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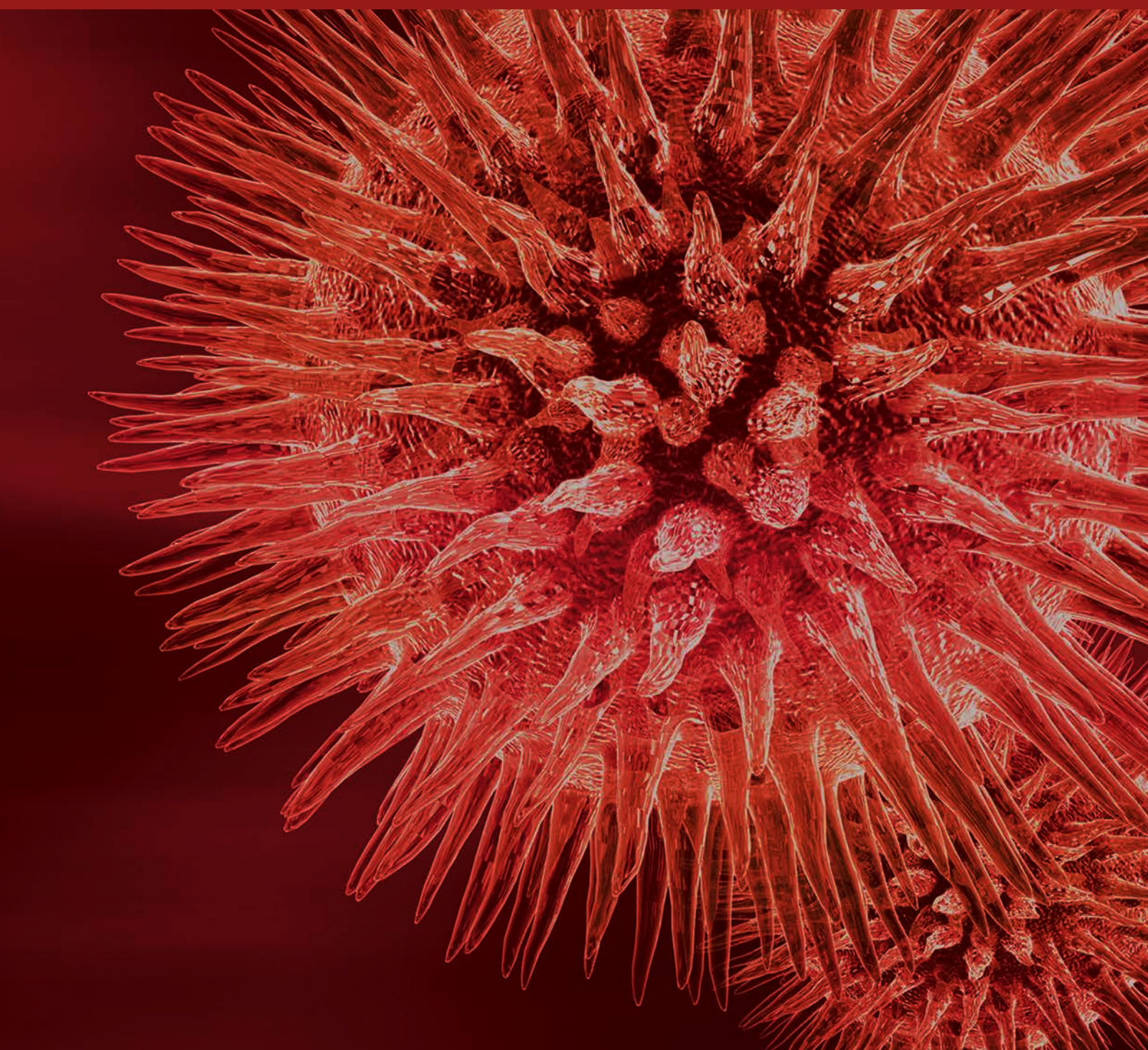
Plant Fungal Pathogenesis

Guest Editors: Jun Yang, Tom Hsiang, Vijai Bhadauria, Xiao-Lin Chen, and Guotian Li



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Editorial
Plant Fungal Pathogenesis

Jun Yang,[1] Tom Hsiang,[2] Vijai Bhadauria,[3] Xiao-Lin Chen,[4] and Guotian Li[5]

[1]*Department of Plant Pathology, China Agricultural University, Beijing 100193, China*
[2]*School of Environmental Sciences, University of Guelph, Guelph, ON, Canada N1G 2W1*
[3]*Semiarid Prairie Agricultural Research Centre, Agriculture and Agri-Food Canada, Swift Current, SK, Canada S9H 3X2*
[4]*College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China*
[5]*Department of Plant Pathology and the Genome Center, University of California, Davis, CA 95616, USA*

Correspondence should be addressed to Jun Yang; yangj@cau.edu.cn

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Fungal plant pathogens can cause enormous losses in yield and quality of field crops, fruits, and other edible plant material, and this becomes increasingly a more important issue to human health and the global economy in this century, with increasing human populations and climate change threats to arable land. Deciphering fungal pathogenesis not only allows us to better understand how fungal pathogens infect host plants but also provides valuable information for the control of plant diseases, including new strategies to prevent, delay, or inhibit fungal development. This special issue summarizes recent novel findings on plant fungal pathogenesis.

Pathogenic fungi differ greatly in their life styles. Some are necrotrophic, while others are hemibiotrophic, biotrophic, or obligately biotrophic. Despite the obvious differences in life styles, fungal pathogens are known to use well-conserved proteins in infection processes. The conserved proteins are therefore potential targets for controlling these fungal diseases. X. Zhang et al. focused on receptor for activated C kinase 1 (Rack1), a conserved protein involved in various biological processes in eukaryotes. They reviewed functions of Rack1 proteins in model and pathogenic fungi. Rack1 proteins are involved in vegetative growth, conidiation, mating, toxin biosynthesis, and stress responses via different pathways including cAMP/PKA and MAPK pathways in different fungi, illustrating how Rack1 proteins are involved in fungal pathogenesis.

In order to infect, pathogenic fungi can develop specialized infection structures, such as appressoria to penetrate host cells. During this process, the peroxisomes play key roles to facilitate full functions of virulence proteins. X.-L. Chen et al. focused on roles of the peroxisomes in the rice blast fungus *Magnaporthe oryzae*. They described molecular mechanisms underlying how the peroxisomes function related to life cycles and metabolic processes. And also, they provided an overview of the relationship between peroxisomes and pathogenicity. This review will be valuable for researchers interested in understanding how the peroxisomes serve as a platform to orchestrate plant host invasion by plant filamentous fungi.

The hemibiotrophic fungus *Colletotrichum higginsianum* is the causal agent of anthracnose diseases on a wide range of cruciferous plants (Brassicaceae), including the model plant *Arabidopsis thaliana*. Also, the *C. higginsianum-A. thaliana* pathosystem is now considered to be an important model for studying fungal pathogenicity, in which both hosts can be efficiently genetically manipulated. The conserved pathogenic factors in various fungal pathogens that have been the subject of much study might also be considered targets for pesticide design. As a pleiotropic regulator of morphogenesis and plant infection, Ste7 MEK possesses highly conserved roles in phytopathogens. Q. Yuan et al. reported that the *C. higginsianum* gene *ChSTE7* is involved in regulation of vegetative growth, appressorial formation, and invasive growth in host tissues. This is an important and conserved virulence factor affecting infection of *C. higginsianum* on cruciferous plants.

Covalent histone modifications, such as methylation and acetylation, provide key epigenetic information in transcriptional regulation and chromatin structure organization for functional responses. Histone methylation provides an

excellent epigenetic mechanism for stable transfer of gene expression profiles to progeny cells. In pathogenic fungi, the SET domain-containing proteins play essential roles in fungal growth and development. Z. Cao et al. demonstrated that MoKMT2H, an Ash1-like histone modification protein, played important roles in conidial germination and pathogenesis in *M. oryzae*. They found that the Δ*Mokmt2h* null mutants are not defective in genome-wide histone methyltransferase modification, vegetative hyphal growth, conidial morphology, conidiation, or disease lesion formation on rice leaves. However, the *MoKMT2H* deletion mutants showed delayed conidial germination and attenuated virulence. Their results suggested that *MoKMT2H* plays an important role in conidial germination in the rice blast fungus.

The defense mechanisms of wheat against *Puccinia striiformis* f. sp. *tritici* (*Pst*) infection are complex, and activation of defense responses is critical in order to prevent the spread of pathogens. The plant cytoskeleton, including microtubules and microfilaments, is a highly dynamic subcellular structure that is associated with plant defense responses. J. Wang et al. focused on the function of microtubule polymerization in wheat against the stripe rust fungus *Pst* CYR23. They detected the frequency of hypersensitive cell deaths and H_2O_2 accumulations in leaves treated with microtubule inhibitor oryzalin before inoculation with strain CYR23. Depolymerization of microtubules reduced the resistance of plants via hypersensitive responses and led to decreased H_2O_2 accumulation, suggesting that microtubules play roles in resistance against the stripe rust fungus in wheat.

Taken together, these research results have furthered our understanding of plant-microbe interactions and may help promote further research in this area.

Jun Yang
Tom Hsiang
Vijai Bhadauria
Xiao-Lin Chen
Guotian Li

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Research Article

An Ash1-Like Protein MoKMT2H Null Mutant Is Delayed for Conidium Germination and Pathogenesis in *Magnaporthe oryzae*

Zhaojun Cao, Yue Yin, Xuan Sun, Jun Han, Qing peng Sun, Min Lu, Jinbao Pan, and Weixiang Wang

Beijing Key Laboratory of New Technique in Agricultural Application College of Plant Science and Technology, Beijing University of Agriculture, Beijing 100026, China

Correspondence should be addressed to Jinbao Pan; buazkp@163.com and Weixiang Wang; wwxbua@163.com

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Ash1 is a known H3K36-specific histone demethylase that is required for normal Hox gene expression and fertility in *Drosophila* and mammals. However, little is known about the expression and function of the fungal ortholog of Ash1 in phytopathogenic fungus *Magnaporthe oryzae*. Here we report that MoKMT2H, an Ash1-like protein, is required for conidium germination and virulence in rice. We obtained *MoKMT2H* null mutant (Δ*MoKMT2H*) using a target gene replacement strategy. In the Δ*MoKMT2H* null mutants, global histone methyltransferase modifications (H3K4me3, H3K9me3, H3K27me3, and H3K36me2/3) of the genome were unaffected. The Δ*MoKMT2H* mutants showed no defect in vegetative hyphal growth, conidium morphology, conidiation, or disease lesion formation on rice leaves. However, the *MoKMT2H* deletion mutants were delayed for conidium germination and consequently had decreased virulence. Taken together, our results indicated that MoKMT2H plays an important role in conidium germination during appressorium formation in the rice blast fungus and perhaps other pathogenic plant fungi.

1. Introduction

Covalent histone modifications, such as methylation and acetylation, provide key epigenetic information in transcriptional regulation and chromatin structure organization for functional responses [1, 2]. The vast array of such modifications gives enormous potential for functional responses. The timing of the appearance of a modification will depend on the signaling conditions within the cell. Histone methylation is catalyzed by histone methyltransferases (HMTs) and provides an excellent epigenetic mechanism for the stable transfer of gene expression profiles to progeny cells [1, 3, 4].

HMTs can be grouped into two divergent families; histone lysine methyltransferases (HKMTs) catalyze the methylation of lysine residues, and protein arginine methyltransferases (PRMTs) catalyze the methylation of arginine residues. HKMTs are conserved in a wide range of eukaryotes, playing roles in cellular signaling pathways related to the cell cycle, transcription, and pathogenesis [1, 5, 6]. In *Fusarium graminearum*, H3K4me is required for the active transcription of genes involved in deoxynivalenol and aurofusarin biosynthesis [7]. The histone H3K4 methyltransferase FgSET1 plays important roles in the response to agents that damage the cell wall by negatively regulating the phosphorylation of FgMgv1 [7, 8]. Another H3K27me3 methyltransferase, KMT6, regulates the development and expression of secondary metabolite gene clusters in *F. graminearum* [9].

The SET domain is an evolutionarily conserved domain found in HMTs. In *Neurospora crassa*, the SET domain-containing protein set-2 encodes a histone H3K36 methyltransferase and is essential for normal growth and development [10–12]. The *Drosophila* trithorax group protein absent, small, or homeotic discs 1 (Ash1) contains a SET domain and is involved in maintaining active transcription of many genes by conferring a relaxed chromatin structure [13–20]. In mammals, Ash1-like (ASH1L) is the Ash1 homolog and occupies most actively transcribed genes and methylates histone H3 in a nonredundant fashion at a subset of genes, including Hox

Table 1: Fungal strains used in this study.

Strain	Description	Reference
P131	The wild-type strain	Peng and Shishiyama (1988) [29]
KO1	The deletion mutant of *MoKMT2H*	This study
KO2	The deletion mutant of *MoKMT2H*	This study
KO3	The deletion mutant of *MoKMT2H*	This study

genes [13, 17, 20, 21]. In human HeLa cells, Ash1 methylates histone H3 at lysine 36 and regulates the development of myelomonocytic lineages from hematopoietic stem cells [22]. However, roles for the fungal Ash1 ortholog, a well-studied H3K36me2 methyltransferase, have not yet been extensively explored in *M. oryzae*.

M. oryzae is a causal agent of rice blast disease, which is the most devastating and persistent disease in cultivated rice [23, 24]. *M. oryzae* can produce a three-celled asexual spore called a conidium as its primary source of inoculum. When a conidium lands on the surface of a leaf from a rice plant, it germinates to form an appressorium as the infection structure. The appressorium can enter into the host plant and grow inside the plant tissues, leading to yield losses or even death of the infected plant [25–28]. Recently, several studies have focused on the molecular mechanism of HKMTs in plant pathogenesis and plant infection [7, 8, 29]. The *M. oryzae* histone H3K4me3 methyltransferase gene *SET1* was targeted for gene disruption by homologous recombination [28]. Chromatin immunoprecipitation-sequence analysis indicated that MoSET1 can regulate global gene expression during infection-related morphogenesis [28]. Widely conserved MoKMT2H in ascomycete fungi is a functional homolog of Ash1, which is implicated in H3K4 and H3K36 methylation. In a previous study, researchers used a wheat-infecting strain to confirm that the *MoKMT2H* gene is involved in infection-related morthogenesis and pathogenicity to wheat and barley cultivars other than rice [28]. Here, we targeted the Ash1-like *MoKMT2H* gene for gene replacement to further determine the function of MoKMT2H in a rice-infecting *M. oryzae* strain. The Δ*MoKMT2H* null mutant has no obvious defect in vegetative hyphal growth, conidium morphology, conidiation, or disease lesion formation on rice and wheat leaves. However, the Δ*MoKMT2H* null mutant was delayed for conidium germination and consequently had decreased virulence. This study offers evidence for the involvement of the histone methyltransferase function of Ashl-like proteins during plant pathogenesis and may provide important implications for discovering new HKMTs in the rice blast fungus and perhaps other pathogenic plant fungi.

2. Materials and Methods

2.1. Antibodies. Antibodies against histone H3 (Abcam, ab1791), H3K36me3 (Abcam, ab9095), H3K36me2 (Abcam, ab9049), H3K4me3 (Abcam, ab8580), H3K27me3 (Upstate, 07-449), and H3K9me3 (Millipore, 07-442) were purchased commercially.

2.2. Fungal Strains and Growth Conditions. The wild-type strain P131 of *Magnaporthe oryzae* was used in this study. P131 was the field isolate [29], which was used to generate mutant strains. The wild-type strain P131 and all the transformant strains generated in this study were listed in Table 1. They were grown on oatmeal tomato agar (OTA) medium and cultured at 25°C under light conditions [29–31]. For extraction of genomic DNA and protein and isolation of protoplasts, fresh mycelia were braked and cultured in liquid complete medium (CM: 0.6% yeast extract, 0,3% enzymatic casein hydrolysate, 0.3% acidic casein hydrolysate, and 1% glucose) and shaken at 150 rpm at 25°C for 36 h. To determine fungal growth, mycelial plugs of ~5 mm in diameter were inoculated on OTA plates. Colony diameter on each plate was measured for ~3 days. Each experiment was repeated three times.

2.3. Molecular Manipulations with Nucleic Acids. For genomic DNA extraction, genomic DNA was isolated using the cetyltrimethylammonium bromide protocol as described [31, 32]. ~1 to 2 g mycelia was harvested and ground into a fine powder and then extracted with 15 mL of extraction buffer (50 mM Tris-HCl, 100 mM EDTA, and 150 mM NaCl). Add 750 μL 20% SDS, mix well, and incubate at 37°C for 1 h. Add 2.25 mL 5 M NaCl and 2 mL CTAB/NaCl buffer (10% CTAB, 0.7 M NaCl), mix well, and then incubate at 65°C for 30 min. Add chloroform : isoamyl alcohol (24 : 1) and mix well. Cell debris was removed by centrifugation at 10,000 rpm for 15 min and genomic DNA was pelleted by isopropyl alcohol. The pellet was washed with 70% alcohol and dissolved in sterilized-distilled water and stored in −20°C. For Southern blot analysis, the extracted genomic DNA was completely digested by the restriction enzyme *Eco*RI digestion, and agarose gel separation and DNA gel blotting were performed following the standard protocols [29, 30, 32]. Hybridization was performed in solution containing 6x SSC, 5x Denhardt's solution, 0.5% SDS, and 100 μL mL^{-1} denatured salmon sperm DNA, at 65°C. Blots were exposed to phosphorimager analyzer (BAS-2040, Fuji Photo Film Co., Ltd., Tokyo, Japan) and visualized by phosphorimager analyzer software.

2.4. Generation of the MoKMT2H Gene Replacement Strains with Split-PCR Strategy. For generating the *MoKMT2H* gene replacement stains, target gene replacement was carried out using the split-PCR strategy [33]. The primers used to amplify the flanking sequences from the genomic DNA of P131 in this study are listed in Table 2. For the first round of PCR, the upstream and downstream flanking sequences

Table 2: PCR primers used in this study.

Primer	Sequence (5′-3′)
LBCK	GCCTGTCTGATTGGA
RBCK	GGGAGGGGGTGATGACGGTC
LB-F	CTGCTGCTTAGGTCGGTAGTCT
LB-R	TTGACCTCCACTAGCTCCAGCCAAGCCATTGGCTGGTTGGTTTGTTGGT
RB-F	GAATAGAGTAGATGCCGACCGGGCGTCACATGCGAACAAGAACCA
RB-R	GGCAAGGCAAGATTGGCTAAGA
HYG-F	CTTGGCTGGAGCTAGTGGAGGT
HYG-R	CCCGGTCGGCATCTACTCTATTC
HYG-F1	CGTTGCAAGACCTGCCTGAA
HYG-R1	GGATGCCTCCGCTCGAAGTA
HYG-LBCK	GACAGACGTCGCGGTGAGTT
HYG-RBCK	TCTGGACCGATGGCTGTGTAG
UPF	GAGAACTCAA GCGTCACTCC
UPR	GAACCAAAAGCATGTTTCT
Pr2937F	CCTTGCCTGTCTGATTGG
Pr2937R	GGAGTGACGCTTGAGTTC

were amplified with the primer pairs LBCK/LB-R and RB-F/RBCK, respectively. For the second round of PCR, the fused 2937-HYG DNA flanking fragments of the left arm and right arm were amplified from the DNA products of the first-round PCR with the primers LB-F/HYG-R1 and RB-R/HYG-F1, respectively. Protoplasts were isolated and the two flanking sequences were transformed into P131 protoplasts using the classic method [32, 33]. For selecting hygromycin-resistant or neomycin-resistant transformants, CM plates were supplemented with 250 μg mL^{-1} hygromycin B (Roche, USA) or 400 μg mL^{-1} neomycin (Ameresco, USA). The selected neomycin-resistant transformants were subjected to PCR with primers UPF/HYG-R and UPR/HYG-F, respectively, and Southern blot analysis.

2.5. Protein Extraction and Western Blot Analysis. For all test strains, fresh mycelia were disturbed finely and transferred to liquid CM and shaken at 150 rpm at 25°C for 36 h. The resulting mycelia (~1.5 g) were harvested and frozen in liquid nitrogen. The frozen mycelia were ground into a fine powder and transferred to 10 mL of protein extraction buffer [50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM ethylene diamine tetraacetic acid (EDTA), 1% triton X-100, and 2 mM phenylmethylsulfonyl fluoride (PMSF)] and 10 μL of protease inhibitor cocktail (Roche, shanghai, China) as described [8]. After homogenization with a vortex shaker, the resulting lysate was centrifuged at 12,000 ×g for 30 min at 4°C. The supernatant was then separated by 13% denaturing polyacrylamide gel (SDS-PAGE), transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA, USA) with a Bio-Rad electroblotting apparatus, and evaluated using the antibodies anti-histone H3, anti-H3K36me3, anti-H3K36me2, anti-H3K4me3, anti-H3K27me3, and anti-H3K9me3, respectively [8]. Incubation with a secondary antibody (Santa Cruz, CA, USA) and chemiluminescent detection were performed as described previously [8, 29].

2.6. Plant Virulence Test and Observation of Infection. For the infection assay, *M. oryzae* conidia were collected from 7-day-old OTA plates and resuspended to ~2×10^4 conidia/mL in 0.025% Tween-20 solution. Four-week-old seedlings of rice (*Oryzae sativa*) cv. Lijiangxintuanheigu and 8-day-old seedlings of barley (*Hordeum vulgare*) cv. E9 were used for spray infection assays [29, 30]. Plant incubation was performed as described previously [29, 30]. Conidia were sprayed onto the barley or rice leaves, which were then incubated in a moist and dark chamber at 28°C. At 9 h and 36 h after inoculation, the leaf samples were observed by microscopy. Lesion formation was examined 7 days after inoculation. For mycelium plug inoculation, the mycelium plugs of the Δ*MoKMT2H* null mutants and the wild-type strain were inoculated onto the abraded rice plants leaves and barley leaves. For conidia droplet inoculation, conidia suspended to ~2×10^5 conidia/mL were inoculated onto the abraded rice plants leaves. Lesions were examined and photographed 5–7 days after inoculation. The mean number of lesions formed on 5 cm leaf tips was determined as described previously [29, 30].

