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Gene targeting in the oil-producing fungus *Mortierella alpina* 1S-4 and construction of a strain producing a valuable polyunsaturated fatty acid by gene targeting

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Abstract

To develop an efficient gene-targeting system in *Mortierella alpina* 1S-4, we identified the *ku80* gene encoding the Ku80 protein, which is involved in the nonhomologous end joining pathway in genomic double-strand break (DSB) repair, and constructed *ku80* gene-disrupted strains via single-crossover homologous recombination. The *Δku80* strain from *M. alpina* 1S-4 showed no negative effects on vegetative growth, formation of spores, or fatty acid productivity and exhibited high sensitivity to methyl methanesulfonate, which causes DSBs. Dihomo-γ-linolenic acid (DGLA)-producing strains were constructed by disruption of the *Δ5-desaturase* gene, encoding a key enzyme of bioconversion of DGLA to ARA, using the *Δku80* strain as a host strain. The significant improvement of gene-targeting efficiency was not observed by disruption of *ku80* gene, but the construction of DGLA-producing strain by disruption of the *Δ5-desaturase* gene was succeeded using the *Δku80* strain as a host strain. This report describes the first study on the identification and disruption of the *ku80* gene in zygomycetes and construction of a DGLA-producing transformant using a gene-targeting system in *M. alpina* 1S-4.

Keywords

1 *Mortierella alpina*, Ku80, homologous recombination, gene targeting, Δ5-desaturase,
2 dihomo-γ-linolenic acid

Introduction

Integration of exogenous DNA into the chromosome in all organisms follows two pathways of DNA double-strand break (DSB) repair: homologous recombination (HR) and nonhomologous end joining (NHEJ) pathways (Kanaar et al. 1998). The repair of DSBs is induced by both exogenous and endogenous triggers and causes detrimental DNA lesions (Haber 2000). In the mechanism of the HR pathway, the homologous region is used as a template and the exogenous DNA is integrated into the chromosome. In contrast, in the mechanism of the NHEJ pathway, the strand ends of the exogenous DNA are directly ligated into DSBs without a requirement of sequence identity. These two mechanisms for DSB repair are independent of each other and are considered to function competitively (Van Dyck et al. 1999). The repair of DSBs requires many associated proteins, such as the Rad protein group including Rad54, Rad51, Rad52, Mre11, and Xrs2 in the HR pathway (Kooistra et al. 2004; Krappmann 2007), and Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA ligase 4 (Lig4), and X-ray repair cross-complementing protein 4 (Xrcc4) in the NHEJ pathway (Critchlow and Jackson 1998; Daley et al. 2005). A pathway similar to HR has been confirmed ubiquitously in various organisms (bacteria, yeast, and human) (Krogh and Symington 2004; Shibata et al. 2001). In addition, important discoveries such as the

Rad51-independent HR and Ku80-independent NHEJ pathways and the occurrence of all nonhomologous chromosomal integration under the control of Lig4 have been reported (Ishibashi et al. 2006).

The yeast *Saccharomyces cerevisiae* mainly utilizes the HR system for DSB repair. Accordingly, gene targeting through the HR pathway in *S. cerevisiae* exhibits quite high efficiency (Schiestl et al. 1994). In contrast, many other organisms, including mammals, plants, insects, and filamentous fungi, predominantly use the NHEJ pathway for DSB repair, and exogenous DNA, even if it consists of a long homologous sequence, can be integrated into nonspecific regions in chromosomes. Disruption of the *ku70*, *ku80*, or *lig4* gene leads to an increase in the frequency of HR in filamentous fungi (Ishibashi et al. 2006; Ishidoh et al. 2014; Krappmann et al. 2006; Mizutani et al. 2008; Ninomiya et al. 2004; Takahashi et al. 2006; Tani et al. 2013). In particular, disruption of the *ku80* and/or *lig4* gene in *Neurospora crassa* and the *lig4* gene in *Aspergillus oryzae* have led to 100% targeting efficiency (Ishibashi et al. 2006; Mizutani et al. 2008; Ninomiya et al. 2004).

