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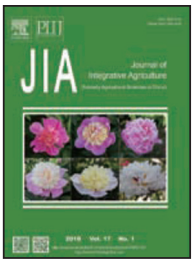

RESEARCH ARTICLE

MAPKs and acetyl-CoA are associated with *Curvularia lunata* pathogenicity and toxin production in maize

NI Xuan, GAO Jin-xin, YU Chuan-jin, WANG Meng, SUN Jia-nan, LI Ya-qian, CHEN Jie

School of Agriculture and Biology, Shanghai Jiao Tong University/State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University/Key Laboratory of Urban Agriculture (South), Ministry of Agriculture, Shanghai 200240, P.R.China

Abstract

Mitogen-activated protein kinase (MAPK) cascades play an important role in extracellular signal transduction and are involved in the pathogenicity of fungal pathogens to host plants. In *Curvularia lunata*, the roles of two MAPK genes, *Clk1* and *Clm1*, have already been studied. *Clk1* is involved in conidia formation and pathogenicity, and *Clm1* is closely related to pathogen cell wall formation and pathogenicity to maize leaves. In this study, a third *C. lunata* MAPK gene, *Clh1*, which is homologous to *hog1*, was successfully cloned. We found that a *Clh1* deletion mutant had lower intracellular glycerol accumulation than the wild-type stain and was unable to grow normally under osmotic stress conditions. Furthermore, the deletion mutants of three *C. lunata* MAPK genes (*Clk1*, *Clm1* and *Clh1*) had lower levels of acetyl-CoA, which is an important intermediate product in the synthesis of melanin and furan toxin, and down-regulated expression of pathogenicity-associated genes. Furthermore, pathogenicity and the ability to produce toxin were restored after adding acetyl-CoA to the culture medium, suggesting that acetyl-CoA is closely involved in the pathogen MAPK signaling pathway.

Keywords: *Curvularia lunata*, MAP kinase, acetyl-CoA, pathology

1. Introduction

Curvularia lunata (Wakker) Boed, is a fungal pathogen of maize and belongs to *Deuteromycotina. Curvularia* leaf spot is the most important maize disease in China and caused a great loss in maize production in northern China in the 1990s (Rizner *et al.* 2003). Although it has been shown that melanin and furan toxin are the most important factors affecting the pathogenicity of *C. lunata* (Liu *et al.* 2009; Gao *et al.* 2014), less research has focused on identifying the genes that are responsible for their production.

Mitogen-activated protein kinase (MAPK) cascades play an important role in regulating the pathogenicity of phytopathogenic fungi (Moriwaki *et al*. 2007; Ding *et al*. 2009). Changes in signals have been closely linked to pathogen virulence factors, stress response, nutrient metabolism, differentiation and cell survival (Bardwell 2007; McClean *et al*. 2007). The Fus3/Kss1 homologous gene *Pmk1* controls the formation of melanized appressoria and plant infection in *Magnaporthe grisea* (Sesma and Osbourn 2004). The *Slt2* homologous gene, *Mgslt2*, is sensitive to glucanase and several fungicides, and in *Mycosphaerella graminicola Mgslt2* gene deletion results in the formation of unmelanized colonies with short aerial hyphae (Mehrabi *et al*. 2006). And the *Hog1* homologous gene *cpmk1* in

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NI Xuan, E-mail: xuanni16@163.com; Corresponsence CHEN Jie, E-mail: Jiechen59@sjtu.edu.cn

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Cryphonectria parasitica is involved in osmosensitivity, and deletion of this gene causes poor conidiation, a reduction in laccase and cryparin production and, most significantly, an obvious decline in pathogenicity to chestnut trees (Park *et al*. 2004). In our previous research, we identified *Clk1*, the Fus3/Kss1 homologous gene, and *Clm1*, the *Slt2* homologous gene, in *C*. *lunata* and showed that *Clk1* is involved in conidia formation and pathogenicity, and *Clm1* is involved in pathogen cell wall formation and pathogenicity to maize leaves (Wang *et al*. 2011; Gao *et al*. 2014). Therefore, *C*. *lunata* MAPKs play a significant role in the generation of melanin and virulence.

