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S.-F. Lu · I. I. Tolstorukov · S. Anamnart
Y. Kaneko · S. Harashima

Cloning, sequencing, and functional analysis of *H-OLE1* gene encoding Δ9-fatty acid desaturase in *Hansenula polymorpha*

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Abstract *H-OLE1*, a gene encoding Δ9-fatty acid desaturase (FAD) in *Hansenula polymorpha* strain CBS 1976, was isolated by hybridization based upon its homology with the *P-OLE1* gene cloned earlier from a related species, *Pichia angusta* IFO 1475. The sequence of the *H-OLE1* gene revealed high structural conservation with Δ9-FADs from various organisms. A putative 451-amino acid polypeptide encoded by the gene, like all other Δ9-FADs, contained two domains: an N-terminal catalytic domain containing three conserved histidine clusters, and a C-terminal cytochrome b_5-like domain which has been suggested to be involved in electron transport in desaturation reactions. The whole *H-OLE1* gene complemented a *H. polymorpha fad1* mutation leading to a defect in Δ9-FAD. However, the unsaturated fatty acid requirement that the *Saccharomyces cerevisiae ole1* mutant displays was complemented by only the open reading frame of *H-OLE1* driven by *S. cerevisiae* glyceroaldehyde-3-phosphate dehydrogenase promoter, but not by the intact *H-OLE1*, suggesting that the *H. polymorpha* Δ9-FAD was compatible with the desaturation system of *S. cerevisiae* whereas the promoter of the *H-OLE1* gene had no activity in heterologous cells. It was shown by Northern hybridization that transcription of the *H-OLE1* gene in *H. polymorpha* was slightly repressed by exogenous Δ9-unsaturated fatty acid. An *H. polymorpha* disruption mutant (ΔH-OLE1) was created by transformation of an *fad1*/*FAD1* diploid with disrupted *H-OLE1::S-LEU2* linear DNA. It was shown by genetic and molecular analyses that input DNA was integrated in several copies into the chromosomal target to replace the mutated *fad1* allele. Gas chromatography analysis showed identical fatty acid compositions in cells of both *fad1* and Δ*HOLE1* disruption mutants.

S.-F. Lu · I. I. Tolstorukov · S. Anamnart · Y. Kaneko
S. Harashima (✉)
Department of Biotechnology, Faculty of Engineering,
Osaka University, 2-1 Yamadaoka, Suita-shi,
Osaka, 565-0871, Japan
e-mail: harashima@gen.bio.eng.osaka-u.ac.jp
Tel.: +81-06-68797420
Fax: +81-06-68797421

I. I. Tolstorukov
State Research Center of Russian Federation for Genetics and
Selection of Industrial Microorganisms, Moscow 13545, Russia

Present addresses:
S.-F. Lu
Institute of Botany, Chinese Academy of Sciences,
20, Nanxincun Xiangshan, Haidian District,
Beijing 100093, China

S. Anamnart
The Institute of Biotechnology and Genetic Engineering,
Chulalongkorn University,
Phya Thai Road, Bangkok 10330, Thailand

Introduction

Hansenula polymorpha, a thermotolerant methylotrophic yeast, has extremely high biotechnological potential. It was developed as a genetic model for studying various unique metabolic pathways and cellular processes including methanol utilization, peroxisome biogenesis and fatty acid metabolism (Hansen and Hollerberg 1996; Anamnart et al. 1998). Fatty acids and unsaturated fatty acids (UFAs) are well known to be vital compounds for cellular organisms. Especially, poly-UFAs such as linoleic acid (18:2), linolenic acid (18:3), and arachidonic acid (20:4) are considered to be highly valued substances in various fields including pharmaceuticals, medicine, and nutrition. They are naturally supplied to humans in vegetables, fish, and meat. More recently, the filamentous fungus *Mortierella alpina* has been developed as a microbial producer of a variety of high-valued poly-UFAs (Jareonkitmongkol et al. 1992, 1993). Yeasts would be obvious further candidates for a microbial producer because of their safety and ease of handling. However, *Saccharomyces cerevisiae*, which is always the first choice for this kind of research, is unfortunately able to synthesize only mono-UFAs, but not poly-UFAs.

In our previous study (Anamnart et al. 1998), we demonstrated that *H. polymorpha*, like higher plants and unlike most other higher eukaryotes, produces 18:2 and

18:3. This observation permitted a new approach to elucidating the biosynthetic pathways and membrane functions of poly-UFAs by applying the wide spectrum of genetic, molecular, and biochemical techniques to this advanced microbial eukaryotic model. The information obtained from those studies could be exploited to create poly-UFA producing strains of this yeast or even *S. cerevisiae* in the future.

Mono- and poly-UFAs are sequentially synthesized from saturated fatty acids in desaturation reactions catalyzed by a number of fatty acid desaturases (FADs), which introduce double bonds at specific positions of the hydrocarbon chain of fatty acids. We have revealed that *H. polymorpha* has an α-linolenic desaturation pathway: stearic acid, a 18:0, is converted to oleic acid [18:1 (Δ9)] by Δ9-fatty acid desaturase (Δ9-FAD), which introduces the first double bond in the Δ9 position. Subsequently, oleic acid is converted to linoleic acid [18:2 (Δ9, 12)] by Δ12-desaturase-catalyzed introduction of the second double bond at the Δ12 position. The third double bond is introduced at the Δ15-position of linoleic acid by Δ15-desaturase, resulting in the generation of α-linolenic acid [18:3 (Δ9, 12, 15)]. In order to understand the genetic system regulating desaturation of fatty acids, a number of UFA-requiring mutants were isolated from *H. polymorpha* strain CBS 1976 (=NCYC 495) (Anamnart et al. 1998) and identified as having mutations in the gene *FAD1* which encodes Δ9-FAD. Results of analysis of these mutants suggested that Δ9-FAD is the key enzyme in desaturation pathways and plays an essential role in lipid metabolism.

Genes encoding Δ9-FAD have been cloned from various organisms (Thiede et al. 1986; Ntambi et al. 1988; Stukey et al. 1989; Sakamoto et al. 1994; Gargano et al. 1995; Meesters and Eggink 1996; Mylona et al. 1996; Nakashima et al. 1996; Luo et al. 1997; Meesters et al. 1997; Fukuchi-Mizutani et al. 1998; Itoh et al. 1998) including *Pichia angusta* IFO 1475 (=CBS 7003), the gene designated *P-OLE1* of which was isolated in this laboratory by polymerase chain reaction (PCR) and DNA hybridization techniques (Anamnart et al. 1997). It was shown that the native *P-OLE1* gene complements the mutation *ole1* for Δ9-FAD in *S. cerevisiae*. During cloning of the *P-OLE1* gene, we counted that *P. angusta* and *H. polymorpha* are actually two closely related strains of one species, *H. polymorpha* according to the old classification (Kreger-van Rij 1984), and *P. angusta* according to the new classification of yeast taxonomy (Kurtzman and Fell 1997). However, it has been shown very recently (Naumov et al. 1997) that *H. polymorpha* strain CBS 1976 (=NCYC 495), widely used in genetic studies, and *P. angusta* strain CBS 7003 (=IFO 1475), from which the *P-OLE1* gene was cloned (Anamnart et al. 1997), are two related but independent species. To establish an adequate gene–host system in our genetic studies on the *H. polymorpha* model, we decided to clone the Δ9-FAD gene (*H-OLE1*) from the genome of *H. polymorpha* CBS 1976. In this paper, cloning of *H-OLE1* gene from *H. polymorpha* and its functional analysis are described.

