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The bZIP transcription factor PsBZP32 is involved in cyst germination, oxidative stress response, and pathogenicity of *Phytophthora sojae*

Yuting Sheng^{1,2†}, Long Lin^{1†}, Han Chen¹, Tianhuizi Pu¹, Xiaoyun Liu¹, Suomeng Dong^{1,3,4}, Wenwu Ye^{1,3,4*}  and Yuanchao Wang^{1,3,4*}

Abstract

The basic leucine zipper (bZIP) transcription factor family, one of the largest and most diverse transcription factor families in eukaryotes, is associated with the development and stress responses of many eukaryotic organisms. However, their biological functions in oomycete plant pathogens are unclear. A genome-wide analysis of bZIP transcription factors in *Phytophthora sojae* showed that PsBZP32, which has a unique bZIP-PAS domain structure, exhibited a high transcription level during the early stages of *P. sojae* infection. We silenced *PsBZP32* in *P. sojae* and found that the transformants showed defective cyst germination and pathogenicity. The transformants could not eliminate host-derived reactive oxygen species during infection and were more sensitive to oxidative stress. Their response to oxidative stress was independent of the *PsBZP32* transcription level; however, subcellular localization and phosphorylation of PsBZP32 were affected by H₂O₂ stress. These results indicate that PsBZP32 is involved in regulation of *P. sojae* cyst germination, oxidative stress response, and pathogenicity.

Keywords: *Phytophthora sojae*, bZIP transcription factor, Cyst germination, Oxidative stress response, Pathogenicity, Phosphorylation

Background

The *Phytophthora* genus includes over 120 described species, which are sometimes referred to as fungus-like organisms but are actually oomycetes in the kingdom Stramenopila (Martin et al. 2014). *Phytophthora* species are notorious plant destroyers, causing multibillion-dollar direct economic losses to agricultural production and natural ecosystems worldwide annually (Erwin and Ribeiro 1996). For example, *Phytophthora infestans* is the etiologic agent of potato late blight, which caused the Irish potato famine of

the 1840s. *Phytophthora sojae* mainly infects soybean, causing ‘damping off’ of seedlings as well as stem- and root-rot of mature plants (Tyler 2007).

Transcription factors (TFs), which control the rate of transcription by binding to specific DNA sequences, play crucial roles in almost all biological processes, such as regulation and coordination of growth, development, cell cycle progression, and a series of physiological and metabolic adaptations to the environment (Karin 1990). *Phytophthora* species or oomycetes may have different and more complex transcriptional regulation networks. For example, according to the Fungal Transcription Factor Database (v. 1.2; <http://ftfd.snu.ac.kr>), the average number of TFs in *Phytophthora* species is 736, almost twofold that (424) in fungal species. In addition, the Myb TFs in *Phytophthora* species have novel diversified

* Correspondence: yeww@njau.edu.cn; wangyc@njau.edu.cn

†Yuting Sheng and Long Lin contributed equally to this work.

¹Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China

Full list of author information is available at the end of the article



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DNA-binding domains and a developmental stage-specific expression profile (Xiang and Judelson 2010). The bZIP TF family has an expanded number of genes in oomycetes, particularly in *Phytophthora* species (Ye et al. 2013). Also, many members are oomycete-specific with novel bZIP motif variants (according to the two conserved amino acid sites, the bZIP basic regions changed from the N-R type to C-R, R-R, V-R, N-K, or other types), indicating novel DNA-binding specificities or interactions with different factors (Gamboa-Melendez et al. 2013; Ye et al. 2013). Several specific *cis*-regulatory elements were identified in *Phytophthora* species, and many are enriched in the promoters of pathogenicity-related genes, including lineage-specific effectors (Seidl et al. 2012). The absence of a TATA box and presence of novel motifs in promoters indicate that the basic transcriptional regulatory elements of oomycetes are likely diverged from that of model systems (Roy et al. 2013).

To date, few studies have been conducted on the biological functions of *Phytophthora* TFs, and more have focused on the Myb- and bZIP-family TFs. In *P. infestans*, the Myb-family TF Myb2R4 is a regulator of the light regime and sporulation, and Myb2R3 is essential for sporulation (Xiang and Judelson 2014). In *P. sojae*, silencing of *PsMYB1*, which encodes a R2R3-type Myb TF, led to defective zoosporogenesis and infection (Zhang et al. 2012). Thirty-eight bZIP-encoding genes were identified in the *P. infestans* genome (Gamboa-Melendez et al. 2013). *Pibzp1*, which interacts with the sporangium-inducing kinase *Pipkz1*, is essential for zoospore motility and infection (Blanco and Judelson 2005). Eight bZIP TFs with novel bZIP motif variants (Asn-to-Cys) have been silenced in *P. infestans*. None were found to be essential for growth or pathogenicity, and only one bZIP (PITG_16038) plays a key role in H₂O₂ detoxification (Gamboa-Melendez et al. 2013). We predicted that 71 candidate bZIP TFs are encoded in the genome of *P. sojae* (Ye et al. 2013); however, the biological functions of these bZIP TFs are unknown.

Among the *P. sojae* bZIP TFs, only PsBZP32 has a Per-ARNT-Sim (PAS) domain together with the conserved bZIP domain. The PAS domain can serve as versatile sensor detecting a range of chemical and physical stimuli and regulating the activity of functionally diverse effector domains (Moglich et al. 2009). For example, in *Neurospora*, the PAS domain-containing TF WC-1 acts as both a blue-light photoreceptor and a positive element in the circadian negative feedback loop under constant darkness (He et al. 2005). In this study, we investigated the function of PsBZP32 on cyst germination, pathogenicity, and oxidative stress responses in *P. sojae*. We also found that the subcellular localization and phosphorylation status of PsBZP32 were regulated when *P. sojae* was under oxidative stress.

Results

PsBZP32 is a candidate bZIP TF with an additional PAS domain

Among the 71 predicted bZIP TFs in *P. sojae* (Ye et al. 2013), only PsBZP32 contained both the conserved bZIP domain and an additional PAS domain. Based on cDNA sequencing, we found that *PsBZP32* gene contained two introns (127 and 76 bp) and a 1176 bp open reading frame (ORF) encoding a 391-amino-acid protein. Conserved homologs of PsBZP32 were identified in species of *Phytophthora*, downy mildew, *Pythium*, and *Saprolegnia* (Fig. 1a). PsBZP32 and its homologs shared a similar domain architecture; i.e., a bZIP and PAS domain (bZIP-PAS; Fig. 1b). Using the Pfam database (<http://pfam.xfam.org>), we found that the bZIP-PAS domain organization was conserved in the proteins of non-oomycetes such as brown algae and diatoms; however, it is likely specific to Stramenopila.

