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GmBTB/POZ, a novel BTB/POZ domain-containing nuclear protein, positively regulates the response of soybean to *Phytophthora sojae* infection

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SUMMARY

Phytophthora sojae is a destructive pathogen of soybean [*Glycine max* (L.) Merr.] which causes stem and root rot on soybean plants worldwide. However, the pathogenesis and molecular mechanism of plant defence responses against *P. sojae* are largely unclear. Herein, we document the underlying mechanisms and function of a novel BTB/POZ protein, GmBTB/POZ, which contains a BTB/POZ domain found in certain animal transcriptional regulators, in host soybean plants in response to *P. sojae*. It is located in the cell nucleus and is transcriptionally up-regulated by *P. sojae*. Overexpression of *GmBTB/POZ* in soybean resulted in enhanced resistance to *P. sojae*. The activities and expression levels of enzymatic superoxide dismutase (SOD) and peroxidase (POD) antioxidants were significantly higher in *GmBTB/POZ*-overexpressing (*GmBTB/POZ*-OE) transgenic soybean plants than in wild-type (WT) plants treated with sterile water or infected with *P. sojae*. The transcript levels of defence-associated genes were also higher in overexpressing plants than in WT on infection. Moreover, salicylic acid (SA) levels and the transcript levels of SA biosynthesis-related genes were markedly higher in *GmBTB/POZ*-OE transgenic soybean than in WT, but there were almost no differences in jasmonic acid (JA) levels or JA biosynthesis-related gene expression between *GmBTB/POZ*-OE and WT soybean lines. Furthermore, exogenous SA application induced the expression of *GmBTB/POZ* and inhibited the increase in *P. sojae* biomass in both WT and *GmBTB/POZ*-OE transgenic soybean plants. Taken together, these results suggest that GmBTB/POZ plays a positive role in *P. sojae* resistance and the defence response in soybean via a process that might be dependent on SA.

Keywords: BTB/POZ domain, enzymatic antioxidants, *Phytophthora sojae*, salicylic acid.

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INTRODUCTION

Plants have evolved to defend themselves against microbial pathogens using multiple defence mechanisms, including chemical or physical barriers, such as preformed antimicrobial compounds or a waxy cuticle, as well as harbouring innate plant immunity systems (Schneider, 2002). These immunity systems have great potential to protect plants from pathogen attack and to enable plants to defend themselves against widespread disease (Vidhyasekaran, 2015). This form of immune defence can be divided into two branches of response that are interconnected: pathogen/microbe-associated molecular patterns (PAMPs or MAMPs)-triggered immunity (PTI), which is based on the perception of PAMPs and effectors by cell surface-localized pattern recognition receptors (PRRs), and effector-triggered immunity (ETI), which is based on host disease resistance (R) proteins (Dangl *et al.*, 2013; Jones and Dangl, 2006). PTI and ETI are associated with the activation of common defence networks mediated by kinases, transcription factors, pathogenesis-related (PR) proteins, reactive oxygen species (ROS) and phytohormones (Baxter *et al.*, 2014; Chandran *et al.*, 2014; Cui *et al.*, 2015). Plant hormones, including salicylic acid (SA) and jasmonic acid (JA), perform a key role in plant immunity systems and major roles in the regulation of plant basal defence responses (Koornneef and Pieterse, 2008; Thaler *et al.*, 2012). SA acts as a crucial signalling element in systemic acquired resistance (SAR) signalling pathways; the SA-mediated pathway is typically activated by biotrophic pathogens (Glazebrook, 2005). Plant defence responses that depend on JA are usually induced by necrotrophic pathogens (Glazebrook, 2005). In general, the SA pathway is prioritized over the JA pathway, and crosstalk between the two signalling pathways is usually antagonistic and might represent an adaptive response in plants (Thaler *et al.*, 2012).

The BTB/POZ domain (Broad Complex, Tramtrack, Bric-a-brac/Pox virus and Zinc finger) was originally identified as a conserved motif present in *Drosophila melanogaster* bric-à-brac, tramtrack and broad complex transcriptional regulators and in

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many pox virus zinc finger proteins (Bardwell and Treisman, 1994; Koonin *et al.*, 1992; Zollman *et al.*, 1994). The BTB/POZ domain is an NH_3-terminal motif of approximately 120 amino acids that is highly conserved and hydrophobic (Oyake *et al.*, 1996). BTB/POZ proteins can also contain other types of domain in addition to the evolutionarily conserved BTB/POZ domain (Csankovszki *et al.*, 2001; David *et al.*, 1998; Stogios *et al.*, 2005). Specifically, many BTB/POZ proteins also contain one or more BTB-BACK-kelch (BBK) motifs (Stogios *et al.*, 2005) and may also contain a secondary protein domain, such as C2H2, ANKYRIN, NPH3 or MATH [meprin and TRAF (tumour necrosis factor receptor-associated factor) homology] motifs (Csankovszki *et al.*, 2001; David *et al.*, 1998; Dong *et al.*, 1996). The BTB/POZ domain is usually found as a single-copy protein domain in proteins containing only one or two other types of domain, thereby classifying them as members of the BTB-zinc finger (BTB-ZF), BTB-NPH3, MATH-BTB and BTB-BACK-PHR (BBP) families of BTB/POZ proteins (Stogios *et al.*, 2005). By contrast, the Skp1 and ElonginC BTB/POZ proteins almost exclusively consist of only the core BTB/POZ domain (Salas-Vidal *et al.*, 2005; Weber *et al.*, 2005).

The BTB/POZ domain is a widely distributed structural motif found in an array of proteins involved in various biological processes, including transcriptional and cytoskeleton regulation and the formation of voltage-gated channels (Ahmad *et al.*, 2003; Bomont *et al.*, 2000; Collins *et al.*, 2001; Ziegelbauer *et al.*, 2001). BTB/POZ proteins have been discovered in organisms from yeasts to humans (Furukawa *et al.*, 2003; Pintard *et al.*, 2004). NPR1 was the first BTB/POZ protein identified in plants: it was discovered in *Arabidopsis thaliana* and Arabidopsis *npr1* mutants were unable to respond to SA or to express PR genes induced by SA (Cao *et al.*, 1997; Delaney *et al.*, 1995). *PR-1* gene expression may also be regulated by the interaction of NPR1 and bZIP transcription factors, thereby enhancing the resistance of host plants to pathogenic microorganisms (Zhang *et al.*, 1999). Furthermore, NPR1 improves the resistance of several plant species to pathogen attack (Lin *et al.*, 2004; Makandar *et al.*, 2006; Meur *et al.*, 2008). It has been shown that a subfamily of nucleus-localized BTB/POZ proteins encoded by *LIGHT-RESPONSE BTB1* (*LRB1*) and *LRB2* in Arabidopsis strongly influences photomorphogenesis (Christians *et al.*, 2012). In barley aleurone cells, GMPOZ, which contains a BTB/POZ domain, is implicated in the regulation of hormone-responsive gene expression (Woodger *et al.*, 2004). To date, over 100 plant BTB/POZ proteins have been identified (Gingerich *et al.*, 2005, 2007). Excluding NPR1, a systematic study of the roles played by BTB/POZ proteins in disease resistance in soybean has not yet been reported.

