The Basic Leucine Zipper Transcription Factor PlBZP32 Associated with the Oxidative Stress Response Is Critical for Pathogenicity of the Lychee Downy Blight Oomycete *Peronophythora litchii*

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ABSTRACT Basic leucine zipper (bZIP) transcription factors are widespread in eukaryotes, including plants, animals, fungi, and oomycetes. However, the functions of bZIPs in oomycetes are rarely known. In this study, we identified a bZIP protein possessing a special bZIP-PAS structure in *Peronophythora litchii*, named *PlBZP32*. We found that *PlBZP32* is upregulated in zoospores, in cysts, and during invasive hyphal growth. We studied the functions of *PlBZP32* using the RNAi technique to suppress the expression of this gene. *PlBZP32*-silenced mutants were more sensitive to oxidative stress, showed a lower cyst germination rate, and produced more sporangia than the wild-type strain SHS3. The *PlBZP32*-silenced mutants were also less invasive on the host plant. Furthermore, we analyzed the activities of extracellular peroxidases and laccases and found that silencing *PlBZP32* decreased the activities of *P. litchii* peroxidase and laccase. To our knowledge, this is the first report that the functions of a bZIP-PAS protein are associated with oxidative stress, asexual development, and pathogenicity in oomycetes.

IMPORTANCE In this study, we utilized the RNAi technique to investigate the functions of PlBZP32, which possesses a basic leucine zipper (bZIP)-PAS structure, and provided insights into the contributions of bZIP transcription factors to oxidative stress, the production of sporangia, the germination of cysts, and the pathogenicity of *Peronophythora litchii*. This study also revealed the role of PlBZP32 in regulating the enzymatic activities of extracellular peroxidases and laccases in the plant-pathogenic oomycete.

KEYWORDS *Peronophythora litchii*, bZIP transcription factor, pathogenicity, peroxidase, laccase

Lychee (or litchi; *Litchi chinensis* Sonn.) is a famous tropical and subtropical fruit with high economic value due to its appearance, taste, and nutrition, and its production is approximately 3.5 million tons worldwide each year (1, 2). Downy blight caused by *Peronophythora litchii* is one of the most destructive diseases of lychee during production and postharvest (3, 4).

In plant-microbe interactions, one of the most rapid plant defense reactions is the oxidative burst, which constitutes the production of reactive oxygen species (ROS), including superoxide and H_2O_2 generated by NADPH oxidases (5, 6). The accumulation of ROS at the site of pathogen invasion can either directly kill the pathogen (7, 8) or function as a second messenger to induce the expression of various plant defense-related genes and to trigger pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) in plants (9–12).

The transcription factors (TFs) of the basic leucine zipper (bZIP) family are multifunctional across pathogenic fungi. For example, MoAtf1 regulates the transcription of genes encoding laccases and peroxidases, the oxygen scavengers, and thus is required for full virulence in the rice blast fungus *Magnaporthe oryzae* (13). MoAP1 also mediates the oxidative stress response and is critical for growth, conidium formation, and pathogenicity in *M. oryzae* (14). In another plant-pathogenic fungus, *Fusarium oxysporum*, HapX mediates iron homeostasis; therefore, it is essential for rhizosphere competence and virulence of this pathogen (15). In the human pathogen *Cryptococcus neoformans*, Gsb1 is required for oxidative stress response, mating, and virulence (16). The Yap1-involved H_2O_2 detoxification is also associated with virulence in *Ustilago maydis* (17), *Candida albicans* (18), *Alternaria alternata* (19), *Verticillium dahliae* (20), *Aspergillus parasiticus* (21), and *Colletotrichum gloeosporioides* (22). However, in *Cochliobolus heterostrophus* and *Aspergillus fumigatus*, CHAP1 and AfYap1 are associated with the sensitivity against H_2O_2 and menadione but are not essential for virulence (23, 24).

Oomycetes, which include many notorious plant pathogens, are fungus-like organisms; however, they are evolutionarily related to brown algae and belong to the kingdom Stramenopila (25). Systematic analysis identified conventional bZIPs and novel bZIP transcription factors in *Phytophthora infestans* (26), *Phytophthora ramorum*, and *Phytophthora sojae* (27, 28). Several bZIPs from *P. sojae* are upregulated by H_2O_2 treatment (28). Using stable gene silencing analyses, several *P. infestans* bZIPs were found to play roles in protecting the pathogen from hydrogen peroxide-induced injury (26). The novel *P. infestans* bZIP PITG_11668 was nucleus localized, suggesting that it is an authentic transcription factor (26). Pibzp1 from *P. infestans* was functionally characterized, which interacts with a protein kinase and is required for zoospore motility and plant infection (29).

A Per-ARNT-Sim (PAS)-containing bZIP transcription factor was annotated in *P. sojae*, named PsBZPc32 (28), but without functional characterization. In this study, we identified the PsBZPc32 ortholog in *P. litchii* named PlbZIP32. We analyzed the sequences of BZP32 orthologs, as well as the transcriptional profile of *PlBZP32*. We further characterized the function of the *PlbZIP32* gene in oxidative stress response, asexual sporulation, and pathogenicity. Our results showed that *PlBZP32* is required for the asexual development, oxidative stress response, and pathogenicity of *P. litchii*.

RESULTS

***PlBZP32* gene belongs to a bZIP transcription factor family and is upregulated in zoospores, cysts, and late stages of infection.** *P. litchii* BZP32 (*PlBZP32*) is the ortholog of *P. sojae* BZPc32, which encodes a bZIP transcription factor and possess a unique bZIP-PAS structure (30). There are 50 bZIP transcription factors in *P. litchii* based on a search with the Batch CD-search tool (CDD; in NCBI) (31), while PlBZP32 is the only one containing a bZIP-PAS structure (see Table S1 in the supplemental material). Here, we also searched the orthologous proteins from *P. infestans*, *P. parasitica*, *P. ramorum*, *Pythium ultimum*, *Albugo candida*, *Saprolegnia parasitica*, and *Thalassiosira pseudonana* for sequence alignment and phylogenetic analysis. PlBZP32 is a 392-amino acid (aa) protein with a bZIP domain located in aa positions 246 to 294 and a PAS domain located in aa positions 304 to 376 (see Fig. S1 in the supplemental material). We found that the bZIP domain-PAS structure is conserved in all the orthologs examined here (Fig. S1). Phylogenetic analysis showed that these kinds of bZIP transcription factors are widespread and conserved in oomycetes and algae, while *Thalassiosira pseudonana* is outside the oomycete group (Fig. 1).

