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Disruption of lig4 improves gene targeting efficiency in the oleaginous fungus Mortierella alpina 1S-4

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Abbreviations: HR, homologous recombination; NHEJ, none homologous end joining; DSBs, DNA double-strand breaks; OA, oleic acid; LA, linoleic acid; GLA, \(\gamma\)-linolenic acid; DGLA, dihomo-\(\gamma\)-linolenic acid; ARA, arachidonic acid.
Highlights

- We identified a lig4 gene encoding DNA ligase 4 in Mortierella alpina 1S-4
- We disrupted the lig4 gene in M. alpina 1S-4 by homologous recombination
- We improved gene targeting efficiency dramatically in M. alpina 1S-4 by disruption of the lig4 gene
Abstract

The oil-producing zygomycete Mortierella alpina 1S-4 is known to accumulate beneficial polyunsaturated fatty acids. We identified the lig4 gene that encodes for a DNA ligase 4 homolog, which functions to repair double strand breaks by non-homologous end joining. We disrupted the lig4 gene to improve the gene targeting efficiency in M. alpina. The M. alpina 1S-4 Δlig4 strains showed no defect in vegetative growth, formation of spores, and fatty acid production, but exhibited high sensitivity to methyl methansulfonate, an agent that causes DNA double-strand breaks. Importantly, gene replacement of ura5 marker by CBXB marker occurred in 67% of Δlig4 strains and the gene targeting efficiency was 21-fold greater than that observed in disruption of the lig4 gene in the M. alpina 1S-4 host strain. Further metabolic engineering of the Δlig4 strains is expected to result in strains that produce higher levels of rare and beneficial polyunsaturated fatty acids and contribute to basic research on the zygomycete.

Keywords

Mortierella alpina; DNA ligase 4 (lig4); homologous recombination; gene targeting
1. Introduction

The integration of exogenous DNA into chromosomes occurs through two pathways that repair DNA double-strand breaks (DSBs), homologous recombination (HR) and non-homologous end joining (NHEJ) (Kanaar et al., 1998). These pathways are independent of one another and often function competitively (Van Dyck et al., 1999). In HR, exogenous DNA is integrated into the chromosome by making use of homologous regions as templates for precise insertion. This process requires the Rad family of proteins, (Rad54, Rad51, Rad52), Mre11, and Xrs2 (Kooistra et al., 2004; Krappmann, 2007). HR is largely conserved among species and has been ubiquitously found from bacteria and yeast to humans (Krogh and Symington, 2004; Shibata et al., 2001).

NHEJ, on the other hand, involves direct ligation of the exogenous DNA strand ends into the DSBs and has no requirement for sequence homology. In general, NHEJ requires Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA ligase 4 (Lig4), and X-ray repair cross-complementing protein 4 (Xrcc4) (Critchlow and Jackson, 1998; Daley et al., 2005). NHEJ is also largely conserved among species. It was reported that a Ku8-dependent and Ku80-independent NHEJ manner was found in *Neurospora*, whereas the Lig4 was a key enzyme related with all
NHEJ pathways (Ishibashi et al., 2006). Most organisms, including mammals, plants, insects, and filamentous fungi, predominantly utilize the NHEJ pathway for DSB repair. Recently, it was reported that disruption of the *ku70*, *ku80* or *lig4* genes leads to an increase in the frequency of HR in various filamentous fungi (Ishidoh et al., 2014; Maruyama and Kitamoto, 2008; Ninomiya et al., 2004). In particular, disruption of the *lig4* gene in *Neurospora crassa* and *Aspergillus oryzae* increased targeting efficiency to 100% (Ishibashi et al., 2006; Mizutani et al., 2008).