Materials and methods

Organisms, plasmids, and DNAs

The auxotrophic strain leu1-1 of *H. polymorpha* derived from CBS 1976 (=NCYC 495) (Gleeson and Sudbery 1988), a gift from J.A.K.W. Kiel, was used for cloning the *H-OLE1* gene. *P. angusta* IFO 1475, *P. pastoris* IFO 0948, IFO 1013, IFO 10777, *P. methanolica* IFO 10704, and *Candida boidinii* IFO 10240 and IFO 10329, were obtained from the Institute for Fermentation, Osaka. *P. pastoris* GS115 was obtained from K. Yoshida. The *H. polymorpha* haploid mutant *fad1*, M1-3-7D (*fad1-1 ura3-1 leu1-1*), diploid mutant D2321-51 (*fad1-2*/*FAD1 leu1-1*/*leu1-1 ura3-1*/*URA3 ade1-1*/*ADE*) and *S. cerevisiae ole1* mutant, SH4348 [*MAT*a *ole1 ura3–52 leu2-3,112 trp1 his1-29*(*HIS4-lacZ*, *ura3–52*)] were used from our stock collection. *Escherichia coli* strain DH5α was used for amplification of plasmid DNA. Plasmid pBluescript II KS^+ (Stratagene, La Jolla, Calif., USA) was used for manipulation of DNA. Plasmids pLSF1, pLSF6, pLSF22, pLSF25 (Fig. 1), and pLSF19 (not shown) were constructed in this study. pLSF1 is pBluescript II KS^+ containing the 3.4-kb *Bam*HI-*Xho*I fragment of *H. polymorpha* genomic DNA with the *H-OLE1* gene inserted between the *Bam*HI and *Xho*I sites of the vector. pLSF6 was constructed by inserting the fragment from pLSF1 into pYO325, a gift from Y. Ohya. pYO325 is pRS305 (Sikorski and Hieter 1989) containing 2 μm DNA. pLSF19 is pUC19 with the *Bgl*II fragment containing the *LEU2* gene from YEp13 inserted into the *Bam*HI site. pLSF22 was constructed by replacement of the *Hin*dIII-*Sma*I fragment of *H-OLE1* gene in pLSF1 by the *Hin*dIII-*Sma*I fragment containing the *S-LEU2* gene from pLSF19. An *S. cerevisiae* multicopy expression vector p520 (a gift from T. Ashikari) in which the *Eco*RI-*Sal*I segment of pYE2211 (Ashikari et al. 1989) was replaced with the *Eco*RI-*Sal*I region of the polycloning site of M13mp18, was used for expression of *H-OLE1* under *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase promoter (GAPp). To construct pLSF25, the PCR product of the entire *H-OLE1* open reading frame (ORF) amplified from the *H-OLE1* gene as the template with oligonucleotides lu-oli11 (5′-GAGCTCAGG-AAAGTTGATGGGAAC-3′) and lu-oli12 (5′-GGATCCTCA-TCTTTTCGTCGTCTC-3′) (Fig. 3) as primers, was inserted into the *Sac*I-*Bam*HI site of p520 under the control of the GAPp.

Media and cultivation

Standard YEPD, MIN, DO (Gleeson and Sudbery 1988) and YEPD/U, DO/U media supplemented with 0.8 mM oleic acid, 18:1, 0.3 mM each of palmitoleic acid, 16:1, linoleic acid,18:2, and α-linolenic acid, 18:3, were used for the growth of *H. polymorpha* strains. To grow *S. cerevisiae ole1* mutants, YEPD and DO media were supplemented with 1 mM oleic acid and designated YEPD/18:1 and DO/18:1. *H. polymorpha* transformants were selected on synthetic DO medium and *S. cerevisiae* transformants on DO/18:1. MAME medium (2.5% maltose, 0.5% malt extract, 2% agar) was used for induction of sporulation in *H. polymorpha* diploids. *E. coli*, yeast *H. polymorpha* and *P. angusta* were cultivated at 37 °C. All other yeast strains were cultivated at 30 °C.

Genetic and analytical methods

Tetrad and random spore analyses of *H. polymorpha* were performed as described (Gleeson and Sudbery 1988). Cellular fatty acid composition was analyzed by gas chromatography (Anamnart et al. 1998). Yeast chromosomal DNA was prepared as described (Hereford et al. 1979; Adams et al. 1997).

Transformation of *H. polymorpha* was carried out by electroporation (Faber et al. 1994). Southern blot analysis and colony hybridization were performed using ECL Random Prime Labelling and Detection Systems, version II (Amersham). Northern blot

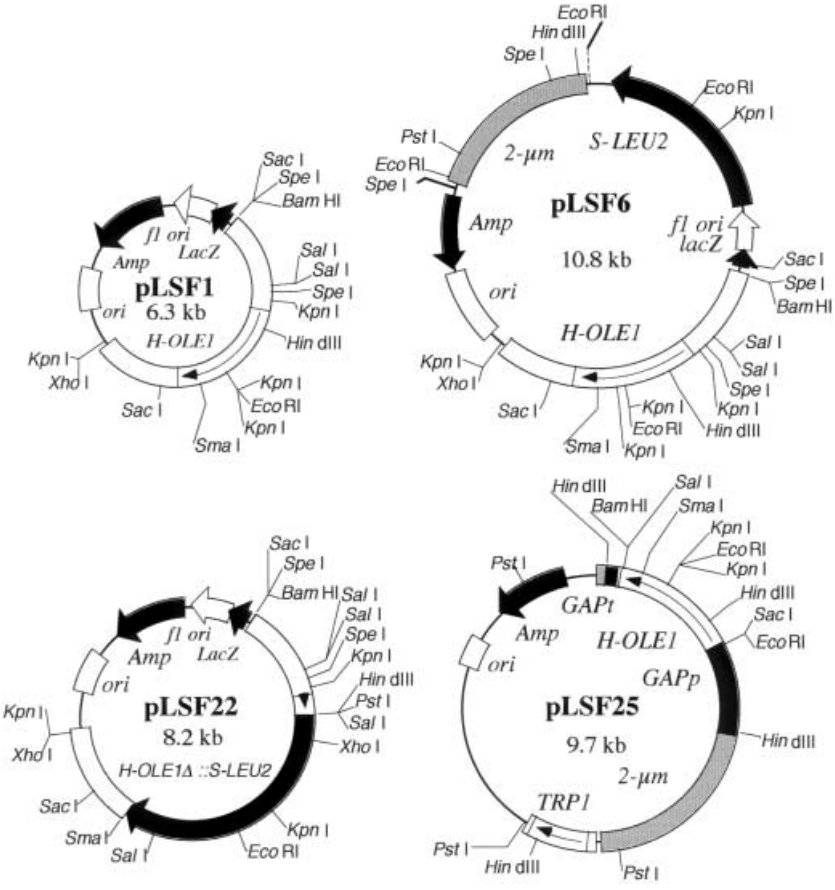

Fig. 1 Structures of the constructed plasmids. pLSF1 and pLSF6 contain the 3.4-kb *Bam*HI-*Xho*I fragment of the *Hansenula polymorpha* genome containing the *H-OLE1* gene. pLSF25 contains the *H-OLE1* ORF between *Saccharomyces cerevisiae* GAPp and GAP terminator sequences. In pLSF22, the *Hin*dIII-*Sma*I fragment in the ORF of the *H-OLE1* in pLSFl was replaced by the *Hin*dIII-*Sma*I fragment containing the *S. cerevisiae LEU2* gene

analysis was carried out as described (Ausubel et al. 1989; Sambrook et al. 1989), with ^{32}P-labeled probe DNA. PCR was performed with the Gene Amp DNA amplification Reagent Kit (Perkin Elmer Cetus, Norwalk, Conn., USA) by the Gene Amp PCR System 2400. Bacterial plasmid DNA was isolated by the alkaline lysis method (Sambrook et al. 1989). DNA for sequence analysis was isolated using the Qiagen Plasmid Mini Kit (Qiagen K. K.). Nucleotide sequences were determined by the dideoxy chain termination method using M13 F or M13 RV primers with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli Taq DNA polymerase FS (Perkin-Elmer) with an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). In some cases, a ΔTth DNA Polymerase Sequencing PRO kit (Toyobo, Osaka, Japan) with ^{32}P-labeled deoxycytidine triphosphate was used.

