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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Identification and characterization of Δ12 and Δ12/Δ15 bifunctional fatty acid desaturases in the oleaginous yeast *Lipomyces starkeyi*

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Abstract

Fatty acid desaturases play vital roles in the synthesis of unsaturated fatty acids. In this study, Δ12 and Δ12/Δ15 fatty acid desaturases of the oleaginous yeast *Lipomyces starkeyi*, termed LsFad2 and LsFad3, respectively, were identified and characterized. *Saccharomyces cerevisiae* expressing *LsFAD2* converted oleic acid (C18:1) to linoleic acid (C18:2), while a strain of *LsFAD3*-expressing *S. cerevisiae* converted oleic acid to linoleic acid, and linoleic acid to α-linolenic acid (C18:3), indicating that LsFad2 and LsFad3 were Δ12 and bifunctional Δ12/Δ15 fatty acid desaturases, respectively. The overexpression of *LsFAD2* in *L. starkeyi* caused an accumulation of linoleic acid and a reduction in oleic acid levels. In contrast, overexpression of *LsFAD3* induced the production of α-linolenic acid. Deletion of *LsFAD2* and *LsFAD3* induced the accumulation of oleic acid and linoleic acid, respectively. Our findings are significant for the commercial production of polyunsaturated fatty acids, such as ω-3 polyunsaturated fatty acids, in *L. starkeyi*.

Keywords Δ12 fatty acid desaturase · Δ15 fatty acid desaturase · Polyunsaturated fatty acids · Oleaginous yeast · Lipid

Introduction

Oleaginous yeasts, such as *Lipomyces starkeyi*, *Yarrowia lipolytica*, *Cryptococcus curvatus*, and *Rhodosporidium toruloides*, are a potential cost-effective means of producing microbial oils without competing with food production. These microorganisms are able to store intracellular lipids to approximately 60% of their cell dry weight (Ageitos et al. 2011; Kosa and Ragauskas 2011; Donot et al. 2014). Oleaginous yeasts boast several advantages for lipid production over other oleaginous microorganisms, such as molds or algae, due to their unicellular form and high growth rates. In addition, oleaginous yeasts are able to produce lipids from a large variety of renewable substrates, including sugars derived from non-edible biomasses. The genetic engineering of oleaginous yeasts is a powerful tool for optimizing both the quantity and quality of produced lipids (Wang et al. 2013; Shi and Zhao 2017). For example, strains of *Y. lipolytica* were engineered to produce the ω-3 fatty acid, eicosapentaenoic acid (EPA) (Xue et al. 2013). *L. starkeyi*, which produces triacylglycerols (TAGs) to more than 70% of its dry cell weight (Angerbauer et al. 2008), is one of the most attractive and well-studied oleaginous yeasts. The complete genome of *L. starkeyi* is available from the Joint Genome Institute (JGI) website (http://genome.jgi.psf.org/Lipst1_1) (Riley et al. 2016). In addition, several genetic engineering tools for *L. starkeyi* have already been developed, including transformation (Calvey et al. 2014), multicopy integration and expression of heterologous genes (Oguro et al. 2015), and efficient gene targeting for gene deletion and integration using non-homologous end-joining-deficient strains (Oguro et al. 2017).

Fatty acids play vital roles in the functions of cytoplasmic and organelle membranes, and in carbon source storage. In *L. starkeyi*, major fatty acid species are saturated fatty acids with 16 carbon atoms (palmitic acid, C16:0) and monounsaturated (single double bond) fatty acids containing 18 carbon atoms (oleic acid, C18:1). In the most studied model yeast, *S. cerevisiae*, palmitic acid (C16:0) is synthesized from

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acetyl-CoA and NADPH by acetyl-CoA carboxylase and fatty acid synthase. Monounsaturated fatty acids are synthesized from saturated fatty acids by Δ9 fatty acid desaturase Ole1p (Stukey et al. 1989; Martin et al. 2007; Henry et al. 2012). Fatty acid elongases convert 16-carbon fatty acids to 18-carbon fatty acids (Toke and Martin 1996; Schneiter et al. 2000).

In addition to palmitic acid (C16:0) and oleic acid (C18:1), *L. starkeyi* contains small amounts of polyunsaturated fatty acids, C18:2 (linoleic acid) and C18:3 (A-linolenic acid), abbreviated here as LA and ALA, respectively. In *L. starkeyi* and many other yeast species, second and third double bonds are introduced by Δ12 and Δ15 fatty acid desaturases, respectively. Previously, the enzymatic activity of *L. starkeyi* Δ12 fatty acid desaturase was measured using microsomal system extracted from *L. starkeyi* cells (Lomascolo et al. 1996). This study yielded the following information: (1) the optimal pH was between pH 7 and 8, (2) thermal stability was low, (3) the enzyme was inhibited by Hg^{2+} and activated by Mg^{2+}, Mn^{2+}, and Zn^{2+}, and (4) oleoyl-CoA was the preferred substrate (Lomascolo et al. 1996). However, the gene(s) encoding Δ12 fatty acid desaturase has (have) not been identified. The identification of Δ12 fatty acid desaturase gene(s) is essential for the modification of lipid content, the production of polyunsaturated fatty acids, and the physiological analyses of unsaturated fatty acids in *L. starkeyi* cells. Some Δ12 and Δ15 fatty acid desaturases have been identified in yeasts, plants, and animals (Watanabe et al. 2004; Wei et al. 2004; Kainou et al. 2006; Wei et al. 2006; Matsuda et al. 2012; Radovanovic et al. 2014; Sangwallek et al. 2014; Angelis et al. 2016; Lee et al. 2016; Sun et al. 2016). In addition, some fatty acid desaturases are reportedly bifunctional, exhibiting both Δ12 and Δ15 desaturase activities (Damude et al. 2006; Zhou et al. 2011; Yan et al. 2013; Cui et al. 2016). Both Δ12 and Δ15 fatty acid desaturases are key enzymes for the production of ω-3 polyunsaturated fatty acids, including EPA and docosahexaenoic acid (DHA).