The oil-producing filamentous fungus, *Mortierella alpina* 1S-4, is capable of producing large amounts of carbon 20 (C20) polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4ω6, ARA) and the eicosapentaenoic acid (20:5ω3, EPA) in triacylglycerols (Sakuradani et al., 2009). This fungus has been used as a model microorganism for the biosynthesis and accumulation of lipid molecules, including PUFAs (Kawashima et al., 1995; Kikukawa et al., 2013; Sakuradani, 2010; Sakuradani et al., 2013). We previously reported techniques for gene manipulation in this fungus (Ando et al., 2009a; Ando et al., 2009b; Takeno et al., 2004a, 2004b; Takeno et al., 2005b) to further understand the selective production of various PUFAs in *M. alpina* 1S-4. Genes were manipulated by means of chemical mutation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as well as molecular breeding through
overexpressing or silencing genes by RNA interference (Sakuradani, 2010; Sakuradani et al., 2013; Takeno et al., 2005a). However, chemical mutation and molecular breeding have difficulty in isolating mutations producing various PUFAs and in controlling the biosynthetic pathways of PUFAs in transformants, respectively. Furthermore, traditional mutagenesis yields undesirable mutations in the genome, and plasmid vectors are randomly integrated, which often unnecessarily affect growth rate and/or fatty acid production of this fungus.

Previously, we disrupted the \textit{ku80} gene by means of biolistic particle bombardment (Ando et al., 2009a) and evaluated the improvement in gene targeting efficiency in the \textit{Δku80} strain. However, significant improvement was not detected (Kikukawa et al., 2015). In this study, we sought to develop a more efficient gene targeting system in \textit{M. alpina} 1S-4 by disrupting the \textit{lig4} gene through double crossing-over HR by the \textit{Agrobacterium tumefaciens}-mediated transformation (ATMT) method. The ATMT method introduces a single-copy of the fragment between the left and right borders of gene disruption vectors into the genome. It was previously reported that the ratio of integration by HR was increased by ATMT method (Gouka et al., 1999; Kito et al., 2008; Michielse et al., 2005a; Michielse et al., 2005b). In the ATMT of \textit{M. alpina} 1S-4, integration at multiple sites in the genome occurs at low frequencies in \textit{M. alpina} 1S-4.
(Ando et al., 2009b).
2. Materials and methods

2.1 Enzymes and chemicals

Restriction enzymes and other DNA-modifying enzymes were obtained from Takara Bio Inc. (Shiga, Japan). All other chemicals were of the highest purity commercially available.

2.2 Strains, media, and growth conditions

*M. alpina* 1S-4 is deposited in the culture collection of the Graduate School of Agriculture of Kyoto University (Sakuradani, 2010) and the uracil auxotrophic (*ura5*<sup>−</sup>) strain with a mutation in the *ura5* gene selected from *M. alpina* 1S-4 was used as the host strain in this study (Takeno et al., 2004b). Czapek-Dox agar medium containing 0.05 mg/ml uracil was used for sporulation of *M. alpina* 1S-4 *ura5*<sup>−</sup> strain (Takeno et al., 2004b). Synthetic complete (SC) medium was used as a uracil-free synthetic medium for cultivation of the transformants derived from *M. alpina* 1S-4 *ura5*<sup>−</sup> strain at 28°C (Takeno et al., 2004b). GY medium (2% glucose and 1% yeast extract) was used for fatty acid composition analysis and extraction of genomic DNA. Uracil-free SC agar plates containing 0.03% Nile Blue A (Sigma-Aldrich, St. Louis, MO) and GY agar
plates containing 10 μg/ml carboxin and 0.03% Nile Blue A were used for selection of transformants with the carboxin resistance CBXB gene cassette (Ando et al., 2009a).

*Escherichia coli* DH5α was used for DNA manipulation and grown on LB agar plates containing 50 μg/ml kanamycin. *Agrobacterium tumefaciens* C58C1 was used for the transformation of the *M. alpina* 1S-4 ura5- strain. LB-Mg agar medium, minimal medium (MM), and induction medium (IM) were used for transformation, cultivation, and infection of *A. tumefaciens*, respectively (Ando et al., 2009b). All solid media were made by addition of 2.0% agar.

