Fungal Biology 123 (2019) 547–554

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

The pH sensing receptor AopalH plays important roles in the nematophagous fungus *Arthrobotrys oligospora*

Juan Li [a, *], Runian Wu [a], Meng Wang [a], James Borneman [b], Jinkui Yang [a], Ke-Qin Zhang [a, **]

[a] State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, Yunnan University, Kunming, 650091, PR China
[b] Department of Microbiology and Plant Pathology, University of California, Riverside, CA, 92521, USA

ARTICLE INFO

Article history:
Received 22 October 2018
Received in revised form
24 April 2019
Accepted 9 May 2019
Available online 18 May 2019

Corresponding Editor: Julian Rutherford

Keywords:
Ambient pH
Conidiation
Nematodes
PacC/Rim101 signaling
Trap formation

ABSTRACT

There is well-conserved PacC/Rim101 signaling among ascomycete fungi to mediate environmental pH sensing. For pathogenic fungi, this pathway not only enables fungi to grow over a wide pH range, but it also determines whether these fungi can successfully colonize and invade the targeted host. Within the pal/PacC pathway, palH is a putative ambient pH sensor with a seven-transmembrane domain. To characterize the function of a palH homolog, AopalH, in the nematophagous fungus Arthrobotrys oligospora, we knocked out the encoding gene of AopalH through homologous recombination, and the transformants exhibited slower growth rates, greater sensitivities to cationic and hyperoxidation stresses, as well as reduced conidiation and reduced trap formation, suggesting that the pH regulatory system has critical functions in nematophagous fungi. Our results provide novel insights into the mechanisms of pH response and regulation in fungi.

1. Introduction

A conserved pH regulatory system in fungi has evolved that enables them to survive in a wide pH range (Arst and Peñalva, 2003; Bertuzzi et al., 2014). In *Aspergillus nidulans*, the pH signaling pathway named pal/PacC includes six dedicated components (palA, palB, palC, palF, palH, and palI) and one zinc-finger transcription factor PacC — this signaling is equivalent to the *RIM*/Rim101p signaling in *Saccharomyces cerevisiae* (Caddick et al., 1986; Peñalva and Arst, 2002; Arst and Peñalva, 2003; Peñalva et al., 2008, 2014). Within this signaling, palH acts upstream of all other Pal proteins with the exception of palI (Calcagno-Pizarelli et al., 2007). In PacC/Rim101 pH-responsive signaling, palH is a putative ambient pH sensor with a seven-transmembrane domain while palI is another pH sensor with a four-transmembrane domain that acts upstream of or in concert with palH (Calcagno-Pizarelli et al., 2007; Lucena-Agell et al., 2016). The cytosolic tail of palH interacts with palF and the pH signal transduces through the palF-palH complex to the downstream components palA and palC as well as the signaling protease palB at alkaline pH (Negreteurtasun et al., 1997; Hervás-Aguilar et al., 2010; Galindo et al., 2012). PalB triggers the zinc finger transcription factor PacC by catalyzing its C-terminal proteolysis (Futai et al., 2001). In general, PacC acts as a transcriptional activator of alkaline-expressed genes and a repressor of acid-expressed genes under alkaline pH (Tilburn et al., 1995). There is abundant evidence indicating that mutations in the five pal genes (palA, palB, palC, palF and palH) and the transcription factor PacC can result in the suppression of alkaline-expressed genes and the activation of acid-expressed genes (Negreteurtasun et al., 1999; Denison, 2000; Peñalva and Arst, 2002; Peñalva et al., 2008).

In pathogenic ascomycete fungi, pH is one of the key environmental factors affecting the activity of virulence factors secreted by pathogens. Thus, the PacC/Rim101 pH-responsive signaling not only plays an important role in ambient pH sensing but it also determines the organism's pathogenicity (Davis, 2009; Cupertino et al., 2012; Cornet and Gaillardin, 2014; Huang et al., 2015). For instance, the two entomophagous fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, can secrete enzymes such as proteases, chitinases, glycosidases, and lipases to kill the host by, for example, host cuticle penetration by hyphae (St Leger et al., 1997). It is suggested that these enzymes require an appropriate pH range to function properly (Wang and Feng, 2014; Zhu et al., 2016). Previous studies have shown that both the cuticle-degrading enzymes in

* Corresponding author. Fax: +86 871 65034838.
** Corresponding author. Fax: +86 871 65034838.
E-mail addresses: juanli@ynu.edu.cn (J. Li), kqzhang@ynu.edu.cn (K.-Q. Zhang).

https://doi.org/10.1016/j.funbio.2019.05.008
1878-6146/

M. anisopliae and oxalic acid in *B. bassiana* were pH-dependent virulence factors (St Leger et al., 1998; Kirkland et al., 2005). Also, the rapid propagation of the pathogen within the host is the result of their successful response to osmotic stress in the hemocoel and in evading host defenses (Lewis et al., 2009; Chen et al., 2014).

Among the pathogenic fungi, nematophagous fungi are a group of natural enemies of nematodes. Nematophagous fungi can attack and kill nematodes through diverse processes (Nordbring-Hertz et al., 2006; Zhang and Hyde, 2014). These fungi have been proposed as potential biological control agents for managing harmful plant-parasitic nematodes (Li et al., 2015). Among the nematophagous fungi, *Arthrobotrys oligospora* is the most-studied model for examining the molecular mechanisms of fungus–nematode interaction (Li et al., 2015). This fungus can capture nematodes by forming special trapping devices named adhesive networks (Nordbring-Hertz et al., 2006). These trapping devices can be induced by the presence of nematodes, resulting in profound morphological transitions and pronounced lifestyle switches from saprophytes to predators. Recently, the genome of *A. oligospora* was sequenced, and a model of the formation of the nematode traps was proposed, which involve multiple signal transduction pathways. The model suggested genes associated with diverse cellular processes such as energy metabolism, biosynthesis of cell wall and adhesive proteins, cell division, peroxisome biogenesis, among others, were involved their formation (Yang et al., 2011). Nematophagous fungi have a worldwide distribution which suggests that the PacC/Rim101 pH-responsive signaling may play important roles enabling these fungi to adapt to wide range of environmental conditions. Moreover, it has been shown that nematophagous fungi can secrete several extracellular enzymes, such as serine proteases, chitinases and collagenases to destroy the cuticle integrity of the nematodes and facilitate fungal penetration and colonization during the infection process (Huang et al., 2004; Yang et al., 2007). Intriguingly, the consensus binding motif, GCCARG, for the pH transcription factor PacC, has been identified in the promoter regions of a subtilisin-like cuticle-degrading enzyme (designated PrC) in the nematophagous fungus *Clonostachys rosea* (Zou et al., 2010b). In transcription factor Δ*pacC* mutants, the expression of prC was downregulated (Zou et al., 2010a). Also, the transcript level of prC was significantly higher under alkaline growth conditions than under acidic growth conditions (Zou et al., 2010a). All of these studies suggests that the pH regulatory pathway may play a pivotal role in nematophagous fungi.