The oil-producing filamentous fungus *Mortierella alpina* 1S-4 is a producer of carbon 20 (C20) polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4ω6, ARA) and eicosapentaenoic acid (20:5ω3, EPA), which are rich in triacylglycerols

(Sakuradani et al. 2009). In addition, the lipid productivity of this fungus reaches 600 mg/g of dried mycelia. For these reasons, the fungus has been used as a model oleaginous microorganism for biosynthesis and accumulation of lipids, including PUFAs (Kawashima et al. 1995; Kikukawa et al. 2013; Sakuradani et al. 2005; Sakuradani et al. 2013; Sakuradani et al. 1999b; Sakuradani et al. 2008). In previous studies, several techniques for gene manipulation in this fungus, such as a host–vector system (Ando et al. 2009a; Takeno et al. 2004a; Takeno et al. 2005b), RNA interference (Takeno et al. 2005a), and transformation systems (Ando et al. 2009b; Takeno et al. 2004b), have been established. By use of such transformation systems, plasmid vectors are integrated randomly into the fungal genome.

To construct a high-producing strain of beneficial PUFAs from this fungus by metabolic engineering, an efficient gene-targeting system using HR is necessary. However, gene targeting by HR in this fungus is rarely attempted, given that NHEJ is predominant and the efficiency of HR is low. In this study, to evaluate function of Ku80 to HR in *M. alpina* 1S-4, we identified the *ku80* gene and constructed *ku80* gene-disrupted strains via single-crossover HR. Moreover, to evaluate the gene-targeting efficiency in the *ku80* gene-disruptant, we constructed of a dihomo-γ-linolenic acid (DGLA)-producing strain by *Δ5-desaturase* (*Δ5ds*)

gene-disruption.

Materials and methods

Enzymes and chemicals

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

Strains, media, and growth conditions

M. alpina 1S-4 is deposited in the Graduate School of Agriculture of Kyoto University, Japan (Sakuradani 2010) and the uracil auxotrophic strain (*ura5*$^{-}$ strain) (Takeno et al. 2004b) was used as a host strain. Czapek–Dox agar medium containing 0.05 mg/ml uracil was used for sporulation of the *ura5*$^{-}$ strain, as described previously (Takeno et al. 2004b). Synthetic complete (SC) medium was used as a uracil-free synthetic medium for cultivation of transformants derived from the *M. alpina* 1S-4 *ura5*$^{-}$ strain at 28°C (Takeno et al. 2004b). GY medium (20 mg/ml glucose and 10 mg/ml yeast extract) was used for fatty acid composition analysis and extraction of genomic DNA. GY agar medium containing 0.75 mg/ml 5-fluoroorotic acid (5-FOA) and 0.05 mg/ml uracil were used to confirm the growth of *ku80*-disrupted transformants (Boeke et al. 1984; Razanamparany and Bégueret 1986; Watrin et al. 1999). GY agar

medium containing 100 μg/ml carboxin was used for selection of *ku80*-disrupted transformants. *Escherichia coli* strain DH5α was used for DNA manipulation and grown on LB agar plates containing 50 μg/ml kanamycin. All solid media contained 2% agar.

Genomic DNA preparation

M. alpina 1S-4 host strain and transformants were cultivated in 100 ml of GY liquid medium at 28°C for 5 days with shaking at 100 rpm. Fungal mycelia were harvested by suction filtration and washed with sterile water. Preparation of genomic DNA was performed by a previously described method (Okuda et al. 2014).