### 2.3 Genomic DNA preparation

*M. alpina* 1S-4 was cultivated in 100 ml of GY liquid medium for 5 days at 28°C with shaking at 100 rpm. Preparation of genomic DNA was performed as previously described (Okuda et al., 2014b).

### 2.4 Cloning and identification of the lig4 gene from *M. alpina* 1S-4

Two highly degenerate primers, Lig4 F1 and Lig4 R1, were synthesized for cloning the *lig4* gene (Table S1). The primers were designed based on the conserved amino acid sequences of Lig4 homologs from *Rhizopus delemar* (accession no. EIE79010), *Canis*
lupus (accession no. XP_542663), Laccaria bicolor (accession no. XP_001874645),
Pan troglodytes (accession no. XP_509726), Schizosaccharomyces pombe (accession no. NP_587888). The sequences of the sense and antisense primers correspond to the regions that encode LIVGGY and TLRFPR peptides, respectively. PCR amplification was carried out in a total volume of 50 μl containing 1 μg of genomic DNA, 0.25 μl of Takara EX Taq polymerase (Takara Bio Inc.), 5 μl of 10 × EX Taq buffer, 200 μM of each dNTP, and 5 pM of each primer, and conditions were as follows: 35 cycles of 1 min at 94ºC, 1 min at 60ºC, and 2 min at 72ºC, followed by a final extension period of 5 min at 72ºC. The resulting 571 bp fragment was cloned into the pT7Blue T-Vector (Novagen, Madison, WI, USA) and the sequence was verified using the Beckman-Coulter CEQ8000 system (Beckman-Coulter, Fullerton, CA, USA). RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) and cDNA was synthesized using the PrimeScript™ High Fidelity RT-PCR Kit (Takara Bio Inc.), according to the manufacturers’ protocols.

To isolate the entire lig4 genomic DNA from M. alpina 1S-4, inverse PCR was performed using Lig4 IPCR F and Lig4 IPCR R primers (Table S1). The BamHI-digested genomic fragment was self-ligated and used as a template for PCR amplification. PCR amplification was carried out in a total volume of 50 μl containing
500 ng of the template, 0.25 µl of Takara EX Taq polymerase (Takara Bio Inc.), 5 µl of 
10 × EX Taq buffer, 200 µM of each dNTP, and 5 pM of each primer, and conditions 
were as follows: initial denaturation of 1 min at 94ºC, 35 cycles of 40 sec at 94ºC, 40 
sec at 57ºC, and 3 min at 72ºC, and an extension period of 10 min at 72ºC. The 
amplified 3.0 kb fragment was cloned into the pT7Blue T-Vector and the sequence was 
confirmed using the Beckman-Coulter CEQ8000 system.

2.5 Construction of lig4 gene targeting plasmid vectors

A T-DNA binary vector for lig4 gene targeting was constructed using the M. alpina 
1S-4 transformation vector pBIG35Zh (Ando et al., 2009b; Okuda et al., 2014a) or 
pBIG3CBZ. pBIG3CBZ was generated by excising the carboxin resistance CBXB gene 
cassette (Ando et al., 2009a) from the M. alpina 1S-4 transformation vector 
pSDZNCBXB using the EcoRI and XbaI restriction sites. The resulting fragment was 
ligated into the pBIG35Zh vector using the same restriction sites, and the resulting 
plasmid was designated pBIG3CBZ. The 5’-lig4 fragment (1.6 kb) and 3’-lig4 fragment 
(1.8 kb) were amplified from the M. alpina 1S-4 genomic DNA template using Lig4 F1 
ApaI and Lig4 R1 ApaI, and Lig4 F2 XbaI and Lig4 R2 Nhel (Table S1), respectively. 
pBIG35ZΔL4 and pBIG3CBZΔL4 were generated by ligating the 3’-lig4 fragment
(XbaI and NheI restriction sites) and the 5’-lig4 fragment (ApaI restriction site) into pBIG35Zh and pBIG3CBZ, respectively (Fig. 1). pBIG35ZΔL4 is used for disruption of the lig4 gene in the host strain, and pBIG3CBZΔL4 is used for estimating the targeting efficiency in a Δlig4 strain. The replacement of the ura5 marker with the CBXB marker in the lig4 locus was confirmed by PCR using the extracted genomic DNA as a template and Lig4 up F and CBXB stop R primers (Table S1). The sequences between left border (LB) and right border (RB) integrate into the genomic DNA of *M. alpina* 1S-4 upon transformation.

