Fungal Genetics and Biology 45 (2008) 1364–1372

Contents lists available at ScienceDirect

Fungal Genetics and Biology

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

PHL1 of *Cercospora zeae-maydis* encodes a member of the photolyase/cryptochrome family involved in UV protection and fungal development ☆

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ARTICLE INFO

Article history:
Received 29 January 2008
Accepted 8 July 2008
Available online 19 July 2008

Keywords:
Cercospora
Gray leaf spot
Photoreactivation
Photolyase
Cryptochrome

ABSTRACT

DNA photolyases harvest light energy to repair genomic lesions induced by UV irradiation, whereas cryptochromes, presumptive descendants of 6-4 DNA photolyases, have evolved in plants and animals as blue-light photoreceptors that function exclusively in signal transduction. Orthologs of 6-4 photolyases are predicted to exist in the genomes of some filamentous fungi, but their function is unknown. In this study, we identified two putative photolyase-encoding genes in the maize foliar pathogen *Cercospora zeae-maydis*: *CPD1*, an ortholog of cyclobutane pyrimidine dimer (CPD) photolyases described in other filamentous fungi, and *PHL1*, a cryptochrome/6-4 photolyase-like gene. Strains disrupted in *PHL1* (Δ*phl1*) displayed abnormalities in development and secondary metabolism but were unaffected in their ability to infect maize leaves. After exposure to lethal doses of UV light, conidia of Δ*phl1* strains were abolished in photoreactivation and displayed reduced expression of *CPD1*, as well as *RAD2* and *RVB2*, orthologs of genes involved in nucleotide excision and chromatin remodeling during DNA damage repair. This study presents the first characterization of a 6-4 photolyase ortholog in a filamentous fungus and provides evidence that *PHL1* regulates responses to UV irradiation.

1. Introduction

As sessile organisms, fungi have developed highly effective strategies to cope with a variety of harmful environmental conditions, including excessive exposure to UV irradiation. UV light induces chromosomal damage, such as the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4 pyrimidine-pyrimidone lesions (6-4 PPs) (Sinha and Hader, 2002), with the formation of CPD lesions predominating over 6-4 PP lesions at a ratio of approximately 10:1 (Douki and Cadet, 2001). UV-induced lesions are repaired by DNA photoylases, specialized enzymes that bind specifically to damaged DNA and harvest light energy to either break the cyclobutane ring formed in CPD lesions or to reverse the photoaddition of the C4 carbonyl of a thymine to the C5–C6 double bond of a neighboring pyrimidine in a 6-4 PP lesion (Weber, 2005). Two distinct classes of photolyases repair UV-induced lesions: the evolutionarily ancient CPD photolyases found in all taxonomic kingdoms, and the 6-4 photolyases, found only in multicellular Eukaryotes (Essen and Klar, 2006). Among plants and animals, homologs of 6-4 photolyases have evolved to form the cryptochrome family of blue-light photoreceptors, members of which regulate a wide variety of biological processes in response to light. Intriguingly, genes predicted to encode proteins homologous to 6-4 photolyases have been identified in the sequenced genomes of several filamentous fungi. Because no photolyase/cryptochrome-like gene has been characterized in fungi, it is unclear whether these genes function as DNA-repair enzymes or photoreceptors in the Fungal kingdom.

The anamorphic fungal genus *Cercospora* is comprised of numerous and diverse plant pathogens that affect important crops throughout the world. Many species of *Cercospora* produce the host non-specific phytotoxin cercosporin, a photosensitizing perylenequinone whose biosynthesis and activation are induced by visible light (Daub and Ehrenshaft, 2000). Recently, a cluster of genes encoding the enzymes required for cercosporin biosynthesis was identified and characterized (Chen et al., 2007), but little is known at the molecular level about the regulation of cercosporin biosynthesis by light. Presumably, the transcriptional activation of genes involved in cercosporin biosynthesis requires the function of a sensory protein such as a photoreceptor.

