Two fatty acid $\Delta^9$-desaturase genes, ole1 and ole2, from Mortierella alpina complement the yeast ole1 mutation

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Genes encoding two distinct fatty acid $\Delta^9$-desaturases were isolated from strains of the oleaginous fungus Mortierella alpina. Two genomic sequences, $\Delta^9$-1 and $\Delta^9$-2, each containing a single intron, were cloned from strain CBS 528.72 while one cDNA clone, LM9, was isolated from strain CBS 210.32. The $\Delta^9$-1 gene encoded a protein of 445 aa which shared 99% identity with the LM9 gene product. These proteins also showed 40–60% identity to the $\Delta^9$-desaturases (Ole1p) of other fungi and contained the three conserved histidine boxes, C-terminal cytochrome b5 fusion and transmembrane domains characteristic of endoplasmic reticulum membrane-bound $\Delta^9$-desaturases. LM9 and $\Delta^9$-1 are therefore considered to represent the same gene (ole1). The ole1 gene was transcriptionally active in all M. alpina strains tested and its function was confirmed by complementation of the Saccharomyces cerevisiae ole1 mutation. Fatty acid analysis of yeast transformants expressing the CBS 210.32 ole1 gene showed an elevated level of oleic acid (18:1) compared to palmitoleic acid (16:1), the major fatty acid component of wild-type S. cerevisiae. This indicated that the M. alpina $\Delta^9$-desaturase had a substrate preference for stearic acid (18:0) rather than palmitic acid (16:0). Genomic clone $\Delta^9$-2 (ole2) also encoded a protein of 445 aa which had 86% identity to the $\Delta^9$-1 and LM9 proteins and whose ORF also complemented the yeast ole1 mutation. The transcript from this gene could only be detected in one of the six M. alpina strains tested, suggesting that its expression may be strain-specific or induced under certain physiological conditions.

Keywords: Mortierella alpina, $\Delta^9$-desaturase genes, yeast complementation, fatty acid desaturation, oleaginous fungus

INTRODUCTION

The oleaginous zygomycete Mortierella alpina produces up to 50% of its cell dry weight as triacylglycerol oil, approximately 40% of which consists of the long-chain polyunsaturated fatty acid (LCPUFA) arachidonic acid (ARA; 20:4, n-6). LCPUFAs are important both nutritionally and pharmacologically and there is much interest in developing microbial processes for their production (Ratledge, 1993; Sancholle & Lösel, 1995; Leman, 1997). They are directly incorporated into membranes of the central nervous system and hence affect brain and nerve development (Willatts et al., 1998). LCPUFAs also act as precursors to a range of hormones, especially the prostaglandins, leukotrienes and thromboxanes, and are therefore thought to play important roles in combating or preventing a number of human diseases (Katayama & Lee, 1993). Oil produced by M. alpina has been screened for toxicity and is

Abbreviations: 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, $\alpha$-linolenic acid; 19:3, $\gamma$-linolenic acid; 20:3, dihomo-$\gamma$-linolenic acid; 20:4, arachidonic acid (ARA); ER, endoplasmic reticulum; LCPUFA, long-chain polyunsaturated fatty acid; RACE, rapid amplification of cDNA ends; UTR, untranslated region. The GenBank/EMBL accession numbers for the sequences reported in this paper are Y18553 and Y18554 (CBS 528.72 ole1 and ole2 genomic sequences, respectively) and AF0085500 (CBS 210.32 ole1 cDNA).
currently used as a supplement in infant formulae in some countries (Hempenius et al., 1997). The pathway for fatty acid desaturation and elongation from stearic acid (18:0) to LCPUFAs in filamentous fungi has been elucidated both by biochemical means and by studying mutant strains isolated by classical mutagenesis (Ratledge, 1993; Certik et al., 1998).

