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An AGC kinase, PgAGC1 regulates virulence in the entomopathogenic oomycete *Pythium guiyangense*

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ABSTRACT

Mosquitoes are the most important medical species by transmitting some of deadly infectious diseases to human. In recent years extensive studies of vector control have been focused on biological control agents due to the grave issue raised by continuous application of chemical compounds. *Pythium guiyangense* X.Q. Su was first isolated from infected larvae of *Aedes albopictus* in 2006 in China and it has been proven to be a promising mosquito control agent. However, the molecular mechanisms of this oomycete pathogenic to mosquitoes are still not clear. In this study, we identified a new gene from the genome of *P. guiyangense*, PgAGC1 that belongs to the AGC kinase group and we found that the transcriptional expression levels of this gene were significantly up-regulated during infection of mosquito *Culex pipiens pallens*. Disruption of the PgAGC1gene via genetic transformation methods affects colony growth and stress responses and results in reduced mortality and infection rates. All the evidence revealed that, besides its role in growth and stress resistance, PgAGC1 is putative determinants of *P. guiyangense* virulence. The results of this study become of particular importance in understanding the mechanisms of oomycete–mosquito interactions.

1. Introduction

Mosquitoes are members of the family *Culicidae*, which can transmit some of the most devastating and harmful infections to human, including malaria, yellow fever, West Nile virus, dengue fever, filariasis, Zika virus and other arbovirus, rendering *Culicidae* the deadliest animal family in the world (WHO, 2017; Musso and Gubler, 2016). To curb nuisance biting by mosquitoes and their transmission of parasitic diseases, chemical insecticides have been commonly used as the solution. However, the application of residual synthetic insecticides has entailed ever-developing resistance, environmental pollution and toxicity to human and non-target organisms (Scholte et al., 2004). It is therefore not surprising that research interests have been shifted from chemical compounds to more benign biological control agents, such as bacteria, nematodes, and entomopathogenic fungi (Scholte et al., 2003). So far, only *Lagenidium giganteum* is currently produced commercially for mosquito control (Scholte et al., 2004).

A new strain of oomycetes, *P. guiyangense* X.Q. Su, was isolated from infected larvae of *Aedes albopictus* from Guizhou, China in 2006 (Su, 2006). Previous experiments have proven that *P. guiyangense* is highly virulent to mosquito larvae, with a wide range of host species (Yu et al., 2008). Field evaluation on the safety of popular plant and animal species suggests that *P. guiyangense*is is safe to non-target organisms (Zhao and Su, 2008). It also adapts different ecological environment and can be easily mass-produced (Zhao and Su, 2008). All these characteristics exhibited by *P. guiyangense* make it a potential vector control agent. Classically, *P. guiyangense* belongs to the genus *Pythium* (kingdom *Stramino-pila*; phylum *Oomycota*; class *Oomycetes*) commonly referred as water molds. *Pythium* species occurred mostly as plant pathogens, including the most aggressive and devastating species, *P. aphanidermatum* (St Leger and Lovett, 2016). Besides these species, *Pythium undulatum* infects fish (Horner et al., 2012) and *Pythium insidiosum* causes chronic skin lesions in mammals

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(Rujirawat et al., 2015), but few studies have been published about this genus infecting mosquitoes. The detailed knowledge of *P. guiyangense* pathogenesis will provide valuable information for understanding interactions between mosquitoes and *Pythium*.

Protein kinases regulate most of the signal transduction in eukaryotic cells by phosphorylation of substrate activity (Manning et al., 2002). The AGC kinases represent one of the most evolutionarily conserved groups (Sobko, 2006). In fungi and multicellular eukaryotes, AGC kinases mediate signaling events that affect growth and morphogenesis (Garcia et al., 2012). Furthermore, various reports show that AGC kinases are one of the major mechanisms by which eukaryotic cells sense and respond to environmental stresses (Alves de Castro et al., 2016; Garcia et al., 2012; Peti and Page, 2013). Additionally, members of AGC kinases are important for virulence in human, plant and insect pathogens (Alves de Castro et al., 2016; Berndt et al., 2010; Fang et al., 2009).

To establish a direct role of the AGC pathway in infection-related morphogenesis in *P. guiyangense*, the gene expression level of the kinase, PgAGC1 was detected, and the results revealed that PgAGC1 was up-regulated during *P. guiyangense* infection of *Culex pipiens pallens*. Silencing of the gene PgAGC1 resulted in significantly reduced mosquito mortality rate in comparison with the wild-type strain of *P. guiyangense*. Moreover, the attenuated PgAGC1 strains exhibited a decreased growth rate and increased resistance to H_2O_2. These investigations demonstrated the critical role of the gene PgAGC1 from *P. guiyangense* in pathogenicity during mosquito larvae infection.

