Fungal Genetics and Biology 94 (2016) 54–68

Contents lists available at ScienceDirect

Fungal Genetics and Biology

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

Regular Articles

Unh1, an *Ustilago maydis* Ndt80-like protein, controls completion of tumor maturation, teliospore development, and meiosis

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ARTICLE INFO

Article history:
Received 14 April 2016
Revised 4 July 2016
Accepted 6 July 2016
Available online 7 July 2016

Keywords:
Ustilago maydis
Plant-induced meiosis
Teliospore maturation
Ndt80-like DNA-binding protein
Melanin biosynthesis

ABSTRACT

In this study, *Ustilago maydis* Ndt80 homolog one, *unh1*, of the obligate sexual pathogen *U. maydis*, is described. Unh1 is the sole Ndt80-like DNA-binding protein in *U. maydis*. In this model basidiomycete, Unh1 plays a role in sexual development, influencing tumor maturation, teliospore development and subsequent meiotic completion. Teliospore formation was reduced in deletion mutants, and those that did form had unpigmented, hyaline cell walls, and germinated without completing meiosis. Constitutively expressing *unh1* in haploid cells resulted in abnormal pigmentation, when grown in both potato dextrose broth and minimal medium, suggesting that pigmentation may be triggered by *unh1* in *U. maydis*. The function of Unh1 in sexual development and pigment production depends on the presence of the Ndt80-like DNA-binding domain, identified within Unh1. In the absence of this domain, or when the binding domain was altered with targeted amino acid changes, ectopic expression of Unh1 failed to complement the *unh1* deletion with regards to pigment production and sexual development. An investigation of *U. maydis* genes with upstream motifs similar to Ndt80 recognition sequences revealed that some have altered transcript levels in Δ*unh1* strains. We propose that the first characterized Ndt80-like DNA-binding protein in a basidiomycete, Unh1, acts as a transcription factor that is required for teliospore maturation and the completion of meiosis in *U. maydis*.

1. Introduction

Ustilago maydis (DC) Corda is an obligate sexual pathogen and the model for biotrophic basidiomycete plant pathogens (Banuett, 1995; Brefort et al., 2009). Its lifecycle consists of three different forms – the haploid saprophytic form, the pathogenic dikaryotic form, and the diploid teliospore (reviewed by Kahmann and Kämper, 2004). Sexually compatible haploids fuse to form the filamentous dikaryon, and at this stage the fungus is an obligate parasite, requiring the plant host for growth and development. The dikaryon invades the plant and grows within and between plant cells, triggering the formation of characteristic tumors (Banuett and Herskowitz, 1996; Martinez-Espinoza et al., 2002; Snetselaar and Mims, 1992). Within these tumors, the branching hyphae fragment into teliospore initials, the initials begin rounding, pigment is deposited and ornamentation occurs leading to darkly pigmented mature teliospores with echinulation (Banuett and Herskowitz, 1996). *U. maydis* teliospores are dormant dispersal agents and the only cell type competent to complete meiosis (Banuett, 1995; Kahmann and Kämper, 2004). Meiosis initiates during the formation of teliospores in the plant, and arrests in late prophase I when the teliospores enter dormancy (Kojic et al., 2013). Following dispersal, teliospores germinate and resume meiosis, completing meiotic divisions and generating haploid cells (Kojic et al., 2013; Saville et al., 2012). Meiosis and teliospore formation are temporally linked in *U. maydis*; however, up to 3% (Christensen, 1931; Kojic et al., 2002), or as high as 10% (Holliday, 1961) of the teliospores fail to complete meiosis and germinate as diploids. Thus, meiosis and teliospore formation are linked but separable events. The obligate *in planta* development required for teliospore formation and meiotic competence indicates the control of these processes in *U. maydis* must respond to signals received while in the plant. This means that part of the key to understanding plant/pathogen interaction during *U. maydis* pathogenesis is elucidating the control pathways that trigger meiosis.

