INTRODUCTION

The phytopathogenic oomycete *Phytophthora sojae* is the causative agent of soybean root rot disease worldwide. Characteristics of the diseased plants are water-soaked decay and damping-off of soybean seedlings, causing plant stand reductions and yield losses of $1–2$ billion each year (Kamoun et al., 2015; Tyler, 2007). The economic impact of *P. sojae*, together with an abundance of available genome and transcriptome data, makes it a suitable model species for the study of oomycete plant pathogens (Tyler, 2007; Ye et al., 2011).

In oomycetes, gene-targeting transgenesis has been typically carried out by regulation at the transcriptional level via internuclear gene silencing (van West et al., 1999, 2008). However, gene silencing-based manipulation is unstable and inefficient. Homologous recombination has been widely used for gene replacement in species across the kingdoms but this approach did not work well before the establishment of the CRISPR/Cas9 system. Recently, genome modification involving CRISPR/Cas9 has been implemented in some oomycetes (Fang & Tyler, 2016; Fang et al., 2017), providing significant technical feasibility for oomycete research. The CRISPR/Cas9 system evolved as an innate component of the adaptive immune response in bacterial and archaeal systems, and is now used as a versatile molecular tool that ensures specific and targeted genome modification in a broad range of eukaryotes. Applications of this genome-editing toolkit include targeted gene mutations and gene replacements, stimulating the inherent potential of functional genomics research of oomycetes in the post-genomic era.

A CRISPR/Cas9-mediated in situ complementation method for *Phytophthora sojae* mutants

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Abstract

*Phytophthora sojae* is an important model species for oomycete functional genomics research. Recently, a CRISPR/Cas9-mediated genome-editing technology has been successfully established in *P. sojae*, which has been rapidly and widely applied in oomycete research. However, there is an emerging consensus in the biological community that a complete functional gene research system is needed such as developed in the investigations in functional complementation carried out in this study. We report the development of an in situ complementation method for accurate restoration of the mutated gene. We targeted a regulatory B-subunit of protein phosphatase 2A (PsPP2Ab1) to verify this knockout and subsequent complementation system. We found that the deletion of *PsPP2Ab1* in *P. sojae* leads to severe defects in vegetative hyphal growth, soybean infection, and loss of the ability to produce sporangia. Subsequently, the reintroduction of *PsPP2Ab1* into the knockout mutant remedied all of the deficiencies. This study demonstrates the successful implementation of an in situ complementation system by CRISPR/Cas9, which will greatly accelerate functional genomics research of oomycetes in the post-genomic era.

KEYWORDS
CRISPR/Cas9, gene complementation, genome editing, oomycete, *Phytophthora sojae*
genomics studies in oomycetes, for instance P. sojae (Ma et al., 2017; Yang et al., 2017), Phytophthora capsici (Miao et al., 2018; Wang et al., 2018), Phytophthora palmivora (Gumtow et al., 2018), and Aphanomyces invadans (Majeed et al., 2018). Nevertheless, with the extensive use of these precision-targeted genome-editing platforms, there are still a few remaining issues for the effective use of editing technologies in oomycetes.

To verify that a mutant phenotype is truly due to the absence of a particular gene, the reintroduction of the native gene into the mutant lacking the gene is required. This can be done by reintroducing a copy of the gene, on a plasmid or at a different location in the genome (Choudhary et al., 2015; Zhou et al., 2016), an approach that has proved to be applicable in oomycetes (Qiu et al., 2020). The advantage of this approach is that only one plasmid is needed and it is relatively easy to express a gene reintegrated at a random location. A disadvantage is that transcription of unrelated parts of the chromosome at the site of insertion may be affected (van Dam & Bos, 2012). Thus, the ability to accurately replace the mutated gene, to test for phenotype restoration, is highly desirable to achieve functional genomic research.

