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MgLig4, a homolog of *Neurospora crassa* Mus-53 (DNA ligase IV), is involved in, but not essential for, non-homologous end-joining events in *Magnaporthe grisea*

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ABSTRACT

In many eukaryotic organisms, the non-homologous end-joining (NHEJ) system is a major pathway for the repair of DNA double-strand breaks (DSBs). DNA ligase IV is a component of the NHEJ system and is strictly required for the NHEJ system in *Saccharomyces cerevisiae* and in *Neurospora crassa*. To investigate the functions of DNA Ligase IV in *Magnaporthe grisea*, we generated deletion mutants of *MGLIG4*, which encodes a homolog of *N. crassa* DNA Ligase IV. Mutants (*mglig4*) showed no defects in asexual or sexual growth, and were fully pathogenic. Compared to the wild-type, *mglig4* exhibited weak sensitivity to a DNA-damaging agent, camptothecin. In addition, the frequency of targeted-gene replacement was relatively elevated in *mglig4*, although this varied in a gene-dependent manner. Surprisingly, non-homologous integration of DNA was frequently observed in *mglig4* transformants. Our results demonstrate that MgLig4 is involved in, but not essential for, the NHEJ system in *M. grisea*.

1. Introduction

DNA double-strand breaks (DSBs) can cause lethal damage to cells and can be caused directly or indirectly by a variety of exogenous and endogenous agents, such as enzymatic degradation, excision of transposons, failure of normal cellular processes, radicals, ionizing radiation, and UV light (Daley et al., 2005; Haber, 2006; Shrivastav et al., 2008). DNA-damaging chemicals including radiomimetic-chemicals which produce reactive oxygen species, UV-mimetic chemicals that create replication blocking lesions, and inhibitors of topoisomerase I/II are also known to produce DSBs (Fasullo et al., 2004; Shrivastav et al., 2008). In eukaryotes, two major systems for repairing DSBs are known: homologous recombination (HR) and non-homologous end-joining (NHEJ). The HR system operates on homologous sequences and is the major repair pathway in *Saccharomyces cerevisiae* (Takita et al., 1997). The NHEJ system involves direct joining of double-strand ends regardless of DNA sequence homology. In many eukaryotes, including humans, plants, insects, and filamentous fungi, the NHEJ system appears to be the main DSBs repair pathway (Pastink et al., 2001). In the mammalian NHEJ system, DSBs repair involve the detection and binding of the DSB ends by Ku70/Ku80 heterodimers followed by the gathering of additional NHEJ factors, including the DNA-dependent protein kinase (DNA-PK), DNA ligase IV-Xrcc4 complex, and polymerases to the DSBs ends to complete the repair (Roberts and Ramsden, 2007). Recently, DNA ligase III was identified as a component of a backup pathway to the DNA-PK/ DNA ligase IV dependent NHEJ system in mammalian cells (Wang et al., 2006). A limited but important member of core NHEJ components such as the Ku70/Ku80 heterodimer and DNA ligase IV, are conserved between fungi and mammalian cells (Critchlow and Jackson, 1998; Ishibashi et al., 2006). In *S. cerevisiae*, Ku70 and Ku80, encoded by *YKU70* and *YKU80*, respectively, are also important for NHEJ, being involved in NHEJ processing and alternative error-prone DNA-repair pathway suppression (Critchlow and Jackson, 1998). However, unlike the mammalian system, DNA ligase IV encoded by *DNL4* is strictly and specifically required for NHEJ system in *S. cerevisiae* (Daley et al., 2005). In many filamentous fungi, Ku70 and Ku80 have important roles in NHEJ system as their deletion, which suppresses NHEJ activity, increases the frequency of HR. For example, the relative frequency of HR was significantly elevated in Ku70 and Ku80 deletion mutants of *N. crassa*, *mus-51* and *mus-52*, respectively (Ninomiya et al., 2004). When vectors with >0.5-kb that were identical to the target sequence were introduced to *mus-51* or *mus-52*, HR frequency of 100% was obtained (Ninomiya et al, 2004; Ishibashi et al., 2006). In *Aspergillus oryzae*,