Meiosis is crucial to the maintenance of genetic diversity in all sexually reproducing organisms. The economically important

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http://dx.doi.org/10.1016/j.fgb.2016.07.006
1087-1845/

smuts and rusts require growth in the host plant to initiate meiosis and this initiation is linked to pathogenic development. In these fungi meiosis is paused at a time analogous to the pachytene checkpoint, or meiotic recombination checkpoint in *Saccharomyces cerevisiae* (Donaldson and Saville, 2008; Kojic et al., 2013; Saville et al., 2012). In *S. cerevisiae* this checkpoint prevents the cell from exiting prophase I and entering into meiotic divisions if recombination has not been completed (Bailis and Roeder, 2000). The progression of meiosis is consistent among sexually reproducing fungi; however, the external cues that induce entry into meiosis and the control of meiotic progression are less conserved (Saville et al., 2012).

A search for *U. maydis* genes potentially involved in meiosis identified *UMAG_02775* (*unh1*) as a probable Ndt80-like transcription factor based upon sequence similarity in the DNA-binding domain (Donaldson and Saville, 2008). These proteins have been characterized in ascomycete fungi where a given species often has multiple Ndt80-like proteins with distinct roles in influencing developmental transitions, including those leading to sexual reproduction (Hutchison and Glass, 2010; Wang et al., 2006). *S. cerevisiae* has a single family member, Ndt80, which is a meiosis-specific transcription factor required for exit from the pachytene checkpoint, leading to meiotic divisions (Chu et al., 1998; Chu and Herskowitz, 1998; Hepworth et al., 1998; Xu et al., 1995). *unh1* has limited sequence similarity to Ndt80-like proteins outside the Ustilaginomycetes, however, its binding domain is conserved. As the only Ndt80-like protein present in *U. maydis*, *unh1* was hypothesized to control progression through meiosis in *U. maydis*. The results of this study support the contention that Unh1 functions as a transcription factor that plays an essential role in sexual development in *U. maydis*, being required for teliospore maturation and the completion of meiosis in *U. maydis*. Therefore, these analyses identify a key component of the largely unknown control pathway for *in planta* teliospore development and meiosis commitment in smut fungi.

2. Materials and methods

2.1. Strain construction and growth conditions

The *U. maydis* strains used in this study are listed in Table 1. Compatible *U. maydis* haploid strains FB1 and FB2 as well as the solo pathogenic diploid strain FB-D12 (Banuett and Herskowitz, 1989) were provided by Flora Banuett (California State University, Long Beach, United States). The haploids were used as wild-type (wt) strains for all experiments. Haploid *U. maydis* cells were grown in YEPS medium (1% yeast extract, 2% sucrose, 2% peptone), potato dextrose broth (PDB; BD Difco), or minimal medium (Holliday, 1974) at 28–30 °C shaking at 250 rpm. Mutants containing the hygromycin resistance cassette were grown on medium supplemented with 300 μg mL^{-1} hygromycin B (BioShop), while those with the carboxin resistance cassette were grown on medium supplemented with 4 μg mL^{-1} carboxin (Sigma). Subcloning efficiency DH5α chemically competent *Escherichia coli* cells (Life Technologies) were transformed with plasmids conferring ampicillin resistance and grown on Lysogeny broth (LB, EMD Millipore) plates or liquid medium (Sambrook and Russell, 2001), containing 100 μg mL^{-1} ampicillin (BioShop), at 37 °C, with liquid cultures shaking at 250 rpm. Plasmids were extracted and purified using the illustra Plasmid Prep Mini Spin Kit (GE Healthcare Life Sciences). Ligations for vector construction were carried out with T4 DNA ligase (New England Biolabs) and incubated at 16 °C for 21 h. To verify correct vector construction, the inserted fragments were sequenced from the plasmid using Big Dye Terminator chemistry V3.1 (ABI) and an automated sequencer (ABI 3730 DNA analyzer). Raw sequences were trimmed using default settings of the SeqManII module of Lasergene v.5.0 (DNASTAR), and aligned using MEGA 5 (Tamura et al., 2011).