In terms of the two well-characterized amino acid sites in the DNA-binding region of the bZIP domain, we identified PsBZP32 as the N-R type of bZIP TF (Fig. 1b), a conventional type of bZIP TF present in diverse animals, fungi, and plants (Gamboa-Melendez et al. 2013; Ye et al. 2013). Digital gene expression profiling (Ye et al. 2011) and reverse transcription–quantitative PCR (RT-qPCR) showed that the transcription levels of *PsBZP32* were higher during the early infection stages than developmental stages of *P. sojae* (Additional file 1: Figure S1a and Fig. 1c), implying that PsBZP32 contributes to pathogenicity.

Silencing of *PsBZP32* affected the cyst germination rate and hyphal morphology in *P. sojae*

To evaluate the biological functions of *PsBZP32*, we performed gene silencing in *P. sojae* by expressing the anti-sense full-length sequence of *PsBZP32* (Additional file 1: Figure S1b). After antibiotic resistance screening, 136 transformants were obtained for confirmation by genomic PCR and RT-qPCR. Among them, four transformants (T14, T43, T81, and T87) were finally selected. PCR analysis of genomic DNA from these four transformants indicated the presence of the exogenous construct (Additional file 1: Figure S1c). RT-qPCR demonstrated that the *PsBZP32* transcription levels in these four transformants were 53–71% of those in the wild-type strain (WT) and the control strain (CK; in which the backbone vector pTOR::GFP was transformed) at the mycelial stage (Fig. 1d).

Compared with WT and CK, the *PsBZP32*-silenced transformants exhibited no significant differences in mycelial growth, sporangium formation, zoospore production, encystment, or oospore production, but showed a difference in the cyst germination process. At 3 h post-encystment of zoospores, the average cyst germination

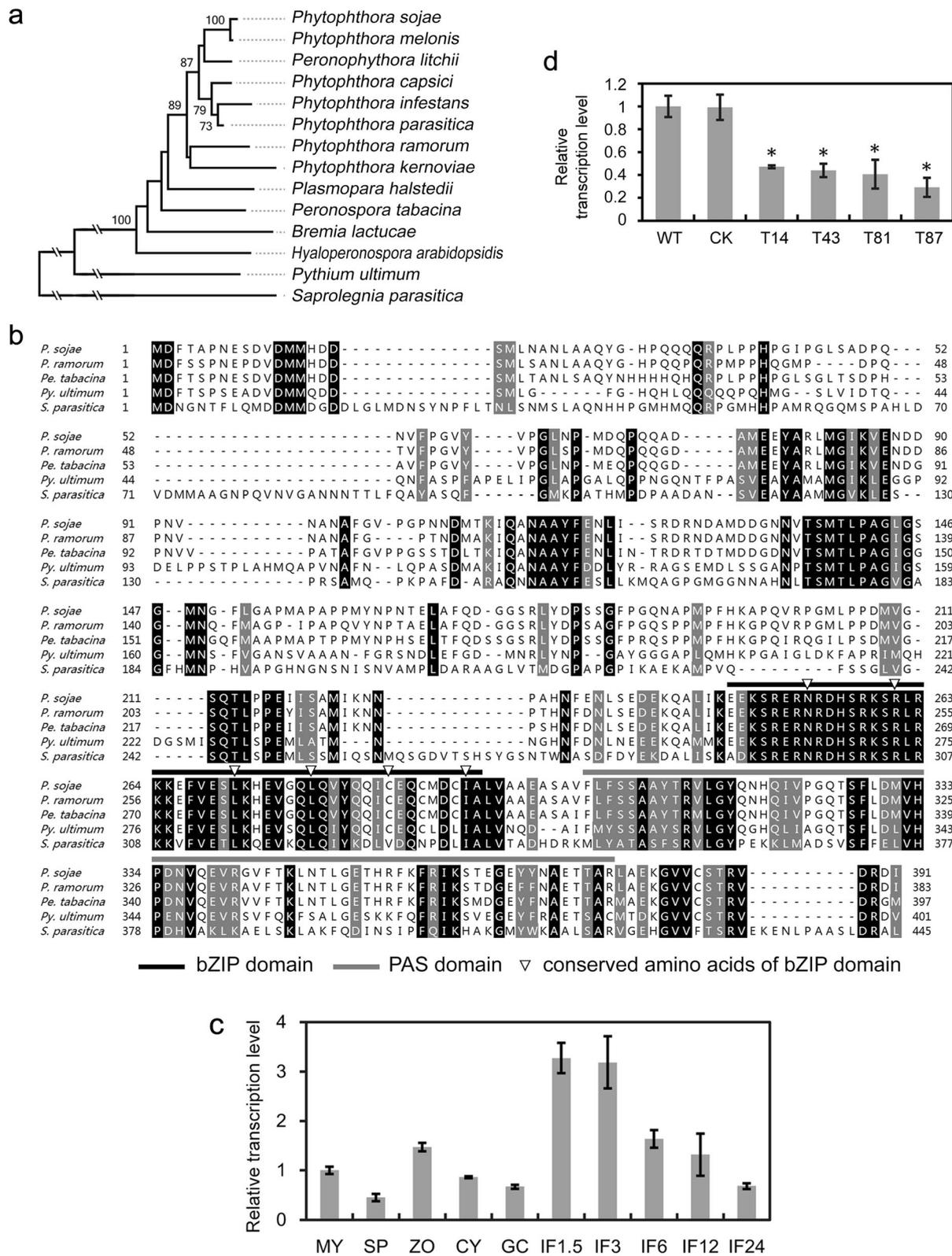


Fig. 1 (See legend on next page.)

