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

Guanghui Kong, a Yubin Chen, a Yizhen Deng, a,b Dinan Feng, a Liqun Jiang, a,c Lang Wan, a Minhui Li, a Zide Jiang, a Pinggen Xi a

a Department of Plant Pathology, Guangdong Province Key Laboratory of Microbial Signals and Disease Control, South China Agricultural University, Guangzhou, China
b Integrative Microbiology Research Centre, South China Agricultural University, Guangzhou, China
c Guangdong Province Key Laboratory of New Technology in Rice Breeding, Rice Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China

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 H2O2 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$_2$O$_2$ 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 *Chlorella variabilis* and *Aspergillus fumigatus*, CHAP1 and AfYap1 are associated with the sensitivity against H$_2$O$_2$ 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$_2$O$_2$ 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). Pbzip1 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 *PIBZP32*. We further characterized the function of the PlbZIP32 gene in oxidative stress response, asexual sporulation, and pathogenicity. Our results showed that *PIBZP32* is required for the asexual development, oxidative stress response, and pathogenicity of *P. litchii*.

**RESULTS**

*PIBZP32* gene belongs to a bZIP transcription factor family and is upregulated in zoospores, cysts, and late stages of infection. *P. litchii* BZP32 (*PIBZP32*) 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 PIBZP32 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. PIBZP32 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 *PIBZP32*, we first examined the transcriptional profile of the *PIBZP32* 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 *PIBZP32* was upregulated in zoospores, cysts, and late stages of infection compared with that of the
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.
containing a Discs-large homologous regions (DHR) domain. The transcriptional level of \( \text{Pl105397} \) in \( \text{PlBZP32} \)-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 \( \text{PlBZP32} \)-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 \( \text{PlBZP32} \) gene did not affect the growth of \( P. \text{litchii} \) when cultured on carrot juice agar (CJA) medium (Fig. 3D and E).

**PIBZP32-silenced mutants were more sensitive to oxidative stress.** To investigate whether PIBZP32 is involved in the response to oxidative stress, the PIBZP32-silenced mutants and wild-type strain were exposed to \( \text{H}_2\text{O}_2 \) concentrations of 2 and 5 mM, respectively. The PIBZP32-silenced mutants displayed a greater growth inhibition rate than the wild-type and CK strains (Fig. 4). These results suggested that PIBZP32 is required for resistance to oxidative stress.

We also analyzed the transcription levels of PIBZP32 after treatment with 5 mM \( \text{H}_2\text{O}_2 \). We found no significant changes in the transcription level of PIBZP32 from 5 minutes to 3 h posttreatment, compared with the untreated control (Fig. 5). These results suggest that the involvement of PIBZP32 in oxidative stress management might be not associated with a transcription-level regulation of this gene.

**Silencing of PIBZP32 impaired the pathogenicity of \( P. \text{litchii} \).** The production and accumulation of reactive oxygen species, the oxidative burst, has been shown to occur in...
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 PlBZP32 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.
**PIBZP32** 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 **PIBZP32**-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 **PIBZP32**-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 **PIBZP32**-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...
domain database) or the SMART database; 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$_2$O$_2$ 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, PIBZP32, 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 PIPAE4 and PIPAES genes using this technique (39). However, we failed to knock out PIBZP32 in P. litchii, suggesting that it might be an essential gene. Therefore, we investigated the function of PIBZP32 by a gene silencing strategy. We obtained only 3 PIBZP32-silenced transformants out of 182 transformants screened by antibiotic resistance. The silencing efficiency of this gene is much lower than that of PIMAPK10, 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 PIBZP32-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 PIBZP32 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 PIBZP32; thus, we assumed that our targeted silencing of PIBZP32 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 PIBZP32 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
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 H2O2. 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 PlBZP32 as a bait in NCBI (BioProject ID PRJNA290406) (30). Sequences of BZP3c2 orthologs in P. sojae (v1.1), P. ramorum (v1.1), P. capsici (v11.10), 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 Guangdong 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(T) 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 (CCTCTGGTGTTCTTCTTC) and PlBZPRTFR (CGTGTTGAGCTTCGTGAACA); and PlActF (TCACGCTATTGTTCGCGGTCCACAC), using the cDNA from 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 experiment with three technical replicates each time.