Cloning and identification of the *ku80* gene from *M. alpina* 1S-4

Two highly degenerate primers, ku80 F and ku80 R (Table 1), were synthesized for cloning of the *ku80* cDNA, based on the amino acid sequences of Ku80 homologs from two filamentous fungi, *Rhizopus delemar* (accession EIE88285) and *Aspergillus clavatus* (accession XP_001272945). The sequences of the primers correspond to regions that encode IAIQMIVT and PFAGDVNTY peptides. PCR amplification was performed in a total volume of 50 μl containing 1 μg of genomic DNA, 0.25 μl of *Takara EX taq* polymerase (Takara Bio), 5 μl of 10× *EX Taq* buffer, 200 μM of each

dNTP, and 5 pM of primers, and performed as 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by one cycle of extension at 72°C for 5 min. The resulting 0.7-kb fragment was cloned into the pT7Blue T-Vector (Novagen, Merck KGaA, Darmstadt, Germany), and the nucleotide sequence was determined with a Beckman Coulter CEQ8000 system (Beckman Coulter, Fullerton, CA, USA). For cDNA synthesis and construction of a cDNA library, RNA extraction reagent Isogen (Nippon Gene, Tokyo, Japan) and a PrimeScript High Fidelity RT-PCR Kit (Takara Bio) were used, following the supplier's instructions.

To isolate whole *ku80* genomic DNA from *M. alpina* 1S-4, inverse PCR was performed with primers, ku80 IPCR F and ku80 IPCR R (Table 1). The *Sal*I/*Xho*I-digested genomic fragment was self-ligated and then used as a template. PCR amplification was performed in a total volume of 50 μl containing 500 ng of the template, 0.25 μl of *Takara EX taq* polymerase (Takara Bio), 5 μl of 10× *EX Taq* buffer, 200 μM of each dNTP, and 5 pM of each primer, and performed as follows: initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 3 min and one cycle of extension period at 72°C for 10 min. The amplified 3.3-kb fragment was cloned into the pT7Blue T-Vector and identified completely with the Beckman Coulter CEQ8000 system.

Construction of the plasmid vector for *ku80* gene-targeting

A binary vector pKSUku80 was constructed for *ku80* gene-targeting on the backbone of pKSU, which is pBluescript II KS (+) (Stratagene, La Jolla, CA, USA) ligated with a *ura5* gene marker cassette derived from the *M. alpina* 1S-4 transformation vector. A 4.3-kb fragment containing a partial *ku80* gene, amplified with ku80 start and ku80 *Xho*I R primers (Table 1) using *M. alpina* 1S-4 genomic DNA as a template, was cloned into the pUC118 using a Mighty Cloning Kit (Blunt End) (Takara bio). The resulting plasmid (pUC118ku80) was digested with *Hin*dIII and the digested partial *ku80* fragment was ligated into pKSU vector digested with the same restriction enzyme. The resulting plasmid was named pKSUku80 (Fig. 2A).

A pKSCD5 vector for the *Δ5ds* gene-targeting was constructed on the backbone of the *M. alpina* 1S-4 transformation vector pKSC. The *CBXB* gene expression cassette from pSBZNCBXB (Ando et al. 2009a), digested with *Eco*RI and *Xba*I, was ligated into pBluescript II KS (+) (Stratagene) digested with the same restriction enzymes, and the resulting plasmid was named pKSC. The partial *Δ5ds* gene (2024-bp) was amplified with two primers, Δ5 *Eco*RI F and Δ5 *Xho*I R primers (Table 1) and *M. alpina* 1S-4 genomic DNA as a template. The resulting fragment was digested with *Eco*RI and *Xho*I

and ligated into the pKSC plasmid digested with the same restriction enzymes. The resulting plasmid was named pKSCD5 (Fig. 5A).