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Fig. 1 PsBZP32 is a conserved bZIP transcription factor with an additional PAS domain. **a** Phylogenetic analysis of PsBZP32 and its homologues in oomycetes. Full-length amino acid sequences were used to generate the phylogenetic neighbor-joining tree in MEGA 6.0. **b** Protein sequence alignment of PsBZP32 and its homologs in *Phytophthora ramorum*, *Peronospora tabacina*, *Pythium ultimum*, and *Saprolegnia parasitica*. **c** Transcription pattern of *PsBZP32* according to RT-qPCR. MY, SP, ZO, CY, and GC represent the asexual life stages of *P. sojae*: vegetative mycelia, sporangia, zoospores, cysts, and germinated cysts, respectively. IF1.5, IF3, IF6, IF12, and IF24 represent the infection stages of *P. sojae*: 1.5, 3, 6, 12, and 24 h post-infection (hpi), respectively. Relative transcription levels were calculated using the MY values as a reference. **d** Relative transcription levels of *PsBZP32* in *PsBZP32*-silenced transformants and control strains. WT, wild-type strain; CK, control strain transformed with empty vector. *, significant difference compared with the WT (two-tailed *t*-test, $P < 0.01$)

rate ranged from 37 to 54% in the *PsBZP32*-silenced transformants, significantly lower than the rates in WT (73%) and CK (70%) (Fig. 2a). At 10 h post-encystment of zoospores, significantly more abnormal hyphae (short with multiple branches) developed from the germinated cysts of the transformants (Fig. 2b), with average rates of 24–55%, significantly higher than the rates in WT and CK (both 2%; Fig. 2c).

PsBZP32 is required for pathogenicity and invasive hyphal colonization of *P. sojae*

Cyst germination is a key step preceding infection of *P. sojae* (Zuo et al. 2005). To explore whether *PsBZP32* is involved in pathogenicity, zoospores of the *PsBZP32*-silenced transformants, WT, and CK were separately inoculated onto etiolated seedlings of the susceptible soybean cultivar Hefeng47. To compensate for the reduced cyst germination rate, double the zoospore number in the *PsBZP32*-silenced transformants compared with WT and CK were used to inoculate soybean seedlings. However, the *PsBZP32*-silenced transformants produced only small necrotic dark-brown spots on

etiolated seedlings at 36 h post-infection (hpi), whereas both WT and CK produced spreading lesions (Fig. 3a).

To identify the mechanism underlying the pathogenicity defect in *PsBZP32*-silenced transformants, we observed zoospore-infected host tissues by microscopy and found that zoospores of WT, CK, and *PsBZP32*-silenced transformants penetrated epidermal cells of etiolated seedlings and produced few short infectious hyphae at 12 hpi (Fig. 3b). All strains induced reactive oxygen species (ROS) accumulation in the epidermal cells of soybean etiolated seedlings at 3 hpi. However, at 12 hpi, ROS accumulated in soybean seedlings inoculated with *PsBZP32*-silenced transformants, while ROS accumulation was negligible in seedlings inoculated with WT or CK (Fig. 3b), suggesting that host-derived ROS scavenging is impaired by silencing of *PsBZP32* in *P. sojae*.

PsBZP32 contributes to oxidative stress resistance, but not at the transcription level

To determine whether *PsBZP32* is involved in resistance to oxidative stress, WT, CK, and *PsBZP32*-silenced transformants were cultured on Plich medium supplemented with 0–5 mM H_2O_2 . Compared with the