Known fungal fatty acid desaturases are all endoplasmic reticulum (ER) membrane-bound enzymes which have their active site on the ER’s cytoplasmic face. The active site comprises three histidine-rich boxes, normally containing eight essential histidine residues, which fold up to form the di-iron binding site in the native protein (Shanklin et al., 1994). Cytochrome \( b_5 \) is used as the electron donor and in the majority of cases the desaturase is a protein fusion with a cytochrome \( b_5 \) domain attached at either the N or C terminus. The substrate for the initial \( \Delta 9 \)-desaturation of 18:0 to oleic acid (18:1) is stearoyl-CoA but some of the subsequent desaturation steps, including A12- and \( \Delta 6 \)-desaturation, occur using the fatty acyl chains of phospholipid molecules (Jackson et al., 1998). \( M. \) \textit{alpina} mutants defective in most of the desaturase activities have been isolated and these have been used, combined in some instances with specific desaturase inhibitors, to alter the fatty acid composition of the fungus (Jareonkitmongkol et al., 1994; Kawashima et al., 1997). An alternative approach to manipulating the LCPUFA biosynthetic pathway in \( M. \) \textit{alpina} is to isolate and either overexpress or disrupt the genes encoding the desaturases and elongases. Indeed, the gene encoding the \( \Delta 5 \)-desaturase which converts dihomo-\( \gamma \)-linolenic acid (20:3) to 20:4 has been isolated from two different strains of \( M. \) \textit{alpina} (Knutzon et al., 1998; Michaelson et al., 1998). The \( M. \) \textit{alpina} \( \Delta 5 \)-desaturase, as predicted, contains three histidine boxes, although one of the essential histidine residues has been replaced with a glutamine, a change which is found in some other desaturases. This enzyme also contains a cytochrome \( b_5 \) domain fused at the N terminus.

The \( \Delta 9 \)-desaturase carries out the first step in the desaturation pathway which leads to the greatest decrease in fatty acid transition temperature compared to subsequent desaturation reactions (Harwood, 1997). Because of this, \( \Delta 9 \)-desaturase activity is important in maintaining membrane fluidity and its expression is therefore highly regulated. In several organisms, including \textit{Saccharomyces cerevisiae}, this control is exerted both at the transcriptional and post-transcriptional level (Choi et al., 1996; Gonzalez & Martin, 1996). The \( \Delta 9 \)-desaturase gene (OLE1) has been isolated from a number of yeasts and filamentous fungi and all have a similar structure (Stukey et al., 1990; Gargano et al., 1995; Meesters & Eggink, 1996; Anamnart et al., 1997; GenBank accession no. AF026401). We have therefore undertaken to isolate the gene encoding this enzyme from \( M. \) \textit{alpina} and to study its expression. Recently, a \( \Delta 9 \)-desaturase gene has been isolated from a patented strain of \( M. \) \textit{alpina} whose gene product displays \( \Delta 9 \)-desaturase activity in \textit{Aspergillus oryzae} and has a high degree of identity to other \( \Delta 9 \)-desaturases (Sakuradani et al., 1999). In this paper, we describe the isolation and characterization of two distinct \( \Delta 9 \)-desaturase genes, ole1 and ole2, from two strains of \( M. \) \textit{alpina} which are freely available from fungal culture collections.