2. Materials and methods

2.1. P. guiyangense *and mosquito strains*

P. guiyangense Su strain (Guiyang Medical University, Guiyang, China), designated the wild-type (WT), was used for this study. Both the WT and transformants were maintained on 10 % vegetable juice (V8) medium in the dark at 25 °C ± 1 °C. The Nanjing laboratory strain of *Cx. pipiens pallens* was obtained from Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China, and was used for conducting the following experiments. The mosquito larvae were fed with duck liver powder and reared at room temperature of 25 °C ± 1 °C with a 16 h light and 8 h dark photoperiod and a relative humidity of 70 %. The adults were provided with 10 % sugar water solution and the female adults were fed with mouse blood once a week before they lay eggs.

2.2. *Bioinformatics analysis of PgAGC1*

The DNA and amino acid sequences of PgAGC1 were obtained from the *P. guiyangense* genome database (unpublished). The PgAGC1 protein sequence was used as the query sequence to blast against the genomes of *Pythium ultimum*, *Phytophthora sojae*, *Ph. ramorum*, *Ph. infestans*, *Hyaloperonospora parasitica*, *Albugo laibachii*, and *Saprolegnia parasitica* using Blastp with a cutoff of 1E-5. The functional domains of PgAGC1 protein were predicted in the Pfam database (http://pfam.xfam,org). The phylogenic tree was constructed using MEGA 5.0 software (Tamura et al., 2011) with the neighbor joining method and 1000 replicates for bootstrap analysis.

2.3. *Plasmid construction and* P. guiyangense *gene transformation*

A 1152 bp fragment was amplified by PCR with Phanta Super-Fidelity DNA Polymerase (Vazyme, NanJing, China) using primers of *PKA03242*-F and *PKA03242*-R (Table S1) from genomic DNA of *P. guiyangense* under the following conditions: 30 s at 95 °C; 15 s at 95 °C, 15 s at 58 °C and 1 min at 72 °C for 32 cycles; and 5 min at 72 °C. After amplification, the restriction enzymes SmaI and KpnI were used to digest the PCR products; then, the fragments were inserted into vector pHAM34. The constructed plasmids were then transferred into *P. guiyangense* wild strain using a PEG-mediated protoplast transformation method, as previously described (Dou et al., 2008; Zhang et al., 2015). The successful PgAGC1 transgenic lines were screened by amplifying the genomes from each line with Pham34-F and Pham34-R primers from Table S1. Total RNA was isolated from all transformants culture hyphae and used to determine the transcript level of PgAGC1 by quantitative RT-PCR (qRT-PCR).

2.4. *Quantitative gene expression analysis of PgAGC1 at infection stages*

After culturing of the *P. guiyangense* wild-type strain on 10 % liquid V8 medium for three days, the mycelia were harvested for DNA and RNA extraction. To obtain a large amount of zoospores, the liquid mycelia were rinsed in deionized distilled water three times and were then induced in the light at 25 °C for 7 h to collect spores by centrifuging. The zoospore suspension was further stored in the dark for 5 h for preparing cystospores.

Infection assays were conducted in plastic cups (capacity of 200 mL) containing 100 mL of deionized distilled water with 25 s-instar larvae of *C. pipiens pallens* and 4 disks (10 mm × 10 mm sized) of liquid *P. guiyangense* mycelia together. Three drops of 4 % duck liver powder were added as required, and each treatment group was replicated at least three times. Infected larvae were collected at 10 h, 20 h and 48 h post-treatment for qRT-PCR.

Genomic DNA of all the lines was extracted according to the protocol previously described (Chen et al., 2014), and total RNA was isolated using the RNA simple Total RNA Kit (Tiangen, China). To determine the relative expression of all strains, we used the SYBR Green Master Mix (Vazyme, NanJing, China) to perform qRT-PCR using RT-F and RT-R primers (Table S1). The qRT-PCR reactions were performed on an ABI PRISM 7500 Real-Time PCR System (Applied Biosystems) under the following conditions: 95 °C for 5 min, 10 s at 95 °C for 10 s, and 60 °C for 34 s for 40 cycles to calculate cycle threshold values, followed by a dissociation program of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s to obtain melt curves. The *P. guiyangense* actin gene was used as an endogenous control, and the relative expression level of the tested gene was calculated with the formula $2^{-\triangle\triangle Ct}$ (Livak and Schmittgen, 2001). To determine the relative transcriptional level, the expression level of *P. guiyangense* (WT) mycelia was assigned a value of 1.0.