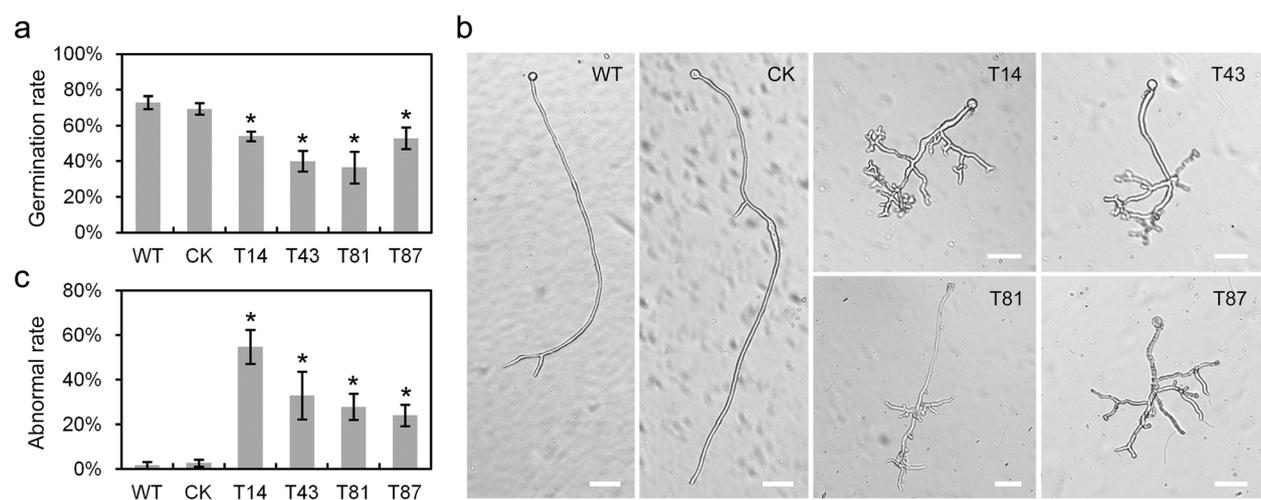
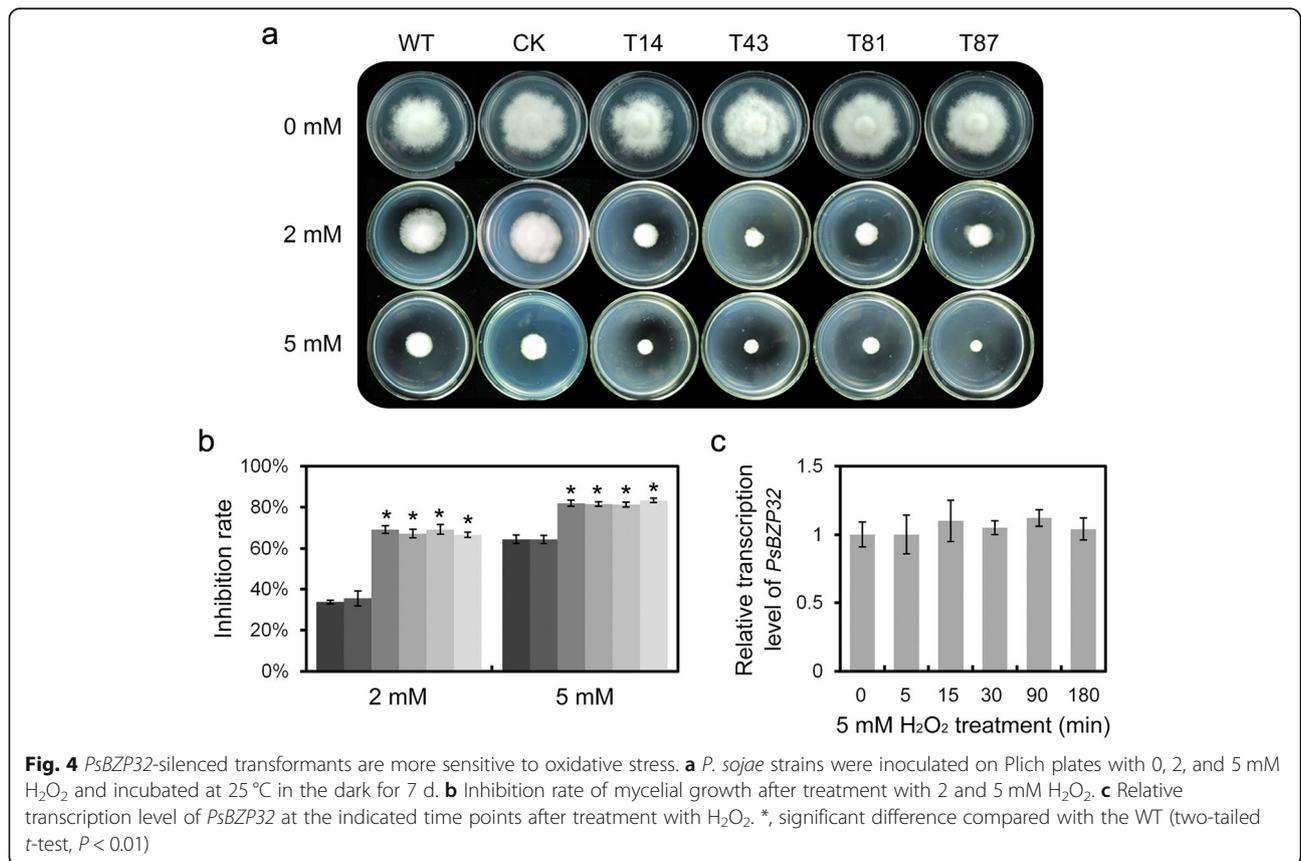
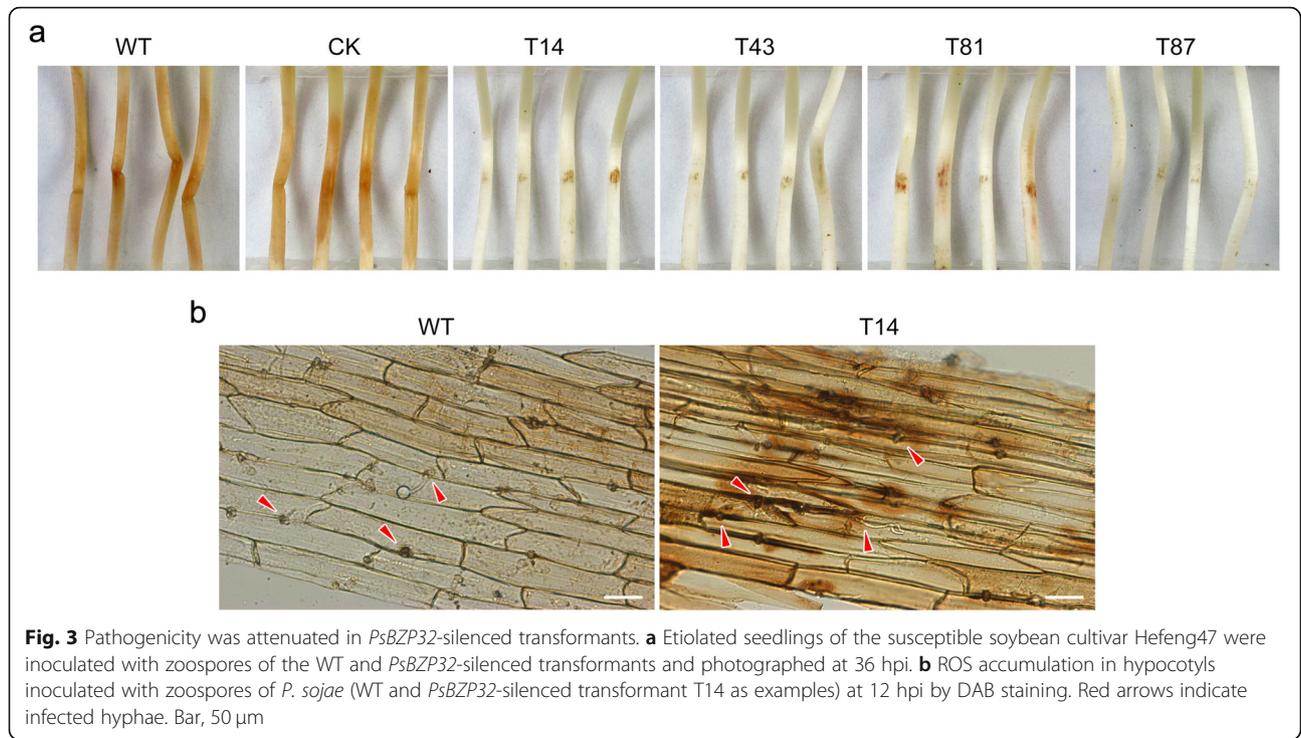


Fig. 2 Cyst germination was affected in *PsBZP32*-silenced transformants. **a** Cyst germination rate at 3 h after encystment. **b** Microscopic observation of germinated cysts at 10 h after encystment. Representative normally germinated hypha of WT and CK, and abnormally germinated hypha (short with multiple branches) of *PsBZP32*-silenced transformants are shown. **c** Abnormal rate of germinated cysts at 10 h after encystment. Bar, 20 μ m. *, significant difference compared with the WT (two-tailed *t*-test, $P < 0.01$)



treatment without H_2O_2 (0 mM), all *P. sojae* strains treated with 2 mM H_2O_2 displayed mycelial growth inhibition, with inhibition rates of 64–68% in the *PsBZP32*-silenced transformants compared with 35% and 36% in WT and CK, respectively. Similarly, when exposed to 5 mM H_2O_2 , the *PsBZP32*-silenced transformants did not grow, whereas WT and CK proliferated at a low rate (Fig. 4a, b). Additionally, the transcription level of *PsBZP32* in WT and CK treated with 5 mM H_2O_2 showed no significant changes up to 180 min (Fig. 4c). Therefore, *PsBZP32* is involved in the vegetative growth of *P. sojae* in response to oxidative stress independently of its transcription level.