To investigate the biological function of *PlBZP32*, we first examined the transcriptional profile of the *PlBZP32* gene in various growth stages, including mycelia, sporangia, zoospores, cysts, germinating cysts, and oospores, and in infection stages, including 1.5, 3, 6, 12, 24, and 48 h postinoculation. Our results showed that *PlBZP32* was upregulated in zoospores, cysts, and late stages of infection compared with that of the
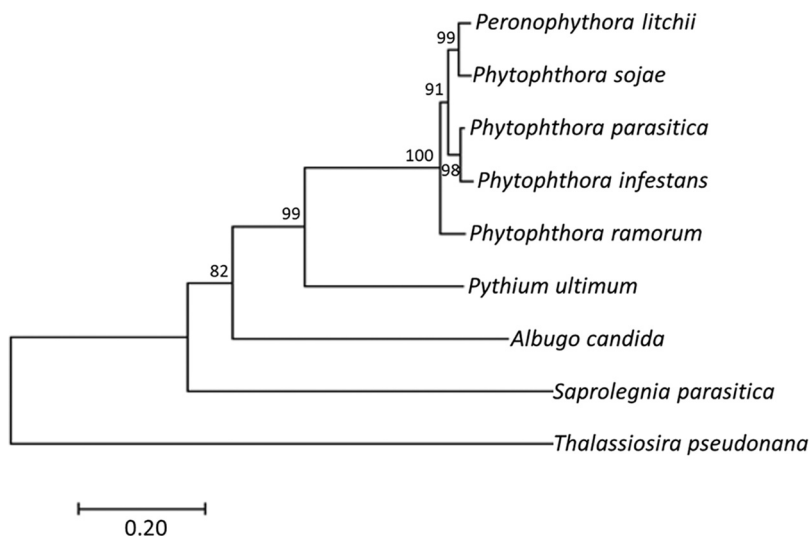
FIG 1 Phylogenetic analysis of PlBZP32 protein and its orthologs. The evolutionary history was inferred using the neighbor-joining method (47). Evolutionary analyses were conducted in MEGA7 (48).

vegetative mycelial growth stage (Fig. 2), suggesting that PlBZP32 might function in these specific stages.

Generation of *PlBZP32*-silenced transformants. To generate the *PlBZP32*-silenced mutants, we used a polyethylene glycol (PEG)-mediated protoplast transformation with a pTORmRFP4 vector carrying an antisense full-length copy of *PlBZP32*. We obtained 182 transformants by antibiotic resistance screening. These transformants were confirmed by genomic PCR and subsequent quantitative reverse transcription PCR (qRT-PCR). Among them, three transformants (T15, T21, and T35) showed significant reduction in *PlBZP32* transcriptional levels in mycelium and zoospore, compared with the wild-type strain SHS3 and the CK strain in which the backbone vector pTORmRFP4 was transformed (Fig. 3A and B). Considering that homology-based transcriptional silencing might cause off-target effects, especially those affecting the expression of neighboring gene(s) (32, 33), we searched the neighboring locus of *PlBZP32* and found one gene (*Pl105397*; GenBank accession number MT396990) 875 nucleotides (nt) away from *PlBZP32*. *Pl105397* encodes a structural maintenance of chromosomes (SMC) protein
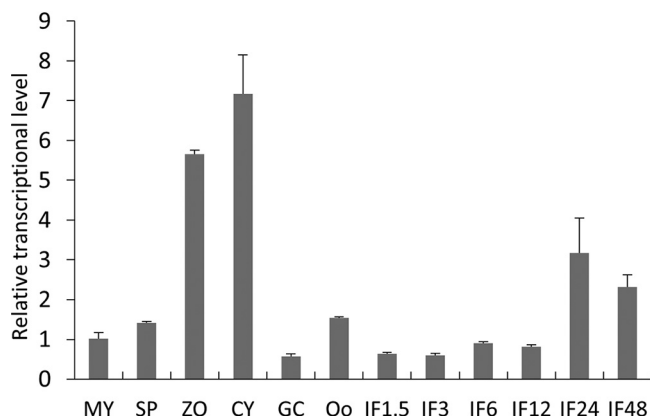

FIG 2 Transcriptional profile of the *PlBZP32* gene. Relative transcriptional levels of *PlBZP32* were determined by qRT-PCR with total RNA extracted from specific stages of the life cycle. MY, mycelia; SP, sporangia; ZO, zoospores; CY, cysts; GC, germinating cysts; Oo, oospores; IF1.5 to IF48, infection stages of 1.5, 3, 6, 12, 24, and 48 h postinoculation. Transcriptional levels were normalized using the MY values as "1," and the *P. litchii ACTIN* gene served as an internal control. The experiment was repeated three times with independent samples.
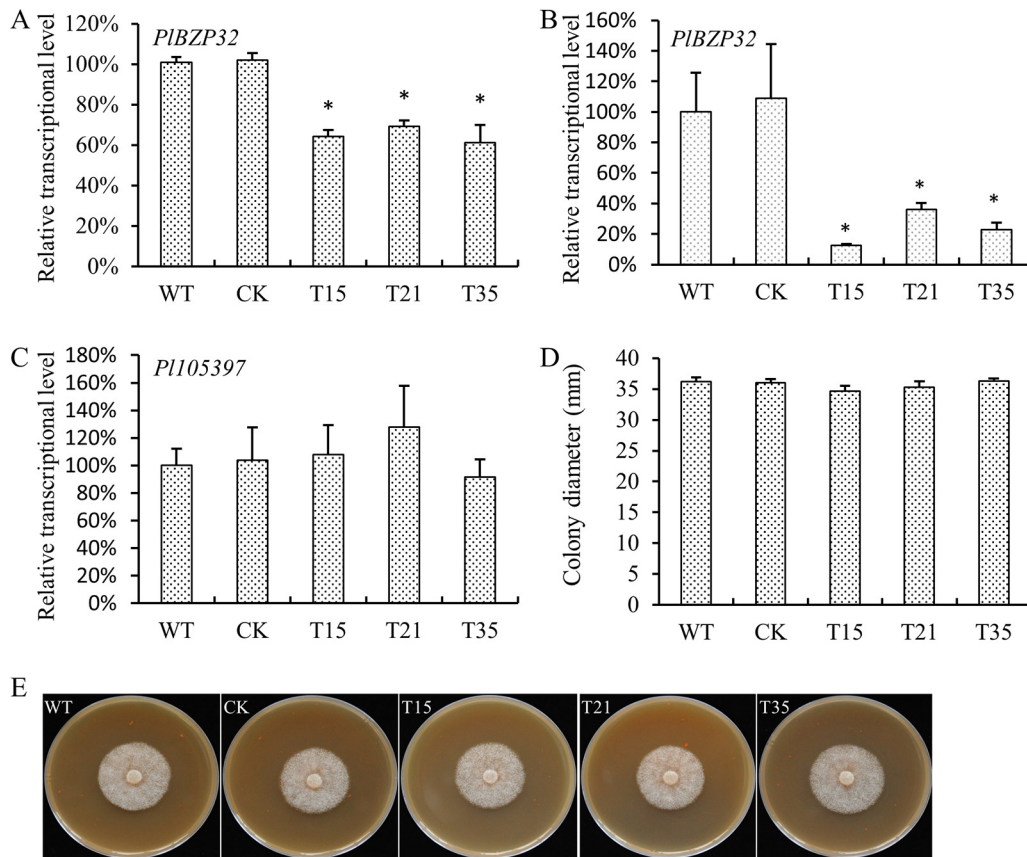
FIG 3 *PlBZP32* was not required for the mycelial growth of *P. litchii*. (A, B) Relative transcriptional levels of *PlBZP32* in wild type (WT), CK (transformed with empty vector), and transformants. *PlBZP32* transcription was normalized to that of the WT value (set as "1.0"). The *P. litchii* ACTIN gene served as an internal control. RNA used in qRT-PCR was extracted from mycelium (A) and zoospore (B). (C) Transcriptional level of *Pl105397* in WT, CK, and *PlBZP32*-silenced transformants. (D) Colony diameter of WT, CK, and the three *PlBZP32*-silenced transformants. Bar charts A to D depict means ± SD derived from 3 independent repeats, each of which contained 3 replicates. Asterisks on the bars denote significant difference ($P < 0.05$) based on Duncan's multiple range test method. (E) WT, CK, and the *PlBZP32*-silenced transformants were inoculated on CJA medium for 3 days at 28°C in darkness and then photographed. These experiments were repeated three times.

