Microbiological Research 210 (2018) 6–11

Contents lists available at ScienceDirect

Microbiological Research

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

The transcription factor-encoding gene *crtf* is involved in *Clonostachys chloroleuca* mycoparasitism on *Sclerotinia sclerotiorum*

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ARTICLE INFO

Keywords:
Clonostachys chloroleuca
Transcription factor
Mycoparasitism
Sclerotinia sclerotiorum
Gene knockout and complementation

ABSTRACT

Clonostachys chloroleuca 67-1 (formerly *C. rosea* 67-1) is a potential biocontrol fungus active against various fungal plant pathogens. From transcriptome sequencing of 67-1 parasitizing sclerotia of *Sclerotinia sclerotiorum*, we identified the transcription factor-encoding gene *crtf* that is significantly up-regulated during mycoparasitism. Transcription factors are widely distributed in fungi and involved in multiple biological processes. However, their role and regulatory mechanisms in mycoparasitism remain poorly understood. In this study, the function of *crtf* during 67-1 mycoparasitism was verified through gene knockout and complementation. The results showed that deletion of *crtf* did not influence fungal morphological characteristics, but the ability of the Δ*crtf* mutant to parasitize sclerotia and suppress soybean Sclerotinia white mold in the greenhouse was markedly diminished compared with the wild type strain. The biocontrol activity of Δ*crtf* recovered wild type levels when complemented with a plasmid expressing the *crtf* gene. These findings suggest that *crtf* plays a crucial role in *C. chloroleuca* mycoparasitism and provide insight into the molecular mechanisms underlying *C. chloroleuca* mycoparasitism on plant pathogenic fungi.

1. Introduction

Transcription factors (TFs) are highly conserved trans-acting proteins involved in the initiation of transcription together with RNA polymerase, and they play crucial roles in the regulation of gene expression. When cells are induced by internal signals associated with growth and development, or external stimulation by environmental stress or the presence of pathogens, TFs become active through a series of signal transduction pathways and integrate with specific cis-acting elements to ensure that target genes are appropriately expressed temporally and spatially to respond to various signals (da Silva Ferreira et al., 2006; Liu et al., 2010; Marinho et al., 2014). Up to now, a total of 61 TF families was reported in 148 fungal species (http://ftfd.snu.ac.kr/index.php?a=view).

TFs are widely distributed in filamentous fungi and involved in multiple biological processes, and the morphological character of fungi can change when transcription factor-encoding genes are deficient. For example, conidiation of *Magnaporthe oryzae* and *Penicillium digitatum* (Zhang et al., 2013; Matheis et al., 2017) and the production of sclerotia in *Botrytis cinerea* (Zhang et al., 2016b) and microsclerotia in *Verticillium dahlia* (Luo et al., 2016) were found to be inhibited. In mutants of *Fusarium oxysporum*, the number of macroconidia was significantly decreased, while the windmill-shaped structures were detected (Zheng et al., 2012). TFs have a major influence on the production of secondary metabolites in fungi. Low-level expression of transcription factor-encoding genes resulted in a remarkable reduction in gliotoxin production in *Aspergillus fumigatus* (Schoberle et al., 2014) and low biosynthesis of arabitol and mannitol in *F. verticillioides* (Malapi-Wight et al., 2013) and melanin in *V. dahlia* (Xiong et al., 2016). However, the yield of some fungal metabolites can increase, as observed for penicillin in *P. chrysogenum* (Cepeda-García et al., 2014) and yellow pigment in *Trichoderma reesei* (Derntl et al., 2017). To some extent, TFs may affect the metabolism of fungal substances such as N-acetylglucosamine, D-galacturonic acid and sulfur-containing compounds. (Piłsyk et al., 2015; Kappel et al., 2016; Zhang et al., 2016a). TFs also play important roles in regulating fungal stress resistance to osmotic and oxidative pressure, antifungal drugs, and light (Michel et al., 2015; Wang et al., 2015; Cetz-Chel et al., 2016; Dankai et al., 2016; Luo et al., 2017).