The subcellular localization of PsBZP32 was modulated by oxidative stress

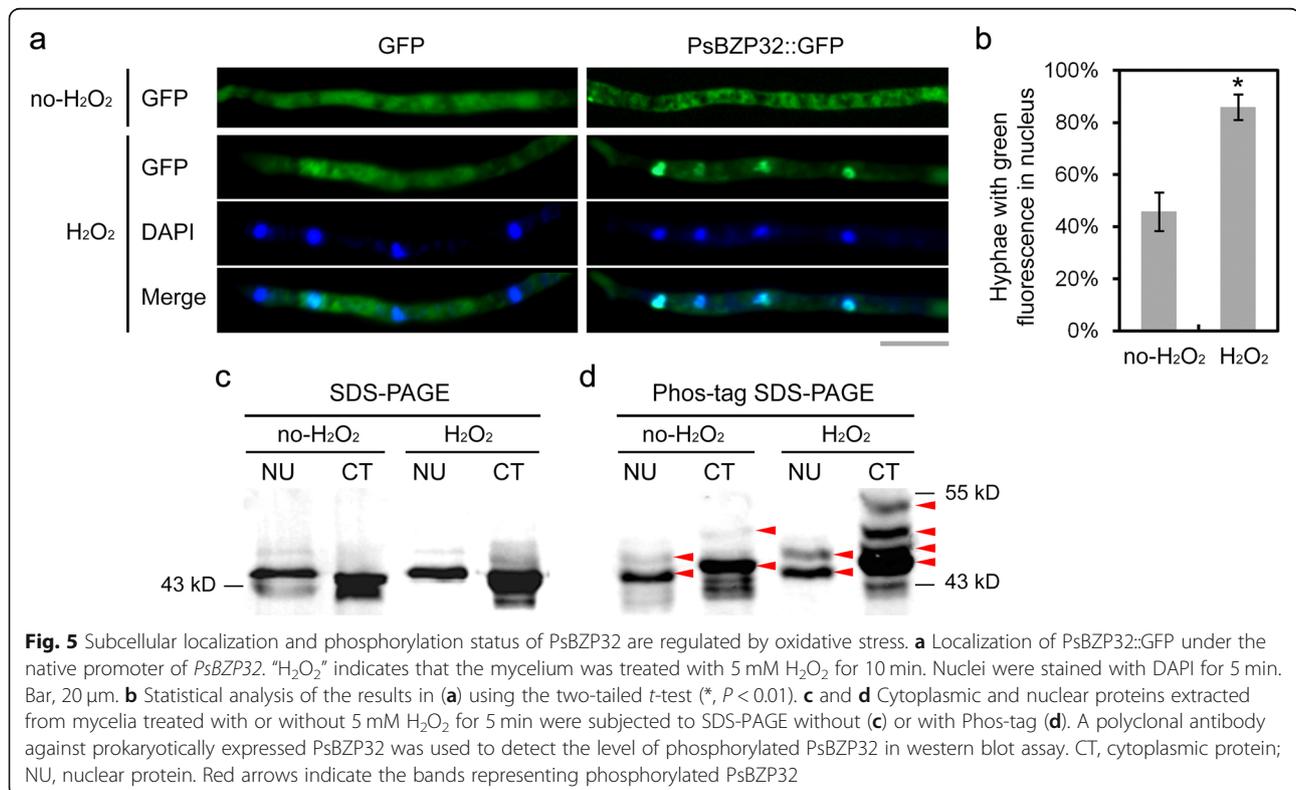
To assess the regulation of PsBZP32 under oxidative stress conditions, the subcellular localization of PsBZP32 was analyzed in the absence and presence of H_2O_2 . A transformant was generated from the WT strain P6497, in which expression of the PsBZP32::GFP fusion protein was driven by the native promoter of *PsBZP32*. The GFP-expressing transformant was used as the control. In the *PsBZP32::GFP*-expressing strain without oxidative stress, green fluorescence was observed throughout the cells in 54% of hyphae versus concentrated in nucleus-like spots in the remaining 46% of hyphae. After treatment with 5 mM H_2O_2 for 10 min, the proportion of

hyphae with green fluorescence concentrated in nucleus-like spots was increased to 86% (Fig. 5a, b). The green fluorescent spots were shown to be nuclei by DAPI staining (Fig. 5a). In contrast, green fluorescence was distributed throughout hyphae cells of the GFP-expressing control strain (Fig. 5a). Therefore, changes in the subcellular localization of PsBZP32 may be related to its role in the oxidative stress response.

PsBZP32 was differentially phosphorylated under oxidative stress

According to KinasePhos (<http://kinasephos.mbc.nctu.edu.tw>), a web server used to predict phosphorylation sites (Huang et al. 2005), nine serine residues, three threonine residues, and four tyrosine residues were predicted in PsBZP32, with a prediction specificity of 95% (Additional file 2: Figure S2), implying that PsBZP32 is phosphorylated.

We next performed Phos-tag SDS-PAGE, normal SDS-PAGE, and western blot analyses of the phosphorylated levels of PsBZP32 in the cytoplasm and nucleus of cells treated with or without H_2O_2 (Fig. 5c, d). In the nuclear protein samples, one slower-migrating band in addition to the main band was detected by Phos-tag SDS-PAGE, compared with normal SDS-PAGE, and this band was more obvious in the H_2O_2 -treated sample. The migrating band indicated that PsBZP32 might be phosphorylated. Similarly, in the cytoplasm samples, one slower-migrating band in



addition to the main band was observed on the Phos-tag gel in the absence of H₂O₂ treatment. However, in the presence of H₂O₂, there were two additional slower-migrating bands on the Phos-tag gel. The difference in the number and migration distance of the migrating bands indicated different phosphorylation forms and levels of the PsBZP32 protein. The phosphorylation level and pattern likely increased when the pathogen was under oxidative stress, and PsBZP32 was present in the cytoplasm.