2.5. *Analysis of colony growth*

All strains were cultured in petri dishes on 10 % V8 juice agar medium at 25 °C for 3 d. Colony diameters were measured after 3 d of incubation, and the morphology of hyphae was observed using an Olympus BX43 phase-contrast microscope (Olympus Corp., Tokyo, Japan). Each treatment contained at least three biological repeats, and then a t-test was used for analyzing the statistical difference between lines.

2.6. *Abiotic stress tolerance test*

To test abiotic stress, mycelium blocks (3 cm × 3 cm) were inoculated onto plates with 2.5 M H_2O_2 or 5 M H_2O_2 (oxidative stress) for 3 d to observe vegetative growth and measure diameter of mycelia. All the experiments were repeated at least three times and significant difference was analyzed with *t*-test.

2.7. Pathogenicity assays

P. guiyangense wild-type strain and three transformants (T3, ST1 and ST3) were cultured in 10 % liquid V8 medium for 5 d to collect mycelia. Pathogenicity assays were accomplished by placing 25 s-instar larvae of *C. pipiens pallens* and 4 disks (10 mm × 10 mm sized) of mycelia together in a plastic cup (capacity of 200 mL) containing 100 mL of deionized distilled water. For infection analysis, the larvae were picked out after 5 d, washed in deionized distilled water, and cultured in the medium to record infection using microscopic examination and to calculate infection rates. For mortality bioassays, the number of dead larvae was recorded once a day up to 15 d. Each treatment was replicated at least three times and t-test was applied for significant difference analysis.

3. Results

3.1. Gene cloning and characterization of PgAGC1

By analyzing the whole genome sequences of *P. guiyangense*, we identified an AGC kinase gene, designated PgAGC1, and amplified a 1152 bp full-length gene sequence from genomic DNA of *P. guiyangense* with the PCR primers *PKA03242*-F and *PKA03242*-R (Table S1). Comparison of the cDNA and genomic sequences of PgAGC1 confirmed that this gene encoded a polypeptide of 383 amino acids without any introns, and it contained a protein kinase domain and a protein kinase C domain (Fig. 1A). Using PgAGC1 protein as the query sequence, we could identify homologs from 7 representative oomycetes including *P. ultimum*, *Phytophthora sojae*, *Ph. ramorum*, *Ph. infestans*, *Hyaloperonospora arabidopsidis*, *A. laibachii*, and *S. parasitica*. PgAGC1 showed high sequence similarity, ranging from 65 to 95 %, to these homologous genes. Based on the phylogenetic analysis, PgAGC1 shared the highest similarity with PYU1_T008365 derived from *P. ultimum*, and then clustered with homologs from *Phytophthora* and other oomycetes (Fig. 1B), suggesting the same phylogenetic relationships within the oomycete species tree. These results indicated that PgAGC1 was widely distributed and highly conserved in this class of oomycetes.

3.2. PgAGC1 is up-regulated at infection stages

We examined the transcript profiles of PgAGC1 at different developmental stages and post-infection times using qRT-PCR. Analysis results showed that the expression levels of PgAGC1

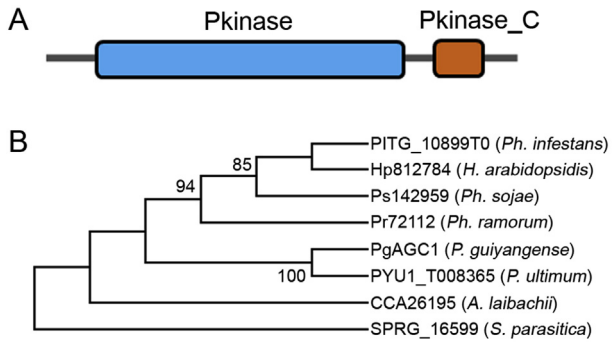

Fig. 1. Bioinformatics analysis of PgAGC1gene. (A) Predicted functional domains of PgAGC1 in *Pythium guiyangense*. The kinase domain was represented by the blue rectangle, and the kinase C domain was represented with the orange rectangle. (B) Phylogenetic relationship of PgAGC1 and homologs from seven different oomycete species. The phylogenetic tree was constructed using the neighbor joining method and 1000 replicates for bootstrap analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

