British Mycological Society promoting fungal science

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

# Efficient gene targeting in *ligase IV*-deficient *Monascus ruber* M7 by perturbing the non-homologous end joining pathway

Yi HE[a,c], Yanchun SHAO[a,c], Fusheng CHEN[a,b,c,d,*]

[a]Key Laboratory of Environment Correlative Dietology, Huazhong Agricultural University, Ministry of Education, Wuhan 430070, Hubei Province, PR China
[b]Hubei Collaborative Innovation Center for Industrial Fermentation, Wuhan 430068, Hubei Province, PR China
[c]College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei Province, PR China
[d]The Food College of SHIHEZI University, Shihezi, 832003, Xinjiang Province, PR China

ARTICLE INFO

*Article history:*
Received 18 May 2014
Received in revised form
6 July 2014
Accepted 7 July 2014
Available online 17 July 2014
*Corresponding Editor:*
Michael Lorenz

*Keywords:*
DNA ligase IV
Gene replacement frequency
Homologous recombination
Genetic manipulation
Mutagen sensitivity

ABSTRACT

Inactivating the non-homologous end joining (NHEJ) pathway is a well established method to increase gene replacement frequency (GRF) in filamentous fungi because NHEJ is predominant for the repair of DNA double strand breaks (DSBs), while gene targeting is based on homologous recombination (HR). DNA ligase IV, a component of the NHEJ system, is strictly required for the NHEJ in *Saccharomyces cerevisiae* and *Neurospora crassa*. To enhance the GRF in *Monascus ruber* M7, we deleted the *Mrlig4* gene encoding a homolog of *N. crassa* DNA ligase IV. The obtained mutant (MrΔlig4) showed no apparent defects in vegetative growth, colony phenotype, microscopic morphology, spore yield, and production of *Monascus* pigments and citrinin compared with the wild-type strain (*M. ruber* M7). Gene targeting of *ku70* and *triA* genes revealed that GRF in the MrΔlig4 strain increased four-fold compared with that in the wild-type strain, reached 68 % and 85 %, respectively. Thus, the MrΔlig4 strain is a promising host for efficient genetic manipulation. In addition, the MrΔlig4 strain is more sensitive than *M. ruber* M7 to a DNA-damaging agent, methyl methanesulfonate.



## Introduction

*Monascus* spp. are well-known industrial fungi, which are mainly used to produce red fermented rice (RFR) that has been extensively used as food colourant, food additive, folk medicine, as well as fermentation starter to brew rice wine and vinegar for many centuries in oriental countries (Lin *et al.* 2008; Lee & Pan 2011, 2012a). Previous chemical investigations revealed that *M.* spp. can produce structural and bioactive diverse secondary metabolites such as monacolins, gamma aminobutyric acid, pigments, and dimerumic acid, which are effective for the management of blood cholesterol, diabetes, blood pressure, obesity, and Alzheimer's disease, and for the prevention of cancer (Lee *et al.* 2006,

* *Corresponding author*. Tel./fax: +86 27 87282927.
E-mail address: chenfs@mail.hzau.edu.cn (F. Chen).
http://dx.doi.org/10.1016/j.funbio.2014.07.003

2008; Shi & Pan 2011, 2012; Feng *et al.* 2012; Hsu & Pan 2012; Lee & Pan 2012b; Lu *et al.* 2013). Recently, with the availability of genomic sequences of several *Monascus* strains, the gene clusters involved in *Monascus* pigments (Balakrishnan *et al.* 2013; Xie *et al.* 2013; Liu *et al.* 2014), citrinin (Sakai *et al.* 2008; Li *et al.* 2012), Monacolin K (Chen *et al.* 2009), and G-protein signalling pathway (Li *et al.* 2010a, 2014; Yang *et al.* 2012) have been studied but many genes are still insufficiently characterized (Shao *et al.* 2014), necessitating efficient gene targeting systems of *M.* spp. for elucidating the function of candidate genes.

In order to obtain efficient targeted integration of DNA fragments *via* homologous recombination (HR), disruption of several non-homologous end joining (NHEJ)-related genes (*ku70*, *ku80* or *lig4*) has been tested in fungi, plants, and animals (Meyer 2008). Efficient gene targeting has been demonstrated in NHEJ mutants of yeasts (Teo & Jackson 1997; van Attikum & Hooykaas 2003), *Neurospora crassa* (Ninomiya *et al.* 2004; Ishibashi *et al.* 2006), *Aspergillus* spp. (Krappmann *et al.* 2006; Nayak *et al.* 2006; Takahashi *et al.* 2006, 2011; Meyer *et al.* 2007; Mizutani *et al.* 2008; Chang *et al.* 2010; Fang *et al.* 2012), *Penicillium* spp. (Snoek *et al.* 2009; de Boer *et al.* 2010; Hoff *et al.* 2010; Li *et al.* 2010b; Bugeja *et al.* 2012), *Magnaporthe grisea* (Kito *et al.* 2008; Villalba *et al.* 2008), *Coprinopsis cinerea* (Nakazawa *et al.* 2011), *Sordaria macrospora* (Pöggeler & Kück 2006), *Cryptococcus neoformans* (Goins *et al.* 2006), and *Botrytis cinerea* (Choquer *et al.* 2008). Compared with the common gene replacement frequency (GRF) lower than 30 % in filamentous fungi (Meyer 2008), the GRFs in some NHEJ-deficient fungi even reached 100 %, and disruption of *lig4* had more improvement on GRF than knocking out *ku70* or *ku80*. Thus, these mutants have great biotechnological applications, and many major breakthroughs related to high-throughput functional genomics, proteomics studies, and natural product discovery have been achieved by using them (Colot *et al.* 2006; Chiang *et al.* 2008; Takahashi *et al.* 2008; Carvalho *et al.* 2010; Kück & Hoff 2010; Verbeke *et al.* 2012).