In this study, we identified and characterized Δ12 and Δ12/Δ15 bifunctional fatty acid desaturases, termed LsFad2 and LsFad3, respectively, in *L. starkeyi*. Genes encoding these desaturases were not essential for *L. starkeyi* growth, but they contributed significantly to the production of polyunsaturated fatty acids, especially at low temperatures. The overexpression of *LsFAD2* or *LsFAD3* induced an accumulation of LA or ALA, respectively, indicating that these genes encoded key enzymes for the production of polyunsaturated fatty acids including ω-3 polyunsaturated fatty acids.

Materials and methods

Strains and media

The *L. starkeyi* and *S. cerevisiae* strains used in this study are listed in Table 1. *L. starkeyi* strains were grown under aerobic conditions at 30 or 20 °C for the indicated time in GY (5% glucose and 0.5% yeast extract) or YPD (2% glucose, 2% peptone, and 1% yeast extract) medium. When necessary, 50 μg/ml zeocin (Nacalai Tesque, Kyoto, Japan) and/or 100 μg/ml hygromycin B (Wako Pure Chemical Industries, Osaka, Japan) were added to the YPD medium. *Escherichia coli* DH5α competent cells were purchased from Nippon Gene (Tokyo, Japan).

Genetic manipulation

Transformation of *L. starkeyi* was performed as described previously (Oguro et al. 2015), and transformants were selected on YPD medium containing 100 μg/ml of hygromycin B. Total DNA was extracted using the GenTLE (Yeast) High Recovery system (Takara Bio, Shiga, Japan). Total RNA was extracted using the NucleoSpin RNA system (Takara Bio) after mechanical cell disruption.

Expression of *LsFAD2* and *LsFAD3* genes in *S. cerevisiae*

pL1091-5/DGA1ΔN (*DGA1ΔN* encodes Dga1p with 29 N-terminal amino acids deleted) was constructed as described previously (Kamisaka et al. 2013). The *L. starkeyi* Δ12 desaturase gene (termed *LsFAD2*) (protein ID: 68481) was amplified by polymerase chain reaction (PCR) with a high-fidelity DNA polymerase (PrimeSTAR GXL; Takara Bio) using *L. starkeyi* cDNA as a template with the following primers: 5′-GCAAGCTTATGTCCACAATAACATAC-3′ (the *Hin*dIII site is underlined) and 5′-ATGCGGCCGC TTACTGAGCCCTTCTT-3′ (the *Not*I site is underlined). The amplified product was excised as a *Hin*dIII-*Not*I fragment and used to construct pL1177-2/*LsFAD2*. The *L. starkeyi* Δ12/Δ15 desaturase gene (termed *LsFAD3*) (protein ID: 310193) sequence was codon-optimized for expression in *S. cerevisiae* and synthesized by GenScript (Piscataway, NJ, USA). This was then excised as a *Hin*dIII-*Not*I fragment and used to construct pL1177-2/*LsFAD3*. The correctness of the constructed vectors was verified by DNA sequencing.

S. cerevisiae BY4741 Δ*snf2* mutant was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA) (Table 1). The yeast cells were transformed by the lithium acetate method (Ito et al. 1983) using a transformation kit (Zymo Research; Orange, CA, USA). The Δ*snf2* mutant was transformed by plasmids pL1091-5/ DGA1ΔN (Kamisaka et al. 2013), pL1177-2 harboring *LsFAD2* or *LsFAD3* (described above), and pL2137-26 (Kainou et al. 2006). pL1091-5, pL1177-2, and pL2137-26 are yeast expression vectors with 2-μm replication origins, an *ADH1* promoter, and a yeast selection marker (*URA3*, *LEU2*, *HIS3*, respectively) (Kainou et al. 2006). The transformed yeast cells were grown under aerobic conditions at 20 °C for 7 days in nitrogen-limited SD medium

Table 1 Yeast strains used in this study

Strain name	Relevant genotype	Reference and source
Lipomyces starkeyi		
Δ*lslig4**	*lslig4*::P*TDH3-Sh ble*-T*TDH3*	Oguro et al. 2017
Δ*lslig4* Δ*lsfad2*	*lslig4*::P*TDH3-Sh ble*-T*TDH3*, *lsfad2*::P*TDH3-hph*-T*TDH3*	This study
Δ*lslig4* Δ*lsfad3*	*lslig4*::P*TDH3-Sh ble*-T*TDH3*, *lsfad3*::P*TDH3-hph*-T*TDH3*	This study
Δ*lslig4* + *LsFAD2*	*lslig4*::P*TDH3-Sh ble*-T*TDH3*, 18S rDNA::P*TDH3-LsFAD2*-T*TDH3*	This study
Δ*lslig4* + *LsFAD3*	*lslig4*::P*TDH3-Sh ble*-T*TDH3*, 18S rDNA::P*TDH3-LsFAD3*-T*TDH3*	This study
Saccharomyces cerevisiae		
BY4741 Δ*snf2*	*Mat a, leu2Δ0, his3Δ1, ura3Δ0, met15Δ0, snf2::kanMX4*	Invitrogen Life Technologies