3. Results

3.1. Target Gene Replacement of MoKMT2H in M. oryzae. MoKMT2H is widely conserved in ascomycete fungi. MoKMT2H is similar to mammalian Ash1l protein, which is required for H3K36me2 methylation. In previous studies, the Δ*MoKMT2H* mutants showed a significant reduction in vegetative growth, germination, appressorium formation, and pathogenicity to host plants [25]. However, the researchers used a wheat-infecting *M. oryzae* strain in their experiments and their tested *M. oryzae* strain cannot infect rice. To further determine the function of MoKMT2H in a rice-infecting *M. oryzae* strain, we performed target gene replacement of *MoKMT2H* with a hygromycin phosphotransferase cassette

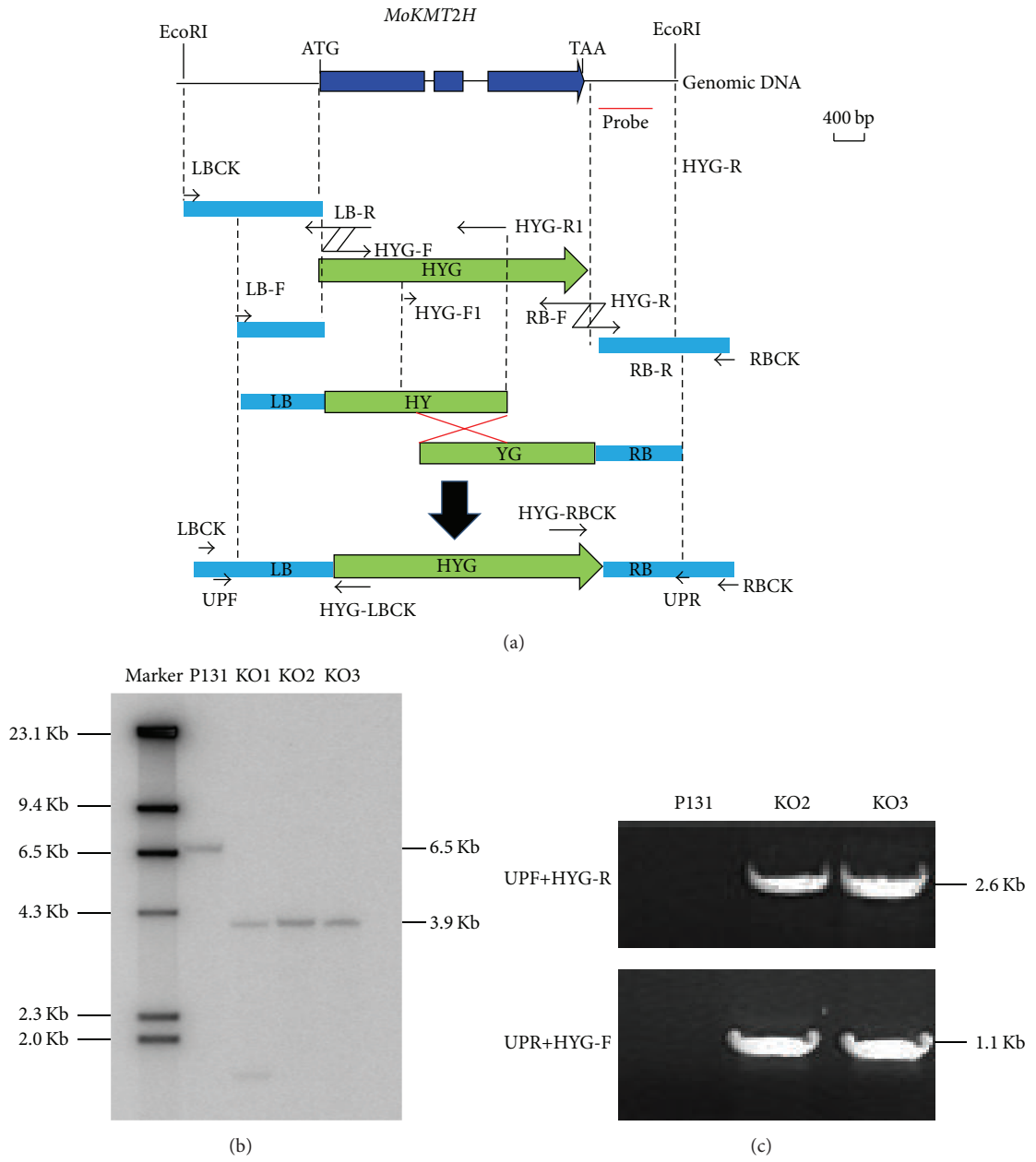

Figure 1: The strategy for *MoKMT2H* gene replacement with PCR. (a) Schematic representation of the genomic DNA of *MoKMT2H*. Blue boxes indicate exons. The ATG start codon and TAA stop codon are indicated. The *Eco*RI restriction enzyme sites are indicated. The red line labeled "probe" shows the region used for Southern blot analysis. The upstream and downstream flanking sequences were amplified with primer pairs LBCK/LB-R and RBCK/RB-F, respectively. The fused DNA fragments with HYG were amplified with LB-F/HYG-R1 and HYG-F1/RB-R. The DNA fragments used for transformants were amplified with LB-F/RB-F. (b) Southern blot analysis of *Eco*RI-digested genomic DNA from wild-type P131 and the neomycin-resistant transformants KO1, KO2, and KO3. Blots were hybridized with probe as indicated in (a). The DNA fragment used for a probe was amplified with primer pair Pr2937F/Pr2937R. (c) The transformants KO2 and KO3 were confirmed by amplification with primer pairs UPF/HYG-R and UPR/HYG-F.

using the split-marker recombination method (Figure 1(a)). Three neomycin-resistant transformants, KO1, KO2, and KO3, were selected for verification by PCR or Southern blot analysis. To further characterize the *MoKMT2H* gene deletion in the selected strains KO1, KO2, and KO3, we performed Southern blot analysis using a DNA fragment as a probe. The whole genomes of KO1, KO2, and KO3 were digested with *Eco*RI. When probed with a 0.3-kb DNA fragment, amplified with primer pairs Pr2937F/Pr2937R (Table 2), the Δ*MoKMT2H* null mutants produced a 3.9-kp band, whereas the wild-type strain had a 6.5-kp band (Figure 1(b)). For PCR verification, we used two primers, UPF/HYG-R and HYG-F/UPR, in our study. When amplified with primer pair UPF/HYG-R, the Δ*MoKMT2H* but not wild-type P131

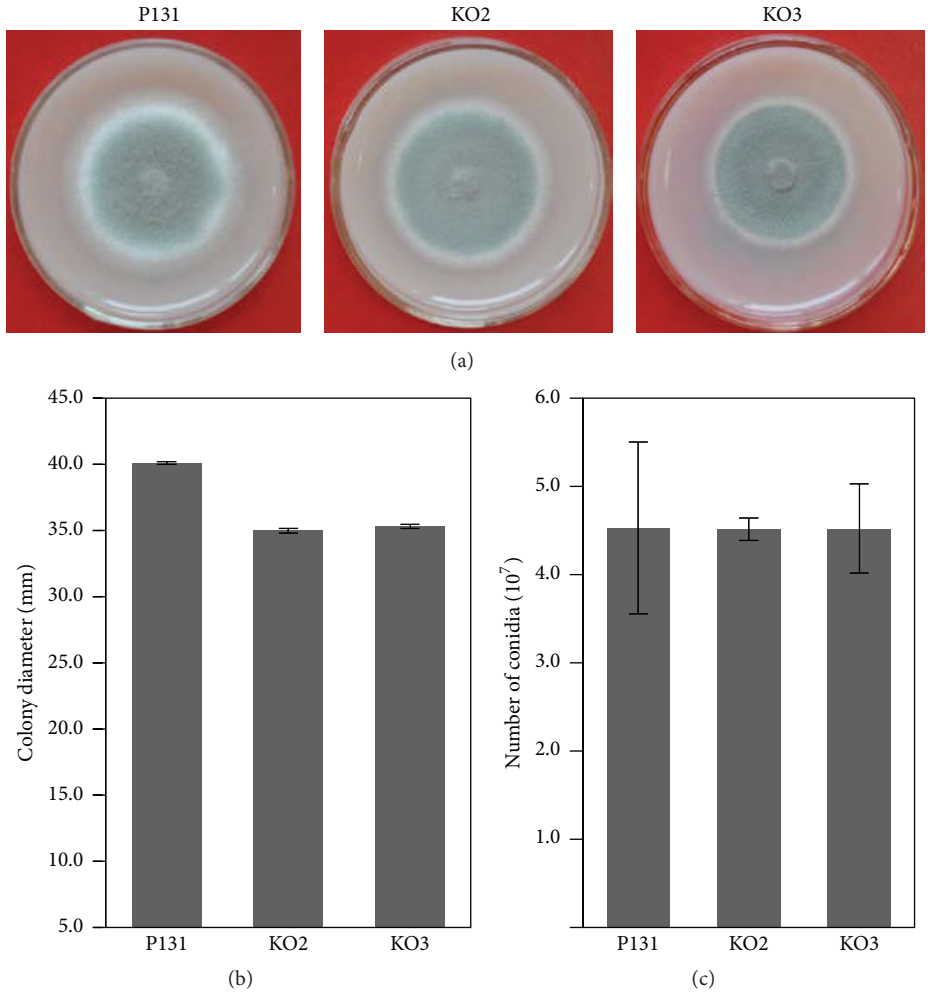

Figure 2: Comparisons of colony morphology, hyphal growth rate, and conidiation capacity of wild type (P131) and the gene deletion mutants of MoKMT2H. (a) Colony morphology of each strain incubated on OTA plates for 5 days at 25°C. (b) Colony diameter of each strain. (c) The average number of conidia for each strain. Values are the mean ± SD from three biological replicates.

strain had a 2.6-kb DNA band, whereas the Δ*MoKMT2H* strain had a 1.1-kb DNA band with primers HYG-F/UPR (Figure 1(c)). These results confirm that the *MoKMT2H* gene was completely replaced.

3.2. MoKMT2H Is Required for Conidium Formation and Pathogenicity on Wound Leaves of Rice. To further characterize the biological and chemical roles of *MoKMT2H* in *M. oryzae*, we compared the colony morphology, hyphal growth rate, conidiation capacity, and virulence on the leaves of rice. The Δ*MoKMT2H* null mutants and wild-type P131 were incubated on OTA plates for 3 days. The Δ*MoKMT2H* null mutants showed no obvious defects in colony morphology (Figure 2(a)), hyphal growth rate (Figure 2(b)), or the capacity for conidium formation (Figure 2(c)) in comparison with the wild-type P131 strain (Figure 2(a)), suggesting that *MoKMT2H* is not required for asexual development in *M. Oryzae. The* Δ*MoKMT2H* null mutant strain KO3 was selected for plant virulence test. To evaluate virulence of the Δ*MoKMT2H* null mutants, conidia suspensions from the Δ*MoKMT2H* null mutants and from wild-type P131 were inoculated onto the surface of barley. The Δ*MoKMT2H* conidium formation was reduced by 61% in comparison with the wild type 9 h after inoculation (Figures 3(a) and 3(b)). Strikingly, at 36 h after inoculation, wild-type P131 formed bulbous infection hyphae and could extend to neighboring host cells, whereas the Δ*MoKMT2H* null mutants had obvious defects in penetration peg formation (Figure 3(a)). To further confirm the plant infection defects in Δ*MoKMT2H* null mutants, conidia suspensions of the Δ*MoKMT2H* null mutants and wild-type P131 were sprayed onto seedlings of rice cultivar LTH. Typical robust lesions of the rice blast were observed for the wild type. However, the lesions of Δ*MoKMT2H* null mutants were not markedly reduced as compared with the wild type (Figure 3(c)). For the barley leaves, typical disease lesions were observed from both the wild type and the Δ*MoKMT2H* null mutant strains following spray inoculation of conidia (Figure 3(d)). To further check whether the Δ*MoKMT2H* null mutants could infect the host cells through wounds, the mycelium plugs of the Δ*MoKMT2H* null mutants and wild type were inoculated

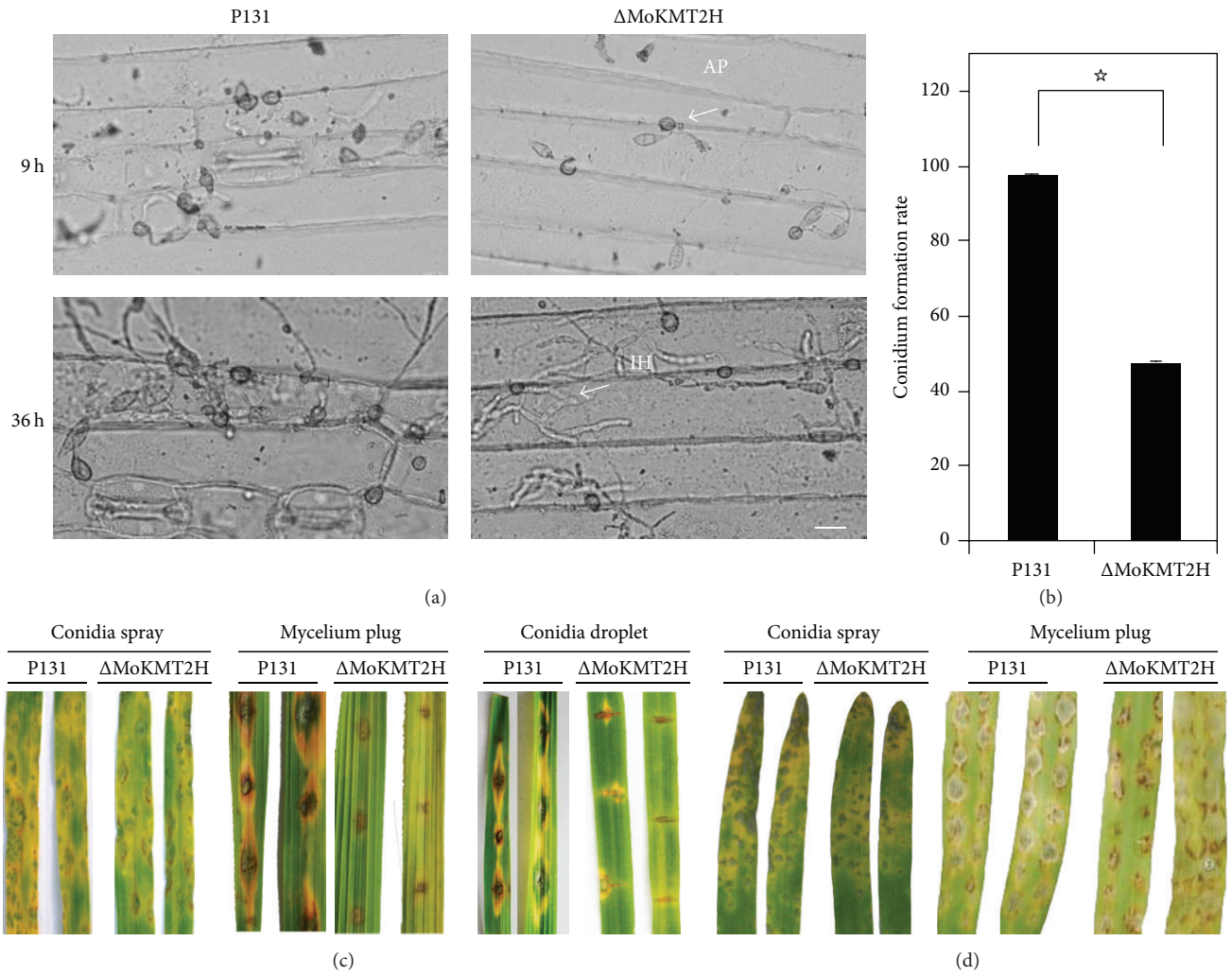

Figure 3: MoKMT2H is required for conidium formation and pathogenicity on rice leaves. (a) Microscopic observation of conidium formation in the wild-type strain and ΔMoKMT2H null mutants on barley leaves at 9 and 36 h after inoculation. Bar 20 μm. AP: appressorium, IH: infectious hyphae. (b) Conidiation of P131 and ΔMoKMT2H null mutants after growth on OTA plates for 7 days. Comparison of the conidium formation rate between P131 and ΔMoKMT2H null mutants at 48 h after conidiation. Values are the mean ± SD from three biological replicates. (c) Conidia spray (left), mycelium plug (middle), and conidia droplet inoculation on rice leaves with conidia from the wild-type P131 and the ΔMoKMT2H null mutants. Typical leaves were observed 7 days after mycelium plug inoculation. Mycelium plug and conidia droplet inoculation was conducted on abraded rice leaves. Typical leaves were observed 5 days after mycelium plug inoculation. (d) Conidia spray (left) and mycelium plug (right) inoculation on barley leaves with conidia from the wild-type P131 and the ΔMoKMT2H null mutants. ☆ indicates $P < 0.05$.

onto abraded rice leaves (Figure 3(c)). Although inoculation with the wild-type strain caused severe and typical lesions on wound rice leaves, the Δ*MoKMT2H* null mutants could not cause any disease lesions, suggesting that *MoKMT2H* was essential for penetration peg formation on wounded rice leaves (Figure 3(c)). To further confirm our observation, we performed conidia droplet inoculation on wounded rice plant leaves. As shown in Figure 3(c), inoculation with the wild-type caused typical disease lesions around the wound sites compared with the Δ*MoKMT2H* null mutants. Therefore, these results suggest that *MoKMT2H* regulates conidium formation and penetration peg formation in wound pathogenesis on rice leaves.

3.3. MoKMT2H Is a Conserved Set Domain-Containing Protein but Is Not Involved in Genome-Wide Histone Methylation. The SET domain, which mediates lysine methylation, regulates chromatin-mediated gene transcription. Published studies show that SET proteins in lower organisms can manipulate host transcription machinery in host-pathogen interactions [10, 11]. MoKMT2H is an Ash1-like protein [12]. Ash1-like orthologs of MoKMT2H are also present in *Drosophila*, *Homo sapiens*, *Mus musculus*, *F. graminearum*, and *M. oryzae* [10–16]. To further identify the homologs of Ash1-like proteins, we assessed the conservation of the SET domains through amino acid sequence alignment (Figure 4). The sequence alignment in Figure 4 highlights the conservation of the

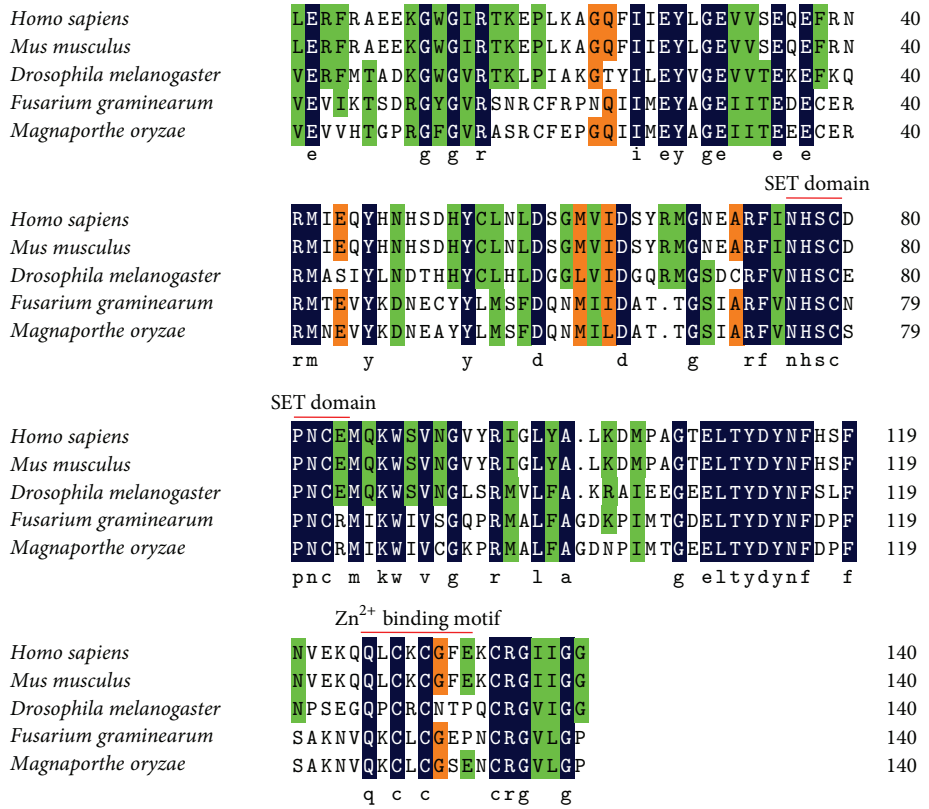

FIGURE 4: Multiple alignment of SET domain of Ashl-like proteins in classic species. The background colors indicate the conservation of amino acid residues. Orange shading color represents ≥50% similarity, green represents ≥75% similarity, and black represents 100% similarity. The conserved residues are indicated by red lines; the SET domain residues in the NHxxxPN motif and the post-SET domain, which consists of the cysteine-rich motif CxCxxxxC that binds Zn^{2+} OR that coordinates Zn^{2+} binding, are indicated.

SET domain residues in the NHxxxPN motif and the post-SET domain containing the Zn^{2+}-binding motif (CxCxxxxC) among these selected Ash1-like proteins. Collectively, these data indicate that MoKMT2H is highly conserved across lower eukaryotes to higher animals, which suggests that these SET domain-containing proteins may have evolved important roles in manipulating chromatin methylation-mediated gene transcription.

Considering that MoKMT2H is a candidate histone methyltransferase, we decided to apply western blot analysis to determine whether the methylation pattern of genome-wide histone lysines in the Δ*MoKMT2H* null mutants was changed. Some groups reported that MoKMT2H is not specific for H3K4, H3K27me3, or H3K20me3 methylation in their KMT null mutants [28]. However, some groups proved that the SET domain of MoKMT2H is much more closely related to H3K36me2-specific methyltransferases than H3K4-specific methyltransferases such as Set1 or trithorax group proteins [20, 22]. Total proteins were extracted from the mycelia of Δ*MoKMT2H* null mutant and wild-type strains. There was no significant reduction in the signal of H3K4me3, H3K9me3, H3K27me3, and H3K36me3 methylation in the Δ*MoKMT2H* null mutant (Figure 5). Moreover, the Δ*MoKMT2H* null strain also displayed no activity on H3K36me2 of the genome-wide chromatin. It is possible that MoKMT2H may act only on some specific target genes during the process of pathogenesis.

4. Discussion

The SET domain, which mediates lysine methylation, is one of the major epigenetic marks and regulates chromatin-mediated gene transcription [10, 12, 17]. The multiple alignment depicts the evolutionary history of the conserved SET domain of MoKMT2H, an Ash1-like protein. The presence of the cysteine-rich (CxCxxxxC) post-SET domain facilitates the binding of Zn^{2+} ions and marks an evolutionary divergence from the homologs in other organisms. In addition, the *S*-adenosylmethionine cofactor-binding site and the post-SET motif-containing region are also highly conserved. In our study, we did not, however, detect a change in genome-wide histone methylation on H3K36me2/3, H3K4me3, H3K9me3, or H3K27me3 in the Δ*MoKMT2H* null strains (Figure 5 and data now shown), which suggests that MoKMT2H may have methylation activity only on specific target genes of histone H3 or other key regulated proteins during the process of plant pathogenesis. Further experiments will focus on the HMT activity on recombinant nucleosomes or core histones with recombinant MoKMT2H SET domain deletions in vitro.

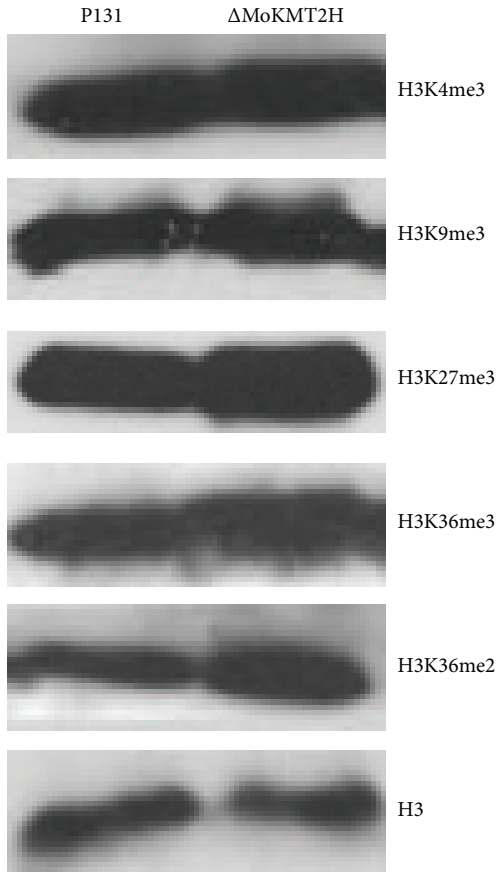

Figure 5: Western blot analysis of multiple histone modifications in the wild type and ΔMoKMT2H null mutants. Total proteins were extracted and separated by 13% SDS-PAGE, followed by immunoblotting with the indicated antibodies. Anti-H3 was used as a loading control.

MoKMT2H is required for conidiation, conidium germination, and appressorium formation [26, 28, 29], and Δ*MoKMT2H* null strains are defective for pathogenicity on wheat cultivar Norin 4 [28]. However, those previously tested wild-type and null mutant strains can only infect wheat. To further determine the biological and chemical activity of MoKMT2H, we generated Δ*MoKMT2H* null strains from the P131 wild-type strain, which forms classical lesions on rice leaves. The most interesting conclusion in this study we obtained when compared with previous studies is that deletion of *MoKMT2H* reduced the rate of conidium formation and plant virulence on wounded rice leaves. More interestingly, spray inoculation of the Δ*MoKMT2H* null strains did not cause any reduction in pathogenicity on rice leaves (Figures 3(c) and 3(d)). These results suggest that MoKMT2H may recognize specific effectors induced by wounds during the plant-pathogen interaction. Our study has thus shown that the Ash1-like protein MoKMT2H is required for conidium germination and pathogenesis in *M. oryzae*. MoKMT2H is widely conserved in ascomycete fungi; however, its biological roles have not been uncovered. Further research will focus on determining whether MoKMT2H has the chemical activity of a histone methyltransferase and isolating the proteins that it interacts with and its downstream target genes during pathogenesis.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Zhaojun Cao and Yue Yin contributed equally to this work. Weixiang Wang designed research, analyzed data, and wrote the paper; Zhaojun Cao and Yue Yin performed research; Min Lu, Qing peng Sun, Jun Han, and Jinbao Pan contributed analytic tools.

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Review Article

Roles of Rack1 Proteins in Fungal Pathogenesis

Xue Zhang,[1] Rashmi Jain,[2,3,4] and Guotian Li[2,3,4]

[1]*Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA*
[2]*Department of Plant Pathology and the Genome Center, University of California, Davis, CA 95616, USA*
[3]*Feedstocks Division, Joint BioEnergy Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA*
[4]*Biological Systems and Engineering Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720, USA*

Correspondence should be addressed to Guotian Li; gtli@ucdavis.edu

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Pathogenic fungi cause diseases on various organisms. Despite their differences in life cycles, fungal pathogens use well-conserved proteins and pathways to regulate developmental and infection processes. In this review, we focus on Rack1, a multifaceted scaffolding protein involved in various biological processes. Rack1 is well conserved in eukaryotes and plays important roles in fungi, though limited studies have been conducted. To accelerate the study of Rack1 proteins in fungi, we review the functions of Rack1 proteins in model and pathogenic fungi and summarize recent progress on how Rack1 proteins are involved in fungal pathogenesis.