In this study, we characterized a homologue of the pH sensing receptor palH, called AopalH, in the nematode-trapping fungus *A. oligospora*. The encoding gene of AopalH was knocked out through homologous recombination, and the knockout strain exhibited a slower growth rate and a greater sensitivity to cationic stress. Also, our results suggested the involvement of AopalH in the regulation of conidation and trap formation in *A. oligospora*.

2. Materials and methods

2.1. Strain and growth conditions

The wild type strain of *A. oligospora* ATCC24927 was purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained on cornmeal agar (CMA) at 28 °C. *S. cerevisiae* (FY834) was grown in yeast extract peptone dextrose (YPD) plates. Plasmid pRS426 was kept in *Escherichia coli* strain DH5α (Takara, Shiga, Japan) (Christianson et al., 1992). 200 μg/mL hygromycin B (Amresco, Solon, USA) was used for selecting transformants on regeneration medium (PDASS, PDA supplying with 0.6 M sucrose, 0.3 g/L yeast extract, 0.3 g/L tryptone, 0.3 g/L peptone) (Zhao et al., 2014). *Caenorhabditis elegans* grown on oatmeal agar medium at room temperature were separated using the Baerman funnel technique (Gray, 1984).

2.2. Phylogenetic analysis of palH homologs

By using the amino acid sequence of palH (GenBank: AAF70858) from *A. nidulans* as a query, a total of 24 palH amino acid sequences from 24 ascomycetes fungi were downloaded from NCBI. MUSCLE v3.5 was used to generate a protein alignment (Edgar, 2004). Gblocks 0.91b was used to remove the ambiguous areas of the alignment with default parameters (Castresana, 2000; Talavera and Castresana, 2007). Finally, an alignment consisting of 322 amino acids was obtained (Supplementary File. S1) and used to perform Neighbor-joining (NJ) analysis with the Kimura-2 parameter distance model in MEGA 6 (Tamura et al., 2013). The reliability of the tree topologies was evaluated using bootstrap analysis with 1000 replicates for NJ (Felsenstein, 1985).

2.3. Gene knockout of AopalH in A. oligospora

The gene encoding the pH signal transduction protein AopalH (GenBank XP_011117902) of *A. oligospora* was knocked out following the method described in (Colot et al., 2006). The amplified hygromycin resistant gene and the 5′ and 3’ flanking fragments of *AopalH* were co-transformed into *S. cerevisiae* strain FY834 to form the disruption vector pRS426-*AopalH*-hph (Table 1). The vector pRS426-*AopalH*-hph was transformed into *A. oligospora* protoplasts (Tunlid et al., 1999). Transformants were confirmed by both PCR and Southern blot analyses.

2.4. Comparison of vegetative growth and conidia yield between the wild type and ΔAopalH strains under different conditions

Mycelial plugs 1 cm in diameter from the wild-type and the *ΔAopalH* mutant strains were incubated on PDA, TYGA, and CMA media for 5 d at 28 °C. In addition, the same size colony of the wild-type and the *ΔAopalH* mutant strains were inoculated onto TG media (1 % Tryptone, 1 % Glucose) at pH 5, pH 7, and pH 9 for 5 d at 28 °C.

To compare their stress response capabilities, the same size mycelial plugs (1 cm in diameter) of the wild-type and the two *ΔAopalH* mutant strains were cultivated on TG media with: 0.1, 0.2, and 0.3 M NaCl; 5, 10, 15 mM H_2O_2; and 0.01, 0.02, 0.03 % SDS. The diameters of colonies were measured every day until the mycelia covered the whole surface of the 6 cm diameter plates.

The sporulation capacity of the wild-type and the *ΔAopalH* mutant strains was also analyzed. The same size mycelial plugs (1 cm in diameter) of the wild-type and the *ΔAopalH* mutant strains were inoculated on CMY media with different pHs (5, 6, 7, 8, 9, and 10) for 14 d at 28 °C. The conidia were collected using 5 mL sterile water, and then filtered to remove the mycelial debris by using four layers of lens paper. The number of conidia per square millimeter of the colonies was determined using a hemocytometer (Xie et al., 2012).

Three replicates of all of the experiments described above were performed.

2.5. Induction of trap formation

To assess trap formation in *A. oligospora* strains, approximately 10^4 of conidia of the wild-type and the *ΔAopalH* mutant strains were inoculated on CMA media with different pHs (5, 6, 7, 8, 9, and 10) and incubated at 28 °C. After 4–5 d of growth, 100-150 adult nematodes were added to the *A. oligospora* mycelia. The traps were counted at specified time intervals using light microscopy

Table 1
List of PCR primers used for homologous recombination in this study.

Primers	Sequence (5′-3′)	Length(bp)	Description
palH-5f	GTAACGCCAGGGTTTTCCCAGTCACGACGTCGGTCGTCGTATAAAGGAATCTTT	2002	Amplify the 5′ flanking region of the palH gene
palH-5r	ATCCACTTAACGTTACTGAAATCTCCAACTCCACAGAGTAGTCGGAGTAGGTG		
palH-3f	CTCCTTCAATATCATCTTCTGTCTCCGACCACCGCCAGACCAAGTCG	2153	Amplify the 3′ flanking region of the palH gene
palH-3r	GCGGATAACAATTTCACACAGGAAACAGCAAATTCATCGCCGAAACCTC		
hphF	GTTGGAGATTTCAGTAACGTTAAGTGGAT	1447	Amplify the *hph* cassette
hphR	GTCGGAGACAGAAGATGATATTGAAGGAGC		
palH-Tf1	TCCTTCCGACCACCTTGTTAC	1972/2,713[a]	Amplify the transformants and wild-type strain
palH-Tr1	AGGGACTACTAGATGTGCCTTCC		
palH-Sf1	AGCAACAGCAGCAACCCAAC	625	Used for making Southern blot probe 1
palH-Sr1	TGGCGGCGTAAGGATGAC		
palH-R1	CCCGAGGCAGTAATCCAGT	547	Used for making Northern blot probe
palH-R2	TGAGAATGCGAGTGCGAGT		
palH-Tf2	GGAATACCAACACGCACAT	1341	Amplify the transformants and wild-type strain
palH-Tr2	TCTCCCTTACGAAACCACTT		
palH-Sf2	AACCCGCTCGTCTGGCTAA	465	Used for making Southern blot probe 2
palH-Sr2	TCGCCCTTCCTCCCTTTATT		

[a] 1972 for transformants and 2713 for wild type strain.