*Δ*lslig4* strain was generated from a *L. starkeyi* CBS1807 strain

containing 0.17% Bacto-yeast nitrogen base without amino acids and ammonium sulfate (Wako Pure Chemical Industries), 5% glucose, 0.25% ammonium sulfate, and 20 μg/ml methionine. In some experiments, 0.2 mg/ml linoleic acid was added to the medium with 0.25% Tergitol NP-40.

Overexpression of *LsFAD2* and *LsFAD3* genes in *L. starkeyi*

The construct for the overexpression of *LsFAD2* or *LsFAD3* is shown in Fig. 1a. pKS-18S-*hph*-*LsFAD2* and pKS-18S-*hph*-*LsFAD3*, which were used for the overexpression of *LsFAD2* and *LsFAD3* genes in *L. starkeyi*, were constructed by the insertion of artificially synthesized DNA between the *Pme*I and *Avr*II sites of pKS-18S-*hph* (Oguro et al. 2015). The *hph* gene encoded hygromycin B phosphotransferase. First, we designed a DNA sequence containing an *LsTDH3* promoter region (P_{TDH3}), the *LsFAD2* or *LsFAD3* gene, and a *TDH3* terminator region (T_{TDH3}), in which *Pme*I and *Avr*II sites were added to the 5′ and 3′ ends, respectively. The synthesized DNA sequences for the overexpression of *LsFAD2* or *LsFAD3* are shown in the Supplemental Materials, Table S1. These synthetic DNA fragments were created and cloned in pUC57 by GENEWIZ (South Plainfield, NJ, USA). Second, pKS-18S-*hph* plasmid was amplified by PCR using PrimeSTAR Max Polymerase (Takara Bio) with the following primers: 5′-ATTGCCCTAGGAACTAGCTCAAGGG ACGTGCTATTCCCAC-3′ (the *Avr*II site is underlined) and 5′-ATTGCGTTTAAACATGTAGCGGGTGGTGATGGT GGAAC-3′ (the *Pme*I site is underlined). The resulting PCR products and synthesized DNA fragments were digested with *Avr*II and *Pme*I and ligated using Ligation High (TOYOBO, Osaka, Japan). The resultants were termed

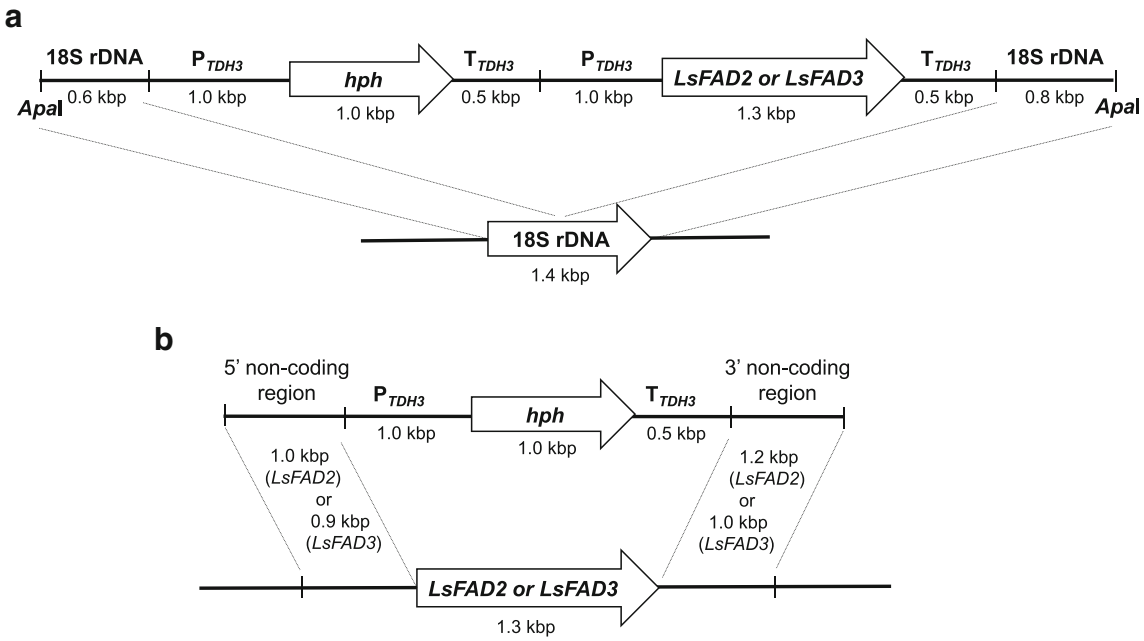

Fig. 1 Construction of **a** overexpression and **b** deletion of *LsFAD2* and *LsFAD3* genes. P_{TDH3} and T_{TDH3} indicate the promoter and terminator regions of the *L. starkeyi* TDH3 gene, respectively. Homologous recombination is indicated by dotted lines

pKS-18S-*hph*-P_{TDH3}-*LsFAD2*-T_{TDH3} and pKS-18S-*hph*-P_{TDH3}-*LsFAD3*-T_{TDH3}, respectively. The Δ*lslig4* strain was transformed with pKS-18S-*hph*-P_{TDH3}-*LsFAD2*-T_{TDH3} or pKS-18S-*hph*-P_{TDH3}-*LsFAD3*-T_{TDH3} digested by *Apa*I, and transformants were selected on YPD medium containing 100 μg/ml hygromycin B.