In this study, we implemented an in situ complementation method in P. sojae using a gene encoding a regulatory B-subunit of protein phosphatase 2A. Protein phosphatase 2A (PP2A) is a subgroup of widely conserved serine/threonine phosphatases and plays diverse roles in transcription, translation, differentiation, cell cycle, and signal transduction (Mumbey & Walter, 1993). We identified a regulatory B-subunit of PP2A holoenzyme in P. sojae, called PsPP2Ab1, which shows similarity to CDC55 in Saccharomyces cerevisiae (Healy et al., 1991). A CRISPR/Cas9-mediated gene knockout and subsequent in situ gene complementation were applied to examine its function in mycelial growth, sporangium formation, and virulence in P. sojae. Our results demonstrate successful in situ complementation and reiterate the added value of genome editing for unravelling gene function in Phytophthora.

2 | RESULTS

2.1 | CRISPR/Cas9-mediated gene knockout of PsPP2Ab1

In S. cerevisiae, the regulatory B-subunit, CDC55, is involved in cell morphogenesis, cell wall synthesis, and cytokinesis, and this B-subunit consists of tandem WD-40 motifs, which are evolutionarily conserved in fungi, animals, and plants (Farkas et al., 2007; Healy et al., 1991; Mayer et al., 1991). To gain a better insight into the occurrence of homologs of this B-subunit in P. sojae and other oomycetes, BLAST searches were conducted using the ScCDC55 protein sequence as a query. Homologs were identified in P. capsici, P. sojae, Phytophthora infestans, Phytophthora ramorum, Pythium ultimum, Saprolegnia parasitica, and S. cerevisiae. Phylogenetic analyses showed that most oomycetes have only one homolog of CDC55 (Figure S1), indicating its potentially key role in oomycete species. Hence, we named this sole B-subunit of PP2A, Ps558953, PsPP2Ab1 in the following studies.

To identify the biological function of PsPP2Ab1 in P. sojae, a CRISPR/Cas9-mediated gene-knockout system was developed. Single guide RNAs (sgRNAs) targeting PsPP2Ab1 were designed by web tools (Fang et al., 2017). Two sgRNAs (sgRNA1 and sgRNA2; Figure 1a) were chosen after sgRNA identification, and off-target and sgRNA secondary structure analyses. Two plasmids, the “all-in-one” plasmid, pYF515, and the donor DNA plasmid that contained the entire hygromycin B phosphotransferase gene (hph), flanked by 1 kb of homology arms surrounding the PsPP2Ab1 gene, were used for cotransformation in P. sojae (Figure 1b). Individual G418-resistant transformants were isolated and subjected to genomic DNA extraction and PCR analyses.

Two rounds of screening were used to check homology-directed repair (HDR) mutants and for validating the replaced region (Figure S2a). PCR amplifications in the first round used primers located inside the PsPP2Ab1 gene to detect deletion events. The second round of PCRs used primers located outside the PsPP2Ab1 homology arms and within the hph gene to detect homologous recombination events. Of 69 primary transformants screened, 15 exhibited no bands of PsPP2Ab1 (Figure S2b), indicating that deletion events may have occurred. Of these potential candidates, six exhibited positive bands in the second round (Figure S2c), indicating that the original PsPP2Ab1 open reading frame (ORF) had been replaced by the hph ORF. Sanger sequencing across the junctions of the flanking sequences and hph was also consistent with the replacement of the PsPP2Ab1 ORF with the hph gene (Figure S3a).