Transformation of *M. alpina* 1S-4

The gene-targeting vectors were introduced into spores of *M. alpina* 1S-4 by biolistic particle bombardment with PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) (Takeno et al. 2004b). Given that linear plasmids are completely integrated into chromosomes by HR (Shiotani and Tsuge 1995), the vectors were digested with restriction enzymes and introduced into the spores: the pKSUku80 vector was digested with *Nco*I and the pKSCD5 vector was digested with *Nru*I. Spores (1.5×10^8) were spread on an agar plate using SC uracil-free medium for transformation with pKSUku80 or GY containing 5-FOA for transformation with pKSCD5. After bombardment, the plates were incubated at 28°C for 5 days. Transformants were transferred to a new GY plate containing 5-FOA.

Mutagen sensitivity

Sensitivity to chemical mutagen toxicity in gene-manipulated transformants was evaluated by spot test (Kato et al. 2004; Mizutani et al. 2008). Methyl methanesulfonate

(MMS) was added to GY agar medium at final concentrations of 0, 0.01, 0.02, 0.025, 0.05, and 0.1%.

Identification of gene-disruption by PCR and Southern blot analysis

The *ku80* gene-disrupted candidates were evaluated by means of colony-PCR using the extracted genomic DNA as a template and the primers ku80 start and ura5 stop R (Table 1). When integration into the genomic *ku80* gene locus was successful, a 4.3-kb fragment was amplified using the primers and genomic DNA from the transformants.

Correct homologous integration in the genomic *ku80* gene was confirmed by Southern blot analysis. The 1.0-kb partial *ku80* gene amplified with primers ku80 F2 and ku80 R2 (Table 1) using *M. alpina* 1S-4 genomic DNA as a template was used as a probe for hybridization. Southern blot hybridization was performed as described previously (Sakuradani et al. 1999a). Genomic DNA (10 μg) digested with *Xho*I was size-fractioned by electrophoresis in 1% agarose gel and transferred to Amersham Hybond-N^+ membrane (GE Healthcare Ltd., Buckinghamshire, UK) using a VacuGene XL Vacuum Blotting System (GE Healthcare). Southern hybridization was performed using the Gene Images AlkPhos Direct Labeling and Detection System (GE

Healthcare).

For Southern blotting analysis of *Δ5ds* gene-disruption, 1.7 kb of the *Δ5ds* gene fragment amplified with primers Δ5 F and Δ5 R (Table 1) using *M. alpina* 1S-4 genomic DNA as a template was used as a probe for hybridization. Genomic DNA (10 μg) was digested with the pair *Nar*I and *Xba*I or *Cla*I and *Xho*I. Southern blot hybridization was then performed as described above.

Fatty acid analysis

Fatty acid production and composition of transformants were analyzed as described previously (Kikukawa et al. 2013). In this study, mycelia of the transformants and the host strain were inoculated into 3 ml of GY liquid medium in a 20-ml Erlenmeyer flask and cultivated at 28°C with reciprocal shaking at 120 rpm for 7 days. The fungal strains after cultivation were harvested by filtration and dried at 120°C for 2 h. The dried cells were directly transmethylated with 10% methanolic HCl at 55°C for 2 h. The resulting fatty acid methyl esters were extracted with *n*-hexane, concentrated, and analyzed with a GC-17A gas chromatograph (GC; Shimadzu, Kyoto, Japan) equipped with an HR-SS-10 capillary column (Shinwa Chemical Industries, Kyoto, Japan). Fatty acids were quantified using tricosanoic acid as an internal standard. All

experiments were performed in triplicate.

Nucleotide sequence accessions

The *ku80* gene from *M. alpina* 1S-4 has been registered in the DNA Data Bank of Japan (DDBJ) database as accession LC009413. The *Δ5ds* genomic gene of *M. alpina* 1S-4 has been deposited in GenBank/EMBL/DDBJ as accession AB188307.