Construction of *LsFAD* deletion mutants

The construct for the deletion of the *LsFAD2* or *LsFAD3* gene is shown in Fig. 1b. Two DNA fragments of 5′- and 3′-non-coding regions of the *LsFAD2* gene (1033 and 1159 bp, respectively) were amplified by PCR using the genomic DNA of *L. starkeyi* and the following primers: 5′-GCTG GGTACCGGGCCCTAAAGTACAGAGCATCGTTTG-3′ and 5′-CGAAGTCGTTTAATTGGCGATGCTCTGCACTG GGTG-3′ (for the 5′-non-coding region of the *LsFAD2* gene) and 5′-GTGGTGGGCGATGTATTTAATTAATTTAATTT ATAACATTGTTTTAACACA-3′ and 5′-CTCG AGGGGGGGCCCGGACACAGACGGATCAAGCGTCT TGACT-3′ (for the 3′-non-coding region of the *LsFAD2* gene), respectively. Two DNA fragments of the 5′- and 3′-non-coding regions of the *LsFAD3* gene (922 and 1039 bp, respectively) were amplified by PCR using the genomic DNA of *L. starkeyi* and the following primers: 5′-CTGG GTACCGGGCCCGTAGAGATCTCACAATTGCTGTC AGAC-3′ and 5′-CGAAGTCGTTTAATTTGTTGTTCTAG TTCAGATATTCCG-3′ (for the 5′-non-coding region of the *LsFAD3* gene) and 5′-GTGGTGGGCGATGTAGCGAG ATCCTATCTTAGACTCTTCCAC-3′ and 5′-CTCG AGGGGGGGCCCAAGAATAATCTCCAAGGAATGAA CATATAGCTAT-3′ (for the 3′-non-coding region of the *LsFAD3* gene), respectively. The *hph* gene cassette containing the *LsTHD3* promoter and terminator regions was amplified by PCR using pKS-18S-*hph* (Oguro et al. 2015) as a template with the following primers: 5′-AATTAAACGACTTC GCCAAACGGAGACCATGCATTC-3′ and 5′-TACA TCGCCCACCACTACCACCTTGTTTCAACAA-3′. pBluescript KS (+) vector was amplified by PCR using pKS-18S-*hph* as a template with the following primers: 5′-GGGC CCCCCCTCGAGGTCGACGGTATCGATAA-3′ and 5′-GGGCCCGGTACCCAGCTTTTGTTCCCTTTAGTG-3′. These PCR fragments (pBluescript KS (+) vector, approximately 1 kbp of the 5′- and 3′-non-coding regions of *LsFAD2* or *LsFAD3* genes, *hph* gene cassette) were linearized using an In-Fusion HD cloning kit (Takara Bio). The resultant plasmid vectors were termed pKS-*LsFAD2*::*hph* and pKS-*LsFAD3*::*hph*, respectively.

Next, pKS-*LsFAD2*::*hph* and pKS-*LsFAD3*::*hph* were digested by *Apa*I and *Pvu*II, respectively, and introduced into *L. starkeyi* Δ*lslig4* to construct *LsFAD2* and *LsFAD3* deletion mutants.

Lipid analysis

The direct transmethylation of all fatty acid residues in yeast cells was carried out using methanolic 10% (*v*/*v*) HCl and methylene chloride, as described previously (Kamisaka et al. 2006). Aliquots of yeast cultures were taken at the indicated times and collected by centrifugation at 1400×*g* for 5 min. Cell pellets were washed once with distilled water and dried at 105 °C for 3 h. The dried pellets were then resuspended in 1 ml 10% methanolic HCl and 0.5 ml methylene chloride. After incubating at 60 °C for 3 h, 1 ml of saturated NaCl and 1 ml of hexane were added to the mixture. The resulting fatty acid methyl esters were analyzed on a gas chromatography-mass spectrometer (GC-MS) (QP2010 SE; Shimadzu, Kyoto, Japan) equipped with a DB-225MS capillary column (30 m × 0.25 mm i.d., Agilent Technologies, Santa Clara, CA, USA). Total fatty acids were quantified using heptadecanoic acid methyl ester as an internal standard.

Real-time quantitative reverse transcription PCR

L. starkeyi cells (Δ*lslig4*, *LsFAD2*- and *LsFAD3*-overexpressing strains) were pre-cultured in YPD medium at 30 °C for 3 days and inoculated into GY medium. The cultures were incubated at 150 rpm, at 20 or 30 °C for 2 or 4 days. After incubation, cells were collected by centrifugation at 11,000×*g* for 2 min and washed with distilled water. Total RNA was extracted using a NucleoSpin RNA system (Takara Bio) and reverse transcription PCR was performed using a PrimeScript II 1st-strand cDNA Synthesis Kit (Takara Bio). cDNA was synthesized from 100 ng of total RNA.