2.2 | CRISPR/Cas9-mediated in situ gene complementation of ∆PsPP2Ab1

To confirm that the disruption of PsPP2Ab1 was responsible for the phenotype described below, an in situ complementation assay was conducted to complete the gene-editing system in P. sojae. After culturing the mutants for at least three generations in antibiotic-free plates for more than 2 weeks, the mutants could no longer grow on G418 antibiotic plates (Figure S4a). PCR analyses showed that the ORF of NPTII was detectable in genomic DNA of two PsPP2Ab1 knockout mutants, but undetectable in cDNA of all PsPP2Ab1 knockout mutants (Figure S4b). These results suggest that these mutants can undergo a second transformation with G418 as the selection marker. To avoid persistent cleavage of the newly transformed PsPP2Ab1 gene from the previous RNA template, the ORF sequence of PsPP2Ab1 with two mutated sgRNA sites was exploited as donor DNA in the complementation system (PsPP2Ab1 M; Figure 1a). This mutated donor DNA vector, along with Cas9 and the new RNA template targeting the hph gene, were co-transformed into the P. sojae knockout line (∆PsPP2Ab1-M1 in this study) (Figure 1c).

Two rounds of screening were used for examining complemented strains (Figure S5a). PCR amplifications in the first round used primers located outside the PsPP2Ab1 ORF to detect deletion events of hph (F4/R4; Figure S5a). Subsequently, the second round of PCRs used primers located outside the PsPP2Ab1 homology arms and within the PsPP2Ab1-M ORF to detect the in situ complementation events (F5/
R5 and F6/R6; Figure S5a). Of 48 primary transformants screened, 10 exhibited no bands of \( hph \) (Figure S5b), indicating that deletion events had occurred. Of these potential candidates, six exhibited positive bands in the second screening round (Figure S5c), indicating that the \( PsPP2Ab1 \)-M ORF was reintroduced. Sanger sequencing across the junctions of the flanking sequences and the \( PsPP2Ab1 \)-M ORF, and the inner \( PsPP2Ab1 \)-M ORF mutated sites were also consistent with reintroduced \( PsPP2Ab1 \)-M ORF (Figure S3b,c), suggesting that \( hph \) was accurately replaced with the mutated \( PsPP2Ab1 \) ORF.

Evaluating all of the strains, analysis of genomic DNA confirmed that \( PsPP2Ab1 \)-M was amplified in the wild-type strain, the empty vector (EV) line, and the complemented strain of \( \Delta PsPP2Ab1 \), but not in the \( \Delta PsPP2Ab1 \) and \( \Delta PsPP2Ab1 \)-EV strains (in which the \( PsPP2Ab1 \) knockout was not successful) (Figure S3d). In addition, the semiquantitative assay showed that \( \Delta PsPP2Ab1 \) and \( \Delta PsPP2Ab1 \)-EV no longer displayed the RNA expression of \( PsPP2Ab1 \) (Figure S3e). In summary, the CRISPR-mediated system was used to achieve not only the gene knockout but also subsequent in situ gene complementation, which builds a solid technological support for genomic functional research in oomycetes.

2.3 | Complementation of \( PsPP2Ab1 \) restored mycelial growth and sporangia formation

To investigate the biological function of \( PsPP2Ab1 \) in different developmental phases, we analysed the phenotypes of \( PsPP2Ab1 \) knockout mutants. We show the results from one representative strain, \( \Delta PsPP2Ab1 \). The recipient wild-type strain (P6497) and the EV line were included as controls. Compared to the wild-type, the \( \Delta PsPP2Ab1 \) mutant formed a slow-growing colony. The average growth rate of the P6497, EV, and complemented (\( \Delta PsPP2Ab1 \)-C) colonies was around 0.29 cm/day. Under the same conditions, the growth rate of the \( \Delta PsPP2Ab1 \) mutant and \( \Delta PsPP2Ab1 \)-EV was only 0.22 cm/day (Figure 2a,b). Intriguingly, the \( \Delta PsPP2Ab1 \) mutant lost the ability to produce sporangia (Figure 2a,c). However, the
PsPP2Ab1 knockout did not affect the capacity of oospore formation in P. sojae (Figure 2a,d).