Results

Identification and phylogenetic analysis of the *ku80* gene from *M. alpina* 1S-4

To isolate the ku80 partial gene fragment, a 0.7-kb gene fragment was amplified by PCR using highly degenerate primers and *M. alpina* 1S-4 genomic DNA as a template. The predicted amino acid sequence encoded by the partial gene fragment showed high similarity to those of Ku80 proteins from other organisms. To identify the whole *ku80* gene from *M. alpina* 1S-4, inverse PCR was performed with *M. alpina* 1S-4 genomic DNA. The open reading frame of *ku80* gene from *M. alpina* 1S-4 was found to consist of 3366 bp. Based on the whole *ku80* gene information from genomic DNA, the full-length cDNA of the *ku80* gene was obtained by PCR. The *ku80* cDNA with 2511-bp length was predicted to encode a protein consisting of 836 amino acids. These results suggested that the *M. alpina* 1S-4 *ku80* genomic gene has nine exons (1–129, 257–311, 400–543, 653–821, 906–1467, 1580–1795, 1877–2043, 2141–3116, 3274–3366) and eight introns. The predicted amino acid sequence of *M. alpina* 1S-4 Ku80 shares low identities with those of metazoa (*Mus musculus*, 21%; *Rattus norvegicus*, 21%; *Homo sapiens*, 25%; *Tigriopus japonicas*, 21%), higher plants (*Hordeum vulgare*, 24%; *Triticum aestivum*, 25%; *Oryza sativa*, 25%; *Arabidopsis thaliana*, 22%), oleaginous yeast (*Rhodosporidium toruloides*, 24%), fungi (*Neurospora crassa*, 29%; *N.*

tetrasperma, 29%; *Lecanicillium* sp., 29%; *Aspergillus oryzae*, 28%; *A. sojae*, 28%; *A. fumigatus*, 29%; *Penicillium digitatum*, 28%). Compared with various Ku80 proteins from these organisms, the Ku80 from *M. alpina* 1S-4 is located in the expected position in the phylogenetic tree (Fig. 1).

Disruption of the *ku80* gene of *M. alpina* 1S-4 with pKSUku80 vector

A vector for *ku80* gene disruption, pSKUku80, digested with *Nco*I was delivered into spores of *M. alpina* 1S-4 *ura5*$^{-}$ strain by biolistic particle bombardment with a PDS-1000/He Particle Delivery System. To confirm integration of a *ura5* gene marker, all transformants grown on an SC uracil-free plate were inoculated onto GY medium containing 5-FOA. Finally, 77 transformants were obtained under these conditions. The transformants were selected by colony PCR with primers ku80 start and ura5 stop R (Table 1), and each genomic DNA as a template. Fragments of approximately 4.3-kb, which were formed presumably by integration via HR, were observed in only two transformants (3 and 6), but not in the host strain (Fig. S1 A and B).

The genome integration patterns of transformants 3 and 6 were confirmed by Southern blot analysis. Their genomic DNAs were digested with *Xho*I and a 1.0-kb fragment consisting of partial *ku80* gene was used as a probe (Fig. 2A). The 5.2-kb

hybridization signal on the host strain was not detected in the two transformants (Fig. 2B). However, the expected 4.0- and 8.3-kb signals resulting from single-crossover HR were detected only in transformant 3. These results suggest that a single pKSUku80 vector was successfully integrated into *ku80* genomic DNA of transformant 3. In contrast, some of the introduced pKSUku80 vectors appear to have been integrated ectopically into the *ku80* gene locus in transformant 6. Thus, transformant 3 was used as a host strain for *Δ5ds* gene disruption in the present research.