1. Introduction

Diseases caused by pathogenic fungi pose a serious threat to human, animal, plant, and ecosystem health [1, 2]. Particularly, plant diseases caused by fungi, for example, rice blast and wheat rust, have long been known as widespread threats to global food security [1, 3]. In pathogenic fungi, key proteins and signal transduction pathways play conserved roles in the regulation of different developmental and infection processes and are therefore valuable targets for controlling these devastating diseases [4, 5].

Heterotrimeric G proteins are involved in environmental sensing of nutrients, pheromones, stresses, and other stimuli in eukaryotes [6]. The heterotrimeric G protein complex consists of Gα, Gβ, and Gγ subunits. In the signal transduction pathway, heterotrimeric G proteins are activated by G protein-coupled receptors (GPCRs) that perceive various signals. In the absence of a stimulus, the GDP-bound Gα monomer and the G$\beta\gamma$ dimer form an inactive complex with the GPCR. Binding of the ligand to the GPCR triggers exchange of GDP with GTP in association with Gα and subsequent dissociation of Gα from G$\beta\gamma$, and the released active form of the G$\beta\gamma$ dimer interacts with other effector proteins to activate downstream signal transduction pathways. One of them is the cyclic adenosine 3′,5′-monophosphate/protein kinase A (cAMP/PKA) pathway. The GPCR activates the adenylyl cyclase, which catalyzes the formation of cAMP that in turn activates the PKA-mediated signaling cascades. cAMP is degraded by the cyclic nucleotide phosphodiesterase, which, together with the adenylyl cyclase, balances the level of cAMP in the cell [7]. Another pathway activated by the GPCR is the mitogen-activated protein kinase (MAPK) pathway, consisting of three sequentially activating kinases, with the upstream MAPK kinase kinases (MAPKKKs) perceiving signals from the receptors and activating MAPK kinases (MAPKKs) that in turn activate the MAPKs through phosphorylation, for example, Fus3/Kss1 and Slt2 in *Saccharomyces cerevisiae* [8]. The phosphorylated MAPKs activate related transcription factors, which in turn regulate genes in different biological processes.

Receptor for activated C kinase 1 (RACK1) was originally identified as the binding protein for activated protein kinase C [9, 10]. Rack1 belongs to the WD-repeat-containing proteins and contains seven WD40 repeats. These seven WD40 repeats assemble into a typical seven-bladed β-propeller structure that provides an interactive platform

for the binding of other proteins [11]. Rack1 proteins are constitutively expressed in eukaryotes. Rack1 has been intensively studied in mammalian cells [12, 13]. Rack1 functions in angiogenesis, tumor growth, cell migration, apoptosis, autophagy, neuronal response, proper function of the circadian clock, defense responses against virus infection, chromatin remodeling, transcriptional and translational regulations, and cAMP/PKA and MAPK pathways [11–15]. Rack1 regulates these biological processes through the control of protein complex assembly [13]. In plants, Rack1 proteins are functional in seed germination, leaf production, flowering, hormonal signaling, and biotic/abiotic stress responses [16–18]. The emerging role of Rack1 as a scaffold protein in the MAPK pathway in *Arabidopsis thaliana* has been recently published [19–21]. In protozoan parasites, Rack1 proteins are critical for effective mammalian parasitization by *Leishmania major* and *Trypanosoma brucei*, which cause leishmaniasis and African trypanosomiasis, respectively [22–24]. The *L. major* LACK (*Leishmania* homologue of mammalian RACK) antigen is also a target of the immune response in mice and therefore a vaccine candidate for human leishmaniasis [22]. In *Plasmodium falciparum*, the most lethal malarial parasite, the Rack1 protein inhibits Ca^{2+} signaling of the mammalian cells and subverts the host intracellular environment, an important mechanism for parasite survival [25]. In summary, Rack1 proteins play important roles across a wide variety of organisms. In this review, we summarize recent progress on Rack1 proteins in model and pathogenic fungi.

2. Rack1 Homologs Are Well Conserved

The Rack1 protein is found in many organisms, including the alga *Chlamydomonas reinhardtii*, yeasts, filamentous fungi, plants, nematodes, insects, and vertebrates, indicating that it is evolutionarily conserved [16, 58]. In fungi, Rack1 proteins are well conserved in most species of Chytridiomycota, Zygomycota, Glomeromycota, Ascomycota, and Basidiomycota (Figure 1). Rack1 proteins are also well conserved in fungi-like microbes, oomycetes, which cause serious plant diseases, such as potato late blight and sudden oak death [59, 60].

Rack1 proteins are functionally conserved. Multiple complementation assays show that Rack1 proteins are functionally interchangeable among different species, or even across kingdoms [26, 49, 52]. For example, the mammalian *RACK1* gene can rescue the defects of *rack1* mutants in *Schizosaccharomyces pombe* and *Aspergillus nidulans* [40, 52]. The *Neurospora crassa cpc-2* and the rat *RACK1* genes are able to complement the *cpc2* mutant in *S. pombe* [26]. *RACK1* genes of *A. nidulans* and yeasts (*S. cerevisiae*, *S. pombe*, and *Candida albicans*) can largely rescue the hyphal growth defects of the *cpcB* deletion mutant in *Aspergillus fumigatus* [49]. Species-specific roles of Rack1 proteins have also been shown in different fungi. For example, the *A. nidulans CpcB* ortholog but not the *RACK1* genes from yeasts can completely rescue the conidiation defect of the *A. fumigatus cpcB* mutant [49]. Taken together, these studies show the well conserved and unique roles of Rack1 proteins in different fungal species.

3. Rack1 Proteins in Model Fungi

3.1. Saccharomyces cerevisiae. In *S. cerevisiae*, a model unicellular eukaryote, the Rack1 homolog, known as Asc1, is involved in multiple biological processes (Table 1). Asc1 is involved in the general amino acid control (GAAC) that provides the cell with sufficient amounts of protein precursors under conditions of amino acid limitation [61]. Amino acid starvation in yeast promotes translation of GCN4, which then causes expression of genes required for synthesis of all 20 amino acids. This response is called GAAC. In the absence of amino acid starvation, Asc1 represses Gcn4 [26], a transcription activator of the GAAC pathway [61]. In *S. pombe*, studies suggest that Cpc2 promotes the GAAC response under the amino acid starvation condition [41].

Asc1 functions as the Gβ subunit for Gpa2 and interacts directly with the Gα subunit as a guanine nucleotide dissociation inhibitor that inhibits the guanine nucleotide exchange activity of Gα. Asc1 negatively regulates glucose-mediated signaling via two pathways. In the cAMP/PKA pathway, Asc1 binds to the effector enzyme adenylyl cyclase (Cyr1) in addition to Gpa2 and represses the production of cAMP in response to glucose stimulation [27]. Secondly, Asc1 is involved in the Kss1 MAPK pathway by binding to Ste20. In the *asc1* mutant, basal phosphorylation of Kss1 is enhanced. Similarly, under pheromone stimulus, the *asc1* mutant shows enhanced phosphorylation of both Fus3 and Kss1 but shows reduced expression of gene *FLO11*, key components for pheromone response, mating, and filamentous growth [8, 28].

Asc1 is involved in cell wall integrity and stress responses [28–31]. The *asc1* mutant is hypersensitive to cell wall damaging agents, iron chelators, and nitrosative stress [29, 31]. Similarly, Cpc2 is involved in stress responses in *S. pombe* [42]. The cell wall integrity defect of the *asc1* mutant most likely results from PKC-independent mechanisms because the double-knockout mutant (*asc1 pkc1*) shows synergistic sensitivity to cell wall stresses [29]. Interestingly, the basal phosphorylation level of the MAPK Slt2 is higher in the *asc1* mutant [32], indicating that Asc1 regulates the *SLT2* MAPK pathway. In addition, Asc1 regulates replication stress-induced formation of P-bodies that consist of many enzymes involved in mRNA turnover [33, 62]. Asc1 also regulates cell size. Compared to the wild-type control, the cell size of the *asc1* mutant is larger in *S. cerevisiae* [28]. The enlarged cell size is also observed in the *cpc2* mutant in *S. pombe* [43].

Asc1 executes its many functions by interacting with different proteins and regulating the transcription and translation of different genes. *De novo* proteome analysis indicates that the *asc1* mutant shows 50% elevated *de novo* biosynthesis of soluble proteins, compared to the wild type. In contrast, expression of insoluble proteins is reduced by nearly a quarter in the *asc1* mutant [31]. The protein level of several transcription factors, including Rap1, Tec1, Phd1, and Flo8, is downregulated but the protein level of Ste12 is upregulated over 6-fold in the *asc1* mutant [31]. Interestingly, all three transcription factors, Flo8, Ste12, and Tec1, positively regulate the expression of gene *FLO11*. Flo8 and Tec1 are downregulated and Ste12 is upregulated in the *asc1* mutant. However, the expression of gene *FLO11* is still downregulated

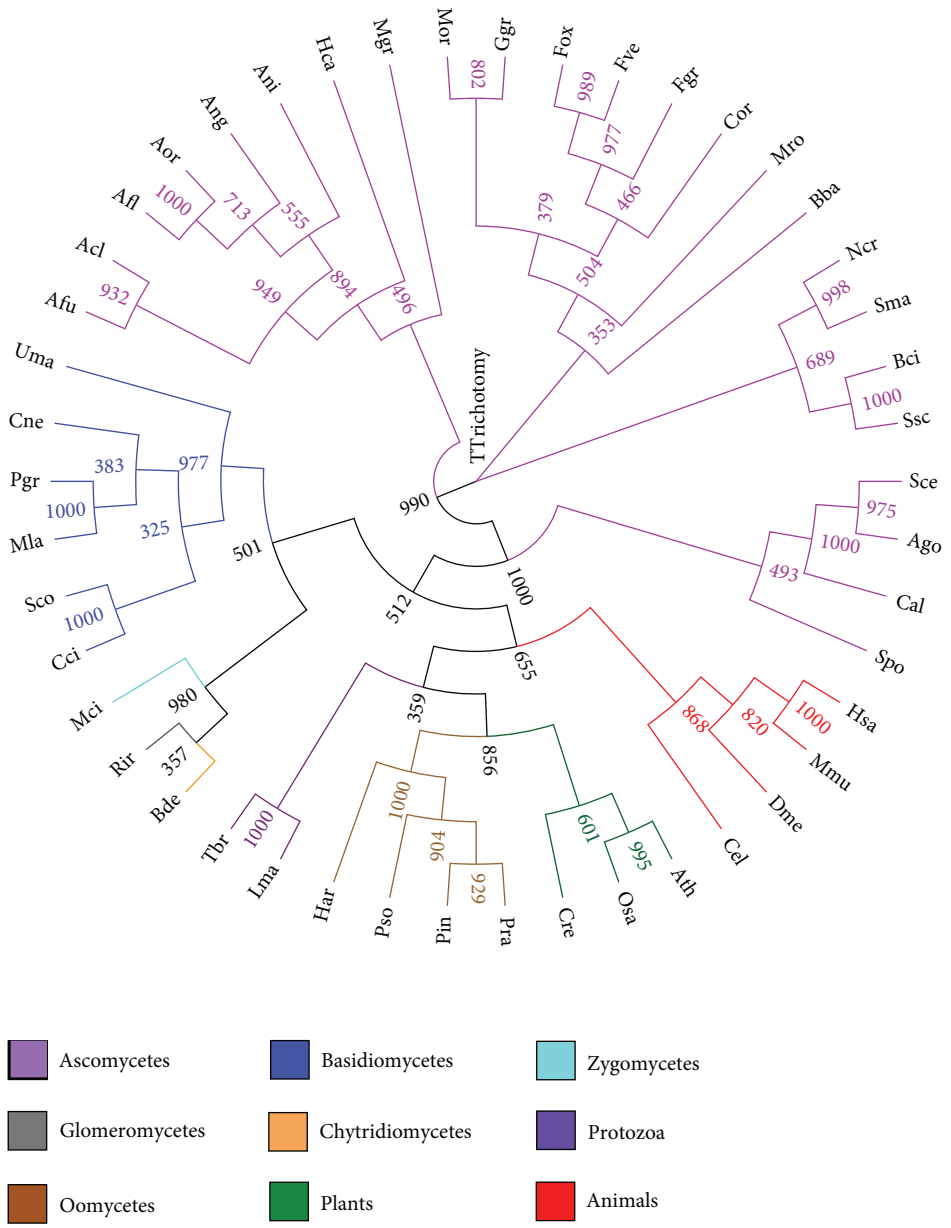

Figure 1: Phylogenetic tree of Rack1 proteins of eukaryotes. The predicted amino acid sequences of Rack1 proteins were aligned with ClustalX multiple sequence alignment program and the phylogenetic tree was visualized using FigTree program. Numbers indicate the 1000-bootstrap value of each branch. The accession number of each Rack1 protein is indicated in parentheses. Acl, *Aspergillus clavatus* (XP_001272169.1); Afl, *Aspergillus flavus* (EED47144.1); Afu, *Aspergillus fumigatus* (EDP50669); Ago, *Ashbya gossypii* (NP_985746.1); Ang, *Aspergillus niger* (XP_001389307.1); Ani, *Aspergillus nidulans* (XP_661767); Aor, *Aspergillus oryzae* (XP_001816748.1); Ath, *Arabidopsis thaliana* (NP_173248.1); Bba, *Beauveria bassiana* (XP_008600266.1); Bci, *Botrytis cinerea* (XP_001551314.1); Bde, *Batrachochytrium dendrobatidis* (XP_006679085.1); Cal, *Candida albicans* (P83774.2); Cci, *Coprinus cinereus* (XP_001830441.1); Cel, *Caenorhabditis elegans* (NP_501859.1); Cne, *Cryptococcus neoformans* (AAX94564.1); Cor, *Colletotrichum orbiculare* (ENH83707.1); Cre, *Chlamydomonas reinhardtii* (XP_001698065.1); Dme, *Drosophila melanogaster* (NP_477269.1); Fgr, *Fusarium graminearum* (XP_011318772.1); Fox, *Fusarium oxysporum* (KNB02916.1); Fve, *Fusarium verticillioides* (EWG39980.1); Ggr, *Gaeumannomyces graminis* (XP_009225866.1); Har, *Hyaloperonospora arabidopsidis* (HpaP813344); Hca, *Histoplasma capsulatum* (EGC41401.1); Hsa, *Homo sapiens* (NP_006089.1); Lma, *Leishmania major* (XP_001684560.1); Mci, *Mucor circinelloides* (OAD00838.1); Mgr, *Mycosphaerella graminicola* (XP_003852899.1); Mla, *Melampsora laricis-populina* (EGG07665.1); Mmu, *Mus musculus* (NP_032169.1); Mor, *Magnaporthe oryzae* (XP_003710816.1); Mro, *Metarhizium robertsii* (XP_007824428.1); Ncr, *Neurospora crassa* (CAA57460.1); Osa, *Oryza sativa* (NP_001043910.1); Pgr, *Puccinia graminis* (EFP89129.2); Pin, *Phytophthora infestans* (XP_002903189.1); Pra, *Phytophthora ramorum* (PSURA_41642); Pso, *Phytophthora sojae* (XP_009531042.1); Rir, *Rhizophagus irregularis* (ESA03866.1); Sce, *Saccharomyces cerevisiae* (NP_013834.1); Sco, *Schizophyllum commune* (XP_003029882.1); Sma, *Sordaria macrospora* (XP_003351440.1); Spo, *Schizosaccharomyces pombe* (AAA56865.2); Ssc, *Sclerotinia sclerotiorum* (EDN91497.1); Tbr, *Trypanosoma brucei* (XP_829201.1); Uma, *Ustilago maydis* (XP_011388829.1).

Table 1: Rack1 proteins in model and pathogenic fungi.

Species	Rack1	Functions	References
Ascomycetes			
Saccharomyces cerevisiae	Asc1	General amino acid control, pheromone response, mating and filamentous growth, pseudohyphal development, translation regulation and fidelity, cell size, cell wall integrity, stress responses, and cAMP/PKA and MAPK pathways	[8, 26–39]
Schizosaccharomyces pombe	Cpc2	Stress responses, cell wall integrity, cell size, mitotic commitment, positive role in the general amino acid control response, cell cycle progression, and meiotic development	[40–43]
Candida albicans[*]	Asc1	Growth, filamentation, adhesive growth, invasion, and virulence	[44, 45]
Neurospora crassa	*cpc-2*	Cross-pathway control, sexual reproduction, and the heterotrimeric G protein complex	[46–48]
Aspergillus fumigatus[*]	CpcB	Growth, conidiation, conidial germination, cell wall integrity, mycotoxin (gliotoxin) production, virulence, and antifungal drug resistance	[49–51]
Aspergillus nidulans	CpcB	Growth, conidiation, conidial germination, sexual development, mycotoxin (sterigmatocystin) production, and cross-pathway control	[50, 52]
Basidiomycetes			
Cryptococcus neoformans[*]	Gib2	Growth, virulence, a scaffolding adaptor protein, and a Gβ-like subunit of the cAMP/PKA pathway	[53–56]
Ustilago maydis[§]	Rak1	Growth, cell wall integrity, cell fusion, filament formation, appressorium formation, virulence, and a ribosomal protein	[53, 57]

[*] Human pathogens.
[§] Plant pathogens.

in the *asc1* mutant and the invasive growth is impaired [28]. Some proteins involved in cell wall biogenesis and overall cell morphology were found to be significantly reduced in the *asc1* mutant. Almost 50% of all proteins found to be regulated in *asc1* cells are involved in energy metabolism. This comprehensive group is composed of proteins taking part in glycolysis, mitochondrial biogenesis and respiration, oxidative stress, and fermentation. The findings of *de novo* proteome analysis are consistent with the phenotypes of the *asc1* mutant [31].

Asc1 is involved in translational regulation in *S. cerevisiae*. The Asc1 protein is a core component of the small (40S) ribosomal subunit [34–36]. Phosphoproteome and Western blotting studies show that the ribosomal protein Asc1 affects the phosphorylation of the eukaryotic translation initiation factors eIF2α and eIF4A and the ribosome-associated complex RAC [28]. Asc1 also participates in nascent peptide-dependent translation arrest at consecutive basic amino acid sequences, and the 40S subunit binding to Rack1 is crucial for translation arrest. Translation arrest of an mRNA by a nascent peptide leads to cleavage of the mRNA, as well as to cotranslational protein degradation [37]. Asc1 is required for efficient translation of short mRNAs with short open reading frames that usually show greater than average translational efficiency in diverse eukaryotes [38]. Asc1 is required to prevent frameshifting at ribosomes stalled at repeated CGA codons. In the absence of Asc1, ribosomes continue translation at CGA codons but undergo substantial frameshifting. Thus, the general translation fidelity of the cell depends upon Asc1-mediated quality control [39]. Taken together, these studies indicate that Asc1 constitutes a ribosomal interface for signal transduction and translational regulation [28].

3.2. Neurospora crassa. *N. crassa* is a model filamentous fungus [63]. In *N. crassa*, cross-pathway control (CPC) was first described by Carsiotis and collaborators [64, 65]. The cross-pathway control in *N. crassa* is the same as the GAAC in *S. cerevisiae*; that is, starvation for any one of several amino acids leads to globally increased synthesis of enzymes of many amino acid biosynthetic pathways in this fungus. *cpc-2* positively regulates the cross-pathway control under conditions of amino acid limitation [46]. Therefore, there is no activation of target amino acid biosynthetic genes in the *cpc-2* mutant under starvation conditions (Table 1). Under nonstarved conditions, mutation of the *cpc-2* gene decreases fungal growth. Formation of protoperithecia during sexual reproduction is impaired in the *cpc-2* mutant [47]. Genetic epistasis between *cpc-2* and components of the G protein pathway has been studied [48]. One Ga subunit (*gna-3*) is epistatic to *cpc-2* during submerged-culture conidiation, while two other Gα subunits (*gna-1*, *gna-2*) are independent of *cpc-2*, and the Gβ (*gnb-1*) and Gγ (*gng-1*) subunits operate

downstream of *cpc-2* during submerged-culture conidiation. In yeast two-hybrid assays, *gna-3* interacts with *cpc-2*. The epistatic studies together with the protein-protein interaction studies indicate that *cpc-2* does work in the heterotrimeric G protein complex.

4. Rack1 Proteins in Pathogenic Fungi

4.1. Candida albicans. *C. albicans* is an opportunistic pathogen and causes life-threatening systemic infections in immunocompromised individuals [66]. Its virulence factors include host recognition biomolecules (adhesins), morphogenetic transitions (the reversible transition between unicellular yeast cell and filamentous growth forms), biofilms, secreted aspartyl proteases, and phospholipases [44, 66]. From transcriptomics and proteomics data, Liu et al. show that the expression of *ASC1* in *C. albicans* is iron-, temperature-, and Gcn4-dependent and is downregulated by amino acid starvation, caspofungin, and farnesol (Table 1) [45]. The *asc1* null mutant displays shorter hyphae on several liquid and solid hypha-inducing media, compared to the wild-type strain [45]. Adhesive growth and invasive growth are impaired in the *asc1* null mutant [44]. The *asc1* null mutant has attenuated virulence. In a mouse model of systematic infection, the *asc1* null mutant is dramatically less virulent than the wild-type strain shown by the fact that 50% of the mice infected with the *asc1* null mutant survived, while the wild-type strain killed all the infected mice. In another assay using a mouse model of disseminated infection, nearly all of the mice inoculated with the wild-type strain died within 16 days. However, mice infected with the *asc1* null mutant were completely asymptomatic [44]. Finally, Fan et al. show that transcription of adhesion-related genes *ALS3*, *ECE1*, and *HWP1* [67] is reduced in the *asc1* null mutant, indicating that Asc1 regulates virulence of *C. albicans* through these important genes [44].

4.2. Aspergillus nidulans and Aspergillus fumigatus. *A. nidulans*, a model filamentous fungus, is also reported as an opportunistic pathogen [68, 69]. *A. fumigatus*, the major causative agent of life-threatening invasive aspergillosis in immunocompromised individuals, is a ubiquitous saprophytic fungus [70]. Thermotolerance, specialized cell wall, melanization, resistance to oxidative stress, and mycotoxins (gliotoxin) are important for virulence of *A. fumigatus*. In addition, the cAMP/PKA pathway regulates the synthesis of several virulence factors [71]. In both *A. nidulans* and *A. fumigatus*, Rack1 proteins, CpcB, play important roles in fungal growth, conidiation, conidial germination, and mycotoxin production (Table 1) [49, 50]. In *A. nidulans*, CpcB represses the cross-pathway control in the presence of amino acids and regulates sexual development [50, 52]. Compared to the wild-type strain, the *cpcB* mutant of *A. nidulans* produces much fewer cleistothecia, and the cleistothecia contain no ascospores, indicating that CpcB is required for proper formation of fruiting bodies and ascosporogenesis [50, 52]. In *A. fumigatus*, CpcB is localized in the cytoplasm. To determine the genetic relationship between CpcB and GpaB (Ga), the *gpaB* mutant and the double-deletion mutant, *cpcB gpaB*, were generated. The double mutant *cpcB gpaB* causes a similar phenotype to the *gpaB* mutant with abnormal multiple septa conidiophores, indicating the overlapping functions of CpcB and GpaB [49]. Cell wall integrity of the *cpcB* mutant is impaired, revealed by transmission electron microscopy examinations [49]. Virulence of the *cpcB* mutant is attenuated in an immunosuppressed mouse model. The study also demonstrates that the G–H residues in WD repeats 1, 2, and 3 and the W–D residue in WD repeat 2 of CpcB are required for normal hyphal growth and conidiation and are also necessary for maintaining normal antifungal drug susceptibility. The *cpcB* mutant displays enhanced drug resistance, which might be due to reduced intracellular drug accumulation and altered ergosterol component. In addition, the first G–H residue of CpcB plays a critical role in the virulence of *A. fumigatus* [51]. The involvement of CpcB in pathogenesis of *A. fumigatus* might be through its connection with the G protein complex and/or its role in maintaining proper cell wall structure.