**METHODS**

**Strains, media and growth conditions.** \( M. \) \textit{alpina} strain CBS 528.72 (ATCC 32222) was obtained from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands, and strain CBS 210.32 was a gift from Professor S. Shimizu, Kyoto University, Japan. Other \( M. \) \textit{alpina} strains used in this study were CBS 224.37, CBS 250.53 and CBS 527.72 (ATCC 32221), all obtained from the CBS, and strain CCF 2639 from the Culture Collection of Fungi, Charles University, Prague, Czech Republic, which was kindly supplied by Professor R. Herbert, University of Dundee. \textit{S. cerevisiae} strain L8-14AC (a ole1a::LEU2 leu2-3-112 ura3-52 his4); Stukey et al., 1989) was supplied by Professor M. Schweizer, Institute of Food Research, Norwich, \textit{S. cerevisiae} strains NCYC 1383 (a his3A1 leu2-3-112 trp1-289 ura3-52), NCYC 1662 (a arg) and \textit{AY925 (a ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can-100) and FY1679-3A (a ura3-52) were kindly supplied by Bruce Pearson, Institute of Food Research, Norwich. PCR-amplified DNA fragments were cloned in \textit{Escherichia coli} strains XL-1 Blue MRF’ (Stratagene), TOP10 (Invitrogen) or DH5a (Promega). Genomic and cDNA libraries were constructed using \textit{E. coli} strains XL-1 Blue MRF’ (Stratagene) and ER1647 (Amer sham Pharmacia Biotech), respectively. In vivo excision of phagemids was carried out using \textit{E. coli} XLORL (Stratagene) or BM25.8 (Amer sham Pharmacia Biotech). \textit{M. alpina} was maintained as vegetative mycelial cultures on potato dextrose agar slopes (PDA; Difco) and liquid cultures were inoculated with mycelial suspensions prepared in potato dextrose broth (PDB; Difco). PDB or GY broth, containing 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.05% (w/v) KCl, 2% (v/v) ACM salts (Mortice et al., 1998), pH 6.5, were used for growing mycelium for nucleic acid extractions. The effects of fatty acid addition to \textit{M. alpina} cultures were studied by supplementing PDB with 0.5% (v/v) ethanol with or without individual fatty acids at a final concentration of 1 mM. Cultures were grown either at 25 or 28°C with shaking at 150 r.p.m. \textit{S. cerevisiae} L8-14AC was maintained on YPD agar supplemented with 0.5 mM palmitoleic acid, 0.5 mM oleic acid and 1% (w/v) tergitol NP-40 (Sigma) and transformants were selected on similarly supplemented yeast nitrogen base medium (YNB), containing 0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose and 1.5% (w/v) agar, which lacked uracil.

**Amplification of \( \Delta 9 \)-desaturase probes and DNA sequencing.** Degenerate primers with homology to conserved histidine-box and cytochrome \( b_5 \) regions of \( \Delta 9 \)-desaturase genes from \textit{S. cerevisiae} (Stukey et al., 1990), \textit{Histoplasma capsulatum} (Gargano et al., 1995) and \textit{Cryptococcus curvatus} (Meesters & Eggink, 1996) were synthesized on an ABI 394 DNA–RNA synthesizer. Primer combinations P3 (5‘-TAYCAYAAYTYTTCAYCAYCA-3‘) and P4 (5‘-TYSCCSCGGRGTNGTC-3‘), and DESfor (5‘-CTKGGYATTYAWCWGCG-3‘) and DESrev (5‘-CAGAASGTSCGRTGGTGC-3‘) were used to amplify fragments from CBS 528.72 genomic DNA. PCR conditions were 94°C hot start for 5 min, 30 cycles of 94°C for 0.5 min, 52°C (primers P3/P4) or 46°C (primers DESfor/DESrev) for 1.5 min, 72°C for 1 min and a final extension at 72°C for 10 min. Degenerate primers Hisfor (5‘-WSICAYMGIAYICAY-
CA-3') and His3rev (5'-YTCRTGRTGRAARTTGTG-3') were used at an annealing temperature of 55 °C to amplify fragments from cDNA reverse-transcribed from total RNA of CBS 210.32 as described by Michaelson et al. (1998). Primers specific to M. alpina gene DA-1, 91for (5'-CATCACAGCA- GGCAATGTTA-3') and 91rev (5'-GGCGCCGACGATGT- CGAGCA-3'), were used at an annealing temperature of 62 °C to amplify a DA-1-specific probe. Primer BR99 (5'-AAACGT- TTTATTACAACAGGC-3') was used in combination with primer 91for at an annealing temperature of 52 °C to generate the remaining part of the DA-1 gene. PCR products were cloned into pCRII, pCR2.1-TOPO (both Stratagene) or pGEM-T (Promega) and the plasmids purified using the Nucleon Phytopure Plant DNA Extraction kit (Amersham Pharmacia Biotech) or by a DNA Extraction kit (Qiagen) where the DNA, dissolved in TE buffer (10 mM HCl, 1 mM EDTA, pH 7) was purified either by CsCl density gradient centrifugation or by using a modification to the Plasmid Midi Purification kit (Qiagen). RT-PCR was carried out using a cDNA first-strand synthesis kit (Amersham Pharmacia Biotech) with an anchored oligo(dT) primer, CN95 (5'-CTTTCTGTATGGTGCGTACCTAGAGCTGCT(5')ts-3'), followed by PCR with gene-specific primers. DNA sequencing was performed using the PRISM dye terminator kit (Perkin Elmer) and automated sequencer models 373A or 377 (Perkin Elmer). DNA sequences were analysed using the University of Wisconsin GCG software package.