Results

Southern blot analysis of various methylotrophic yeasts with the *P-OLE1* gene probe

DNAs of *H. polymorpha* CBS 1976, *P. angusta* IFO 1475, and several other representative species of methylotrophic yeasts were tested by probing Southern blots with the entire ORF of the *P-OLE1* gene (Fig. 2). The amplification was performed with the *P-OLE1* gene as the template and OLI 393 (5′-CTCGAGAGGAAAGTTGATGGGA-3′) and OLI 394 (5′-AGATCTTCATATTTTCGTCGTCT-3′) (Anamnart et al. 1997) as primers. Strong hybridization signals (Fig. 2) were obtained from *H. polymorpha* CBS 1976, *P. angusta* IFO 1475 and all the *P. pastoris* strains analyzed. Very weak signals were obtained from *P. methanolica* DNA and no signals were detected from the two *C. boidinii* strains used. It was noted that in all four independent *P. pastoris* strains, the same 7.4-kb *Eco*RI fragment was hybridized (Fig. 2A), whereas certain restriction fragment length polymorphisms were found between the *OLE1* genes of *H. polymorpha* and *P. angusta* (Fig. 2B). The findings that the putative gene for Δ9-FAD in *H. polymorpha* differs from but exhibits high homology with *P-OLE1*, and is present in a single copy in the *H. polymorpha* chromosomal DNA, made it feasible to clone the gene from the genome.

Restriction mapping, cloning, and sequencing of *H-OLE1* gene

A restriction map of the *H. polymorpha* chromosomal locus containing the putative *H-OLE1* gene (Fig. 2C) was constructed based upon the data of the Southern blot analysis using the *P-OLE1* ORF shown partially in Fig. 2A and B. It was expected that a 3.4-kb *Bam*HI-*Xho*I chromosomal fragment contains the whole *H-OLE1* gene. In this fragment, the nontranslated flanking regions appear to consist of 1 kb each, which is about 0.6 kb longer than that in the *P-OLE1* gene cloned earlier (Anamnart et al. 1997).

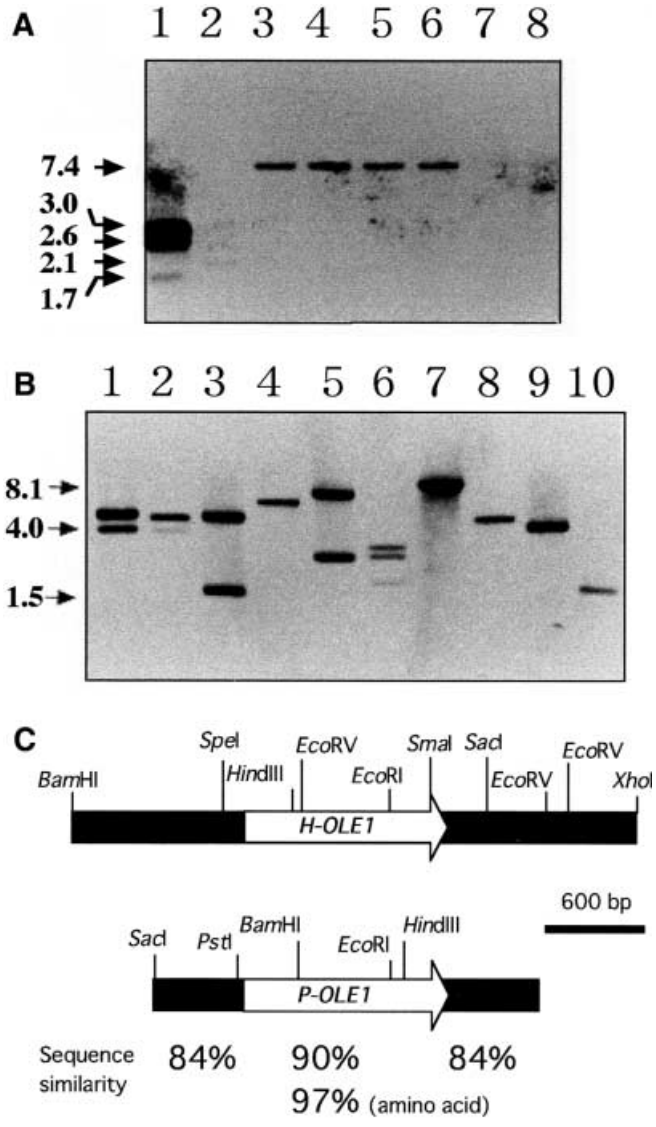

Fig. 2A–C Southern blot analysis of the genomic DNAs of various methylotrophic yeasts probed with the ORF of the *P-OLE1* gene (**A, B**) and restriction maps of *H-OLE1* and *P-OLE1* genes (**C**). **A** *Eco*RI-digested DNA of *H. polymorpha* CBS 1976 (*lane 1*), *Pichia methanolica* IFO 10704 (*lane 2*), *P. pastoris* IFO 1013 (*lane 3*), *P. pastoris* IFO 10777 (*lane 4*), *P. pastoris* IFO 0948 (*lane 5*), *P. pastoris* GS115 (*lane 6*), *Candida boidinii* IFO 10329 (*lane 7*), and *C. boidinii* IFO 10240 (*lane 8*). **B** DNA of *P. angusta* IFO 1475 (*lanes 1, 3, 5, 7, 9*) and *H. polymorpha* CBS 1976 (*lanes 2, 4, 6, 8, 10*). *Hin*dIII-digested (*lanes 1, 2*), *Bam*HI-digested (*lanes 3, 4*), *Eco*RI-digested (*lanes 5, 6*), *Bgl*II-digested (*lanes 7, 8*), and *Eco*RV-digested (*lanes 9, 10*). *Arrows* (**A, B**) indicate the sizes of the fragments. **C** Restriction map of 3.4-kb *Bam*HI-*Xho*I chromosomal fragment bearing the putative *H-OLE1* gene was constructed on the basis of the data from Southern blot analysis. Restriction map of the 2.3-kb region with the *P-OLE1* gene was reconstructed from the sequencing data (Anamnart et al. 1997). The *arrows* indicate the determined (for *P-OLE1*) and expected (for *H-OLE1*) ORFs and transcriptional directions. Nucleotide homology of and the deduced amino acid sequences of *H-OLE1* and *P-OLE1* according to the data from Fig. 3 are indicated as percentages

The genomic DNA of *H. polymorpha* was digested with *Bam*HI and *Xho*I and separated by electrophoresis. A 3.4-kb fragment was purified and inserted between the *Bam*HI and *Xho*I sites of pBluescript II KS$^+$. Two among approximately 700 *E. coli* transformants showing a hybridization signal with the *P-OLE1* ORF were identified and found to harbor identical plasmids (pLSF1, Fig. 1) containing the 3.4-kb insertion. The nucleotide sequence of the cloned fragment was determined and analyzed (Fig. 3). This analysis revealed four in-frame ATG codons and an ORF of 1353 bp counting from the first start ATG, which exhibited 90% homology with *P-OLE1* ORF (Anamnart et al. 1997) at nucleotide level and 97% homology at amino acid level (Fig. 2C).

