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Homeobox transcription factor CoHox3 is essential for appressorium formation in the plant pathogenic fungus *Colletotrichum orbiculare*

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ABSTRACT

The plant pathogenic fungus *Colletotrichum orbiculare*, which is the causal agent of cucumber anthracnose disease, forms dome-shaped, melanized appressoria as a host invasion structure. We found 10 conserved homeobox transcription factors based on genome analysis. In this study, we functionally analyzed one of the *C. orbiculare* homeobox gene named *CoHox3* (*Colletotrichum orbiculare* Homeobox transcription factor 3) during the infection process. *CoHox3* knockout mutants were non-pathogenic on host leaves (inoculated with 1.0×10^4 conidia/mL). Conidia of *CoHox3* mutants germinated and/or formed lightly pigmented structures, but did not form mature appressoria on glass slides, artificial membranes, or host leaves. Only 0.3–0.6% of germinated conidia of *CoHox3* mutants formed infection hyphae on host leaves. Green fluorescent protein-based expression experiments showed that germinated conidia of the *CoHox3* mutant did not express *CoGAS1* gene, an appressorium-specific gene, although wild-type strain 104-T expressed the *CoGAS1* gene during appressorium formation. When conidia of the *CoHox3* mutant was incubated on glass slides, nuclear division was significantly later than that of wild-type, which formed appressorium. Thus, homeobox transcription factor CoHox3 is required to form normal appressoria.

1. Introduction

Colletotrichum orbiculare is the causal agent of anthracnose of cucumber. *Colletotrichum* spp. form special infection structures during host plant infection. Conidia attach to the surface of the host plant. An attached conidium germinates to form a germ tube with a dome-shaped and melanized appressorium. Mature appressoria penetrate into the host plant and invasive hyphae elongate from them (Kubo & Takano, 2013). To understand morphogenesis and control plant pathogens, it is important to reveal this infection morphogenesis. A few studies showed that this morphogenesis process would be controlled by homeobox gene.

Homeobox transcription factors are essential for the morphogenesis of various organisms. The homeobox was discovered as a shared sequence of about 180 bp in homeotic genes of *Drosophila melanogaster* (Burglin & Affolter, 2015; McGinnis, Garber, Wirz, Kuroiwa, & Gehring, 1984; Scott & Weiner, 1984). Homeobox genes are conserved in various organism, including animals, plants, and fungi, and play key roles in the morphogenesis of the organism. In plant pathogens, however, the function of homeobox genes in pathogenicity have only been the subject of a few reports.

It is known that Ste12 in plant pathogens is functionary conserved and is required to penetrate into the host plant (Hoi & Dumas, 2010; Rispail & Di Pietro, 2010). In the case of plant pathogens, a homolog of Ste12 was first analyzed homeobox gene in rice blast fungus *Magnaporthe oryzae* and named MST12 (Park, Xue, Zheng, Lam, & Xu, 2002). Ste12 was first isolated from the yeast *Saccharomyces cerevisiae* (Errede & Ammerer, 1989), and it has functions in mating and invasive growth. *Saccharomyces cerevisiae* Ste12 functions downstream of MAPKs Fus3 and Kss1. Ste12 includes an N-terminally located homeodomain-like motif Ste (InterPro number IPR003120), which is one of the DNA binding motifs. In *M. oryzae*, mutants lacking *MST12* showed a phenotype similar to wild-type such as vegetative growth, conidiation, conidial germination, and appressorium formation. However, when *mst12* mutants were inoculated on host rice and barley leaves,

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mutants formed no lesions, and they failed to penetrate onion epidermal cells. In cucumber anthracnose fungus *C. orbiculare*, CST1 is a homolog of *S. cerevisiae* Ste12, and it is also related to penetration of the host plant (Tsuji, Fujii, Tsuge, Shiraishi, & Kubo, 2003). The *cst1* mutants formed no penetration peg on host leaves and artificial cellulose membrane. In bean anthracnose pathogen *C. lindemuthianum*, deletion mutant of CLSTE12 that is homologous to yeast Ste12 was non-pathogenic (Hoi et al., 2007). *CLSTE12* mutants formed penetration pores from mature appressoria on bean leaves similar to wild-type, but they lost penetrating ability. *CLSTE12* mutants also showed reduced pectinase activity and conidial adhesion to polystyrene. In gray mold pathogen *Botrytis cinerea*, the *Bc-ste12* mutant showed reduced infection (Schamber, Leroch, Diwo, Mendgen, & Hahn, 2010). On host leaves, the *Bc-ste12* mutant formed malformed appressoria and penetrated very rarely. In addition, the *Bc-ste12* mutant showed lack of sclerotia formation.

Among the other homeobox genes, it is noteworthy that *Mohox7* in *M. oryzae* is involved in infection structure morphogenesis. The *Mohox7* mutant failed to form appressoria on the germ tube and tips of hyphae (Kim et al., 2009). The *Mohox7* mutant also was unable to form lesions on intact host leaves but caused disease on wounded leaves. Microscopic analysis showed that the *Mohox7* mutant formed no normal appressoria but formed non-melanized swellings and hooking structures. It is possible that the function of Mohox7 is conserved in plant pathogenic fungi as with Ste12.

In this study, we found 10 homeobox genes in *C. orbiculare* based on genome analysis. We then analyzed knockout mutant of *CoHox3*, a homolog of *M. oryzae MoHOX7*, among the 10 homeobox genes of the *C. orbiculare*. If *CoHox3* mutant lost normal appressoria formation ability like the phenotype of the Mohox7, CoHox3 would be a homeobox transcription factor that is required to pathogenicity, and is functionally conserved among plant pathogens.