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A. sojae, *A. fumigatus*, *A. nidulans*, and *A. niger*, deletion of Ku70 or Ku80 homologs also resulted in a high efficiency of targeted-gene replacement (TGR), although it was not as high as that seen in *N. crassa* (da Silva Ferreira et al., 2006; Krappmann et al., 2006; Nayak et al., 2006; Takahashi et al., 2006; Meyer et al., 2007). In *M. grisea*, a deletion mutant of a Ku80 homolog, *mgku80*, showed significantly higher TGR (78–100%) than the wild-type strain (average 8%) (Villalba et al., 2008). However, the frequency of HR event in *mgku80* was dependent on the target gene, e.g., targeted replacement of the *ACE1* gene did not occur in *mgku80* (Villalba et al., 2008). Previous work on DNA ligase IV, Mus-53, in *Neurospora crassa* has shown that Mus-53 is required for all NHEJ pathways (Ishibashi et al., 2006). The *mus-53* mutant showed a very high frequency of TGR (100%) when it was introduced with a vector possessing as few as 100 base pairs that were identical to those of the target gene (Ishibashi et al., 2006). In contrast, no transformant was obtained when the identical region was <50 bp in *mus-53* (Ishibashi et al., 2006). In addition, transformation efficiency of *mus-53* was approximately four to five times lower than that of the wild-type (Ishibashi et al., 2006). These results strongly indicated that *mus-53* was protected from non-homologous integration, and only mus-53 transformants generated via the HR pathway survived after transformation (Ishibashi et al., 2006).

Here, we report the phenotypes, sensitivity to DNA-damaging agents, and frequency of TGR of DNA ligase IV deletion mutants of *M. grisea, mglig4*. The *mglig4* mutants showed no defects in colony phenotype, pathogenicity, or fertility, but were more sensitive to the DNA-damaging agent camptothecin than the wild-type. The frequency of TGR in *mglig4* was greater than that in the wild-type, and was gene-dependent. Interestingly, ectopic integration of DNA was often observed in *mglig4* mutants. Our results indicate that MgLig4 is involved in, but not strictly required for, NHEJ in *M. grisea*.

2. Materials and methods

2.1. Fungal strains, media, DNA manipulations, and molecular analyses

Magnaporthe grisea wild-type strains Guy11 (Leung et al., 1988) and P2 were used. P2 is infertile Japanese rice pathogenic isolate. For storage, both strains were cultured at 25 °C for 8 days on paper discs placed on oatmeal medium (3% ground oatmeal, 0.5% glucose, 1.6% agar), before being desiccated and kept at −20 °C as described (Nishimura et al., 2003). For DNA extraction, mycelia were cultured in 50 mL YG medium (0.5% yeast extract and 2% glucose) for 5 days at 25 °C with rotary shaking at 100 rpm. Genomic DNA was extracted by the CTAB method as described (Xu and Leslie, 1996). Briefly, harvested mycelia were ground in liquid nitrogen, incubated at 65 °C in CTAB buffer (2% CTAB, 100 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.7 M NaCl) for 30 min, then incubated in the same volume of CIA buffer (chloroform to isoamyl alcohol ratio 23:1 vol/vol) at room temperature for 30 min. Ethanol was added to the supernatant collected after centrifugation of the mycelia/buffer mixture to precipitate DNA. For labeling and detection of the probes for Southern hybridization, we used the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare) according to the manufacturer's instructions. For PCR (polymerase chain reaction) amplification, Takara Ex Taq (Takara, Japan), a Taq polymerase, was used. We tested the fertility of Guy11-derived mutants (mating type *MAT1-2*) by incubating the fungi on oatmeal medium with the fertile wild-type strain 4000-R-10 (mating type *MAT1-1*) under fluorescent light as described (Nishimura et al., 2003). Homology search was done by BLASTP program (Altschul et al., 1990). Amino acid alignment was conducted with the CLUSTALW algorithm (Thompson et al., 1994). Phylogenetic analysis was performed using Phyml software (Guindon and Gascuel, 2003) with the maximum likelihood method. The tree was plotted with MEGA ver. 4.0 software (Tamura et al., 2007).