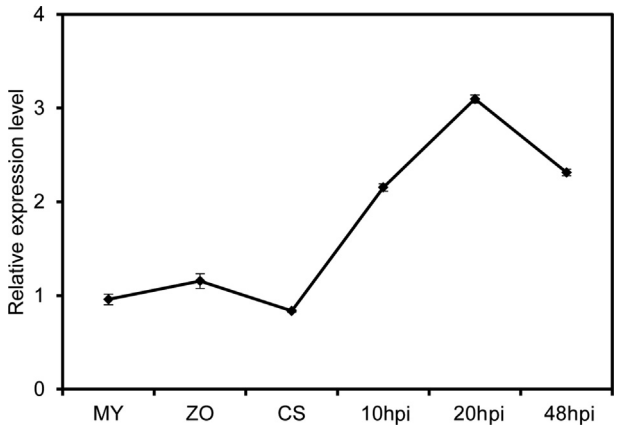

Fig. 2. Relative transcript levels of PgAGC1 at developmental stages and post-infection. MY, ZO and CS indicated as mycelium, zoospore and cystospore respectively. hpi: hours of post infection. Relative transcriptional expression level was normalized with *P. guiyangense* actin gene. Bars indicate standard errors.

were similar in mycelia, zoospores and cytospores, suggesting that this gene might possess similar functions throughout the development cycle. However, during the early stage of *P. guiyangense* infection of *Cx. Pipiens pallens* (10 hpi), PgAGC1 was significantly up-regulated and reached the maximum expression level at 20 hpi, then dropped down at the late stage of infection (Fig. 2), strongly implying that PgAGC1 might be a virulence factor during the interaction between *P. guiyangense* and mosquito larvae.

3.3. Silencing of PgAGC1 in P. guiyangense

To gain insight into the functions of PgAGC1 in *P. guiyangense*, genetic transformation was conducted by inserting a 476 bp fragment of PgAGC1 amplified with Insertion-F and Insertion-R primers from Table S1 into the plasmid vector pHAM34,which was then

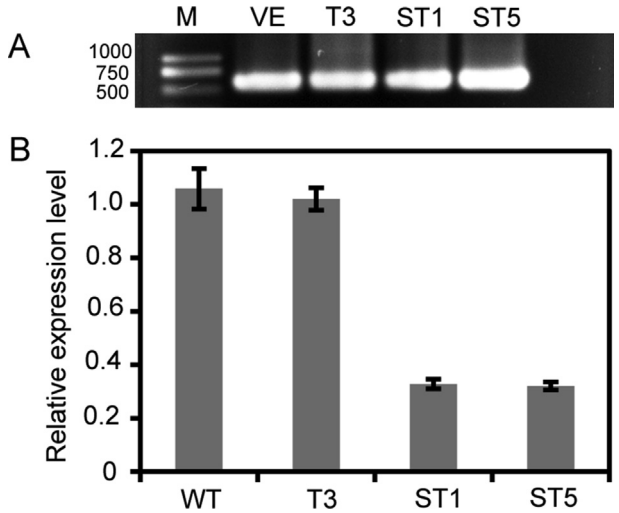

Fig. 3. Establishment of PgAGC1 gene silencing strains. (A) PCR verification of successful insertion of PgAGC1 into genomic DNA. All PCR reactions were performed with Pham34 primers and transgene should yield an amplified fragment 623 in length. M: molecular marker; VE: pHAM34 vector incorporated with PgAGC1 gene; T3: control strain; ST1 and ST5: two putative PgAGC1 silenced strains. (B) Relative levels of PgAGC1 transcript in *P. guiyangense* transformants measured using qRT-PCR. Relative expression levels were standardized using *P. guiyangense* actin gene. T3 strain showed no significant difference from the wild type (WT) and served as control. All the experiments were repeated at least three times with similar results. Bars show standard error.

transformed into the protoplast of *P. guiyangense* for gene silencing. The successful transformation was primarily identified via genomic PCR analysis with Pham34 primers (Table S1), and the results were shown in Fig. 3A. We further evaluated the expression profiles of the targeted PgAGC1 gene in all generated transgenic lines, and then obtained three mutants, designed as T3, ST1, ST5 from ~200 transformants. Transcription levels in transgenic lines showed that T3 had similar expression level with the wild-type and used as the control strain for further studies, while the gene transcript levels were reduced to 20–30 % of the WT value in ST1 and ST5, suggesting that PgAGC1 gene in ST1 and ST5 lines was significantly silenced (Fig. 3B). Thus we concluded that PgAGC1 was specifically disrupted in ST1 and ST5 strains, and these lines were used for further explore the biological functions of the PgAGC1 gene.