☆ Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the products, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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doi:10.1016/j.fgb.2008.07.005

The focus of this study was to identify and characterize a cryptochrome/6-4 photolyase-like gene in the maize foliar pathogen *Cercospora zeae-maydis*. We cloned and disrupted *PHL1*, a gene highly homologous to photolyase- and cryptochrome-encoding genes from plants and animals. Disruption of *PHL1* completely abolished photoreactivation and impaired the expression of *CPD1*, a second putative photolyase identified in this study, as well as at least two other genes involved in DNA damage repair. Disruption of *PHL1* caused abnormalities in development and secondary metabolism, suggesting additional signaling functions for *PHL1*. We hypothesize that the fungal family of photolyase/cryptochrome-like genes such as *PHL1* have evolved regulatory functions that distinguish them from 6-4 photolyases in higher plants and animals.

2. Materials and methods

2.1. Fungal strains and growth conditions

Strain SCOH1-5 of *Cercospora zeae-maydis* was used as the wild type in this study. Strains Δ*phl1-1* and Δ*phl1-2* (disrupted in *PHL1*) were constructed as described below. Strains WT-GUS and phl1-GUS were constructed by transforming strains SCOH1-5 and strain Δ*phl1-2*, respectively, with plasmid pDB23, a vector constructed by cloning a geneticin-resistance cassette from pKS-GEN (Bluhm et al., 2008), into the EcoRI site of vector pNOM102 (GenBank Accession No. Z32701). To promote conidiation, strains were maintained on V8 agar in constant darkness and transferred every 5 days. For analyses of cercosporin biosynthesis, fungi were grown on 0.2× potato dextrose agar (0.2× PDA; BD Biosciences, Sparks, MD). Tissue for genomic DNA was collected from cultures grown in liquid YEPD medium (0.5% yeast extract, 1.0% peptone, and 3% dextrose); tissue for RNA extractions was prepared by inoculating conidial suspensions onto cellophane membranes overlaid on either 0.2× PDA or V8-agar plates as described by Cooley et al., (2005). Light was provided by two General Electric (GE) F40CW-RS-MM (cool white) bulbs 16 in. above the plates. Light intensity at the plates was 15–17 μmol/m^2/s as determined with a LiCor integrating spectrophotometer (model LI 188B). Experiments were monitored continuously to verify that exposure to light did not affect the temperature of the plates.

2.2. Nucleic acid manipulations

Plasmids were purified with the Wizard Plus SV Minipreps DNA purification system (Promega; Madison, WI). Fungal genomic DNA was extracted by a modified CTAB protocol (Proctor et al., 1997), and Southern analyses were performed following standard protocols (Sambrook and Russell, 2001). RNA was extracted with Trizol reagent (Invitrogen; Carlasbad, CA) and purified with an RNeasy miniprep purification kit (Qiagen; Valencia, CA). For analyses of gene expression, cDNA was generated with the Stratascript RT-PCR system (Stratagene) using random primers as template. DNA sequences were determined by the Purdue University Genomics Center (West Lafayette, IN).