**Nucleic acid manipulations.** High molecular mass genomic DNA was isolated from 4-d-old, PDB-grown M. alpina mycelium which had been freeze-ground with liquid nitrogen. DNA was isolated either by the Nucleon Phytopure Plant DNA Extraction kit (Amersham Pharmacia Biotech) or by a DNA Extraction kit (Qiagen). Total RNA was isolated using the Ready-To-Go T-primed First-Strand kit (Amersham Pharmacia Biotech) with freeze-ground mycelium or the TRIzol reagent (Life Technologies) with freeze-dried mycelium. The two PCR products, 310 and 1098 bp respectively, were then fused using primers A and D to generate a 1.3 kb intronless fragment. All PCR products were cloned and sequenced using standard procedures for either capillary or vacuum transfer of nucleic acids to nylon membranes. In all cases, hybridization was carried out at 65 °C in Puregene HYB-9 DNA hybridization solution (Flowgen) and blots were subsequently washed in 2× SSC (1× SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7) for 5 min, 0.5% (w/v) SDS and in 0.1× SSC, 0.5% (w/v) SDS at 65 °C. Signals were detected with a Fuji BAS 1500 phosphorimager or by autoradiography on X-ray film. On Northern blots, signals were standardized for fluctuations in RNA loading against the histone H4 transcript (P. Wongwathanarat and others, unpublished results).

**Library construction and screening.** A genomic library was constructed in ZAP Express (Stratagene) with BamHI-digested DNA prepared from strain CBS 528.72 following the manufacturer’s instructions. The library was screened with PCR-amplified DA-9-desaturase probes which had been labelled with [α-32P]dATP using the Megaprime DNA labelling kit (Amersham Pharmacia Biotech). Approximately 5×10^6 p.f.u. was used in the primary screen and positive plaques subjected to a secondary screen before in vivo excision of the pBK-CMV phagemids with ExAssist helper phage from E. coli XLORL (Stratagene). A cDNA library was constructed in zMOS2lox (Amersham Pharmacia Biotech) using EcoRI end-adapted cDNA synthesized from strain CBS 210.32 as described previously (Michaelson et al., 1998). About 1×10^5 p.f.u. from this library was screened with DA-9-desaturase probes which had been labelled with [α-32P]dCTP and positive clones in vivo excised in E. coli BM25.8 (Amersham Pharmacia Biotech), a P1 cre recombinase host (Michaelson et al., 1998). The five termini of positive cDNA clones were confirmed or their missing sequences completed by 5'-RACE (rapid amplification of cDNA ends) using the terminal transferase RACE method (Boehringer Mannheim) with cDNA as template and the nested primers RA15′ (5'- AGAGTCGATGTAACCTGCTTG-3') and RA25′ (5'- GATAACGAATCCGAGG-3') which contains an EcoRI site (underlined) for cloning purposes and primer cDNA3en (5'-AATCTGAGAATTCCGGCGCCG-3') which is complementary to the NotI anchor region (underlined) at the 3’ end of the first-strand cDNA.

