. (F) GUS activity analysis of GmSPOD1 promoter expression. GUS activity was measured using a 4-methylumbelliferyl-D-glucuronide assay. The x-axis numbers correspond to the numbers 1–5 in (E). The data represent the means ±SD of three independent experiments. (This figure is available in color at JXB online.)
Based on our data, we propose a model for the pathway regulating the defense response against *P. sojae* infection in soybean (Fig. 7J). According to this model, the bHLH TF GmPIB1 is a positive regulator of the response to *P. sojae* infection. During *P. sojae* infection, GmPIB1 transcription is activated and this TF binds to the promoter of *GmSPOD1*, thereby directly inhibiting its expression. Subsequently, the reduced expression of *GmSPOD1* leads to decreased intracellular ROS levels and enhanced resistance to *P. sojae* in soybean plants. Our findings provide important insights into the mechanism underlying the response of soybean to *P. sojae* infection and offer a strategy for designing and breeding *P. sojae*-resistant soybean by genetically manipulating a bHLH gene.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Nucleotide and amino acid sequences of GmPIB1 cDNA.

Fig. S2. Resistance analysis of GmPIB1 transgenic soybean hairy roots.
Fig. S3. Expression and purification of fusion protein.
Table S1. List of primers used in this study.

Acknowledgements

This work was supported by NSFC (31171577, 31671719), National Key Research and Development Program of China (2017YFD0101300), Outstanding Talents and Innovative Team of Agricultural Scientific Research, Young and Middle-aged scientific and Technological innovation leader (MOST), Academic backbone of NEAU (17XG21), Natural Science Foundation of Heilongjiang Province (JC201308, C20150101), and Changjiang Scholar Candidates Program for Provincial Universities in Heilongjiang (2013CJHB003).

Author contributions

PX, SZ, QC, and LD designed the research. QC, LD, TG, TL, NL, and LW performed the research. XC and JW analysed the data. PX, SZ, QC, and LD wrote the article.

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