Discussion

We reported an expanded number (71) of bZIP candidates in oomycetes, but only PsBZP32 contains an additional PAS domain (Ye et al. 2013). PAS domain-containing proteins occur in all kingdoms of life, and this domain mediates protein–protein interactions to sense diverse signals (Moglich et al. 2009). According to our bioinformatics analysis, this bZIP-PAS protein is conserved and specific to Stramenopila. In this study and a recent study, the gene silencing assays indicated that the bZIP-PAS protein PsBZP32 plays a key role in the cyst germination, oxidative stress response, and pathogenicity of *P. sojae*, while PIBZP32 in *Peronophythora litchii* has similar biological functions (Kong et al. 2020). Therefore, the bZIP-PAS proteins may have conserved functions in oomycete plant pathogens.

Zoospores, the main vector for dispersal of *P. sojae* in the field, can swim chemotactically toward isoflavones released by soybean root. When a zoospore encounters the root, it transforms into an adhesive cyst, which then germinates to produce hyphae and initiate infection (Tyler 2007). Silencing of *PsBZP32* reduced the cyst germination rate and resulted in abnormally germinated cysts. The abnormally germinated cysts lost polarized growth and developed into multi-branched hyphae. By contrast, *PsBZP32*-silenced transformants showed normal mycelial growth, sporangium formation, zoospore production, encystment, and oospore production. Therefore, PsBZP32 may regulate *P. sojae* development during cyst germination.

PsBZP32-silenced transformants showed defective pathogenicity, producing only necrotic dark brown spots at the inoculation site. They could not eliminate host-derived ROS during infection and were hypersensitive to H₂O₂. Therefore, PsBZP32 is essential for ROS detoxification at the growth and infection stages of *P. sojae*. In *Pe. litchii*, PIBZP32 can regulate the activities of extracellular peroxidases and laccases (Kong et al. 2020), and the heat shock TF PsHSF1 in *P. sojae* also has these functions (Sheng et al. 2015). However, silencing of *PsBZP32* did not attenuate extracellular peroxidase and laccase activities. Therefore, PsBZP32 may regulate ROS detoxification via other pathways.

PsBZP32 showed no significant change in its transcription level under H₂O₂ stress, indicating that it regulates the oxidative stress response independently of its transcription level. The subcellular localization of bZIP is important for its transcriptional regulation (Llorca et al. 2014). MoAP1, a bZIP TF that mediates the oxidative stress response in *Magnaporthe oryzae*, was more concentrated in the nucleus in the presence than absence of H₂O₂ (Guo et al. 2011). Also, PsBZP32 translocated from the cytoplasm to the nucleus under H₂O₂ treatment. The subcellular localization and activity of bZIPs are regulated by protein modifications, including phosphorylation (Llorca et al. 2014). Subcellular localization of MoCRZ1, a calcineurin-responsive TF in *M. oryzae*, is regulated by phosphorylation. When dephosphorylated by calcineurin, it translocates from the cytoplasm to the nucleus to regulate Ca²⁺/calcineurin-dependent gene expression. Next, MoCRZ1 is phosphorylated in the nucleus and translocated to the cytoplasm (Choi et al. 2009; Kim et al. 2010). We found that the phosphorylation status of PsBZP32 in the cytoplasm, but not in the nucleus, was changed by H₂O₂. However, the relationship between PsBZP32 localization and phosphorylation is unclear.

CRISPR/Cas9-mediated gene knockout in *P. sojae* was established in 2016 (Fang and Tyler 2016). In this study, we performed this assay at least twice in order to knockout *PsBZP32* in *P. sojae*, although over two hundred transformants were obtained from antibiotic resistance screening, no one could be confirmed to obtain *PsBZP32* knockout. In *Pe. litchii*, the researchers also failed to knock out *PIBZP32* (Kong et al. 2020). In addition, we generated *PsBZP32*-overexpressing transformants, but they showed only 1.5-to-3-fold expression levels and no different phenotype compared with WT. These results indicated that expression level of BZP32 may be accurately regulated and the functions of BZP32 may be essential for the survival or basic development of oomycetes. Considering that gene silencing might cause off-target effects, we confirmed that the transformed antisense sequence of *PsBZP32* had no potential target (> 21 bp identical sequence) in *P. sojae* genome based on bioinformatics analysis, and in *Pe. litchii*, *PIBZP32*-silencing did not affect the expression of the neighboring gene *PI105397* (Kong et al. 2020). However, due to the technical limitations on *BZP32* knockout (and complementation), the accurate biological functions of these proteins still demand further investigation.

Conclusions

We identified a bZIP-PAS type TF, PsBZP32, which exhibited a high expression level during early infection of *P. sojae*. Silencing of *PsBZP32* led to a defect in cyst germination and pathogenicity in *P. sojae*. *PsBZP32*-

silenced *P. sojae* transformants could not eliminate host-derived ROS during infection and were more sensitive to oxidative stress. The response of *PsBZP32*-silenced transformants to oxidative stress was independent of the transcription level of *PsBZP32*; however, the subcellular localization and phosphorylation of *PsBZP32* were influenced by H_2O_2 . Therefore, *PsBZP32* is an important regulator of the cyst germination, oxidative stress response, and pathogenicity of *P. sojae*. Further work should focus on the mechanism underlying the change in the subcellular localization of *PsBZP32* in response to oxidative stress and the function of the PAS domain in signal sensing. Also, the target genes of *PsBZP32* need to be investigated to understand transcriptional regulation during oxidative stress in oomycetes.