3.4. *PgAGC1 is required for vegetative growth of* P. guiyangense

Accumulated evidence has suggested that AGC kinases mediate signal pathways affecting fungi growth and morphology (Garcia et al., 2012; Peti and Page, 2013). We also tested whether PgAGC1 regulated mycelium growth and hyphal morphology via growing WT, T3, ST1 and ST5 strains of *P. guiyangense* in 10 % V8 juice agar medium at 25 °C for 3 d. We then observed morphological changes of hyphae and measured the diameters of mycelium of these strains; all data are summarized in Fig. 4. Our results demonstrated that the non-silenced line T3 showed the same hyphal morphology and similar growth rate as that of WT (Fig. 4A). However, both silenced lines ST3 and ST5, displayed ~35 % reduced growth rates in comparison with that of WT ($p < 0.01$) (Fig. 4C). In addition, under microscopic examination, the hyphal branches of the two silenced lines appeared more curved than those in the WT strain (Fig. 4B). Taken together, our studies implied that PgAGC1 gene plays important roles in the development of *P. guiyangense*.

3.5. *Silencing of PgAGC1 results in a reduced stress response*

AGC kinases are also regulators of the signaling pathway involved in stress responses (Sobko, 2006). Abiotic stress conditions included potentially harmful osmotic, oxidative, UV and thermal conditions. To explore the role of PgAGC1 in response to oxidative stresses, we cultured the WT, T3, and silenced ST1 and ST5 strains in medium containing 2.5 M H_2O_2 or 5 M H_2O_2, and then we analyzed the inhibition rates of mycelium. Our research showed that ST1 and ST5 displayed higher inhibition rates than those of WT and T3 with significant difference ($P < 0.01$) when they were exposed to H_2O_2, and the inhibition rates of ST1 and ST5 increased dramatically in response to a higher concentration of H_2O_2 (Fig. 5). All the evidence supported that PgAGC1 might mediate the signal pathway to resist the oxidative stresses in *P. guiyangense*.

3.6. *Silencing of PgAGC1 attenuates virulence in* Cx. pipiens pallens

To determine the effect of silenced PgAGC1 on pathogenicity during mosquito infection, we performed bioassays for the WT and transgenic lines of *P. guiyangense*. Early second-instar of *Cx. pipiens pallens* were inoculated with 4 disks (10 mm × 10 mm sized) of mycelia of *P. guiyangense*, as outlined in the methods. The infection rates after inoculation with *P. guiyangense* mycelium for 5 d were analyzed and Fig. 6A showed that the infection rates of WT and T3 were similar without significant difference. However, the infection