To verify whether the in situ complemented strains recovered these defective phenotypes, we analysed the phenotypes of complemented knockout strains simultaneously. We also show the results from one representative strain, ∆PsPP2Ab1-C, along with the empty vector line, ∆PsPP2Ab1-EV, in which the knockout mutant was not successfully complemented with PsPP2Ab1. Our results show that the complemented strain displayed an identical growth rate and sporangium formation compared to the wild-type strain (Figure 2a,b,c). In addition, all the knockout mutants (ΔPsPP2Ab1-M1 to M6) and in situ complemented lines (ΔPsPP2Ab1-C1 to C6) generated in this study displayed consistency in growth rates (Figure S6), which further confirms the reliability of this CRISPR-mediated gene knockout and in situ complementation system. Taken together, the loss of PsPP2Ab1 led to severe defects in growth and sporangium formation, and subsequent in situ complementation compensated for these deficiencies in the developmental phases.

2.4 | Complementation of PsPP2Ab1 recovered virulence

To test the effects of the PsPP2Ab1 deletion and complementation during plant infection, plugs of mycelium of all the strains were inoculated onto hypocotyls (Hefeng 47). The P6497, EV, and ∆PsPP2Ab1 strains produced typical water-soaked decay around the disease lesions on the infected seedlings, whereas the ΔPsPP2Ab1 mutant and the ΔPsPP2Ab1-EV showed significantly decreased lesion size (Figure 3a). Measurements of the relative P. sojae

![Figure 2](image-url) Mycelial growth and sporangium production of PsPP2Ab1-knockout and complemented strains. (a) Growth characteristics after 5 days on V8 medium and microscopic visualization of oospores and sporangia of the wild-type (P6497), empty vector control line (EV), PsPP2Ab1-knockout (ΔPsPP2Ab1), empty vector control line of ΔPsPP2Ab1 (ΔPsPP2Ab1-EV), and complemented strain (ΔPsPP2Ab1-C). (b) Growth rates in cm/day on V8 medium. (c) The relative numbers of sporangia and (d) oospores with the number in wild-type strain P6497 set at 1. All experiments were repeated three times with similar results. Scale bar, 50 μm. **Significant difference at p < .01
biomass in infected soybean seedlings revealed hardly any pathogen biomass in the ∆PsPP2Ab1- or ∆PsPP2Ab1-EV-infected tissues with levels less than 5% compared to the levels found in P6497-, EV-, and ∆PsPP2Ab1-C-infected tissues (Figure 3b). The infection assay was also performed using wounded seedlings and similar results were obtained; the ∆PsPP2Ab1 mutant and the ∆PsPP2Ab1-EV also developed smaller lesions than the P6497, EV, and ∆PsPP2Ab1-C strains (Figure 3a,c). Overall, the mutants inoculated on wounded seedlings displayed more severe disease symptoms than on unwounded seedlings (Figure 3), suggesting the loss of virulence is partly associated with penetration. To examine whether all the knockout mutants and in situ complemented lines exhibited similar disease phenotypes, we also analysed the virulence of all strains generated in this study. All the knockout mutants (∆PsPP2Ab1-M1 to M6) and the complemented strains (∆PsPP2Ab1-C1 to C6) displayed consistency in virulence (Figure S7), which further confirms the reliability

![Figure 3](image-url)

**FIGURE 3** Pathogenicity test of PsPP2Ab1-knockout and complemented strains. (a) Lesions on soybean (cultivar Hefeng 47) at 72 hr postinoculation (hpi) of 4-day-old aetiolated hypocotyls with hyphae of the wild-type (P6497), empty vector control line (EV), PsPP2Ab1-knockout (∆PsPP2Ab1), empty vector control line of ∆PsPP2Ab1 (∆PsPP2Ab1-EV), and complemented strain (∆PsPP2Ab1-C). Experiments were repeated three times with similar results. In the panels labelled “Wounded”, the hypocotyls were wounded prior to inoculation with hyphae. (b, c) Relative pathogen biomass in inoculated unwounded or wounded hypocotyls expressed as the ratio between the amounts of Phytophthora sojae DNA and soybean DNA detected at 72 hpi with the ratio P6497/soybean set at 1. **Significant difference at \( p < .01 \)
of this CRISPR-mediated gene knockout and in situ complementation system. The above data show that the pathogenic impairment of the PsPP2Ab1 knockout mutant was recovered by the in situ complementation.