Real-time quantitative PCR (qRT-PCR) was performed using LightCycler 480 SYBR Green I Master and LightCycler 480 II (Roche, Basel, Switzerland). qRT-PCR conditions were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 10 s. The melting program was 95 °C for 5 s and 65 °C for 1 min. Primer pairs used were as follows: 5′-GCCGGAGTACACAATCAAAGA-3′ and 5′-TAAGAGAGCGGTCGAAGCAG-3′ for the *LsFAD2* gene, 5′-AGGGCGCGACAGCTACTAT-3′ and 5′-ATGA TGCTGTGGAACAGGTG-3′ for the *LsFAD3* gene, and 5′-ACCCAGATTGTCTTTGAGACG-3′ and 5′-GACA GCCTGGATGGAGACA-3′ for the *LsACT1* gene (ID of JGI data base 67392). Gene expression data from qRT-PCR were analyzed using the ΔΔCt method (Livak and Schmittgen 2001). Gene expression ratios were normalized to actin (*LsACT1* gene) using the $2^{-\Delta\Delta Ct}$ method and determined by the following equations: ΔCt = Ct (target gene) − Ct (actin).

Nucleotide sequence accession number

The synthesized DNA sequence of *LsFAD3* for the heterologous expression in *S. cerevisiae* was deposited in DDBJ/EMBL/GenBank under the accession number LC387462.

Results

Identification of putative Δ12 desaturases in *L. starkeyi*

Two putative Δ12 desaturases of *L. starkeyi*, termed LsFad2 and LsFad3, were identified by searching the BLAST database (https://blast.ncbi.nlm.nih.gov/) using amino acid sequences of *Fusarium verticillioides* Δ12 desaturase (GenBank accession no. ABB88515.1, Damude et al. 2006) and Δ12/Δ15 bifunctional desaturase (GenBank accession no. ABB88516.1, Damude et al. 2006). The protein IDs of LsFad2 and LsFad3 were 68481 and 310193 in the JGI database, respectively. The length of the *LsFAD2* and *LsFAD3* genes was 1263 and 1330 bp, respectively, and both genes contained one putative intron region (*LsFAD2*: 54 bp, *LsFAD3*: 52 bp). The *LsFAD2* and *LsFAD3* genes encoded proteins containing 402 and 425 amino acids, respectively. LsFad2 showed a low amino acid sequence similarity to *F. verticillioides* Δ12 fatty acid desaturase (identity, 50%) and Δ12/Δ15 bifunctional fatty acid desaturase (identity, 44%). Likewise, LsFad3 showed low amino acid sequence similarity with *F. verticillioides* Δ12 (identity, 44%) and Δ12/Δ15 bifunctional (identity, 48%) fatty acid desaturases (Table 2). The LsFad2 and LsFad3 also showed low similarity with each other (identity, 50%). Among these four proteins, there is some consensus in the amino acid sequences, as indicated by the asterisk in Fig. 2. Both LsFad2 and LsFad3 contained three histidine-rich motifs, H-box 1 to 3 (Fig. 2), which are conserved in many fatty acid desaturases that act as potential ligands for non-heme iron atoms and are essential for desaturase activity (Shanklin et al. 1994; Avelange-Macherel et al. 1995; Shanklin et al. 1997; Shanklin and Cahoon 1998). Based on our analyses of the deduced amino acid sequences of LsFad2 and LsFad3 using TMHMM Server ver. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Möller et al. 2001), LsFad2 and LsFad3 were predicted to have more than three and five transmembrane helices, respectively (Fig. 2). We also compared the deduced amino acid sequences of LsFad2 and LsFad3 to those of other fatty acid desaturases, including *Pichia pastoris*, *Mortierella alpina*, and *Caenorhabditis elegans* (Table 2). Both LsFad2 and LsFad3 showed similarities with Δ12 and Δ12/Δ15 fatty acid desaturases isolated from yeast and fungi but showed little similarity with *C. elegans* Δ12 fatty acid desaturase. Since it is not possible to fully characterize the desaturase activities of LsFad2 and LsFad3 based only on their deduced amino acid sequences, both proteins were expressed in *S. cerevisiae* and *L. starkeyi* in order to obtain empirical activity data.