2.6 Transformation of *M. alpina* 1S-4

Spores of the *M. alpina* 1S-4 uracil auxotrophic strain was freshly prepared from cultures growing on Czapek-Dox agar medium supplemented with 0.05 mg/ml uracil, and the spore suspension was filtered through Miracloth (Calbiochem) (Takeno et al., 2004b).

Transformation of *M. alpina* 1S-4 was performed using the ATMT method as previously described (Ando et al., 2009b) with slight modification. After co-cultivation of *A. tumefaciens* cells and *M. alpina* 1S-4 spores, the membranes were transferred to uracil-free SC agar plates containing 0.03% Nile Blue A or GY agar plates containing
10 μg/ml carboxin. After incubation for 4 days at 28°C, aerial hyphae of fungal colonies were transferred to fresh uracil-free SC agar plates or GY agar plates containing 100 μg/ml carboxin.

2.7 Selection of lig4 gene-disrupted strains by PCR

*M. alpina* 1S-4 transformed with pBIG35ZΔL4 were cultured on fresh uracil-free SC agar medium at least three times. Candidates containing *lig4* gene disruption were then identified by first amplifying the *lig4* genomic gene locus using *Lig4* up F and *Lig4* down R primers (Table S1) and genomic DNA prepared from the transformants. Then, the resulting fragment was used as a template for another round of PCR amplification using three primer sets, *Lig4* F1 *Apa*I and *Lig4* R1 *Apa*I, *Lig4* in F and *Lig4* in R, and *Lig4* F1 *Apa*I and *ura5* 400 R (Table S1 and Fig. S1).

2.8 Southern blot analysis

Southern blot hybridization was carried out as previously described (Ando et al., 2009b). Genomic DNA (10 μg) was digested with *Sac*I and *Xho*I, size-fractioned by electrophoresis (1% agarose), and transferred to Amersham Hybond-N+ membrane (GE Healthcare Ltd., Buckinghamshire, UK). Probes used for hybridization correspond to a
0.8 kb region of the 5′-fragment of lig4 gene and a 0.6 kb region of the ura5 gene, and were generated using Lig4 F2 and Lig4 R1 ApaI, and ura5 start F and ura5 stop R primer sets (Table S1), respectively. Southern hybridization was performed with Amersham Alkphos Direct Labeling Reagents (GE Healthcare) and Amersham CDP-Star Detection Reagent (GE Healthcare), and chemiluminescence was detected by ImageQuant LAS 4000 mini system (GE Healthcare).

2.9 Mutagen sensitivity of Δlig4::ura5 strain

Sensitivity to chemical mutagens was evaluated by the spot test (Kato et al., 2004; Mizutani et al., 2008). Methyl methanesulfonate (MMS), at 0, 0.01, 0.02, 0.03, 0.04, and 0.005% [w/v], was added to SC agar medium supplemented with 0.05 mg/ml uracil. Spores were spotted and grown on the agar plates for 8 days.

2.10 Fatty acid analysis

Analysis of the fatty acid production and compositions of the transformants was performed as previously described (Kikukawa et al., 2013) with slight modification. The mycelia of the Δlig4 strain and its host strain were inoculated into 10 ml of GY liquid medium and cultivated for 7 days at 28°C with reciprocal shaking at 300 rpm. The
fungal strains were harvested by filtration and dried for 3 h at 120°C. The dried cells were directly transmethylated with 10% methanolic HCl for 2 h at 55°C. The resulting fatty acid methyl esters (FAMEs) were extracted using n-hexane, concentrated, and analyzed by GC-2010 Plus gas chromatography (GC; Shimadzu, Kyoto, Japan) equipped with a TC-70 capillary column (GL Science Inc., Tokyo, Japan). Fatty acid quantification was calculated using tricosanoic acid as an internal standard. All experiments were performed three times.

