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The HAP complex in *Fusarium verticillioides* is a key regulator of growth, morphogenesis, secondary metabolism, and pathogenesis

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ABSTRACT

Among eukaryotic organisms, the HAP complex is a conserved, multimeric transcription factor that regulates gene expression by binding to the consensus sequence CCAAT. In filamentous fungi, the HAP complex has been linked to primary and secondary metabolism, but its role in pathogenesis has not been investigated extensively. The overarching goal of this study was to elucidate the role of the HAP complex in *Fusarium verticillioides*, a ubiquitous and damaging pathogen of maize. To this end, orthologs of core HAP complex genes (*FvHAP2*, *FvHAP3*, and *FvHAP5*) were identified and deleted in *F. verticillioides* via a reverse genetics approach. Deletion of *FvHAP2*, *FvHAP3*, or *FvHAP5* resulted in an indistinguishable phenotype among the deletion strains, including reduced radial growth and conidiation, altered colony morphology, and derepression of pigmentation. Additionally, disruption of the HAP complex impaired infection and colonization of maize stalks. Deletion strains were hypersensitive to osmotic and oxidative stress, which suggests the HAP complex of *F. verticillioides* may mediate responses to environmental stress during pathogenesis. This study directly implicates the HAP complex in primary and secondary metabolism in *F. verticillioides* and provides one of the first links between the HAP complex and virulence in a plant pathogenic fungus.

1. Introduction

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland) is a ubiquitous and versatile pathogen of maize, attacking stalks, kernels, and seedlings (White, 1999). Fusarium stalk rot caused by *F. verticillioides* is one of the most important and damaging diseases of maize (Afolabi et al., 2008; Shim et al., 2006). Characterized by discoloration and breakdown of pith tissue, the disease causes premature plant death and lodging that severely reduce yields (Afolabi et al., 2008; Yamamura and Shim, 2008). Additionally, *F. verticillioides* produces fumonisin mycotoxins, a group of polyketide-derived secondary metabolites linked to acute and chronic toxicoses in humans and livestock (Bluhm and Woloshuk, 2005; Lazzaro et al., 2012; Rheeder et al., 2002). Although recent work has helped elucidate molecular mechanisms underlying kernel pathogenesis and fumonisin biosynthesis (Woloshuk and Shim, 2013), the current understanding of how *F. verticillioides* causes pathogenesis in the stalk environment is fragmentary (Yamamura and Shim, 2008).

F. verticillioides gains access to maize stalks via wounds created by insects or mechanical damage, direct penetration of root or stalk tissue, natural entry points (i.e. nodes), or infected seed (Afolabi et al., 2008; Gatch and Munkvold, 2002; Wilke et al., 2007). Interestingly, *F. verticillioides* is commonly associated with maize as an asymptomatic endophyte, and thus pathogenesis is not necessarily the default outcome of the host-fungus interaction (Bacon et al., 2008). One possibility is that stress-induced changes in host physiology trigger the switch from an endophytic to pathogenic lifestyle in *F. verticillioides*, although the underlying mechanisms are unknown. In commercial maize production systems, environmental stresses, e.g., physical injury, foliar disease, flooding, high temperature, and drought stress, are associated with increased incidence and severity of stalk rot (Dodd, 1980). Presumably, the ability of *F. verticillioides* to associate with maize alternatively as an asymptomatic endophyte or a virulent pathogen requires widespread transcriptional programming within the fungus.

Among eukaryotes, the HAP complex regulates gene expression by binding the consensus sequence CCAAT (Mantovani, 1999; McNabb and Pinto, 2005). In *Saccharomyces cerevisiae*, the HAP complex is minimally composed of three subunits: Hap2p, Hap3p, and Hap5p, which collectively form the core DNA-binding protein (Hahn and Guarente, 1988; Hahn et al., 1988; McNabb et al., 1995; McNabb and Pinto, 2005). In fungi, the HAP complex has been

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linked to fundamentally important biological processes, including nutrient acquisition, oxidative stress responses, and asexual reproduction (McNabb et al., 1995; Steidl et al., 1999; Thön et al., 2010). Additional subunits, Hap4p (yeast) and HapX (filamentous fungi), have been shown to interact with the core complex to mediate a subset of biological functions (Forsburg and Guarente, 1989; Hortschansky et al., 2007; McNabb and Pinto, 2005). Notably, HapX was recently shown to have a role in virulence in the vascular wilt pathogen *Fusarium oxysporum* (López-Berges et al., 2012). However, the role of the core HAP complex in plant pathogenesis has not been extensively investigated among filamentous ascomycetes.

The overarching goal of this study was to elucidate the role of the HAP complex of *F. verticillioides* in growth, development, and stalk rot pathogenesis. To this end, genes encoding orthologs of the HAP complex core subunits (*FvHAP2*, *FvHAP3*, and *FvHAP5*) were identified and targeted for deletion. Deletion of *FvHAP2*, *FvHAP3*, or *FvHAP5* resulted in indistinguishable phenotypes, characterized by reduced growth and conidiation, altered hyphal morphology, and misregulation of secondary metabolism. Additionally, disruption of the HAP complex impaired pathogenesis in maize stalks and increased sensitivity to osmotic and oxidative stress. Thus, the HAP complex of *F. verticillioides* is involved in regulating pathogenesis and secondary metabolism, a finding that provides one of the first direct links between the HAP complex and virulence in a plant pathogenic fungus.