Characterization of LsFad2 and LsFad3 using a heterologous expression system in *S. cerevisiae*

To characterize the enzymatic specificity of LsFad2 and LsFad3, these proteins were expressed in *S. cerevisiae* and cultured with or without LA, as described in the "Materials and methods" section. The main fatty acids in *S. cerevisiae* are palmitoleic acid (C16:1) and oleic acid (C18:1). Polyunsaturated fatty acids, including LA and ALA, were not detected in control cells (Table 3). *S. cerevisiae* cells that overexpressed *LsFAD2*

Table 2 Homology of LsFad2 and LsFad3 with other desaturases

Species Function (GenBank accession No.)	***Lipomyces starkeyi*** Identity (Similarity)		**References**
	LsFad2	LsFad3	
Candida parapsilosis Δ12/Δ15 fatty acid desaturase (CAY39362.1)	54% (82%)	46% (78%)	Bucek et al. 2014
Pichia pastoris Δ12 fatty acid desaturase (AAX20125.1)	54% (81%)	45% (78%)	Wei et al. 2006
Pichia pastoris Δ15 fatty acid desaturase (ABL63813.1)	54% (81%)	41% (76%)	Zhang et al. 2008
Fusarium verticillioides Δ12 fatty acid desaturase (ABB88515.1)	50% (77%)	44% (75%)	Damude et al. 2006
Fusarium verticillioides Δ12/Δ15 fatty acid desaturase (ABB88516.1)	44% (76%)	48% (76%)	Damude et al. 2006
Mortierella alpina Δ12 fatty acid desaturase (BAA81754.1)	49% (77%)	39% (76%)	Sakuradani et al. 1999
Rhodosporidium kratochvilovae Δ12/Δ15 fatty acid desaturase (AHZ13213.1)	45% (74%)	43% (78%)	Cui et al. 2016
Coprinopsis cinerea Δ12/Δ15 fatty acid desaturase (BAF45335.1)	41% (70%)	36% (70%)	Zhang et al. 2007
Caenorhabditis elegans Δ12 fatty acid desaturase (AAA67369.1)	28% (65%)	26% (63%)	Spychalla et al. 1997

Fig. 2 Sequence alignment of *L. starkeyi* LsFad2 and LsFad3 and *F. verticillioides* Δ12 fatty acid desaturase (FvD12D) and bifunctional Δ12/15 fatty acid desaturase (FvD12/15D). Amino acid sequences were compared using ClustalW (DNA Data Bank of Japan; http://www.ddbj.nig.ac.jp/) using default settings. Histidine-rich motifs (H-box) are indicated in bold and circled. Putative transmembrane helices are underlined

accumulated LA with reduced levels of oleic acid. In contrast, *S. cerevisiae* cells overexpressing *LsFAD3* produced both LA and ALA. These results indicated that *LsFAD2* and *LsFAD3* encoded Δ12 fatty acid desaturase and Δ12/Δ15

Table 3 Fatty acids composition of *S. cerevisiae* cells overexpressing *LsFAD2* or *LsFAD3*

	Fatty acid composition (%)						
	C16:0 (palmitic acid)	C16:1 (palmitoleic acid)	C18:0 (stearic acid)	C18:1 (oleic acid)	C18:2 (linoleic acid)	C18:3 (α-linolenic acid)	Others
Control	14.7 ± 1.1	47.6 ± 0.4	5.2 ± 0.1	27.6 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	4.8 ± 0.3
+*LsFAD2*	14.6 ± 0.4	44.7 ± 0.9	6.1 ± 0.2	20.8 ± 0.4	9.1 ± 0.3	0.0 ± 0.0	4.5 ± 0.4
+*LsFAD3*	14.2 ± 0.4	44.3 ± 0.2	6.0 ± 0.0	29.4 ± 0.4	0.4 ± 0.0	1.3 ± 0.0	4.2 ± 0.1
Control +LA	15.4 ± 0.5	49.5 ± 0.5	4.8 ± 0.1	23.0 ± 0.8	1.5 ± 0.4	0.0 ± 0.0	5.7 ± 0.8
+*LsFAD2* +LA	15.7 ± 0.1	40.4 ± 0.5	5.5 ± 0.1	17.6 ± 0.8	15.4 ± 0.5	0.1 ± 0.0	5.4 ± 0.1
+*LsFAD3* +LA	15.1 ± 0.3	40.6 ± 0.6	5.2 ± 0.2	23.6 ± 1.2	6.4 ± 0.4	4.0 ± 0.0	5.1 ± 0.3

LA: linoleic acid

Results are means ± S.D. (n = 3)

bifunctional fatty acid desaturase, respectively. *S. cerevisiae* control cells cultured in a medium containing LA were shown to contain LA in their lipids, indicating that additive fatty acids were absorbed and employed in the synthesis of lipids. In media containing LA, *S. cerevisiae* cells overexpressing *LsFAD3* converted added LA to ALA and accumulated significantly more ALA than did cells overexpressing *LsFAD3* in the absence of LA in the initial culture medium (Table 3). These results indicated that added LA was converted to ALA by LsFad3.

Overexpression and deletion of *LsFAD2* and *LsFAD3* genes in *L. starkeyi*

Next, we constructed *L. starkeyi* strains that overexpressed or lacked *LsFAD*s, as described in the "Materials and methods" section. The Δ*lslig4* strain, which is a non-homologous end-joining-deficient strain, exhibited high homologous recombination efficiency (Oguro et al. 2017) and was used as a parent (control) strain in this study. The *LsFAD2* and *LsFAD3* genes were overexpressed using the *LsTDH3* promoter (P_{TDH3}) and a multicopy integration system, which introduced genes into the ribosomal DNA locus (Oguro et al. 2015). Using qRT-PCR and the genomic DNA of each overexpression strain, we confirmed that cells overexpressing *LsFAD2* contained two copies of P_{TDH3}-*LsFAD2*-T_{TDH3} cassettes in their genomes. Cells overexpressing *LsFAD3* had four copies of P_{TDH3}-*LsFAD3*-T_{TDH3} cassettes in their genomes (data not shown). The mRNA levels of the *LsFAD2* gene in cells overexpressing the *LsFAD2* gene and cultured at 20 or 30 °C for 2 days were approximately 4- to 5-fold higher than those of control cells (Fig. 3a). The mRNA levels of the *LsFAD3* gene in cells overexpressing *LsFAD3* were approximately 15- to 21-fold higher than those of control cells (Fig. 3b). The expression levels of *LsFAD2* and *LsFAD3* were higher during the log-phase of growth (cultured for 2 days) than in the stationary phase (cultured for 4 days) in cells overexpressing *LsFAD2* and *LsFAD3*, respectively.

