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The N-terminus region of the putative C_2H_2 transcription factor Ada1 harbors a species-specific activation motif that regulates asexual reproduction in *Fusarium verticillioides*

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ABSTRACT

Fusarium verticillioides is an important plant pathogenic fungus causing maize ear and stalk rots. In addition, the fungus is directly associated with fumonisin contamination of food and feeds. Here, we report the functional characterization of Ada1, a putative Cys2-His2 zinc finger transcription factor with a high level of similarity to *Aspergillus nidulans* FlbC, which is required for the activation of the key regulator of conidiation *brlA*. *ADA1* is predicted to encode a protein with two DNA binding motifs at the C terminus and a putative activator domain at the N terminus region. Deletion of the *flbC* gene in *A. nidulans* results in "fluffy" cotton-like colonies, with a defect in transition from vegetative growth to asexual development. In this study we show that Ada1 plays a key role in asexual development in *F. verticillioides*. Conidia production was significantly reduced in the knockout mutant (Δada1), in which aberrant conidia and conidiophores were also observed. We identified genes that are predicted to be downstream of *ADA1*, based on *A. nidulans* conidiation signaling pathway. Among them, the deletion of *stuA* homologue, *FvSTUA*, resulted in near absence of conidia production. To further investigate the functional conservation of this transcription factor, we complemented the Δada1 strain with *A. nidulans flbC*, *F. verticillioides ADA1*, and chimeric constructs. *A. nidulans flbC* failed to restore conidia production similar to the wild-type level. However, the Ada1N-terminal domain, which contains a putative activator, fused to *A. nidulans* FlbC C-terminal motif successfully complemented the Δada1 mutant. Taken together, Ada1 is an important transcriptional regulator of asexual development in *F. verticillioides* and that the N-terminus domain is critical for proper function of this transcription factor.

1. Introduction

Asexual sporulation in fungi is a highly efficient reproduction mechanism. It allows the production of a large number of asexual spores – conidia – that are not only effective propagation and dispersion tool but also the primary means for animal and plant infection. In addition, they play an important role in protection and survival in unfavorable environmental conditions. Adams et al. (1998) proposed that temporal and spatial regulation of gene expression, cell specialization, and intracellular communication is required for this critical developmental process to take place. The genetic regulation of conidiation has been extensively studied and elucidated in the fungal model organisms *Aspergillus nidulans* and *Neurospora crassa* (for review see Adams et al., 1998; Springer, 1993). In *A. nidulans*, ten putative transcription factors (TFs), specifically *sfgA*, *brlA*, *flbB*, *flbD*, *flbC*, *abaA*, *stuA*, *vosA*, *medA* and *wetA*, have been identified to be expressed at different conidiation stages and carefully orchestrate this important developmental process (Adams et al., 1998; Busby et al., 1996; Etxebeste et al., 2010; Seo et al., 2006). In filamentous fungi, various extracellular stimuli have been shown to activate signal transduction pathways. G-protein signaling, cAMP signaling and MAP kinase pathways are key signal transduction pathways known to regulate fungal growth, development and pathogenicity (Calvo et al., 2002; Lengeler et al., 2000). Notably, TFs are generally recognized as the downstream component in these pathways, directly responsible for activation or repression of target genes.

While a significant progress has been made, we still have a limited understanding of the function of TFs in filamentous fungi (Li et al., 2007). TFs activate and/or suppress gene expression by stabilizing or blocking the recruitment of RNA polymerase for transcriptional initiation. In addition, they associate with enzymes that catalyze the modification of chromatin condensation, which allows genes to become more or less accessible for transcription

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(Harbison et al., 2004). Changes in chromatin structure by histone modifications activate or silence gene transcription in a reversible manner. During fungal growth and asexual development, clusters of genes responsible for synthesizing secondary metabolites are silenced by histone deacetylation and methylation (Strauss and Reyes-Dominguez, 2011). However, under conditions conducive for sexual development and secondary metabolite biosynthesis, a recently defined VeA–LaeA–VelB complex removes heterochromatin marks thus promoting expression of genes associated with secondary metabolism and sexual development in *A. nidulans* (Bayram et al., 2008).

TFs with zinc finger domains are present in several organisms ranging from bacteria to humans. C_2H_2 TFs harbor cysteine and histidine residues that are bound and stabilized by a zinc ion (Iuchi et al., 2007). The Fungal Transcription Factor Database (http://ftfd.snu.ac.kr) has predicted a large number of putative C_2H_2 TFs found in fungal genomes, with some species, such as *Magnaporthe oryzea*, harboring over 200 C_2H_2 putative TF genes (Park et al., 2008). In select fungal species, it has been predicted that members of this TF family are involved in pathogenicity, cell differentiation, carbon utilization, and development (Flaherty et al., 2003; Kwon et al., 2010; Schumacher et al., 2008). For instance, *A. nidulans* C_2H_2 TF *brlA* is the key regulator responsible for the transition from vegetative growth to conidiogenesis in this model fungus (Adams et al., 1998; Mah and Yu, 2006). Adams et al. (1998) have shown that six genes make up a genetic cascade that is required for the proper activation of *brlA*. Among those, *flbC* encodes a putative TF with two C_2H_2 DNA-binding domains at the C terminus, and a potential activator domain at the N-terminus region (Kwon et al., 2010). *flbC* deletion mutants display a defect in transition from growth to asexual development, which results in reduced conidiation (Adams et al., 1998; Kwon et al., 2010).