**Construction of yeast expression vectors.** The LM9 and DA-2 ORFs were cloned into the yeast expression vector pVT100-U which contains the 2µ origin of replication, the URA3 selection marker and the alcohol dehydrogenase (ADH1) promoter and terminator regions (Vernet et al., 1987). A synthetic, intronless version of DA-2 with BamHI and HindIII sites at the 5’ end and a BamHI site at the 3’ end was created by overlap extension PCR using genomic clone DA-2 as template and the following primer combinations: primer A (5’-AAGATCCAAGCTTTTAAAAATGGCCAACCTCCTCCCTCCCACA-3’) and primer B (5’-CCATAACCGCTGTATCGAATTACCAAAGGC-3’), and primer C (5’-GGCTTGATTATCTCGACGATAACCAAGCCTTTG-3’) and primer D (5’-GGATTTCTCATTCTTCCTTGGATGTCGACCATATA-3’). Since the start and stop codons and the overlap regions are single-underlined and the relevant restriction sites are double-underlined. The two PCR products, 310 and 1098 bp respectively, were then fused using primers A and D to generate a 1.3 kb intronless fragment. All PCR products were cloned into pVT100-U, transformed into yeast and screened for the presence of the 2µ origin of replication and the ADH1 and HindIII and BamHI and directionally cloned into pVT100-U. The sequence of the insert was checked using primers from the ADH1 promoter and terminator regions: primer ADH1 (5’-GCTATCAAATATAAAACAG-3’) and primer ADH2 (5’-GAAATCTCGCTTATTTAGGA-3’), respectively.

Since LM9 is a cDNA clone, PCR was carried out with this as template to create a HindIII site at the 5’ end of the ORF and an XbaI site at the 3’ end using primer E (5’-AATCTAGAAGCTTTAAAAATGGAATCGACCTCTTC-CCCTCTC-3’) and primer F (5’-ATCACTAGAATTTCGGCGCTTTGCTGTACGATTCCGCCTG-3’) at an annealing temperature of 58 °C. The 1366 bp PCR fragment was digested with HindIII and XbaI, directionally cloned into pVT100-U and the sequence of the insert checked using primers ADH1 and ADH2.

**Yeast transformation and fatty acid analysis.** pVT100-U containing either the LM9 or DA-2 ORF was transformed into the S. cerevisiae olel mutant strain L8-14C by the lithium acetate/single-stranded carrier DNA/PEG whole cell method of Gietz et al. (1995). Undigested vector DNA (200 ng) in sterile TE buffer (pH 8.0) and 250 µg single-stranded herring sperm carrier DNA were incubated with 50 µl competent yeast cells for each transformation. Following lithium acetate/PEG treatment and heat shock at 42 °C for 15 min, the cells were transformed in vivo excised in E. coli BM25.8 (Amersham Pharmacia Biotech), a P1 cre recombinase host (Michaelson et al., 1998). The 5’ termini of positive cDNA clones were confirmed or their missing sequences completed by 5’-RACE (rapid amplification of cDNA ends) using the terminal transferase RACE method (Boehringer Mannheim) with cDNA as template and the nested primers RA15′ (5’- AGAGTCGATGTAACCTGCTTG-3') and RA25′ (5’- GATAACGAATCCGAGG-3') which contains an EcoRI site (underlined) for cloning purposes and primer cDNA3en (5’-AATCTGAGAATTCCGGCGCCG-3') which is complementary to the NotI anchor region (underlined) at the 3’ end of the first-strand cDNA.

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RESULTS AND DISCUSSION

Isolation of Δ9-desaturase genes from M. alpina strains CBS 528.72 and CBS 210.32

Degenerate primer combination DESfor/DESrev, covering a region containing histidine boxes 1 and 2, generated several fragments with CBS 528.72 genomic DNA as template, two of which, 594 and 554 bp in size, showed 40–60% amino acid identity when translated to known fungal Δ9-desaturases. Both fragments contained consensus sequences for introns (described below) of 155 and 115 bp, respectively, which disrupted the ORF at the same position in the deduced proteins. These protein fragments showed about 85% identity to each other, suggesting that there could be two Δ9-desaturase genes in this strain of M. alpina. A smaller fragment of 232 bp, generated with specific primers 91for and 91rev, which were derived from the 594 bp fragment sequence, still contained the 155 bp intron and was subsequently used to probe a genomic library from CBS 528.72. Degenerate primer combination P3/P4, which was designed to amplify a region further downstream between histidine box 3 and the haem-binding consensus sequence of the cytochrome b5 domain, generated a fragment of 347 bp that also had sequence homology to Δ9-desaturase genes. This fragment was also used to screen the CBS 528.72 genomic library. Similarly, degenerate primer combination His2for/His3rev, annealing to histidine boxes 2 and 3 respectively, amplified a 426 bp fragment from CBS 210.32 cDNA which on translation had 40–60% amino acid identity to fungal Δ9-desaturases and this was used to probe a cDNA library from CBS 210.32.