In our previous study, we found that GRFs were doubled and tripled compared with that in wild-type strain when deleting *ku70* and *ku80* in *Monascus ruber* M7, respectively, but their GRFs were still very circumscribed, only reached 42 % and 62 %, respectively (He *et al.* 2013). In this study, we cloned a *lig4* like gene, *Mrlig4*, encoding a protein sharing 51.1 % identity with MUS-53 (DNA ligase IV protein, GenBank No: BAF34364.1) in *N. crassa* (Ishibashi *et al.* 2006) from *M. ruber* M7 genome. Subsequently, we constructed *Mrlig4*-deleted mutant of *M. ruber* M7 (MrΔlig4) and analysed its GRFs through knocking out two different genes, *ku70* (GenBank No: KC192955) and *triA* (GenBank No: KC561929, a gene encodes a putative acetyltransferase involved in *Monascus* pigments biosynthesis). Results revealed that GRF in MrΔlig4 increased to four times comparing with that in the wild-type strain. Thus, *Mrlig4* is an important gene participating in the NHEJ pathway, like *ku70* and *ku80* genes. Moreover, comparing with the wild-type strain, MrΔlig4 showed higher sensitivity to methyl methanesulfonate (MMS) but not to UV-light, and there were no obvious differences between these two strains in morphological features and production of pigments and citrinin.

## Materials and methods

### *Fungal strains, culture media, and growth conditions*

All strains used in this study are described in Table 1. *Monascus ruber* M7 was used as the wild-type strain. For phenotypic characterization, four kinds of media including potato dextrose agar (PDA) medium, malt extract agar medium (MA), Czapek yeast extract agar medium (CYA), and glycerol nitrate agar medium (G25N) were utilized (He *et al.* 2013). Hygromycin B-resistant transformants and neomycin-resistant transformants were selected on PDA medium containing 30 μg $mL^{-1}$ hygromycin B (Sigma–Aldrich, Shanghai, China) and 15 μg $mL^{-1}$ G418 (Sigma–Aldrich, Shanghai, China), respectively. *M. ruber* M7 and all mutant strains were cultivated at 28 °C.

### *Genomic DNA extraction*

Fungal genomic DNA was extracted from mycelia growing on PDA plates covered with cellophane membranes by the cetyltrimethylammonium bromide (CTAB) method (Shao *et al.* 2009).

### *Cloning of the* Mrlig4 *gene*

A pair of primers, Mrlig4 F2-Mrlig4 R2 (Table 2), was designed to amplify the putative *Mrlig4* gene using Oligo 6 software (http://www.oligo.net/). The PCR protocol was as follows: initial denaturation at 94 °C for 5 min followed by 30 amplification cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 3.5 min with a final extension step at 72 °C for 10 min using T100 Thermal Cycler (Bio-Rad, Hercules, USA).

### *Deletion of the* Mrlig4 *gene*

For target gene deletion, a strategy was designed to replace the putative *Mrlig4* coding region with hygromycin B resistance gene (*hph*) using double-joint PCR method (Yu *et al.* 2004) (Fig 1A). Briefly, a 782-bp of 5′ flanking region and a 698-bp of

**Table 1 – *M. ruber* strains constructed and used in this study.**

| Strain | Parent | Genotype | Source |
|---|---|---|---|
| M7 | M7 | Wild-type | Red fermented rice[a] |
| MrΔku70 | M7 | *Δku70::neo* | Previous study[b] |
| MrΔku80 | M7 | *Δku80::hph* | Previous study[b] |
| MrΔlig4 | M7 | *Δlig4::hph* | This study |
| MrΔku80Δku70 | MrΔku80 | *Δku80::hphΔku70::neo* | Previous study[b] |
| MrΔlig4Δku70 | MrΔlig4 | *Δlig4::hphΔku70::neo* | This study |
| MrΔlig4ΔtriA | MrΔlig4 | *Δlig4::hphΔtriA::neo* | This study |

a *M. ruber* M7 strain was isolated by Chen & Hu (2005). It has been stored in Culture Collection of State Key Laboratory of Agricultural Microbiology (part of China Center for Type Culture Collection (CCTCC), Wuhan, China) and gained the classification number CCAM 070120.
b These three strains were constructed by He *et al.* (2013).