Soybean BTB/POZ protein interacts with GmLHP1, as revealed in a bimolecular fluorescence complementation (BiFC) assay (Fig. S1, see Supporting Information), and GmLHP1 may play a crucial role in the response to *P. sojae* (Cheng *et al.*, unpublished data). In this study, we isolated the soybean *BTB/POZ* gene, encoding a protein containing a BTB/POZ domain that is found in certain animal transcriptional regulators, which was designated as *GmBTB/POZ* [GenBank accession no. XM_006578889; National Center for Biotechnology Information (NCBI) protein no. XP_006578952]. GmBTB/POZ is localized to the nucleus and fails to activate transcription in yeast cells. To further elucidate the role of GmBTB/POZ in plant defence, we investigated the effect of *GmBTB/POZ* overexpression on *Phytophthora* root rot resistance in soybean. Several physiological and biochemical analyses showed that GmBTB/POZ plays an important role in the pathogenic process of *P. sojae* in soybean by enhancing SA levels, providing evidence that GmBTB/POZ is directly involved in the defence response of soybean to *P. sojae* attack.

RESULTS

Expression of *GmBTB/POZ* is induced by *P. sojae*

We obtained the full-length cDNA sequence of *GmBTB/POZ* (GenBank accession no. XM_006578889) from total RNA isolated from soybean cultivar 'Suinong 10'. Sequence analysis suggested that the full-length *GmBTB/POZ* cDNA is 1108 bp, containing a 777-bp open reading frame (ORF), encoding a 258-amino-acid peptide (Fig. S2, see Supporting Information) with a predicted molecular mass of 29.099 kDa. Phylogenetic analysis and multiple sequence alignment demonstrated that GmBTB/POZ shares 48.99%–81.78% identity in overall amino acid sequence with its other plant species orthologues, including *Capsella rubella* CrBTB/POZ (XP_006288349.1), *Populus euphratica* PeBTB/POZ (XP_011027431.1), *Populus trichocarpa* PtBTB/POZ (XP_002300959.1), *Brassica rapa* BrBTB/POZ (XP_009134091.1), *Pyrus × bretschneideri* PbBTB/POZ (XP_009354024.1), *Cucumis sativus* CsBTB/POZ (XP_004139801.1), *Cucumis melo* CmBTB/POZ (XP_008451217.1), *Ziziphus jujuba* ZjBTB/POZ (XP_015885781.1), *Jatropha curcas* JcBTB/POZ (XP_012078336.1), *Morus notabilis* MnBTB/POZ (XP_010105442.1), *Lupinus angustifolius* LaBTB/POZ (XP_019464120.1), *Cicer arietinum* CaBTB/POZ (XP_004502546.1), *Citrus clementina* CcBTB/POZ (XP_020212618.1), *Phaseolus vulgaris* PvBTB/POZ (XP_007137484.1), *Vigna angularis* VaBTB/POZ (XP_017420040.1) and *Vigna radiata* VrBTB/POZ (XP_014498765.1) (Figs S3, S4, see Supporting Information).

To evaluate the expression profiles of *GmBTB/POZ*, we performed quantitative reverse transcription-polymerase chain reaction (RT-PCR) to examine *GmBTB/POZ* transcript levels in the soybean resistant cultivar 'Suinong 10' and the soybean susceptible cultivar 'Dongnong 50'. As shown in Fig. 1A, the expression levels of *GmBTB/POZ* in the plant tissues of Suinong 10 were much higher than those in the plant tissues of Dongnong 50. We further explored the gene expression pattern of *GmBTB/POZ* during infection with zoospores of *P. sojae*, which revealed that *GmBTB/POZ* expression was induced by *P. sojae* in the

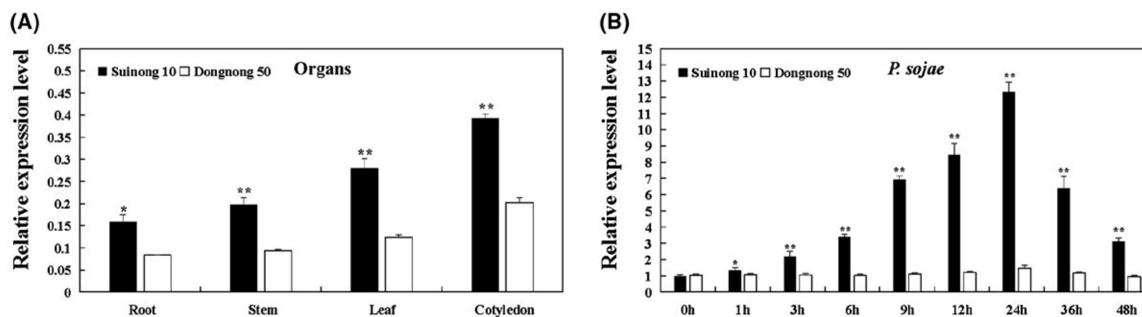

Fig. 1 Expression patterns of *GmBTB/POZ* in *Phytophthora sojae*-resistant and *P. sojae*-susceptible soybean cultivars. (A) The tissue-specific expression patterns of *GmBTB/POZ* in resistant cultivar 'Suinong 10' and susceptible cultivar 'Dongnong 50' under normal conditions. (B) Relative expression of *GmBTB/POZ* in soybean cultivars 'Suinong 10' and 'Dongnong 50' on *P. sojae* infection. The infected samples were collected at 0, 1, 3, 6, 9, 12, 24, 36 and 48 h after *P. sojae* infection. The relative expression levels of *GmBTB/POZ* were compared with those of mock-treated plants (plants treated with sterile water) at the same time point. Fourteen-day-old soybean plants were used for analysis. The housekeeping gene of soybean *GmEF1* (NM_001248778) was used as an internal control to normalize the data. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analysed using Student's *t*-test (*$P < 0.05$, **$P < 0.01$). Bars indicate the standard error of the mean.

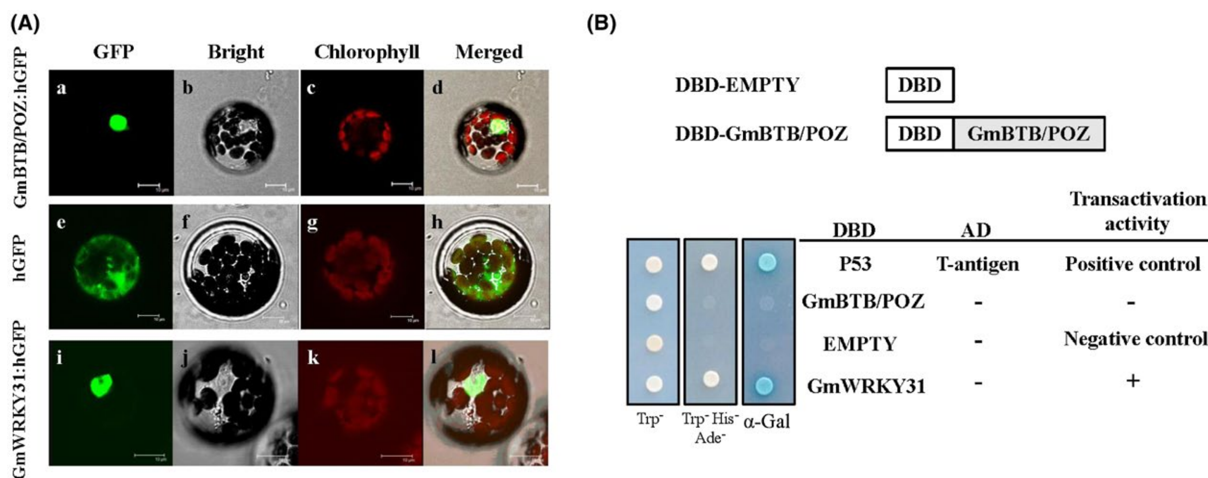

Fig. 2 Subcellular localization and transcriptional activation analysis of GmBTB/POZ. (A) Subcellular localization was investigated in Arabidopsis protoplasts via confocal microscopy. Images of bright-field (b, f, j), green fluorescent protein (GFP) fluorescence (green) only (a, e, i), chlorophyll autofluorescence (red) only (c, g, k) and their combination (d, h, l) are shown. Scale bars indicate 10 μm. (B) The open reading frame (ORF) of *GmBTB/POZ* was amplified into the pGBKT7 (GAL4 DBD) vector to generate the DBD-GmBTB/POZ constructs. The yeast strain AH109 was transformed with pGBKT7-53+pGADT7-T, pGBKT7-*GmBTB/POZ*, pGBKT7-*GmWRKY31* and pGBKT7. The transformed cells were grown on synthetic dropout medium without tryptophan [SD (-Trp)], SD medium without Trp, histidine and adenine [SD (-Trp/-His/-Ade)] and SD medium without Trp, His and Ade but with α-galactosidase [SD (-Trp/-His/-Ade/α-gal)] for 3 days at 30 °C. Transcriptional activation was monitored by the detection of yeast growth and performance of an α-Gal assay. [Colour figure can be viewed at wileyonlinelibrary.com]

plant tissues of Suinong 10, reaching a peak at 24 h, followed by a steep decline (Fig. 1B). By contrast, in the plant tissues of Dongnong 50, there was almost no change in *GmBTB/POZ* transcript abundance after treatment (Fig. 1B), demonstrating the differential expression pattern of *GmBTB/POZ* between the resistant and susceptible cultivars.