4.3. Cryptococcus neoformans. *C. neoformans* is an encapsulated yeast-like basidiomycetous fungus. *C. neoformans* has a worldwide distribution [72]. It is found in soil, bird excrement, and trees. It is an opportunistic pathogen and causes meningoencephalitis in immunocompromised individuals [73]. Fungal capsulation and melanization, among others, are important for its virulence. The major virulence factors of the pathogen are regulated by the cAMP/PKA pathway [53]. In *C. neoformans*, the Rack1 protein Gib2 is required for full virulence (Table 1) [53–55]. Gib2 functions as a scaffolding adaptor protein and interacts with Gα (Gpa1) and Gγ (Gpg1 and Gpg2) proteins. Gib2 also interacts with the protein kinase C1 Smg1, a phosphodiesterase Pde2, and the small G protein Ras1, which are all involved in the cAMP/PKA pathway. Gib2 positively regulates the cAMP/PKA pathway, for overexpression of Gib2 rescues the defects of the *gpa1* mutant in both melanization and capsule formation and restores the cAMP level by relieving the inhibitory effect of Ras1 on the adenylyl cyclase Cac1 in the *gpa1* mutant [54, 55]. In a murine model of cryptococcosis, disruption of the *GIB2* gene results in severe attenuation of virulence [54]. In summary, Gib2 is involved in virulence by acting as a scaffolding adaptor protein of the cAMP/PKA pathway in *C. neoformans*.

4.4. Ustilago maydis. *U. maydis*, a dimorphic basidiomycete, causes corn smut and serves as a model for obligate biotrophic fungal pathogens [74]. Infection of maize by *U. maydis* requires that haploid yeast cells of compatible mating types fuse and establish dikaryotic filamentous hyphae. The morphological transition from budding to filamentous hyphae is important for fungal virulence and is regulated by the conserved cAMP/PKA and MAPK pathways. Loss of Rack1 protein, Rak1, causes slow growth, hypersensitivity to cell wall stress, attenuated cell fusion, and attenuated virulence in *U. maydis* (Table 1) [57]. In infection assays, more than 80% of plants infected with the wild-type strain showed tumor formation but no tumors could be observed in *rak1*-infected maize plants. Filament formation and appressorium formation are impaired in the mutant. The attenuated cell

fusion likely results from the reduced expression of *rop1*, a transcriptional activator of the pheromone response factor (prf1) that regulates pheromone (mfa) and pheromone-receptor genes. Rak1 is a ribosome-associated protein and interacts with a large number of proteins including 32 ribosomal proteins shown in protein pull-down assays. These proteins are involved in metabolism, energy production, cell division, DNA replication, rRNA processing, and UDP-galactose translocation, which are possibly involved in fungal pathogenesis [75]. The constitutive expression of *rop1* or constitutive activation of the pheromone-responsive MAPK pathway could rescue the defect of the *rak1* mutant in conjugation tube formation. Prf1 is regulated at the post-transcriptional level by the cAMP/PKA pathway and the MAPK pathway, indicating that Rak1 possibly functions in the cAMP/PKA and MAPK pathways [74]. However, no interaction between Rak1 and any Gα subunits has been established, and additional studies are needed to determine how Rak1 participates in the cAMP/PKA and MAPK pathways that are required for pathogenicity of *U. maydis*.

5. Concluding Remarks and Perspectives

Rack1 proteins play important roles in fungal pathogenesis. However, compared to the intensive studies on Rack1 proteins in human, plants, and yeasts [12, 16, 39], limited studies have been conducted in a few pathogenic fungi. In these fungi, Rack1 proteins affect various virulence factors through conserved signal transduction pathways (Table 1) [76, 77]. In other fungal pathogens, such as *Penicillium marneffei* and *Histoplasma capsulatum*, Rack1 proteins are found to be associated with stress responses or dimorphic transition [78, 79]. In plant pathogens *Fusarium verticillioides* and *F. graminearum*, WD40 proteins are required for virulence and other important functions, though the Rack1 proteins have not been characterized [80]. In addition, protein-protein interaction network analyses indicate that WD40 proteins are highly connected in the malaria parasite, with 1,928 potential interactions, supporting the role of WD40-containing proteins, including Rack1 proteins, as hubs in cellular networks [81]. Given the conserved and versatile roles of Rack1 proteins in eukaryotes [34], it is important to explore the unique and dynamic roles of Rack1 proteins in different pathogenic fungi.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Roles of Peroxisomes in the Rice Blast Fungus

Xiao-Lin Chen, Zhao Wang, and Caiyun Liu

State Key Laboratory of Agricultural Microbiology, The Provincial Key Lab of Plant Pathology of Hubei Province, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

Correspondence should be addressed to Xiao-Lin Chen; chenxiaolin@mail.hzau.edu.cn

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The rice blast fungus, *Magnaporthe oryzae*, is a model plant pathogenic fungus and is a severe threat to global rice production. Over the past two decades, it has been found that the peroxisomes play indispensable roles during *M. oryzae* infection. Given the importance of the peroxisomes for virulence, we review recent advances of the peroxisomes roles during *M. oryzae* infection processes. We firstly introduce the molecular mechanisms and life cycles of the peroxisomes. And then, metabolic functions related to the peroxisomes are also discussed. Finally, we provide an overview of the relationship between peroxisomes and pathogenicity.

1. Introduction

Peroxisomes are single membrane-bound microbodies which existed in all eukaryotic cells. In different organisms and environmental conditions, their abundance can be changed rapidly, and functions could be different. The abundance of peroxisomes is coordinated by several cellular processes, including peroxisome biogenesis, peroxisome proliferation, and peroxisome degradation [1]. In budding yeast, the genes involved in those cellular processes include a set of *PEX* genes, which encode peroxins [2]. Up to now, over 30 *PEX* genes have been found in different organisms [2].

There are several important metabolic processes that take place in peroxisomes, which involve fatty acids β-oxidation, glyoxylate cycle, hydrogen peroxide detoxification and secondary metabolite biosynthesis, and so forth [1]. The roles of peroxisomes have been extensively uncovered in yeast, filamentous fungi, plant, and human [3]. Some peroxisome functions are species specific, such as the methanol assimilation in yeast [4], the glyoxylate cycle in plant seeds [5], and the plasmalogens biosynthesis in mammal [6]. In filamentous fungi, they can develop a special compartment, the peroxisome-derived woronin body, to seal the septal pore when suffered cellular wounding [7].

The molecular mechanisms of peroxisome life cycle have been extensively studied in yeast [2]. Peroxisome studies in the filamentous fungi are also increased rapidly, especially in the model filamentous fungi *Aspergillus nidulans* [8, 9] and *Neurospora crassa* [10–12], the plant pathogens *Colletotrichum orbiculare* [13–15] and *Magnaporthe oryzae* [16–20], and the human pathogens *Candida albicans* [21, 22], *Aspergillus fumigatus* [23, 24], and *Cryptococcus neoformans* [25, 26]. An important aspect is that the peroxisomes are found to play key roles in fungal pathogenicity towards their host, including plants and human. In all these pathogenic fungi, the roles of peroxisomes in *M. oryzae* have received extensive concern.

Over the past two decades, many components involving roles of peroxisomes have been identified in *M. oryzae* (Table 1). This review focuses on recent advances in our understanding of peroxisomes in *M. oryzae*, including a description of the peroxisome life cycle during fungal infection, and an overview of their remarkable functions relevant to pathogenesis.

2. Life Cycle of the Peroxisomes

The peroxisome life cycle mainly includes peroxisome biogenesis, peroxisome proliferation, and peroxisome degradation [27]. The peroxisomes are thought to be born originally from the endoplasmic reticulum (ER) [27]. Basically, the biogenesis process contains peroxisomal membrane proteins (PMPs) acquisition and peroxisomal matrix proteins import. Peroxisome proliferation can be achieved by either

Table 1: Peroxisome-related genes identified in *M. oryzae*.

Gene	Functions	References
Peroxisome biogenesis		
MoPEX5	Receptor of PTS1 peroxisomal matrix proteins	[18]
MoPEX6	Peroxisomal matrix protein import	[28]
MoPEX7	Receptor of PTS2 peroxisomal matrix proteins	[17, 18]
MoPEX19	Peroxisomal membrane proteins import	[16]
Peroxisome fission		
MoPEX11A/11B/11C	Peroxisome elongation	[20]
PEF1	Peroxisome division	[33]
MoFis1	Peroxisome division	[36]
Pexophagy		
MoSNX41	Pexophagy and retrieval trafficking	[19]
MoPEX14	Pexophagy	[19]
Woronin body		
HEX1	Seal the septal pore	[45]
β-oxidation		
MFP1	Peroxisome β-oxidation	[47]
PTH2	Acetyl CoA transportation	[48]
CRAT2	Acetyl CoA transportation	[48]
MoCRC1	Carnitine-acylcarnitine carrier	[49]
Glyoxylate cycle		
ICL1	Isocitrate lyase/glyoxylate cycle	[50]
Redox homeostasis		
AGT1	Synthesis of the pyruvate	[51]

de novo formation from ER or/and peroxisome fission. When the peroxisomes have finished their mission, they can be degraded by pexophagy, an autophagic process [27].

In *M. oryzae*, there are several *PEX* genes involving peroxisome life cycle that have been characterized, including *MoPEX5*, *MoPEX6*, *MoPEX7*, *MoPEX14*, *MoPEX19,* and *MoPEX11* family genes. *MoPEX5* and *MoPEX7* are involved in matrix proteins import [17, 18], *MoPEX6* participates in receptors import for recycling [28], *MoPex14* functions as a matrix docking protein [19], *MoPEX19* functions as chaperone and receptor for importing of both matrix proteins and PMPs [16], and *MoPEX11* family genes are involved in peroxisomal fission processes [20].

2.1. Peroxisome Biogenesis. In yeast, during peroxisome biogenesis, peroxisome membrane proteins (PMPs) should firstly be inserted into membranes, which are mediated by *PEX3*, *PEX16,* and *PEX19* [29–31]. Then the peroxisomal matrix proteins, which are synthesized in the cytoplasm, are translocated into the peroxisomes by peroxisome membrane docking complex [32]. Most of the peroxisomal matrix proteins contain either PTS1 (type I peroxisomal targeting signal) at the C-terminus or PTS2 (type II peroxisomal targeting signal) at the N-terminal and can be recognized by shuttle receptors Pex5 or Pex7-mediated complex, respectively. The cargos on the PTS1 and PTS2 receptors are accepted by the Pex13/Pex14/Pex17 docking complex, and then the receptors are recycled by the ubiquitin system. The ubiquitinated receptors can be extracted into cytoplasm by AAA+ ATPases Pex1 and Pex6 [27].

In *M. oryzae*, MoPex19 is the ortholog of yeast PMPs receptor Pex19. Pex19 protein is located in the cytoplasm and newly formed peroxisomes, which is consistent with its PMPs receptor function that shuttles the PMPs between the cytosol and peroxisomal membrane [30]. Deletion of *MoPEX19* will lead to PMPs mislocalization. PMP47 is a representative PMP which is normally distributed in the peroxisomes in the wild type strain, while in the *MoPEX19* deletion mutants, its localization pattern is changed, which is distributed in the cytoplasm [16]. This demonstrated that the PMP47 cannot be imported into the peroxisomes. Moreover, in the Δ*mopex19* mutants, peroxisomal structures and peroxisome-derived woronin bodies are both absent [16], indicating that the MoPex19 is essential for biogenesis of peroxisomes and woronin bodies.

M. oryzae MoPex5 and MoPex7 are also proved to function as receptors of peroxisomal matrix proteins, which are involved in importing of the matrix proteins into peroxisomes [17, 18]. Disruption of *MoPEX5* and *MoPEX7* will block the PTS1 and PTS2 peroxisomal import pathways, respectively. In the wild type strain, RFP-PTS1 and GFP-PTS2 are normally distributed in the punctuate peroxisomes, while in the Δ*mopex5* mutants, RFP-PTS1 is dispersed in cytoplasm but GFP-PTS2 is still located in peroxisomes. In contrast, in the Δ*mopex7* mutants, RFP-PTS1 is still located in peroxisomes but GFP-PTS2 is dispersed in cytoplasm.

These results demonstrated the MoPex5-mediated PTS1 peroxisomal import pathway and MoPex7-mediated PTS2 peroxisomal import pathway separately function in the rice blast fungus [17, 18]. The thiolase MoThl1 is a candidate PTS2 protein; it is failed to be located at the peroxisomes in the Δ*mopex7* mutants [17], which further supports the role of MoPex7 in the PTS2 peroxisomal protein import pathway.

The function of Pex6 ortholog in *M. oryzae*, MoPex6, was also evaluated [28]. In *MoPEX6* disruption mutants, the GFP-SRL protein is diffused in the cytoplasm, failed to be localized in the punctuate peroxisomes in mycelia, conidia, germ tubes, and appressoria [28], indicating the PTS peroxisomal import pathway is blocked. This result is consistent with the cellular function of Pex6, which is involved in recycling of matrix protein receptors (Pex5 and Pex7) during peroxisome biogenesis.

In the *MoPEX14* disruption mutants, the GFP-SRL protein is also mislocalized to the cytoplasm, while when the $PEX14_{61\text{-}361}$ or $PEX14_{1\text{-}258}$ is expressed in the Δ*mopex14* mutants, the punctate localization of GFP-SRL can be restored [19]. These data confirmed the functions of *MoPex14*, which act as a matrix docking protein to facilitate peroxisomal protein import and peroxisome biogenesis.

2.2. Peroxisome Proliferation. Peroxisomes can proliferate rapidly according to suitable environment stimulation. The proliferation processes can be achieved by *de novo* biogenesis from the ER, or by fission from the preexisting peroxisomes. In yeast, the peroxisome fission processes mainly consist of several steps. At the beginning, the mature peroxisomes are elongated by the functions of the peroxisomal membrane protein Pex11. Then the matrix proteins can be imported into the elongated peroxisomes, and the fission machinery can also be imported into appropriate place for fission. The dynamin-like protein Dnm1 is located at the constriction sites and leads to membrane fission processes by GTP hydrolysis. At last, the daughter peroxisomes can be produced from the fission processes, which is achieved by cooperation of several proteins, including Fis1, Dnm1, and the adaptors Mdv1 or Caf4 [27].

The peroxisome fission process in *M. oryzae* is identified recently, by several independent studies. There are three members of *PEX11* family genes in *M. oryzae* genome, named *MoPEX11A*, *MoPEX11B,* and *MoPEX11C*, respectively [20]. However, it seems that only *MoPEX11A* plays vital role in peroxisome fission. The *MoPEX11A* deletion mutant exhibits decreased but enlarged peroxisomes compared to the wild type, which demonstrated the *MoPEX11A* is important for peroxisome elongation and proliferation. In contrast, the *MoPEX11B* and *MoPEX11C* deletion mutants are normal in both number and size of the peroxisomes [20], indicating they could not be key regulators during peroxisomal proliferation.

There is only one counterpart of Mdv1/Caf4 protein in *M. oryzae*, named *PEF1* [peroxisome fission protein 1] [33]. This gene may play dual roles of Mdv1 and Caf4, because deletion of *PEF1* will lead to evident peroxisomal fission defect during the fission inducing conditions. The Δ*pef1* mutant forms string-linked peroxisomes, in contrast to the punctuate structures in normal cells [33]. Similar situation can be found in the Δ*mdv1*Δ*caf4* double mutant or the Δ*fis1* and Δ*dnm1* mutants in yeast [34]. The phenotypic defect indicates that the daughter peroxisomes cannot be cut from the elongated peroxisomes in Δ*pef1*. Pef1 can bind to Fis1 with its N-terminal extension (NTE) region and to Dnm1 with its C-terminal WD40 repeat region. With the help of adaptors Mdv1 or Caf4, the outer membrane protein Fis1 can recruit Dnm1 to peroxisomes for fission [35]. Pef1 can be well colocalized with MoFis1, which is recently reported to play important roles in mitochondria fission in *M. oryzae* [36]. This is intelligible, because in *S. cerevisiae*, the peroxisome fission machinery can be also used to facilitate mitochondria fission process.

2.3. Peroxisome Degradation. Peroxisome abundance can be rapidly cleared by a selective autophagic process, which is known as the pexophagy. In yeast, there are two different peroxisome degradation modes, macropexophagy and micropexophagy [37]. The macropexophagy sequesters peroxisomes to form a pexophagosome, which leads the peroxisomes to vacuole for degradation. The micropexophagy encloses peroxisomes by vacuolar membrane protrusions and the micropexophagy specific membrane apparatus (MIPA) to vacuole for degradation [37]. There are several *ATG* and *PEX* genes reported to be involved in perophagy [37].

In *Pichia pastoris*, *ATG30* is required for both of macropexophagy and micropexophagy [38], but no *ATG30* homolog gene can be found in *M. oryzae*. In *P. pastoris* and *C. orbiculare*, *ATG26* plays key roles in pexophagy, and the *CoATG26* is essential for infection process [13, 39]. However, In *M. oryzae*, *MoATG26* is not involved in pexophagy and is dispensable for virulence [19].

In *S. cerevisiae*, the pexophagy process can be mediated by Atg20/Snx42 [40]. By the assistance of sorting nexins Snx41 and Atg24/Snx4, Atg20/Snx42 can be also involved in endosomal retrieval trafficking [40]. However, only one protein [MoSnx41] with high similarity to Snx41 and Snx42/Atg20 can be found in *M. oryzae*. Studies have found that the *MoSNX41* plays key roles in conidiation and pathogenesis. Deletion of *MoSNX41* leads to pexophagy deficiency, demonstrating this gene is involved in pexophagy in *M. oryzae*. The yeast ScSnx42 can restore the pexophagy deficiency of the Δ*mosnx41* mutant but failed to recover the defects of conidiation and pathogenesis, indicating the pexophagy process is dispensable for development and pathogenicity in *M. oryzae*. The function of MoSnx41 in conidiation and pathogenesis is mediated by Snx41-dependent retrieval trafficking, but not pexophagy [19]. As a peroxisomal membrane protein, *PEX14* is also involved in pexophagy of *Hansenula polymorpha* [41]. In *M. oryzae*, the MoPex14 has also been proved to be essential for pexophagy, but dispensable for pathogenicity.

2.4. Peroxisome Differentiation. Filamentous fungi can form a special structure, called woronin body (WB), which is differentiated from the peroxisomes [42]. It is accepted that the peroxisome-related woronin bodies are used to seal the septal pore when suffering cellular wounding [43]. The formation mechanism of woronin bodies has been well

studied in *Neurospora crassa* [44]. Hexagonal peroxisome Hex1 is the major structural protein in woronin body, which can be imported into the peroxisome matrix and assembled to form a woronin body core structure. This core structure can recruit WB sorting complex protein [WSC] into the peroxisome membrane. Then the nascent WB can be budded from the mother peroxisomes.

In *M. oryzae*, the *HEX1* homolog gene has been cloned and the roles of the woronin bodies have also been uncovered by analyses of the *MoHEX1* functions. The woronin body is proved to be required for development, appressoria formation, and infection hyphae growth and therefore is essential for pathogenicity in *M. oryzae* [45]. Ultrastructural analyses proved that the woronin bodies are located adjacent to septa of mycelia, germ tubes, and infection hyphae, but they are less observed in conidia. The *HEX1* deletion mutant is lack of woronin bodies, and when the cells are damaged, the cellular materials will bleed out through the septal pores. The *M. oryzae* Hex1 protein contains the PTS1 peroxisome targeting signal, which can help it locate into the peroxisomes. For the woronin bodies being formed from the peroxisomes, defects in peroxisome formation cycles could lead to defect in woronin body formation. Consistent to this hypothesis, nearly all disruption of the genes involving in peroxisome life cycle can result in woronin body deficiency [45].

2.5. Regulation of Peroxisome Dynamics. The peroxisomes in the cell are dynamics according to environmental conditions and development stages. Occupancy of the peroxisomes in a cell is determined by several processes, including peroxisome biogenesis, peroxisome proliferation [*de novo* formation and fission], and peroxisome degradation (pexophagy). *De novo* biogenesis and fission process are both used to increase peroxisomes numbers. However, how to coordinate *de novo* formation and peroxisomal fission remains unclear. It is possible that both of the ER formation and fission of the preexisting peroxisomes might exist in normal conditions. It is supposed that, upon the peroxisome fission inducing conditions, such as in fatty acids condition, the peroxisomes are needed to be largely produced in a short time and the *de novo* formation process may be not enough or effective. In contrast, the peroxisome fission cycles would fulfill peroxisome demands in a limited time. During infection of *M. oryzae*, the lipid stores should be utilized less than 12 h. The rapid lipolysis of lipid bodies produced mass fatty acids, which in turn induces peroxisome fission process (*de novo* biogenesis efficiency could also be elevated), and massive peroxisomes are produced in short time, thus promoting fatty acids utilization and facilitating infection. When fatty acids are utilized, the peroxisomes should be decreased in a short time, and then they can be degraded by pexophagy. In *C. orbiculare*, the CoAtg26-dependent pexophagy is used to recycle cellular amino acids of the appressoria for infection [13]. How the peroxisome fission and pexophagy are activated and suspended remains obscure.

The Snf1/AMPK pathway plays central role in response to nutrient stress in *M. oryzae*. A recent study demonstrated that the MoSnf1 pathway can regulate peroxisomal maintenance and lipid metabolism by responding to nutrient-free environment [46]. In Δ*mosnf1* mutant, the peroxisomes are significantly decreased during appressoria formation. Accordingly, the Δ*mosnf1* mutant is also defect in lipid droplet mobilization, fails to generate enormous turgor, and loses its virulence [46]. Other regulatory mechanisms should also be uncovered in the future.

3. Metabolic Functions of Peroxisomes

Multiple metabolic processes occur in the peroxisomes, which makes the peroxisomes play crucial role in fungal development and pathogenesis. Peroxisomal β-oxidation of fatty acids ubiquitously existed for all eukaryotes. The reactive oxygen species [ROS] homeostasis can be also mediated by peroxisomes. Peroxisomes can be involved in many other metabolic pathways, including glyoxylate cycle in plants and fungi [52], penicillin biosynthesis in *Penicillium chrysogenum* [53], and melanin biogenesis in filamentous fungi [54].

3.1. Fatty Acid β-Oxidation. The β-oxidation metabolism is mainly used to degrade fatty acids for nutrient and energy utilization [55]. This metabolic process involves four major enzymes, acyl-CoA oxidase, 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase. Through a four-step pathway mediated by these enzymes, the acetyl-CoA is produced [55]. The acetyl-CoA can be fed to the glyoxylate cycle and gluconeogenesis to produce nutrient or metabolites.

In *M. oryzae*, the Mfp1/Fox2 protein has been shown to play an important role in fatty acid metabolism and pathogenesis [47]. The Δ*mfp1* mutants cannot grow on olive oil or oleic acid as sole carbon sources, indicating its roles in fatty acids metabolism. The expression level of *MFP1* is significantly induced in the olive oil condition. The Mfp1GFP fusion protein is well colocalized with FoxA-RFP in the peroxisome-like punctuate structures [47]. Because the *A. nidulans* FoxA has been proved to be a β-oxidation enzyme and can be located in the peroxisomes, the *M. oryzae* Mfp1 should also be located in the peroxisomes and is required for fatty acid β-oxidation.

In the past, β-oxidation is thought to occur exclusively in peroxisomes in the filamentous fungi, while it can occur in both peroxisomes and mitochondria in mammal. However, recent studies demonstrated that the mitochondrial β-oxidation is also functional and important for the infection of the fungal pathogens [56]. In *M. oryzae*, Enoyl-CoA hydratase Ech1 is an important mitochondrial β-oxidation enzyme, which is important for conidial germination, appressoria penetration, and host colonization. The Δ*ech1* mutant cannot utilize C14 fatty acid and also cannot well utilize C16 and C18 fatty acids. Consequently, Δ*ech1* is reduced in melanization and sensitive to oxidative stress. Generally, the short- and medium-chain fatty acids (less than 20C) can be oxidized in mitochondria, while long-chain fatty acids (over 20C) should be degraded in peroxisomes to shorter chain fatty acids for full oxidation in mitochondria [56]. Thus, the mitochondrial β-oxidation and peroxisomal β-oxidation can

collaborate with each other for different length fatty acid oxidation in *M. oryzae*.

Acetyl CoA is the main product of fatty acid β-oxidation in the appressorium of *M. oryzae*, which must be transported into different cellular compartments for consumption. The acetyl CoA transportation is catalysed by carnitine acetyl transferases (CATs). In budding yeast *S. cerevisiae*, Cat2 is involved in transferring acetyl CoA to the cytoplasm, while Cat1 is used to transfer acetyl CoA into mitochondria for utilization by the tricarboxylic acid cycle [57]. Two *CAT* genes, *PTH2* and *CRAT2,* have been studied in *M. oryzae* [48]. The results demonstrated that *PTH2* plays major role in acetyl CoA transfer. Pth2-GFP protein is colocalized with the peroxisome marker protein and is abundant in appressoria. The Δ*pth2* is reduced in melanin deposition, defect in host penetration, and essential for pathogenesis [48]. Further analysis found that the Δ*pth2* mutant cannot utilize some lipid substrates. In contrast, the Δ*crat2* displays no evident defect in those mentioned phenotypes [48]. The carnitine-acylcarnitine carrier protein Crc1 functions in transferring the acetyl CoA cross the mitochondrial membrane. The *M. oryzae MoCRC1* deletion mutant is severely reduced in appressorial penetration and invasive growth. *MoCRC1* is also needed for utilization of olive oil [49].