2.2. Generation of MgLIG4 deletion mutants (mglig4)

Primers used are listed in Table 1. The Mglig4-F1/R1 and Mglig4-F2/R2 primers pairs amplified 0.9-kb each of the 5- and 3-flanking regions, respectively, of *MgLIG4* (GenBank Accession No. AB353135, MGG_12899. 6) (Fig. 1A). The amplified regions were cloned into XbaI– BamHI and EcoRI–HindIII sites of pAhd5 (a kind gift from Dr. Jin-Rong Xu, Purdue University, USA; Park et al., 2002), respectively, and the resultant construct was designated pKOMGLIG4 (Fig. 1A). To generate *MgLIG4* deletion mutants, we introduced a 3.2-kb DNA fragment amplified from pKOMGLIG4 with Mglig4-F1/R2 primers (Fig. 1A) into the wild-type strain Guy11 by the PEG transformation method (Leung et al., 1990). Replacement of *MgLIG4* in the transformants was screened by PCR using the Mglig4-IF1/IR1 primer pair (Fig. 1A) and confirmed by Southern hybridization analysis. For the first hybridization, a 0.9-kb DNA fragment generated by EcoRI and HindIII digestion of pKOMGLIG4 was used as a probe (probe 1 in Fig. 1A). For the second hybridization, a 0.6-kb DNA fragment

Table 1
Primers used in this study

Name	Sequence (5′ > 3′)
Vector construction	
Mglig4-F1	TCTAGACGTCAATCGACCCTAGGAAA
Mglig4-R1	GGATCCTTTTTAATCGGCAGCCTTTG
Mglig4-F2	GAATTCGGCGGTGTTTATAGGTGGAA
Mglig4-R2	AAGCTTGGGGAAGCAACCTGTACTCA
Pmglig4-F1	GTCGCATTGAATGGATGGCT
Pmglig4-R1	CTCCTTGTACAGTACCTCAG
Mgwc1-F1	AGATCTTTGTCTTGGCCATCTTACCC
Mgwc1-R1	AGATCTGAGCTGCGTACCGTAAT
Mgwc1-F2	AAGCTTCGATTTGCGAGGAGTAGGAC
Mgwc1-R2	AAGCTTTTTGTTCTCTCCCCTCATGG
Mgwc1-F3	GCAAAAAACCTGGCTATAGA
Mgwc1-R3	GGCATTATGGTGATAATATA
MagB-F1	ACTAGTCAACGCTACTGGGCATTT
MagB-R1	CTGCAGCTTCTCCTCCGTGCTCATTC
MagB-F2	GGATCCTGGTCCTCTTGTGAGCCTCT
MagB-R2	GAATTCAAGAAGCGGAAGAATCAGCA
Mrgs2-F1	GGAATTCACAACCCATTTTATTCTCCCC
Mrgs2-R1	GGGATCCACAGCCTCATCCAGTTCCGA
Mrgs2-F2	AAGATCTACTCAGTCACCGTACGATAC
Mrgs2-R2	AACTAGTATCCATTGCCGTGATGAGAG
Alb1-F1	CAGAGATGGTCTCAGTCACC
Alb1-R1	TCAGACTCGGGACGGAGCAG
Screening	
Mglig4-IF1	CACGATTGCGGGAGCACTT
Mglig4-IR1	GTTCGTGAGGAGACAGCCTC
Mglig4-IF2	AATCGTCCCCGTAATCACTCC
Mglig4-IR2	CATCTCGCCGTCCAAAATCAG
Mrgs2-F3	TCGTCTGCTTGGTTGACTTG
Mrgs2-R3	TTGAGGTTTTGGGGCTAGTG
Mrgs2-IF1	AACTCCACATTGGACGGAAG
Mrgs2-IR1	GTGTCCCCTGTGACCAGTCT
MagB-IF1	GGTTGCGGAATGAGCAC
MagB-IR1	CTTGAACGCTTGAAGCACTC
MagB-SCR	CAAGTACCTGATCCATACCC
CAM-R	AACGATCGGGGAAATTCG
Hph-F1	AACTCACCGCGACGTCTGTC
Hph-R1	TTGTCCGTCAGGACATTGTT
Hph-F2	AGCTGCGCCGATGGTTTCTACAA
Hph-R2	GGGGCGTCGGTTTCCACTATCG
Ble-F1	GACTTCGTGGAGGACGACTT
Ble-R1	GACACGACCTCCGACCACT
Bar-F1	GTCGACAGAAGATGATATTG
Bar-R1	GTCGACCTAAATCTCGGTGA
Bsd-F1	GTCAATATGCCTTTGTCTCAAGAAGAATCC
Bsd-R1	ATGCTCGGTTCACTAGTAACAGAAAGTAGC