(Olympus, Tokyo, Japan). Data from individual treatments were expressed as mean ± SE. Differences were determined by ANOVA using GraphPad Prism software (version 6.0, GraphPad Software, USA), and P values < 0.05 were considered significant.

2.6. Northern blot analysis

25 μg of RNA was resolved on a 1.2 % formaldehyde agarose gel and blotted onto a nylon membrane. The membrane was UV-cross-linked and hybridized overnight at 65 °C with either [α-^{32}P] dATP-labeled probes in Ultrahyb buffer (PerfectHyb™ Plus Hybridization Buffer 1×, Ambion, Austin, Texas, USA). Blots were washed twice with 0.5 × SSC and 0.1 % SDS for 20 min at 65 °C. DNA probes were obtained by random-primer labeling (GE Rediprime™ II DNA Labeling System, Amersham Pharmacia Biotech, USA) of specific DNA fragments generated by using the PCR primers palH-R1 and palH-R2. *GAPDH* RNA were used as loading controls.

3. Results

3.1. Identification and phylogenetic analysis of AopalH in A. oligospora

Using the amino acid sequence of palH (GenBank: AAF70858) from *A. nidulans* as a query (Negreteurtasun et al., 1999), a single copy gene encoding the AopalH protein was identified in the *A. oligospora* genome. This gene encodes a polypeptide of 997 amino acids that contains three introns. In addition, seven strong transmembrane regions were predicated by using the TMpred program (results not shown), which is consistent with the gene from *A. nidulans*. The amino acid sequence of AopalH shared a high degree of identity to palH orthologs from other ascomycetes fungi. Phylogenetic analysis showed that these palH proteins was consistent with the taxonomic relationships of these species, which were inferred by analysis of other gene sequences (Fig. 1) (James et al., 2006). The palH proteins identified from the three nematode-trapping fungi *A. oligospora, Drechslerella stenobrocha* and *Dactylellina haptotyla*, which belong to Orbiliomycetes, formed one congruent clade while those from Eurotiomycetes, Sordariomycetes and Leotiomycetes formed three separate clades (Fig. 1).

3.2. Screening and confirmation of the ΔAopalH mutants

The encoding gene of AopalH in *A. oligospora* was knocked out through homologous recombination (Fig. 2A). Compared to a 2713-bp fragment from the wild-type strain, a 1972-bp fragment was amplified from 9 transformants using the primers palH-Tf1/palH-Tr1, while no fragment was obtained from the transformants by using the primers palH-Tf2/palH-Tr2 (Fig. 2B, C, Table 1). Southern blot analysis detected a single band in the wild-type strain and the transformants using probe P1 and the restriction enzyme ApaI, respectively, and the sizes were consistent with their predicted values (Fig. 2D and Table 1). However, only one band was observed in the transformants using probe P2 and the restriction enzyme ApaI, and no band was obtained in the wild-type strain (Fig. 2E and Table 1). We also assessed the transcript levels of AopalH during the process of trap formation by Northern blot analysis. After the addition of a nematode extract (NE), the transcript level of AopalH increased when compared to the no-NE control, suggesting that AopalH is likely involved in trap formation (Fig. 2F).

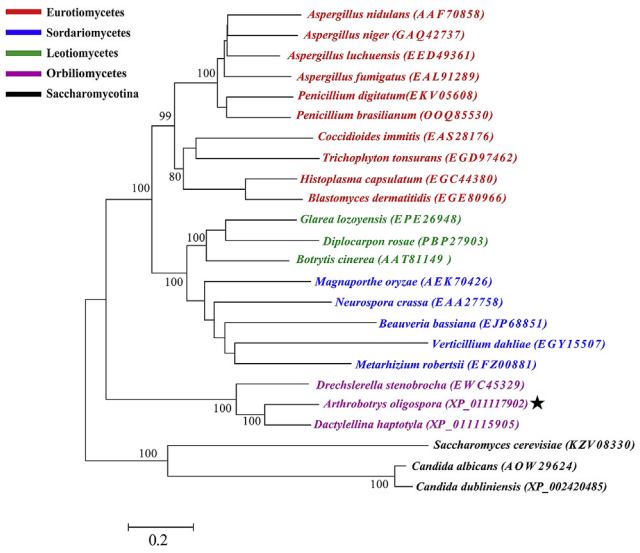

Fig. 1. Phylogenetic analysis of palH homologs from 22 different filamentous fungi. Phylogenetic analyses were performed on an alignment of 322 amino acids from 24 ascomycetes fungi using MEGA 6 (Tamura et al., 2013). The Neighbor-joining (NJ) algorithm using the Kimura-2 parameter distance model and pairwise deletion option for gaps was used. The reliability of the tree topologies was evaluated using bootstrap analysis with 1000 replicates for NJ (Felsenstein, 1985). GenBank accession numbers are shown in brackets, and the palH proteins from *Candida albicans, Saccharomyces cerevisiae and Candida dubliniensis* were used as outgroups.