containing a Discs-large homologous regions (DHR) domain. The transcriptional level of *Pl105397* in *PlBZP32*-silenced transformants was also examined, and no significant difference was achieved compared with wild-type and CK strains (Fig. 3C).

We checked the growth of these *PlBZP32*-silenced transformants in comparison with wild-type and CK strains. We measured the colony diameter of these strains at 3 days after inoculation and found that the silencing of the *PlBZP32* gene did not affect the growth of *P. litchii* when cultured on carrot juice agar (CJA) medium (Fig. 3D and E).

PlBZP32-silenced mutants were more sensitive to oxidative stress. To investigate whether PlBZP32 is involved in the response to oxidative stress, the *PlBZP32*-silenced mutants and wild-type strain were exposed to H_2O_2 concentrations of 2 and 5 mM, respectively. The *PlBZP32*-silenced mutants displayed a greater growth inhibition rate than the wild-type and CK strains (Fig. 4). These results suggested that *PlBZP32* is required for resistance to oxidative stress.

We also analyzed the transcription levels of *PlBZP32* after treatment with 5 mM H_2O_2. We found no significant changes in the transcription level of *PlBZP32* from 5 minutes to 3 h posttreatment, compared with the untreated control (Fig. 5). These results suggests that the involvement of PlBZP32 in oxidative stress management might be not associated with a transcription-level regulation of this gene.

Silencing of PlBZP32 impaired the pathogenicity of P. litchii. The production and accumulation of reactive oxygen species, the oxidative burst, has been shown to occur
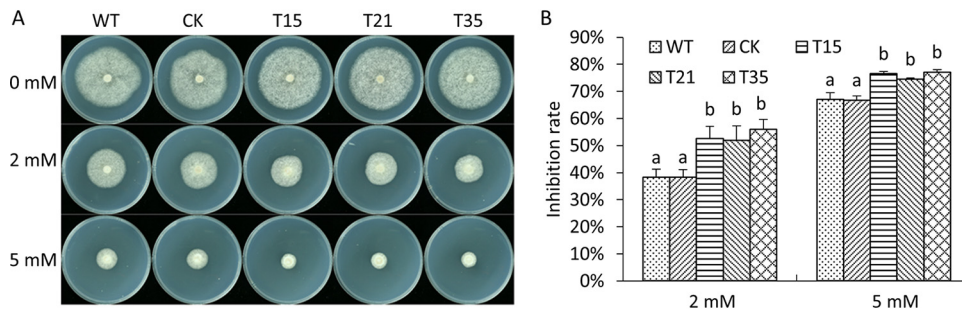
FIG 4 The *PlBZP32*-silenced mutants were hypersensitive to H$_2$O$_2$. (A) The wild type (WT), CK, and *PlBZP32*-silenced mutants were inoculated on Plich medium with or without 2 or 5 mM H$_2$O$_2$ and cultured at 25°C for 7 days. (B) The colony diameters of the tested strains were measured. Wild-type and CK strains were used as controls. The growth inhibition rate was calculated using the following formula: (the diameter of control − the diameter of treated strain)/(the diameter of control) ×100%. The bar chart depicts means ± SD derived from 3 independent repeats, and different letters represent a significant difference ($P < 0.05$) based on Duncan's multiple range test method.

in plant-pathogen interactions, such as H$_2$O$_2$ and O^{2-}, directly acting as antimicrobial agents (34, 35). Given that silencing of *PlBZP32* decreased the resistance of *P. litchii* to H$_2$O$_2$, we next analyzed whether *PlBZP32* is involved in the pathogenicity of this pathogen. *PlBZP32*-silenced mutants and the wild-type strain were inoculated on lychee leaves. At 12, 24, and 36 h after inoculation, we measured the diameter of lesions. The results showed that the lesions caused by *PlBZP32*-silenced mutants were significantly smaller than that caused by wild-type and CK strains (Fig. 6), indicating that *PlBZP32* is required for the full pathogenicity of *P. litchii*.

Silencing of PlBZ*P32* affected the production of sporangia and germination of cysts. Transcriptional profiling analyses showed that the *PlBZP32* gene was upregulated in asexual stages (zoospore and cyst) of *P. litchii*, so we also analyzed the production of sporangia and the germination rate of cysts. Our results showed that *PlBZP32*-silenced mutants produced approximately 40% to 50% more sporangia than wild-type and CK strains (Fig. 7A and B) and each sporangiophore of *PlBZP32*-silenced mutants produced more branch tips and sporangia (Fig. 7C and D). We tested the germination rate of cysts and found it was significantly decreased in the *PlBZP32*-silenced mutants. The germination rate of cysts is less than 50% in *PlBZP32*-silenced mutants, whereas it was approximately 80% in wild-type or nonsilenced strains (Fig. 8). However, the silencing of *PlBZP32* did not significantly affect the length and germination of sporangia (see Fig. S2 and S3 in the supplemental material). Thus, *PlBZP32* may negatively regulate the sporangium production and positively regulate the cyst germination.
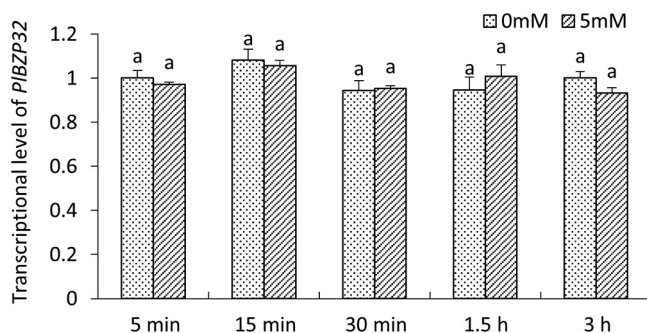