While the role of TFs is critical in eukaryotic transcription regulation, only a few have been characterized in the corn pathogen *Fusarium verticillioides* (Sacc.) Nirenberg (teleomorph: *Gibberella moniliformis* Wineland) (Brown et al., 2007; Flaherty et al., 2003; Flaherty and Woloshuk, 2004; Kim and Woloshuk, 2008). This ascomycete causes stalk and ear rots on corn worldwide leading to yield losses (Munkvold and Desjardins, 1997; White, 1999), but more importantly produces fumonisins on infested grains (Rheeder et al., 2002). Consumption of fumonisin-contaminated corn has been linked to cancer and a variety of detrimental health effects in humans and animals (Gelderblom et al., 1988; Marasas, 2001; Minorsky, 2002; Missmer et al., 2006). *F. verticillioides* is also known to be allergenic to humans, and capable of systemically infecting patients with cancer and HIV (Guarro and Gene, 1995; Petmy et al., 2002). Furthermore, it has the ability to produce skin lesions (Shaoxi et al., 1996), and has been associated with ulcerative keratitis (Naiker and Odhav, 2004). Due to these concerns, the US Food and Drug Administration has established several parameters to monitor fumonisin levels in feed and foodstuff (Park and Troxell, 2002).

Studies have shown a genetic linkage between fumonisin production and asexual conidiation in *F. verticillioides* (Choi and Shim, 2008; Glenn et al., 2004; Sagaram et al., 2007; Shim and Woloshuk, 2001; Shin et al., 2013). *In silico* analysis revealed that *F. verticillioides* does not posses a homologue to *A. nidulans* BrlA, but it does encode a putative C_2H_2 TF similar to FlbC, which we designated Ada1. The aim of this study was to investigate the role of Ada1 in regulating conidiation and fumonisin production in *F. verticillioides*. Ada1 and *A. nidulans* FlbC shares 96% identity at the C-terminus motif and 46% identity overall. The lack of *brlA* homologue in *F. verticillioides*, along with the observation that *brlA* homologues have only been found in members of the Class Eurotiomycetes, specifically in *Aspergillus* and *Penicillium* species (Chung et al., 2011), led us to postulate a divergence of regulatory mechanism for asexual development and secondary metabolism in fungi.

2. Materials and methods

2.1. Fungal strain, culture media, and growth conditions

F. verticillioides wild-type strain 7600 (Fungal Genetics stock Center, Kansas City, KS) was stored at −80 °C in a 30% glycerol solution. Conidia used for inoculum were obtained by growing the fungal strains on V8 juice agar at 25 °C for 7 days. For growth studies, the strains were inoculated on 100 ml of YEPD medium with constant shaking (100 rpm) at 25 °C. Cultures were harvested, oven dried at 100 °C for 24 h, and weighed for biomass quantification. For microconidia production assays, strains (0.5 cm-diameter agar blocks) were inoculated on 0.2x PDA, KCl, V8 agar plates and cracked-corn medium (Sagaram et al., 2007), incubated at 25 °C under 14 h light/10 h dark cycle for 7 days. Conidia were harvested using sterile water (5 ml) and counted with a haemocytometer.

2.2. Nucleic acid manipulation and quantitative real time RT- PCR (qRT-PCR)

F. verticillioides genomic DNA was extracted as previously described (Shim and Woloshuk, 2001). The primers used in this study are listed in Table A.1. Southern analyses were performed as previously described (Sagaram and Shim, 2007), and the probes were ^{32}P-labeled with the Prime-It Random Primer Labeling kit (Stratagene, La Jolla, CA, USA). For gene expression analyses, the strains were inoculated on 100 ml of YEPD medium with constant shaking (100 rpm) and maintained at 25 °C, with a 14-h light/10-h dark cycle. Cultures were harvested at 24, 48 and 72 h post inoculation. qRT-PCR analyses were performed in a Cepheid Smart Cycler system with a QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) with 150 ng of total RNA as template for each sample. *F. verticillioides* β-tubulin gene (*TUB2*) was used for normalization. qRT-PCR studies were carried out with 30 min of reverse transcription at 50 °C followed by 15 min of pre-denaturation at 95 °C and 35 cycles of 15 s at 95 °C and 30 s at 62 °C.

2.3. Gene-deletion, complementation and chimeric constructs, and fungal transformation

The *ADA1* gene-disruption cassette was constructed using the split-marker recombination strategy (Shin et al., 2013) (Fig. 1A). DNA fragments corresponding to the 5′ (1089 bp) and the 3′ (1282 bp) flanking regions of *ADA1* gene were first PCR amplified. Then, partial *HPH* fragments (designated *HP* [766 bp] and *PH* [924 bp]) were amplified as previously described (Fu et al., 2006). In a 1:1 M ratio, 5′ flanking region-*PH* fragment and 3′ flanking region-*HP* fragment were joined by PCR. For *FvSTUA* deletion construct, we amplified the 5′ and 3′ flanking region of *FvSTUA* using primer pairs FvStu-5′for/FvStu-5′rev and FvStu-3′for/FvStu-3′rev, respectively. Split-marker recombination strategy was performed as previously described.

To complement the *ADA1*-deletion mutant (Δada1), the *ADA1* gene was amplified from the wild-type strain and transformed into the Δada1 strain (Fig. 1B). Plasmid pBS-G containing geneticin (G418)-resistance gene (*GEN*) was amplified with primers M13F and M13R. *ADA1* gene including 1 Kb 5′ UTR and 1 Kb 3′ UTR was amplified from genomic DNA using Expand Long Polymerase (Roche Molecular Biochemicals, IN, USA). *ADA1* and *GEN* were co-transformed into the *F. verticillioides* Δada1 strain, and colonies resistant to both hygromycin and geneticin were evaluated for phenotypic complementation.

To investigate the functional role of *ADA1* and *flbC* N- and C-terminus regions, we assembled three complementation constructs (Fig. 1C–E). A *flbC* complemented strain (Δada1-flbC) was gener-

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