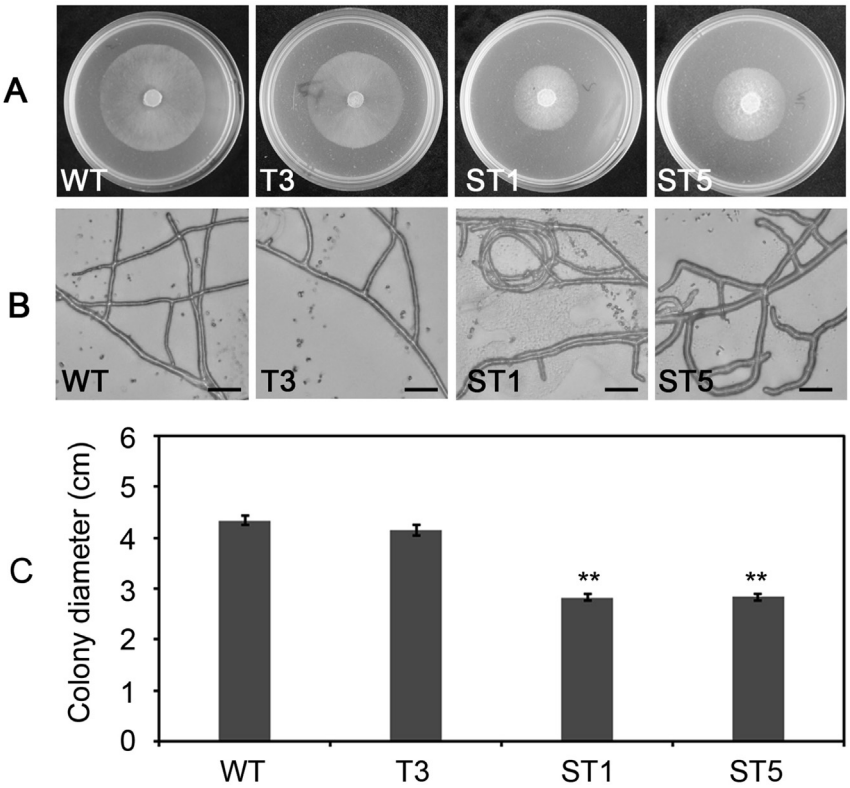

Fig. 4. PgAGC1 is required for vegetative growth of *P. guiyangense*. All photos and measurement were made 3 d after incubation. (A). Cultured colonies of wild type and mutants of *P. guiyangense* on 10 % V8 medium. (B). Hyphae morphological changes of *P. guiyangens* transformants observed under microscope. Bars indicate 10um. (C). Measurement of colony diameter for *P. guiyangens* wild-type and mutant strains. Bars were standard errors. ** indicates significant difference ($p < 0.01$) between the silenced strain (ST1or ST5) and the WT.

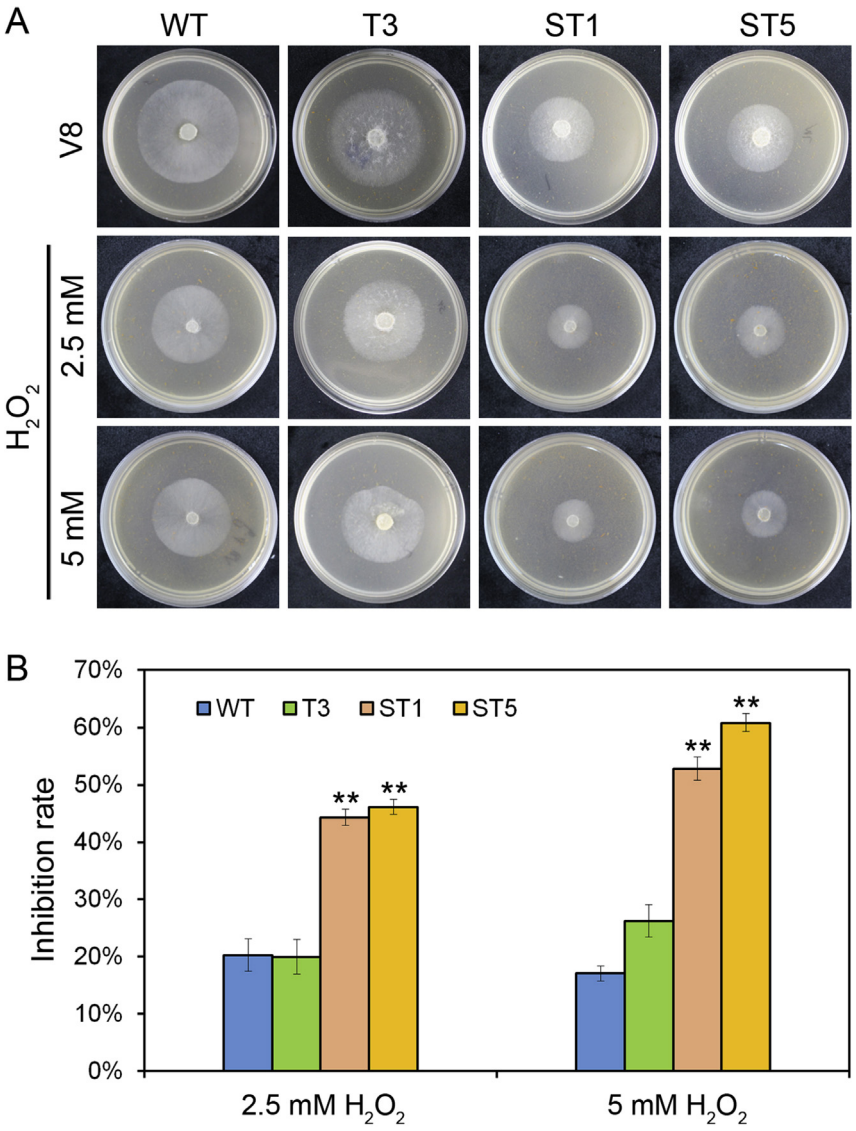

Fig. 5. PgAGC1 regulated the stress responses of *P. guiyangense*. Photos were taken 3 d after inoculation. All experiments were replicated at least three times. (A) Colony growth of *P. guiyangens* transgenic lines under different H_2O_2 stress conditions. (B) Inhibition rates of colony growth in the mutant strains. The colony diameters of T3, ST1 and ST5 under H_2O_2 were all compared with those of the wild-type to obtain the inhibition rates. Bars highlight the standard errors calculated from at least three repeats. Asterisks suggest the statistical significant difference ($p < 0.01$) between transgenic lines and the wild-type strain.