3.2. Glyoxylate Cycle. The glyoxylate cycle is a metabolic pathway which can be normally found in plants and fungi [52]. This pathway can assimilate acetyl CoA for gluconeogenesis and eventually generate glucose. The glyoxylate cycle is mainly induced when the fatty acids and acetate should be used [52]. The fatty acid β-oxidation pathway produces massive acetyl CoA, which will be processed by glyoxylate cycle. In this pathway, the acetyl CoA can be converted to glyoxylate by isocitrate lyase, and then the glyoxylate can be further converted to malate by malate synthase. The malate can be further metabolized to hexoses by gluconeogenesis.

The isocitrate lyase (Icl1) and malate synthase (Mls1) are two of principal enzymes involving glyoxylate cycle [52]. In *M. oryzae*, *ICL1* is highly expressed during appressoria formation and penetration stages, indicating that the glyoxylate cycle should be induced in these stages [50]. Defect in peroxisome biogenesis will lead to loss functions of glyoxylate cycle. For example, disruption of *MoPEX19* will result in failure in acetate utilization [16].

3.3. Redox Homeostasis. Oxidative reactions are theme of the peroxisome metabolism, which generates massive reactive oxidative species (ROS), especially the hydrogen peroxide (H_2O_2) [58]. In order to eliminate harmful ROS, ROS scavenging becomes an important peroxisomal metabolism. To detoxification, the hydrogen peroxide can be scavenged by catalases and peroxidases, which are abundant in peroxisomes. A number of catalases and peroxidases are predicted in genome of *M. oryzae*, and some of which (such as *CATB* and *CPXB*) have been reported to function in host ROS detoxification [59, 60]. However, no catalase or peroxidase has been revealed to take part in intracellular peroxisome ROS detoxification. Glutathione S-transferases (GSTs) and peroxiredoxins (PRXs) are other antioxidant enzymes existing in peroxisomes [61]. There is also no GST or PRX protein reported to play roles in peroxisome ROS detoxification. In *Alternaria brassicicola* and *A. fumigatus*, a transmembrane protein TmpL has been identified and proved to be important for reduction of intracellular ROS and detoxification of external ROS and thus is important for fungal infection. TmpL can be located in the woronin body, and its expression level is evidently elevated in conidiation and initial invasive growth stages [62].

During peroxisome β-oxidation process, acetyl-CoA formation will accompany mass formation of FADH2 and NADH. To keep peroxisome redox homeostasis, the FADH2 and NADH should be eliminated. For the NADH, it can be reoxidated to NAD^+. This reaction can be catalyzed by the peroxisomal lactate dehydrogenase, which can mediate production of lactate from pyruvate. In *M. oryzae*, the pyruvate is generated by the alanine: glyoxylate aminotransferase 1 (AGT1), which transfers the alanine amino group to glyoxylate and results in formation of the pyruvate. AGT1GFP is colocalized with RFP-MTS1 fusion protein, demonstrating that AGT1 is located in the peroxisomes [51]. The Δ*agt1* mutant cannot form appressoria on artificial inductive surface. When the NAD^+ and pyruvate were added during conidia germination on artificial inductive surface, the appressorium formation can be restored [51]. Thus, the AGT-mediated pyruvate generation can function as one of factors to maintain redox homeostasis during appressoria formation.

3.4. Melanin Biosynthesis. In filamentous fungi, peroxisomes not only are crucial for the primary metabolism, but also play important roles in the biosynthesis of secondary metabolites. Many plant pathogenic fungi can produce melanin to protect the conidia to survive in different environment and to facilitate host penetration during infection. The dihydroxy naphthalene (DHN) melanin is well studied and found to be essential for appressorial mediated penetration in *M. oryzae*. The fungal appressorium contains a distinct melanin layer located between the cell wall and the membrane, which can be used to generate turgor for penetration. The DHN melanin is synthesized by the polyketide pathway, through which the peroxisomes-derived acetyl-CoA can be used to produce the 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). The 1,3,6,8-THN is then used to synthesize the DHN melanin, catalyzed by a series of enzymes, including Alb1, Rsy1, and Buf1 [63].

Because the acetyl-CoA is mainly produced in peroxisomes, defects in peroxisome formation would lead to block in melanin synthesis. Consistent with this prediction, all disruptions of the peroxisome biogenesis-related genes can result in melanin deficiency.

3.5. Cell Wall Biosynthesis. The fungal cell wall mainly consists of chitin, β-1,3-glucan, β-1,6-glucan, and mannoproteins [64]. This rigid structure can protect fungal from extracellular stresses and is flexible for adaptation to development and environment [64]. It is believed that fungal cell wall chitin and glucan are derived from acetyl-CoA, and the defects

in peroxisomes will lead to deficiency in cell wall integrity. Consistent with this hypothesis, the *M. oryzae MoPEX5*, *MoPEX6,* and *MoPEX19* deletion mutants are all sensitive to the cell wall-perturbing agents, such as Congo Red and Calcofluor White [16, 18, 28].

4. Peroxisome and Pathogenicity

Functions of peroxisomes have been studied in kinds of organisms, including the filamentous fungi. In fungal pathogens, an intriguing feature is their roles in pathogenicity. It is reported that peroxisomes are required for virulence of almost all fungal pathogens, such as the plant pathogens *C. orbiculare* and *M. oryzae*, the insect pathogen *M. robertsii*, and the human pathogens *C. albicans*, *A. fumigatus* and *C. neoformans*.

During infection, *M. oryzae* can form a specialized structure known as appressorium. Along with the formation and maturation of appressoria, the lipid stores are mobilized and utilized. The lipid stores are firstly coupled to lipolysis, resulting in triglycerides and glycerol; the latter can accumulate enormous turgor pressure. Meanwhile, the triglycerides can be adopted by the peroxisomes for subsequent fatty acids β-oxidation to produce acetyl CoA and ATP. The acetyl CoA can be utilized by the glyoxylate cycle and gluconeogenesis pathway for glucan and chitin biosynthesis; they can also be utilized for melanin biosynthesis. Therefore, the peroxisomes mediated cellular processes and metabolisms can provide key factors, such as melanin and cell wall integrity, and play key roles during *M. oryzae* infection (Figure 1). Detailed peroxisome-related roles during *M. oryzae* infection will be described below.

4.1. Peroxisome Biogenesis and Pathogenesis. Defects in peroxisomal biogenesis will lead to severe disorder of peroxisomal metabolisms, including fatty acids β-oxidation, glyoxylate cycle, melanin, and cell wall biosynthesis. Consequently, the fungal development and pathogenicity will be severely affected. During germination and appressorial development, the expression of *MoPEX19* was evidently elevated. Deletion of this gene will lead to deficiency in glyoxylate cycle and severe defects in development and complete loss of pathogenicity [16]. The Δ*mopex7* mutant is reduced in utilization of short-chain fatty acids and reduced its capacity in conidiation [17]. The *MoPEX5* seems to play more important role than *MoPEX7*, because the Δ*mopex5* mutant exhibits more severe defects than Δ*mopex7*, such as failure to utilize some fatty acids, generation of less turgor, and more sensitivity to H_2O_2 pressure. Moreover, distinct defects in developments are also detected in Δ*mopex5*. This phenomenon demonstrated that the PEX5-mediated PTS1 peroxisomal import pathway could be more important than the PEX7-mediated PTS2 peroxisomal import pathway [18]. However, both of the Δ*mopex5* and Δ*mopex7* mutants lose their pathogenicity. *MoPEX6* is also required for long-chain fatty acids utilization and is essential for pathogenicity. The Δ*mopex6* mutant forms nonmelanized appressoria; as a result, it cannot form the penetration peg and infection hyphae. Additionally, mycelia of Δ*mopex6* are more sensitive to Calcofluor White, suggesting the cell wall of the mutant is defect [28].

4.2. Peroxisome Fission and Pathogenesis. Block in peroxisome proliferation can result in failure to increase peroxisome number and impact the peroxisomal metabolism. In *M. oryzae*, deletion of *MoPEX11A* and *PEF1* can both severely reduce the fatty acids utilization and virulence capacity [20]. However, in contrast to totally loss of virulence in Δ*mopex5*, Δ*mopex6*, Δ*mopex7,* or Δ*mopex19*, the reduction of the virulence in Δ*mopex11A* and Δ*pef1* is evidently slighter [20, 33]. Other phenotypic defects, such as the melanin layer formation, turgor generation, cell wall integrity, and ROS tolerance, are also slighter in Δ*mopex11A* and Δ*pef1* [20, 33]. These indicate that the *de novo* formation, another way for peroxisome proliferation, can still function or compensate the defects of the peroxisome fission in the Δ*mopex11A* and Δ*pef1* mutants.

4.3. Pexophagy and Pathogenesis. In *C. orbiculare*, the Atg26-mediated pexophagy has been proved to be essential for pathogenicity, by rapidly removing redundant peroxisomes after appressoria maturation [13]. The recycling of cellular components required for invasive growth could be the primary cause. However, it seems that the pexophagy process could be dispensable for pathogenicity in *M oryzae*. It has been proved that the *Magnaporthe MoATG26* gene is not involved in pexophagy and is dispensable for virulence [19]. Another gene, *MoSNX41*, obtains the ability to regulate pexophagy in *M. oryzae*, and it plays important roles in pathogenesis. However, its pathogenicity-related function is not relevant to roles in pexophagy, because the yeast ScSnx42 (homolog of MoSnx41) can restore the pexophagy deficiency of Δ*mosnx41*, but cannot recover the defects of pathogenesis [19]. The function of MoSnx41 in conidiation and pathogenesis could be related to Snx41-dependent retrieval trafficking pathway, which may function in gamma-glutamyl cycle and GSH antioxidant production.

4.4. Woronin Body and Pathogenesis. Failing to form woronin body would result in multiple phenotypic defects in *M. oryzae*. The Δ*hex1* mutant is normal in mycelial growth, conidiation, and mating processes, but it forms abnormal appressoria, delayed in host penetration and severely blocked in infection hyphae growth [45]. As a result, Δ*hex1* is severely reduced in virulence. Besides, lack of *HEX1* will also result in failure to survive in nitrogen starvation condition, which could explain why the mutant cannot survive in host cells with kinds of cellular damage. The peroxisome β-oxidation is not affected in Δ*hex1*, indicating the function of woronin body is distinct from the peroxisomes, although it is derived from the latter [45].

4.5. Fatty Acid β-Oxidation and Pathogenesis. Peroxisomal β-oxidation is one of the chief catabolic processes during fungal infection, which can produce acetyl CoA and energy, as well as glycerol. The glycerol is used to form appressoria turgor,

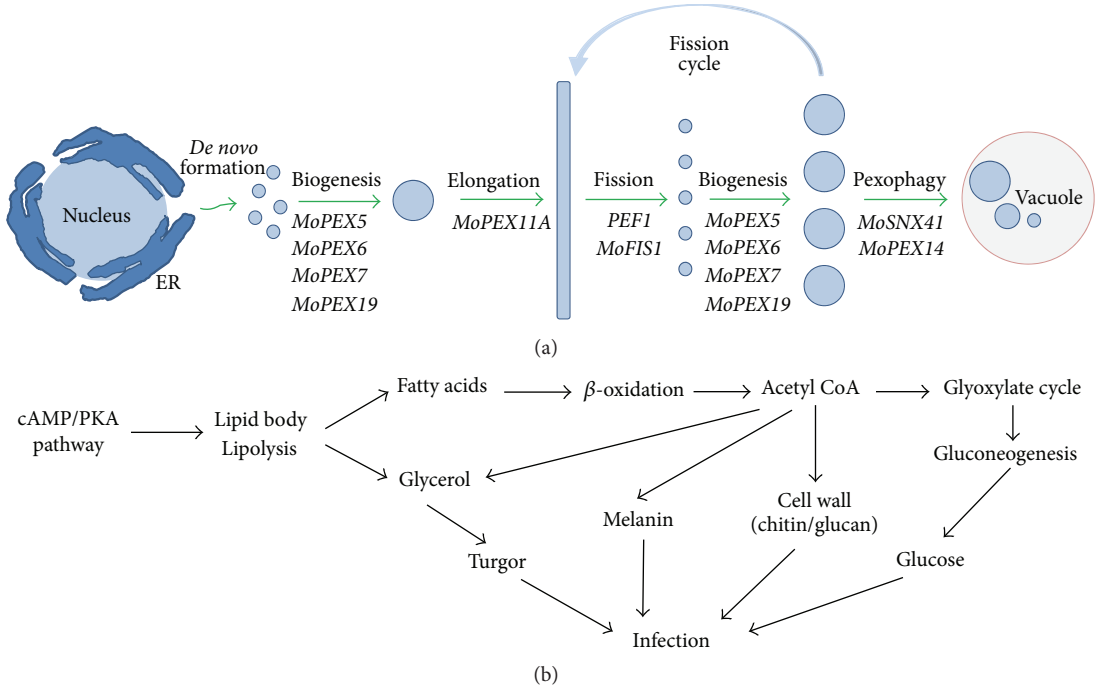

Figure 1: Life cycle and functions of the peroxisomes in *M. oryzae*. (a) Model of life cycle of the peroxisomes. The peroxisomes are synthesized from the ER and then mature through peroxisome biogenesis process. During the fission inducing condition, the matured peroxisomes can be elongated, and then the daughter peroxisomes can be produced by the fission process, the newly formed peroxisomes will mature through the biogenesis process, and the matured peroxisomes can be elongated again for another fission cycle. When the fission inducing condition is removed, the redundant peroxisomes can be eliminated through pexophagy process. (b) Function of the peroxisomes during fungal infection. When the conidia attach the host surface, the cAMP/PKA signaling pathway is activated, leading to mobilization of the lipid stores by lipolysis, and produces the fatty acids and glycerol. The glycerol will be used to generate turgor. The fatty acids will be taken by the peroxisomes for β-oxidation, which can produce mass of acetyl CoA. Acetyl CoA can be used by the glyoxylate cycle and gluconeogenesis to produce glucose for infection or be used to synthesize melanin and cell wall components. Together with the glycerol generated turgor, these products can help the fungus to penetrate the cuticle and colonize in the host cells.

and the acetyl CoA can be used by the glyoxylate cycle to produce nutrient; it also can be used to synthesize melanin and cell wall contents, or other purposes. All of the mentioned products are critical for fungal infection. In *M. oryzae*, the Mfp1 protein involving in peroxisomal β-oxidation is proved to play important roles in fatty acid metabolism and pathogenesis [47]. Defects in peroxisome biogenesis will severely block fatty acids β-oxidation, and all peroxisome biogenesis and peroxisome fission-related genes are important for fatty acids β-oxidation. For example, the *PEX6* disruption mutant is defect in olive oil utilization, cell wall integrity, and appressorial melanization and is lost in penetration capacity [28]. Block in acetyl CoA transportation will lead to similar defect in those mentioned cellular processes. The Δ*pth2* mutant produces less melanin than the wild type and fails to penetrate the host cells and thus is essential for pathogenesis [48]. It cannot grow on lipid substrates. *MoCRC1* is also required for olive oil utilization. The Δ*mocrc1* deletion mutant is severely reduced in penetration and invasive growth. The β-oxidation can also occur in mitochondria [49]. However, it seems like that the peroxisomal β-oxidation is important for appressoria-mediated penetration, while the mitochondrial β-oxidation functions in conidial viability and keeping redox homeostasis during host colonization.

4.6. Glyoxylate Cycle and Pathogenesis. The peroxisomal β-oxidation produced acetyl CoA should be used by the glyoxylate cycle to provide a mechanism for glucose generation. Glyoxylate cycle enzymes, such as Icl1, are required for full virulence of *M. oryzae*. The expression of *ICL1* is significantly elevated during conidial germination, appressorium formation, and penetration peg formation stages. Correspondingly, the Δ*icl1* mutant is delayed in conidial germination and appressorium formation and retards in cuticle penetration [50].

4.7. Redox Homeostasis and Pathogenesis. Accompanied with the degradation of fatty acids, redox homeostasis will be broken, where it is harmful to the fungi and must be rebalanced quickly. Failure in removing redundant oxides may lead to reducing of infection. For example, acetyl-CoA formation resulted in mass NADH, which can be eliminated by reoxidating it to NAD^+. This reaction is catalyzed by peroxisomal lactate dehydrogenase and needs pyruvate. The

pyruvate is generated by the alanine: glyoxylate aminotransferase 1 (Agt1) in *M. oryzae*. The Δ*agt1* mutant fails to penetration via appressoria and totally lost its pathogenicity [51].

5. Conclusions and Perspective

As a model plant pathogen, the rice blast fungus *M. oryzae* gains more attention on role of peroxisomes than other pathogens. Considerable progress has been made for us to understand life cycle and functions of the peroxisomes in the filamentous fungi. Knowledge gained from past studies will provide comprehensive understanding in the peroxisomes and may lead to develop novel targets for new drugs against pathogenic fungi. The mechanistic details the peroxisome life cycle and functions are developing rapidly, but how these processes can be well tuned according to the developmental stages and environmental conditions is largely unknown. In the future, efforts should be done to elucidate these regulatory mechanisms.

A large number of *PEX* genes can be found in the genome of *M. oryzae*; the precise roles of these should be further characterized in the future. Another challenge is to reveal the mechanism of *de novo* synthesis and uncover its roles during appressorium formation. The connections between the peroxisomes and other cellular processes or structures should also be addressed. Genome-wide screening of peroxisome-related genes and global analysis of the *PEX* genes can help us to systematically investigate functions and mechanisms of the peroxisomes. The omics approaches can help us to establish the peroxisomal regulatory networks.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Microtubule Polymerization Functions in Hypersensitive Response and Accumulation of H_2O_2 in Wheat Induced by the Stripe Rust

Juan Wang, Yang Wang, Xinjie Liu, Yuanliu Xu, and Qing Ma

State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling 712100, China

Correspondence should be addressed to Qing Ma; maqing@nwafu.edu.cn

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The plant cytoskeleton, including microtubules and microfilaments, is one of the important factors in determining the polarity of cell division and growth, as well as the interaction of plants with invading pathogens. In defense responses of wheat against the stripe rust (*Puccinia striiformis* f. sp. *tritici*) infection, hypersensitive response is the most crucial event to prevent the spread of pathogens. In order to reveal the effect of microtubules on the hypersensitive cell death and H_2O_2 accumulation in the interaction of wheat (*Triticum aestivum*) cv. Suwon 11 with an incompatible race, CYR23, wheat leaves were treated with microtubule inhibitor, oryzalin, before inoculation. The results showed that the frequency of infection sites with hypersensitive response occurrence was significantly reduced, and hypersensitive cell death in wheat leaves was suppressed compared to the control. In addition, the frequency and the incidence of infected cells with H_2O_2 accumulation were also reduced after the treatment with oryzalin. Those results indicated that microtubules are related to hypersensitive response and H_2O_2 accumulation in wheat induced by the stripe rust, and depolymerization of microtubules reduces the resistance of plants to pathogen infection in incompatible interaction, suggesting that microtubules play a potential role in the expression of resistance of wheat against the stripe rust fungus.

1. Introduction

In general, plants are subjected to the attack of a vast number of potential pathogens during their lifetime. As a result, they have evolved intricate defense mechanisms including hypersensitive response (HR) and the accumulation of reactive oxygen species (ROS) [1] to recognize and defend the attack of these invading pathogens. The localized hypersensitive cell death, accompanied by the restriction of pathogen growth, is an ubiquitous expression of plant resistance to pathogens [2]. Typically, HR occurs during successful defense in the host plants, usually leaving only small necrotic spots. Meanwhile, ROS plays important roles in defense response during plant-pathogen interactions [3–5]. Generation of ROS, especially hydrogen peroxide (H_2O_2), has been reported as one of the earliest responses of plant cells to the attack of various pathogens [3, 6, 7]. H_2O_2 accumulation can inhibit fungal growth [8] and is also involved in the occurrence of HR during the early infection stage [7] as well as regulates a myriad of cellular signaling pathways [9]. Understanding the resistance mechanisms of plants against the invasion of pathogens is critical to develop novel and sustainable disease control approaches.

The plant cytoskeleton, including microtubules and microfilaments, is a highly dynamic subcellular structure that is associated with the plant defense response. For example, cytoskeletal elements are responsible for cytoplasmic aggregation, organelle movements, papilla formation, H_2O_2 production, and HR-cell death beneath the infection site [2, 10–12]. Evidence for a crucial role of the cytoskeleton in plant defense has been provided by using drugs that alter the polymerization-depolymerization dynamics of microtubules (colchicine, taxol, or oryzalin) and microfilaments (cytochalasins, latrunculin, or phalloidin). Effects of cytoskeleton inhibitors on defense response of plants during pathogen infection have been studied in several plant-microbe systems. During the interaction between cowpea and cowpea rust fungus, *Uromyces vignae*, cytochalasin treatment

greatly delayed the generation of HR [12]. In *Linum usitatissimum-Melampsora lini* system, the inhibition of HR was also observed after treatment with antimicrotubule agent oryzalin [13]. Moreover, when wheat cells were attacked by nonhost pathogen *Sphaerotheca fuliginea*, oryzalin treatment inhibited the occurrence of HR and allowed *S. fuliginea* to penetrate and form haustoria in mesophyll cells of the wheat [14]. Interestingly, reorganization of microtubules during defense responses varies in different experimental systems. Microtubules were observed gathering around the infection sites upon fungal infection [12, 13, 15, 16] and even were generally disrupted upon perception of an oomycete infection signal [17, 18]. In contrast, microtubules inhibitors propyzamide and oryzalin did not affect the entry rate of fungi into barley (*Hordeum vulgare*) leaf epidermal cells [19]. So it is difficult to deduce common roles for microtubules during plant-microbe systems.

Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), occurs worldwide and is one of the most destructive diseases of wheat in many cool and temperate regions, especially in China [20]. The attack of the rust fungus triggers HR and H_2O_2 accumulated in the affected leaf mesophyll cells of the resistant wheat cultivars [21, 22]. It is reasonable to assume that the more we understand the resistance mechanisms of the wheat against the stripe rust, the more likely we are able to find new ways to control the disease. In the present paper, to provide experimental evidence for a role of microtubules, we focus on the effects of oryzalin on hypersensitive cell death and H_2O_2 accumulation in the interaction between wheat cultivar Suwon 11 and an incompatible race CYR23 of *Pst*.

2. Materials and Methods

2.1. Plant Cultivars and Pathogen. Wheat (*Triticum aestivum* L.) cultivar Suwon 11 and a Chinese race of *Pst*, CYR23, were used in this study. Suwon 11 is highly resistant to race CYR23. The seedlings were grown in 10 cm plastic pots in growth chamber with a 16 h : 8 h (light : dark) photoperiod (60 mmol m^{-2} s^{-1} photon flux density) at 16°C with 60% relative humidity (RH). Seven-day-old seedlings at the primary leaf stage were inoculated with fresh urediniospores of CYR23 using a fine paintbrush. After inoculation, the seedlings were kept at 100% RH in constant dark for 24 h at 12°C before being cultivated in the growth chamber. Specimens of inoculated wheat leaf tissues were taken at 12, 24, 48, 72, and 96 hours after inoculation (hai). Three independent biological replications were collected at each time point.

2.2. Treatment with Oryzalin. Oryzalin (Sigma-Aldrich, St. Louis, MO, USA) was used as inhibitor of microtubules [23]. The chemical was dissolved in dimethylsulfoxide (DMSO) as a 100 mmol stock solution, stored at −20°C, and diluted with distilled water prior to use. For inhibitor treatment, 400 μg mL^{-1} oryzalin solution was injected into the primary leaves of seven-day-old wheat seedlings by pressure infiltration with a needleless syringe, and 1% DMSO was used as control treatment. We confirmed that 1% DMSO did not affect fungal development or the penetration efficiency of *Pst* (data not shown). After injection, leaves were inoculated with fresh urediniospores of CYR23. Specimens of inoculated wheat leaf tissues were taken at 12, 24, 48, 72, and 96 hai.

2.3. Detection of Inhibitor Effects on Hypersensitive Response. Detection of hypersensitive cell death was carried out using a whole leaf transparent fluorescence staining method [24]. Wheat leaf segments of 3 cm long were clipped from the center of inoculated leaves. Leaf sections were fixed and decolorized in a boiling mixture of lactophenol : ethanol (1 : 2, v/v) for 1.5 min and stored overnight at room temperature (20°C). For Calcofluor staining, the cleared leaf segments were washed twice with 50% ethanol (v/v) for 15 min. The leaves were then rinsed twice with distilled water and soaked in 0.05 M NaOH twice. After washing 3 times with distilled water, the specimens were incubated in Tris-HCl buffer (0.1 M, pH 8.5) for 30 min and then stained with 0.1% (w/v) Calcofluor M2R (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. After washing 4 times (10 min each) with water and once (30 min) with 25% (v/v) aqueous glycerol, cleared leaf segments were mounted on glass slides in microscopy solution and examined with fluorescent microscopy. To investigate the effects of the microtubule depolymerization on the hypersensitive cell death of wheat, the number of penetration sites displaying necrosis was calculated. The formation of substomatal vesicles was defined as a penetration site or infection site. At least 50 penetration sites on each of the four leaf segments were scored for each of the time points. All the specimens were examined under a Nikon 80i fluorescent microscope (Nikon Corporation, Japan).