Subcellular localization and transcriptional activation ability of GmBTB/POZ

To examine the subcellular localization of GmBTB/POZ, the expression of the GmBTB/POZ-GFP fusion protein was analysed in Arabidopsis protoplasts (Fig. 2A). The green fluorescent protein (GFP) signal was observed in the entire cell of protoplasts harbouring the 35S:GFP vector alone, whereas the GmBTB/POZ-GFP green fluorescent signal was strongly displayed in the nucleus in transformed cells, similar to the signal produced by GmWRKY31-GFP, reported by Fan *et al.* (2017), clearly indicating that GmBTB/POZ is a nucleus-localized protein (Fig. 2A).

The BTB/POZ domain is a highly conserved structural motif involved in transcriptional regulation (Aravind and Koonin, 1999; Collins *et al.*, 2001; Hu *et al.*, 2010). To test the activation of the transcription function of GmBTB/POZ, we performed a transient expression assay in yeast cells using a GAL4-responsive reporter

system. As shown in Fig. 2B, transformed yeast cells harbouring DBD-P53+T-antigen (pGBKT7-53+pGADT7-T, positive control) and DBD-GmWRKY31 (pGBKT7-GmWRKY31), which exhibited transcriptional activation ability in our previous studies, grew well in synthetic dropout medium without tryptophan, histidine and adenine [SD (-Trp/-His/-Ade)] and showed α-galactosidase (α-gal) activity, whereas yeast cells containing DBD-GmBTB/POZ (pGBKT7-GmBTB/POZ) or empty (pGBKT7, negative control) exhibited no α-gal activity. These data confirm that GmBTB/POZ has no transcriptional activation activity.

GmBTB/POZ-overexpressing (GmBTB/POZ-OE) transgenic soybean plants exhibit enhanced resistance to *P. sojae*

To determine the role of GmBTB/POZ in the response to *P. sojae*, we transformed soybean plants with a construct harbouring GmBTB/POZ-Myc driven by the 35S promoter via *Agrobacterium*-mediated transformation. The expression of recombinant GmBTB/POZ-Myc protein was confirmed by immunoblotting using an anti-Myc antibody (Fig. 3B). At 96 h post-inoculation (hpi), wild-type (WT) soybean roots inoculated with *P. sojae* zoospores exhibited more serious symptoms, with watery and rotting lesions, than those of *GmBTB/POZ*-OE soybean lines (Fig. 3A). Moreover, we analysed the relative biomass of *P. sojae* in soybean roots based on the presence of the transcript level of the *P. sojae TEF1* gene (EU079791) (Fig. 3C); the results indicated that overexpression of *GmBTB/POZ* enhances the resistance to *P. sojae* after root infection. Furthermore, the cotyledons of WT inoculated with *P. sojae* zoospores exhibited more serious symptoms than those of *GmBTB/POZ*-OE soybean lines (Fig. 4A), and the lesion area of *GmBTB/POZ*-transformed soybean plants was much smaller than that of WT lines (Fig. 4B). In addition, the relative biomass of *P. sojae* was significantly lower in *GmBTB/POZ*-transformed

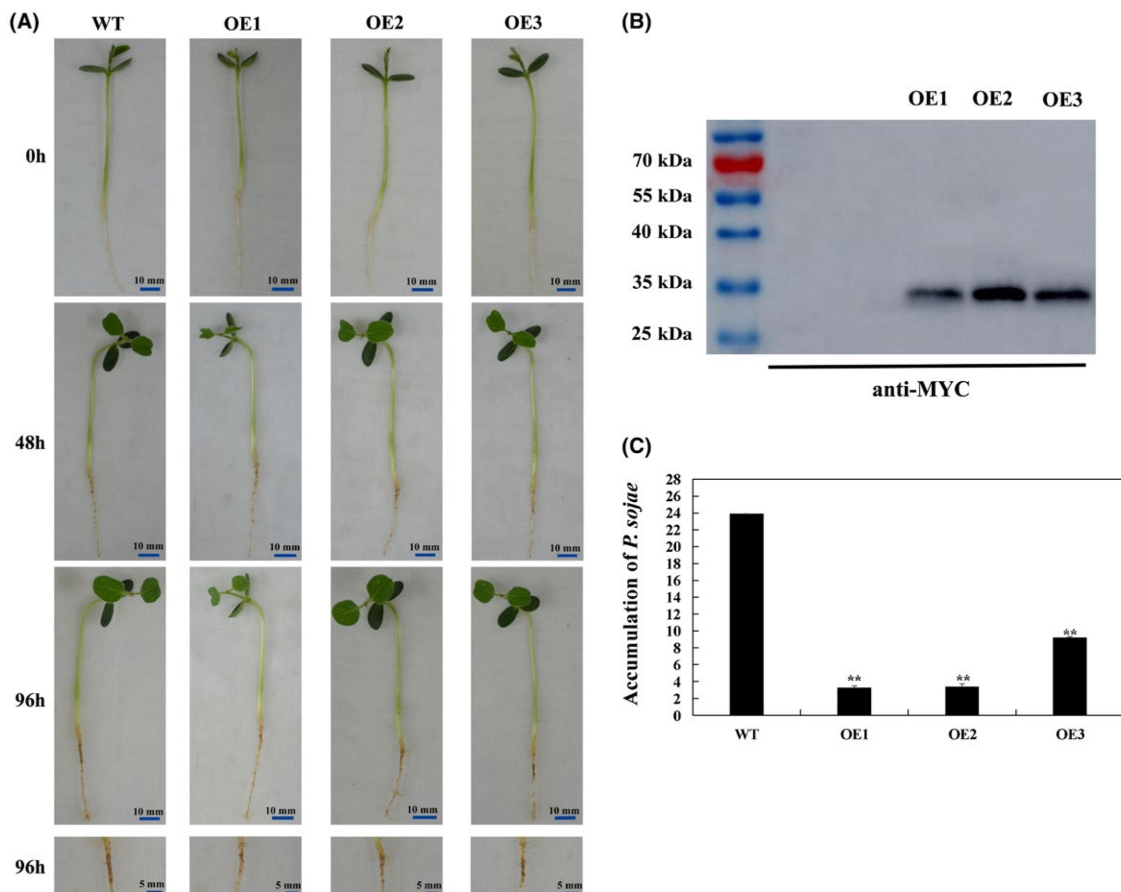

Fig. 3 Resistance analysis of *GmBTB/POZ* transgenic soybean plants. (A) Disease symptoms on the roots of *GmBTB/POZ*-overexpressing (*GmBTB/POZ*-OE) and wild-type (WT) plants at 96 h after inoculation with *Phytophthora sojae*. (B) Immunoblot analysis of the expression of GmBTB/POZ in three positive overexpressing transgenic soybean lines (OE1, OE2 and OE3). The corresponding protein bands of GmBTB/POZ-Myc (total molecular mass of 34 kDa) were detected. (C) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of the *P. sojae* relative biomass in three *GmBTB/POZ*-OE soybean plants and WT soybean plants based on the transcript level of the *P. sojae TEF1* gene (EU079791). The experiment was performed on three biological replicates, each with three technical replicates, and statistically analysed using Student's *t*-test (*$P < 0.05$, **$P < 0.01$). Bars indicate the standard error of the mean. [Colour figure can be viewed at wileyonlinelibrary.com]