Growth characteristics and mutagen sensitivity

Given that Ku70, Ku80, and Lig4 proteins are involved in DSB repair through NHEJ in diverse organisms (Hopfner et al. 2002; Lisby and Rothstein 2004) and telomere maintenance in some organisms (Hande 2004), the growth characteristics and mutagen sensitivity of *M. alpina* 1S-4 *Δku80* strain were investigated. The growth rate of the *Δku80* strain did not decrease, compared with that of the wild strain both on plate medium and in liquid medium (data not shown). In addition, the germination rate of its spores was similar to that of the wild strain (data not shown). Furthermore, given that the fatty acid productivity and composition of the *Δku80* strain were similar to those of the wild strain, we infer that *ku80* gene disruption did not affect fatty acid productivity

(Table 3). The sensitivity to chemical mutagens causing DSBs, MMS, of *M. alpina* 1S-4 *Δku80* strain was evaluated as described previously (Ishibashi et al. 2006; Ninomiya et al. 2004). The *Δku80* strain showed no sensitivity to low (≤0.02%) concentrations of MMS, but showed high sensitivity to 0.05% MMS (Fig. 3).

Construction and characterization of *Δ5ds* gene disruptant with pKSCD5 vector

To evaluate the improvement of gene-targeting efficiency in the *Δku80* strain and construct a strain producing valuable PUFAs by use of gene targeting, *Δ5ds* gene disruption causing an increase in DGLA production and a decrease in ARA production (Fig. 4) was performed in the *Δku80* strain as a host strain (Fig. 5A). A vector for *Δ5ds* gene disruption, pKSCD5, which contains the *CBXB* marker, was digested with *Nru*I to enhance gene targeting efficiency and was introduced into spores of the *Δku80* strain on GY medium containing 100 μg/ml carboxin by biolistic particle bombardment. After bombardment, the spores were cultivated at 28°C for 5 days. Finally, 32 stable transformants were obtained.

All stable transformants, the wild type, and the *Δku80* strain were cultivated in 3 ml of GY medium at 28°C for 7 days with reciprocal shaking, and their fatty acid productivities were determined by GC analysis. The ratio of DGLA to total fatty acids

reached 36.8% in transformant 15, whereas that of ARA was only 3.4% (Fig. 6 and Table 3). Transformant 15 exhibited the same fatty acid composition as that of the *Δ5ds* gene-defective mutant S14 isolated previously (Jareonkitmongkol et al. 1993) (Table 3). To confirm the disruption of the *Δ5ds* gene in transformant 15, Southern blot analyses were performed with genomic DNAs prepared from the *Δku80* strain and transformant 15 (Fig. 5B). When the genomic DNAs were digested with *Cla*I and *Xho*I, the 4.3-kb hybridization signal corresponding to the original *Δ5ds* open reading frame was not detected in transformant 15, but the 3.3- and 7.5-kb signals were detected. When the genomic DNAs were digested with *Nar*I and *Xba*I, the 3.7-kb signal was not detected in transformant 15, but the expected 4.2- and 6.0-kb signals were detected. These results showed that the *Δ5ds* gene in transformant 15 was successfully disrupted by integration of the pKSCD5 vector. Unexpected signals in transformant 15, however, were observed on the Southern blot. This finding may mean that several pKSCD5 vectors had been introduced into random sites in the genomic DNA of the *Δku80* strain by biolistic particle bombardment.

Discussion

To improve gene-targeting efficiency in *M. alpina* 1S-4, we cloned and identified the *ku80* gene encoding the Ku80 protein, which forms a Ku-protein complex with Ku70 protein, and is involved in the NHEJ pathway. Ku80 homolog proteins of other organisms were classified by kingdom (Fig. 1). However, the predicted translation product of the *ku80* gene of this fungus shares low (<30%) identities with those of other organisms. We constructed a *ku80* gene disruptant (transformant 3 in Fig. 2B) via HR using the pKSUku80 vector. In transformant 6, we speculated that some of the introduced pKSUku80 vectors were integrated ectopically into the *ku80* gene locus (Fig. 2B). In general, one of the problems of biolistic particle bombardment is the delivery of many plasmids into cells. However, in view of the result for transformant 3, the biolistic particle bombardment method is applicable to the integration of a single vector into the genome via HR. The *Δku80* strain showed no marked differences in vegetative growth, formation of spores, or fatty acid productivity compared with the host strain (Table 3). Thus, we expect the *Δku80* strain to be a superior host strain for metabolic engineering for PUFA production. Furthermore, the *Δku80* strain exhibited a sensitivity to 0.05% MMS (Fig. 3) similar to those of *N. crassa*, *A. fumigatus*, and *A. aculeatus* (da Silva Ferreira et al. 2006; Ninomiya et al. 2004; Tani et al. 2013). Such sensitivity indicates