2.4. Detection of Inhibitor Effects on H_2O_2. The detection of H_2O_2 was analyzed histochemically using the 3,3-diaminobenzidine (DAB; Amresco, Solon, OH, USA) staining method [7, 21]. The inoculated primary leaves were cut and the cut ends were immersed in a solution containing 1 mg mL^{-1} DAB dissolved in HCl-acidified (pH 3.8) distilled water. Leaves were incubated for additional 8 h to allow DAB uptake and react with H_2O_2. After incubation, inoculated leaves were cut into 1.5 cm long segments and then fixed and decolorized in boiling 95% ethanol for 10 min before being cleared in saturated chloral hydrate. Subsequently, leaf segments were stored in microscopy solution (50% glycerol) and examined under differential interference contrast (DIC) optics with a Nikon 80i microscope (Nikon Corporation, Japan).

3. Results

3.1. Oryzalin Treatment Had No Effect on Infectious Development of Pst. Although pharmacological study generally represents a common approach to tackle the role of cytoskeleton in plant-microbe interactions, the anticytoskeletal drugs applied may also damage the microbial cytoskeleton that plays an important role during plant colonization. To determine the effects of oryzalin (400 μg mL^{-1}) on the development of *Pst*, we compared the infectious development of *Pst*

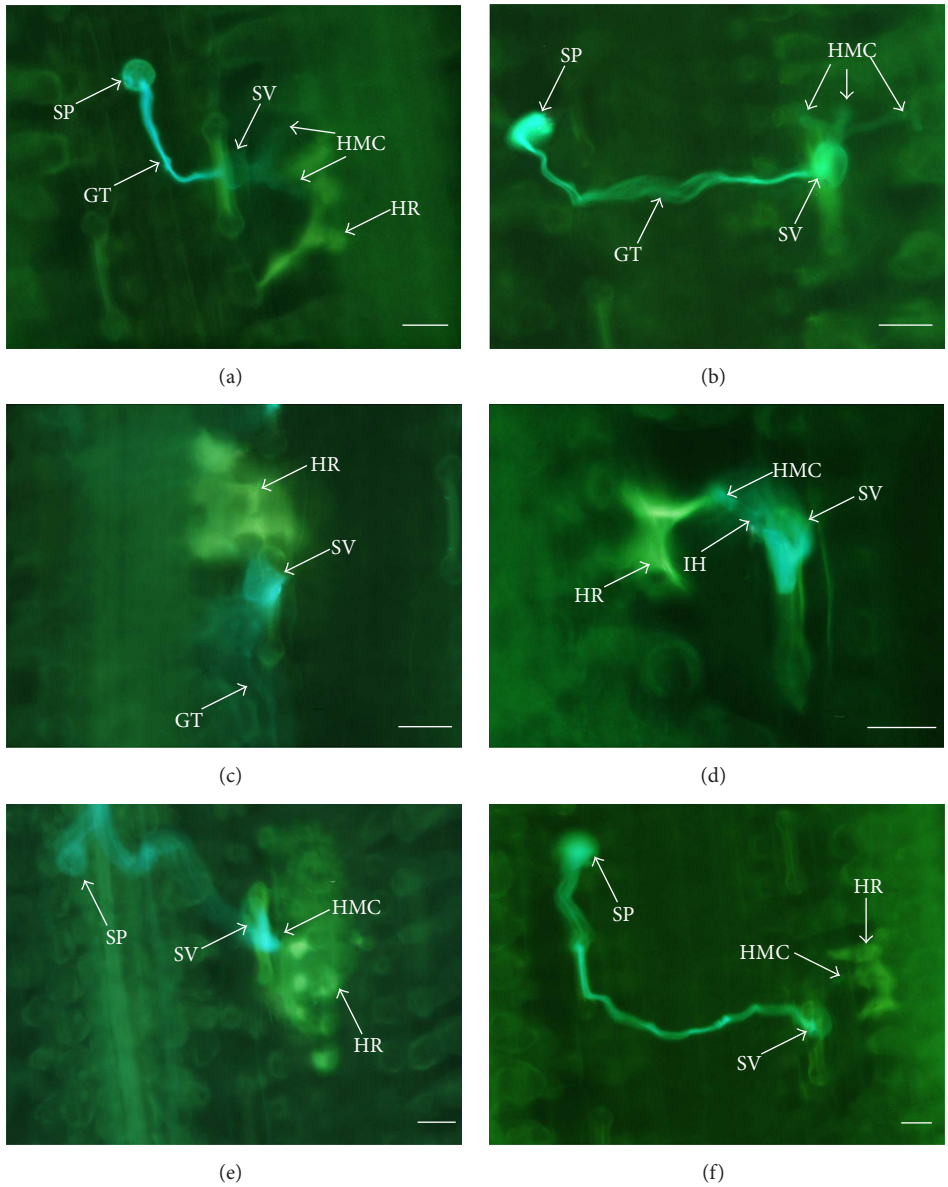

Figure 1: Fluorescence micrographs of hypersensitive cell death localization in incompatible interaction between wheat and *Pst* (race CYR23) in DMSO-only (control) and oryzalin treatments. (a) In control, haustorial mother cells formed and mesophyll cells showed HR reaction, 24 hai. (b) More than two haustorial mother cells formed treated with oryzalin, 24 hai. (c) Conspicuous HR in mesophyll cells was observed in control, 48 hai. (d) The apex of the infection hypha formed a haustorial mother cell. HR was induced by HMC and the whole cells started to lose original shape treated with oryzalin, 48 hai. (e) In control, many HR cells were visualized in mesophyll cells, 96 hai. (f) Slight HR-like cells appeared in the mesophyll cells treated with oryzalin treatment, 96 hai. GT: germ tube; HMC: haustorial mother cell; HR: hypersensitive response; IH: infection hypha; SP: spore; and SV: substomatal vesicle. Scale bars = 50 μm.

inoculated on oryzalin treated leaves with that of the control (leaves treated with 1% DMSO).

Both on the control (1% DMSO) and on oryzalin treated leaves, urediniospores germinated normally, and germ tubes grew on the leaf surface until they reached stomas, where the tip of the germ tube swelled and entered into stomatal cavity through stomatal aperture. A substomatal vesicle was formed within the cavity and then developed into 1–3 infectious hyphae. Growth of the infection hyphae made them get in touch with the mesophyll cells, which induced the development of a haustorial mother cell. Our results indicated that treatment with 400 μg mL^{-1} oryzalin solution did not affect the infectious development of *Pst* on wheat leaves.

3.2. Oryzalin Treatment Increased the Susceptibility of Resistant Wheat Plants to Pst. A few uredia were observed on sites with necrosis in leaves pretreated with the microtubule inhibitor oryzalin (infection type 2 or middle resistance) 15 days after inoculation. However, only some necrotic elongated spots without uredia production were found in control

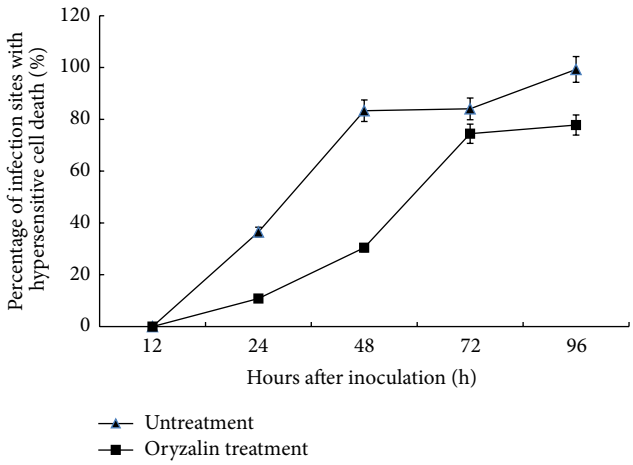

Figure 2: Incidence of mesophyll cells of wheat leaves at infection sites exhibiting hypersensitive cell death after inoculation with *Pst* (race CYR23) in DMSO-only and oryzalin treatments. Bars represent standard deviation. Replicate experiments led to similar results.

wheat leaves (infection type 0 or nearly immune reaction). This indicated that the resistance level of wheat cultivar Suwon 11 to CYR 23 was decreased upon microtubules depolymerization, suggesting that microtubules may play an important role in the incompatible interaction between wheat and *Pst*.

3.3. Oryzalin Inhibited the Hypersensitive Response in Wheat during Wheat-Pst Interaction. In wheat-*Pst* incompatible interaction, the fungal development was remarkedly restricted in infection sites by hypersensitive response of the mesophyll cells. Microscopically, in the control leaves, the HR induced by haustorial mother cells was obvious in mesophyll cells at 24 hai (Figure 1(a)). However, only a few slight fluorescence stainings could be observed at the infection sites in oryzalin treated leaves, and, occasionally, HR could not be detected although three or more haustorial mother cells were formed at infection sites (Figure 1(b)). Although, both in the control and in the oryzalin treated leaves, the ratios of penetration sites with HR were increased significantly at 48 hai in comparison with 24 hai, the extent of HR in mesophyll cells was much less in the treated leaves than that in the control (Figures 1(c) and 1(d)). With incubation time advancing, the number of penetration sites with necrotic mesophyll cells continued to increase, and almost every infection site was necrotic in the control leaves at 96 hai (Figure 1(e)). However, in oryzalin treated leaves with advancing incubation time, 96 hai, less penetration sites with necrotic mesophyll cells were detected, and the extension of necrosis was also smaller than that of the control (Figure 1(f)).

The percentage of penetration sites with mesophyll necrotic cells was significantly lower in the oryzalin treatment than in the control over the whole examination period (Figure 2). There were 36% infection sites that had necrosis in the control, but only about 11% in the oryzalin treated specimens at 24 hai. The percentage of incidence of hypersensitive cell death in the control leaves increased rapidly to 83% at 48 hai, followed by a slight increase at 72 hai, and reached approximately 100% at 96 hai. In contrast, in treatment with oryzalin, the percentage of hypersensitive cell death was only 30% at 48 hai but markedly increased to 74% at 72 hai and finally reached 77% at 96 hai. These results showed that hypersensitive response occurrence induced by *Pst* infection was reduced by oryzalin treatment, indicating that normal hypersensitive cell death was suppressed after depolymerization of microtubules in wheat mesophyll cells, especially in the early period of pathogen infection.

3.4. Oryzalin Treatment Suppressed H_2O_2 Accumulation during Wheat-Pst Interaction. After *Pst* hyphae entering through the opening stomata, in the solvent-only control, H_2O_2 accumulation was first observed both in the mesophyll cells and in the guard cells as indicated by reddish-brown staining due to DAB polymerization at 24 hai (Figure 3(a)). Up to 48 hai, stronger reddish-brown DAB staining was detected and more mesophyll cells with DAB staining appeared (Figure 3(c)). At 96 hai, both mesophyll cells and adjacent cells showed strong DAB staining (Figure 3(e)). On the contrary, in oryzalin treated specimens, DAB staining was restricted mainly in the guard cells at 24 hai (Figure 3(b)), and the DAB staining in guard cells became weaker at 48 hai when haustorial mother cells were formed (Figure 3(d)). Although obvious DAB staining was detected both in mesophyll cells and in guard cells at 96 hai, the stain was much weaker than that of the control at the same time point (Figure 3(f)).

During the examined time period, the oryzalin treated specimens had significantly lower percentage of penetration sites with DAB staining in the incompatible interaction between Suwon 11 and CYR23 in comparison with the control, although both of them showed similar trends (Figure 4). In the specimens treated with DMSO only, the percentage of infection sites with DAB staining was 60% at 12 hai, reached the peak of approximately 70% at 24 hai, and then decreased sharply to 20% at 48 hai, followed by an increase to 30% at 72 hai, and kept the same level to 96 hai. In contrast to the control, the numbers in the oryzalin treated specimens at the same experimental time points were 17%, 45%, 5%, and 20% (Figure 4). Those results clearly showed that the microtubules depolymerization drug oryzalin suppressed H_2O_2 accumulation during wheat-*Pst* interaction.

4. Discussion

In this study we found that the microtubule polymerization inhibitor, oryzalin, caused a reduction in the occurrence of hypersensitive response and accumulation of H_2O_2 in wheat cultivar Suwon 11 inoculated with the incompatible *Pst* race CYR23, which increased the susceptibility of wheat to the rust fungus compared to normal. In our previous study, we found that cytochalasin A, an inhibitor of actin polymerization, reduced the incidence of hypersensitive cell death and delayed accumulation of H_2O_2 in wheat leaves infected with *Pst* [25]. Meanwhile, our results revealed that the cytoskeleton in mesophyll cells has a potential role in

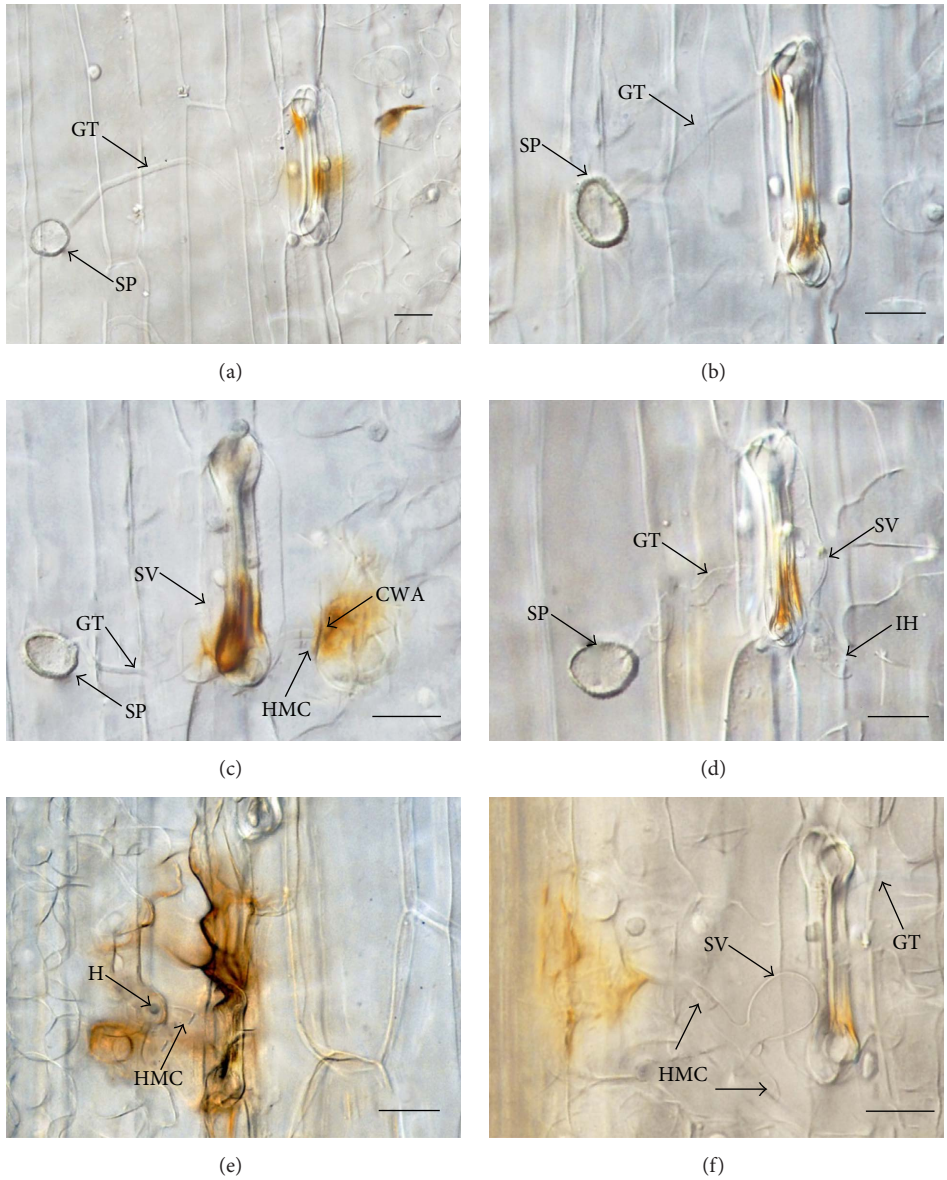

Figure 3: Micrographs of differential interference contrast (DIC) of H_2O_2 accumulation in wheat against *Pst* (race CYR 23) in DMSO-only and oryzalin treatments. (a) Mesophyll cells and guard cells showing DAB staining in control, 24 hai. (b) Positive DAB staining was detected mainly in guard cells treated with oryzalin, 24 hai. (c) Guard cells showing obvious and stronger reddish-brown H_2O_2 accumulation and mesophyll cells exhibiting the extension of plant cell wall apposition (CWA) in control, 48 hai. (d) Weaker DAB staining detected in guard cells treated with oryzalin, 48 hai. (e) Mesophyll cells and guard cell exhibit intensive H_2O_2 accumulation in control, 96 hai. (f) Mesophyll cells and guard cell showed slight H_2O_2 accumulation in oryzalin treatment at 96 hai. GT; germ tube; HMC; haustorial mother cell; SP; spore; and SV; substomatal vesicle. Scale bars = 25 μm.

HR generation and H_2O_2 accumulation and was involved in plant defense responses. Moreover, depolymerizations of microtubules and microfilaments suppressed the defense reactions and promoted the infection of stripe rust fungus in wheat [14, 26], suggesting that intact microtubules and microfilaments networks are necessary for wheat defending invaded the stripe rust fungus.

The microtubule inhibitor oryzalin provides an acceptable approach to study the role of microtubules in plant-pathogen interaction. Our results in this study indicated that depolymerization of microtubules inhibited HR of plant cells in response to pathogen attack. Similarly, the delay of HR after treatment with oryzalin was observed in a range of incompatible plant-pathogen interactions, including cowpea-cowpea rust fungus [12] and flax-flax rust fungus [13]. H_2O_2 generation and accumulation during the early infection stage were often associated with early plant defense responses [7]. H_2O_2 accumulation was only detected in guard cells before 48 hai in oryzalin treatment specimens instead of 24 hai in the control, which indicated that oryzalin treatment

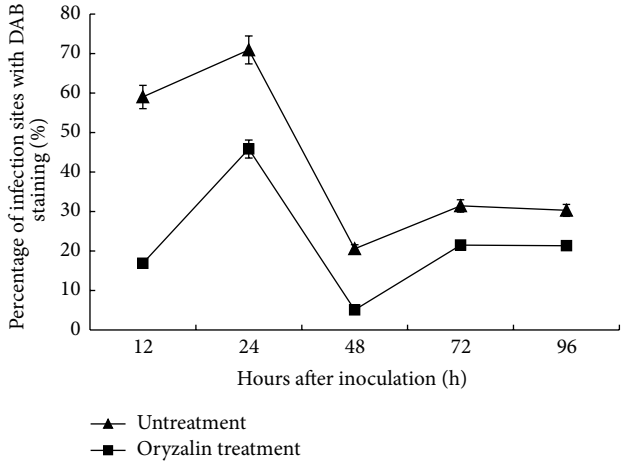

Figure 4: Percentage of mesophyll cells of wheat at interaction sites exhibiting H_2O_2 accumulation after inoculation with *Pst* (race CYR23) in DMSO-only and oryzalin treatments. At least 50 infection sites of each of four leaf pieces were scored for each time point. Bars represent standard deviation. Replicate experiments led to similar results.

delayed the accumulation of H_2O_2 in wheat against rust fungus attack. Meanwhile, we also found that the burst of H_2O_2 was restrained after treatment with oryzalin. The data further confirmed that microtubules are necessary for H_2O_2 accumulation. In addition, according to the results, oryzalin also inhibited the hypersensitive response in wheat during wheat-*Pst* interaction. Thus, microtubules may play an essential role in resistance response of wheat against the stripe rust.

Moreover, the role of microtubules in HR remains controversial. Oryzalin allowed incompatible oomycete hyphae to spread in the manner of a compatible interaction [27]. However, disruption of microtubules by oryzalin, cell death, and nuclear movements were not affected during the infection of cowpea-cowpea rust fungus [12]. Therefore, we suggest that the role of microtubules in induction of HR varies between different interaction systems.

Traditionally, the plant microtubules are essential players for many different cellular events such as growth, division, cell motility, production of the ER body, vesicular sorting, signal transduction, and cell wall deposition [28]. For the cytoskeleton response to pathogen attack, the role of the microtubules has been reported in different plant-microbe interactions. In barley-*Erysiphe* and flax-*Melampsora* interactions, radial arrays of microtubules formed beneath the appressoria [15, 16]. Treatment with microtubule inhibitors delayed onset of the hypersensitive response in the flax-*Melampsora* system [23]. Moreover, microtubules were identified as a central component in the control of protoplast volume during the response to hyperosmotic stress [29] and the membrane fluidity in cold sensing [30]. In addition, microtubules might act as a negative regulator of ion channel activity or as stress-focusing elements that collect and convey membrane perturbations to a channel [31].

Pathogens are able to suppress the host defenses by secreting effector proteins. In turn, plants evolved resistance proteins, which allow recognition of these effectors. This leads to effector-triggered immunity (ETI) and activation of the hypersensitive response (HR) [32]. ETI or HR involves the production of reactive oxygen species (ROS) and the transcriptional activation of genes, encoding antimicrobial pathogenesis-related (PR) proteins. The signaling pathways of ETI are fine-tuned by plant signaling molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) [33, 34]. The hormone SA plays a major role in plant resistance to hemi/biotrophic pathogens [34]. Multiple regulator proteins control microtubule dynamics. Different regulators use different mechanisms to regulate microtubule dynamics. MAP65, a microtubule-associated protein conserved in higher eukaryotes, binds to microtubule to stop microtubule depolymerization [35]. In addition, mutants accumulate in *Arabidopsis thaliana* MAP65-3 increased levels of SA and constitutively express genes encoding PR proteins in the leaves, indicating that AtMAP65-3 exerts a role in negatively regulating plant defense responses [36]. Therefore, the focus of future work in this field should be studying the functions of microtubule-associated proteins in controlling microtubule dynamics that take part in the resistant response of wheat against *Pst*.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Juan Wang and Yang Wang contributed equally to this paper.

Acknowledgments

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Research Article

ChSte7 Is Required for Vegetative Growth and Various Plant Infection Processes in *Colletotrichum higginsianum*

Qinfeng Yuan, Meijuan Chen, Yaqin Yan, Qiongnan Gu, Junbin Huang, and Lu Zheng

The Key Laboratory of Plant Pathology of Hubei Province, Huazhong Agricultural University, Wuhan, Hubei 430070, China

Correspondence should be addressed to Lu Zheng; luzheng@mail.hzau.edu.cn

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Colletotrichum higginsianum is an important hemibiotrophic phytopathogen that causes crucifer anthracnose in various regions of the world. In many plant-pathogenic fungi, the Ste11-Ste7-Fus3/Kss1 kinase pathway is essential to pathogenicity and various plant infection processes. To date, the role of ChSte7 in *C. higginsianum* encoding a MEK orthologue of Ste7 in *Saccharomyces cerevisiae* has not been elucidated. In this report, we investigated the function of ChSte7 in the pathogen. The ChSte7 is predicted to encode a 522-amino-acid protein with a S_TKc conserved domain that shares 44% identity with Ste7 in *S. cerevisiae*. ChSte7 disruption mutants showed white colonies with irregularly shaped edges and extremely decreased growth rates and biomass productions. The ChSte7 disruption mutants did not form appressoria and showed defects in pathogenicity on leaves of *Arabidopsis thaliana*. When inoculated onto wounded leaf tissues, the ChSte7 disruption mutants grew only on the surface of host tissues but failed to cause lesions beyond the wound site. In contrast, both the wild-type and complementation strains showed normal morphology, produced appressoria, and caused necrosis on leaves of *Arabidopsis*. Analysis with qRT-PCR suggested that ChSte7 was highly expressed during the late stages of infection. Taken together, our results demonstrate that ChSte7 is involved in regulation of vegetative growth, appressorial formation of *C. higginsianum*, and postinvasive growth in host tissues.