2. Materials and methods

2.1. Identification of homeobox transcription factors

Homeobox proteins of *C. orbiculare* containing the homeobox domain (IPR001356, IPR009057) or STE-like domain (IPR003120) were identified using InterPro (http://www.ebi.ac.uk/interpro/).

2.2. Fungal strains and culture conditions

Colletotrichum orbiculare strain 104-T (stock culture from the Laboratory of Plant Pathology, University of Shiga Prefecture) was used as a wild-type strain. All *C. orbiculare* strains were maintained on potato dextrose agar (PDA) medium (3.9% (w/v), Nissui Pharmaceutical Co., Ltd., Tokyo) at 24 °C.

2.3. Disruption of CoHox3 gene

CoHox3 gene disruption vector was constructed by fusion-PCR using a similar method to Izumitsu et al. (2009). All primers used in this study are listed in Table 1. The 1-kb fragment of 3′ untranslated region of *CoHox3* was amplified by PCR using TaKaRa Ex Taq® (Takara Bio Inc., Kusatsu) and primers dis-CoHox3-LF and dis-CoHox3-LR. The 1-kb fragment of 5′ untranslated region of *CoHox3* also was amplified by PCR using primers dis-CoHox3-RF and dis-CoHox3-RR. The amplified products and hygromycin B phosphotransferase (*hph*) gene cassette were used for fusion PCR. Products amplified by fusion PCR with primers dis-CoHox3-LF and dis-CoHox3-RR were applied as *CoHox3* gene disruption vector.

2.4. Fungal transformation

To obtain protoplasts of the wild-type strain 104-T, hyphae were incubated for 3 d in 50 mL of potato dextrose broth (PDB) medium (4.2% (w/v), Becton, Dickinson and Company Sparks, Franklin Lakes). Harvested mycelia were treated with cell wall degrading enzyme solution (50 mg/mL glucanex, 10 mg/mL kitalase) for 1 h. Protoplasts were abstracted from enzyme solution, and STC solution (1.2 M Sorbitol, 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$) added. The 100 μL protoplast solution (1×10^7 protoplasts) was added to the *CoHox3* gene disruption vector, and incubated for 15 min on ice. Protoplast solution was added to 1 mL of 40% polyethylene glycol solution (40% Polyethylene glycol, 1.2 M Sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5), 3% 0.2 N NaOH (w/v)) and incubated for 15 min at room temperature. This solution was mixed with 10 mL regeneration medium including 100 μg/mL hygromycin B (Wako Pure Chemical Industries, Ltd., Osaka) on a plate. Colonies of transformants growing were picked to PDA medium.

2.5. Plasmid construction

To construct vectors except for the plasmid pZBchH2B-mCherry described below, an In-Fusion® HD Cloning Kit (Takara Bio USA, Inc., Mountain View) was used (Zhu, Cai, Hall, & Freeman, 2007). To construct the *CoHox3* complement vector pZNCH3C, a 8.3-kb fragment of *CoHox3* gene containing untranslated regions of the promoter and terminator were amplified by PCR using Takara PrimeSTAR® GXL DNA Polymerase (Takara) with primers comp-CoHox3-F and comp-CoHox3-R. The resulting *CoHox3* gene fragment was designed to contain a 15-bp overlap with the ends of the *Eco*RV-linearized donor vector pZNat1 bearing a nourseothricin-resistance gene (*NAT*) cassette (Izumitsu et al., 2009). The gene fragment was inserted into the *Eco*RV site of pZNat1 using the In-fusion reaction and transformation both in accordance with the manufacturer's instructions. Transformation experiments of *Escherichia coli* were performed using DH5α competent cells (Takara). The resulting plasmid was named pZNCH3C. To construct

Table 1
Primers used in this study.

Name	Sequence
dis-CoHox3-LF	CAAGGCATTAAGCATCCGAGGGCTG
dis-CoHox3-LR	CTGAGCAAACTGGCCTCAGGCATTTGAGAAGCACGAC CGTGGCGGATGGAGCAGTG
dis-CoHox3-RF	GATCAAAAAGTGCTCATCATTGGAAAACGTTCTTCGAA CGACGCACTACGATCGGGAGC
dis-CoHox3-RR	GGACGCGGCTTGTTCTACACAGAG
HPH-F	GTGCTTCTCAAATGCCTGAG
HPH-R	CGAAGAACGTTTTCCAATG
chk-CoHox3-INF (1F)	CCTTACATGTCCACGTCTGGCAT
chk-CoHox3-INR (1R)	GCGAAAACATTAGCCTTTGACCG
chk-CoHox3-LF (2F)	AGACAGACAAGAGTATCCGCAGAG
chk-CoHox3-RR (3R)	AGTTAGTGCAAAACCTCTTCCTCG
chk-HPH-F (2R)	TGTAGAAGTACTCGCCGATAGTGG
chk-HPH-R (3F)	GTTGCCTAAATGAACCATCTTGTC
CoGAS1p-inf-F	tggaattctgcagatATGACGAAGTTTGCCCTGGCGTACC
CoGAS1p-inf-R	gcccttgctcaccatTTTTGACTAAAGGTGAGTTGAAG
eGFP-F	atggtgagcaagggcgagg
eGFP-inf-R	gccagtgtgatggatTTACTTGTACAGCTCGTCCATGCC
comp-CoHox3-F	tggaattctgcagatCCTCCAACCCGCCCAAACAATGC
comp-CoHox3-R	gccagtgtgatggatGGATGGACGCGGCTTGTTCTACACAGAG
CtmCherry-F	GATATCATTTAAATGGTGGTGGTGGTGGTatggtgagcaagg gcgagg
mCherry-R	ctacttgtacagctcgtccatgc
BchH2B-prom-F	TGAACACGACCAACTGGGAATGC
BchH2B-mCherry-R	TTCGTGGATGAAGAGTACTTGGTAAC

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