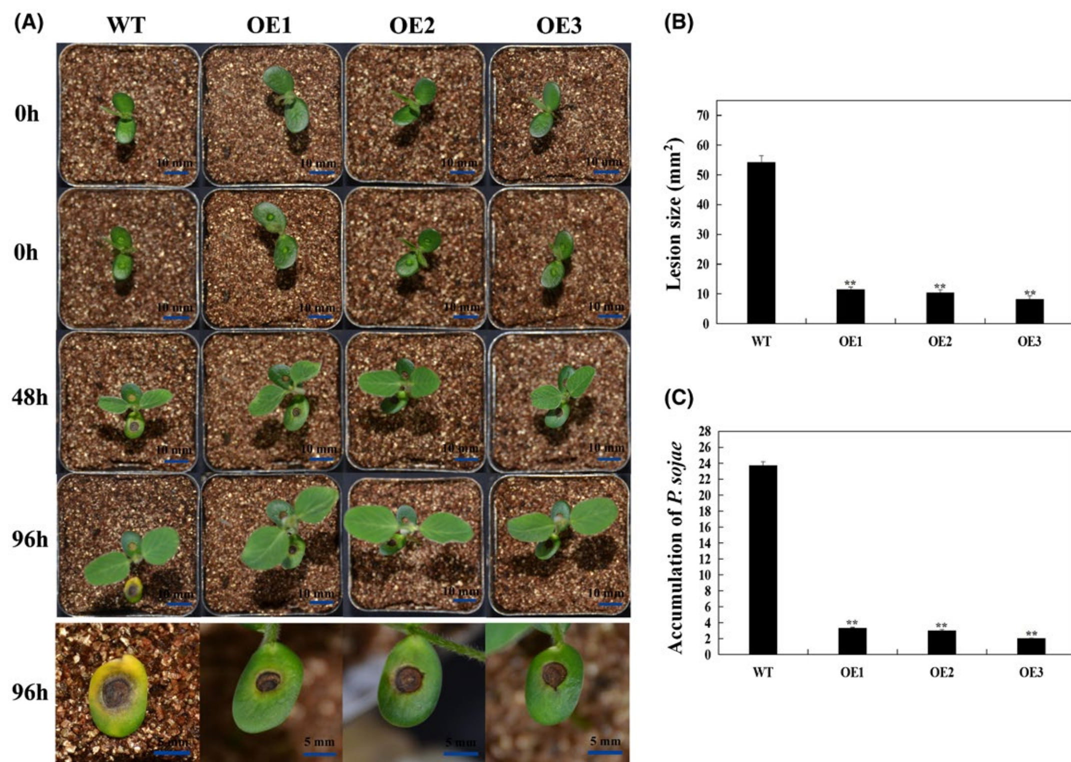

Fig. 4 GmBTB/POZ enhances resistance to *Phytophthora sojae* in transgenic soybean cotyledons. (A) Disease symptoms on living cotyledons of *GmBTB/POZ*-overexpressing (*GmBTB/POZ*-OE) and wild-type (WT) plants at 96 h after inoculation with *P. sojae*. (B) Lesion size measured from photographed cotyledons of *GmBTB/POZ*-OE transgenic and WT plants at 96 h post-inoculation (hpi). The lesion size of each independent soybean line ($n = 3$) was calculated, and the lesion sizes are shown in the columns based on a comparison with the average lesion area on WT soybean. (C) Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of the relative biomass of *P. sojae* in *GmBTB/POZ* transgenic lines and WT soybean based on *P. sojae TEF1* transcript levels. The experiment was performed on three biological replicates, each with three technical replicates, and statistically analysed using Student's *t*-test (*$P < 0.05$, **$P < 0.01$). Bars indicate the standard error of the mean. [Colour figure can be viewed at wileyonlinelibrary.com]

plants than in WT in infected living cotyledons after 96 h. The results indicate that the overexpression of *GmBTB/POZ* in soybean plants could enhance the resistance to *P. sojae* in soybean.

Soybean GmBTB/POZ-regulated defence response to *P. sojae* involves the antioxidant defence system and oxidative stress damage

Plants experience various types of environmental stress which can lead to the production of ROS. Excess ROS concentrations induce oxidative damage or apoptotic cell death (Hückelhoven and Kogel, 2003; Yu and Liu, 2003). Plant responses to pathogen attack are closely related to the formation of ROS (Nandinip *et al.,* 2008; Soosaar *et al.,* 2005). We therefore analysed the relative ROS levels in WT and *GmBTB/POZ*-OE soybean plants at 0, 12, 24 and 48 hpi. The changes in ROS levels displayed a similar tendency in WT and *GmBTB/POZ*-OE lines, whereas the relative ROS levels in *GmBTB/POZ*-OE lines were significantly lower than in WT at all time points during the incubation period (Fig. 5A). We also analysed the hydrogen peroxide (H_2O_2) contents at 24 hpi via 3,3′-diaminobenzidine (DAB) staining in soybean leaves (Fig. 5B). The amount of DAB staining was clearly lower in *GmBTB/POZ*-OE plants relative to WT. The results show that GmBTB/POZ might play a crucial role as an antioxidant in protecting soybean from *P. sojae* infection.

Plants have well-developed antioxidant defence systems that efficiently scavenge ROS, involving the antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD) (Du *et al.,* 2001). We therefore analysed SOD and POD activity, as well as the expression of *GmSOD1* (NM_001248369) and *GmPOD* (XM_006575142), in infected soybean plants. SOD and POD activity and the expression levels of related genes were much higher in *GmBTB/POZ*-OE transgenic soybean plants than in WT under both mock treatment and at 24 hpi (Fig. 6). In general, the SOD and POD activity are influenced to a certain extent by the expression levels of the corresponding enzymes (Badawi *et al.,* 2004; McKersie and Jones, 1999; Melchiorre *et al.,* 2009; Wang *et al.,* 2015). Accordingly, we deduced that the antioxidant enzymatic activities were increased because of the higher expression level of the corresponding enzymatic genes in *GmBTB/POZ*-OE soybean plants, and thus may eliminate ROS as a plant defence response to *P. sojae* infection.