Clone LM9 was isolated from the CBS 210.32 cDNA library and contained a 166 kb EcoRI insert which encoded a protein of 445 aa with 98% identity to the Δ9-desaturase from M. alpina 1S-4 described by Sakuradani et al. (1999) and 40–60% identity to other fungal Δ9-desaturases. This protein displayed the three conserved histidine boxes, C-terminal cytochrome b5 fusion and transmembrane domains characteristic of ER membrane-bound Δ9-desaturases (Fig. 1a). The poly(A) tail and part of the 3′-untranslated region (3′-UTR) were missing from this clone. Several attempts at 5′-RACE using gene-specific nested primers RA15′ and RA25′ only extended the 5′ end of the cDNA by 32 bp, suggesting that the transcription start site was close to this point. 3′-RACE with primers RA13′ and cDNA3en completed the 3′-UTR and included the poly(A) tail. A consensus poly(A) addition signal, AATTTA (Gurr et al., 1987), was present in this gene 160 bp downstream from the TAG stop codon. On assembling the complete LM9 sequence, the total length of the cDNA was 174 kb. On Southern blots of CBS 210.32 genomic DNA, digested with either BamHI or HindIII and probed with fragment His2for/His3rev, at least two strongly hybridizing bands were seen per track, indicating that this strain may also have more than one Δ9-desaturase gene (data not shown).

The 232 bp 91for/91rev and 347 bp P3/P4 genomic probes did not cross-react with each other under stringent hybridization conditions but hybridized to
Table 1. Fatty acid composition of ole1 and ole2 transformants of S. cerevisiae L8-14C

Cultures were grown at 25 °C for 4 d in unsupplemented YNB medium. Fatty acids were transmethylated directly from washed, freeze-dried cells and fractionated by GC. The values given are the relative fatty acid compositions as a percentage of the total fatty acid content as determined by GC peak area. Values for the wild-type OLE1+ strains represent the mean of four determinations while those for the ole1 and ole2 transformants represent the mean from two independent yeast transformants (± SD).

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Fatty acid composition (%)</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>16:1</td>
<td>18:0</td>
<td>18:1</td>
<td>18:1/16:1 ratio</td>
</tr>
<tr>
<td>Wild-type OLE1+ strains*</td>
<td>18.6±2.7</td>
<td>39.5±4.1</td>
<td>5.9±1.2</td>
<td>21.6±4.2</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>L8-14C+pVT100-U: CBS 210.32 ole1</td>
<td>19.9±0.9</td>
<td>5.8±0.8</td>
<td>2.5±1.1</td>
<td>53.6±0.5</td>
<td>9.4±2.0</td>
</tr>
<tr>
<td>L8-14C+pVT100-U: CBS 528.72 ole2</td>
<td>32.0±1.6</td>
<td>3.4±0.8</td>
<td>48±0.4</td>
<td>43.8±4.0</td>
<td>13.9±6.3</td>
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*Mean values from four haploid, wild-type OLE1+ strains (NCYC 1383, NCYC 1662, AY925 and FY1679-3A) grown under identical conditions.

