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ScienceDirect
Fungal Genetics and Biology 45 (2008) 68–75
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Improved gene targeting in *Magnaporthe grisea* by inactivation of *MgKU80* required for non-homologous end joining ☆

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Received 7 March 2007; accepted 19 June 2007
Available online 10 July 2007

Abstract

The ascomycete *Magnaporthe grisea* is a model species for the study of plant fungal interactions. As in many filamentous fungi, targeted gene replacement occurs at low frequency in *M. grisea* (average 7%). *mus52/KU80* is a gene essential for non-homologous end joining (NHEJ) of DNA double-strand breaks. Its deletion increases the frequency of targeted gene replacement in fungi [Ninomiya, Y., Suzuki, K., Ishii, C., Inoue, H., 2004. Highly efficient gene replacements in *Neurospora* strains deficient for non-homologous end joining. Proc. Natl. Acad. Sci. USA 101(33), 12248–53]. *M. grisea KU80* deletion mutants were constructed and displayed wild-type phenotypes regarding pathogenicity, growth, sporulation and mating. *MgADE4* targeted gene replacement frequency was increased in Δ*ku80* mutants (80% vs 5%) and high frequencies (>80%) were observed at seven other loci. However, the deletion of *MgKU80* did not increase the frequency of *ACE1* replacement indicating that this locus has an intrinsic reduced ability for gene replacement. These results open the way to large-scale reverse genetics experiments in *M. grisea* facilitating the study of the infection process.

Keywords: *Magnaporthe grisea*; *KU80*; Homologous recombination; Non-homologous end joining

1. Introduction

The recent development of genomics in fungi (Galagan et al., 2005; Xu et al., 2006) has highlighted a significant number of candidate genes potentially involved in pathogenicity on plants (Dean et al., 2005; Xu et al., 2006). Their study is hampered by the difficulty in constructing null mutants by targeted gene replacement, since its frequency is low (<5%) in most filamentous fungi (Weld et al., 2006). Targeted gene replacement requires the cellular machinery involved in the repair of DNA double-strand breaks through two main pathways: (1) repair by homologous recombination, and (2) repair of DNA double-strand breaks independently of sequence homology using the non-homologous end joining pathway (NHEJ, Aylon and Kupiec, 2004). In the yeast *Saccharomyces cerevisiae*, homologous recombination is the main pathway involved in repairing DNA double-strand breaks (Takita et al., 1997), whereas in mammals NHEJ is the most active pathway (Tachibana, 2004). NHEJ involves the binding of a Ku complex (K70–Ku80 heterodimer) to broken DNA ends. A DNA-dependent protein kinase (DNA-PKcs) is recruited by the Ku complex and phosphorylates the DNA exonuclease Artemis. This complex stimulates the binding of DNA ligase IV—Xrcc4 at the broken DNA ends (Hefferin and Tomkinson, 2005). The Ku70 and Ku80 proteins were also identified in insects, plants, and fungi (Hefferin and

☆ Submitted for the "Technical Advances" section of Fungal Genetic and Biology.

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doi:10.1016/j.fgb.2007.06.006

Tomkinson, 2005). In the ascomycete *Neurospora crassa*, these proteins are encoded by *mus-51* and *mus-52*, respectively. The deletion of these genes leads to a large increase in the frequency of targeted gene replacement suggesting that the NHEJ pathway competes with homologous recombination for the integration of exogenous DNA fragments (Ninomiya et al., 2004; Ishibashi et al., 2006). Increased frequency (70–100%) of targeted gene replacement was also observed in other fungal *KU70* or *KU80* deletion mutants (*Aspergillus fumigatus*, da Silva Ferreira et al., 2006 and Krappmann et al., 2006; *Cryptococcus neoformans*, Goins et al., 2006; *Aspergillus sojae/oryzae*, Takahashi et al., 2006; *Aspergillus nidulans*, Nayak et al., 2006; *Sordaria macrospora*, Pöggeler and Kück, 2006). These Δ*ku70* and Δ*ku80* mutants had a normal growth, mycelium morphology, ascospores or basidiospores (*C. neoformans*) and were as pathogenic on mice as corresponding wild-type strains (*A. fumigatus* and *C. neoformans*). These mutants have stimulated large-scale gene knock-out experiments in *N. crass*a (Colot et al., 2006). Extending these techniques to plant pathogenic fungi would facilitate deciphering mechanisms involved in pathogenicity through large-scale reverse genetics experiments.