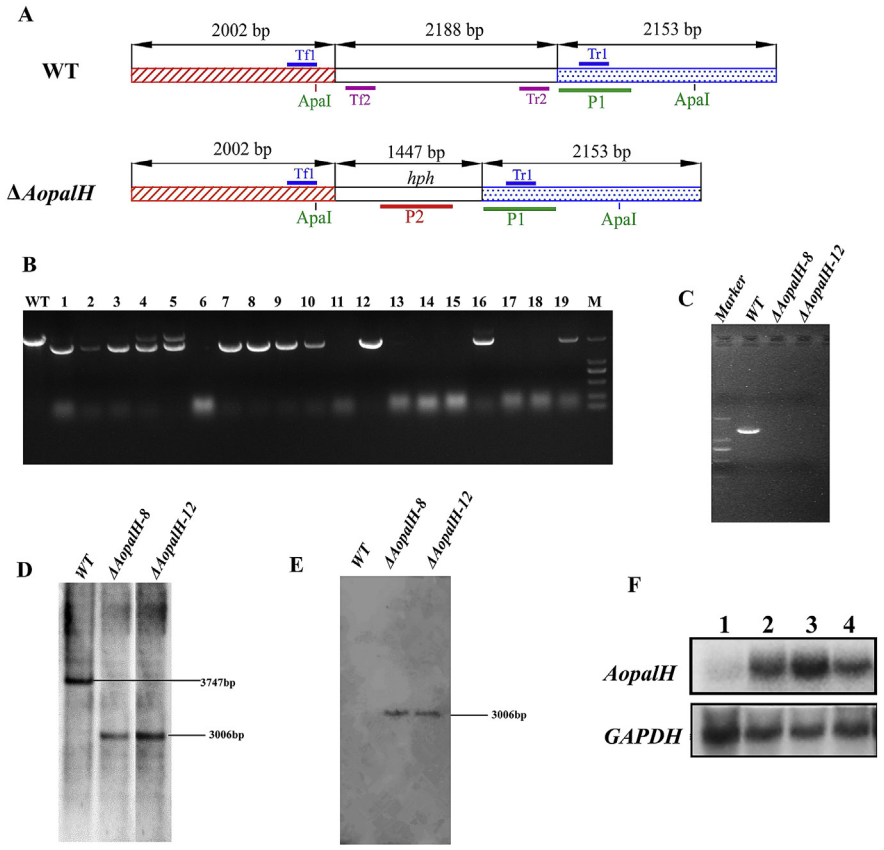

Fig. 2. Deletion of the *AopalH* gene in *A. oligospora*. A. A diagram of the replacement of the *AopalH* gene using homologous recombination. The restriction site of ApaI, probe 1 (P1) and probe 2 (P2) used for the Southern blot analysis are shown. The fragments amplified by using the primers palH-Tf1 (Tf1)/palH-Tr1 (Tr1) and palH-Tf2 (Tf2)/palH-Tr2 (Tr2) were used to distinguish transformants from wild-type strain. B. Transformants were confirmed by agarose gel electrophoresis of PCR amplicons created using the primers palH-Tf1/palH-Tr1. WT, the wild-type strain; M, marker (DL2000). C. *AopalH* gene was analyzed by agarose gel electrophoresis of PCR amplicons created using the primers palH-Tf2/palH-Tr2. WT, the wild-type strain; marker (DL2000). D. Southern blot analysis of the wild-type strain (WT) and *ΔAopalH* mutants using probe P1 and restriction enzyme ApaI. E. Southern blot analysis of the wild-type strain (WT) and *ΔAopalH* mutants using probe P2 and restriction enzyme ApaI. F. Northern blot analysis of the *AopalH* gene during trap formation in *A. oligospora*. 1, *A. oligospora* without NE induction; 2, 3, 4 are *A. oligospora* induced by NE 10 h, 24 h, and 40 h after NE treatment, respectively.

3.3. *Effect of* ΔAopalH *mutation on hyphal growth*

The growth rate and colony morphology of the wild-type strain and *ΔAopalH* mutants (*ΔAopalH*-8 and *ΔAopalH-12*) were compared. On TYGA and CMA media, the *ΔAopalH* strains grew slower. On the CMA medium, the aerial hyphae were much less abundant in the mutants. However, no obvious differences between the wild-type strain and the *ΔAopalH* mutants were observed on PDA medium (Fig. 3).

To investigate whether growth rate was influenced by AopalH in different pH environments, colony growth rates of the wild-type and the two *ΔAopalH* strains (*ΔAopalH*-8 and *ΔAopalH-12)* were evaluated on TG medium under acidic (pH 5), neutral (pH 7) and alkaline (pH 9) conditions. At pH 5, the two mutant strains and the wild-type strain all showed slower growth rates. However, at the neutral and alkaline pHs, the two mutant strains grew significantly slower than the wild-type strain (Fig. 4).

3.4. *Effect of different stress conditions on the growth of the wild-type strain and* ΔAopalH *mutants*

The growth rate and colony morphology of the wild-type strain and the *ΔAopalH* mutants were compared on TG medium with different stress factors. No obvious differences were observed in growth between the *ΔAopalH* and wild-type strains on media with various amounts of SDS and H_2O_2 (Fig. 5). However, the mutants

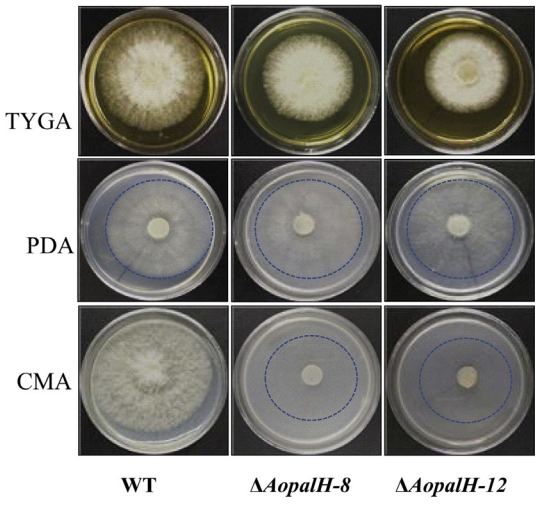

Fig. 3. Comparison of the growth rates and colony morphologies of the wild-type strain and the *ΔAopalH* mutants on PDA, TYGA, and CMA media. Fungi were grown on PDA for 5 d at 28 °C. Colony edges are marked by blue dotted lines. The *ΔAopalH* strains (*ΔAopalH*-8 and *ΔAopalH-12)* grew slower than the wild-type strain on TYGA and CMA media, but there were no differences on PDA media. The mutant strains had fewer aerial hyphae than the wild-type strain on CMA media. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