2.5 | Suppression of PsCDC14 expression in ΔPsPP2Ab1 was restored in complemented strain

Considering that the PsPP2Ab1-knockout mutant is defective in sporangium formation, we examined whether PsPP2Ab1 is engaged in regulating genes whose expression is associated with sporangium formation in P. sojae. In P. sojae and P. infestans, CDC14 regulates nuclear behavior at an early stage of sporulation, is a vitally important gene during sporangium formation, and is up-regulated in sporangial stages (Ah Fong & Judelson, 2003; Zhao et al., 2011). To investigate whether PsCDC14 is regulated by PsPP2Ab1, the relative mRNA level of PsCDC14 in sporangial stages was compared to the wild-type strain and the PsPP2Ab1-knockout mutant using quantitative reverse transcription PCR (RT-qPCR) assays, with PsPP2Ab1 and a constitutively expressed ribosome gene (PsRibo1) included as controls. Compared to P6497, the relative mRNA levels of PsPP2Ab1 in ΔPsPP2Ab1 and ΔPsPP2Ab1-EV were reduced to less than 1%, whereas in ΔPsPP2Ab1-C they reverted to wild-type levels (Figure 4a), confirming successful knockout and complementation of PsPP2Ab1. Importantly, the expression level of PsCDC14 exhibited a significant reduction (80%) in ΔPsPP2Ab1 and ΔPsPP2Ab1-EV while in ΔPsPP2Ab1-C it showed a return to the wild-type level (Figure 4c). PsRibo1 exhibited no changes in any strain (Figure 4b). Collectively, the data indicate that PsPP2Ab1 may be involved in regulating the expression of PsCDC14 during the sporangium formation process, and that the in situ reintroduction of PsPP2Ab1 can compensate for its own expression levels but also its downstream effects.

3 | DISCUSSION

A substantial number of whole-genome sequences of oomycetes are now available, and further sequences are being determined (Kamoun et al., 2015). Consequently, there are ever-increasing technical demands for genome engineering in functional genomics studies. Recently, revolutionary progress in the CRISPR/Cas9-induced gene-editing system has been made in oomycetes (Fang & Tyler, 2016; Fang et al., 2017). Here, we have advanced the CRISPR/Cas9-mediated in situ complementation technology for the homologous gene-knockout system in P. sojae.

The complementation technology reported in this study demonstrates that a gene can be reintroduced by inserting it accurately into a designated location, which is almost identical to the wild-type situation. A PCR strategy containing two rounds of screening mutants was conducted in this system. As a consequence, one can easily identify desired transformants in a simple yet reliable manner when analysing single colonies after transformation. Hence, in this research a convenient and accurate gene knockout and in situ complementation system was established for P. sojae.

An earlier study of gene-editing systems introduced the ORF of NPTII as the donor DNA but also as a selective marker gene (Fang & Tyler, 2016). In this study, the ORF of hph was selected as the donor DNA with NPTII kept as a selective marker gene. As the bacterial hph is a foreign sequence to oomycetes, the in situ gene complementation can be accomplished without interfering with the function of the selection marker. For in situ complementation, the recombinant hph sequence in knockout mutants in our trial gene system was re-targeted to regain PsPP2Ab1; thus, the in situ complementation of any gene would be possible by targeting this hph sequence so other genes are not disturbed. From another perspective, hygromycin-resistant transformants can be obtained in P. capsici, P. parasitica, and P. infestans (Bailey et al., 1991; Judelson et al., 1991), but this does not apply to all Phytophthora species, for instance P. sojae. Even for those
in which it is possible to use hygromycin as selection marker, it could be that expression of the hph gene from the promoters of insertion locations may not be sufficient for hygromycin selection. Therefore, the ORF of hph was inserted after the promoter of PsPP2Ab1 and hph sequence was involved only as the donor DNA in this system.