FIG 5 Transcription of *PlBZP32* during H$_2$O$_2$ treatment. *P. litchii* mycelia were treated with 5 mM H$_2$O$_2$ for 5 min, 15 min, 30 min, 1.5 h, and 3 h, before collection for total RNA extraction and qRT-PCR analysis. Expression of the *PlBZP32* gene in the untreated mycelia collected at 5 min was used as a reference and normalized to 1.0. The bar chart depicts means ± SD derived from 3 independent repeats. The same letter on the top of bars represents no significant difference ($P > 0.05$), based on Duncan's multiple range test method.
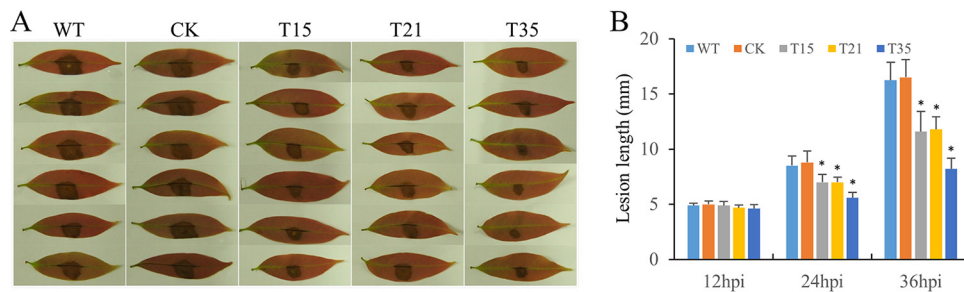
FIG 6 *PlBZP32* was required for full virulence of *P. litchii*. (A) Wild type (WT), CK, and three *PlBZP32*-silenced mutants (T15, T21, and T35) were inoculated on lychee leaves and kept at 25°C in the dark. Photographs were taken 36 h postinoculation (hpi). Representative images for each instance were displayed. (B) Lesion length was measured at 12, 24, and 36 hpi. The values are means ± SD derived from three independent biological repeats (*n* = 12 leaves for each strain). Asterisks denote a significant difference (*P* < 0.05) based on Duncan's multiple range test method.

***PlBZP32* disruption attenuates the activities of extracellular peroxidases and laccases.** Peroxidase and laccase have been reported to be involved in the resistance of ROS and, thus, critical for fungal pathogenicity (14, 36). Given that the *PlBZP32*-silenced mutants displayed reduced pathogenicity compared with the wild-type (WT) and nonsilenced strains, we further analyzed the peroxidase and laccase activities in these strains.

Extracellular peroxidase activity was assessed based on Congo red (CR) degradation (37). As shown in Fig. 9A and C, diameters of the degradation halos caused by the three *PlBZP32*-silenced mutants were reduced compared with the controls. On the other hand, the activities of the extracellular laccase were measured by an oxidation assay of ABTS [2,2′-azinobis (3-ethylbenzothiazolinesulfonic acid)] (37). Three *PlBZP32*-silenced mutants showed a significantly decreased accumulation of ABTS, as visualized by dark purple staining around the mycelial mat, compared with the nonsilenced and wild-type strains (Fig. 9B and D).

DISCUSSION

In oomycetes, bZIP transcription factors are a large protein family. Fifty bZIP transcription factors were identified in *P. litchii*; 71 bZIP transcription factors were predicted from *P. sojae*, of which 45 were confirmed by CDD (NCBI conserved
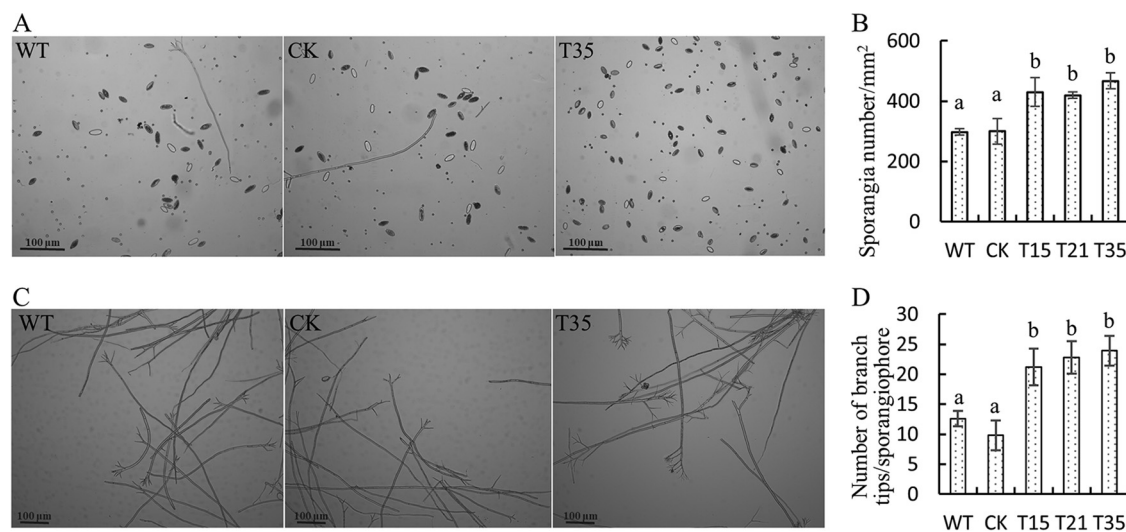
FIG 7 *PlBZP32*-silenced mutants produced more sporangia. (A, B) Sporangia were quantified at 5 days after inoculation. Bar chart represents mean ± SD (C, D). Branch tips of each sporangiophore were counted. These results are derived from three independent biological repeats, each of which contained 5 technical replicates. Different letters represent a significant difference (*P* < 0.05) based on Duncan's multiple range test method.
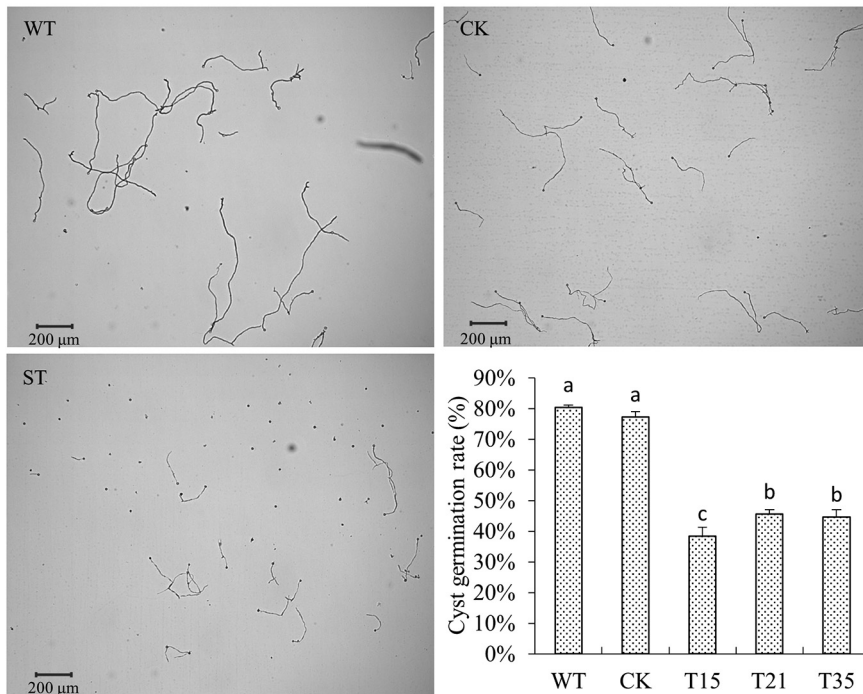
FIG 8 Silencing of *PlBZP32* impaired cyst germination of *P. litchii*. Cyst germination was quantified at 2 h after cyst formation. In the bar graph, mean values present the cyst germination rates of *PlBZP32*-silenced mutants or control strains. Different letters represent a significant difference ($P < 0.05$) based on Duncan's multiple range test method. This experiment was repeated three times. Scale bars, 200 μm.