Methods

P. sojae growth conditions and tissue harvest

P. sojae strain P6497 (race 2) provided by Prof. Brett Tyler (Oregon State University, USA) was used as the WT. WT and all transgenic lines in this study were routinely grown on 10% V8 medium at 25 °C in the dark (Erwin and Ribeiro 1996). Samples from different asexual life stages (mycelia, sporangia, zoospores, cysts, germinated cysts) and infection stages (*P. sojae*-soybean interaction for 1.5, 3, 6, 12, and 24 h) were prepared as described (Ye et al. 2011). To prepare H_2O_2 -treated samples, *P. sojae* mycelia cultured in 10% V8 liquid medium at 25 °C in the dark for 3 d were washed with Plich liquid medium (van West et al. 1999) and incubated in 20 mL Plich liquid medium supplemented with 5 mM H_2O_2 . Mycelia were harvested after incubation for 0, 5, 15, 30, 90, and 180 min. Tissues were immediately frozen in liquid nitrogen followed by grinding to extract RNA for gene expression analysis by RT-qPCR as described previously (Ye et al. 2011).

Vector construction and transformation in *P. sojae*

The pTOR::GFP vector, provided by Prof. Franicne Govers (Wageningen University, The Netherlands), was used to generate constructs for *P. sojae* transformation. The full-length *PsBZP32* ORF was amplified from cDNA of *P. sojae* strain P6497 using the primers *PsBZP32-XbaI-F* (GCTCTAGAATGGACTTCACGGCGCCCAA) and *PsBZP32-EcoRI-R* (CGGAATTCTTAAATGTCGCGGTCCACGCGGTC) and PrimeStar polymerase (Takara Bio Inc., Otsu, Japan). The amplified product was digested with *XbaI* and *EcoRI*, and then ligated in the antisense orientation into the digested pTOR::GFP vector. The sequenced plasmid was used for *P. sojae* transformation to generate *PsBZP32*-silenced transformants as described (Hua et al. 2008). The backbone vector pTOR::GFP was used to generate the control strain of *P. sojae*. The *P. sojae* transformants were screened for

the *PsBZP32* transgene by amplifying their genomic DNA as described (Hua et al. 2008). Total RNA was extracted from the *PsBZP32*-silenced transformants, and RT-qPCR was performed to analyze transcription levels.

The full-length *PsBZP32* (with termination codon deleted) ORF was amplified from cDNA of P6497 using the primers *PsBZP32-ClaI-F* (ccATCGATATGGACTT-CACGGCGCCCAA) and *PsBZP32-BsiwI-R* (gcCGTACGAATGTCGCGGTCCACGCGGTC) and PrimeStar polymerase (Takara Bio Inc.). The amplified product was ligated in the sense orientation into the digested pTOR::GFP vector. The promoter sequence of *PsBZP32*, 922 bp upstream of the *PsBZP32* start codon, was amplified using the primers p*PsBZP32-SnaBI-F* (TACGTATTCAACCCCGGCCATCGCCA) and p*PsBZP32-ClaI-R* (ATCGATGGCTGGGTGTGGCGTCTCCT), and the amplified product was ligated into the digested pTOR::GFP vector to replace the pHam34 promoter. The p*PsBZP32::PsBZP32::GFP* plasmid was sequenced and transformed into *P. sojae* for *PsBZP32* localization analysis.

Cyst germination assay

Zoospores were induced and harvested into 1.5 mL tubes (Ye et al. 2013). Tubes containing at least 20 zoospores/ μ L in a 500 μ L suspension were vortexed for 90 s to induce encystment. Droplets (5 μ L) of the cyst suspension were spotted on 0.5% V8 medium and incubated at 25 °C for 3 h to induce germination. The germination of at least 100 cysts per strain was examined, and all assays were replicated three times, each in triplicate.

Zoospores in 0.5% V8 liquid medium were incubated at 25 °C for 10 h following encystment. The phenotypes of 100 germinated cysts were examined using the Olympus 1X71 inverted microscope. The abnormal rate of germinated cysts was analyzed by two-tailed *t*-test ($P < 0.01$).

Oxidative stress sensitivity assay

The sensitivity of *PsBZP32*-silenced transformants to H_2O_2 was evaluated on modified Plich medium plates with 0, 2, and 5 mM H_2O_2 . Fresh 5 mm hyphal plugs from the WT, CK, and *PsBZP32*-silenced transformants were transferred from 10% V8 plates to modified Plich medium without or with 2 or 5 mM H_2O_2 and incubated at 25 °C for 7 d. The diameter of each line was measured, and the growth rate and inhibition rate were calculated. The inhibition rate was calculated as (growth rate on plates without H_2O_2 – growth rate on plates with H_2O_2) \div growth rate on plates without H_2O_2 . Data were subjected to statistical analysis by two-tailed *t*-test ($P < 0.01$). The experiment was repeated three times, each in triplicate.

Pathogenicity assay

Zoospores and mycelia were inoculated onto hypocotyls of etiolated soybean seedlings of cultivar Hefeng47, which is compatible with *P. sojae* strain P6497 (Li et al. 2013), and incubated at 25 °C in the dark for 36 h; the result was then photographed. Zoospores and mycelia were inoculated on leaves of Hefeng47 and incubated at 25 °C in the dark for 3 d; the result was then photographed. Before inoculation, hypocotyls of etiolated soybean seedlings and leaves of cultivar Hefeng47 were scratched using a pipette tip for the wound-inoculation assay. All experiments were repeated three times; each line was inoculated onto at least four seedlings or leaves.

Staining

To visualize the accumulation of H₂O₂ on hypocotyls of etiolated seedlings at 12 hpi, 3, 3-diamino benzidine (DAB; D8001, Sigma, St. Louis, MI, USA) staining was performed. Briefly, etiolated seedlings inoculated with zoospores for 12 h were collected, placed in 1 mg/mL DAB for 16 h in the dark at room temperature, and decolorized in ethanol (96%) for 48 h (Molina and Kahmann 2007). The epidermis of DAB-stained soybean hypocotyls was visualized using the Olympus 1X71 inverted microscope.

DAPI staining was performed to visualize nuclei. Mycelia were incubated in 10 µg/mL DAPI for 5 min in the dark at room temperature, washed in sterilized distilled water twice, and observed under the Olympus 1X71 inverted microscope using the DAPI filter for blue fluorescence. To measure the localization of PsBZP32::GFP in each treatment, 100 hyphae tip (or hyphae fragments closed to tip) displaying green fluorescence anywhere were selected to calculate the proportion of those with green fluorescence in nucleus; each experiment was performed at least three times using independent samples.