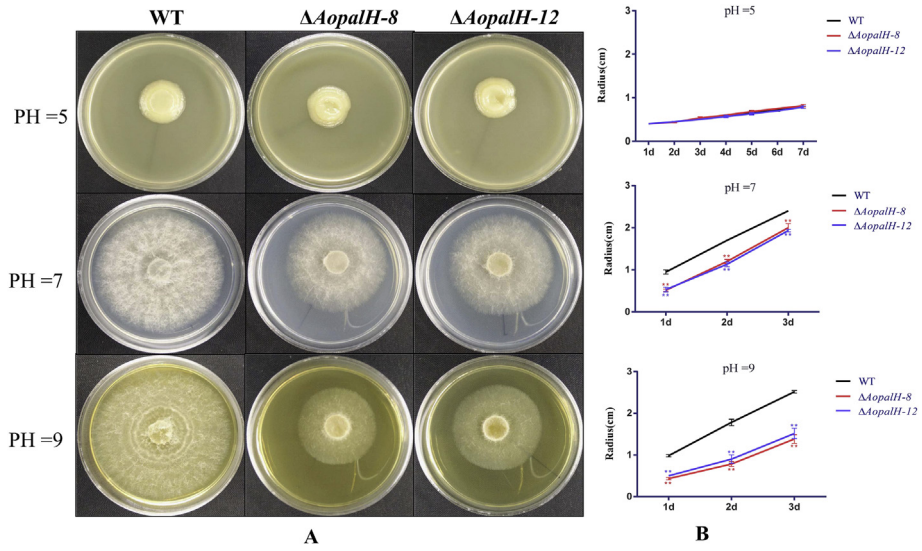

Fig. 4. Comparison of the growth rates and colony morphologies of the wild-type strain and the *ΔAopalH* mutant strains on TG media with different pHs. Fungi were grown on PDA for 5 d at 28 °C. Colony growth rates of the wild-type strain and the two *ΔAopalH* mutant strains (*ΔAopalH*-8 and *ΔAopalH-12)* were evaluated on TG media with different pHs. At pH 5, the two mutant strains and the wild-type strain both exhibited slow growth rates. However, at the neutral and alkaline pHs, the two mutant strains grew significantly slower than the wild-type strain, which was determined by ANOVA using GraphPad Prism software (version 6.0, GraphPad Software, USA), **P < 0.01.

exhibited significantly slower growth rates than the wild-type strain on media with 0.2 M NaCl and 5 mM H_2O_2, while the growth rate of the wild-type and mutant strains were both reduced on media with 0.3 M NaCl (Fig. 5). This indicated that the wild-type and mutant strains have different responses to cationic and hyperoxidation stresses.

3.5. *Effect of* ΔAopalH *mutation on conidiation and trap formation*

The growth rates and capacities to produce conidia and traps of the wild-type and mutant strains in response to different Na^+ concentrations were investigated (Supplementary File S1-S2). The capacity of the wild-type and mutant strains to produce conidia

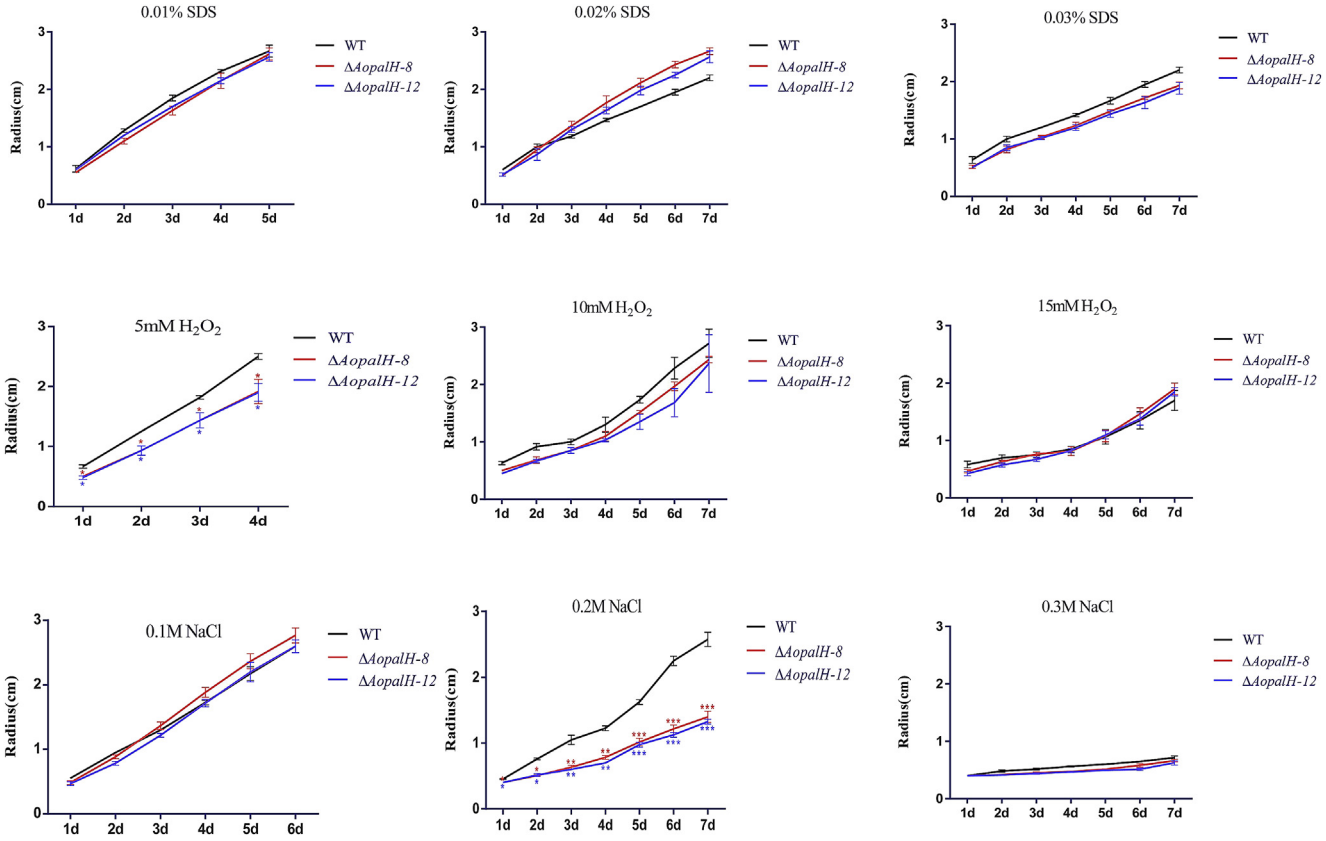

Fig. 5. Comparison of the abilities of the wild-type and *ΔAopalH* mutant strains to grow under different stresses. The growth conditions of the wild-type and *ΔAopalH* mutants on TG media supplemented with: 0.1, 0.2, and 0.3 M NaCl; 5, 10, 15 mM H_2O_2; and 0.01, 0.02, 0.03 % SDS. Differences were determined by ANOVA using GraphPad Prism software (version 6.0, GraphPad Software, USA), *P < 0.05; **P < 0.01.

and traps were gradually decreased with increasing Na^+ concentrations.