2.11 Nucleotide sequence accession number

The *M. alpina* 1S-4 lig4 gene is registered in the DNA Data Bank of Japan (DDBJ) database under the accession number LC009448.

3. Results

3.1 Identification and phylogenetic analysis of the *M. alpina* 1S-4 lig4 homolog

The open reading frame (ORF) of the *M. alpina* lig4 gene consists of 3,309 bp of DNA spanning ten exons (1-103, 217-691, 791-901, 1,009-1,212, 1,312-1,354, 1,449-1,659, 1,776-1,881, 1,974-2,079, 2,178-2,872, and 2,964-3,309). The predicted *M. alpina* Lig4 protein consists of 799 amino acids and shares low sequence identity with

The phylogenetic tree of various homologous Lig4 proteins from these organisms indicated that Lig4 homologs are clustered in each organism group, such as fungi, mammals, and plants, whereas the Lig4 protein (MaLig4) from *M. alpina* 1S-4 was remotely related to those from other organisms (Fig. 2).

3.2 Identification and characterization of Δlig4 mutants

To generate a Δlig4 strain (Δlig4::ura5) of *M. alpina* 1S-4, spores were transformed with pBIG35ZΔL4 (Fig. 1) using the ATMT method (Fig. 3A). Then, the *lig4* disruptants were selected by PCR analysis. The primers Lig4 F1 *Apa*I and Lig4 R1 *Apa*I resulting in the 1.6 kb fragment of 5′-*lig4* upstream region were used for control. When disruption of the *lig4* gene locus occurs, the 0.2 kb fragment from the intact *lig4* gene is no longer amplified by the Lig4 in F and Lig4 in R primers. Instead, a 2.5 kb fragment is amplified in reactions using Lig4 F1 *Apa*I and ura5 400 R primers, indicating that the fragment is successfully integrated into the *lig4* gene locus (Fig.
S1A). A schematic showing the location of the primers used to identify gene disruption and a representative transformant showing the results of the PCR assay are shown in Fig. S1A and S1B. We screened 93 transformants by PCR and found that 3 transformants showed lig4 gene disruption.

We then investigated whether a single homologous integration of the disruption construct into the lig4 gene correctly occurred by Southern blot analysis. Locations of the probes used for the Southern blot analysis are depicted in Fig. 3A. After digestion of genomic DNA with XhoI and detection with probe A, we observed the expected 2.9 kb fragment in the host lig4 gene whereas the gene disrupted mutant had a 1.0 kb fragment (Fig. 3B). We also observed the expected 1.9 kb fragment in lig4 disrupted genomic DNA digested with XhoI, and the 10.7 kb fragment in SacI digested genomic DNA when probe B was used (Fig. 3B). These results suggested that gene replacement with ura5 marker had successfully occurred at the lig4 gene locus in the host strain of M. alpina 1S-4. The targeting efficiency of the lig4 gene in the uracil auxotrophic strain was calculated to be 3.2% (Table 1).

3.3 Estimate of gene targeting efficiency in the Δlig4 mutant

To evaluate the improvement of the gene targeting efficiency in the Δlig4::ura5
strain, we performed an assay to measure the replacement of *ura5* marker with the *CBXB* marker in the *Δlig4* locus (Fig. 4A). The pBIG3CBZΔL4 vector was introduced into spores of the *Δlig4::ura5* strain grown on GY medium containing 100 μg/ml carboxin by the ATMT method. Integration of the *CBXB* gene was measured by colony PCR where successful replacement resulted in the amplification of a 3.5 kb fragment (Fig. 4A and 4B). Of the three transformants analyzed, two showed the expected replacement in the *Δlig4* locus. We also confirmed replacement by sequencing using the Beckman-Coulter CEQ8000 system (data not shown). We calculated the targeting efficiency to be 66.7%, which is 20.7-fold greater than the isolation of the *Δlig4* strain from *M. alpina* 1S-4 host strain (Table 1).