domain database) or the SMART database (28); 22 bZIPs were identified in *P. ramorum* (27); and 38 bZIPs were identified in *P. infestans* (26). Based on transcriptional analysis, several *P. sojae* infection-associated bZIPs were regulated by H_2O_2 treatment (28). Several bZIP proteins from *P. infestans* were functionally characterized and only Pibzp1 was found essential for virulence (26, 29). In our study, we identified and analyzed a bZIP transcription factor, PlBZP32, which is an ortholog of *P. sojae* BZPc32 and possesses a bZIP-PAS structure.

CRISPR/Cas9-mediated gene disruption in *P. sojae* was established in 2016 (38), and our group also succeeded in disrupting *PlPAE4* and *PlPAE5* genes using this technique (39). However, we failed to knock out *PlBZP32* in *P. litchii*, suggesting that it might be an essential gene. Therefore, we investigated the function of *PlBZP32* by a gene silencing strategy. We obtained only 3 *PlBZP32*-silenced transformants out of 182 transformants screened by antibiotic resistance. The silencing efficiency of this gene is much lower than that of *PlMAPK10*, as reported by our group previously (40). Out of all the G418-resistant transformants, only around 37% contain the empty vector, based on PCR verification. This gene was downregulated by 31% to 39% in the mycelia of the three *PlBZP32*-silenced transformants and 65% to 87% in the zoospores. The efficiency of downregulating this gene expression seems higher in zoospores than in mycelia, likely because the transcriptional level of *PlBZP32* is highly expressed in zoospores compared to mycelia. The gene silencing strategy might affect other genes with high identity in the sequence. However, other *P. litchii* BZP-encoding genes showed less than 51% identity with *PlBZP32*; thus, we assumed that our targeted silencing of *PlBZP32* may not affect the expression of other BZP-encoding genes. On the other hand, homology-based transcriptional silencing may also cause off-target effects on the neighboring gene(s) (32, 33), but we managed to confirm that it did not happen to the annotated gene *Pl105397* that was proximal to the *PlBZP32* locus.

The production of sporangia and germination of cysts are very important for the asexual reproduction, dissemination, and infection of *P. litchii*. In this study, the
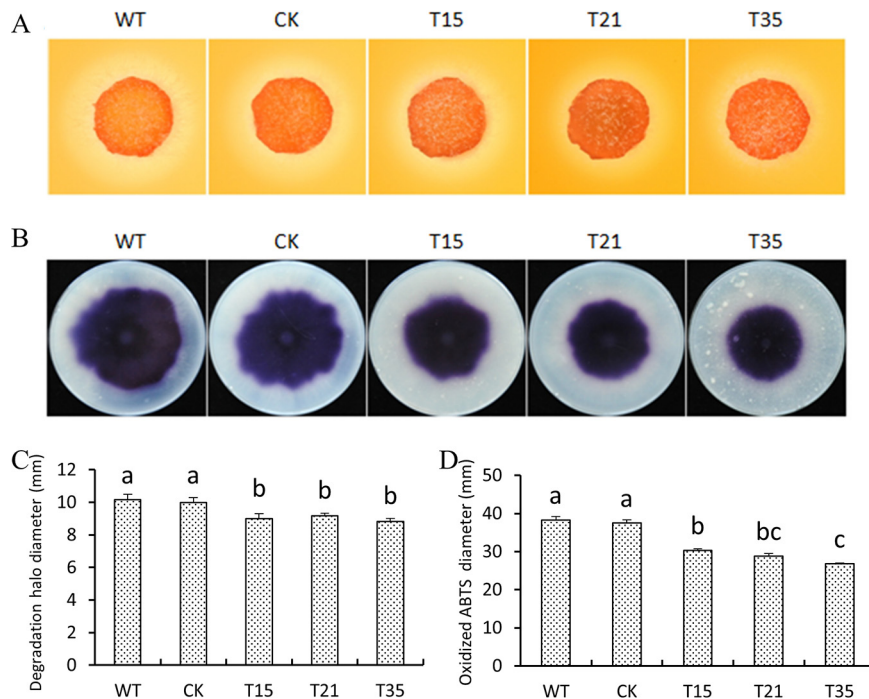
FIG 9 *PlBZP32* regulated activities of extracellular peroxidases and laccases. (A) Peroxidase activity assay. Mycelial mats of WT, CK, and three *PlBZP32*-silenced transformants were inoculated onto solid Plich medium containing Congo red at a final concentration of 500 μg/ml, as an indicator. The discoloration of Congo red was observed after incubation for 1 day. (B) Laccase activity assay. Mycelial mats of the aforementioned strains were inoculated on lima bean agar (LBA) media containing 0.2 mM ABTS for 10 days. (C) The discoloration halo diameters were measured at 1 day postinoculation. (D) The diameters of oxidized ABTS (dark purple) were measured at 10 days postinoculation. Different letters in (C) and (D) bar charts represent a significant difference ($P < 0.05$) based on Duncan's multiple range test method. These two experiments contains three independent biological repeats, each of which contained three replicas.