The wild-type strain and *ΔAopalH* mutants were incubated on CMY media with different pHs for 14 d at 28 °C to allow conidiation. Our analyses showed that conidiation was significantly reduced in the absence of the *AopalH* gene (Fig. 6; Supplementary File S1).

In the wild-type strain, trap structures were observed 12 h after inoculation. A large number of traps were produced 24 h after inoculation and almost all of the nematodes were captured and degraded after 48 h. In contrast, the *ΔAopalH* mutants did not form traps at 24 h, and only a few of the traps were produced on or around the nematodes at 48 h (Fig. 6). The trap numbers were also significantly reduced in the *ΔAopalH* mutants at all of the pHs tested (Supplementary File S1).

4. Discussion

In this study, a homologous gene of *palH*, *AopalH,* was examined to characterize its role in the nematode-trapping fungus *A. oligospora*. *AopalH* has high amino acid sequence identity with *palH* homologs from other fungi, especially those from two nematode-trapping fungi, *D. stenobrocha* and *D. haptotyla* (Meerupati et al., 2013; Liu et al., 2014). Phylogenetic analysis showed that the palH orthologs from nematode-trapping fungi formed a distinct clade separated from the other non-nematophagous filamentous fungi (Fig. 1), which was consistent with their taxonomic relationships.

We also determined that the capacity to produce conidia and traps was significantly inhibited when the *AopalH* gene was knocked out (Fig. 6). Northern blot analysis showed that the transcript level of the *AopalH* gene was up-regulated after the addition of NE in *A. oligospora* (Fig. 2F), suggesting that the PacC/Rim101 pH-responsive signaling, and specifically the putative ambient pH sensor AopalH, may play a critical role in trap formation of *A. oligospora*. In comparison with the wild-type strain, the *ΔAopalH* strains exhibited slower growth rates on TYGA and CMA meidia (Fig. 3). Also, at neutral and alkaline pHs, the *ΔAopalH* mutants grew slower than the wild type strain (Fig. 4). These results indicated that the absence of AopalH impacted the vegetative growth of *A. oligospora*. In addition, the mutant strains showed lower growth rates than the wild-type strain on media with 0.2 M NaCl and 5 mM H_2O_2 (Fig. 5), suggesting that AopalH might also influence *A. oligospora*'s response to cationic and hyperoxidation stresses. However, this difference in growth rates was not observed at 0.3 M NaCl, because all of the strains did not grow well under these conditions. All of these phenotypes indicate that the pH sensing receptor AopalH likely plays a pivotal role in conidiation, trap devices, vegetative growth as well as cationic and hyperoxidation stress response in *A. oligospora*.

The pal/PacC mediated pH signal transduction pathway has been reported to regulate several biological processes in filamentous fungi. For example, in *A. nidilans*, genes involved in cellulolytic processes are regulated by PacC/Rim101 pH-responsive signaling (Xia et al., 2010). Also, the deletion of individual genes in the Pal/Pac signaling pathway can result in drastically reduced expression of some enzymes required for the syntheses of oxalic and lactic acids in *B. bassiana* (Zhu et al., 2016). In this study, we identified for the first time that this pH regulatory pathway may play critical roles in fungal conidiation and trap formation in *A. oligospora*. Previously, an orthologue of the transcription factor PacC, which acts downstream of PalH in PacC/Rim101 pH-responsive signaling, has been functionally identified in another nematophagous fungus, *C. rosea* (Zou et al., 2010). In *C. rosea*, disruption of *pacC* resulted in a slower growth rate at alkaline pH, altered filamentation, reduced conidiation and attenuated virulence to nematodes, suggesting that PacC functions as a positive regulator of virulence to nematodes in *C. rosea* (Zou et al., 2010). In our study, the disruption of *AopalH* in *A. oligospora* reduced conidiation and prevented the formation of traps. Although the mechanisms used by these two fungi to attack nematodes are different, the pH regulatory systems in both

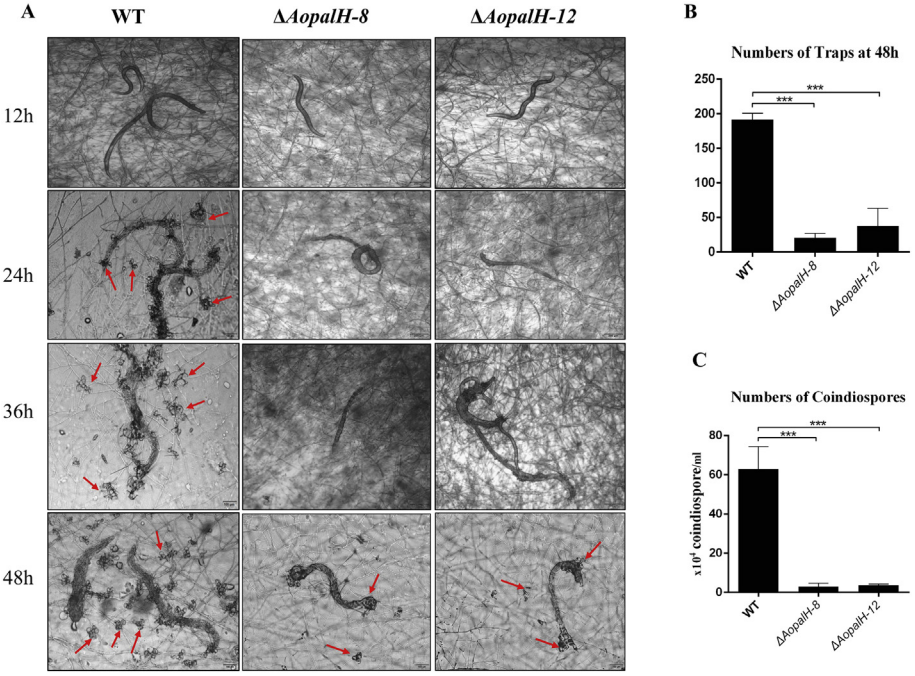

Fig. 6. Comparison of trap formation and conidiation between the wild-type and the *ΔAopalH* mutant strains. A. Wild-type and *ΔAopalH* mutant strains 12 h, 24 h, 36 h and 48 h after being induced by nematode extract (NE). B. Induction of trap formation in the wild type and mutant strains. C. Sporulation of the wild-type and *ΔAopalH* mutant strains. Differences were determined by ANOVA using GraphPad Prism software. ***P < 0.001 versus WT (wild type). The results are the means ± SE of three independent experiments.