3.4 Mutagen sensitivity, growth, and fatty acid productivity of the *Δlig4::ura5* strain

The *Δlig4::ura5* strain showed sufficient sporulation, and the spores germinated on MMS-free SC agar medium. However, compared to the host strain, the *Δlig4::ura5* strain grew at a significantly decreased rate when grown on SC agar plates containing MMS (Fig. 5). The *Δlig4::ura5* strain was not capable of growing on the plates containing MMS concentrations higher than 0.03%, whereas the host strain was able to grow at 0.03% MMS. Furthermore, we compared the production of fatty acids and
growth rates of the Δlig4::ura5 strain to those of host strain. The Δlig4::ura5 and host strains showed similar growth rates and slightly higher fatty acid production in GY liquid medium (Table 2), and both also formed white aerial hyphae (under condition of 0% MMS in Fig. 5). The Δlig4::ura5 strain showed similar PUFA composition mainly consisting of linoleic acid (LA), γ-linoleic acid (GLA), dihomo-γ-linolenic acid (DGLA), and ARA, to that of the host strain (Table 2).
4. Discussion

We cloned and identified the lig4 gene encoding DNA ligase 4 (Lig4), a protein involved in NHEJ of all species, in M. alpina 1S-4. Homologous Lig4 proteins identified in other organisms were classified by kingdom and showed that the Lig4 homolog (MaLig4) from M. alpina 1S-4 shares low identities (about 30%) with those of other organisms (Fig. 2).

We then developed a method to disrupt the lig4 gene (the Δlig4::ura5 strain) in M. alpina 1S-4 through HR with pBIG35ZΔL4 vector. By using the ATMT method, we obtained Δlig4::ura5 strains with 3.2% efficiency which is a slightly higher than the biolistic particle bombardment method used for ku80 gene disruption (Kikukawa et al., 2015). Additionally, the targeting efficiency of the replacement of ura5 marker by the CBXB marker in the Δlig4 locus in the Δlig4::ura5 strain was calculated as 66.7%, which is 20.7-fold higher than disruption of the lig4 gene with pBIG35ZΔL4 in M. alpina 1S-4 host strain (Table 1). Compared with that of the Δku80 strain, the targeting efficiency was drastically improved in Δlig4 strain (Kikukawa et al., 2015). The disruption of ku80 was performed through single crossing-over HR, resulting in incomplete ku80 disruptant. It was suggested that the incomplete Ku80 protein formed a Ku-protein complex with the Ku70 protein and reduced targeting efficiency. One
explanation for this improvement of targeting efficiency is that disruption of lig4 gene through double crossing-over HR led to complete inactivation of Lig4. However, unlike N. crassa, A. oryzae, and A. luchuensis, the targeting efficiency did not reach 100% in the Δlig4::ura5 strain (Ishibashi et al., 2006; Mizutani et al., 2008; Takahashi et al., 2011). It is unclear why the gene targeting efficiency in several organisms, including this fungus, did not improve to 100% (Kito et al., 2008; Maruyama and Kitamoto, 2008; Tashiro et al., 2013). Depending on the target gene locus, the targeting efficiency is quite different because of the mosaic nature of active and inactive domains in eukaryotic chromosomes (Wolffe and Guschin, 2000). Therefore, we need to evaluate the gene targeting efficiency by disruption of various genes related to fatty acid synthesis in order to collect more statistical data.