Complementation of *H. polymorpha fad1* and *S. cerevisiae ole1 mutants* lacking Δ9-FAD

An *H. polymorpha* mutant for Δ9-FAD (Anamnart et al. 1998), M1-3-7D (*fad1-1 leu1-1 ura3-1*), was transformed with plasmid pLSF6 (Fig. 1) bearing the cloned *Bam*HI-*Xho*I fragment and the *S-LEU2* gene. Since UFA-containing media drastically reduced the efficiency of the *H. polymorpha* transformation, Leu$^+$ Ufa$^+$ double selection (DO-Leu medium) was employed. Nine out of the 19 Leu$^+$ Ufa$^+$ transformants obtained were analyzed. Transformant cells were plated on the nonselective YEPD/U medium and the phenotypes of a number of single colonies were determined by replication onto a series of diagnostic plates with and without UFA: YECD/U, YEPD, DO/U-Leu, and DO-Leu. All of the transformants analyzed were found to be unstable with respect to the Ufa$^+$ and Leu$^+$ phenotypes. Among the colonies initially exhibiting the Leu$^+$ Ufa$^+$ phenotype (see Tl and T2 on Fig. 4A), at least 8% of clones of each were found to have become Leu$^-$ Ufa$^-$ (see Dl and D2 on Fig. 4A).

As a control, vector pYO325 was introduced into diploid strain D2321-51 (*fad1-2*/*FAD1 leu1-1*/*leu1-1 ura3-1*/*URA3 ade1-1*/*ADE1*). Three among more than 1,000 Leu$^+$ transformants were subjected to random spore analysis and were plated onto selective DO/U-Leu medium. When the stability of the Leu$^+$ Ufa$^+$ and Leu$^+$ Ufa$^-$ meiotic segregants was analyzed after plating onto nonselective YEPD/U plates, no Ufa$^+$ Leu$^+$ haploid progeny producing Ufa$^-$ Leu$^-$ derivatives were isolated, while Ufa$^-$ Leu$^+$ and Ufa$^+$ Leu$^-$ derivatives were observed, indicating that the vector alone does not complement the *fad1* mutation. As an example, the phenotype of the segregant TV, Leu$^+$ Ufa$^-$ [*fad1-2* (*S-LEU2*)], was shown in Fig. 4A.

The results of Southern blot analysis of *H. polymorpha* transformants for plasmid pLSF6 are presented in Fig. 5A. Undigested DNA of recipient M1-3-7D, R (lane l), two initial Leu$^+$ Ufa$^+$ transformants T1 and T2 (lanes 2 and 3), and two Leu$^-$ Ufa$^-$ derivatives Dl and D2 (lanes 4 and 5) were probed with pLSF6. The results clearly demonstrated the presence of the plasmid in both initial transformants and its absence in Leu$^-$ Ufa$^-$ derivatives, as well as the recipient.

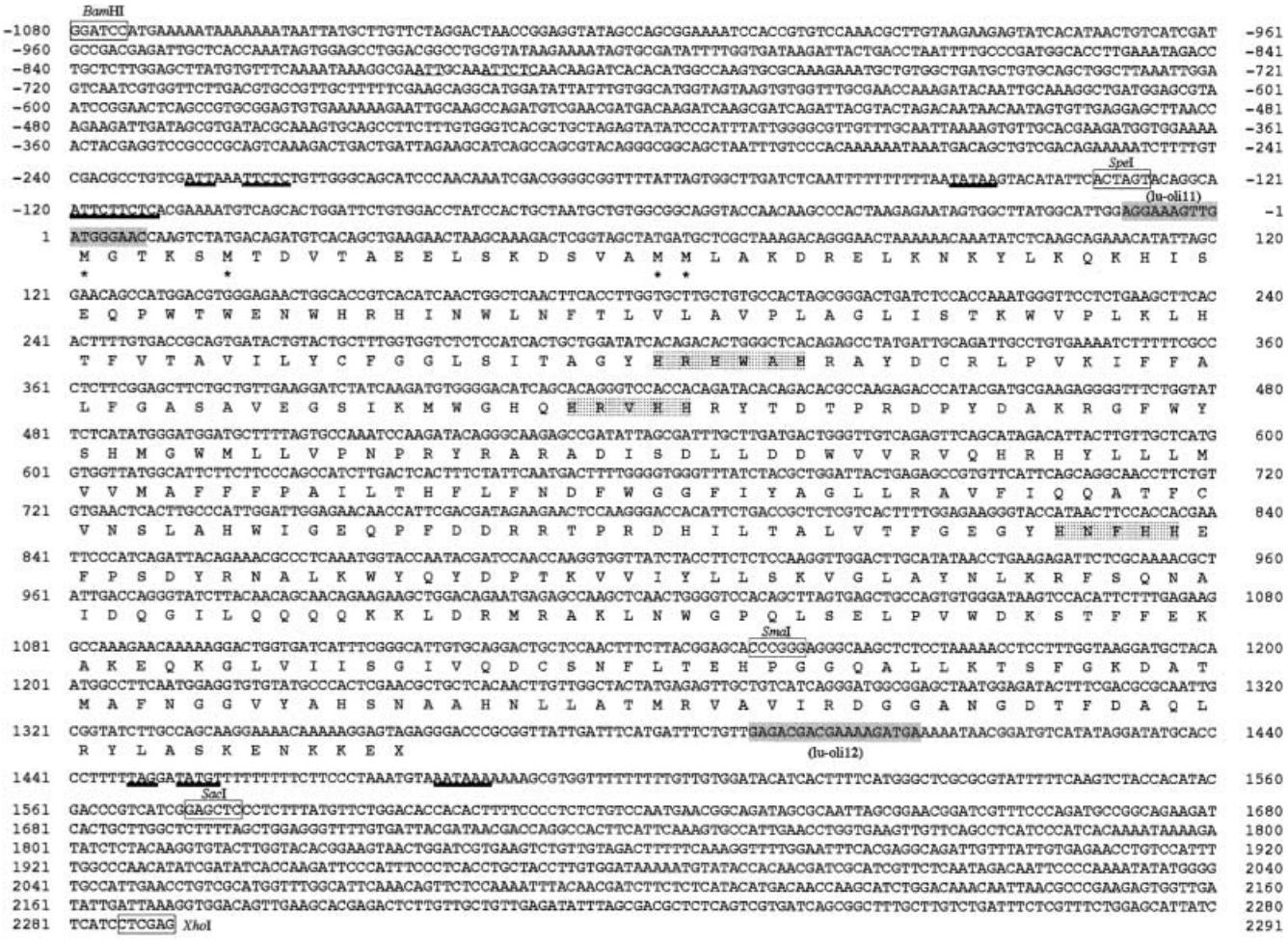

Fig. 3 The nucleotide sequence of the 3.4-kb, *Bam*HI-*Xho*I fragment of the *H. polymorpha* genome and the deduced amino acid sequence of the Δ9-FAD polypeptide. Nucleotides are numbered relative to the A residue of the first ATG as +1. *Asterisks* indicate four in-frame ATG codons. *X* indicates the stop codon of translation. The TATA box (−148) and the putative ROX1-binding sites in the 5′ end, as well as the putative transcriptional termination signal, TAG...TATGT and AATAAA in the 3′ nontranslated regions, are *underlined*. *HxxxxH* and *HxxHH* below the nucleotide sequence indicate histidine clusters in the Δ9-FAD polypeptide. lu-oli11 and lu-oli12 are the oligonucleotides used for PCR to amplify the *H-OLE1* ORF. The nucleotide sequence has been deposited in the DDBJ/EMBL/GenBank data library and assigned the accession number AB024576

An *S. cerevisiae* Δ9-FAD mutant, *ole1* (SH4348), was transformed with hybrid plasmid pLSF6 and vector pYO325. In both cases, more than 1,000 Leu^+ transformants were selected on DO/18:1–Leu plates. Unexpectedly, neither plasmid pLSF6, bearing the *H-OLE1* gene, nor the vector alone, produced any Ufa^+ transformants able to grow on YEPD or DO-Leu media (see ST1 and ST1V, Fig. 4B). Nevertheless, mitotic and Southern blot analyses showed that Leu^+ transformants with plasmid pLSF6 (ST1) as well as transformants with vector pYO325 (ST1V) contained plasmids of the expected sizes (Fig. 5B, lanes 2 and 3).