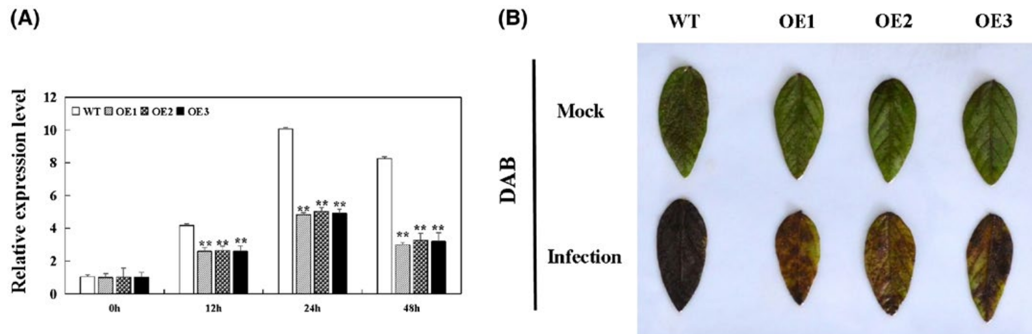

Fig. 5 Reactive oxygen species (ROS) levels and 3,3′-diaminobenzidine (DAB) staining analysis. (A) Relative expression levels of ROS in *GmBTB/POZ*-overexpressing (*GmBTB/POZ*-OE) and wild-type (WT) lines at 0, 12, 24 and 48 h post-inoculation (hpi). (B) H_2O_2 levels were detected by DAB staining in plants infected with *P. sojae* at 24 hpi. Values are relative to the value of mock-treated (treated with sterile water) plants at the same time point. The results from 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 the standard error of the mean. [Colour figure can be viewed at wileyonlinelibrary.com]

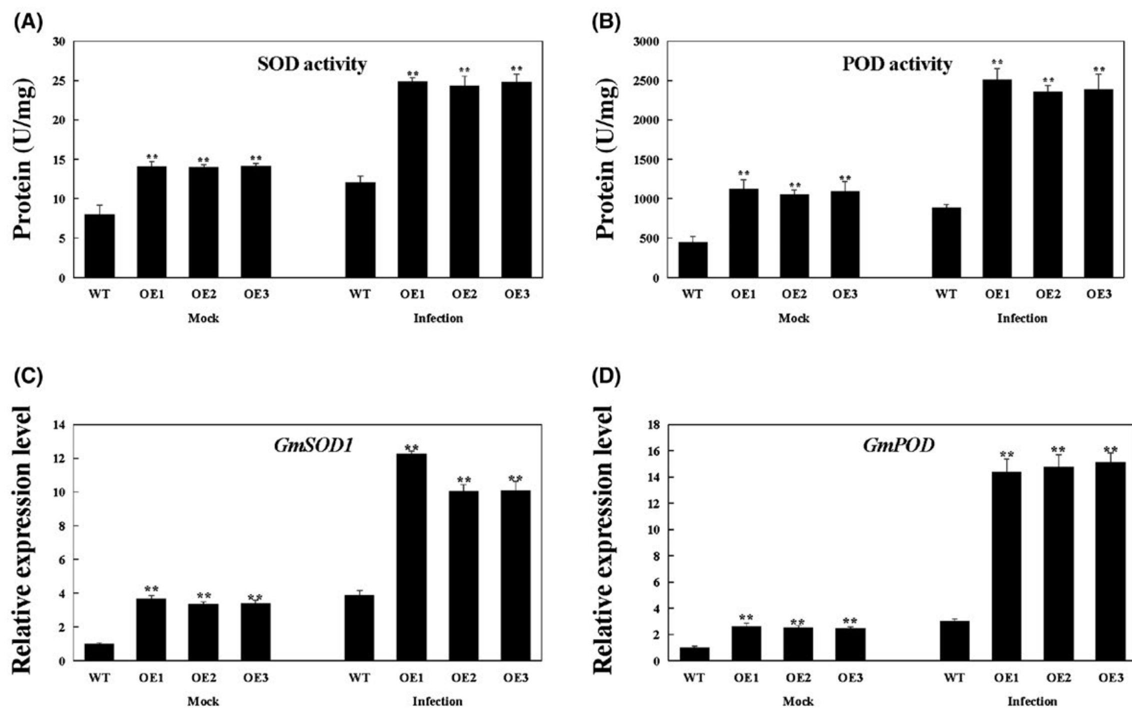

Fig. 6 Analysis of antioxidant enzyme activity (A, B) and the relative expression of genes (C, D) under mock treatment and infected by *Phytophthora sojae* at 24 h post-inoculation (hpi). The activity of the control sample [mock-treated wild-type (WT) plants] was set to unity. The experiment was performed on three biological replicates, each with three technical replicates, and statistically analysed using Student's *t*-test (*$P < 0.05$, **$P < 0.01$). Bars indicate the standard error of the mean. POD, peroxidase; SOD, superoxide dismutase.

GmBTB/POZ regulates defence-associated gene expression in response to *P. sojae* infection

To evaluate the gene expression efficiency of *GmBTB/POZ* as an indicator of the defence response to *P. sojae* infection, we monitored *GmBTB/POZ* transcript levels by quantitative RT-PCR. *GmBTB/POZ* transcript levels were significantly higher in *GmBTB/POZ*-OE plants compared with WT plants under both mock treatment and after *P. sojae* inoculation at 24 hpi (Fig. 7).

PR proteins are crucial components involved in the plant defence response to invading pathogens and can affect the defence reactions of plants (Lcvan and Eavan, 1999; Loon *et al.*, 2006). For example, MPK3 and MPK6 are two PR proteins that have been implicated to play critical roles in plant defence responses against pathogens (Colcombet and Hirt, 2008; Rasmussen *et al.*, 2012). It has been demonstrated previously that phenylalanine ammonia-lyase (PAL) positively regulates

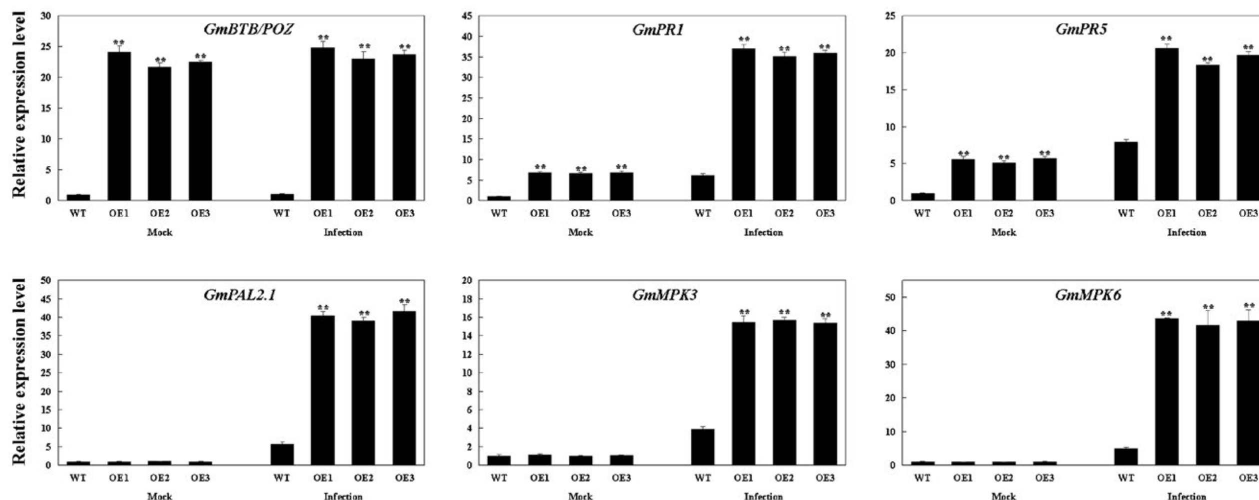

Fig. 7 Relative expression levels of defence-associated genes in soybean plants under mock treatment and infected by *Phytophthora sojae* at 24 h post-inoculation (hpi). The housekeeping gene of soybean *GmEF1* was used as an internal control to normalize the data. The expression level of the control sample [mock-treated wild-type (WT) plants] was set to unity. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analysed using Student's *t*-test ($^*P < 0.05$, $^{**}P < 0.01$). Bars indicate the standard error of the mean.

the defence response to pathogen infection in soybean (Zhang *et al.*, 2017). To investigate the potential defence mechanism of the GmBTB/POZ-regulated resistance to *P. sojae*, we examined the expression of some candidate defence-related genes, such as the PR genes *GmPR1* (AF136636) and *GmPR5* (M21297), pathogen-responsive mitogen-activated protein kinase genes *GmMPK3* (Glyma11g15700) and *GmMPK6* (Glyma02g15690), and, finally, the PAL gene *GmPAL2.1* (NM_001250027). The transcript abundances of these resistance genes were significantly higher in *GmBTB/POZ*-OE plants than in WT at 24 hpi (Fig. 7). The results suggest that GmBTB/POZ improves defence against *P. sojae* by affecting defence-related gene expression.