1. Introduction

The hemibiotrophic ascomycete fungus *Colletotrichum higginsianum* is the causal agent of anthracnose disease on a wide range of cruciferous plants, such as *Brassica*, *Raphanus*, and the model plant *Arabidopsis thaliana* [1]. For example, in South China, this fungus usually causes typical water-soaked lesions on leaves of Chinese cabbage (*B. parachinensis*), leading to 30–40% yield loss yearly [2]. To invade host tissue, conidia first attach to plant surfaces and germinate to form melanized appressoria. After that, *C. higginsianum* penetrates the plant cell with high turgor pressure generated in the melanized appressorium, and then large bulbous biotrophic hyphae form in the first infected cell. Finally, the fungus differentiates secondary hyphae to kill host tissues [3].

The *C. higginsianum-A. thaliana* pathosystem provides an attractive model for dissecting fungal pathogenicity and plant resistance, in which both partners can be genetically manipulated [4]. Genome and transcriptome analyses of *C. higginsianum* infecting *A. thaliana* indicate that this fungus has many virulence factors [5]. To date, just limited molecular determinants of virulence in *C. higginsianum* have been reported. ChEC effectors are focally secreted from appressorial penetration pores before host invasion, revealing new levels of functional complexity for *C. higginsianum* [6]. Arginine biosynthesis was shown to be critical for early stages of plant infection by *C. higginsianum* [7]. Ch-MEL1 is required for both appressorial formation and melanin production in *C. higginsianum* as well as postinvasive growth in host tissues [8]. Chpma2 deletion mutants form fully melanized appressoria but entirely fail to penetrate the host tissue and are nonpathogenic [9]. However, more virulence factors in the model phytopathogen remain to be elucidated and characterized.

Signal transduction is a highly conserved system that enables eukaryotes to sense and respond to extracellular conditions. The mitogen-activated protein kinase (MAPK) cascade is one of the ubiquitous signaling systems in eukaryotes. The cascade is universally composed of three kinase proteins, MAPK-extracellular regulated kinase kinase (MEKK),

MAPK-extracellular regulated kinase (MEK), and MAPK [10]. Upon perception of an appropriate external stimulus, MEKK phosphorylates MEK, which then phosphorylates MAPK, resulting in enzymatic activation and eventual relay of signal to ultimately activate physiological responses [11, 12]. These pathways are involved in a variety of developmental processes in yeasts and filamentous fungi [12, 13]. The signaling model in yeast, *Saccharomyces cerevisiae* involving the Ste11-Ste7-Fus3/Kss1 cascade, has been characterized for pheromone responses and filamentous growth pathways [14]. In several phytopathogenic filamentous fungi, MAPK genes have been frequently annotated as virulence factors, such as Pmk1 in *Magnaporthe oryzae* [15–17], Cmk1 in *C. orbicular* [18], Kpp2 (Ubc3) in *Ustilago maydis* [19, 20], and Bmp1 in *Botrytis cinerea* [21]. The results of previous studies indicate that there are some differences in this signaling system between fungal plant pathogens and yeasts. In *S. cerevisiae*, certain protein kinases act in more than one pathway (e.g., the MEK Ste7 and MEKK Ste11 participate in two and three pathways, resp.); however, the Ste11-Ste7-Kss1 cascade is unique to pathogenesis in plant-pathogenic fungi [12, 13]. With the exception of differences in signaling systems, it is suggested that there are significant differences in the input/output of this cascade, not only between yeasts and plant pathogens, but also among different pathogens [15, 18–20]. The inputs and outputs of these cascades are probably dependent on the fungal species, which suggests that the components of the MAPK cascades should be separately characterized for individual fungal plant pathogens.

The Ste11-type MEKK and Ste7-type MEK are two important upstream kinases of the Fus3/Kss1-type MAPK cascade which has been considered a master regulator of pathogenesis in plant pathogens [22, 23]. In previous work, the Ste7 homologues in many pathogens were found to be essential for appressorial formation in pathogenesis [24–28]. In this study, we identified and characterized ChSte7 encoding a MAPKK orthologue of the yeast Ste7 in *C. higginsianum*. Deletion of ChSte7 resulted in significant reduction in vegetative growth and loss of ability to form appressoria. Most interestingly, wounding inoculation assays and microscopic observations indicated that the ChSte7 deletion mutants were also defective in their invasive growth inside the host plant tissues. Otherwise, ChSte7 was highly expressed in the postinvasive growth phase. All these data support the involvement of ChSte7 in regulation of vegetative growth, appressorial formation, and postinvasive growth in host tissues.

2. Materials and Methods

2.1. Strains, Plasmids, and Plants. The wild-type strain IMI349061 of *C. higginsianum* (Table 1), originating from diseased tissues of *B. campestris*, was kindly provided by Professor Yangdou Wei from the University of Saskatchewan, Canada. Plasmids pMD18T-HYG with hph cassette and pNeo3300III with neocassette used for gene disruption and complementation vector construction [29] were stored at −80°C in 20% glycerol (v/v) as bacterial suspensions.

Arabidopsis thaliana ecotype Col-0 was used in virulence assays. *Arabidopsis* seeds were sown on the surface of peat-based compost and placed in growth chamber with 16/8 h photoperiod and day and night temperatures of 22 and 18°C, severally. Lighting provided a photosynthetic photon flux rate of 40 μmol m^{-2}s^{-1} (400–700 nm), and the chamber was maintained at 65–80% relative humidity.

2.2. Disruption and Complementation of Target Gene ChSte7. To replace ChSte7, an 870 bp fragment of upstream flanking sequence and 950 bp fragment of downstream flanking sequence of the gene were amplified, respectively, with primer pairs, ChSte7F1FP/ChSte7F1RP and ChSte7F2FP/ChSte7F2RP (Table 2). PCR products of the upstream flanking sequence digested with HindIII/SalI and the downstream flanking sequence digested with XbaI/KpnI were ligated into the corresponding restriction sites of vector pMD18T-HYG, resulting in the initial vector F1-HYG-F2. The vector F1-HYG-F2 was then digested with HindIII and KpnI and ligated with pNeo3300III to form the gene disruption vector pNeo3300IIIChSte7-Ko. The vector, pNeo3300IIIChSte7-Ko, was transformed into *Agrobacterium tumefaciens* EHA105 by electroporation, and then conidia of *C. higginsianum* wild-type strain were transformed with vector pNeo3300IIIChSte7-Ko based on the *A. tumefaciens*-mediated transformation (ATMT) protocol described by Li et al. [30]. To obtain ChSte7 disruption mutants, transformants were grown on potato dextrose agar (PDA) supplemented with 50 μg/mL of hygromycin (Merck, Germany) and 500 μg/mL of cephalosporin (Amresco, USA) and then subcultured on PDA supplemented with 150 μg/mL antibiotic G418 (Amresco, USA). Gene disruption transformants were confirmed by PCR amplification with two pairs of primers, ChSte7-KF/ChSte7-KR and HphSP/HphAP (Table 2), and RT-PCR with primers ChSte7-KF and ChSte7-KR (Table 2).

To confirm that phenotypes of the ChSte7 disruption mutants were due to the targeted gene disruption, one disruption mutant Δ*ChSte7-26* (Table 1) was complemented with a full length sequence of ChSte7. Since the upstream sequence of ChSte7 was not found in sequencing scaffolds of the *C. higginsianum* assembly, high-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) was used to amplify the upstream sequence of ChSte7 (corresponding to the promoter region). For hiTAIL-PCR, genomic DNA was used as a template in successive reactions with nested RB-specific primers RB-0a, RB-1a, and RB-2a together with the degenerate primers LAD1-1, LAD1-2, LAD1-3, LAD1-4, and AC1 following thermal cycling settings for hiTAIL-PCR described by Liu and Chen [31]. The 3882 bp fragment including the 1728 bp ORF of ChSte7, 1674 bp upstream, and 480 bp downstream was amplified from genomic DNA of the wild-type strain with primer pair Ste7comFP and Ste7comRP (Table 2) and cloned into the HindIII site of vector pCAMBIA3300III, generating the complementation plasmid pNeo3300IIIChSte7-Com. To obtain the ChSte7 complementation transformants, conidia of Δ*ChSte7-26* were transformed with vector pNeo3300IIIChSte7-Com by the ATMT method. The complementation transformants were screened on PDA containing 150 μg/mL G418, and gene fragments were detected by PCR and RT-PCR analyses.

Table 1: Strains used in this study.

Strain	Description	Reference
Ch-1	*Colletotrichum higginsianum* IMI349063	[4]
Δ*ChSte7-26*	ChSte7 disruption mutant from Ch-1	This study
Δ*ChSte7-48*	ChSte7 disruption mutant from Ch-1	This study
CΔ*ChSte7-26-20*	ChSte7 complementation strain from Δ*ChSte7-26*	This study
EHA105	*Agrobacterium tumefaciens* competent cell	[8]
DH5*α*	*Escherichia coli* competent cell	[8]

Table 2: Primers used in this study.

Primer	Sequence (5′ to 3′)
ChSte7FP	ATGGCCGATCCTTTTGCGC
ChSte7RP	CTAGTTGGAGGTACCATTGTACACATCG
ChSte7F1Fp	CCCAAGCTTGGATACTGTCCATCACTTCATCGCC
ChSte7F1Rp	ACGCGTCGACCCATGCATGATCTGGAGCTCACG
ChSte7F2Fp	GCTCTAGAAAAGTGATGAACGCTTGGGCA
ChSte7F2Rp	GGGGTACCGACCCTCCCGTCTCCTTCG
ChSte7-KF	TCGGGTAAGTTTGGCTTTGTTTCA
ChSte7-KR	CTAGTTGGAGGTACCATTGTACACATCG
HphSp	TTCTGCGGGCGATTTGTG
HphAp	AGCGTCTCCGACCTGATG
ChSte7comFP	CCCAAGCTTGCTTAACCATCGGCAACTTCATG
ChSte7comRP	CCCAAGCTTTAGGATAGGAGGCCCTTCCCTGACT
TubulinS	AGAAAGCCTTGCGACGGAACA
TubulinA	CCTCCAGGGTTTCCAGATTA
qRT-STE7F	CAAGAAAGAGATGCGTAAG
qRT-STE7R	GCCGTAGAAGTTGACAATA
qRT-tubulinF	ATGCAGATGTCGTAGAGA
qRT-tubulinR	ACTGTTGTTGAGCCTTAC

Restriction enzyme cutting sites are underlined.

2.3. Phenotypic Analysis. Mycelia of the wild-type strain and mutants were inoculated onto PDA plates and cultured in darkness for 7 days at 25°C for growth rate and conidiation testing. Mycelia were harvested by suction filtration from 7-day-old cultures grown in 100 mL potato dextrose broth (PDB) at 25°C with shaking at 150 rpm, dried at 60°C, and weighed. Hyphae picked from edge of the colony on PDA were examined by light microscopy (Nikon, Tokyo, Japan). Conidia were harvested with sterile distilled water and passed through four layers of lens paper to remove debris and mycelia. The conidial suspension was adjusted to a concentration of 1×10^6 spores/mL with sterile deionized water and suspension droplets (10 μL) were spotted on microscope plastic coverslips (Thermo Fisher Scientific, MA, USA) placed in 9 cm diameter petri dishes, and conidial germination and appressorial formation were examined by light microscopy after 12 h.

2.4. Pathogenicity Assay and Infection Observation. Conidial suspensions at the concentration of 1×10^6 spores/mL were prepared as stated above and used for plant inoculation. Intact plants of *Arabidopsis* were used to assess the virulence of the disruption and complementation transformants of ChSte7. Conidial suspensions were sprayed onto the upper and lower surfaces of *Arabidopsis* leaves from 4- to 5-week-old plants. After sealing the plants inside plastic propagators lined with wet tissue paper to provide high humidity, inoculated plants were incubated at 25°C in a controlled environment chamber (18 h photoperiod). Lesion formation was examined at 5 days after inoculation (dpi).

To observe infection structures of wild-type strain and mutants, *Arabidopsis* leaves from 4- to 5-week-old plants were spotted with 10 μL droplets of the prepared conidial suspension on either side of each midvein. Inoculated leaves were incubated in complete darkness at 25°C. Inoculated leaf tissues collected after 4 days of incubation were cleared in a solution of methanol : chloroform : glacial acetic acid (6 : 3 : 1), then rehydrated and stained with 1% trypan blue in glycerol, and viewed by light microscopy.

To assess the ability of the ChSte7 disruption mutants to grow invasively inside the host plant tissues independent of penetration, conidial suspensions were also spotted on wounded site of the *Arabidopsis* leaves. Wounding experiments were carried out by pricking the detached leaves with a fine sterile needle prior to inoculation and placing

conidial suspensions directly on the wound sites. Lesion formation was examined at 4 dpi and inoculated leaf tissues were cleared, rehydrated and stained, and finally viewed by light microscopy.

2.5. DNA/RNA Manipulation, RT-PCR, and qRT-PCR Analysis. Total genomic DNA (gDNA) was isolated from *C. higginsianum* wild-type strain with CTAB following Sambrook et al. [32]. Hyphae harvested from PDB, conidia obtained from PDA plates, and *Arabidopsis* leaves sprayed with conidial suspension at concentration of 1×10^6 spores/mL at 5, 20, 40, 65, and 90 h after incubation (hpi) were collected, flash-frozen in liquid nitrogen, and stored at −80°C until required. RNA isolation was carried out using TRIzol® Plus RNA Purification Kit (Invitrogen, Carlsbad, USA), and potential DNA contamination was removed by DNase I treatment (RNase Free) (Takara, Dalian, China) following manufacturer's instructions. First-strand cDNA was synthesized by using Revert Aid first-strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) following the manufacturer's instructions. Expressions of ChSte7 in disruption mutants and the complementation strain were examined by RT-PCR, and a 1208 bp fragment was amplified with gene-specific primers ChSte7-KF and ChSte7-KR (Table 2). The *C. higginsianum* β-tubulin sequence amplified with primers TubulinS and TubulinA (Table 2) was used as the reference gene. PCR conditions were 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and with a final extension at 72°C for 5 min. Expression of ChSte7 at different development stages of the fungus *in vitro* or *in planta* was analyzed by qRT-PCR with ChSte7 gene-specific primers qRT-STE7F/qRT-STE7R (Table 2). The *C. higginsianum* β-tubulin as the reference gene was amplified with primers qRT-tubulinF and qRT-tubulinR (Table 2). PCR conditions were 50 cycles of 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s and with a final extension from 65°C to 95°C (0.5°C/5 s). PCR reactions were run on a PTC-200 DNA Engine Peltier Thermal Cycler (Bio-Rad, Hercules, USA).

2.6. Bioinformatics. The full sequence of ChSte7 was downloaded from the *C. higginsianum* genome database (http://www.broadinstitute.org/annotation/genome/colletotrichum_group/MultiHome.html). To confirm sequence validity, the primers ChSte7FP and ChSte7 RP (Table 2) were designed and used for the amplification of the ChSte7 gene. All primers used in this work were designed with primer premier 5.0 (http://www.premierbiosoft.com/primerdesign/). Open reading frames were further analyzed using the gene prediction program FGENESH (Softberry Inc., Mount Kisco, NY, USA). Protein domain and motif predictions were performed with SMART software (http://smart.embl-heidelberg.de/). The Ste7 protein sequences from different organisms were obtained from the GenBank database, using the BLAST algorithm. Sequence alignments were performed using the Clustal X (version 2.0, http://www.clustal.org/clustal2/), and the phylogenetic tree was generated by the Mega software (version 5.0, http://www.megasoftware.net/index.php).

2.7. Statistical Analysis. The data of all quantitative assays were analyzed with DPS statistical analysis software (version 3.01, China Agric. Press, Beijing, China), using analysis of variance and the test of Least Significant Difference (LSD) at $P = 0.05$.

3. Results

3.1. Identification and Characterization of ChSte7. The protein sequence of Ste7 from *S. cerevisiae* was used as the query to blast (BLASTp) against available *C. higginsianum* genome. We identified a locus CH063_02455 named as ChSte7 which encodes the Ste7 homologue and is predicted to encode a 522-amino-acid protein that shares 44% identity with Ste7 in *S. cerevisiae*. Sequence analysis with SMART revealed that ChSte7 contained a S_TKc conserved domain (serine/threonine protein kinases, catalytic domain) (Figure 1(a)). Phylogenetic analysis of ChSte7 to other Ste7 proteins revealed that ChSte7 from *C. higginsianum* was most similar to Ste7 proteins of *C. gloeosporioides* and *C. orbiculare* and most distant from those of *Bipolaris maydis* and *S. cerevisiae* (with identities still close to or above 44%) (Figure 1(b)). This result indicates that Ste7 proteins are conserved in fungi.

3.2. ChSte7 Is Highly Expressed during Invasive Growth. To gain insight into the functions of ChSte7, we first examined the gene expression profile at different stages of *C. higginsianum* using qRT-PCR. In comparison to the conidiation stage, the expression levels of ChSte7 were significantly increased in vegetative and invasive growth stages (5 to 90 hpi) (Figure 2). The expression of ChSte7 was highest in the vegetative stage while no expression was found in the conidiation stage. Lower expressions were detected during the early stages of infection at 5–40 hpi. However, the expression of ChSte7 increased significantly during late infection until 90 hpi. These observations suggest that ChSte7 is highly expressed in the late infection and may play a key role during necrotroph in *C. higginsianum*.

3.3. Targeted Disruption and Complementation of ChSte7. A gene disruption vector, pNeo3300IIIChSte7-Ko (Figure 3(a)), containing hygromycin B phosphotransferase (hph) gene and both 5′ and 3′ flanking regions of ChSte7, was constructed and then transformed into the wild-type strain. The transformants were first selected on hygromycin-containing media and then selected on G418-containing media to avoid random insertion. Fifty candidate disruption transformants without resistance to G418 among 348 hygromycin-resistant transformants were obtained. Two candidate disruption transformants Δ*ChSte7-26* and Δ*ChSte7-48* were found lacking the 1208 bp ChSte7 fragment compared to the wild-type strain after PCR amplification with STE7SP/STE7AP (Table 2), but an 887-bp hph fragment was obtained by PCR amplification with hphF/hphR (Table 2) in the two candidate transformants (Figure 3(b)). Null mutation of the ChSte7 gene was further confirmed by RT-PCR analysis, since the ChSte7 transcript was not detected in these two targeted disruption transformants (Figure 3(c)). These results demonstrated that the

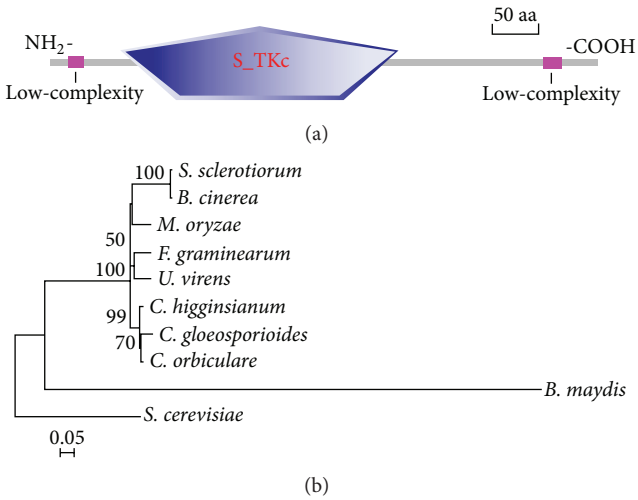

Figure 1: Functional domain identification and phylogenetic tree. (a) A conserved S_TKc domain (serine/threonine protein kinases, catalytic domain) and two low-complexity regions in ChSte7 were predicted using the SMART website. (b) Sequence alignments were performed using Clustal X 2.0 program and the calculated phylogenetic tree was viewed using Mega 5.0 program. Neighbor-joining tree was constructed with 1000 bootstrap replicates of phylogenetic relationships between Ste7 homologues in fungi. All 10 protein sequences of the Ste7 homologues were downloaded from the NCBI database and the accession numbers of Ste7 homologues are shown as follows: *S. sclerotiorum* (*Sclerotinia sclerotiorum* XP_001588345.1), *B. cinerea* (*Botrytis cinerea* XP_001557712.1), *M. oryzae* (*Magnaporthe oryzae* ELQ32975.1), *F. graminearum* (*Fusarium graminearum* XP_011318809.1), *U. virens* (*Ustilaginoidea virens* KDB12657.1), *C. higginsianum* (*Colletotrichum higginsianum* CCF40893.1), *C. gloeosporioides* (*Colletotrichum gloeosporioides* AAD55385.1), *C. orbiculare* (*Colletotrichum orbiculare* ENH81835.1), *B. maydis* (*Bipolaris maydis* EMD86379.1), and *S. cerevisiae* (*Saccharomyces cerevisiae* CAA98732.1).

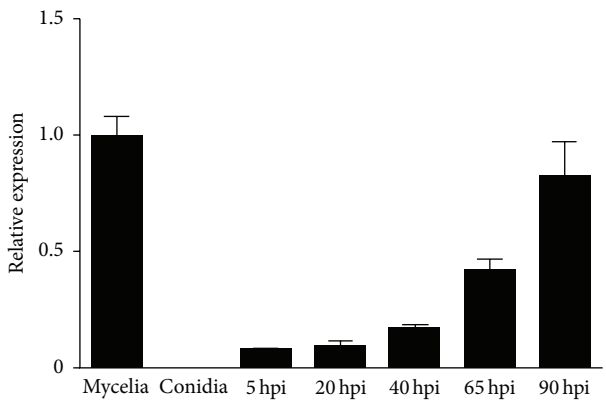

Figure 2: Expression profiles of ChSte7 assayed by qRT-PCR. RNA was extracted from mycelia and conidia, as well as infected *Arabidopsis* seedlings at different times of inoculation (5, 20, 40, 65, and 90 hpi). β-tubulin gene was used as an internal control. Relative abundance of ChSte7 transcripts during infectious growth was normalized by comparing with vegetative growth in potato dextrose broth (relative transcript level = 1). Three independent biological experiments with three replicates in each treatment were performed.

ChSte7 gene was deleted in the ChSte7 disruption transformants Δ*ChSte7-26* and Δ*ChSte7-48*. To investigate whether altered growth phenotypes and the loss of virulence in ChSte7 disruption transformants could be restored by reintroduction of a wild-type copy of ChSte7, we transformed Δ*ChSte7-26* with plasmid pNeo3300IIIChSte7-Com. Subsequently, complementation transformant CΔ*ChSte7-26-20* was confirmed by PCR and RT-PCR analysis (Figures 3(b) and 3(c)) and chosen for further phenotype analysis.

3.4. ChSte7 Plays a Crucial Role in Vegetative Growth and Colony Morphology. To explore the role of ChSte7 in vegetative growth and colony morphology, the Δ*ChSte7* mutants and the complementation transformant together with the wild-type strain were cultured on PDA plates for 7 days. The Δ*ChSte7* mutants showed a significantly reduced growth rate and biomass while conidial production was consistent with the wild-type and complementation strain (Table 3). The growth rate and biomass of the Δ*ChSte7* mutants were reduced to approximately 55.8–58.1% and 31.6–38.8%, respectively, compared with those of the wild-type strain. The Δ*ChSte7* mutants showed irregular-shape colonies, as well as increased aerial hyphae (Figure 4(a)). Moreover, the Δ*ChSte7* mutants produced wavy and twisted hyphae with increased branching (Figure 4(b)). These results suggest that ChSte7 plays an important role in hyphal growth and morphology.

3.5. ChSte7 Is Essential for Appressorial Formation and Pathogenicity in C. higginsianum. In order to investigate whether ChSte7 is essential for pathogenicity, we first tested the ability to form appressoria by placing droplets of conidial suspensions on artificial hydrophobic surface. The wild-type strain started to form many appressoria by 12 h after inoculation. In contrast, the Δ*ChSte7* mutants germinated poorly (Table 3) and did not form any appressoria, even after 24 h (Figure 4(c)). Moreover, Δ*ChSte7* mutants often formed long germ tubes. The ChSte7 complementation strain CΔ*ChSte7-26-20* regained the ability to form normal appressoria (Figure 4(c)).

To test the pathogenicity of mutants, conidial suspensions of all strains were inoculated onto nonwounded *Arabidopsis* leaves. The Δ*ChSte7* mutants were nonpathogenic while the wild-type and CΔ*ChSte7-26-20* strain were virulent and formed typical necrotic lesions on leaves (Figure 5(a)).