Transformation of P. litchii. The full-length open reading frame of PlBZP32 was amplified with primers PlBZP32-BsiWI-F (AAAGTGACGATGCGACTCGTGCGCTAATG) and PlBZP32-ClAI-R (AAAATCGATAATGTCGCGGTCCACAC), using the cDNA from P. litchii strain SHS3 as the template. The amplified PlBZP32 fragment was ligated into the pTORmRFP4 vector digested with Clal and BsiWI in antisense orientation (45, 46).

PlBZP32-silenced mutants were generated using an established protocol (44). Preliminary transformants 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-BsiWIF. The gene silencing efficiency was evaluated with real-time RT-PCR, using the primers, PlBZPRTF/PlBZP32-ClaI-R and PlActR/PlActF, 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 the 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 PI10S397 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
This research was supported by grants to G.K. from the National Natural Science Foundation of China (31701771) and the Natural Science Foundation of Guangdong Province, China (2017A030310310); grants to P.X. from the Natural Science Foundation of China (31701771 and the Natural Science Foundation of Guangdong Province, China).
of Guangdong Province, China (2019A1515010977 and 2017A020208039) and an earmarked fund to Z.J. from the China Agriculture Research System (CARS-32-11).

We thank Steve Whisson for providing the plasmid pTORMRFp4. We thank Ziqing Zhu for technical assistance and Wenwu Ye for sequence analysis.

REFERENCES

1. Jiang YM, Wang Y, Song L, Liu H, Lichter A, Kerchofechuuen O, Joyce DC, Shi J. 2006. Positive- and negative-characteristics of 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 Peronospora litchii, the causal organism of litchi downy blight. Mycologia 72:73–74. https://doi.org/10.1080/00275514.1980.12021242.

4. Nirl, Xue J, Wu P, Wang D, Lin L, Jiang Y, Duan X, Wei X. 2013. Antifungal activity of hypomyecin against Peronospora 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. Döke N, Miura Y, Sanchez LM, Park HJ, Bahn Y, Kang HA. 2017. A novel bZIP protein, Gsb1, transduces oxidative and osmotic stress signals. FEBS J 284:241–256. https://doi.org/10.1111/1742-4658.13402.

7. López-Berges MS, Capilla J, Turrà D, Schafferer L, Matthijs S, Joehl C, Kong et al. 2015. Arabidopsis vcr1 encodes a novel transcription factor that mediates the oxidative burst and pathogen response. Mol Plant Microbe Interact 28:1230–1239. https://doi.org/10.1094/MPMI-01-16-0030-R.

8. Guo M, Guo W, Chen Y, Dong S, Zhang X, Zheng X, Zheng Y. 2009. The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defense—a review. Gene 498:45–51. https://doi.org/10.1016/j.gene.2012.04.080.

9. Torres MA, Dangl JL. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol 6:3109–3122. https://doi.org/10.1016/j.pbi.2005.05.014.

10. Kao C, Leu L. 1980. Sporangium germination of Peronospora litchii, the causal organism of litchi downy blight. Mycologia 72:73–74. https://doi.org/10.1080/00275514.1980.12021242.

11. Zhang H, Fang Q, Zhang Z, Wang Y, Zheng X. 2009. The role of litchi downy blight. Mycologia 72:73–74. https://doi.org/10.1080/00275514.1980.12021242.
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, Dai T, Dou D, Gijzen M, 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, Li W, Jiang L, S itu J, Xi P, Li M, Jiang Z. 2019. Pectin acetyl esterase 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, S itu J, Deng YZ, Wan L, Xu D, Chen Y, Xi P, Jiang Z. 2018. PIMAPK10, 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(−ΔΔC(T)) Method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262.

44. Jiang L, Ye W, S itu 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 PIM90 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.molev.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.