This study revealed a functional regulatory B-subunit of PP2A in P. sojae that participates in sporangium formation. Strains of ΔPsPP2Ab1 complemented by this new in situ gene reintroduction method displayed identical phenotypes to the wild-type strain, confirming its crucial biological function. The first case of complementation of CRISPR-Cas9 knockout mutants was reported in P. capsici (Wang et al., 2019). The current research more precisely demonstrates that a complemented gene can be replaced into the genome of oomycetes in a site-specific manner, representing one of the best protocols for gene complementation in terms of functional genomics research.

In eukaryotic species, regulatory B-subunits of PP2A can be clustered into four distinct groups that have a lack of sequence similarity and structural association, although they recognize similar segments of the A-subunit (Janssens & Goris, 2001). The Arabidopsis thaliana genome contains three regulatory B-subunit families including the 55 kD B, the 52–74 kD BI, and the 72–130 kD BII subunit families, which are commonly referred to as the "specificity unit" that determines the target specificity of the trimeric PP2A holoenzyme (Farkas et al., 1997). In S. cerevisiae, the regulatory B-subunits are responsible for both the substrate specificity and the localization of the PP2A complexes. There are two types of regulatory B-subunits in the S. cerevisiae genome: the B- or BI-subunit encoded by the CDC55 and RTS1, respectively (Gentry & Hallberg, 2002).

In this research, the sole B-subunit of PP2A encoded by PsPP2Ab1 in the P. sojae genome belongs to 55 kD B-subunit group, which exists in all eukaryotic kingdoms. Furthermore, this regulatory B-subunit has been shown to be important for sporangium formation in P. sojae. One interesting finding was that this regulatory B-subunit appears to be highly expressed in sporangial stages (Figure 8). The knockout of PP2A affected the expression level of a cell division cycle gene, PsCDC14, which was also up-regulated during sporangial stages (Zhao et al., 2011). These data corroborate previous findings that CDC14 genes are required for sporangium formation in oomycetes (Ah Fong & Judelson, 2003; Zhao et al., 2011). In S. cerevisiae, the phosphorylated PP2A-subunit regulates timely CDC14 activation during metaphase (Jativa et al., 2019). Thus, the regulatory mechanism underlying PsPP2Ab1 and PsCDC14 remains an important research subject in oomycetes.

In summary, a CRISPR-mediated gene knockout and in situ complementation system was successfully established and applied to verify that a conserved PP2A regulatory B-subunit plays a vital role in developmental phases and growth, and in regulating a cell division cycle gene of P. sojae. This new genome-engineering system should greatly enhance applications in oomycete functional gene research and we envision the wide application of this enabling technology in the gene-editing field in oomycetes.

4 | EXPERIMENTAL PROCEDURES

4.1 | P. sojae strains and culture conditions

The genome-sequenced P. sojae strain P6497 (race 2), generously provided by Dr Brett Tyler (Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR, USA), was used as the wild-type strain in this study. The wild-type strain and all transgenic lines were routinely grown on 10% (vol/vol) V8 agar medium at 25 °C in the dark. The asexual life stages, such as vegetative hyphae, sporulating hyphae, and zoospores, were collected as previously described (Hua et al., 2008). The growth rates of the transformants were calculated using cultures growing on 10% (vol/vol) V8 agar medium. Sporulating hyphae were prepared by repeatedly washing 2-day-old mycelia grown in 10% (vol/vol) V8 broth with sterile distilled water (SDW), followed by incubation in the dark at 25 °C for 4–8 hr until sporangia developed.