Nucleic acid extraction and RT-qPCR

Genomic DNA was isolated from hyphae of *P. sojae* strain P6497 and all transgenic lines grown in 10% V8 liquid medium using the Plant DNA Kit (Omega Bio-tek, USA) and subjected to PCR. Total RNA was extracted from mycelia of the WT at various developmental and infection stages. Also, mycelia were treated with H₂O₂, and RNA was extracted with the Abion Extraction Kit (Life Technology, USA), following the manufacturer's procedures, and quantified using a spectrophotometer (ND-1000; Nanodrop, USA). The total RNA integrity was confirmed by agarose gel electrophoresis.

RT-qPCR was performed to determine the transcription levels of target genes. First-strand cDNA was synthesized using M-MLV reverse transcriptase (RNase-

free, Invitrogen, Shanghai, China) and oligo (dT)18 primer (Invitrogen). A 20 µL reaction volume was prepared using the SYBR Prime Script RT-PCR kit (Takara Bio Inc.) according to the manufacturer's instructions. qPCR was performed on the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, USA) following the manufacturer's instructions. Relative transcription levels were analyzed using 7500 System Sequence Detection Software. Primers used were designed using Primer 3.0 Input (v. 0.4.0) and commercially synthesized (Invitrogen). The primers used to amplify PsBZP32 were PsBZP32-qRT-F (CGTCTGCGCAAGAAAGAGTTC) and PsBZP32-qRT-R (GCGCTCGAGAAAAGGAACAC). *actA* (PsACTA-qRT-F: ACTGCACCTTCCAGACCATC; PsACTA-qRT-R: CCACCACCTTGATCTTCA TG) from *P. sojae* was amplified as a constitutively expressed endogenous control, and the transcription levels of target genes relative to that of *actA* were calculated by the 2^{-ΔΔCT} method. All experiments were performed at least three times using independent RNA samples.

Immunodetection

Hyphae cultured in 10% V8 liquid medium at 25 °C in the dark for 3 d were washed twice with SDS and transferred to SDW in the presence or absence of 5 mM H₂O₂ for 5 min. Hyphal tissues were immediately frozen in liquid nitrogen followed by grinding for cytoplasmic and nuclear protein extraction. Cytoplasmic protein extraction was performed using 1× PBS, 1% protease inhibitor cocktail (Sigma, P8340), 1% phosphatase inhibitor cocktail (Sigma, P0044, P5726), and 1 mM phenylmethylsulfonyl fluoride (ST506; Beyotime, Shanghai, China). Nuclear protein extraction was performed as described previously (Xu et al. 2012). Protein samples were added to SDS-PAGE sample loading buffer (Beyotime, P0015L), boiled for 5 min, subjected to 12% SDS-PAGE and Phos-tag SDS-PAGE, and transferred onto Immobilon-P membranes (IPVH00010; Millipore, USA) for western blotting using a rabbit polyclonal antibody specific to PsBZP32 (provided by Zoobio, Nanjing, China) and a rabbit anti-mouse IgG (926–32,211; Odyssey, Licor, USA). Phos-tag SDS-PAGE gels were prepared according to the instructions of the manufacturer of Phos-tag Acrylamide (304–93,526; Poppard, China). Anti-PsBZP32 was prepared using *Escherichia coli*-expressed PsBZP32 as the antigen.

Bioinformatics analysis

Protein sequences of PsBZP32 homologs in non-*P. sojae* species were identified by BLASTP searches (McGinnis and Madden 2004) of the genomic databases of NCBI (<http://www.ncbi.nlm.nih.gov>), EumicrobeDB (<https://>

www.eumicrobedb.org), and Joint Genome Institute (JGI) (<http://www.jgi.doe.gov/>). The obtained sequences were submitted to NCBI-CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd>) and SMART (<http://smart.embl-heidelberg.de/>) to predict conserved functional domains (Schultz et al. 1998; Letunic et al. 2015). Sequences were aligned using Clustal W (Thompson et al. 1994), and phylogenetic trees were constructed in MEGA 6.0 (Tamura et al. 2007) using neighbor-joining algorithms and 1000 bootstrap replications.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-020-00078-3>.

Additional file 1: Figure S1. Transcription pattern of *PsBZP32* and generation of *PsBZP32*-silenced transformants. **a** Transcription pattern of *PsBZP32* obtained from the *P. sojae* Digital Gene Expression database (Ye et al. 2011). MY, SP, ZO, CY, and GC represent the asexual life stages of *P. sojae*: vegetative mycelia, sporangia, zoospores, cysts, and germinated cysts, respectively. IF1.5, IF3, IF6, IF12, and IF24 represent the infection stages of *P. sojae*: 1.5, 3, 6, 12, and 24 hpi, respectively. **b** Schematic diagram of the construct used to silence *PsBZP32* in *P. sojae*. A reverse fragment of full-length *PsBZP32* was expressed with the strong promoter pHAM34 to silence *PsBZP32*. tHAM34 indicates the terminator. Reversed *PsBZP32* was inserted between pHAM34 and tHAM34 in the pTOR vector. **c** Genomic DNA PCR (using pTOR primers pTOR-F and pTOR-R indicated in **b**). The exogenous fragment was integrated into the *P. sojae* genome.

Additional file 2: Figure S2. Phosphorylation sites predicted from the *PsBZP32* protein sequence. Prediction was performed using KinasePhos (<http://kinasephos.mbc.nctu.edu.tw>).

Abbreviations

bZIP: Basic leucine zipper; ORF: Open reading frame; PAS: Per-ARNT-Sim; TF: Transcription factor; RT-qPCR: Reverse transcription quantitative PCR; WT: Wild-type strain; CK: Control strain transformed with empty vector; ROS: Reactive oxygen species

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Authors' contributions

YW, WY, and SD conceived and designed the experiments. YS, LL, HC, TP, and XL carried out the experiments. All authors analyzed the data. YS, LL, and WY written the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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Competing interests

The authors declare that they have no conflict of interest.

Author details

¹Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China. ²Present address: College of Agriculture, Ludong University, Yantai 264000, China. ³The Key Laboratory of Plant Immunity, Nanjing Agricultural University, Nanjing 210095, China. ⁴The Key Laboratory of Integrated Management of Crop Diseases and Pests (Ministry of Education), Nanjing 210095, China.

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