that the NHEJ pathway in this strain is repressed. To evaluate the improvement of gene-targeting efficiency and construct a beneficial PUFA-producing strain, the disruption of *Δ5ds* gene was performed using the *Δku80* strain as a host strain. The *Δ5ds* gene disruptant, transformant 15, produced a large amount of DGLA. The DGLA productivity of transformant 15 was at the same level (Table 3) as that of a *Δ5ds* gene-defective mutant obtained by chemical mutagenesis (Jareonkitmongkol et al. 1993). However, chemical mutagens cause mutation in multiple locations in the genome and often suppress growth, spore germination, and PUFA production. The ARA production of the *Δ5ds* gene disruptant was drastically decreased. Though we isolated a single colony from spore of the *Δ5ds* gene disruptant to avoid contamination of intact spores, quite low level of ARA remained. Given that the pKSCD5 vector was integrated via single-crossover HR, the incomplete Δ5-desaturase may act in catalyzing the conversion of DGLA to ARA. For this reason, future gene targeting should be performed via double-crossover HR. Southern blot analysis using the genomic DNA from the *Δ5ds* gene disruptant suggested that several vectors were integrated into ectopic sites on the chromosome. To obtain a complete disruptant with a single plasmid, more transformants should be isolated and checked, or gene targeting should be performed by an alternative transformation method such as an *A. tumefaciens*-mediated

method, introducing a single vector into a spore of the host strain.

Only one of the 77 transformants was detected as a *ku80*-gene disruptant (Table 2). Using of the *ku80* gene-disrupted strain as a host, one of the 32 transformants was detected as *Δ5ds*-gene disruptant. In previous research, *Δ6ds*-gene disruption was attempted in *Mortierellaceae*, *M. isabellina*, by biolistic particle bombardment and more than 70 transformants were obtained. However, none was disrupted in its *Δ6ds*-gene (Zhang et al. 2007). In this study, we found that we obtained at least one of 32 transformant by using *Δku80* strain. The efficiencies of gene targeting in *Δku80* strains from *A. sojae*, *A. oryzae*, *A. fumigatus*, *A. niger*, and *Lecanicillium* sp. were significantly improved (da Silva Ferreira et al. 2006; Honda et al. 2011; Ishidoh et al. 2014; Takahashi et al. 2006). However, the efficiency in the *Δku80* strain from *M. alpina* 1S-4 was hardly improved. In a previous study in *N. crassa*, chromosomal integration of exogenous DNA was achieved via two types of HR and two types of NHEJ, the Ku80-dependent major pathway and the Ku80-independent minor pathway (Ishibashi et al. 2006). In *M. alpina* 1S-4, it is suggested that the Ku80-independent pathway may play a major pathway of NHEJ and reduce gene-targeting efficiency. When the *Δku80* strain was used as a host, the incomplete Ku80 protein formed a Ku-protein complex with the Ku70 protein, and the pKSCD5 vector was integrated

ectopically via the NHEJ pathway. The loss of Lig4 activity involved in both the major and minor NHEJ pathways raised the targeting efficiency to 100% in *A. oryzae* and *A. luchuensis* (Mizutani et al. 2008; Takahashi et al. 2011). Further improvement in targeting efficiency in the *Δku80* strain, such as by simultaneous disruption of the *lig4* gene, might facilitate metabolic engineering and reverse-genetic studies in *M. alpina* 1S-4.