TFs are important for fungal pathogenicity, and their absence can reduce the intensity of infection of plant fungal pathogens such as *F. oxysporum* in common bean seedlings, *P. digitatum* in citrus fruits, and *V. longisporum* in tomato (Garcia-Sanchez et al., 2010; Timpner et al.,

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https://doi.org/10.1016/j.micres.2018.03.002
Received 18 September 2017; Received in revised form 28 February 2018; Accepted 3 March 2018
Available online 08 March 2018
0944-5013/

2013; Vilanova et al., 2016). Weakened penetration by *F. graminearum* and *B. cinerea* were also observed in wheat spikelets and bean leaves, respectively (Zhao et al., 2011; Schumacher et al., 2014). TFs are also involved in parasitism and antagonism of biocontrol fungi. Zou et al. (2010) demonstrated that disruption of a TF gene attenuated the virulence of *Clonostachys rosea* to nematodes, and Hussain et al. (2016) found that a TF gene-deficient mutant of the nematophagous fungus *Hirsutella minnesotensis* displayed reduced endoparasitic ability. Similar results were obtained in the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* for which gene-deficient mutants were unable to form appressorium and evade host immunity, hence the biocontrol activity against insects was lost (Li et al., 2015; Ying et al., 2014; Huang et al., 2015; Shang et al., 2015). However, the role of TFs in mycoparasitism remains unclear.

C. rosea is a promising biocontrol fungus that is capable of infecting fungal pathogens and nematodes (Cota et al., 2008; Schöneberg et al., 2015; Keyser et al., 2016). In a previous study, we found that the TF gene *crtf* was markedly upregulated in *C. chloroleuca* 67-1 (formerly *C. rosea* 67-1) under induction of sclerotia (Sun et al., 2015b). Herein, the role of *crtf* in *C. chloroleuca* mycoparasitism was further investigated using gene knockout and complementation approaches. The research provide a foundation for further understanding of the molecular mechanisms underlying *C. chloroleuca* mycoparasitism.

2. Material and methods

2.1. Strains, gene and plasmids

C. chloroleuca 67-1 (ACCC 39160) was originally isolated from a vegetable yard in Ledong Farm in Hainan Province, China (Zhang et al., 2004). *S. sclerotiorum* Ss-H (ACCC 39161) was isolated from *Sclerotinia*-infected stems of soybean in Heilongjiang Province. Sclerotia of *S. sclerotiorum* were cultured on carrot medium as previously described (Sun et al., 2015a,b).

The TF gene *crtf* that is highly upregulated in 67-1 during mycoparasitization on sclerotia of *S. sclerotiorum* was investigated in this study, and its gene sequence was deposited in GenBank under accession number KY701729.

The plasmids pKH-KO and pKN were used to construct *crtf* gene knockout and complementation vectors, respectively (Liu et al., 2016). pKH-KO contains two uracil-specific excision reagent (USER) cloning sites, a *trpc* promoter and a hygromycin B (*hph*) resistance gene, and pKN carries G418 resistance gene.

2.2. Bioinformatic analysis

The sequence of *crtf* was compared using NCBI Blast (http://www.ncbi.nlm.nih.gov/blast/), and the transcription factor family was analyzed using Fungal Transcription Factor Database (http://ftfd.snu.ac.kr/index.php?a=view). The molecular weight and isoelectric point of Crtf protein were calculated by using ExPASy program. The transmembrane regions were detected by using TMHMM Server v 2.0 software, and the signal peptide was predicted using SignalP 4.1. The expression levels of *crtf* in 67-1 during mycoparasitizing on sclerotia were also analyzed.