Lig4 protein is involved in NHEJ in phylogenetically diverse organisms (Hopfner et al., 2002; Lisby and Rothstein, 2004) and in telomere maintenance in some organisms (Hande, 2004). The lig4 disruption in some organisms led to sensitivity to MMS (Ishibashi et al., 2006; Ninomiya et al., 2004). The Δlig4::ura5 strain exhibited high sensitivity to MMS (Fig. 5), and the sensitivity was similar to that of N. crassa, A. oryzae, and A. kawachii (Ishibashi et al., 2006; Mizutani et al., 2008; Tashiro et al., 2013). The high sensitivity of the Δlig4::ura5 strain to MMS suggests repression of the
NHEJ pathway in this strain.

This study is the first report to describe the identification and characterization of the \( \text{lig4} \) gene from Mucoromycotina. The \( \Delta \text{lig4}::\text{ura5} \) strain showed no defect in vegetative growth, formation of spores, and fatty acid productivity (Table 2) making it suitable for subsequent molecular breeding. Additionally, we demonstrated improvement in gene targeting efficiency in the \( \Delta \text{lig4} \) strain, which is expected to facilitate metabolic engineering for production of beneficial PUFAs and reverse genetic studies for basic research in \( M. \text{alpina} \) 1S-4.
5. Conclusion

We successfully improved the efficiency of gene targeting in *M. alpina* 1S-4 by disrupting the *lig4* gene. These novel Δ*lig4* mutants display similar growth rate, sporulation, and production of PUFA to that of the host strain. This study is the first report to describe the identification and characterization of *lig4* gene from Mucoromycotina. Our efficient gene targeting system may be used to generate various gene disrupted strains to produce beneficial PUFAs and may also reveal new mechanisms of lipid biosynthesis and lipid accumulation.

Conflict of Interest

The authors declare no conflict of interests.

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Figure legends

Fig. 1. The pBIG35ZΔL4 or pBIG3CBZΔL4 vector used for lig4 gene-disruption. LB, left border; RB, right border; His550p, M. alpina 1S-4 histone H4.1 promoter short fragment; ura5, orotate phosphoribosyltransferase gene of M. alpina 1S-4; CBXB, the mutated sdhB gene for carboxin resistance; SdhBt, M. alpina 1S-4 sdhB translation terminator; NPTIII, neomycin phosphotransferase III gene; TrfA, trfA locus, which produces two proteins that promote replication of the plasmid; ColEI ori, ColEI origin of replication; oriV, pRK2 origin of replication.

Fig. 2. The phylogenetic analysis of Lig4 homologs. The tree was created by using the neighbor-joining (NJ) method with 10,000 bootstrap replicates by the sequence analysis software GENETYX ver. 11.0 (Genetyx corp., Tokyo, Japan). Aspergillus nidulans (AnLig4), Q5BH83; Aspergillus oryzae (AoLig4), BAE62914; Aspergillus fumigatus (AfLig4), EAL91408; Cryptococcus neoformans (CnLig4), AAW46139; Coprinopsis cinerea (CcLig4), Q7Z7W5; Mus musculus (MmLig4), NP_795927; Rattus norvegicus (RnLig4), NP_001099565; Homo sapiens (HsLig4), NP_001091738; Arabidopsis thaliana (AtLig4), NP_568851; Oryza sativa (OsLig4), Q7X7E9; Mortierella alpina 1S-4 (MaLig4), LC009448.

Fig. 3. Schematic for disruption of the lig4 gene showing the probe locations for Southern blot analysis and confirmation of Δlig4 strains by Southern blot. (A) The figure illustrates homologous integration of the pBIG35ZΔL4 vector into the lig4 genomic gene locus in M. alpina 1S-4. Gray short bar indicates the position hybridized
by probe. *Dotted lines* indicate the position and base length of hybridization signals. (B) Southern hybridization analysis of the *Δlig4* gene strains. *XhoI-* or *SacI-*digested genomic DNA was hybridized with the probes shown in A. The asterisks demonstrate signals of an endogenous *ura5* gene. Lane 1, host (*ura5*) strain; Lane 2, *Δlig4* strain.