The *U. maydis unh1* deletion constructs were created following the PCR-based method established by Kämper (2004). *E. coli* containing plasmids pMF1-c (carboxin resistance) and pMF1-h (hygromycin resistance) (Brachmann et al., 2004), were obtained from Jörg Kämper (Karlsruhe Institute of Technology, Karlsruhe, Germany). The 5′ and 3′ regions flanking the *unh1* gene were amplified from genomic DNA by PCR using Finnzymes Phusion according to the manufacturer's suggested protocol (Thermo Fischer Scientific). The 5′ and 3′ flanking regions of the genes were amplified with primers *unh1*_LF_Out-F and *unh1*_LF_SfiI-R and *unh1*_RF_Out-R and *unh1*_RF_SfiI-F (Table S1), respectively. The products were purified using the QIAquick Gel Extraction Kit (Qiagen) following the microcentrifuge protocol. The flanking regions were digested with *Sfi*I; pMF1-h and pMF1-c were digested with *Sfi*I and *Bsa*I (New England Biolabs). The digested products were purified by gel extraction. DNA concentration was determined using a Nanodrop 8000 Spectrophotometer (Thermo Scientific) and equal concentrations of each flank were ligated to either the hygromycin B or carboxin cassette. The ligation product was gel extracted and amplified by PCR with Finnzymes Phusion using the nested primers, *unh1*_LF_Nest-F and *unh1*_RF_Nest-R (Table S1). The hygromycin B resistance construct was transformed into FB1 and the carboxin resistance construct was transformed into FB2, replacing *unh1* by homologous recombination. Transformation was carried out by the method of Wang et al. (1988), as modified in Morrison et al. (2012). This resulted in the creation of the deletion strains FB1Δ*unh1* and FB2Δ*unh1* (Table 1). Integration of the construct at the *unh1* locus was confirmed through PCR and Southern blot analysis using the DIG High Prime DNA labeling and Detection Starter Kit 1 (Roche). The absence of *unh1* transcripts in the deletion mutants was assessed by RT-PCR using primers Unh1-F and Unh1-R (Table S1).

The *unh1* deletion was complemented with wild-type *unh1* (p*unh1*), *unh1* with residues K641 through A824 deleted (p*unh1*ΔKA) and *unh1* containing R to A point mutations at residues 647, 720 and 761 (p*unh1*3A). *U. maydis* strains p*unh1*, p*unh1*ΔKA and p*unh1*3A were created in the FB1 Δ*unh1* strain. The wild-type *unh1* gene was obtained by amplification of cDNA isolated from *U. maydis* teliospores with the primers PublexUnh1-F and PublexUnh1-R (Table S1) using Platinum Taq

Table 1
Strains used in this study.

Name	Strain	Relevant genotype[a]	Source
Wild-type (wt)	FB1	*a1 b1*	Banuett and Herskowitz (1989)
	FB2	*a2 b2*	Banuett and Herskowitz (1989)
Solopathogenic diploid	FB-D12	*a1/a2 b1/b2 pan−/+ ade −/+*	Banuett and Herskowitz (1989)
Δ*unh1*	FB1Δ*unh1*	*a1 b1* Δ*unh1*::*hph*	This publication
	FB2Δ*unh1*	*a2 b2* Δ*unh1*::*carbR*	This publication
p*unh1*	FB1Δ*unh1* otef: *unh1*	*a1 b1* Δ*unh1*::*hph* otef: *unh1*::*carbR*	This publication
p*unh1*ΔKA	FB1Δ*unh1* otef: $unh1^{\Delta 641-824}$	*a1 b1* Δ*unh1*::*hph* otef: $unh1^{\Delta 641-824}$::*carbR*	This publication
p*unh1*3A	FB1Δ*unh1* otef: $unh1^{RA647,720,761}$	*a1 b1* Δ*unh1*::*hph* otef: $unh1^{RA647,\ 720,\ 761}$:: *carbR*	This publication

[a] unh1 is UMAG_02775.