However, when the *H-OLE1* ORF was introduced into the expression vector p520 under the control of the *S. cerevisiae* GAPp, complementation of the *S. cerevisiae ole1* mutation was observed. Trp^+ colonies were selected on DO/18:1–Trp medium after transformation of the *S. cerevisiae ole1* mutant SH4348 with plasmid pLSF25 (*GAPp-H-OLE1*) and vector p520. It was found that all transformants with pLSF25 (see ST2 on Fig. 4B), unlike the two transformants with vector p520 and plasmid pLSF6 (ST2V and ST1, Fig. 4B), grew on UFA-free media, i.e., YEPD and DO-Trp.

The results of Southern blot analysis of an *S. cerevisiae* transformant with pLSF25 (ST2) and its Trp^- Ufa^-derivative (SD2) (Fig. 5C, lanes 2 and 3) agreed with the idea that the Ufa^+ phenotype in *S. cerevisiae* cells was conferred by plasmid pLSF25 which carries the *H-OLE1* ORF driven by the *S. cerevisiae* GAPp.

Transcription of the *H-OLE1* gene in *H. polymorpha* cells

Northern blot analysis of total RNA isolated from *H. polymorpha* wild-type cells (strain leu1-1) was conducted using the *H-OLE1* gene as the probe. The 1.7-kb *Spe*I-*Sac*II fragment of the *H-OLE1* gene was isolated from pLSF1 for use as a probe. The 0.7-kb *Sal*I

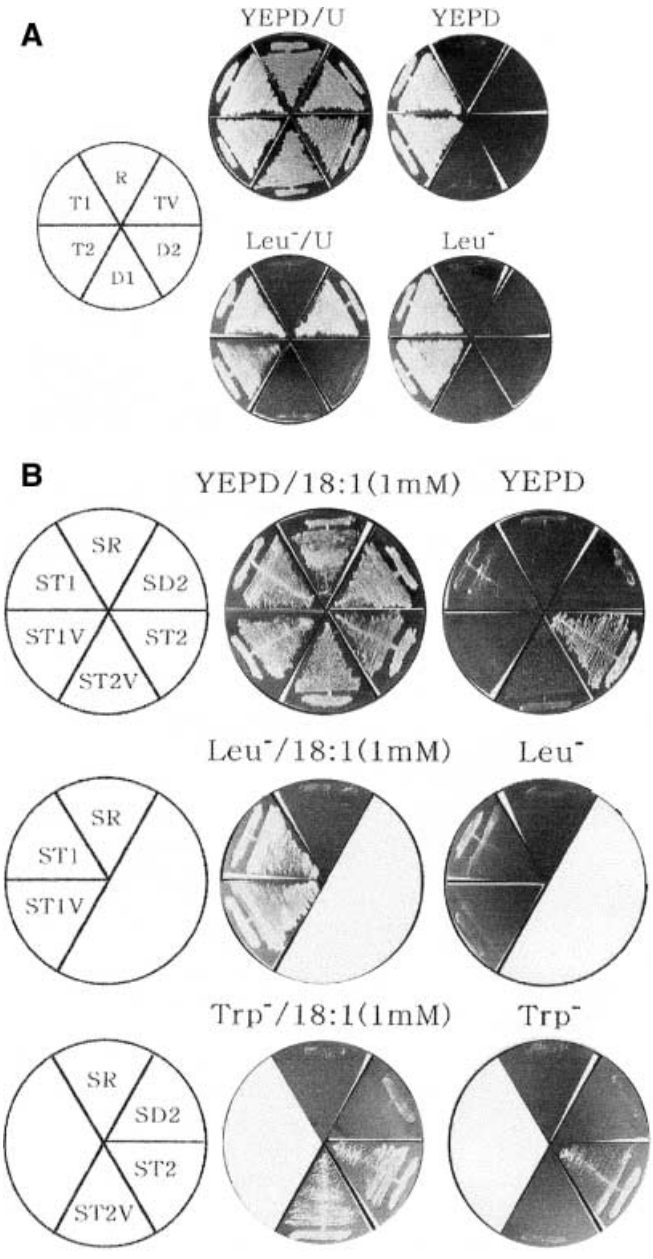

Fig. 4A, B Phenotypic analyses of *H. polymorpha* and *S. cerevisiae* recipients and transformants. **A** Growth, on nonselective YEPD/U and a series of selective plates, of *H. polymorpha* recipient *fad1*, M1-3-7D (R), two independent Leu$^+$ Ufa$^+$ transformants with plasmid pLSF6 (T1 and T2), two Leu$^-$ Ufa$^-$ mitotic derivatives (D1 and D2) obtained after growth of transformants T1 and T2 on nonselective medium YEPD/U, and a haploid *fad1* segregant from diploid D2321-51 harboring vector pYO325 (TV). **B** Growth, on nonselective YEPD/18:1 and a series of selective plates, of *S. cerevisiae* recipient *ole1*, SH4348 (SR), Leu$^+$ transformants with pLSF6 (ST1) and pYO325 (ST1V), Trp$^+$ transformants with pLSF25 (ST2) and p520 (ST2V), Trp$^-$ Ufa$^-$ mitotic derivative of the transformant ST2 (SD2)

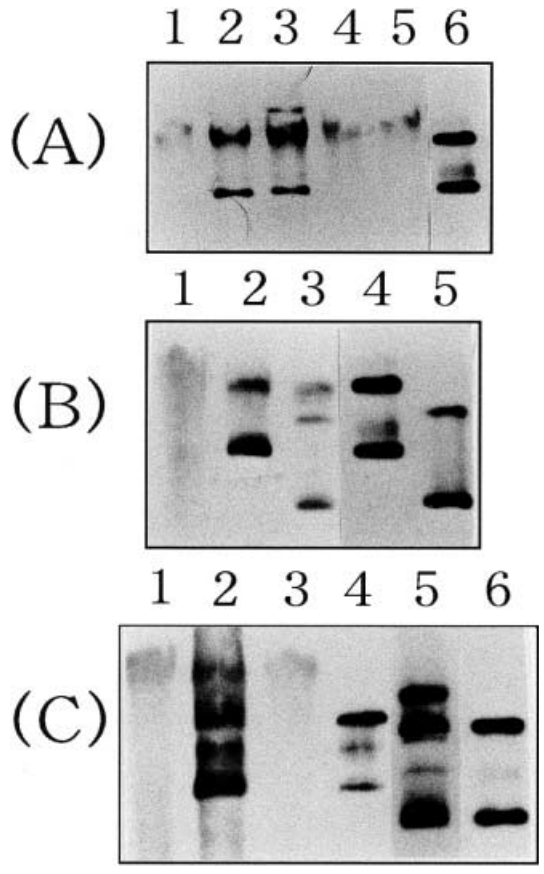

Fig. 5A–C Southern blotting of undigested total DNA of *H. polymorpha* and *S. cerevisiae* transformants. **A** DNAs of *H. polymorpha* *fad1* recipient M1-3-7D, R (*lane 1*), Leu$^+$ Ufa$^+$ transformants Tl and T2 (*lanes 2, 3*), Leu$^-$ Ufa$^-$ derivatives D1 and D2 (*lanes 4, 5*) and plasmid pLSF6 (*lane 6*) were probed with pLSF6. **B** DNAs of *S. cerevisiae ole1* recipient SH4348 (SR) (*lane 1*), Leu$^+$ transformants ST1 with pLSF6 (*lane 2*) and ST1V with pYO325 (*lane 3*), plasmid pLSF6 (*lane 4*), and pYO325 (*lane 5*) were probed with pLSF6. **C** DNAs of *S. cerevisiae ole1* recipient SH4348, SR (*lane 1*), Trp$^+$ Ufa$^+$ transformant ST2 with pLSF25 (*lane 2*), its Trp$^-$ Ufa$^-$ derivative SD2 (*lane 3*), plasmids pLSF25 (*lane 4*), Trp$^+$ transformant ST2V with p520 (*lane 5*), and p520 (*lane 6*) were probed with pLSF25