To verify whether the loss of virulence of the Δ*ChSte7* mutants was attributable to the defect in appressorial formation, we investigated the formation of different infection structures by Δ*ChSte7* mutants on *Arabidopsis* leaves. At 4 dpi, high frequencies of appressorial formation and penetration on the surface of *Arabidopsis* leaves were found in both the wild-type and CΔ*ChSte7-26-20* strain (above 90%; Figures 5(b) and 5(c)). The Δ*ChSte7* mutants germinated but did not form appressoria on the plant surface, and also no invasive hyphae were observed in the epidermal cells (Figures 5(b) and 5(c)), indicating that loss of pathogenicity of the Δ*ChSte7* mutants was caused, at least in part, by defects in appressorial formation. These observations showed that ChSte7 is required in the early stage of the infectious process,

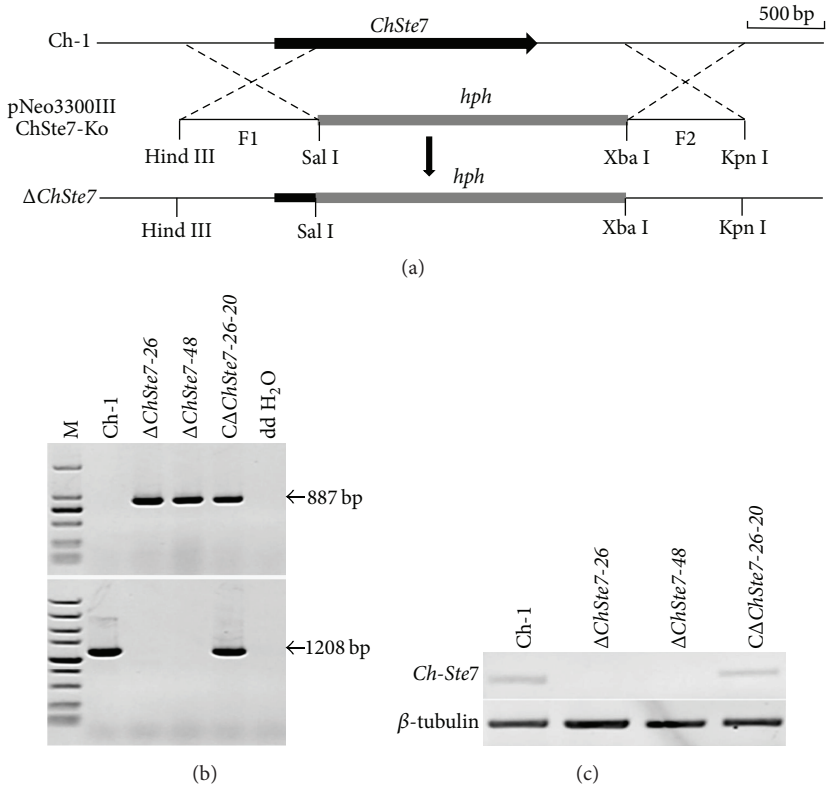

Figure 3: Targeted disruption and complementation of ChSte7 gene. (a) ChSte7 locus and gene deletion vector. (b) PCR analysis of wild-type strain (Ch-1), ChSte7 disruption mutants (Δ*ChSte7-26* and Δ*ChSte7-48*), and complementation strain (CΔ*ChSte7-26-20*). Markers (M) in the top and bottom images are DL2000 and DL5000, respectively. The band of 877 bp in hph gene was amplified in the ChSte7 disruption mutants and complementation strain while a 1208 bp fragment in ChSte7 gene was obtained in the wild-type and complementation strains. (c) RT-PCR analysis of wild-type strain, ChSte7 disruption mutants, and complementation strain.

Table 3: Growth rate, biomass, conidiation, and conidial germination of the ChSte7 disruption mutants and complementation strain of *C. higginsianum*.

Strain	Growth rate (mm/d)	Mycelial dry weight (mg/mL)	Conidiation (10^6/plate)	Germination rate (%)
Ch-1	4.3 ± 0.2^a	2.9 ± 0.1^a	28.0 ± 2.0^a	75.5 ± 6.9^a
Δ*ChSte7-26*	2.4 ± 0.1^b	1.1 ± 0.2^b	26.7 ± 1.5^a	6.2 ± 0.6^b
Δ*ChSte7-48*	2.5 ± 0.1^b	0.9 ± 0.2^b	27.0 ± 0.7^a	7.5 ± 2.0^b
CΔ*ChSte7-26-20*	4.2 ± 0.1^a	2.9 ± 0.2^a	24.7 ± 0.6^a	76.5 ± 3.3^a

Numbers indicated by the same letter in the same column are not significantly different at $P = 0.05$ in a test of LSD.

which corresponds with the results of appressorial formation experiments.

3.6. ChSte7 Disruption Mutants Are Avirulent on Wounded Leaf Tissues. To assess the ability of the Δ*ChSte7* mutants to grow invasively inside the host plant tissues independent of penetration, conidia of the Δ*ChSte7* mutants were directly inoculated on the wound sites of *Arabidopsis* leaves. At 4 dpi, the Δ*ChSte7* mutants could not form lesions on wounded leaf tissues while the wild-type and the CΔ*ChSte7-26-20* strain caused dark necrotic lesions on inoculation sites (Figure 6(a)). Microscopic observation showed that conidia of the Δ*ChSte7* mutants never formed appressoria on *Arabidopsis* leaves, and germinating conidia could not form invasive hyphae to enter into the wound sites but did produce aerial hyphae beyond or around wound sites (Figures 6(b) and 6(c)). The results indicated that Δ*ChSte7* mutants lost the ability for invasive growth in the host tissues. Therefore, we concluded that the loss of virulence in Δ*ChSte7* mutants was attributable to the defects in both appressorial formation and invasive growth.

4. Discussion

The MAPK cascade is one of the most important signaling systems in the regulation of morphogenesis and stress responses. Most filamentous fungi including *C. higginsianum* possess three conserved MAPK cascades. One of the cascades, the Ste11-Ste7-Fus3/Kss1-type cascade, has been considered as a master regulator of pathogenesis in

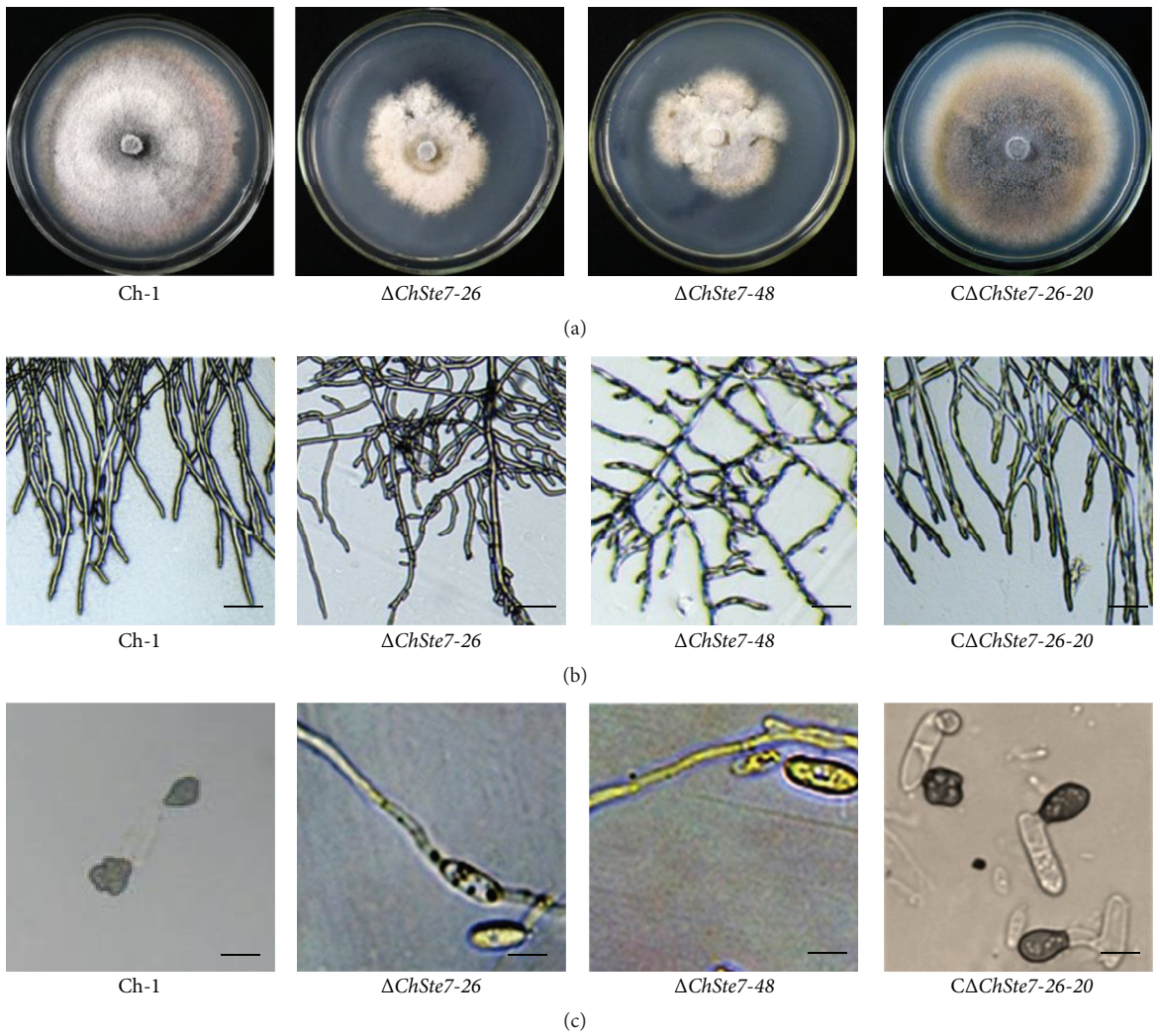

Figure 4: Mycelial growth and appressorial formation of ChSte7 disruption mutants and complementation strain. (a) The ChSte7 disruption caused abnormal colony. Wild-type strain Ch-1, ChSte7 disruption mutants Δ*ChSte7-26* and Δ*ChSte7-48*, and complementation strain CΔ*ChSte7-26-20* were grown on PDA plates for 7 days. (b) Increased furcation of mycelial tips and twisted hyphae were observed for the Δ*ChSte7* mutants. Hyphae of Ch-1, ChSte7 disruption mutants, and complementation strain picked from the edge of the colonies were examined by light microscopy. Scale bar = 10 μm. (c) Appressorial formation was altered in the ChSte7 disruption mutants. Conidial suspensions of Ch-1, ChSte7 disruption mutants, and complementation strain in distilled water were incubated on hydrophobic surface at 25°C for 24 h. Scale bar = 5 μm.

plant pathogens [13, 22, 23, 33]. The similar phenotypes of mutants in this cascade were observed in previous studies for *M. oryzae* [15, 16, 27, 28], *B. cinerea* [21, 34], *C. lagenarium* [18, 35], *U. maydis* [19, 20], and other plant pathogens. These findings implied that Ste7-type MEK was an essential component of the Ste11-Ste7-Fus3/Kss1-type cascade. The Fus3/Kss1-type cascade has been well characterized in some model species of fungal plant pathogens; however, two upstream kinases, Ste11-type MEKK and Ste7-type MEK, have only been characterized in a few systems. In this study, we identified and characterized Ste7-type MEK in *C. higginsianum*, in order to elucidate the functional roles of Ste7, using disruption mutant strains. Consequently, we found Ste7-type MEK in *C. higginsianum* to be involved in various stages of development and morphogenesis in lifecycles, such as hyphae development, appressorial formation, and postinvasive growth. This is the first description of roles of Ste7-type MEK in the model phytopathogen *C. higginsianum*.

Homologues of Ste7 MEK are involved in appressorial formation, an important infection-related morphogenetic process, in *M. oryzae* [27, 28], *Colletotrichum* spp. [18, 25, 35], *B. cinerea* [21, 34], and *U. maydis* [19, 20, 26] as well as *B. maydis* [24, 36, 37]. We suggest that the Fus3/Kss1-type MAPK cascade has highly conserved roles in appressorial formation among plant pathogens. If the involvement of ChSte7 was confined to the initial penetration into the host, then the disruption mutant should be able to infect a host with a mechanically breached outer surface. However, ChSte7

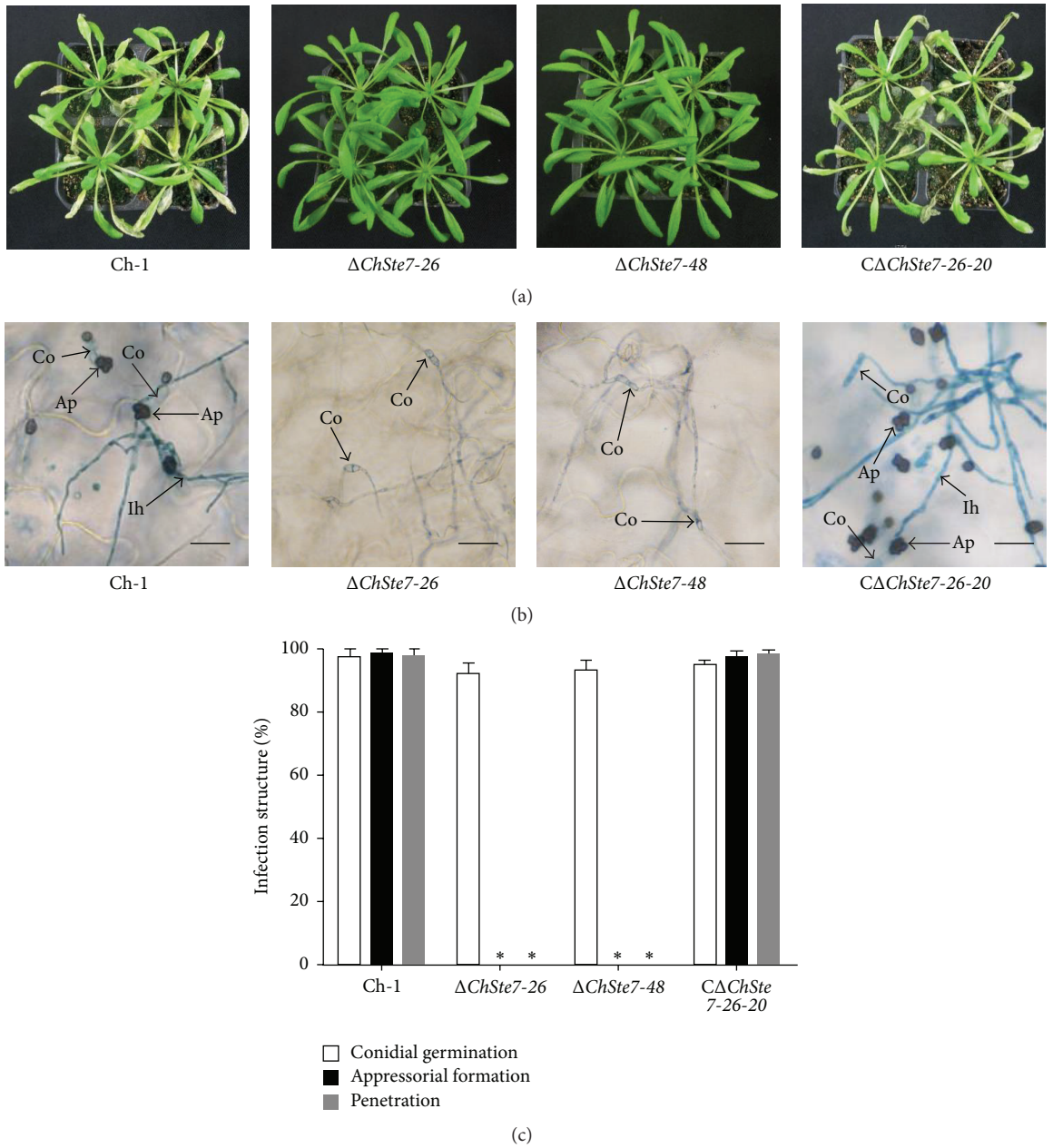

Figure 5: Deficiency in pathogenicity and appressorial formation of ChSte7 disruption mutants on leaves of *Arabidopsis* plants. (a) The ChSte7 disruption attenuated pathogenicity on *Arabidopsis* leaves. Conidia suspensions of wild-type strain Ch-1, ChSte7 disruption mutants Δ*ChSte7-26* and Δ*ChSte7-48*, and complementation strain CΔ*ChSte7-26-20* were sprayed onto the *Arabidopsis* leaves incubated at 25°C for 5 days. (b) Conidial suspensions of wild-type strain, ChSte7 disruption mutants, and complementation strain were inoculated onto the surfaces of *Arabidopsis* leaves and incubated for 4 days. The appressoria of the ChSte7 disruption mutants were not formed, whereas the appressoria of CΔ*ChSte7-26-20* and Ch-1 penetrated epidermal cells and produced abundant invasive hyphae. Ap, appressoria; Co, conidia; Ih, invasive hyphae. Scale bar = 20 μm. (c) Development of infection structures by wild-type strain, ChSte7 disruption mutants, and complementation strain on *Arabidopsis* leaves at 4 dpi. Ratings for infection structures were given as a percentage of the preceding structure. In each experiment, at least 300 appressoria per strain were examined. Means and standard deviations were calculated from three repeated experiments. Asterisks indicate values significantly different from the wild-type (LSD, $P < 0.05$), and standard error bars are shown.

disruption mutants of *C. higginsianum* were unable to form lesions on wounded *Arabidopsis* leaves. Moreover, ChSte7 was highly expressed in the late infection but not in early infection stage. We suggest that Ste7-type MEK might be a crucial factor in pathogenicity of other necrotrophic fungi.

In this study, the results showed that the Ste7-type MEK was not involved in conidiation but did affect conidial germination in *C. higginsianum*. The ChSte7 disruption mutants produced as many conidia as the wild-type strain, but the conidia of ChSte7 disruption mutants germinated poorly

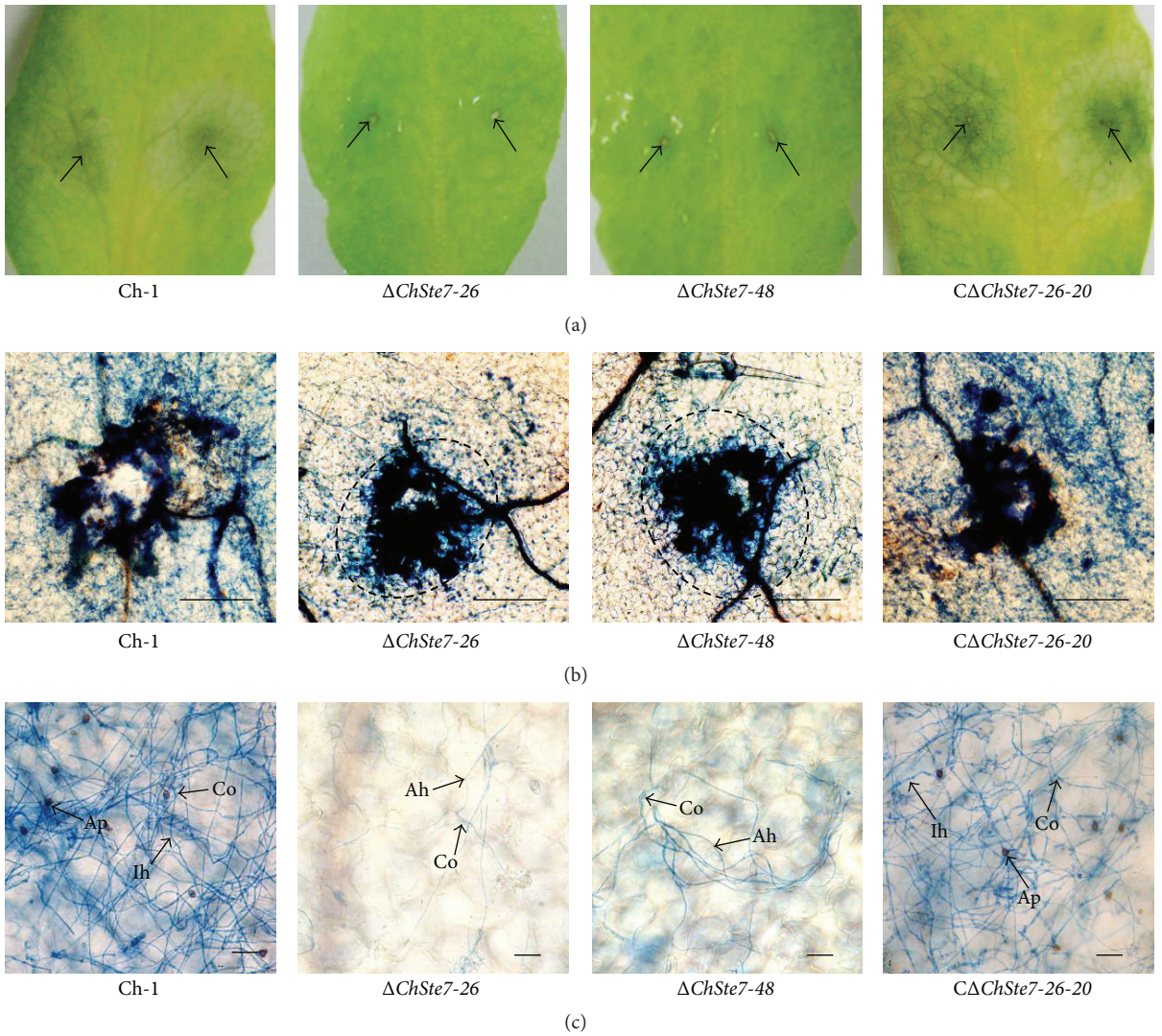

Figure 6: Loss of the ability for invasive growth of ChSte7 disruption mutants in leaves of *Arabidopsis*. (a) Pathogenicity assays were performed on wounded leaves of *Arabidopsis* plants using conidia suspensions of wild-type strain Ch-1, ChSte7 disruption mutants Δ*ChSte7-26* and Δ*ChSte7-48*, and complementation strain CΔ*ChSte7-26-20*. At 4 dpi, the ChSte7 disruption mutants caused no symptoms on wounded tissues, while wild-type and complementation strain caused water-soaked lesions on wounded leaf tissues. Arrows indicate wounded sites on leaves. (b) Leaf tissues from wound sites were viewed by light microscopy. Hyphae from germinating conidia of the ChSte7 disruption mutants grew above the epithelial cells of *Arabidopsis* leaves and could not enter into the wound sites. Scale bar = 100 μm. (c) Near the wound sites, appressoria of the ChSte7 disruption mutants were not formed on leaves, and germinating conidia could not form invasive hyphae to enter into the wound sites but produce aerial hyphae beyond or around wound sites. Ah, aerial hyphae; Ap, appressoria; Co, conidia; Ih, invasive hyphae. Scale bar = 10 μm.

(6%) on plastic coverslips. However, the germination rate of conidia of ChSte7 disruption mutants was more than 92% at 5 dpi on *Arabidopsis* leaves. We speculate that the inhibition of conidial germination in ChSte7 disruption mutants might be overcome on leaf surfaces by host signals and nutrients. Previous studies indicated that the Fus3/Kss1-type MAPK cascade regulated conidiation in *M. oryzae* [15, 27] and other *Colletotrichum* spp. [18, 25, 35] as well as *B. maydis* [25, 36, 37]. On the other hand, the level of regulation of conidial germination was significantly different among these species; no conidia germinated in *B. maydis* [25, 36, 37], a few conidia germinated in *M. oryzae* [15, 27], and a few conidia germinated in water but most conidia of *Colletotrichum* spp. germinated in nutrient-rich conditions [18, 25, 36]. In contrast, this cascade of *B. cinerea* seemed not to be involved in conidiation but affected conidial germination in pure water [21]. In *U. maydis*, both formation and germination of teliospores were also regulated by this cascade [19, 20, 26]. Therefore, the Ste11-Ste7-Fus3/Kss1-type MAPK cascade could regulate morphogenesis of sexual or asexual spores in the species mentioned above, although the regulated stage, spore formation or germination, differed depending on the species.

In *S. cerevisiae*, Ste7 MEK is essential for signal transduction from Ste11 MEKK to Kss1/Fus3 MAPK [12, 38, 39]. However, Ste11 MEKK also acts upstream of another MAPK cascade, the Hog1-type MAPK cascade. In this pathway, called the "Sho1 branch," Ste11 MEKK transmits the signal to downstream proteins via Pbs2 MEK, not Ste7 MEK [40, 41]. On the other hand, previous studies indicated that this crosstalk of MAPK cascades should not occur in filamentous fungi, including plant pathogens, because Pbs2-type MEK in filamentous fungi lacks the proline-rich motif that is required for binding to the Sho1 sensor protein [42–44]. Hence, it is suggested that Ste7-type MEK may transmit all signals from Ste11-type MEKK to Fus3/Kss1 MAPK, without any crosstalk with another MAPK cascade in a phytopathogen such as *C. higginsianum*, unlike in *S. cerevisiae*.

Overall, as a pleiotropic regulator of morphogenesis and plant infection, Ste7 MEK has highly conserved roles in phytopathogens, even those that are phylogenetically diverse. Moreover, there are some functional differences of this gene in several fungal species, which suggest that the regulation of Ste7 MEK varies and may be related to genetic distances between organisms.

5. Conclusions

It can be concluded that the *C. higginsianum* gene ChSte7 is involved in regulation of vegetative growth, appressorial formation, and postinvasive growth in host tissues. This is an important and conserved virulence factor affecting the infection of *C. higginsianum* on cruciferous plants.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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