rates of the two silenced lines ST1 and ST5 were dramatically reduced to 40–41 % in comparison with those of WT and T3, showing significant difference ($P < 0.01$) (Fig. 6A). We also recorded *Cx. pipiens pallens* larval mortality daily for up to 15 d post-inoculation. Cumulative survival curves of larvae after treatment were plotted in Fig. 6B, and a t test was used to assess significant differences between strains. The cumulative survivals of WT and the non-silenced line T3 were similar, and no significant difference was identified. For the two silenced lines ST1 and ST5, they started to display significant survival differences with WT at 5 dpi, and the differences continued to increase over time. The cumulative survivals reduced to 72–75 % in ST1 and ST5 compared to 61 % in WT and 63 % in T3 ($P < 0.01$). These data indicated that PgAGC1 was involved in *P. guiyangense* pathogenicity.

4. Discussion

Mosquitoes are the greatest threat to public health because they are vectors of enormously crippling diseases including malaria, West Nile fever, dengue fever, and other parasitic infections (Feng et al., 2018). The grave issues of insecticide resistance and environmental impact raised by selective application of chemical compounds have led to a necessity for alternative biological control agents (St Leger and Lovett, 2016). Unfortunately, the available literature on entomopathogenic fungi for mosquito control is still limited, and most of the focus is only on several species belonging to the genera *Lagenidium*, *Coelomomyces*, *Beauveria*, and *Metarhizium*, and only *L. giganteum* has been commercialized as a mosquito control agent (Scholte et al., 2004). *P. guiyangense*, belonging to the genus *Pythium*, was first isolated from dead mosquito larvae, and further investigations confirmed that it is a promising mosquito pathogen, because it was very effective in killing many mosquito species, strongly adapt to environmental changes and easily mass-produced (Huang and Su, 2007). However, the molecular mechanisms underlying vector biology and host–pathogen interactions for this species are still not clear.

Our study group identified a new gene, PgAGC1, which contains a protein kinase domain and a protein kinase C domain, from the