fragment of the *P. pastoris* gene encoding GAP purified from plasmid pGAP-9 (Waterham et al. 1997) was used as the internal control. Transcription was examined for cells grown for 6–8 h in 10 ml minimal media containing leucine with or without the addition of 1 mM Δ9 monoUFA, palmitoleic or oleic acid. We found that the *H-OLE1* gene was expressed in *H. polymorpha* cells at a low level and produced a 1.5-kb transcript (Fig. 6). The transcription of the gene was repressed to approximately half, i.e., 0.46-fold, by the addition of 16:1 and 0.65-fold by 18:1, as measured by NIH image software.

Disruption of the *H-OLE1 gene*

In order to obtain a deletion mutant for the *H-OLE1* gene in *H. polymorpha*, we disrupted the gene by targeted integration. A heterozygous *fad1-2*/*FAD1* diploid, D2321-51 (*leu1-1*/*leu1-1*) was transformed with the 4.4-kb linear *Sac*I fragment of pLSF22 (Fig. 1), and Leu$^+$ transformants were selected on DO-Leu plates. The linear DNA used contained a fragment of the *H-OLE1*

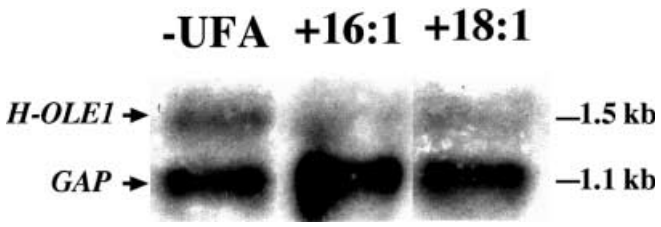

Fig. 6 Northern blot analysis of *H-OLE1* expression in *H. polymorpha* wild-type cells (strain leu1) grown either without (−Ufa) or with the addition of 1 mM palmitoleic (+16:1) or oleic (+18:1) acid. The *Spe*I-*Sac*II fragment of the *H-OLE1* gene and the 0.7-kb *Sal*I fragment of *the P. pastoris GAP* gene (22) were used as probes

gene in which the *Sma*I-*Hin*dIII deletion was replaced by the *S-LEU2* gene which has been shown to have vector activity in *H. polymorpha* cells with both selective and ARS features (Berardi and Thomas 1990). This *H-OLE1::S-LEU2* cassette can either form a replicative plasmid in *H. polymorpha* cells, integrate randomly into a genome, or integrate with low frequency into a chromosomal target by homologous recombination (Beburov et al. 1990). Supposing *H-OLEI* is identical to *FAD1*, if the targeted integration were to occur, either allele, *FAD1* or mutant *fad1-2*, would be replaced by the disrupted *H-OLE1::S-LEU2* DNA. However, since no *H-OLE1::S-LEU2*/*fad1-2* cells could grow on the UFA-free (DO-Leu) medium, only replacement of the mutant *fad1-2* allele could be obtained. It is clear from the genotype of the diploid recipient that a viable targeted transformant must have the *H-OLE1 leu1-1*/*H-OLE1::LEU2 leu1-1* genotype and produce only two types of meiotic progeny: Ufa^+ Leu^- (*H-OLE1 leu1-1*) and Ufa^- Leu^+ (*H-OLE1::S-LEU2 leu1-1*). On the other hand, other types of meiotic progeny, i.e., those exhibiting Ufa^+ Leu^+ (*H-OLE1*$^+$*S-LEU2*$^+$) and Ufa^- Leu^- (*fad1 leu1-1*) phenotype in addition to aforementioned progenies, must also be observed in cases of nonhomologous integration or formation of replicating plasmids.

Among approximately 10^4 Leu^+ colonies, 104 fast-growing transformants (supposed to be integrative) were selected and found to be stable for the Leu^+ phenotype. Twenty-five of the 104 Leu^+ transformants revealed both the dramatic decrease of spore viability on DO-Leu plates and the absence of Leu^+ Ufa^+ segregants in random spore analysis (more than 10^3 spores were analyzed for each transformant). One of the 25 transformants, no. 67, was dissected. It was observed that all 13 tetrads exhibited 2 Ufa^+ Leu^-:2 Ufa^- Leu^+ segregation as expected for the *H-OLE1 leu1*/*H-OLE1::S-LEU2 leu1* diploid, suggesting that the mutant allele, *fad1-2*, was replaced by the input DNA (*H-OLE1::S-LEU2*).

Southern blot analysis of *Bam*HI-*Xho*I-digested DNA of recipient D2321-51, a diploid transformant no. 67 (T) and one complete tetrad (Sl–S4) from the transformant, was performed using pLSF22 as the probe (Fig. 7A). Cosegregation between the UFA requirements of the two segregants, S2 and S4, and the absence of the 3.4-kb *Bam*HI-*Xho*I fragment in their genomes was found, confirming that the mutant allele *fad1-2* was replaced by the *H-OLE1::S-LEU2* cassette.

Unexpectedly, a 2.8-kb fragment in the chromosomal DNA of the transformant (T) and disruption mutants (S2 and S4) appeared, in addition to the 1.6-kb and 3.5-kb *Bam*HI-*Xho*I fragments which were expected in the case of a single copy integration of the cassette into the *H-OLE1* locus. A simple explanation of this result is tandem multicopy integration of the input DNA into the chromosome target (Fig. 7B).