**Fig. 4.** Evaluation of gene targeting efficiency. (A) The figure illustrates homologous integration of the pBIG3CBZΔL4 vector into the *Δlig4* genomic gene locus in the *Δlig4::ura5* strain. *Black arrows*, a and b indicate the position of oligonucleotide primers used for PCR. (B) Confirmation of the replacement of the *ura5* marker to the *CBXB* marker by PCR. PCR amplification was carried out with Lig4 up F (a) and CBXB stop R (b) primers and results in a 3.5 kb product. Lane 1, the *Δlig4::ura5* strain used as a host strain; Lane 2, the strain having the *Δlig4* locus replaced with the *CBXB* marker from the *Δlig4::ura5* strain; Lane M, marker.

**Fig. 5.** Sensitivity of *M. alpina* 1S-4 host strain and *Δlig4* strain to methyl methanesulfonate (MMS). Spores were cultivated on SC agar plates with or without MMS for 8 days and colony diameter was measured.
Fig. 1 Kikukawa et al.

- pBIG35ZΔL4 (13.2 kb)
- or
- pBIG3CBZΔL4 (13.1 kb)

Diagram:
- ColEl ori
- oriV
- NPTIII
- TrfA
- RB
- 3’-lig4
- SdhBt
- ura5 or CBXB
- His550 p
- 5’-lig4
- LB
Fig. 2  Kikukawa et al.
Host strain genomic DNA

$\text{lig4}$-targeting DNA with the $\text{ura5}$ marker

$\Delta\text{lig4}$ locus with the $\text{ura5}$ marker

Fig. 3A  Kikukawa et al.
Fig. 3B  Kikukawa et al.
A

**lig4-targeting DNA with the ura5 marker**

Host strain genomic DNA

**Δlig4 locus with the CBXB marker**
Fig. 4B  Kikukawa et al.

3.5 kb
Fig. 5  Kikukawa et al.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
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<tbody>
<tr>
<td>Lig4 F1</td>
<td>YTIATYGTBGTBTA</td>
</tr>
<tr>
<td>Lig4 R1</td>
<td>AAYYTIMGITCHYCCIMG</td>
</tr>
<tr>
<td>Lig4 IPCR F</td>
<td>CCAGAGAGGTATGCTGCACCTATTTACACCAACCAATCC</td>
</tr>
<tr>
<td>Lig4 IPCR R</td>
<td>GCCAAACATCTCGAACGTAATTCTGATCCCCACTTTGCAAGGTC</td>
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<tr>
<td>Lig4 F1 Apa</td>
<td>GCGGGCCCTGACCTAACAGCCCCGACAATAC</td>
</tr>
<tr>
<td>Lig4 R1 Apa</td>
<td>GCGGGGCAAATATCATGTATACACCAACATGTCAG</td>
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<tr>
<td>Lig4 F2 Xba</td>
<td>GCTCTAGAAGACAGCCGTCACTCATACG</td>
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<tr>
<td>Lig4 R2 Nhe</td>
<td>GCGCTAGCAACTACTCTCGACACCCGCAAC</td>
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<tr>
<td>Lig4 down R</td>
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<tr>
<td>ura5 stop R</td>
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<tr>
<td>CBXB stop R</td>
<td>GATTACTCCAAAGCCATGTC</td>
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The underlined sequences show synthesized restriction enzyme sites.
**Fig. S1.** Schematic for disruption of the *lig4* gene showing PCR primer location and confirmation of Δ*lig4* strains by PCR. (A) The figure illustrates integration of the pBIG35ZΔL4 vector into the *lig4* genomic gene locus in *M. alpina* 1S-4. Black arrows, a ~ e, indicate the position of oligonucleotide primers used for PCR: a, Lig4 F1 ApaI; b, Lig4 R1 ApaI; c, Lig4 in F; d, Lig4 in R; e, ura5 400 R. (B) Selection of Δ*lig4* strains by PCR. Lane 1, primers a and b; Lane 2, primers c and d; Lane 3, primers a and e.