GmBTB/POZ is a positive regulator of SA-dependent signalling during the defence response to *P. sojae*

Notably, *GmPR1* and *GmPR5* were constitutively induced in *GmBTB/POZ*-OE soybean plants under mock treatment, indicating that the defence response of these plants was activated (Fig. 7). Increased production of SA has been associated with constitutively activated defence responses (Chen and Klessig, 1991). Furthermore, research has shown that SA and JA are functional signalling molecules for disease resistance in plants (Dong, 1998). To dissect whether the GmBTB/POZ-regulated defence response is dependent on these phytohormones, we measured both SA and JA content in *GmBTB/POZ* transgenic and WT plants. In addition, the transcript levels of *GmICS1* (XM_003522145) and *GmAOS* (NM_001249516) were measured, which play crucial roles in SA and JA biosynthesis, respectively. As shown in Fig. 8A,B, SA levels were significantly higher in *GmBTB/POZ*-OE leaves than in WT leaves, whereas there was no significant difference in JA levels between the two groups. Consistent with the SA and JA measurements, *GmICS1* transcript levels were markedly higher in the *GmBTB/POZ*-OE group than in the WT group, whereas *GmAOS* expression differed little between the *GmBTB/POZ*-OE plant group and the WT plant group (Fig. 8C,D). It is important to note that two different pathways of SA biosynthesis have been proposed in plants (Chen *et al.*, 2009): the first is the chorismate pathway controlled by isochorismate synthase (ICS) (Catinot *et al.*, 2008; Chen *et al.*, 2009; Wildermuth *et al.*, 2001), whereas the second is the PAL-controlled phenylpropanoid pathway (Huang *et al.*, 2010). The results revealed that the expression levels of *GmPAL2.1* were not constitutively induced in non-infected *GmBTB/POZ*-OE soybean plants (Fig. 7). The soybean genome contains eight GmPAL members (Rawal *et al.*, 2013; Schlueter, 2010). To determine whether there were gene expression level changes for any of the other *GmPAL* genes in the *GmBTB/POZ*-OE group, the expression levels of *GmPAL1.1* (Glyma.19G182300), *GmPAL1.2* (Glyma.03G181700), *GmPAL1.3* (Glyma.03G181600), *GmPAL2.2* (Glyma.20G180800), *GmPAL2.3* (Glyma.13G145000), *GmPAL2.4* (Glyma.10G209800) and *GmPAL3.1* (Glyma.02G309300) were also analysed. The results showed that the transcripts of *GmPAL1.1*, *GmPAL1.2* and *GmPAL1.3* changed significantly in each of the *GmBTB/POZ*-OE lines compared with WT soybean plants, whereas there were no significant differences in the expression levels for each of *GmPAL2.2*, *GmPAL2.3*, *GmPAL2.4* and *GmPAL3.1* (Fig. S5, see Supporting Information). These results indicate that GmBTB/POZ may play a role in regulating both the ICS and PAL pathways of SA accumulation.

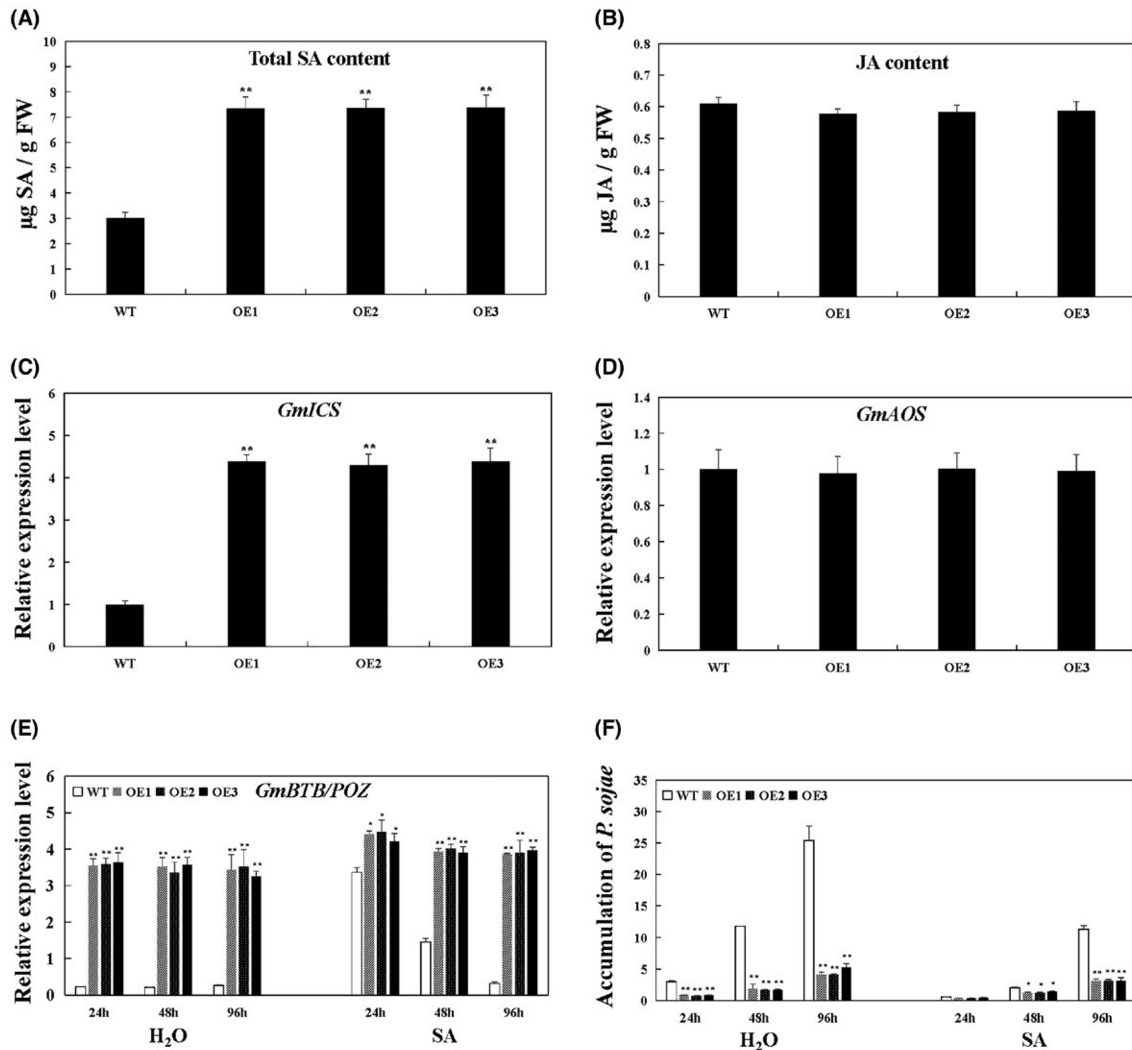

Fig. 8 Investigation of the relationship between GmBTB/POZ and the salicylic acid (SA) pathway in soybean. (A) SA contents in leaves of transgenic and wild-type (WT) soybean. FW, fresh weight. (B) Jasmonic acid (JA) contents in leaves of transgenic and WT soybean. (C) Relative transcript level of *GmICS* in *GmBTB/POZ*-overexpressing (*GmBTB/POZ*-OE) transgenic and WT soybean. The level of the control sample (WT plants) was set to unity. (D) Relative transcript level of *GmAOS* in *GmBTB/POZ*-OE transgenic and WT soybean. The level in the control sample (WT plants) was set to unity. (E) Expression patterns of *GmBTB/POZ* in WT and *GmBTB/POZ* transgenic soybean with treatment of H_2O or SA (0.5 mM). (F) Relative biomass of *Phytophthora sojae* in infected cotyledons after 24, 48 and 96 h of H_2O or SA (0.5 mM) treatment based on *P. sojae TEF1* transcript levels. The housekeeping gene of soybean *GmEF1* was used as an internal control to normalize the data. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analysed using Student's *t*-test (*$P < 0.05$, **$P < 0.01$). Bars indicate the standard error of the mean.