nematophagous fungi have critical functions. Our results highlight the complexity of pH regulatory pathways in fungi and that multiple abiotic and biological factors are involved in regulating conidiation and trap formation.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We are grateful to Prof. Jianping Xu of the Department of Biology, McMaster University, for valuable comments and critical discussions. The research described here is jointly supported by the National Natural Science Foundation of China (approved nos. 31560025 and 31760538), General Program of the Applied Basic Research Programs of Yunnan Province (approval no. 2016FB044), the Department of Science and Technology of Yunnan Province (2017HB006), the NSFC-Yunnan Joint Fund (U1402265), the Program for Excellent Young Talents of Yunnan University (to Jinkui Yang). We also thank the anonymous reviewers for their valuable suggestions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.05.008.

References

Arst, H.N., Peñalva, M.A., 2003. pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. Trends Genet. 19 (4), 224–231.
Bertuzzi, M., Schrettl, M., Alcazar-Fuoli, L., Cairns, T.C., Muñoz, A., Walker, L.A., Herbst, S., Safari, M., Cheverton, A.M., Chen, D., 2014. The pH-responsive PacC transcription factor of *Aspergillus fumigatus* governs epithelial entry and tissue invasion during pulmonary aspergillosis. PLoS Pathog. 10 (10), e1004413.
Caddick, M.X., Brownlee, A.G., Arst, H.N., 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol. Gen. Genet. 203 (2), 346.
Calcagno-Pizarelli, A.M., Negrete-Urtasun, S., Denison, S.H., Rudnicka, J.D., Bussink, H.J., Múnera-Huertas, T., Stanton, L., Hervás-Aguilar, A., Espeso, E.A., Tilburn, J., 2007. Establishment of the ambient pH signaling complex in *Aspergillus nidulans*: PalI assists plasma membrane localization of PalH. Eukaryot. Cell 6 (12), 2365–2375.
Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17 (4), 540–552.
Chen, Y., Zhu, J., Ying, S.H., Feng, M.G., 2014. Three mitogen-activated protein kinases required for cell wall integrity contribute greatly to biocontrol potential of a fungal entomopathogen. PLoS One 9 (2), e87948.
Christianson, T.W., Sikorski, R.S., Dante, M., Shero, J.H., Hieter, P., 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene 110 (1), 119–122.
Colot, H.V., Park, G., Turner, G.E., Ringelberg, C., Crew, C.M., Litvinkova, L., Weiss, R.L., Borkovich, K.A., Dunlap, J.C., 2006. A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. U. S. A. 103 (27), 10352.
Cornet, M., Gaillardin, C., 2014. pH signaling in human fungal pathogens: a new target for antifungal strategies. Eukaryot. Cell 13 (3), 342–352.
Cupertino, F.B., Freitas, F.Z., Paula, R.M.D., Bertolini, M.C., 2012. Ambient pH controls glycogen levels by regulating glycogen synthase gene expression in *Neurospora crassa*. New insights into the pH signaling pathway. PLoS One 7 (8), e44258.
Davis, D.A., 2009. How human pathogenic fungi sense and adapt to pH: the link to virulence. Curr. Opin. Microbiol. 12 (4), 365–370.
Denison, S.H., 2000. pH regulation of gene expression in fungi. Fungal Genetics & Biology Fg & B 29 (2), 61.
Edgar, R., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32 (5), 1792–1797.
Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39 (4), 783–791.
Futai, E., Kubo, T., Sorimachi, H., Suzuki, K., Maeda, T., 2001. Molecular cloning of PalBH, a mammalian homologue of the *Aspergillus* atypical calpain PalB. Biochim. Biophys. Acta 1517 (2), 316–319.
Galindo, A., Calcagno-Pizarelli, A.M., Peñalva, M.Á., 2012. An ordered pathway for the assembly of fungal ESCRT-containing ambient pH signalling complexes at the plasma membrane. J. Cell Sci. 125 (7), 1784–1795.
Gray, N., 1984. Ecology of nematophagous fungi: comparison of the soil sprinkling method with the Baermann funnel technique in the isolation of endoparasites. Soil Biol. Biochem. 16 (1), 81–83.
Hervás-Aguilar, A., Galindo, A., Peñalva, M.A., 2010. Receptor-independent ambient pH signaling by ubiquitin attachment to fungal arrestin-like PalF. J. Biol. Chem. 285 (23), 18095–18102.
Huang, W., Shang, Y., Chen, P., Gao, Q., Wang, C., 2015. MrpacC regulates sporulation, insect cuticle penetration and immune evasion in *Metarhizium robertsii*. Environ. Microbiol. 17 (4), 994–1008.
Huang, X., Zhao, N., Zhang, K., 2004. Extracellular enzymes serving as virulence factors in nematophagous fungi involved in infection of the host. Res. Microbiol. 155 (10), 811–816.
James, T.Y., Kauff, F., Schoch, C.L., Matheny, P.B., Hofstetter, V., Cox, C.J., Celio, G., Gueidan, C., Fraker, E., Miadlikowska, J., 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. Nature 443 (7113), 818–822.
Kirkland, B.H., Eisa, A., Keyhani, N.O., 2005. Oxalic acid as a fungal acaracidal virulence factor. J. Med. Entomol. 42 (3), 346–351.
Lewis, M.W., Robalino, I.V., Keyhani, N.O., 2009. Uptake of the fluorescent probe FM4-64 by hyphae and haemolymph-derived *in vivo* hyphal bodies of the entomopathogenic fungus *Beauveria bassiana*. Microbiology 155 (9), 3110–3120.
Li, J., Zou, C., Xu, J., Ji, X., Niu, X., Yang, J., Huang, X., Zhang, K.-Q., 2015. Molecular mechanisms of nematode-nematophagous microbe interactions: basis for biological control of plant-parasitic nematodes. Annu. Rev. Phytopathol. 53, 67–95.
Liu, K., Zhang, W., Lai, Y., Xiang, M., Wang, X., Zhang, X., Liu, X., 2014. *Drechslerella stenobrocha* genome illustrates the mechanism of constricting rings and the origin of nematode predation in fungi. BMC Genomics 15, 114.
Lucena-Agell, D., Hervás-Aguilar, A., Múnera-Huertas, T., Pougovkina, O., Rudnicka, J., Galindo, A., Tilburn, J., Arst Jr., H.N., Peñalvaet, M.A., 2016. Mutational analysis of the *Aspergillus* ambient pH receptor PalH underscores its potential as a target for antifungal compounds. Mol. Microbiol. 101 (6), 982–1002.
Meerupati, T., Andersson, K.M., Friman, E., Kumar, D., Tunlid, A., Ahren, D., 2013. Genomic mechanisms accounting for the adaptation to parasitism in nematode-trapping fungi. PLoS Genet. 9 (11), e1003909.
Negreteurtasun, S., Denison, S.H., Jr, A.H., 1997. Characterization of the pH signal transduction pathway gene palA of *Aspergillus nidulans* and identification of possible homologs. J. Bacteriol. 179 (5), 1832–1835.
Negreteurtasun, S., Reiter, W., Diez, E., Denison, S.H., Tilburn, J., Espeso, E.A., Peñalva, M.A., Arst, J.H., 1999. Ambient pH signal transduction in *Aspergillus*: completion of gene characterization. Mol. Microbiol. 34 (5), 994–1003.
Nordbring-Hertz, B., Jansson, H.-B., Tunlid, A., 2006. Nematophagous fungi. Encyclopedia of Life Sciences, pp. 1–11.
Peñalva, M.A., Arst, H.N., 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol. Mol. Biol. Rev. 66 (3), 426.
Peñalva, M.A., Bignell, E., Arst, H.N., Tilburn, J., 2008. Ambient pH gene regulation in fungi: making connections. Trends Microbiol. 16 (6), 291.
Peñalva, M.A., Lucena-Agell, D., Arst Jr., H.N., 2014. Liaison alcaline: pals entice non-endosomal ESCRTs to the plasma membrane for pH signaling. Curr. Opin. Microbiol. 22 (8), 49–59.
St Leger, R.J., Joshi, L., Roberts, D., 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhizium anisopliae*. Appl. Environ. Microbiol. 64 (2), 709–713.
St Leger, R.J., Joshi, L., Roberts, D.W., 1997. Adaptation of proteases and carbohydrates of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. Microbiology 143 (Pt 6), 1983–1992.
Talavera, G., Castresana, J., 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst. Biol. 56 (4), 564–577.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30 (12), 2725–2729.
Tilburn, J., Sarkar, S., Widdick, D.A., Espeso, E.A., Orejas, M., Mungroo, J., Peñalva, M.A., Arst, H.N., 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. 14 (4), 779.
Tunlid, A., Ahman, J., Oliver, R.P., 1999. Transformation of the nematode-trapping fungus *Arthrobotrys oligospora*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 173 (1), 111–116.
Wang, C., Feng, M.G., 2014. Advances in fundamental and applied studies in China of fungal biocontrol agents for use against arthropod pests. Biol. Control 68 (1), 129–135.
Xia, H., Yuan, X.Y., Wilhelmus, K.R., 2010. A fungal pH-responsive signaling pathway regulating Aspergillus adaptation and invasion into the cornea. Investig. Ophthalmol. Vis. Sci. 51 (3), 1517–1523.
Xie, X.Q., Li, F., Ying, S.H., Feng, M.G., 2012. Additive contributions of two manganese-cored superoxide dismutases (MnSODs) to antioxidation, UV tolerance and virulence of *Beauveria bassiana*. PLoS One 7 (1), e30298.
Yang, J., Tian, B., Liang, L., Zhang, K.-Q., 2007. Extracellular enzymes and the pathogenesis of nematophagous fungi. Appl. Microbiol. Biotechnol. 75 (1), 21–31.