2.3. Gene knockout

The plasmid pKH-KO was digested with *Pac*I (NEB, MA, USA) at 37 °C for 12 h, and subsequently with *Pac*I and Nt.BbvCI (NEB) for 1 h. The *crtf*-specific primer pairs *TUF*/*TU*R and *TDF*/*TD*R were designed (Table 1) and upstream and downstream regions were amplified from the 67-1 genome (Sun et al., 2015a). Purified pHK-KO, up- and downstream regions, and USER were mixed and incubated at 37 °C for 20 min, then 25 °C for 20 min, then transferred into *Escherichia coli* DH5α (TransGen Biotech, Beijing, China) for propagation.

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)
*Tnei*F	ATGTCGAAGACTTTTATCGGCAATGTCAAG
*Tnei*R	TTAATCCTCAGTGATATCTCCGTACTCCCA
FTF	GGCAACGAGGAGTCCCTCAAGACTGGCAATG
FTR	CAATTCCCTGCCTTCCACAGCGACCCGCT
TUF	GGTCTTAAUCTGGACTACAGTTGAAAACAATT
TUR	GGCATTAAUGGTGAATCCGAACAGGAACCGAT
TDF	GGACTTAAUGTCATATGACCTTGAAAAAGTTG
TDR	GGGTTTAAUAACAGGCGTCTCGTCCCGCTGAG
HBTF	GGATCCCCCGGGCTGCAGGAATTCAGAAACCGCCAAAATGGTCAAGGC
HBTR	GAGGTCGACGGTATCGATAAGCTTATACCTTACAACACCAACAGGCGT

Recombinant vector was verified using electrophoresis and DNA sequencing.

Protoplasts of 67-1 and vector were transformed according to the method described previously (Sun et al., 2017). Colonies on Potato dextrose agar (PDA) plates containing 300 μg/ml hygromycin B were picked and transferred onto PDA for 3 generations. Transformants were then incubated on resistant plates to verify genetic stability.

2.4. PCR validation

DNA from mutant strains was extracted using a Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd, Hangzhou, China), and the *crtf* ORF was amplified using primer pairs *Tnei*F/*Tnei*R (Table 1) with the following program: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min, and a final extension at 72 °C for 10 min. Mutants were verified using electrophoresis, from which the target ORF could be amplified was considered false-positive. Positive Δ*crtf* transformants not yielding the *crtf* ORF and fragments beyond either end of upstream and downstream regions (~5090 bp) were eventually obtained (Fig. 2A).

2.5. Southern blotting

Homologous recombination of *hph* in Δ*crtf* mutants was detected using Southern blotting. DNA was extracted from mutants, digested with *Hin*dIII (NEB), and separated using a 0.7% agarose gel. Fragments were transferred onto a Hybond-N^+ nylon membrane (Amersham, NJ, USA) and hybridized with a DNA probe labeled using a PCR DIG Probe Synthesis Kit (Roche, Penzberg, Germany). Chemiluminescence was conducted using a DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche) according to the manufacturer's instructions.

2.6. Gene complementation

Full-length *crtf* was amplified from the 67-1 genome with primers *HBTF*/*HBTR* (Table 1). The plasmid pKN was digested with *Hin*dIII (NEB) and *Eco*RI (NEB) and ligated together with full-length *crtf* using a pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech) to construct the complementation vector. The vector was verified and gene-deficient mutants were recovered using the methods described above. Genetic stability of the Δ*crtf*+ complementary strains was verified on PDA plates containing 300 μg/ml G418, and the *crtf* ORF in the mutants was amplified using PCR to confirm the accuracy of the complementary strains.

2.7. Mycoparasitism on the sclerotia of S. sclerotiorum

S. sclerotiorum sclerotia were surface-sterilized with 1% NaClO for 3 min, washed with sterile water three times, then immersed into spore suspensions of wild type (WT) 67-1, Δ*crtf* and Δ*crtf*+ mutants at a concentration of 1×10^7 spores/ml for 10 min. Sclerotia were placed