Pre-treatment with SA increases resistance to *P. sojae* infection in *GmBTB/POZ* transgenic soybean plants

To further confirm the relationship between GmBTB/POZ and the SA pathway in soybean, we analysed the expression of *GmBTB/POZ* in WT and *GmBTB/POZ* transgenic soybean lines treated with H_2O (control) and SA (0.5 mM). As shown in Fig. 8E, *GmBTB/POZ* transcript abundance was much higher in *GmBTB/POZ* transgenic soybean than in WT plants on treatment with H_2O at 24, 48 and 96 h. In plants on SA treatment, *GmBTB/POZ* expression was significantly up-regulated at 48 and 96 h in *GmBTB/POZ*-OE plants vs. WT. To further determine whether the GmBTB/POZ-regulated resistance response was related to SA accumulation, we tested the relative biomass of *P. sojae* in *GmBTB/POZ*-OE and WT plants with application of exogenous SA. As expected, SA-treated plants displayed clearly reduced pathogen biomass compared with H_2O-treated plants (Fig. 8F). More interestingly, the differences in the relative biomass of *P. sojae* between *GmBTB/POZ*-OE and WT lines were not obvious at 24 hpi, after which the difference became increasingly noticeable (Fig. 8F). Therefore, it is possible to speculate that *GmBTB/POZ* improves the defence

resistance to *P. sojae* probably by affecting SA accumulation. Moreover, NPR1 is the key node of the SA signalling pathway whose expression is affected by the binding of SA and can mediate the SA-dependent activation of *PR* genes (Cao *et al.,* 1997; Delaney *et al.,* 1995; Rochon *et al.,* 2006). In the present study, the transcript levels of *GmNPR1* (NM_001251745) in *GmBTB/POZ*-OE were significantly higher than those in WT plants (Fig. S5). In summary, these data suggest that GmBTB/POZ plays an integral role in the resistance of soybean to *P. sojae* via a mechanism that is mainly linked to the SA signalling pathway.

DISCUSSION

BTB/POZ proteins are involved in the modulation of various biological processes, including transcriptional regulation (Ahmad *et al.,* 2003; Melnick *et al.,* 2000), cytoskeleton regulation (Bomont *et al.,* 2000; Ziegelbauer *et al.,* 2001) and protein ubiquitination/degradation (Kobayashi *et al.,* 2004; Xu *et al.,* 2003). However, except for NPR1, the potential functions of BTB/POZ proteins in soybean in the plant response to pathogen infection have remained largely uncharacterized. In this study, we identified *GmBTB/POZ*, a novel *BTB/POZ* gene in soybean, and determined that overexpression of *GmBTB/POZ* serves to increase resistance to *P. sojae*.

Changes in gene expression induced by pathogen infection play important roles in the responses of plants to pathogens (Buscaill and Rivas, 2014; Koh *et al.,* 2005; Tsuda and Somssich, 2015). In this work, we analysed the effects of *GmBTB/POZ* expression in the leaves of *P. sojae*-resistant and *P. sojae*-susceptible soybean cultivars. We detected significant induction of *GmBTB/POZ* in 'Suinong 10', with significant differences in expression in the resistant vs. susceptible cultivar, suggesting that GmBTB/POZ plays an important role in the response of soybean to *P. sojae*. Transgenic soybean overexpressing *GmBTB/POZ* displayed significantly higher resistance to *P. sojae* compared with WT.

ROS function as signalling molecules and are involved in the regulation of many growth and developmental processes in plants and in responses to environmental cues (Hirt, 2006; Mittler, 2002). Furthermore, ROS are involved in host–pathogen interactions, including interactions with fungal and oomycete pathogens, and the scavenging of excess ROS can improve plant resistance to many pathogens (Shetty *et al.,* 2003, 2008). Thus, we tested the relative ROS levels in *GmBTB/POZ*-OE and WT lines during infection with *P. sojae*, and these levels were significantly lower in *GmBTB/POZ*-OE plants than in WT. Moreover, DAB staining showed that H_2O_2 levels in leaves when quantified at 24 hpi were clearly lower in the *GmBTB/POZ*-OE group relative to the WT group. Further analysis indicated that GmBTB/POZ positively regulates the activities and expression of the enzymatic antioxidants SOD and POD. Within a cell, SOD provides the first line of defence against ROS (Alscher *et al.,* 2002). Conversely, POD serves as an efficient scavenger of ROS, preventing cellular damage (Tewari *et al.,* 2006). Therefore, these findings suggest that GmBTB/POZ improves antioxidant enzymatic activity in soybean, thereby eliminating ROS in response to *P. sojae*, and thus providing sufficient protection against oxidative damage.

Phytohormones, such as SA and JA, mediate the activation of sophisticated plant defence mechanisms to ward off pathogen attack (Alazem and Lin, 2015; Robert-Seilaniantz *et al.,* 2011; Vlot *et al.,* 2009). *PR1* and *PR5* usually act as effector genes for SAR, a process mediated by SA; high expression levels of these genes indicate that SA signalling has been activated (He *et al.,* 2007; Ward *et al.,* 1991). Herein, we determined that the SA marker genes, such as *GmNPR1*, *GmPR1* and *GmPR5*, were constitutively induced in the group of *GmBTB/POZ*-OE soybean plants. Moreover, SA accumulation and the transcript abundance of SA biosynthesis genes were much higher in *GmBTB/POZ*-OE leaves than in WT. By contrast, JA levels and *GmAOS* expression differed little between *GmBTB/POZ* transgenic and WT plants. These results suggest that GmBTB/POZ plays an important role in the regulation of SA-dependent defence signalling and downstream defence gene expression, but not JA signalling. Interestingly, exogenous application of SA also demonstrated that SA positively regulated *GmBTB/POZ* expression. Combining the results of SA accumulation and the transcript abundance of SA synthesis genes in *GmBTB/POZ*-OE soybean plants, these data suggest that *GmBTB/POZ* up-regulates the related genes for SA synthesis, and SA enhances the expression of *GmBTB/POZ*, creating a positive feedback loop of the SA signal. These results are similar to previous reports indicating that *EDS1* and *PAD4* are located upstream of SA synthesis genes and may promote the synthesis of SA, and also suggesting that there is a positive feedback mechanism involving SA that enhances the expression of *EDS1* and *PAD4* (Brodersen *et al.,* 2006; Shah, 2003). Furthermore, exogenous application of SA limited the increase in biomass of *P. sojae*. These findings suggest that GmBTB/POZ plays a positive role in *P. sojae* resistance and that this defence response might be dependent on SA.

Taken together, we speculate that GmBTB/POZ plays a crucial role in soybean resistance to *P. sojae*, which depends mainly on the SA signalling pathway. In addition, some reports have suggested that many BTB/POZ proteins serve as transcription factors, repressors and transcriptional activators (Ahmad *et al.,* 2003; Cao *et al.,* 1997; Hu *et al.,* 2010; Melnick *et al.,* 2000). Our results showed that GmBTB/POZ is a nuclear-localized protein with no transcriptional activator activity. However, it is by no means clear whether, like some BTB/POZ domain proteins, GmBTB/POZ functions as a transcriptional repressor. The BTB/POZ domain usually also acts as a protein–protein interaction module that can both self-associate and interact with non-BTB/POZ proteins (Geyer *et al.,* 2003; Huynh and Bardwell, 1998).