PlBZP32-silencing mutants produced more sporangia but with reduced germination rate of cysts, and the sporangial germination rate was not affected by silencing of *PlBZP32*. These results suggested that *PlBZP32* might be a negative regulator in the production of sporangia and a positive regulator in the germination of cysts.

We further found that PlBZP32 is required for the full virulence of *P. litchii* and its resistance to oxidative stress caused by H_2O_2. This may indicate that PlBZP32 regulates *P. litchii* virulence via resistance to oxidative stress. In supporting this hypothesis, we found that the enzymatic activities of ROS scavenger peroxidases and laccases were both decreased in the *PlBZP32*-silencing mutants. This finding is consistent with the report of *M. oryzae* MoAP1 (14). However, the direct target genes of the PlBZP32 transcription factor in regulating oxidative resistance and/or pathogenicity of *P. litchii* are unclear.

PAS domain-containing proteins detect a wide range of physical and chemical stimuli and associate with a series of signal transduction systems (41). PlBZP32 contains a PAS domain immediately following the bZIP domain, suggesting that it might interact with some small ligand through a different pathway than other bZIP transcription factors. Further study is needed for characterizing the function of the PAS domain in bZIP transcription factors.

Overall, our study identified a bZIP transcription factor responsible for *P. litchii* development, oxidative stress response, and pathogenicity. At present, we do not fully understand its regulatory mechanism, particularly the function of its PAS domain (and the corresponding ligands/signal molecules) and its downstream target genes. It is also worth further investigating the function diversity (if any) among oomycete species.

MATERIALS AND METHODS

Bioinformatics analysis. The *P. litchii BZP32* (GenBank accession number MT396989) sequence was obtained by a BLAST search using *PsBZPc32* as a bait in NCBI (BioProject ID PRJNA290406) (30). Sequences of BZPc32 orthologs in *P. sojae* (v1.1), *P. ramorum* (v1.1), *P. capsici* (v11.0), *P. infestans*, and *T. pseudonana* (v3.0) genomes were obtained from the DOE Joint Genome Institute (JGI) database (genome.jgi.doe.gov), and the BZP32 ortholog in *Pythium ultimum* was obtained from the *Pythium* Genome Database (pythium.plantbiology.msu.edu). Orthologs of PlBZP32 in *P. parasitica* and *Albugo candida* were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). All of these sequences were listed in Table S2. The sequence alignment (Muscle algorithm) and phylogenetic tree (neighbor-joining algorithm with 1,000 bootstrap replications) were constructed using the MEGA7 program (http://megasoftware.net).

***P. litchii* strain and culture conditions.** *P. litchii* strain SHS3 (wild type) was isolated from Guang-dong Province, China, and cultured on carrot juice agar (CJA) medium (juice from 200 g carrot for 1 liter medium, 15 g agar/liter for solid media) at 25°C in the dark. The *PlBZP32*-silenced transformants were maintained on CJA medium containing 50 μg/ml G418.

Analysis of transcriptional profile of *PlBZP32*. Total RNA was extracted using the total RNA kit (catalog [cat.] number R6834-01; Omega,). Samples included mycelia, sporangia, zoospores, cysts, germinating cysts, oospores, and mycelial mats inoculated on expanded tender lychee leaves of 8 to 10 days old for 1.5, 3, 6, 12, and 24 h (40, 42). The first-strand cDNA was synthesized from total RNA by oligo(dT) priming using a Moloney murine leukemia virus (MMLV) reverse transcriptase kit (number S28025-014; Invitrogen, Carlsbad, CA). The transcription profile of *PlBZP32* was analyzed with qRT-PCR, with *ACTIN* (GenBank accession number MT396988) as an internal control. The primers pairs PlBZPRTF (CCTCTGCGGTGTTCCTTTTC) and PlBZPRTR (CGTGTTGAGCTTCGTGAACA); and PlActF (TCACGCTATTGTTC GTCTGG) and PlActR (TCATCTCCTGGTCGAAGTCC) were used to amplify *PlBZP32* and *ACTIN*, respectively. The relative fold change was calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method (43).

Sporangia were harvested by flooding the mycelia, which had been cultured on CJA media for 5 days, with sterile water, then filtering the subsequent suspension through a 100-μm strainer (44). The suspension was incubated with sterile distilled water at 16°C for 2 h for releasing zoospores. For oospore collection, *P. litchii* strains were inoculated on CJA medium which was covered by an Amersham Hybond membrane. After 10 days, the aerial mycelia were separated from the medium by removing the Amersham Hybond membrane. Then, oospores were scraped from the medium surface and collected.

Transformation of *P. litchii*. The full-length open reading frame of *PlBZP32* was amplified with primers PlBZP32-*Bsiw*I-F (AAACGTACGATGGACTTCACGTCGCCTAATG) and PlBZP32-ClaI-R (AAAATCGATA ATGTCGCGGTCCACAC), using the cDNA from *P. litchii* strain SHS3 as the template. The amplified *PlBZP32* fragment was ligated into the pTORmRFP4 vector digested with ClaI and BsiWI in antisense orientation (45, 46).

PlBZP32-silenced mutants were generated using an established protocol (44). Preliminary transform-ants were screened on CJA media containing 50 μg/ml G418. PCR screening for *PlBZP32*-silencing transformants was performed with primers pHAM34-F (GCTTTTGCGTCCTACCATCCG) and PlBZP32-BsiWI-F. The gene silencing efficiency was evaluated with real-time RT-PCR, using the primers, PlBZPRTF/PlBZPRTR and PlActF/PlActR, as listed above.

Pathogenicity assays on lychee leaves. The 8- to 10-day-old soft expanded lychee leaves were collected from the same plant in an orchard in South China Agricultural University, Guangzhou, Guangdong Province, China. The 5-mm hyphal plugs of the *PlBZP32*-silenced mutants were inoculated on the abaxial side of lychee leaves in a dark box and then were placed in climate room under 80% humidity in the dark at 25°C for 36 h (44). The virulence of each transformant was tested with the wild-type strain (SHS3) and CK strain as positive controls. Lesion length (the longest diameter of the lesion) was measured at 36 h postinoculation. At least three independent repeats were performed for each instance with 12 leaves/repeats.

Extracellular enzyme activity assays. The detection of peroxidase secretion and laccase activity was performed following the reported procedure (37). The experiments were repeated three times independently, with three technical replicates each time.