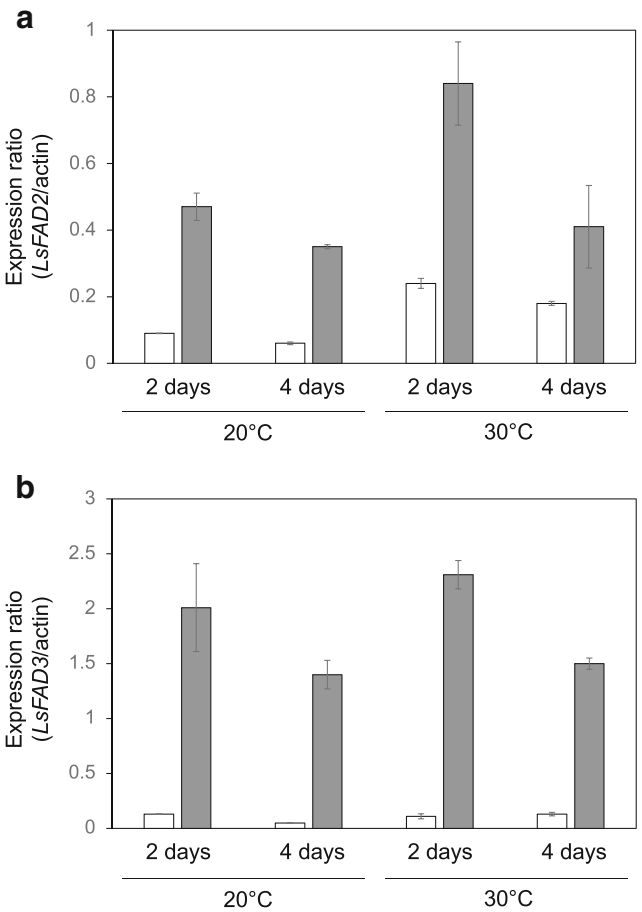

Fig. 3 Expression levels of *LsFAD2* and *LsFAD3* genes. **a** Expression levels of *LsFAD2* in control cells (empty bars) and cells overexpressing *LsFAD2* (shaded bars) that had been cultured at 20 or 30 °C for 2 or 4 days. **b** Expression levels of *LsFAD3* in control cells (empty bars) and cell overexpressing *LsFAD3* (shaded bars) cultured at 20 or 30 °C for 2 or 4 days. The expression level of the actin gene was defined as unity. Error bars indicate standard deviation, $n = 3$

In addition to palmitic and oleic acids, *L. starkeyi* (Δ*lslig4*) cells produced LA (15.4%) and a small amount of ALA (0.2%) at 30 °C (Table 4). In cells cultured at 30 °C, the LA content was dramatically increased by the overexpression of *LsFAD2*, and the production of LA and ALA was completely abolished by deletion of the *LsFAD2* gene. In contrast, at 20 °C, Δ*lsfad2* cells produced only small amounts of both LA and ALA, suggesting that LsFad3 contributed to the production of polyunsaturated fatty acids at low temperatures. Cells overexpressing *LsFAD3* accumulated more ALA compared to control cells. The ALA content of the *LsFAD3* overexpressing strain cultured at 20 °C was approximately 3-fold higher than that of the same strain cultured at 30 °C.

Cells cultured at 20 °C contained higher levels of both LA and ALA than cells cultured at 30 °C (Table 4). In particular, the ALA content of control cells cultured at 20 °C was approximately 60-fold higher than that of control cells cultured at 30 °C. These results suggest that *L. starkeyi* produces polyunsaturated fatty acids as an adaptation to low temperatures. However, the expression levels of both *LsFAD2* and *LsFAD3* genes were not enhanced under low temperature conditions (Fig. 3). This is discussed in greater detail below.