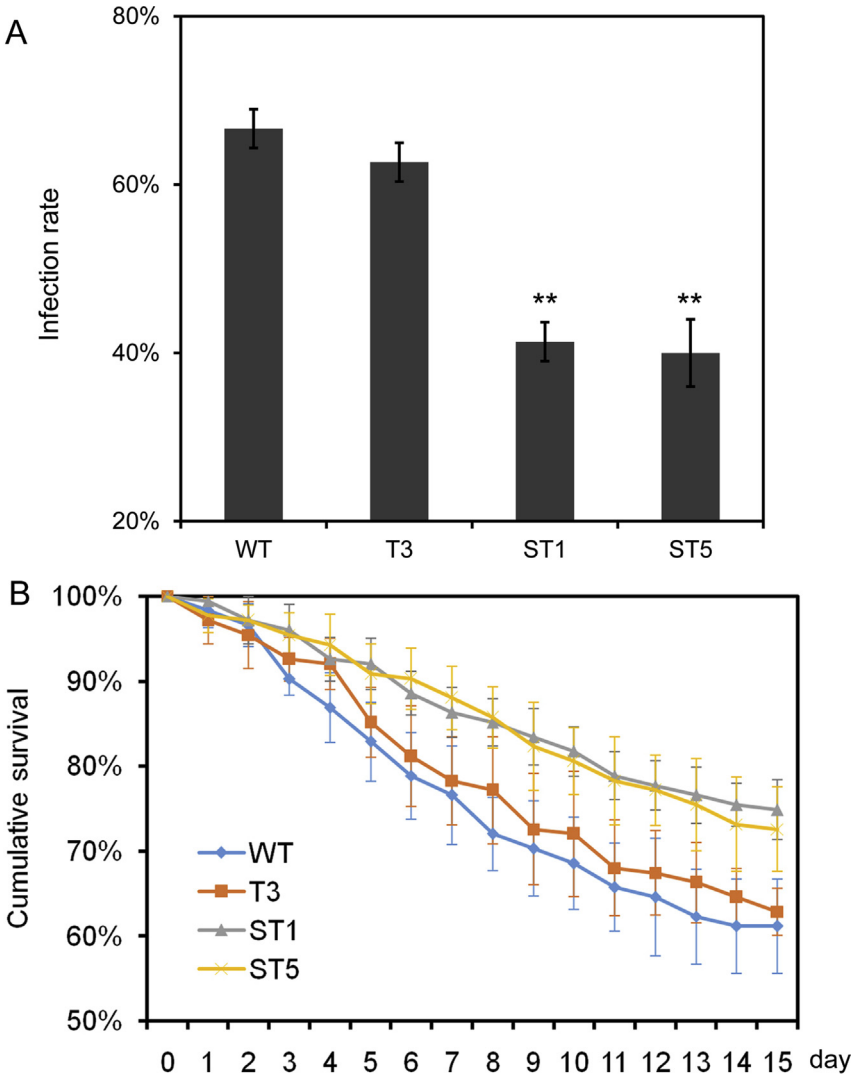

Fig. 6. PgAGC1 is a virulence factor in *P. guiyangense*. (A) The mosquito infection rates after treatment with the wild-type and transgenic strains of *P. guiyangens*. Asterisks indicate significant difference (**p < 0.01) between each transgenic line and the wild-type. Bars represent the standard error from at least three independent replicates. (B) The cumulative survival curves of Cx. *Pipiens pallens* after incubation with *P. guiyangens* transformants. The mortality was recorded daily up to 15 d. Different colored bars show standard errors calculated from at least three repeats.

genome of *P. guiyangense*, and we assigned it to the AGC kinase group. We also found that this gene is conserved among seven oomycetes, including *P. ultimum*, *Ph. sojae*, *Ph. ramorum*, *Ph. infestans*, *H. arabidopsidis*, *A. laibachii*, and *S. parasitica*. Phylogenetic analysis showed that PgAGC1 clustered with homologs from *Phytophthora* and other oomycetes (Fig. 1B), suggesting the same phylogenetic relationships within the oomycete species tree. The biological functions of this gene are still not known, although PgAGC1 exists widely in oomycetes.

Using *P. guiyangense* and *Cx. Pipiens pallens* as a model system, we found that PgAGC1 was up-regulated at infection stages (Fig. 2). To further explore the functions of PgAGC1 in *P. guiyangense*, we established a genetic transformation system to silence genes and obtained three mutant strains designated T3 (control strain without gene silencing), ST1 and ST5 (two attenuated strains) (Fig. 3). Further studies demonstrated that PgAGC1 was required for vegetative growth of *P. guiyangense*, which was supported by reduced growth rates and curved morphology of mycelium in ST1 and ST5 strains (Fig. 4). Under H_2O_2 stresses, the disruption of PgAGC1 exhibited defective vegetative growth of *P. guiyangense* (Fig. 5). Lacking this gene also resulted in reduced mortality and infection rates during *P. guiyangense* infaction of *Cx. pipiens pallens* (Fig. 6).

The physiological roles of diverse AGC kinases have been extensively studied in human, plants and animals, and all the experiments have agreed that these kinases mediate a large number of cellular processes, such as cell growth, response to environmental stresses, and host immunity (Alves de Castro et al., 2016; Peti and Page, 2013; Sobko, 2006); however, the kinase functions are only known for a few fungal pathogens (Alves de Castro et al., 2016; Berndt et al., 2010; Fang et al., 2009; Mitchell and Dean, 1995). Reports from the fungal pathogens *Cryptococcus neoformans*, *Magnaporthe grisea* and *Aspergillus fumigatus* and *Metarhizium anisopliae* elucidated that the mutations of AGC kinases show multi-stress phenotypes and affect virulence (Alves de Castro et al., 2016; Berndt et al., 2010; Fang et al., 2009). For example, Aga1, an AGC kinase from *Ustilago maydis*, the causative agent of corn smut disease, is an essential virulence factor that affects appressoria formation (Berndt et al., 2010). In another model system of insect fungal pathogenesis, *M. anisopliae*, the role of cAMP signal

transduction in virulence was studied, and the results revealed that the PKA mutant (DMaPKA1) showed greatly reduced virulence (Fang et al., 2009). All this evidence suggested that AGC kinases might be important pathogenic factors during pathogen infection, which was consistent with the results of PgAGC1 in *P. guiyangense*. Our findings in this study are of utmost importance for understanding the molecular mechanisms of the interactions between *P. guiyangense* and mosquitoes.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated during this study are included in this main paper and Additional files.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

AX conceived and designed the experiments, jointly performed data analysis and wrote the manuscript. CW, JW, YC, YD, and ZT performed the experiments. DS contributed to data analysis and writing manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2018.11.006.

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