In summary, this report is the first to date to describe the identification and disruption of the *ku80* gene in Mucoromycotina fungi. We succeeded in gene targeting in *M. alpina* 1S-4. On the other hand, though a *ku80* gene disruptant showed normal growth, germination, and lipid production, gene-targeting efficiency was hardly improved. On the other hand, we achieved to construct a *Δ5ds*-gene disruptant using the *Δku80* strain as a host strain. This gene-targeting system may contribute to the construction of various PUFA-producing strains via metabolic engineering.

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Figure legends

Fig. 1 Phylogenetic tree of Ku80 proteins. The tree was created by the neighbor-joining (NJ) method with 10,000 bootstrap replicates using the sequence analysis software GENETYX 11.0 (Genetyx corp., Tokyo, Japan). MmKu80 (*Mus musculus*), AAH51660; RnKu80 (*Rattus norvegicus*), NP_803154; HsKu80 (*Homo sapiens*), P13010; TjKu80 (*Tigriopus japonicus*), AIL94178; HvKu80 (*Hordeum vulgare* subsp. *vulgare*), AEO86624; TaKu80 (*Triticum aestivum*), ADO00729; OsKu80 (*Oryza sativa* Japonica Group), Q75IP6; AtKu80 (*Arabidopsis thaliana*), AEE32242; MaKu80 (*Mortierella alpina* 1S-4), LC009413; RtKu80 (*Rhodosporidium toruloides*), AIA21644; NcKu80 (*Neurospora crassa*), AFM68948; NtKu80 (*N. tetrasperma* FGSC 2508), EGO57771; *Lecanicillium* sp. Ku80 (*Lecanicillium* sp. HF627), AHY22503; AoKu80 (*Aspergillus oryzae*), BAE78503; AsKu80 (*A. sojae*), BAE78504; AfKu80 (*A. fumigatus* Af293), Q4WI96; PdKu80 (*Penicillium digitatum*), AGT79985.

Fig. 2 Construction scheme of *ku80* gene disruptant and confirmation by Southern blot analysis. (A) The figure illustrates the homologous integration of pKSUku80 vector into the *ku80* genomic gene locus in *M. alpina* 1S-4. *Gray short bar* indicates the position hybridized by the probe. *Dotted lines* indicate the position and base lengths of hybridization signals. (B) Southern hybridization analysis of *ku80* gene-disrupted candidates. *Xho*I-digested genomic DNAs from transformants 3 and 6 and host strain were hybridized with the probe.

Fig. 3 Sensitivity of *M. alpina* 1S-4 wild strain, host strain (uracil auxotrophic mutant),

and *Δku80* strain to methyl methanesulfonate (MMS). Their spores were spotted and grown on GY agar plate without MMS or containing 0.05% MMS for 4 days.

Fig. 4 Biosynthetic flow of ARA. ARA is biosynthesized by desaturation at the Δ5-position of DGLA by Δ5-desaturase. LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; ARA, arachidonic acid; Δ6, Δ6-desaturase; GLELO, Δ6-elongase; Δ5, Δ5-desaturase.

Fig. 5 Construction scheme of *Δ5ds* gene disruptant and confirmation by Southern blot analysis. (A) The figure illustrates the homologous integration of the pKSCD5 vector into the *Δ5ds* genomic gene locus in *M. alpina* 1S-4. *Gray short bar* indicates the position hybridized by the probe. *Dotted lines* indicate the position and base lengths of hybridization signals. (B) Southern hybridization analysis of *Δ5ds* gene-disrupted candidates. *Nar*I- and *Xba*I- or *Cra*I- and *Xho*I-digested genomic DNAs were hybridized with the probe. Lane #15 and *Δku80* indicate a *Δ5ds* gene-disrupted candidate and the host strain (*ku80*-disruptant), respectively.

Fig. 6 GC chromatograms of fatty acid methyl esters prepared from total lipids of the *Δku80* strain (A) used as a host strain and the *Δ5ds* gene disruptant #15 (B).