2. Materials and methods

2.1. Fungal strains and culture conditions

All fungal strains used in this study are listed in Table 1. *F. verticillioides* strain 7600 (NRRL 20956; Leslie, 1991; Ma et al., 2010) was the wild type and parental strain in this study. All strains were stored as mycelia in 30% (v/v) glycerol at −80 °C. Working cultures of *F. verticillioides* were maintained on 0.2× PDA (potato dextrose agar; Leslie and Summerell, 2006). Pigmentation was assessed in 10% ICI (0.6 mM NH_4NO_3; Geissman et al., 1966) or 10% ICI-Gln (0.6 mM Glutamine; Wiemann et al., 2009).

To assay vegetative growth, *F. verticillioides* strains were center inoculated (5×10^3 conidia in 10 µl sterile distilled water) onto PDA, YMA (yeast extract malt extract agar; Harris, 2005), CM (complete medium; Correll et al., 1987), or MM (minimal medium; Correll et al., 1987) and incubated at room temperature in the dark. Colony diameters were measured three and six days after inoculation. To assay conidiation, conidia (2×10^5 in 200 µl sterile distilled water) of *F. verticillioides* strains were spread onto SNA (Spezieller Nährstoffarmer Agar; Leslie and Summerell, 2006) and incubated at room temperature in the dark. After seven days, three agar plugs (1 cm in diameter) were collected in 5 ml of sterile distilled water; conidia were released by vortexing and counted with a hemacytometer. For radial growth and conidiation assays, two measurements were averaged per plate and data presented represent the mean across a minimum of three replicates. Experiments were repeated at least three times with similar results.

Table 1
Fusarium verticillioides strains used in this study.

Strain	Genotype
7600	Wild type
FV010	Δ*fvhap2::hph*
FV011	Δ*fvhap2::hph*
FV012	Δ*fvhap2::hph;FvHAP2::neo*
FV013	Δ*fvhap3::hph*
FV014	Δ*fvhap3::hph*
FV015	Δ*fvhap3::hph;FvHAP3::neo*
FV016	Δ*fvhap5::hph*
FV017	Δ*fvhap5::hph*
FV018	Δ*fvhap5::hph;FvHAP5::neo*
FV019	Δ*fvhap3::hph;ScHAP3::neo*

2.2. Nucleic acid manipulations

Plasmid DNA was isolated with a GeneJET Plasmid Miniprep Kit (Thermo Scientific Fermentas, Pittsburg, PA, USA) or via alkaline lysis midiprep performed essentially as described by Sambrook and Russell (2001). Fungal DNA was isolated with a modified cetyltrimethylammonium bromide (CTAB) method (Saghai-Maroof et al., 1984). Mini-preparation of fungal DNA for PCR was performed as previously described (Ridenour et al., 2012). Standard procedures were followed for Southern blotting (Sambrook and Russell, 2001). All primers were obtained from Integrated DNA Technologies (Coralville, IA, USA) and are listed in Supplementary Table S1.

2.3. Bioinfomatics and phylogenetics

Sequence data were obtained through web portals of the Broad Institute, the Joint Genome Institute, and the National Center for Biotechnology Information (http://www.broadinstitute.org, http://www.jgi.doe.gov, and http://www.ncbi.nlm.nih.gov, respectively). Protein domain analyses were conducted with SMART (Letunic et al., 2012). Sequence logos were generated in the MEME Suite web server (http://meme.nbcr.net; Bailey et al., 2009). SWISS-MODEL was used for protein structural modeling (http://www.swissmodel.expasy.org; Schwede et al., 2003; Arnold et al., 2006) and models were visualized with Jmol (http://jmol.sourceforge.net; Hanson, 2010). Promoter analyses were conducted with the dna-pattern program of the RSAT (Regulatory Sequence Analysis Tools) package (http://rsat.ulb.ac.be/; Thomas-Chollier et al., 2011) and the Single Site Analysis (SSA) program in the oPOSSUM-3 package (http://opossum.cisreg.ca/oPOSSUM3/; Kwon et al., 2012). For phylogenetic reconstruction, a data set of Hap2, Hap3, and Hap5-like proteins was assembled from public data repositories. Sequences were initially aligned with Muscle (Edgar, 2004), and ambiguously aligned regions were removed using Gblocks (Castresana, 2000; Talavera and Castresana, 2007). Parsimony analysis was conducted with SeaView Version 4.4.2 (Gouy et al., 2010) using the PHYLIP protpars algorithm (Felsenstein, 1989).

2.4. Targeted gene deletion and complementation

For targeted gene deletion, a split-marker approach was used to replace the endogenous *FvHAP2*, *FvHAP3*, and *FvHAP5* genes with a hygromycin resistance cassette (Catlett et al., 2003; Yu et al., 2004). Gene deletion constructs were generated as previously described (Ridenour et al., 2012), and primer sequences are listed in Supplementary Table S1. Briefly, a region upstream of each gene (5′ flank) and a region downstream of each gene (3′ flank) were amplified from *F. verticillioides* genomic DNA with the following primer pairs: FV00807_F1/FV00807_F2 (*FvHAP2* 5′ flank) and FV00807_F3/FV00807_F4 (*FvHAP2* 3′ flank); FV08087_F1/FV08087_F2 (*FvHAP3* 5′ flank) and FV08087_F3/FV08087_F4 (*FvHAP3* 3′ flank); and FV09223_F1/FV09223_F2 (*FvHAP5* 5′ flank) and FV09223_F3/FV09223_F4 (*FvHAP5* 3′ flank). The overlapping split-marker fragments, HY and YG, were amplified from a hygromycin resistance cassette in pCB1003 (Carroll et al., 1994) with the primer pairs M13F/HY and YG/M13R primers, respectively. The 5′ flank for each gene and the HY marker fragment were then joined

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