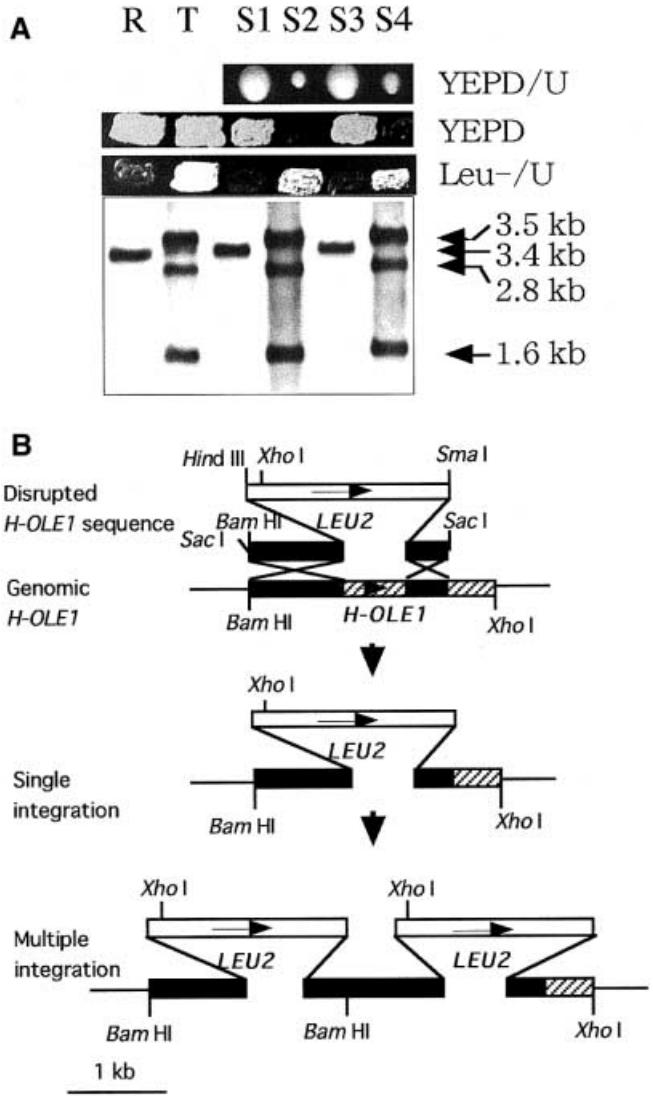

Fig. 7A, B Disruption of the *H. polymorpha H-OLE1* gene. **A** Southern blot analysis of *Bam*HI-*Xho*I-digested DNA of the diploid recipient D2321-51 (R), diploid transformant no. 67 (T), and segregants from one tetrad of the transformant (S1–S4) probed with plasmid pLSF22 (Fig. 1). Sizes of fragments are indicated in kilobase pairs. At the *top*, growth of single spore cultures on YEPD/U agar and phenotypes of the analyzed cultures on UFA-free YEPD and UFA-supplemented DO/U-Leu media are shown. **B** Schemes of a single-copy (*middle*) and tandem (*bottom*) integration of the *H-OLE1:: S-LEU2* cassette into the *H-OLE1* locus

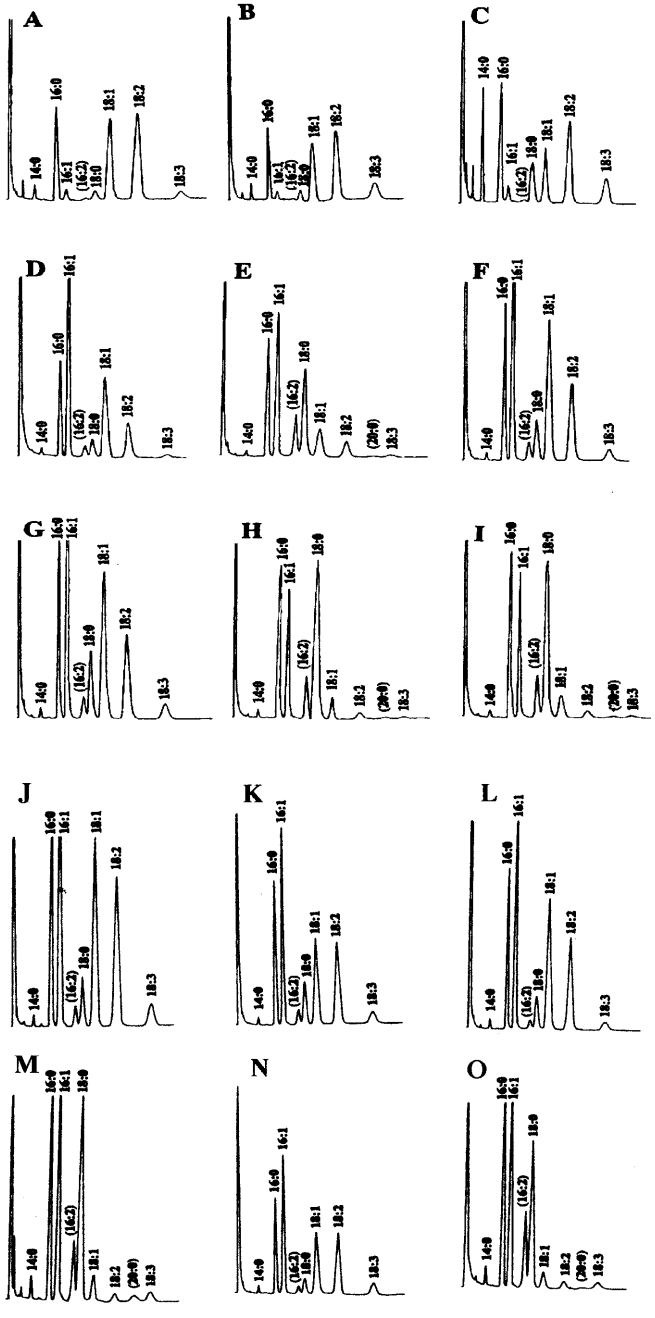

Fig. 8A–O Cellular fatty acid compositions of *H. polymorpha* strains grown in UFA-free medium (**A–C**) and medium supplemented with 0.2 mM 16:1 (**D–O**). Wild-type strain leu1-1 (**A, D**), Ufa^- mutant *fad1-2*, M1-3-7D (**E**), Ufa^+ transformants of M1-3-7D, T1 (**B, F**) and T2 (**C, G**), with plasmid pLSF6, Ufa^- derivatives D1 (**H**) and D2 (**I**) of transformants T1 and T2, Ufa^+ diploid D2321-51 (*fad1-2*/*FAD1*) (**J**), targeting diploid transformant no. 67 (*H-OLE1:S-LEU2*/*FAD1*) (**K**), four segregants from transformant no. 67, Ufa^+ segregants S1, S3 (**L, N**), and Ufa^- deletion mutants S2, S4 (**M, O**). The peak indicated *parentheses*, described as 16:2, was identified by gas chromatography–mass spectroscopy after fractionation of the compound (our unpublished data)

Fatty acid compositions of *H. polymorpha* *H-OLE1* disruptants

Δ9-FAD activity in cells of *H. polymorpha* strains was determined by gas chromatographic analysis of cellular fatty acids in cultures grown in liquid media supplemented with 0.2 mM palmitoleic acid, 16:1 (Fig. 8). In some cases, the wild-type strain and Ufa^+ transformants were grown in UFA-free media. The fatty acid profiles were presented for wild-type strain (leu1) (Fig. 8A, D), Ufa^- recipient *fad1* (M1-3-7D) (Fig. 8E), two Ufa^+ transformants (T1 and T2) obtained after transformation of the *fad1* mutant (M1-3-7D) with plasmid pLSF6 bearing *H-OLE1* gene (Fig. 8B, C, F, G), two Leu^- Ufa^- derivatives from the Tl and T2 transformants (D1 and D2) (Fig. 8H, I), diploid D2321-51 (Fig. 8J), transformant no. 67 (Fig. 8K), and four segregants (S1–S4) (Fig. 8L, M, N, O) from the transformant. All Ufa^+, wild-type strains and transformants grown in UFA-free media (Fig. 8A–C) had similar fatty acid compositions, characterized by a low concentration of C18 saturated (stearic) acid, 18:0, and high amounts of mono- and poly-C18 UFA, 18:1, 18:2, and 18:3, as expected for strains with active Δ9-FAD. These strains, as well as both Ufa^+ diploid cultures (recipient D2321-51 and transformant no. 67) and Ufa^+ meiotic progeny of the transformant, grown in a medium supplemented with 16:1 (Fig. 8D, F, G, J–L, N) had indistinguishable fatty acid profiles, characterized by the highest peak of exogenously added 16:1, a low amount of 18:0, and high amounts of 18:1 and 18:2. In contrast, all Ufa^- strains grown with 16:1 exhibited distinct but similar fatty acid profiles: a mutant *fad1* (M1-3-7D), Ufa^- derivatives D1 and D2 from the transformants Tl and T2, and the disruptants S2 and S4 (Fig. 8E, H, I, M, O), demonstrated high levels of 18:0 and low but significant amounts of 16:2 and 18:1 in addition to the high amount of 16:1 fed into the medium.