The ascomycete *Magnaporthe grisea* is responsible for the rice blast disease and this fungus is a model organism for the study of plant fungal interactions. Its genomic sequence is known (Dean et al., 2005) and extensive studies have been performed to identify the molecular mechanisms involved in the infection process (Xu et al., 2007). However, targeted gene replacement occurs at low frequency (average 7%) in this fungal species (Talbot and Foster, 2001). In this study, we assayed the effect of the inactivation of *MgKU80* involved in the NHEJ pathway on the frequency of targeted gene replacement in *M. grisea*. The *MgKU80* gene orthologous to *N. crassa mus-52* (Ninomiya et al., 2004) was identified in *M. grisea* genome. *MgKU80* was deleted in two different *M. grisea* isolates (P1.2, Guy11) by targeted gene replacement. This deletion increases the frequency of targeted gene replacement at eight independent loci tested (>80%), but not at the *ACE1* locus.

2. Material and methods

2.1. Fungal strains and growth conditions

Wild-type *M. grisea* isolates (P1.2 and Guy11) pathogenic on rice and barley were obtained from the Centre de Coopération Internationale pour la Recherche Agronomique et le Développement (CIRAD). For sporulation, Guy11 and P1.2 were grown on "Rice-Guy11" agar (20 g/L rice flour, 10 g/L glucose, 2 g/L KH_2PO_4, 3 g/L KNO_3, agar 15 g/L, adjusted to pH 6) and "Rice-P1.2" agar (20 g/L rice flour, 2 g/L yeast extract, agar 15 g/L), respectively. Other media have the following composition. TNK-CP derived from Tanaka-B medium (Ou, 1985): 10 g/L glucose, 2 g/L yeast extract, 2 g/L $NaNO_3$, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.004 g/L $FeSO_4{\cdot}7H_2O$, microelements as Tanaka-B, adjusted to pH 5.5–5.8. TNK-CP-Hyg contains 0.12 g/L hygromycin (Sigma–Aldrich, Saint-Louis, USA). TNK-MM: TNK-CP without yeast extract supplied with thiamine 1 mg/L and biotine 1 μg/L after sterilization. TNK-CP-Su-Hyg: same as TNK-CP supplied with 200 g/L sucrose instead of glucose and 0.24 g/L hygromycin. SW-Basta derived from Sweigard et al. (1997): 1.6 g/L YNB w/o amino-acid, 2 g/L asparagine, 1 g/L NH_4NO_3, 1 g/L glucose, 0.035 g/L glufosinate (Basta, Cluzeau Info Labo, Ste Foy la Grande, France) adjusted to pH 6. All solid media contained agar at 15 g/L except TNK-MM supplied with 8 g/L agarose.

2.2. Construction of the gene replacement cassettes

Upstream and downstream regions of the genes of interest were obtained by PCR using genomic DNA (100 ng) as template, primers pairs $KO1^{gene}/KO2^{gene}$ and $KO3^{gene}/KO4^{gene}$ and 0.5 U of DNA polymerase Phusion (Finnzymes, Espoo, Finland). The primers are listed in (Supplementary Table S1) and the strategy used for constructing the gene replacement cassettes is derived from Kämper (2004). Purified amplicons were digested by SfiI whose restriction sites are present in primers $KO2^{gene}$ (SfiIa) and $KO3^{gene}$ (SfiIb). *BAR* or *HPH* cassettes (Sweigard et al., 1997) conferring resistance to glufosinate or hygromycin were excised from plasmid pFV9 or pFV8, respectively ($pB2KS^{-}$ vectors carrying *BAR* or *HPH* cassettes flanked by SfiIa and SfiIb sites). Following ligation, the gene replacement cassette was obtained by amplification using primers $KO5^{gene}$ and $KO6^{gene}$. For the *SER3* gene, $KO2^{gene}$ and $KO3^{gene}$ contained a BamHI and a PstI restriction site, respectively, and the *HPH* cassette was excised from a pUC-HPH vector by digestion with these two enzymes. The *ACE1* deletion cassette was constructed by Böhnert et al. (2004).

2.3. M. grisea transformation

Three micrograms of transforming DNA suspended in 20 μl water were mixed with 20 μl STC (200 g/L sucrose, 50 mM Tris–HCl, pH 7.5, 50 mM $CaCl_2{\cdot}2H_2O$). Frozen protoplasts (10^7 in 200 μl) prepared as described (Böhnert et al., 2004) were thawed on ice and added to the buffered DNA. After 15-min incubation at room temperature, 1 mL of 60% PEG-4000 (NBS Biologicals, Huntingdon, UK) in 0.6 M sorbitol, 10 mM MOPS was slowly added to the protoplasts. Following gentle mixing and 20 min incubation at room temperature, 3 mL of TB3 (200 g/L sucrose, 3 g/L yeast extract) were added. The suspension was shaken (110 rpm) at 26 °C for 3 h (hygromycin) or overnight (Basta/glufosinate). The protoplasts were concentrated to 1 mL by centrifugation at 3500 rpm, mixed to 4 ml of melted TB3-agar containing 5 g/L bacteriological agar (Oxoid, Basingstoke, UK), overlaid onto either TNK-CP-Su-Hyg or SW-Basta plates and incubated at 30 °C for 5–7 days.