2.3. Identification of PHL1, CPD1, RAD2, and RVB1

To identify *PHL1*, degenerate PCR primers PHL1degF (CKCATCTKGTRTTTGAGAAGGA) and PHL1degR (GTGCAGCTSAGCCAYTGCCAGTT) were designed by aligning amino acid and nucleotide sequences of predicted genes from *Magnaporthe grisea, Fusarium graminearum, Aspergillus clavatus*, and *Ustilago maydis*. Genomic DNA of *C. zeae-maydis* was amplified with primers PHL1degF and PHL1degR to generate a 1.1-kb product that was cloned into pGEM-T EZ (Promega) and sequenced. The remainder of the gene, designated *PHL1*, was obtained by genome-walker PCR (Clontech; Mountain View, CA). To walk upstream from the product obtained by degenerate PCR, we amplified genome-walker libraries first with primers phl5p1 (AGCTTCGCCGGCCATGTGCATG ACCT) and phl5p2 (CACGAGCATAAGCGTCCGTGTCCTT) to obtain 914 bp of the gene followed by amplification with primers phl15p3 (TACAACAAGGCCTCCCAACGCACGG) and phl15p4 (ATGCGAT GCGATGCGCTGCTGCCGAT) to obtain 385 bp of the gene. Walking downstream from the original 1.1-kb product was accomplished first by amplifying libraries with primers phl3p1 (AGCACTA GGCTGGCAATTCGGCCAAA) and phl3p2 (CTTCATCCCCTGG CACCTCCCTTCCAA) to obtain 684 bp of *PHL1* followed by amplification with primers phl3p3 (TGATGGTGATGGGTCGGAGACGAAG) and phl3p4 (GGATGGATTGGCGTTGTATCCGAAA) to obtain 1275 bp of the gene. In total, we sequenced 3895 bp of the *PHL1* locus, including the entire open reading frame of *PHL1*, 837-bp upstream from the putative start codon, and 870-bp downstream from the putative stop codon. The entire sequence of the *PHL1* locus was deposited in GenBank (Accession No. EU443730).

To identify *CPD1*, degenerate PCR primers CPDdegF (GAASTNGCMTGGCGRGARTTTTAC) and CPDdegR (ATCATRCGN AGNCGGTTGTGCAT) were designed by aligning amino acid sequences of CPD photolyases from *Bipolaris oryzae* (*PHR1*; AB126091) and *Neurospora crassa* (*phr*; X58713) as well as predicted genes from *Mycosphaerella graminicola*, *Magnaporthe grisea*, *Fusarium graminearum*, and *Aspergillus clavatus*. A 219-bp product amplified from genomic DNA of *C. zeae-maydis* with primers CPDdegF and CPDdegR was cloned into pGEM-T-EZ, sequenced, and submitted to GenBank (Accession No. EU814871).

To identify orthologs of other genes involved in DNA repair, we performed homology-based searches of proteins predicted to be encoded by 27,551 ESTs generated from *C. zeae-maydis* (Bluhm et al., unpublished). Cluster consensus sequence 496_1 (GenBank Accession No. EU443731), a 903-bp sequence obtained by analyzing three overlapping ESTs from two distinct clones, was highly similar to *RAD2* from *Aspergillus clavatus* (XM_001267902). Singlet EST 466_0 (GenBank Accession No. EU443732), a 723-bp sequence, was predicted to encode a protein highly similar to Ruv-B of *Escherichia coli* (AAA24613).

2.4. Disruption of PHL1

For functional analysis of *PHL1*, we targeted the gene for disruption via single homologous recombination. To build the disruption construct, 535 bp of the open reading frame was amplified with primers PHL1F1 (GGCGAGATCGAAGAGCCTGTTG) and PHL1F2 (TTTGGCCGAATTGCCAGCCTA) and cloned into pGEM-T EZ to create pDB68. Then, a NotI fragment from pDB68 was cloned into pKS-HYG (Bluhm and Woloshuk, 2005) to create pDB71. For transformation, a 2.0-kb product containing a 1.4-kb hygromycin-resistance cassette and 535 bp of *PHL1* sequence was amplified from pDB71 with primers PHL1F1 and HYG-F (GATATTGAAGGA GCATTTTTTGGGCT). Protoplasts of *C. zeae-maydis* were prepared and transformed as described by Shim and Dunkle (2003). Hygromycin-resistant colonies were screened by PCR with primers A1 (CATTTGGTGTTTGAGAAGGACACGGA) and H3 (CGGCAATTTCGATGATGCAGCTTG) to identify two independent strains disrupted in *PHL1* (Δ*phl1-1* and Δ*phl1-2*). For Southern analysis, genomic DNA was probed with the hygromycin-resistance cassette from pKS-HYG.

2.5. Quantification of cercosporin biosynthesis

Freshly harvested conidia of each strain were inoculated onto 0.2× PDA plates and incubated at 22 °C under either constant light