Data availability. Sequences of *P. litchii ACTIN*, *PlBZP32*, and *Pl105397* were submitted to GenBank under accession numbers MT396988, MT396989, and MT396990, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, JPG file, 0.9 MB.

FIG S2, TIF file, 0.1 MB.

FIG S3, TIF file, 0.2 MB.

TABLE S1, XLSX file, 0.1 MB.

TABLE S2, XLSX file, 0.01 MB.

ACKNOWLEDGMENTS

REFERENCES

1. Jiang YM, Wang Y, Song L, Liu H, Lichter A, Kerdchoechuen O, Joyce DC, Shi J. 2006. Postharvest characteristics and handling of litchi fruit—an overview. Aust J Exp Agric 46:1541–1556. https://doi.org/10.1071/EA05108.

2. Qi W, Chen H, Luo T, Song F. 2019. Development status, trend and suggestion of litchi industry in mainland China. Guangdong Nong Ye Ke Xue 46:132–139.

3. Kao C, Leu L. 1980. Sporangium germination of *Peronophythora litchii*, the causal organism of litchi downy blight. Mycologia 72:737–748. https://doi.org/10.1080/00275514.1980.12021242.

4. Xu L, Xue J, Wu P, Wang D, Lin L, Jiang Y, Duan X, Wei X. 2013. Antifungal activity of hypothemycin against *Peronophythora litchii* in vitro and in vivo. J Agric Food Chem 61:10091–10095. https://doi.org/10.1021/jf4030882.

5. Apostol I, Heinstein PF, Low PS. 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: role in defense and signal transduction. Plant Physiol 90:109–116. https://doi.org/10.1104/pp.90.1.109.

6. Doke N, Miura Y, Sanchez LM, Park HJ, Noritake T, Yoshioka H, Kawakita K. 1996. The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defence—a review. Gene 179:45–51. https://doi.org/10.1016/s0378-1119(96)00423-4.

7. Lamb C, Dixon RA. 1997. The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275. https://doi.org/10.1146/annurev.arplant.48.1.251.

8. Mellersh DG, Foulds IV, Higgins VJ, Heath MC. 2002. H_2O_2 plays different roles in determining penetration failure in three diverse plant-fungal interactions. Plant J 29:257–268. https://doi.org/10.1046/j.0960-7412.2001.01215.x.

9. Torres MA, Dangl JL. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol 8:397–403. https://doi.org/10.1016/j.pbi.2005.05.014.

10. Nurnberger T, Brunner F, Kemmerling B, Piater L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev 198:249–266. https://doi.org/10.1111/j.0105-2896.2004.0119.x.

11. Zhang H, Fang Q, Zhang Z, Wang Y, Zheng X. 2009. The role of respiratory burst oxidase homologues in elicitor-induced stomatal closure and hypersensitive response in *Nicotiana benthamiana*. J Exp Bot 60:3109–3122. https://doi.org/10.1093/jxb/erp146.

12. Gan Y, Zhang L, Zhang Z, Dong S, Li J, Wang Y, Zheng X. 2009. The LCB2 subunit of the sphingolip biosynthesis enzyme serine palmitoyltransferase can function as an attenuator of the hypersensitive response and Bax-induced cell death. New Phytol 181:127–146. https://doi.org/10.1111/j.1469-8137.2008.02642.x.

13. Guo M, Guo W, Chen Y, Dong S, Zhang X, Zhang H, Song W, Wang W, Wang Q, Lv R, Zhang Z, Wang Y, Zheng X. 2010. The basic leucine zipper transcription factor Moatf1 mediates oxidative stress responses and is necessary for full virulence of the rice blast fungus *Magnaporthe oryzae*. Mol Plant Microbe Interact 23:1053–1068. https://doi.org/10.1094/MPMI-23-8-1053.

14. Guo M, Chen Y, Du Y, Dong Y, Guo W, Zhai S, Zhang H, Dong S, Zhang Z, Wang Y, Wang P, Zheng X. 2011. The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. PLoS Pathog 7:e1001302. https://doi.org/10.1371/journal.ppat.1001302.

15. López-Berges MS, Capilla J, Turrà D, Schafferer L, Matthijs S, Jöchl C, Cornelis P, Guarro J, Haas H, Pietro AD. 2012. HapX-mediated iron homeostasis is essential for rhizosphere competence and virulence of the soilborne pathogen *Fusarium oxysporum*. Plant Cell 24:3805–3822. https://doi.org/10.1105/tpc.112.098624.

16. Cheon SA, Thak EJ, Bahn Y, Kang HA. 2017. A novel bZIP protein, Gsb1, is required for oxidative stress response, mating, and virulence in the human pathogen *Cryptococcus neoformans*. Sci Rep 7:4044. https://doi.org/10.1038/s41598-017-04290-8.

17. Molina L, Kahmann R. 2007. An *Ustilago maydis* gene involved in H_2O_2 detoxification is required for virulence. Plant Cell 19:2293–2309. https://doi.org/10.1105/tpc.107.052332.

18. Enjalbert B, MacCallum DM, Odds FC, Brown A. 2007. Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. Infect Immun 75:2143–2151. https://doi.org/10.1128/IAI.01680-06.

19. Lin CH, Yang SL, Chung KR. 2009. The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* in citrus. Mol Plant Microbe Interact 22:942–952. https://doi.org/10.1094/MPMI-22-8-0942.

20. Wang Y, Deng C, Tian L, Xiong D, Tian C, Klosterman SJ. 2018. The transcription factor VdHapX controls iron homeostasis and is crucial for virulence in the vascular pathogen *Verticillium dahliae*. mSphere 3:e00400-18. https://doi.org/10.1128/mSphere.00400-18.

21. Wee J, Hong S, Roze LV, Day DM, Chanda A, Linz JE. 2017. The fungal bZIP transcription factor AtfB controls virulence-associated processes in *Aspergillus parasiticus*. Toxins 9:287. https://doi.org/10.3390/toxins9090287.

22. Li X, Wu Y, Liu Z, Zhang C. 2017. The function and transcriptome analysis of a bZIP transcription factor CgAP1 in *Colletotrichum gloeosporioides*. Microbiol Res 197:39–48. https://doi.org/10.1016/j.micres.2017.01.006.

23. Lev S, Hadar R, Amedeo P, Baker SE, Yoder OC, Horwitz BA. 2005. Activation of an AP1-like transcription factor of the maize pathogen *Cochliobolus heterostrophus* in response to oxidative stress and plant signals. Eukaryot Cell 4:443–454. https://doi.org/10.1128/EC.4.2.443-454.2005.