Yang, J., Wang, L., Ji, X., Feng, Y., Li, X., Zou, C., Xu, J., Ren, Y., Mi, Q., Wu, J., Liu, S., Liu, Y., Huang, X., Wang, H., Niu, X., Li, J., Liang, L., Luo, Y., Ji, K., Zhou, W., Yu, Z., Li, G., Liu, Y., Li, L., Qiao, M., Feng, L., Zhang, K.Q., 2011. Genomic and proteomic analyses of the fungus *Arthrobotrys oligospor*a provide insights into nematode-trap formation. PLoS Pathog. 7 (9), e1002179.

Zhang, K.-Q., Hyde, K.D., 2014. Nematode-trapping Fungi. Springer Science & Business.

Zhao, X.Y., Wang, Y., Zhao, Y., Huang, Y., Zhang, K.-Q., Yang, J.K., 2014. Malate synthase gene AoMls in the nematode-trapping fungus *Arthrobotrys oligospora* contributes to conidiation, trap formation, and pathogenicity. Appl. Microbiol. Biotechnol. 98 (6), 2555–2563.

Zhu, J., Ying, S.H., Feng, M.G., 2016. The Pal pathway required for ambient pH adaptation regulates growth, conidiation, and osmotolerance of *Beauveria bassiana* in a pH-dependent manner. Appl. Microbiol. Biotechnol. 100 (10), 4423–4433.

Zou, C., Tu, H., Liu, X., Tao, N., Zhang, K.-Q., 2010. PacC in the nematophagous fungus *Clonostachys rosea* controls virulence to nematodes. Environ. Microbiol. 12 (7), 1868–1877.

Zou, C.G., Tao, N., Liu, W.J., Yang, J.K., Huang, X.W., Liu, X.Y., Tu, H.H., Gan, Z.W., Zhang, K.Q., 2010b. Regulation of subtilisin-like protease prC expression by nematode cuticle in the nematophagous fungus *Clonostachys rosea*. Environ. Microbiol. 12 (12), 3243–3252.

Zou, C.G., Tu, H.H., Liu, X.Y., Tao, N., Zhang, K.Q., 2010a. PacC in the nematophagous fungus *Clonostachys rosea* controls virulence to nematodes. Environ. Microbiol. 12 (7), 1868–1877.