4.2 | Targeted gene deletion and complementation

Gene deletion mutants were generated using the CRISPR-mediated gene replacement strategy (Fang & Tyler, 2016). The hph gene, ligated with two 1.0-kb fragments flanking the target gene, was used as donor DNA in HDR (Figure 1b). For complementation, the knockout mutant was transformed using NPTII as a selection marker. The entire gene-coding region with mutated sgRNA sites, inserted into two 1.0-kb fragments flanking the target gene, was used as the donor DNA (Figure 1c). We transformed P. sojae using polyethylene glycol-mediated protoplast transformation (Hua et al., 2008). Putative transformants were selected by growth on 10% (vol/vol) V8 medium containing 50 μg/ml geneticin and screened by PCR using the primer combinations as shown in Figure S2a.

The primer set F1/R1 (Table S1), located within the PsPP2A1 ORF, was used to screen for the deletion of PsPP2Ab1 in the genome of putative transformants. The primer sets F2/R2 and F3/R3 (Table S1) were used to detect homologous recombination events. In the complementation process, the primer set F4/R4 (Table S1) was used to screen for the deletion of hph in the genome of putative transformants. The primer sets F5/R5 and F6/R6 (Table S1) were used to detect homologous recombination events.

4.3 | RNA extraction and gene expression analyses

Total RNAs from distinct stages of the life cycle of P. sojae (vegetative hyphae, sporulating hyphae, zoospores, cysts, germinated cysts, ...
and stages of infection) were extracted as described previously (Hua et al., 2008; Yang et al., 2013). RNA integrity was tested via agarose gel electrophoresis. Before cDNA synthesis, all RNA samples were treated with DNase I using a DNase kit, following the manufacturer’s protocol. First-strand cDNA was synthesized from 1–5 µg total RNA using oligo(dT) primers with an M-MLV reverse transcriptase kit (Takara Bio, Inc.), following the manufacturer’s protocol. Semiquantitative RT-PCR was performed to amplify the actin A gene (ACT-RTF/R) and PsPP2Ab1 (PsPP2Ab1-QRT-F/R). SYBR Green RT-qPCR assays were used to measure PsPP2Ab1 expression using the primer pair PsPP2Ab1-QRT-F/R (Table S1). All reactions were performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The results were analyzed using the ABI 7500 sequence detection software. Relative gene expression levels were determined using actin A (primers ACT-RTF/R; Table S1) levels as internal controls. The means and standard deviations were calculated using data from three replicates.

4.4 | Growth, oospore, and sporangium production

To determine the growth rate, all strains were grown on V8 medium at 25 °C in the dark. The colony diameters on V8 medium were measured over 4 days and used to calculate the average growth rates (mm/day).

To monitor and quantify oospore production, strains were grown on lima bean agar (LBA) medium at 25 °C in the dark. After 10 days the cultures were examined by microscopy. Three blocks of 1 cm² were cut from the medium and in each block three random fields at 40x magnification were selected for counting the number of oospores.

To quantify sporangium production, 10% (vol/vol) V8 broth was inoculated with three mycelial disks (7 mm in diameter) cut from a culture and incubated for 48 hr at 25 °C in the dark. The mycelia were rinsed twice with sterile distilled water, and then flooded with sterile distilled water and left for 8 hr to stimulate sporangia formation. Then this was gently mixed in a blender to obtain a homogenous suspension. Subsequently, three samples of 100 µl were taken and the number of sporangia in each sample was counted by microscopic examination at 40x magnification. All assays were repeated at least three times.

4.5 | Virulence assays

The soybean cultivar Hefeng 47, which is susceptible to P. sojae strain P6497, was grown in plastic pots containing vermiculite at 25 °C for 4 days in the dark. Zoospores were retrieved as described previously (Hua et al., 2008) and diluted to a concentration of 100 zoospores/10 µl. Aetiolated seedlings were inoculated by pipetting 10 µl zoospore suspension on the hypocotyls, and maintained in a climate-controlled room at 25 °C and 80% relative humidity in the dark. Symptoms were evaluated at 3 days postinoculation (dpi), and photographs were taken. Virulence was quantified by determining the ratio of P. sojae DNA to soybean DNA in the infected plants, as measured by quantitative PCR. All assays were repeated independently at least three times.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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