24. Lessing F, Kniemeyer O, Wozniok I, Loeffler J, Kurzai O, Haertl A, Brakhage AA. 2007. The *Aspergillus fumigatus* transcriptional regulator AfYap1 represents the major regulator for defense against reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. Eukaryot Cell 6:2290–2302. https://doi.org/10.1128/EC.00267-07.

25. Cavalier-Smith T, Chao EE, Lewis R. 2018. Multigene phylogeny and cell evolution of chromist infrakingdom Rhizaria: contrasting cell organisation of sister phyla Cercozoa and Retaria. Protoplasma 255:1517–1574. https://doi.org/10.1007/s00709-018-1241-1.

26. Gamboa-Meléndez H, Huerta AI, Judelson HS. 2013. bZIP transcription factors in the oomycete *Phytophthora infestans* with novel DNA-binding domains are involved in defense against oxidative stress. Eukaryot Cell 12:1403–1412. https://doi.org/10.1128/EC.00141-13.

27. Rayko E, Maumus F, Maheswari U, Jabbari K, Bowler C. 2010. Transcription factor families inferred from genome sequences of photosynthetic stramenopiles. New Phytol 188:52–66. https://doi.org/10.1111/j.1469-8137.2010.03371.x.

28. Ye W, Wang Y, Dong S, Tyler BM, Wang Y. 2013. Phylogenetic and transcriptional analysis of an expanded bZIP transcription factor family in *Phytophthora sojae*. BMC Genomics 14:839. https://doi.org/10.1186/1471-2164-14-839.

29. Blanco FA, Judelson HS. 2005. A bZIP transcription factor from *Phytophthora* interacts with a protein kinase and is required for zoospore motility and plant infection. Mol Microbiol 56:638–648. https://doi.org/10.1111/j.1365-2958.2005.04575.x.

30. Ye W, Wang Y, Shen D, Li D, Pu T, Jiang Z, Zhang Z, Zheng X, Tyler BM, Wang Y. 2016. Sequencing of the litchi downy blight pathogen reveals it is a *Phytophthora* species with downy mildew-like characteristics. Mol Plant Microbe Interact 29:573–583. https://doi.org/10.1094/MPMI-03-16-0056-R.

31. Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Geer LY, Bryant SH. 2017. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res 45:D200–D203. https://doi.org/10.1093/nar/gkw1129.

32. Abrahamian M, Ah-Fong AM, Davis C, Andreeva K, Judelson HS. 2016. Gene expression and silencing studies in *Phytophthora infestans* reveal

infection-specific nutrient transporters and a role for the nitrate reductase pathway in plant pathogenesis. PLoS Pathog 12:e1006097. https://doi.org/10.1371/journal.ppat.1006097.

33. Vu AL, Leesutthiphonchai W, Ah-Fong AMV, Judelson HS. 2019. Defining transgene insertion sites and off-target effects of homology-based gene silencing informs the application of functional genomics tools in *Phytophthora infestans*. Mol Plant Microbe Interact 32:915–927. https://doi.org/10.1094/MPMI-09-18-0265-TA.

34. Low PS, Merida JR. 1996. The oxidative burst in plant defense: function and signal transduction. Physiol Plant 96:533–542. https://doi.org/10.1111/j.1399-3054.1996.tb00469.x.

35. Chen X, Wang X, Zhang Z, Wang Y, Zheng X. 2008. Differences in the induction of the oxidative burst in compatible and incompatible interactions of soybean and *Phytophthora sojae*. Physiol Mol Plant Pathol 73:16–24. https://doi.org/10.1016/j.pmpp.2008.10.002.

36. Mayer AM, Staples RC. 2002. Laccase: new functions for an old enzyme. Phytochemistry 60:551–565. https://doi.org/10.1016/s0031-9422(02)00171-1.

37. Sheng Y, Wang Y, Meijer HJ, Yang X, Hua C, Ye W, Tao K, Liu X, Govers F, Wang Y. 2015. The heat shock transcription factor PsHSF1 of *Phytophthora sojae* is required for oxidative stress tolerance and detoxifying the plant oxidative burst. Environ Microbiol 17:1351–1364. https://doi.org/10.1111/1462-2920.12609.

38. Fang Y, Tyler BM. 2016. Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9. Mol Plant Pathol 17:127–139. https://doi.org/10.1111/mpp.12318.

39. Kong G, Wan L, Deng YZ, Yang W, Li W, Jiang L, Situ J, Xi P, Li M, Jiang Z. 2019. Pectin acetylesterase PAE5 is associated with the virulence of plant pathogenic oomycete *Peronophythora litchii*. Physiol Mol Plant Pathol 106:16–22. https://doi.org/10.1016/j.pmpp.2018.11.006.

40. Jiang L, Situ J, Deng YZ, Wan L, Xu D, Chen Y, Xi P, Jiang Z. 2018. PlMAPK10, a mitogen-activated protein kinase (MAPK) in *Peronophythora litchii*, is required for mycelial growth, sporulation, laccase activity, and plant infection. Front Microbiol 9:426. https://doi.org/10.3389/fmicb.2018.00426.

41. Möglich A, Ayers RA, Moffat K. 2009. Structure and signaling mechanism of Per-ARNT-Sim domains. Structure 17:1282–1294. https://doi.org/10.1016/j.str.2009.08.011.

42. Ye W, Wang X, Tao K, Lu Y, Dai T, Dong S, Dou D, Gijzen M, Wang Y. 2011. Digital gene expression profiling of the *Phytophthora sojae* transcriptome. Mol Plant Microbe Interact 24:1530–1539. https://doi.org/10.1094/MPMI-05-11-0106.

43. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262.

44. Jiang L, Ye W, Situ J, Chen Y, Yang X, Kong G, Liu Y, Tinashe RJ, Xi P, Wang Y, Jiang Z. 2017. A Puf RNA-binding protein encoding gene *PlM90* regulates the sexual and asexual life stages of the litchi downy blight pathogen *Peronophythora litchii*. Fungal Genet Biol 98:39–45. https://doi.org/10.1016/j.fgb.2016.12.002.

45. Whisson SC, Boevink PC, Moleleki L, Avrova AO, Morales JG, Gilroy EM, Armstrong MR, Grouffaud S, van West P, Chapman S, Hein I, Toth IK, Pritchard L, Birch PR. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450:115–118. https://doi.org/10.1038/nature06203.

46. Hua C, Meijer HJG, de Keijzer J, Zhao W, Wang Y, Govers F. 2013. GK4, a G-protein-coupled receptor with a phosphatidylinositol phosphate kinase domain in *Phytophthora infestans*, is involved in sporangia development and virulence. Mol Microbiol 88:352–370. https://doi.org/10.1111/mmi.12190.

47. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.

48. Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054.