Δ9-desaturase genes from Mortierella alpina

BamHI fragments of approximately 2.9 and 3.4 kb, respectively, on Southern blots of CBS 528.72 genomic DNA (data not shown). On probing the genomic library with each fragment, positive clones were purified, insert sizes confirmed by BamHI digestion and the inserts sequenced. Clone Δ9-1, isolated using the 91for/91rev probe, contained a 2903 bp BamHI fragment whose encoded protein shared 99% amino acid identity with the LM9 protein. This clone did not contain the complete ORF since the BamHI site is located about 260 bp upstream of the stop codon in LM9. The remaining part of the Δ9-1 gene was isolated by PCR using primers specific to the Δ9-1 intron (primer 91for) and the LM9 3’-UTR (primer BR99) with CBS 528.72 genomic DNA as template. The DNA sequence of this PCR fragment was 100% identical to that of the genomic Δ9-1 clone up to the BamHI site and 99% identical to the LM9 sequence downstream of this site. Clones Δ9-1 and LM9 were therefore considered to represent the same gene, designated ole1. The 155 bp intron in Δ9-1 has the relatively rare 5’ splice site GCAAGT also found in the M. alpina 15S Δ9-desaturase gene (Sakuradani et al., 1999), ends with CAG at the 3’ splice site and contains the consensus lariat sequence TGCTAAC 42 nt from the 3’ end (Gurr et al., 1987). This intron disrupts the ORF in a highly conserved region of Δ9-desaturases and its removal was confirmed in vivo by RT-PCR analysis and sequencing using primers DESfor and DESrev which flank the intron. The structural organization of the CBS 528.72 ole1 gene is illustrated in Fig. 1(b).

Genomic clone Δ9-2 was isolated using P3/P4 as probe and contained a 3342 bp BamHI insert which also encoded a protein of 445 aa. This protein showed less identity (86%) to the M. alpina ole1 gene product. The single intron of 115 bp disrupted the ORF in the same position as in Δ9-1 but had the more common 5’ splice site GTATGT, a 3’ splice site, TAG, and consensus lariat sequences CATCAAC and TCTCAAC, 42 and 18 bp, respectively, from the 3’ end (Gurr et al., 1987). A less common poly(A) addition signal, AT(A)6TAATAA, was located 23 bp downstream from the TAG stop codon. The organization of this gene, designated ole2, is outlined in Fig. 1(c).

Functional characterization of the M. alpina Δ9-desaturase genes in S. cerevisiae

To confirm the in vivo function of the two putative Δ9-desaturase genes, the CBS 210.32 ole1 and CBS 528.72 ole2 ORFs were expressed in L8-14C, an ole1 mutant of S. cerevisiae (Stukey et al., 1989). Each ORF was cloned into the yeast expression vector pVT100-U (Vernet et al., 1987) with the consensus sequence (A)n immediately 5’ of the ATG start codon of each gene, a sequence which is associated with highly expressed S. cerevisiae genes (Hamilton et al., 1987). Both ORFs formed transcripts in yeast transformants, as determined by Northern analysis (data not shown), and complemented the ole1 mutation since the transformants grew without 16:1 and 18:1 supplementation. Fatty acid analysis of the transformants showed that both 16:1 and 18:1 were produced but that the ratio of 18:1 to 16:1 was higher than in wild-type S. cerevisiae (Table 1). M. alpina only produces negligible amounts of 16:1 and this result confirms that the M. alpina Δ9-desaturases had a substrate preference for 18:0 compared with 16:0, unlike the S. cerevisiae enzyme. There also appeared to be a difference in fatty acid composition between the ole1 (LM9) and ole2 (Δ9-2) transformants, indicating that the 14% difference in amino acid identity between the two proteins may have some significance for Δ9-desaturase activity. ole2 (Δ9-2) yeast transformants fed with a range of unsaturated fatty acids failed to desaturate these further, confirming that the ole2 protein only had Δ9-desaturase activity (data not shown).
Fig. 2. Expression of (a) ole1 and (b) ole2 in six strains of *M. alpina*. Total RNA (20 µg), extracted from 4-d-old PDB cultures grown at 25 °C, was Northern-blotted. The membranes were first probed with either (a) the 232 bp ole1 fragment 91for/91rev or (b) the 347 bp ole2 fragment P3/P4, both from CBS 528.72, and exposed for 72 h on a phosphorimage plate. The same membranes were then probed with a 208 bp fragment from the *M. alpina* histone H4.1 ORF and the phosphorimage plate exposed for 5 h to determine RNA loading.