Further studies are needed to identify whether GmBTB/POZ can form dimers or polymers and how this complex takes part in the regulation of pathogen defence responses. Such studies should shed light on the functional mechanism of GmBTB/POZ in biotic stress responses in soybean.

EXPERIMENTAL PROCEDURES

Plant materials and pathogen inoculation

Resistant cultivar 'Suinong 10', which is resistant to the dominant physiological race 1 of *P. sojae* in Heilongjiang, China (Zhang *et al.,* 2010), was used for expression analysis and gene isolation in this study. 'Suinong 10' soybean plants were grown at 22 °C in a 16-h/8-h light/dark photoperiod with 70% relative humidity.

Susceptible cultivar 'Dongnong 50', a soybean cultivar with susceptibility to the dominant physiological race 1 of *P. sojae*, was obtained from the Key Laboratory of Soybean Biology in the Chinese Ministry of Education, Harbin, China, and used for *P. sojae* treatment and gene transformation experiments in this study.

Phytophthora sojae race 1 (PSR01) was isolated from infected soybean plants in Heilongjiang, China (Zhang *et al.,* 2010) and cultivated at 25 °C for 7 days on V8 juice agar in a polystyrene dish.

GmBTB/POZ cloning, sequence analysis and vector construction

'Suinong 10' mRNA was used to clone the full-length cDNA of *GmBTB/POZ*. The PCR products were ligated into the PMD18-T vector (TaKaRa, Dalian, China). Phylogenetic analysis and amino acid sequence alignment of GmBTB/POZ and other BTB/POZs were performed using Molecular Evolutionary Genetics Analysis (MEGA) software 5.1 and DNAMAN, respectively. The ORF of *GmBTB/POZ* was cloned into the vector pCAMBIA3301 with the *bar* gene and 4 × Myc tag as the selectable marker under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter to overexpress the *GmBTB/POZ* gene. The primers used for gene isolation are shown in Table S1 (see Supporting Information).

Soybean genetic transformation

Soybean 'Dongnong 50' was transformed via the *Agrobacterium*-mediated transformation method described by Paz *et al.* (2004). The 35S:*GmBTB/POZ* recombinant plasmid was transferred into *Agrobacterium tumefaciens* strain LBA4404 via the freeze–thaw method as described by Holsters *et al.* (1978). Strain LBA4404 containing recombinant vector 35S:GmBTB/POZ with growth phases (OD_{600} = 0.6) and concentration (OD_{600} = 0.5) was used to infect the cotyledonary nodes of 'Dongnong 50' soybean as the explants. Then, the explants were cultured for 7 days on shoot induction medium. *GmBTB/POZ*-OE plants were identified by PCR and immunoblotting using Myc antibody (Abmart, code number M20002M).

Quantitative RT-PCR

The extraction of total RNA and reverse transcription were performed using TRIzol reagent (Invitrogen, Shanghai, China) and ReverTra Ace Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. qRT-PCR was employed to measure the gene expression levels using a real-time RT-PCR kit (Toyobo, Osaka, Japan) with a LightCycler® 96 System (Roche, California, USA). The gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with *GmEF1* (GenBank accession no. NM_001248778) as the internal control. The qPCR analyses were performed with three technical replicates. The primers used for expression analysis are shown in Table S1.

Subcellular localization

To determine the subcellular localization of GmBTB/POZ, the ORF of *GmBTB/POZ* was ligated into the vector pCAMBIA1302 under the control of the 35S promoter. The recombinant plasmid, 35S:GmBTB/POZ-GFP, was introduced into Arabidopsis protoplasts by polyethylene glycol (PEG)-mediated transfection (Yoo *et al.,* 2007). The GFP signals were imaged using a TCS SP2 confocal spectral microscope imaging system (Leica, Solms, Germany). The primers used for subcellular localization analysis are shown in Table S1.

Transcriptional activation analysis in yeast cells

The ORF of *GmBTB/POZ* was amplified into pGBKT7, generating the fusion construct pGBKT7-*GmBTB/POZ.* The yeast strain AH109 was transformed with pGBKT7-53+pGADT7-T, pGBKT7-*GmBTB/POZ*, pGBKT7-*GmWRKY31* and pGBKT7. The transformed cells were grown on SD (-Trp), SD (-Trp/-His/-Ade) and SD (-Trp/-His/-Ade/α-gal). The transactivation activity of proteins was detected by the growth status and α-gal activity. The primers used for transcriptional activation analysis are shown in Table S1.

Assessment of plant disease responses

To assay for plant resistance to pathogen infection, artificial inoculation procedures were performed as described by Dou *et al.* (2003) and Morrison and Thorne (1978) with minor modifications. Soybean roots and living cotyledons at the V1 stage were inoculated with *P. sojae* zoospores (approximately 1×10^5 spores/mL). Disease symptoms on infected roots and infected cotyledons were photographed with a Nikon D7000 camera. The lesions of the challenged cotyledons were measured by ImageJ software (https://imagej.nih.gov/ij/index.html).

Measurement of ROS generation and DAB staining

ROS levels were detected according to the instructions supplied with the Reactive Oxygen Species Assay Kit (Beyotime Institute

of Biotechnology, Haimen, China), and fluorescence was assayed as described by Qian *et al.* (2009). H_2O_2 levels were visually detected with DAB as described by Zhu *et al.* (2014). The hypocotyls of soybean plants were treated with *P. sojae* zoospores to investigate the response of *GmBTB/POZ*-OE soybean lines to oxidative stress. The artificial inoculation was performed according to the method described by Ward *et al.* (1979) and Morris *et al.* (1991).

Determination of antioxidant enzyme activity and plant hormone levels

SOD and POD enzymes were extracted from approximately 0.1 g of leaves using 0.8 mL of ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ascorbate and 2% polyvinylpyrrolidone (PVP). The enzyme activities were measured as described by Wang *et al.* (2011). SA and JA levels were determined as described by Pan *et al.* (2010) and Zhu *et al.* (2014).

Statistical analysis

All experiments in this study were performed at least three times. Statistical significance between different measurements was examined by Student's *t*-test. A difference was considered to be statistically significant when $^{*}P < 0.05$ or $^{**}P < 0.01$. Bars indicate the standard error of the mean.

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AUTHOR CONTRIBUTIONS

P.X., S.Z. and C.Z. designed the research. C.Z., H.G., R.L. and D.H. performed the research. L.W. and J.W. analysed the data. P.X., S.Z. and C.Z. wrote the article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1 Bimolecular fluorescence complementation (BiFC) analyses of GmBTB/POZ interactions with GmLHP1. GmBTB/POZ-YFP^N+ and GmLHP1-YFP^C were co-transfected into Arabidopsis protoplasts. Scale bars indicate 10 μm.

Fig. S2 Nucleotide and amino acid sequences of *GmBTB/POZ*. The BTB/POZ domain is shown in shadow.

Fig. S3 Phylogenetic analysis of GmBTB/POZ with orthologues from other plant species.

Fig. S4 Alignment of amino acid sequences of GmBTB/POZ with orthologues from other plant species.

Fig. S5 The expression analysis of *GmPAL*s and *GmNPR1* in *GmBTB/POZ*-overexpressing (*GmBTB/POZ*-OE) transgenic and wild-type (WT) plants. The expression level of the control sample was set to unity. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analysed using Student's *t*-test ($*P < 0.05$, $**P < 0.01$). Bars indicate the standard error of the mean.

Table S1 Primer sequences used in this study.