Discussion

Methylotrophic yeast *H. polymorpha* is becoming an attractive model for the study of various metabolic pathways, genes, and regulatory mechanisms unknown in the more advanced yeast species *S. cerevisiae*. The poly-UFA biosynthetic pathway is an example of these unique biological processes. To understand how UFAs are synthesized, what the key reactions of the pathway are, how UFA biosynthesis is regulated, and what roles the various UFAs play in eukaryotic cells, we undertook the study of UFA auxotrophic mutants and cloning of genes encoding FADs (Anamnart et al. 1997, 1998). Here, we first demonstrated structural polymorphism of putative *OLE1* genes in various species of methylotrophic yeast, *H. polymorpha*, *P. angusta*, *P. methanolica*, and *C. boidinii* (Fig. 2), which are thought to be closely related (Harder and Brooke 1990). Structural divergence between *P. angusta* IFO 1475 and *H. polymorpha* CBS 1976 genes prompted us to perform clon-

ing, sequencing, and functional analysis of the gene from *H. polymorpha* strain CBS 1976.

Results (Figs. 4A, 5A, 8) of a genetic complementation test with the *H. polymorpha* mutant *fad1* showed the presence of an active gene *H-OLE1*, encoding Δ9-FAD in the cloned DNA fragment. The putative 451-amino-acid polypeptide encoded by *H-OLE1* has a mass of 52 kDa and an estimated isoelectric point (pI) of 9.67. The peptide consists of 51% hydrophobic, 19% neutral, and 30% hydrophilic residues. The *H-OLE1* protein, like other yeast Δ9-FADs, contains two domains, the N-terminal catalytic and C-terminal cytochrome b_5-like domains. The latter has been suggested to be involved in electron transport and to act as an electron donor in desaturation reactions (Michell and Martin 1995). The hydropathy profile of the *H-OLE1* protein shows two membrane-spanning domains which are found in eukaryotic iron-containing FADs (Shanklin et al. 1994).

The N-terminal catalytic domain of the *H-OLE1* protein contains three conserved histidine clusters (Fig. 3) which have been identified in various membrane-bound desaturases and monooxygenases (Shanklin et al. 1994; Van de Loo et al. 1995). The distances between BoxI–Box2–Box3 in the *H-OLE1* polypeptide were found to be 31 and 132 amino acids (Fig. 3). These features were exactly the same as those of *P-OLE1* polypeptide. It has been suggested that the histidine-rich boxes of the enzymes could be located on the cytoplasmic surface of the membrane (Murata and Wada 1995) and form an active Fe–O–Fe binding center (Shanklin et al. 1994; Van de Loo et al. 1995). Shanklin et al. (1994) have suggested the important role of consistent positioning of the clusters in the function of the potential membrane-spanning domains.

A structural comparison of the genes (Figs. 2C, 3) (Anamnart et al. 1997) shows that the upstream nontranslated region of the sequenced *H-OLE1* gene is 0.6 kb longer than that of the *P-OLE1* gene. Sequence information of this longer upstream region may be important from the viewpoint of transcriptional regulation of the yeast Δ9-FADs. The transcription of, for example, the genes for Δ9-FAD in *S. cerevisiae* (McDonough et al. 1992), *Yarrowia lipolytica*, *P. angusta* (Anamnart et al. 1997) and, as shown in Fig. 6, *H. polymorpha*, are repressed by exogenous Δ9-UFA. On the other hand, FAR, a positive regulatory element was described at the approximately −580 nt position of the *S. cerevisiae OLE1* gene (Choi et al. 1996). Analysis of 1080 bp in the sequenced upstream promoter of the *H-OLE1* gene (Fig. 3) did not allow the identification of a FAR-like sequence. This could explain the negative result of a complementation experiment when plasmid pLSF6 containing the native *H-OLE1* gene was introduced into the *S. cerevisiae ole1* host (Fig. 4B). However, the FAR element was not found even in the cloned region 500 bp upstream of the *P-OLE1* gene which has been shown to be active in the *S. cerevisiae* host (Anamnart et al. 1997). These results suggest that the FAR element might not be crucial for the positive transcriptional regulation of *OLE1* genes in heterologous hosts and the difference in promoter activity of *P-OLE1* and *H-OLE1* in the *S. cerevisiae* host is determined by divergence of nonspecific sequences which exhibit less homology with each other than the ORFs do (Fig. 2C).

An *H. polymorpha* disruptant Δ*H-ole1*, was constructed for the first time in this study by gene replacement of a diploid recipient having the *fad1-2*/*FAD1* genotype (Figs. 2C, 7B): one clone, no. 67, was found to be a heterozygous diploid Δ*H-OLE1::S-LEU2*/*H-OLE1* yielding UFA-requiring haploid progeny in which the mutation *fad1-2* was replaced by the input DNA fragment (Fig. 7). A tandem multicopy integration was detected, which could be explained as the result of crossing over following the formation of head-to-tail oligomeric molecules. With increasing frequency, (1) the formation of autonomously replicating plasmids, (2) random nonhomologous and (3) homologous integration are known as the main events in the transformation of both circular and linear DNAs into *H. polymorpha* cells (Roggenkamp et al. 1986; Beburov et al. 1990; Faber et al. 1994). Random integration of plasmid multimers into the genome has been described in this yeast (Roggenkamp et al. 1986; Clare et al. 1991; Bogdanova et al. 1995; Gatzke et al. 1995). Tandem multicopy integration of linear DNA into *H. polymorpha* and *P. pastoris* chromosomal targets has also been described (Clare et al. 1991; Sierkstra et al. 1991).

Comparative analysis of the cellular fatty acid composition in a Δ*H-ole1* (Δ*fad1*) disruptant and *fad1-2* mutants grown in media containing palmitoleic acid (16:1)with that of the wild-type strain and transformants harboring the whole *H-OLE1* gene (Fig. 8), not only confirmed our conclusions about the identity of the *H-OLE1* and *FAD1* genes but also revealed the more complex but interesting characteristics of UFA biosynthetic pathways in *H. polymorpha* cells. In addition to the main flow of the desaturation pathway described earlier (Anamnart et al. 1998)

$$18{:}0 \rightarrow 18{:}1(\Delta 9) \rightarrow 18{:}2(\Delta 9, 12) \rightarrow 18{:}3(\Delta 9, 12, 15)\ ,$$

several other reactions of desaturation and elongation can be postulated to explain the unexpected appearance of several peaks such as 16:2, 18:1, and 18:2 in gas chromatography fatty acid profiles of mutants (Fig. 8). Desaturation of exogenously added 16:1 (Δ9) to 16:2 (Δ9, 12) catalyzed by Δ12-FAD might be the simplest explanation for the appearance of the 16:2 compound. It is thought that this reaction takes place even in wild-type cells, but the presence of low concentrations of the 16:1 precursor did not allow observation of the 16:2 product in cultures grown on UFA-free media. It is clear that 18:1 and 18:2 are probably not synthesized in Δ*H-ole1* (Δ*fad1*)-disruptant cells from the saturated precursor (18:0), since no Δ9-FAD is presented. Nevertheless, significant amounts of these compounds were still detected in the cells of the disruptants (Fig. 8M, O) and the absolute requirement of UFA for cell division was noted (Fig. 7A). An elongation reaction

$$16{:}1(\Delta 9) \rightarrow 18{:}1(\Delta 11), 16{:}2(\Delta 9,12) \rightarrow 18{:}2(\Delta 11,14)\ ,$$

or elongation followed by a desaturation reaction

$$16{:}1(\Delta 9) \rightarrow 18{:}1(\Delta 11) \rightarrow 18{:}2(\Delta 11,15)\ ,$$

could be suggested to explain the appearance of such 18:1 and 18:2 peaks. However, a further interesting mechanism also remains to be clarified to interpret the presence of a fatty acid with a retention time corresponding to the 18:3 standard in cells of Δ*H-ole1* disruptants (Fig. 8).

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