Acetyl coenzyme A (acetyl-CoA) is generated by the β-oxidation of fatty acids, and acetyl-CoA originating from peroxisomal fatty acid β-oxidation is considered to be essential for the biosynthesis of melanin in appressorial cells. In *C*. *orbiculare*, the melanin biosynthetic pathway starts with the synthesis of 1,3,6,8-T4HN from malonyl-CoA (Asakura *et al*. 2012), which is derived from acetyl-CoA, and melanin synthesis in the appressoria is largely dependent upon acetyl-CoA originating from MFE1-dependent fatty acid β-oxidation (Wang *et al*. 2007). The generation of acetyl-CoA in *M*. *grisea* is required for completion of the prepenetration phase of host plant infection (Bhadauria *et al*. 2012). These findings suggest that acetyl-CoA and β-oxidation are essential to virulence and melanin synthesis and that defects in the production of acetyl-CoA would affect the pathogenicity of *C*. *lunata*. However, so far there is no evidence that directly links acetyl-CoA level to the generation of *C*. *lunata* virulence factors.

Based on previous work, it has been hypothesized that there is some relationship between MAPK signaling and acetyl-CoA in building-up *C*. *lunata* pathogenicity. In order to understand this relationship, we conducted a series of gene knockout experiments to uncover the contribution of acetyl-CoA to the expression of *Clh1*, toxin production and pathogenicity in *C*. *lunata*.

2. Materials and methods

2.1. Fungal strains, plasmid, bacterial strain and host plant

C. *lunata* strain CX-3 is a wild-type strain that causes typical leaf spots in maize. The *ΔClk1* and *ΔClm1* strains have deletions in the *C*. *lunata Clk1* and *Clm1* MAPK genes, respectively (Wang and Chen 2011; Gao *et al*. 2013). The plasmid 1300qh, which carries a hygromycin B resistance gene selective marker, was used for gene disruption. *Escherichia coli* DH5α maintained in Luria-Bertani medium was used for cloning and propagating the plasmid. Huangzao 4 maize was used as the host plant for pathogenicity assays.

2.2. DNA/RNA extraction and cDNA synthesis

To obtain mycelia for DNA and RNA extraction, *C*. *lunata* was cultured in potato-dextrose medium with shaking at 120 r min^{-1} for 72 h at 28°C, and then filtered through two layers of sterile gauze, washed with sterilized Mili-Q water, and ground in liquid nitrogen. Genomic DNA was isolated using the CTAB method (Sambrook *et al*. 1983). Total RNA was extracted using the RNAprep Pure Plant Kit (TianGen Biotech (Beijing) Co., Ltd., China). The concentration of total RNA was determined using the NANODROP2000 (Thermo Scientific, USA). First-strand cDNA was synthesized using the PrimeScript™ RT Reagent Kit according to the manufacturer's instructions (TaKaRa, Japan).

2.3. Cloning and disruption of the *Clh1* gene

All primers used in this study (Table 1) were designed using Primer 5.0 and synthesized by Biosune Biotech (Shanghai, China). The whole sequences of *C*. *lunata* were obtained. The full-length *Clh1* gene sequence, including the introns, was obtained by Blastn searches against GenBank. The 660-bp up-stream flanking sequence, Clh1_u, of *Clh1* was amplified with the Clh1_u_f/Clh1_u_r primer pair from *C*. *lunata* CX-3 genomic DNA, and the 874-bp down-stream flanking sequence, Clh1_d, was amplified with the Clh1_d_f and Clh1_d_r primer pair (Fig. 1-A). The PCR products were confirmed by DNA sequencing.

To generate the *Clh1* disruption construct, the One Step Cloning Kit (QcbioScience&Technologies Co., Shanghai) was used (Fig. 2). The up- and down-stream sequences of the *Clh1* gene were inserted into the 1300qh vector to generate the plasmid *1300qh::Clh1*. The final disruption construct was transformed into *E*. *coli* DH5α competent cells.

2.4. Transformation

Preparation of *E*. *coli* DH5α competent cells and transformation of *C*. *lunata* were performed according to previously described methods (Liu *et al*. 2010). Transformants were selected on CYA medium supplemented with 300 μg mL^{-1} hygromycin B. To identify the gene-deletion mutants, the Hyg_F/Hyg_R and Clh1_4F/Clh1_4R primer pairs were used.

2.5. Southern blot analysis

Southern blot analysis was performed on genomic DNA isolated from *C*. *lunata* wild-type strain CX-3 and putative *Clh1* disruption mutants. DNA aliquots of 5 μg were digested with *Hin*dIII at 37°C for 24 h, separated by agarose gel electrophoresis. To generate the probe, the region