**Δ9-desaturase gene expression in *M. alpina***

Transcription of the ole1 and ole2 genes was examined in a number of *M. alpina* strains by Northern analysis. In all six strains studied, the ole1 transcript was detected but showed strain to strain variation in amount relative to the histone H4 loading control (Fig. 2a). The two strains from which the ole1 gene had been isolated, CBS 210.32 and CBS 528.72, both produced significant amounts of ole1 mRNA. On the other hand, expression of the ole2 gene could only be detected in strain CBS 527.72 (Fig. 2b). The cDNA version of this gene was absent from the CBS 210.32 cDNA library and the failure to detect ole2 transcript by RT-PCR with RNA extracted from strains CBS 210.32 or CBS 528.72 confirmed that ole2 was inactive or extremely poorly expressed in these two strains of *M. alpina*. Sequence comparisons of the CBS 528.72 ole1 and ole2 promoter regions and 5′-UTRs showed that there was no homology between the ole1 and ole2 5′ regions and this is most likely the basis of this differential gene expression. Preliminary PCR analysis of genomic DNA from CBS 210.32 and a third strain of *M. alpina*, CBS 527.72, which was the only strain to show ole2 gene expression (Fig. 2b), using ole2-specific primers revealed that both strains possessed an ole2 gene. The 528 bp fragment which was amplified had 97% DNA sequence identity with the corresponding region of the CBS 528.72 ole2 gene and contained a 118 nt intron at the same position (data not shown). This suggests that most, if not all, strains of *M. alpina* contain both ole1 and ole2 genes.

The presence of two distinct Δ9-desaturase genes in one species is not unique. Two linked genes encoding Δ9-desaturases, which show differential expression, have been identified in *Drosophila* (Wicker-Thomas et al., 1997), mouse (Tabo et al., 1998) and *Arabidopsis* (Fukuchi-Mizutani et al., 1998). Sesame and rose also possess two Δ9-desaturase genes which are differentially expressed under specific growth conditions but their linkage has not yet been determined (Fukuchi-Mizutani et al., 1995; Yukawa et al., 1996). Humans, on the other hand, have two Δ9-desaturase genes but one is an inactive, intronless pseudogene which contains several mutations (Zhang et al., 1998). The need for two distinct Δ9-desaturases in *M. alpina* is not known but in other systems it appears to be related to differentiation and gene expression in specific organs or tissues (Wicker-Thomas et al., 1997; Fukushima-Mizutani et al., 1998).

Supplementation of CBS 210.32 cultures with a variety of unsaturated fatty acids containing a Δ9-unsaturated bond reduced ole1 transcript levels, with 18:1, 18:2 and γ-18:3 having the most pronounced effect (Fig. 3). This repression has been observed in several fungi (Choi et al., 1996; Meesters & Eggink, 1996). In *S. cerevisiae* the
regulatory sequences responsible for transcriptional control, the 111 bp FAR (fatty-acid-regulated) element containing repeated CCCGGG motifs and the sequence GGGTGTAGC, have been identified in the ole1 promoter (Choi et al., 1996). In addition, an as yet undefined sequence in the 5′-UTR is required for post-transcriptional regulation of the S. cerevisiae ole1 gene (Gonzalez & Martin, 1996). Similar sequences could not be found in the promoter regions or 5′-UTRs of the M. alpina ole1 and ole2 genes.

Conclusions

(1) Two distinct A9-desaturase genes, ole1 and ole2, have been isolated from the oleaginous fungus M. alpina. The ole1 gene product showed 98% amino acid identity to that of the M. alpina A9-desaturase gene described by Sakuradani et al. (1999) and 40–60% amino acid identity to other fungal A9-desaturases. The ole2 gene product had lower identity (86%) to the ole1 gene product.

(2) Both ole1 and ole2 ORFs complemented the ole1 mutation in S. cerevisiae and showed a substrate preference for 18:0 compared with 16:0.

(3) The ole1 gene was expressed in all strains of M. alpina which were studied and showed transcriptional regulation in response to supplementation with A9-unsaturated fatty acids.

(4) Transcription of the ole2 gene was only detected in one of the six strains of M. alpina which were examined, suggesting that gene expression may be strain-specific or induced under certain physiological conditions.

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