Discussion

Fatty acid desaturases are attractive for the production of polyunsaturated fatty acids, such as ω-3 fatty acids. Although *L. starkeyi* is one of the most studied oleaginous yeasts, the genes encoding its fatty acid desaturases have not been reported. In this study, we identified and characterized two novel fatty acid desaturases, a Δ12 fatty acid desaturase (LsFad2) and a Δ12/Δ15 bifunctional fatty acid desaturase (LsFad3), using heterologous expression in the model yeast *S. cerevisiae* and overexpression and deletion analyses of genes encoding these desaturases in *L. starkeyi*. In the *L. starkeyi* Δ*lsfad2* and *LsFAD3*-overexpressing *S. cerevisiae* cells, a large proportion of LA was converted to ALA (Tables 3 and 4). The high conversion efficiency of LA to ALA by LsFad3 (*L. starkeyi* Δ*lsfad2* cells 75%, *LsFAD3* overexpression *S. cerevisiae* cells 82%), which was calculated as [ALA / (ALA + LA) × 100%] of cells cultured at 20 °C, suggested that LsFad3 may convert LA to ALA without completely releasing its substrate after conversion of oleic acid to LA. The same phenomenon was seen in other bifunctional Δ12/Δ15 fatty acid desaturases, including a fatty acid desaturase isolated from *F. moniliforme* (Damude et al. 2006). Both LsFad2 and LsFad3 contributed to the production of LA and ALA under low temperature conditions. Interestingly, although the expression levels of these fatty acid desaturase genes were not enhanced at low temperatures (Fig. 3), both LA and ALA accumulated under those conditions. Previously, Lomascolo et al. reported a low thermal stability for *L. starkeyi* Δ12 desaturase (Lomascolo et al. 1996). We hypothesize that the contradiction between the expression levels of fatty acid desaturase genes and the amount of polyunsaturated fatty acids is the reason for the observed thermal stability of LsFad2 and LsFad3, which would be inactivated shortly

Table 4 Fatty acids composition of *L. starkeyi* strains: *LsFAD2* or *LsFAD3* overexpressing strains (+*LsFAD2* or +*LsFAD3*), and *lsfad2* or *lsfad3* deletion mutants (*Δlsfad2* or *Δlsfad3*).

	Fatty acid composition (%)						
	C16:0 (palmitic acid)	C16:1 (palmitoleic acid)	C18:0 (stearic acid)	C18:1 (oleic acid)	C18:2 (linoleic acid)	C18:3 (α-linolenic acid)	Others
30°C							
Control (*Δlig4*)	38.2 ± 0.6	3.6 ± 0.2	3.7 ± 0.2	36.4 ± 1.4	15.4 ± 2.6	0.2 ± 0.1	2.5 ± 0.4
+*LsFAD2*	36.0 ± 0.7	3.2 ± 0.1	2.9 ± 0.0	19.5 ± 1.8	34.6 ± 2.7	1.1 ± 0.1	2.7 ± 0.3
+*LsFAD3*	33.6 ± 0.3	3.4 ± 0.1	3.3 ± 0.3	27.8 ± 1.8	20.2 ± 1.4	8.8 ± 0.4	2.9 ± 0.4
Δlsfad2	38.5 ± 0.7	3.8 ± 0.1	3.5 ± 0.1	52.1 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 0.1
Δlsfad3	32.3 ± 0.3	2.7 ± 0.2	3.2 ± 0.1	25.2 ± 0.2	34.0 ± 0.1	0.2 ± 0.0	2.4 ± 0.3
20°C							
Control (*Δlig4*)	24.3 ± 0.5	3.1 ± 0.1	2.4 ± 0.0	32.8 ± 0.3	22.5 ± 0.3	12.8 ± 0.3	2.0 ± 0.5
+*LsFAD2*	23.3 ± 0.9	2.0 ± 0.1	3.0 ± 1.1	13.8 ± 0.6	40.3 ± 1.3	16.0 ± 0.9	1.5 ± 0.3
+*LsFAD3*	22.5 ± 0.3	2.8 ± 0.1	2.4 ± 0.4	32.1 ± 0.9	14.3 ± 1.6	24.9 ± 1.4	1.0 ± 0.2
Δlsfad2	26.2 ± 0.3	4.4 ± 0.2	2.4 ± 0.0	63.8 ± 0.2	0.5 ± 0.0	1.5 ± 0.1	1.3 ± 0.2
Δlsfad3	22.3 ± 0.1	2.3 ± 0.2	2.0 ± 0.1	28.3 ± 0.5	44.4 ± 0.1	0.0 ± 0.0	0.7 ± 0.5

L. starkeyi cells were cultured at 30°C or 20°C for 2 days in 50ml of the medium containing 5% glucose and 0.5% yeast extract.

Results are means ± S.D. (n = 3)

after their expression at higher temperatures. In yeast, polyunsaturated fatty acids play vital roles in stress tolerance in low temperatures (Angelis et al. 2016), alkaline pH (Yazawa et al. 2009), and other conditions. As the most studied yeast, *S. cerevisiae*, lacks polyunsaturated fatty acids; we think that *L. starkeyi* strains that have modified their fatty acid compositions are useful for analysis of the physiological roles of polyunsaturated fatty acids in eukaryotic microbes.

Our ongoing efforts focus on the biochemical analysis of LsFad2 and LsFad3, including their substrate specificity and temperature stability, and the physiological importance of polyunsaturated fatty acids in *L. starkeyi*. It was reported that Δ12 desaturases of *L. starkeyi* and *Candida lipolytica* exhibit desaturase activity toward oleoyl-CoA (Lomascolo et al. 1996; Horváth et al. 1991), whereas the substrates of Δ12 desaturases and Δ15 desaturases are phosphatidylcholine in plants (Miquel and Browse 1992; Ohlrogge and Browse 1995). Detailed biochemical analyses of LsFad2 and LsFad3 are necessary to understand their physiological roles. In addition, we expect that both LsFad2 and LsFad3 will be applicable to the bioproduction of polyunsaturated fatty acids, including ω-3 fatty acids.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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